Red Blood Cell Lipids Form Immiscible Liquids

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Monolayers at the air-water interface were prepared from lipids extracted from human red blood cells. Epifluorescence microscopy was used to show that monolayers simulating the inner and outer leaflets of the red cell membrane form immiscible liquid phases with critical points at surface pressures of 21 and 29 dyn/cm. At these pressures the monolayer lipid density is comparable to that in the red cell membrane. This suggests that lipid bilayers of a red blood cell are near a miscibility critical point, which should significantly affect the biophysical properties of the red cell membrane. [S0031-9007(98)07922-8]

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Epifluorescence microscope studies have shown that mixtures of cholesterol and phospholipids form immiscible liquid phases in monolayers at the air-water interface [1–4]. There is indirect evidence that immiscibility also occurs in bilayers of these lipids [5–7]. These results raise the question of whether immiscible liquid domains exist in biological membranes. To this end, we extracted the lipids from membranes of human red blood cells (erythrocytes). Red blood cell membranes contain over 250 lipid species [8], most of which are arranged asymmetrically across the membrane [9]. The major headgroup classes of the extracted erythrocyte lipids were separated by thin layer chromatography, then reconstituted with 50 mol % cholesterol so as to mimic compositions of the inner and outer erythrocyte membrane leaflets. Monolayers of these lipids at the air-water interface are found to form immiscible liquid phases.

Figures 1 and 2 show epifluorescence micrographs of monolayers of reconstituted lipids simulating the inner and outer leaflets. Contrast between phases is achieved by 0.2 mol % of a fluorescent probe that is preferentially excluded from the cholesterol-rich phase [4]. Two immiscible liquid phases coexist to surface pressures greater than 20 dyn/cm. Domain shapes characteristic of proximity to a critical point are observed at 21 dyn/cm in the simulated inner leaflet [Fig. 1(b)] and at 29 dyn/cm in the outer leaflet [Figs. 2(c) and 2(d)]. The fingering of the domains in Fig. 2(f) indicates the monolayer is close to the critical composition. "Fingered" domains are circular domains from which stripes emanate. At higher pressures the monolayers are homogeneous as in Figs. 1(c) and 2(e). The domain shape changes are reversible.

It is helpful to consider these results in terms of a simple thermodynamic model. Mixtures of phospholipids alone have not generally been found to exhibit liquid-liquid immiscibility in monolayers. However, immiscibility is seen in binary mixtures of cholesterol with the phospholipids phosphatidylcholine [1–4], phosphatidylethanolamine [10], or phosphatidylserine [11]. Cholesterol and egg sphingomyelin also exhibit immiscibility (data not shown). Thus, all of the red cell's major lipid components can potentially contribute to the observed immiscibility. This leads to the question of how binary properties manifest themselves in a multicomponent mixture. Consider the simplest possible thermodynamic model for the Gibbs free energy G of a multicomponent lipid mixture exhibiting a liquid-liquid critical point:

$$G = \sum_i (\mu_i^0 X_i + kTX_i \ln X_i) + \sum_{i \neq j} 2kT_{ij} X_i X_j . \quad (1)$$

Here X_i is the mole fraction of component i, T_{ij} is the critical temperature of an ij pair, μ_i^0 is the chemical potential of a pure component i, T is temperature, and k is the Boltzmann constant. The critical temperatures T_{ij} depend on the monolayer pressure π and contraction parameters α_{ij}.

$$T_{ij}(\pi) = T + \alpha_{ij}[\pi - \pi_c(ij)]/2k . \quad (2)$$

In experiments on binary mixtures at room temperature T, the surface pressure is changed until a critical pressure $\pi_c(ij)$ is reached. The change in molecular area due to nonideal mixing of i and j is taken to be $\alpha_{ij} X_i X_j$. Experimental values of α_{ij} for cholesterol-phospholipid pairs are large, of magnitude -10 to -40 Å^2 [12]. This corresponds to a large effect of surface pressure on monolayer critical temperature, for example, -5 to -10 °C/dyn/cm [13].

