Cholesterol−Phospholipid Complexes in Membranes

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One of the more resistant problems in structural biology has been to define the structure and function of the liquid region of biological membranes. This is related to the fact that liquids generally do not have well-defined structures. Furthermore, in biological membranes these liquids are typically composed of hundreds of different lipid components. One approach to this problem has been to study the thermodynamic phase behavior of simple mixtures of lipids in monolayers and bilayers and to extrapolate to more complex mixtures. During the course of such studies we have made an unexpected discovery, namely, that mixtures of dihydrocholesterol (C) and phospholipids (P) in monolayers sometimes exhibit *two* upper miscibility critical points. While this is highly unusual for any liquid mixture,[1] there is a substantial literature that deals with this phenomenon from a theoretical point of view.[2−5] Two upper critical points can arise when the components of a liquid mixture react to form a chemically distinct product, or "complex". The present work outlines evidence for complex formation in lipid monolayers at the air−water interface composed of C and P. Dihydrocholesterol rather than cholesterol was used since it is more resistant to oxidation.[6]

The experimental phase diagram in Figure 1a was obtained for a mixture of C (mole fraction X), and P (mole fraction $1 - X$). The phospholipid was a 2:1 molar mixture of DMPS (dimyristoylphosphatidylserine) and DMPC (dimyristoylphosphatidylcholine). This phase diagram was obtained using epifluorescence microscopy and methods described previously.[7] There are several special experimental details to note. (i) The domains formed in the α two-phase coexistence region of Figure 1a were of the usual size, and easily resolved microscopically, as shown in Figure 2a. Domains formed in the β two-phase coexistence region of Figure 1a were small, as shown in Figure 2c. An electric field was used to fuse these small domains to improve their observability.[10] The field was removed for subsequent determination of phase boundaries. (ii) Locations of the critical regions can be established unambiguously by the observation of the superstructure stripe phase that is only seen close to the critical pressure and composition.[11] Epifluorescence micrographs of the stripe phase for each of the critical regions are shown in parts b and d of Figure 2. (iii) The lipid mixture used contains three principal components, C, DMPC, and DMPS. Even so, we discuss the phase diagram in Figure 1a as though it were a binary mixture and as though the phase boundaries were binodal curves.[12] This particular lipid mixture was chosen for illustration because a relatively sharp miscibility cusp is evident at the special composition of 33 mol % C. We have observed similar phase diagrams for a number of other lipid mixtures, but the special compositions are sometimes nowhere near 33 mol % C. As shown in Figure 1b, a binary mixture of C and DMPS also shows two upper critical points but the critical pressure for the α-region is so low (approximately 2 mN/m) that it is difficult to determine the nearby phase boundary very accurately. The occurrence of two upper critical points is thus not dependent on the use of a ternary lipid mixture compared to that of a binary mixture.

