Critical and Off-Critical Miscibility Transitions in Model Extracellular and Cytoplasmic Myelin Lipid Monolayers

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Critical and Off-Critical Miscibility Transitions in Model Extracellular and Cytoplasmic Myelin Lipid Monolayers

Y. Min,† T. F. Alig,‡ D. W. Lee,† J. M. Boggs,§* J. N. Israelachvili, † and J. A. Zasadzinski‡

ABSTRACT Monolayers based on the composition of the cytoplasmic (CYT) or extracellular (EXT) sides of the myelin bilayer form coexisting immiscible liquid phases similar to the liquid-ordered/liquid-disordered phases in phospholipid/cholesterol monolayers. Increasing the temperature or surface pressure causes the two liquid phases to mix, although in significantly different fashion for the CYT and EXT monolayers. The cerebroside-rich EXT monolayer is near a critical composition and the phase transition in the cerebroside-free cytoplasmic side occurs abruptly without domain coalescence; hence, the cytoplasmic monolayer is not near a critical composition, although the domains exhibit shape instabilities within 1–2 mN/m of the transition. The change in mixing pressure decreases significantly with temperature for the EXT monolayer, with \( d\Pi_{\text{ext}}/dT \sim 1.5 \text{ mN/m}^\circ\text{C} \), but the mixing pressure of the CYT monolayer varies little with temperature. This is due to the differences in the nonideality of cholesterol interactions with cerebrosides (EXT) relative to phospholipids (CYT). EXT monolayers on the composition of white matter from marmosets with experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis, remain phase-separated at higher surface pressures than control, while EAE CYT monolayers are similar to control. Myelin basic protein, when added to the CYT monolayer, increases lipid miscibility in CYT monolayers; likely done by altering the dipole density difference between the two phases.

INTRODUCTION

The myelin sheath is formed by concentrically wrapped extensions of oligodendrocyte cell membranes that encircle the axons of the central nervous system (1,2). As a result, the sheath consists of repeat units of double bilayers separated by 3–4-nm-thick aqueous gaps. These gaps were originally the cytoplasmic and extracellular spaces of the oligodendrocytes (1). The cytoplasmic (CYT) and extracellular (EXT) monolayers of the myelin bilayer have significantly different lipid and protein distributions, reflective of their origins in the oligodendrocyte (3–5). The cerebrosides reside in what was originally the extracellular side of the oligodendrocyte bilayer, while the cytoplasmic side of the bilayer contains more phosphatidylethanolamine and phosphatidylcholine (see Tables 1 and 2). These differences in composition give rise to different interactions between the different sides of myelin bilayers, which likely are important in forming and maintaining the multilamellar structure of the myelin sheath (5,6). In addition to the lipid asymmetry, myelin basic protein (MBP, 20–30% of total protein by weight) is found only on the cytoplasmic side of the membrane and acts as an intermembrane adhesion protein, creating electrostatic and hydrophobic bridging forces between the negatively charged cytoplasmic membrane surfaces (5,7,8). The strength of these forces depends on a balance between lipid and MBP net charge (5).

The multilamellar myelin sheath forms a capacitor surrounding the axons, which allows for faster and more efficient conduction of nerve impulses than unmyelinated nerves (1,9). Fast nerve transmission requires a low myelin capacitance, which itself requires a low effective dielectric constant that is promoted by the much lower dielectric constant of lipid hydrocarbon chains (\( \varepsilon_{hc} \sim 2 \)) relative to water (\( \varepsilon_w \approx 80 \)). To take full advantage of the lipid dielectric constant, the myelin sheath must remain tightly wrapped (5), which requires a net attraction between the myelin bilayers. Multiple sclerosis (MS) is the most common progressive neurological disorder in young adults and is characterized by the appearance of lesions in the myelin membrane, reflecting loss of bilayer adhesion, swelling across the water gaps and eventual disintegration of the myelin sheath structure (10–12). Disruptions in the myelin sheath increase the capacitance as water replaces lipid and can lead to changes in nerve signal conduction, resulting in the sensory and motor disabilities accompanying MS. In experimental allergic encephalomyelitis (EAE) in the marmoset, an accepted animal model for MS (10), changes occur in the overall myelin lipid composition (14) (Table 1), which can affect the adhesive interactions between myelin membranes (5) and as we show here, also affect the lateral organization of the lipid membrane.