The same method is used in the present study of a multicomponent mixture. At the putative critical composition, the pressure is changed until the critical pressure π_c is found. Conducting experiments at physiological temperature (37 °C) rather than room temperature (23 °C) is expected to lower the critical pressure by only 1–3 dyn/cm, assuming the average values of the α_{ij} are similar [10,14].

Figure 3(a) gives a schematic ternary phase diagram for two ideally mixing phospholipids (components 1 and 2) and cholesterol (component 3). Two liquid phases coexist within the broad range of compositions and temperatures beneath the surface drawn in Fig. 3(a). The binary mixture of components 1 and 3 is assumed to have the

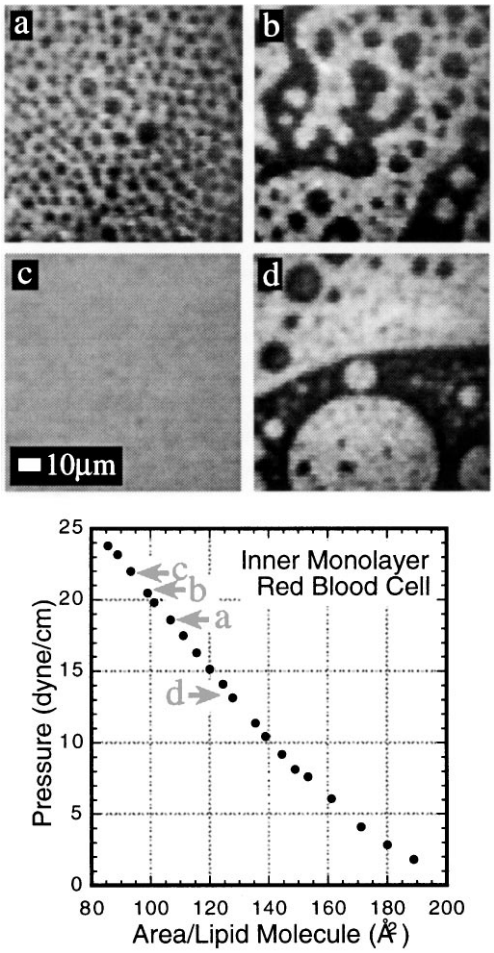

FIG. 1. Top: Epifluorescence micrographs of a monolayer simulating the erythrocyte membrane inner leaflet (Table I, expt 1) as it is laterally compressed at an air-water interface at room temperature (23 °C). At surface pressures below 1 dyn/cm (or mN/m), gas and liquid phases coexist (not shown). As the monolayer is compressed further, immiscible liquid phases appear. (a) 18.5 dyn/cm: 5–10 μm circular domains of dark, liquid phase within a bright, liquid phase. Domains exhibit Brownian motion. (b) 20.8 dyn/cm: fingering characteristic of critical point behavior. (c) 21.9 dyn/cm: homogeneous liquid. (d) 13.2 dyn/cm: circular domains reappear as the surface pressure decreases. Bottom: the pressure vs area/molecule isotherm is similar to those of cholesterol-phospholipid lipid monolayers [3]. Area/molecule errors are estimated to be less than a factor of 2. Errors in pressures are ± 1 dyn/cm throughout. Experiments are standard [14] and concluded within $\sim$10 min to minimize air oxidation. Because of this time constraint, it is virtually impossible to observe an inflection point in the isotherm near the critical point (point b). Even under ideal conditions, the inflection point is difficult to detect [3].