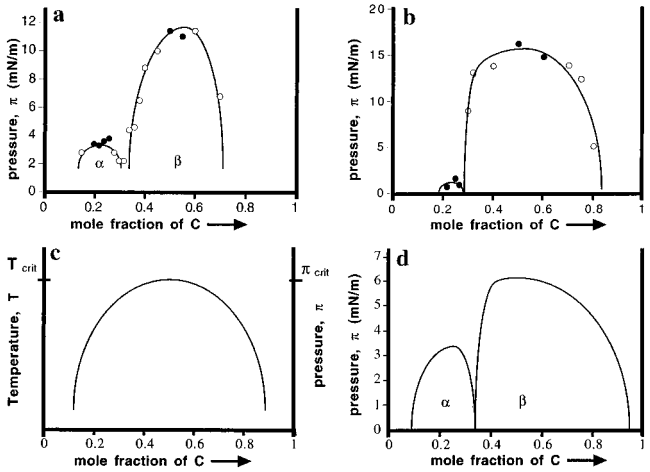

Figure 1. Phase diagrams showing liquid−liquid miscibility critical points. There are two phases below the curves, and one above. In (a) and (d), the two-phase coexistence region corresponding to low C mole fraction is denoted by α, and the two-phase coexistence region corresponding to high C mole fraction is denoted by β. (a) Phase diagram for a mixture of C (mole fraction X) and P (mole fraction $1 - X$). The P is a 2:1 molar mixture of DMPS and DMPC. Plotted data points represent the transition pressures that mark the disappearance of two-phase coexistence during monolayer compression. (b) Phase diagram for a binary mixture of C and DMPS. In both (a) and (b), the experimental data points define the phase boundary between a two-phase coexistence region at low pressures and a one-phase region at higher pressures. The filled circles represent proximity to the stripe superstructure phase; this in turn represents proximity to a miscibility critical point. Open circles give phase boundaries without a stripe superstructure phase. (c) Schematic phase diagram of a cholesterol−phospholipid binary mixture. The critical pressure π_c is the monolayer pressure at which the critical temperature is room temperature. (d) Calculated phase diagram for a binary mixture of C and P, based on an adaptation of the work in refs 2 and 3. In the notation of Talanquer,[3] and in energy units of room-temperature kT, this calculation uses $a = 3$ for P−P_2C repulsion, $b = 5$ for C−P_2C repulsion, $c = 3$ for P−C repulsion, and $K = 2$ for the complex formation equilibrium constant, all at $\pi = 0$. The assumed pressure dependencies are $\partial a/\partial\pi = -0.1$ m/mN, $\partial b/\partial\pi = -0.1$ m/mN, $\partial c/\partial\pi = 0$, and $\partial \ln K/\partial\pi = -1$ m/mN.

Figure 1c gives a schematic phase diagram describing a number of special binary mixtures of C and P having 1:1 critical compositions.[13] These binary mixtures of P and C undergo a contraction in area when mixed at constant pressure.[14] A simple thermodynamic model shows how this contraction results in a decrease in critical temperature with increasing pressure.[13] In our experiments monolayer pressure (π) is changed until the critical temperature is equal to room temperature. This is illustrated by the two ordinate scales. When temperature is fixed, one can pass

(1) Francis, A. W. *Liquid-liquid equilibriums*; Interscience Publishers: New York, 1963; p 16.
(2) Corrales, L. R.; Wheeler, J. C. *J. Chem. Phys* **1989**, *91*, 7097−7112.
(3) Talanquer, V. *J. Chem. Phys.* **1992**, *96*, 5408−5421.
(4) Hueda, Y.; Costas, M. E.; Scott, R. L. *J. Phys. Chem. B* **1997**, *101*, 8676−8682.
(5) Châtellier, X.; Andelman, D. *J. Phys. Chem.* **1996**, *100*, 9444−9455.
(6) (a) Benvegnu, D. J.; McConnell, H. M. *J. Phys. Chem.* **1993**, *97*, 6686−6691. (b) S. L. Keller in this laboratory has made a similar observation.
(7) (a) Subramaniam, S.; McConnell, H. M. *J. Phys. Chem.* **1987**, *91*, 1715−1718. (b) Hirshfeld, C. L.; Seul, M. *J. Phys.* (Paris) **1990**, *51*, 1537−1552.
(8) McConnell, H. M. *Annu. Rev. Phys. Chem.* **1991**, *42*, 171−195.
(9) Möhwald, H. *Annu. Rev. Phys. Chem.* **1990**, *41*, 441−476.
(10) Lee, K. Y. C.; Klingler, J. F.; McConnell, H. M. *Science* **1994**, *263*, 655−658.
(11) (a) Seul, M. *Europhys. Lett.* **1994**, *28*, 557−563. (b) Seul, M.; Chen, V. S. *Phys. Rev. Lett.* **1993**, *70*, 1658−1661. (c) Rice, P. A.; McConnell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3452−3455.
(12) Some mixtures of C and P have been shown to act as pseudobinary mixtures, that is, mixtures of C and an "average" P. See: Hagen, J. P.; McConnell, H. M. *Biochim. Biophys. Acta* **1996**, *1280*, 169−172.