We have used Inouye and Kirshner’s (4) data on the distribution of lipid species between the cytoplasmic and...
extracellular sides of the myelin bilayer to create model myelin monolayers based on the total lipid compositions determined from the white matter of the marmoset (14). In marmosets with EAE, the lipid composition of the white matter changes from that of control marmosets (Table 1) (14). We modeled the EAE extracellular and cytoplasmic monolayers assuming that the lipid distribution between the monolayers was the same as for the control myelin, but with the overall lipid composition reflective of the changes that occurred in EAE (4). Fluorescence microscopy shows that the extracellular and cytoplasmic monolayers have significantly different domain organization and miscibility phase transitions from each other, and from monolayers with the overall lipid composition (6,14–16).

We show for the first time (to our knowledge) that the cerebroside-rich extracellular monolayer in both normal and EAE myelin passes near liquid–liquid miscibility critical point, exhibiting membrane coalescence, stripe formation, and dramatic membrane shape fluctuations near the mixing pressure over the temperature range of 14–37°C. The mixing pressure decreases linearly with temperature from >40 mN/m at 14°C to <10 mN/m at 37°C for the control mixture; for simple phospholipid/cholesterol mixtures or red blood cell lipids the mixing pressure is invariant with temperature (17). This change in temperature dependence is likely due to the more ideal mixing between cerebrosides and cholesterol relative to phospholipids and cholesterol (18). EXT monolayers with the EAE composition remain phase-separated at even higher pressures, 50 mN/m at 20°C to ~25 mN/m at 37°C, also showing critical behavior near the miscibility transition.

The cytoplasmic (CYT) monolayer also shows liquid–liquid immiscibility, but is sufficiently far from a critical composition that the domains remain distinct and separated up to the miscibility transition pressure. The miscibility pressure is insensitive to temperature from 15 to 37°C, ranging from 20 to 30 mN/m, similar to model phospholipid-cholesterol mixtures. Only within 1–2 mN/m of the miscibility transition do the domains undergo polygonal shape instabilities predicted by theory when the electrostatic repulsive forces exceed the forces due to line tension (19). These instabilities suggest that the line tension decreases faster than the dipole density difference as the miscibility transition is approached (20). However, there is little difference in domain morphology or miscibility transitions between CYT monolayers with the EAE and control lipid compositions.

The addition of myelin basic protein to the CYT monolayer induces a concentration-dependent decrease in the miscibility pressure to a minimum value at a certain MBP concentration. Higher MBP concentrations cause the mixing pressure to increase again. This suggests that the protein induces mixing between lipid domains by neutralizing an excess of anionic lipid species located in one or the other phases. Increasing the MBP concentration past the amount needed to neutralize the anionic lipids promotes phase separation, suggesting that an excess of MBP causes charge reversal within the domains, which again promotes phase separation and an increase in repulsion between bilayers (5).

**MATERIALS AND METHODS**

Table 1 shows the (6,14) differences in total lipid composition between normal (control) and EAE myelin in the white matter of the marmoset. Inouye and Kirschner (see their Table V (4)) estimated the lipid fractions associated with the cytoplasmic and extracellular monolayers for rat central nervous system (CNS) myelin (Table 1). We estimated the composition of the cytoplasmic and extracellular monolayers of control and EAE myelin using this same distribution (Table 2), which was taken to be the same for both control and EAE monolayers. To approximate the acyl chain distribution in EXT and CYT compositions, phosphatidylserine (porcine brain, PS), sphingomyelin (porcine brain SM), phosphatidylcholine (porcine brain PC), phosphatidylethanolamine (porcine brain PE), cerebrosides (porcine brain CER), cerebroside sulfatide (porcine brain SCER), and cholesterol (ovine wool), were purchased from Avanti Polar Lipids (Alabaster, AL) and stored in chloroform until used. The predominant fatty acid chain lengths of the PC, PE, and PS are 16:0, 18:0, 18:1, and 20:4. Sodium nitrate, calcium nitrate, and MOPS (morpholine-propanesulfonic