TABLE I. Molar lipid compositions were mixed to approximate the compositions of the inner and outer leaflets of the red blood cell membrane. Concentrations are given for cholesterol (Chol), the four other major lipid species [sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS)], glycolipids (Gly), and the fluorescent probe Texas Red-DMPE (dimyristoylphosphatidylethanolamine). (See Ref. [15].) Experiments 1, 2, 3, and 4 displayed critical pressures, π_c (dyn/cm or mN/m) as in Figs. 1 and 2. Of these, experiments 1 and 4 were best estimates of the inner and outer red blood cell leaflet compositions. Experiments 2 and 3 were variations on the inner leaflet 1. Experiments 5 through 10 were variations on the outer leaflet 4. These six exhibited immiscible liquid phases and a transition at high pressure (>20 dyn/cm) to a homogeneous liquid, but no fingering or stripe phase (No). Lipids extracted [16] from the red blood cell membrane but not yet separated by headgroup contained the sum of all the cholesterol and the four major lipid species of the inner and outer leaflets, with no glycolipids. Monolayers of this "total" mixture also exhibited immiscible liquid phases and a transition at high pressure (>15 dyn/cm) to a homogeneous liquid, but no critical behavior.

Expt	Chol	SM	PC	PE	PS	Gly	TR	π_c
Inner red blood cell leaflet								
1	46.2	5.3	7.4	28.4	12.5	0	0.2	22
2	40.9	5.8	8.1	31.2	13.8	0	0.2	25
3	51.5	4.8	6.7	25.6	11.2	0	0.2	23
Outer red blood cell leaflet								
4	43.4	22.5	23.4	6.3	0	4.2	0.2	29
5	45.3	25.7	26.7	2.1	0	0	0.2	No
6	40.4	28.0	29.1	2.3	0	0	0.2	No
7	36.1	29.9	31.2	2.6	0	0	0.2	No
8	32.9	31.9	33.2	1.8	0	0	0.2	No
9	25.3	35.2	36.5	2.8	0	0	0.2	No
10	82.1	4.6	4.8	0.4	0	7.9	0.2	No

same critical pressure π_{13} as the binary mixture of 2 and 3, π_{23}. Joining these binary critical points is a calculated *line* of critical points. Thus critical properties hold for substantial variation in lipid composition along this critical line. A lipid mixture of $n > 3$ components with interactions described by Eq. (1) has an $n - 1$ dimensional surface of critical points. In this theoretical scenario, critical behavior is sensitive to cholesterol concentration and relatively insensitive to substantial variations in phospholipid composition (cf. Table I).

Figure 3(b) gives a theoretical phase diagram for a binary mixture of components 1, 3 or 2, 3. Superimposed on this phase diagram are the superstructure phases H and S. The hexagonal (H) and stripe (S) phases have length scales D set by a competition between long-range intermolecular dipolar repulsions and interdomain line tension λ.

$$D \approx de^{\lambda/m^2}. \tag{3}$$

Here m is the difference in dipole density in the two phases and d is of the order of a nearest neighbor intermolecular distance. Near the critical point the widths of the stripes are equal to $de(\pi/2)e^{\lambda/m^2}$ [4]. The line tension λ and the dipole energy difference term m are related to the pressure difference $(\pi - \pi_c)$ by critical exponents $\mu \approx 1$ and $\beta \approx 0.25$, respectively [14,17,18]. Hence, as π approaches π_c, D decreases. D is the stripe width or circular domain radius. Indeed, in a binary mixture of cholesterol and phosphatidylcholine, we have observed that the stripe width approaches zero as the surface