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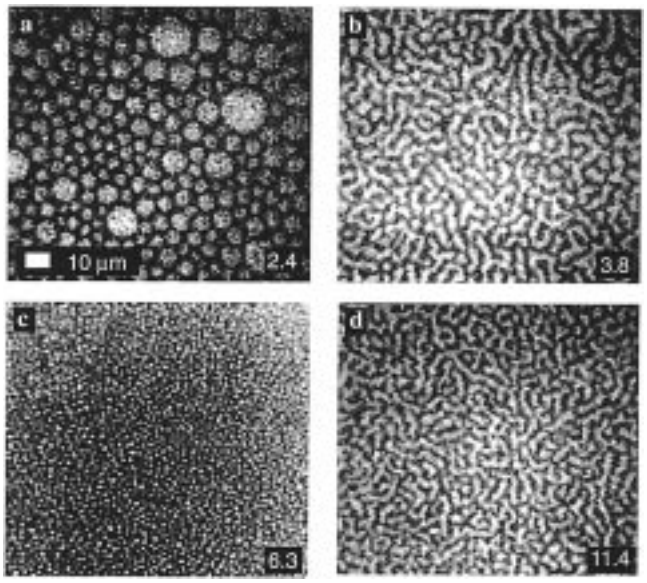

Figure 2. Epifluorescence micrographs of a lipid monolayer consisting of DMPC, DMPS, and C at various pressures at an air−water interface at room temperature (23 °C). (a) and (b) Images of a monolayer consisting of 25 mol % C, 49.9 mol % DMPS, 24.9 mol % DMPC, and 0.2 mol % TR-DMPE (Texas red dimyristoylphosphatidylethanolamine). TR-DMPE is preferentially excluded from the C-rich phase, thereby providing contrast between phases. (a) 2.4 mN/m: 5−10 μm circular domains of bright, C-poor liquid phase within a dark, C-rich liquid phase. These domains exhibit Brownian motion and are characteristic of domains formed in the α two-phase coexistence region of Figure 1a and b. (b) 3.8 mN/m. Stripe phase characteristic of proximity to a miscibility critical point. (c) and (d) Images of a monolayer consisting of 50 mol % C, 32.2 mol % DMPS, 16.6 mol % DMPC, and 0.2 mol % TR-DMPE. (c) 6.3 mN/m. These small white domains are less than 2 μm in diameter and are formed in the β two-phase coexistence region of Figure 1a and b. (d) 11.4 mN/m. Stripe phase characteristic of proximity to a miscibility critical point.

through the critical point by changing the pressure, or when the pressure is fixed, one can pass through the critical point by changing the temperature. Figure 1d is an adaptation of work by Corrales and Wheeler,[2] and by Talanquer,[3] where it is imagined that there is a chemical reaction between P and C to produce a complex P_2C. In this adaptation we have used a mean field description of the pressure dependence of the critical temperatures for the two hypothetical nonreacting binary mixtures, (P, P_2C) and (P_2C, C). The equilibrium constant for the reaction $2P + C \rightarrow P_2C$ is also treated as pressure-dependent. It is necessary that all three pressure-dependent interaction energies be on the order of $\Delta A\pi$, where ΔA is the observed contraction in area associated with mixtures of P and C.[14]

The simplest interpretation of the observed phase diagrams is that complexes of C and P are formed with stoichiometry P_nC_m, where n and m are integers. In this "chemical reaction" model, the region of the cusp in the phase diagram represents a composition of particularly high stability, in that this composition resists dissociation into C and P at lower pressures. The calculations do not preclude the possibility that the phases with these special compositions have some sort of short-range order. However, there is extensive evidence that the phases considered are two-dimensional liquids;[6,15] thus, crystalline order is not likely.

There is earlier but controversial evidence for special P:C stoichiometries in bilayers.[16−18] There is only indirect evidence for the coexistence of liquid phases in bilayers containing phospholipids and cholesterol.[19−21] Some of the most suggestive findings on bilayers have come from studies using various spectroscopic probes that sometimes show remarkably abrupt changes as a function of bilayer composition.[22] In the calculated phase diagram of Figure 1d, there is a major change in membrane properties as one changes lipid composition in the vicinity of the special composition. Below the critical pressures, there is a jump in the composition of the minor phase as one passes from one immiscibility region to another ($\alpha \leftrightarrow \beta$).[23] Even above the critical pressures, the calculated complex concentration profile is sharply peaked near the special composition. We suggest that these factors are the origin of the special compositions reported in the literature.[24−27] A superlattice model with short-range order may be equivalent to a molecular complex model with complexes having appropriate stoichiometries.[28]