**TABLE 1** Lipid mole fractions in control and EAE marmoset central nervous system (CNS) white matter determined by high-performance liquid chromatography (14)

<table>
<thead>
<tr>
<th>Lipid species</th>
<th>CNS white matter (mol %)</th>
<th>EAE CNS white matter (mol %)</th>
<th>Fraction in EXT monolayer (IR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>33</td>
<td>38</td>
<td>0.67</td>
</tr>
<tr>
<td>Hydroxylated cerebrosides</td>
<td>14</td>
<td>15</td>
<td>1.0</td>
</tr>
<tr>
<td>Nonhydroxylated cerebrosides</td>
<td>2.3</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Cerebroside sulfatide</td>
<td>6.4</td>
<td>3.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>2.8</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>21</td>
<td>15</td>
<td>0.6</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>16</td>
<td>17</td>
<td>0.4</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>3.1</td>
<td>7.1</td>
<td>0.2</td>
</tr>
</tbody>
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Extracellular fraction of each lipid shown in the last column was estimated from Inouye and Kirschner (Table V of Inouye and Kirschner (4)).

**TABLE 2** Lipid mole fractions used for the control and EAE model monolayers determined from the overall lipid composition of marmoset white matter taken from healthy and EAE animals (14)

<table>
<thead>
<tr>
<th>Lipid species</th>
<th>Control CYT</th>
<th>Control EXT</th>
<th>EAE CYT</th>
<th>EAE EXT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>32</td>
<td>33</td>
<td>37</td>
<td>38</td>
</tr>
<tr>
<td>Hydroxylated cerebrosides</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>Nonhydroxylated cerebrosides</td>
<td>0</td>
<td>3.5</td>
<td>0</td>
<td>3.6</td>
</tr>
<tr>
<td>Cerebroside sulfatide</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>5.6</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>6.2</td>
<td>4.2</td>
<td>2.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>26</td>
<td>18</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>29</td>
<td>9.0</td>
<td>33</td>
<td>9.6</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>7.0</td>
<td>1.0</td>
<td>7.4*</td>
<td>6.9*</td>
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The lipid distribution between the monolayers was that suggested in Inouye and Kirschner (4).

*The phosphatidylserine concentration in the EAE monolayers was adjusted to give a similar overall charge density to the EAE and control monolayers.
acid) sodium salt were purchased from Sigma-Aldrich (St. Louis, MO). TR-DHPE (Texas Red 1,2-dihexadecanoyl-sn-glycéro-3-phosphoethanolamine, triethylammonium salt) was purchased from Invitrogen (Carlsbad, CA). Myelin basic protein (MBP) was isolated from bovine brain white matter as previously described (21).

The myelin lipids were mixed in a 11:5:4 (v/v) hexane/chloroform/ethanol solution at 1 mg/ml total lipid. Monolayers were spread dropwise onto a pH 7.2 MOPS buffer (150 mM sodium nitrate/10 mM MOPS sodium salt/2 mM calcium nitrate) in a custom-built, temperature-controlled (±0.2°C) Langmuir trough and compressed and/or expanded with a fixed barrier rate. The surface pressure was measured with a filter paper Wilhelmy plate (Riegler & Kirstein, Potsdam, Germany) with an accuracy of ±1 mN/m. An Optiphot optical microscope (Nikon, Tokyo, Japan) was positioned above the trough with a 40× extra-long working distance objective (Nikon) designed for fluorescent light. Full-length movies and individual frames were recorded directly to computer (Moviestar, Mountain View, CA). After-Effect software (Adobe, San Jose, CA) was used to process the recorded video images and the domain sizes and distributions were analyzed using Image J (http://rsbweb.nih.gov/ij/).