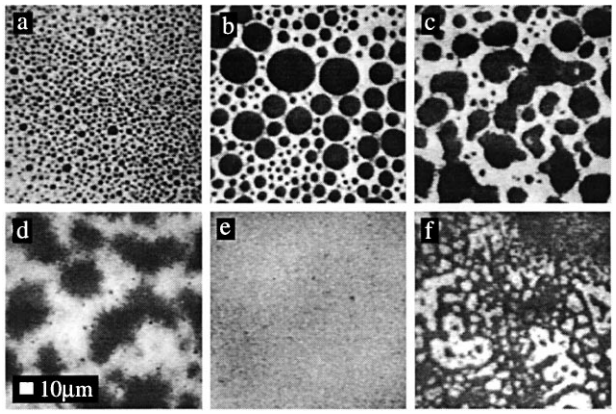

FIG. 2. Epifluorescence micrographs of a lipid monolayer simulating the erythrocyte membrane outer leaflet (Table I, expt 4). The phases and isotherm are similar to Fig. 1. (a) 12.1 dyn/cm (or mN/m): circular liquid domains $<10\ \mu$m. (b) 26.1 dyn/cm: coalescence of domains. (c) 28.4 dyn/cm: fingering characteristic of critical point behavior. (d) 29.1 dyn/cm: critical point. (e) 34.5 dyn/cm: homogeneous liquid. (f) 28.8 dyn/cm: immiscible liquid phases reappear as pressure decreases. The domains exhibit fingering characteristic of proximity to a critical point. At lower pressures, the domains are circular.

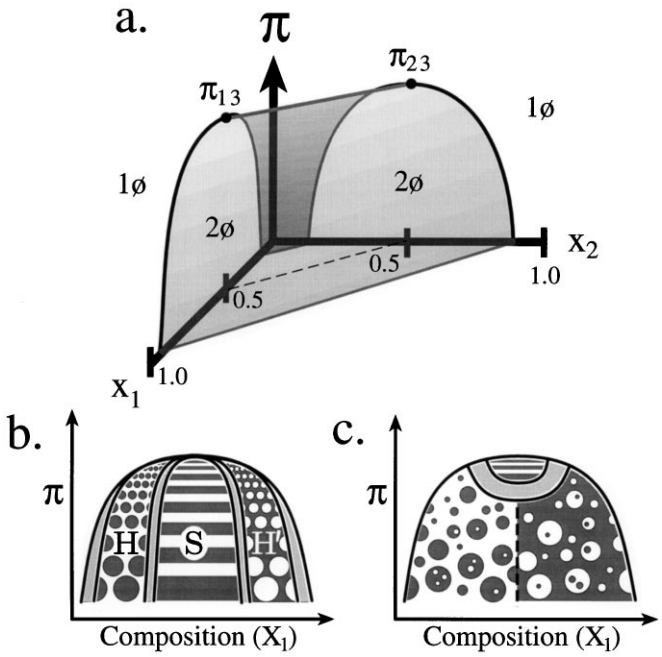

FIG. 3. (a) Schematic ternary phase diagram with mole fractions X_1, X_2, and X_3, where $X_1 + X_2 + X_3 = 1$. Components 1 and 2 are phospholipids and 3 is cholesterol. At pressures $\pi < \pi_{13} = \pi_{23}$, two immiscible liquid phases (2ϕ) coexist under the surface shown. Above this surface, there is one homogeneous liquid phase (1ϕ). In this model, all pure components are assumed to have equal chemical potentials μ_i^0, and surface areas, with area contraction parameters $\alpha_{13} = \alpha_{23}, \alpha_{12} = 0$. Binary phase diagrams for 1,3 and 2,3 appear above the X_1 and X_2 axes. Both pairs are assumed to have equal critical pressures $\pi_{13} = \pi_{23}$. A line of critical points connects the two binary critical points. (b) Schematic theoretical phase diagram of cholesterol-phospholipid binary mixture. Below the critical pressure and within the two phase region three superstructure morphologies are expected, two hexagonal phases (H and H') and a stripe phase (S) [31]. The boundary between the stripe phase and the hexagonal phase is adapted from [32]. Coexistence regions of adjacent phases are in grey. The equilibrium widths of the stripes and the radii of circles depend on the proximity to the critical point, as sketched. (c) Schematic experimental phase diagram of cholesterol-phospholipid binary mixture. Stripes are observed only within a few dyn/cm of the critical point [19]. At lower surface pressures, domains are primarily black circles on a white background or reversed. Fingering is observed in the grey region, at the transition between circular domains and stripes.