There is a distinction to be made between the model described here and the superlattice models proposed earlier for lipid mixtures in bilayers.[18,24−27,29] At low pressures in the monolayers, the complexes can form essentially pure phases in which the other components have almost no solubility. This is similar to superlattice models in that there is a single phase with specific stoichiometry. However, in monolayers these complexes are modeled to be formed cooperatively at higher pressures, where they are completely miscible with the other lipid components above the critical pressure. We suggest that such complexes are present in lipid bilayers and biological membranes even when the overall lipid stoichiometry differs from that of the complex. The complexes may be present as separate molecular entities and need not form a separate macroscopic phase. Cholesterol−phospholipid molecular complexes should therefore persist even above possible miscibility critical points in bilayers and biological membranes.[30]

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(13) Hagen, J. P.; McConnell, H. M. *Biochim. Biophys. Acta* **1997**, *1329*, 7−11. The pressure dependence of the critical temperature in these mixtures can also be described in terms of a weak 1:1 complex with a pressure-dependent equilibrium constant.

(14) Ghosh, D.; Tinoco, J. *Biochim. Biophys. Acta* **1972**, *266*, 41−49.

(15) Stone, H. A.; McConnell, H. M. *Proc. R. Soc. London A* **1995**, *448*, 97−111.

(16) (a) Gershfeld, N. L. *Biophys. J.* **1978**, *22*, 469−488. (b) Tajima, K.; Gershfeld, N. L. *Biophys. J.* **1978**, *22*, 489−500.

(17) Hinz, H.; Sturtevant, J. M. *J. Biol. Chem.* **1972**, *247*, 3697−3700.

(18) Engelman, D. M.; Rothman, J. E. *J. Biol. Chem.* **1972**, *247*, 3694−3697. See also: Presti, F. T.; Pace, R. J.; Chan, S. I. *Biochemistry* **1982**, *21*, 3831−3835.

(19) Ipsen, J. H.; Karlstrom, G.; Mouritsen, O. G.; Wennerstrom, H.; Zuckermann, M. J. *Biochim. Biophys. Acta* **1987**, *905*, 162−172.

(20) Jain, M. K. *Introduction to biological membranes*; John Wiley: New York, 1988.

(21) Recktenwald, D. J.; McConnell, H. M. *Biochemistry* **1981**, *20*, 4505−4510.

(22) The spin-label TEMPO shows remarkable variations of solubility as a function of composition in bilayer membranes composed of DMPC and cardiolipin. See: Berclaz, T.; McConnell, H. M. *Biochemistry* **1981**, *20*, 0, 6635−6640.

(23) Other physical properties, such as domain size in the case of monolayers studied here, also change abruptly as composition is changed in the region of the miscibility cusp. Domain size changes are doubtless related to changes in interdomain line tension and dipole density. See refs 6, 7, and 13.

(24) Virtanen, J. A.; Ruonala, M.; Vauhkonen, M.; Somerharju, P. *Biochemistry* **1995**, *34*, 11568−11581.

(25) Wang, M. M.; Sugar, I. P.; Chong, P. L. *Biochemistry* **1998**, *37*, 11797−11805.

(26) Tang, D.; van Der Meer, W.; Cheng, S.-Y. S. *Biophys. J.* **1995**, *68*, 1944−51.

(27) Parassassi, T.; Giusti, A. M.; Raimondi, M.; Gratton, E. *Biophys. J.* **1995**, *68*, 1895−1902.

(28) A quasi-crystal model in which molecules at lattice sites have an interaction energy that is orientation dependent also yields two upper critical points. See ref 4.

(29) Sugar, I. P.; Tang, D.; Chong, P. L. *J. Phys. Chem.* **1994**, *98*, 7201−7210.

(30) Keller, S. L.; Pitcher, W. H., III; Huestis, W. H.; McConnell, H. M. *Phys. Rev. Lett.* **1998**, *81*, 5019−5022.