RESULTS AND DISCUSSION

Fig. 1 shows fluorescence images of the CYT and EXT model monolayers based on the control and EAE lipid compositions at 23°C and liftoff (Π = 0 mN/m). There is distinct phase separation into two immiscible phases as shown by the segregation of the fluorescent lipid dye. Based on analogies to simple lipid/cholesterol monolayers (17,22–25), as well as the deformability and fluctuations of the domain shapes (see Figs. 5 and 6, later in article), we ascribed the dark phase to be a cholesterol-rich liquid phase and the bright phase a cholesterol-poor liquid phase (17,22,26). In simpler binary and ternary phospholipid/cholesterol mixtures, the cholesterol-rich phase also contains more of the saturated lipid species (17,22) and is known as the liquid-disordered phase (Ld; also often referred to as the Lo phase).

The basis for phase separation is the preference for cholesterol to intercalate into the ordered (all trans) saturated lipid alkyl chains as opposed to disordered (gauche) or unsaturated chains (27). In phosphatidylcholine/cholesterol mixtures, increasing the cholesterol content leads to an increase in bilayer thickness by reducing the tilt of the phospholipid molecules (27); phase separation occurs to minimize the regions of hydrophobic mismatch present at the Leo-Ld interface. The difference in the chain lengths, l_o and l_d between the Lo and Ld phases, leads to a line tension, λ, in which \( \gamma_o \approx \frac{\Delta m}{2} \gamma_{ad} \), is the interfacial tension of the hydrocarbon-air interface. The difference in composition and packing density between the two phases causes a difference in the average dipole density, \( \Delta m \), which leads to an electrostatic repulsion within the domains and between domains (26). The average areas per molecule in the ordered or disordered phases are \( A_o \) or \( A_d \) (of ~50 Å²), with charges \( \pm Q \) separated by a distance \( \delta_o \) or \( \delta_d \) (of ~0.5 nm), which leads to a dipole density difference:

\[
\Delta m \approx Q \left( \frac{\delta_o}{A_o} - \frac{\delta_d}{A_d} \right).
\]

In both EXT and CYT monolayers, the bright phase was continuous and the dark phase formed discrete circular domains, consistent with simpler lipid mixtures containing <40 mol% cholesterol (23–25) and with red blood cell membranes (17). Both EAE and control EXT monolayers had an area fraction, \( f_{avg} \approx 40\% \) of dark Lo phase, while for both EAE and control CYT monolayers, \( f_{avg} \approx 20\% \). From Table 2, if we assign the hydroxylated and

FIGURE 1 Distributions of domain areas of model monolayers of compositions reflecting (a) control cytoplasmic (CYT), (b) control extracellular (EXT), (c) EAE CYT, and (d) EAE EXT lipid mixtures at liftoff (Π = 0 mN/m). The average domain area and area fraction of the dark, fluorescent dye-excluding domains size was analyzed from five randomly selected frames of movies taken during compression of the film (see also the Supporting Material). The area fraction of the dark domains (f_{avg}) in the EXT monolayers is approximately twice that of the CYT lipid mixtures. In analogy to other lipid/cholesterol mixtures, the dark discrete domains are liquid-ordered (Lo) phase in which the saturated lipids and cholesterol are concentrated. The bright, continuous phase is the liquid-disordered (Ld) phase in which the remaining cholesterol, unsaturated and charged lipids reside.
nonhydroxylated cerebrosides (15), and the saturated fractions of the uncharged phospholipids to the $L_0$ domains, and sphingomyelin, cerebroside sulfatide, and the charged and unsaturated phospholipids to the $L_d$ domains (17,20,22,28,29), we expect to see a higher fraction of $L_0$ phase in the EXT monolayers. The fraction of $L_0$ phase should decrease in the following order: EAE EXT > control EXT > EAE CYT > control CYT, as observed. There is a much greater difference between the CYT and EXT monolayers than between the EAE and control monolayers of either CYT or EXT.