pressure is raised to the critical pressure. As the critical pressure is approached from below, preexisting circular domains show fingering and then transform to the stripe phase when the composition is close to the critical composition (data not shown). At low monolayer pressures $\lambda \approx 1.8 \times 10^{-7}$ dyn and $m^2 \approx 10^{-8}$ cgs esu and D is extremely large [18]. Theoretically, at equilibrium a stripe phase should appear over a large region of the phase diagram [Fig. 3(b)]. In contrast, stripe phases are experimentally observed only within 1–2 dyn/cm of the critical point [Fig. 3(c)] [19]. This is related to the fact that the rate of domain size equilibration is slow and that stripes produced experimentally are limited in length [20]. In our experience, these conclusions apply equally well to multicomponent mixtures. Figure 3(c) illustrates the experimental relations between circular domains, fingering domains, and the stripe phase.

The erythrocyte lipid bilayer may be near a critical point since stripe phase fingering is observed in erythrocyte lipid monolayers at comparable molecular densities. We observe critical behavior at molecular areas of $\sim$60 Å^2 for the simulated erythrocyte outer leaflet and $\sim$100 Å^2 for the inner leaflet. (A transition from two liquid phases to one liquid phase is found at $\sim$40 Å^2 for the total lipid mixture.) Uncertainties in these areas due to the chemical assay used [21] are estimated to be less than a factor of 2. The average area of a lipid in an erythrocyte membrane of a phospholipid bilayer is comparable, $\sim$40 or $\sim$60 Å^2, respectively [22,23].

Additional comparison between monolayers and the erythrocyte membrane is provided by the work of Demel *et al.* [24]. These investigators compared the activity of phospholipases on erythrocyte membranes to their activity on lipid monolayers at various pressures. They concluded that a monolayer pressure between 31 and 34.8 dyn/cm yields a susceptibility to this lipase activity equivalent to the susceptibility of erythrocyte membranes to lipase activity. This surface pressure is slightly higher than the pressure at which we observe critical behavior in the simulated inner and outer red blood cell leaflets.

The proximity of red blood cell lipids to a liquid-liquid immiscibility critical point should play a significant role in the cellular physical properties. For example, theoretically, membrane lipid composition and curvature

are coupled near a critical point, influencing cell shape and deformability [25–27]. Since the compressibility of liquids diverges at critical points [17], the lateral compressibility of membranes should be large. Indeed, the response of erythrocyte lipids to membrane receptor ligands such as prostaglandins [28] and acetylcholine [29] suggests a protein-mediated lateral compression or extension of the erythrocyte bilayer. The major role of proteins on the lipid phase behavior is likely to be through their effect on two-dimensional lipid density [30].

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[16] Lipids were extracted from the entire bilayer of erythrocytes of two healthy human volunteers. Lipid species were separated by headgroup (and not by acyl chains) by thin layer chromatography and were assayed for concentration [21]. The assayed concentrations may be in error by as much as a factor of 2. Lipids were then mixed in compositions that approximate the reported inner and outer erythrocyte compositions [22]. Globopentaosylceramide (Sigma) was substituted for erythrocyte glycolipids. Lipids with different acyl chains were mixed equally although the acyl distribution of some lipid species may be asymmetric across the erythrocyte membrane [33]. Molecular weights were estimated as 730 (SM), 760 (PC), 750 (PE), 840 (PS), 1310 (gly), and 1380 (Texas Red-DMPE). By weight, erythrocyte lipids are 24% cholesterol, 11% glycolipids, and 60% phospholipids [22]. Erythrocyte phospholipid headgroups are approximately 30 mol % PC; 30% PE; 25% SM; and 15% PS [22]. The outer leaflet contains 82% of the SM, 76% of the PC, 20% of the PE, and none of the PS [22]. All the glycolipids and by assumption half the cholesterol reside in the outer leaflet.
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