Figs. 2 and 3 show representative fluorescence images of monolayers of the control and EAE compositions of both the outer (EXT) and inner (CYT) sides of the myelin membrane (Table 2) at 23°C (Fig. 2) and 37°C (Fig. 3) as a function of surface pressure, $\Pi$. The dark, $L_0$ domains increased in size and area fraction with increasing surface pressure for all monolayers up to a miscibility transition at which the two phases mix. The miscibility transition pressure decreased with temperature for the EXT monolayers (see Fig. 7 later in article); the dark $L_0$ domains in the EXT monolayer persisted to 40 mN/m (control) or 50 mN/m (EAE) at 23°C (Fig. 2), while the monolayer was homogeneous at 10 mN/m (control) or 20 mN/m (EAE) at 37°C (Fig. 3). For both EAE and control CYT monolayers the liquid-liquid miscibility transition was between 20 and 30 mN/m for all temperatures from 15 to 37°C (see Fig. 6 later in article). The domain shapes and size distributions of the CYT and EXT monolayers are distinctly different under all conditions, with smaller differences between the control and EAE compositions.

The size and shape of the $L_0$ domains is determined by a competition between the line tension, $\lambda$, and the dipole density difference, $\Delta m$, between the $L_0$ and $L_d$ phases (26). The line tension minimizes the domain perimeter per domain area, leading to larger, but fewer, domains. The dipole density difference, on the other hand, leads to a repulsive electrostatic energy that acts to minimize the size of each domain and keeps the domains separated. The domain size distribution is determined by their ratio (19,26), which sets the minimum energy domain radius, $R_m$. 

**FIGURE 2** Fluorescence images of healthy (control) and diseased (EAE), inner (CYT) and outer (EXT) myelin monolayers containing 1 wt % TR-DHPE on a MOPS buffer subphase at $T \approx 20°C$ and pH $\approx 7.2$. All of four model myelin monolayers show a continuous bright $L_d$ phase-separating discrete, dark $L_0$ phase domains. During compression, the two phases become homogeneous at the miscibility pressure, $\Pi_{crit}$ (see Fig. 6). The large stripes present in control EXT monolayers at $\Pi \approx 30$ mN/m indicate proximity to a critical composition (see Fig. 4).

**FIGURE 3** Fluorescence images of control and EAE, inner (CYT) and outer (EXT) myelin monolayers containing 1 wt % TR-DHPE on a MOPS buffer subphase at $T \approx 37°C$ and pH $\approx 7.2$. The EXT monolayers show a dramatic decrease in the miscibility transition pressure as the temperature increases, the EAE monolayers are in a single homogeneous phase for $\Pi = 10$, and the EAE EXT monolayer is in a single phase by $\Pi = 20$ (see Fig. 6). For the control and EAE CYT monolayers, the miscibility transition remains between 20 and 30 mN/m. All of the monolayers collapse by ejecting small bilayer fragments at $\Pi \approx 40$ mN/m.

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The value \( \delta \) is a cutoff distance of \( \approx 0.5 \text{ nm} \), \( e \) is the dielectric constant of water near the interface (40–80), and \( e \) is the exponential, 2.7183. The mean sizes of the domains are larger for the EXT than the CYT monolayers, which implies that \( \lambda/\langle \Delta m \rangle^2 \) is also larger for the EXT monolayer compositions than the CYT compositions (20,25,26,30–32). However, we cannot say whether this is due to an increase in \( \lambda \) or a decrease in \( \langle \Delta m \rangle^2 \).

As the miscibility transition pressure is approached, the differences between the EXT and CYT monolayers are more obvious. For the EXT lipid mixtures at 23°C, Fig. 4 shows that the domains begin to coalesce and change shape at \( \approx 27 \text{ mN/m} \), well below the miscibility transition pressure of 34–37 mN/m (see Movie S1 in the Supporting Material). As the surface pressure increases, the domains undergo a transition from circular to rectangular stripes (33 mN/m) and the domain boundaries grow fuzzy and indistinct at the transition (34 mN/m). Further increases in \( P \) cause the domains to slowly fade from view, suggesting a low diffusivity of the fluorescent lipid and significant pretransitional effects in the homogeneous phase; the remnants of the domains are visible even at \( \approx 40 \text{ mN/m} \) (see Movie S1).

This progression of domain coalescence, stripe formation, and indistinct and fluctuating boundaries is consistent with the EXT lipid mixture being close to a critical composition (17,25,31) over the range of temperature of 14–37°C. To our knowledge, this is the first time cerebrosides have been shown to form a critical mixture with cholesterol.

For the CYT composition, the miscibility transition is much different (Fig. 5, and Movie S2). Even within \( \approx 1 \text{ mN/m} \) of the transition at 23.1 mN/m, the domains are still discrete and circular, with little domain coalescence. Between 23.6 and 23.8 mN/m, the domains begin to change shape (arrows). The larger individual domains undergo a transition from circular to various polygonal shapes with multiple arms (19). For example, the circular domain at the red arrow at 23.6 mN/m transforms into a five-armed star at 23.8 mN/m, before mixing and fading into a homogeneous phase at 24.3 mN/m. The diffusion in the homogeneous phase for the CYT monolayer is much greater than in the near-critical EXT monolayers. The radius \( R_n \) at which isolated circular shapes become unstable with respect to a shape with \( n \)-fold symmetry is predicted to be (19)

\[
R_n = \frac{e^{\lambda \delta}}{8} \exp \left( \frac{4\pi e \lambda}{\langle \Delta m \rangle} \right) = e^{(\lambda - 3)R_o}. \tag{4}
\]
For the transition to elongated, elliptical domains of twofold symmetry, \( Z_2 \approx 10 / 3 \), or \( R_2 \approx 1.4 R_o \). \( Z_n \) and hence, \( R_o \) values increase with \( n \) in a complicated way for higher values of \( n \) (19). Higher-order polygons (\( n = 3, 4, \) and \( 5 \)) are formed from the larger domains in Fig. 5 (arrows). These shape transitions show that these domains, which were stable at lower surface pressures, became unstable at higher surface pressures; hence, \( R_o \) must decrease near the miscibility transition, which implies that \( \lambda \) decreases faster than \( \Delta m \) as the miscibility transition pressure is approached, similar to other phospholipid/cholesterol mixtures (20,31).

In addition to the differences in morphology at the miscibility transition, the temperature dependence of the miscibility pressure is quite different for the EXT and CYT compositions (Fig. 6). Note that
\[
d\Pi_{crit}/dT \sim -1.5 \; \text{mN/m}^{-1} \text{C}
\]

for the both the control and EAE EXT mixtures and the miscibility transition varies from \(-42 \; \text{mN/m} \) at \( 15^\circ \text{C} \) to \(-8 \; \text{mN/m} \) at \( 37^\circ \text{C} \) for the control EXT and \( 50 \; \text{mN/m} \) at \( 20^\circ \text{C} \) to \(-20 \; \text{mN/m} \) at \( 37^\circ \text{C} \). This difference in miscibility pressure is the greatest difference between the control and EAE EXT monolayers. The miscibility transition is essentially temperature-independent (\( d\Pi_{crit}/dT \sim 0 \)) for both the control and EAE CYT mixtures and remains between 20 and 30 mN/m from 15 to \( 37^\circ \text{C} \).

This difference in temperature dependence can be due to the differences in monolayer composition. Keller et al. (17) show that the simplest model for the Gibbs free energy, \( G \), of a multicomponent lipid mixture exhibiting a liquid-liquid critical point is
\[
G = \sum_i (\mu_i + k_B T_i \ln X_i) + \sum_{ij} 2 k_B T_{ij} X_i X_j, \tag{5}
\]

in which \( X_i \) is the mole fraction of component \( i \), \( T_{ij} \) is the critical temperature of an \( i/j \) pair, \( \mu_i \) is the chemical potential of pure component \( i \), \( T \) is temperature, and \( k_B \) is the Boltzmann constant. In this model, the critical temperatures, \( T_{ij} \), depend on the monolayer surface pressure, \( \Pi \), and a contraction parameter, \( \alpha_{ij} \) (17):
\[
T_{ij}(\Pi) = T + \alpha_{ij}(\Pi - \Pi_c(ij))/2k_B. \tag{6}
\]

Here, \( \Pi_c(ij) \) is the critical pressure corresponding to \( T_{ij} \). The contraction parameter is a measure of nonideality; the changes in molecular area due to nonideal mixing of \( i \) and \( j \) is \( \alpha_{ij}X_i X_j \). In this model,
\[
d\Pi_{crit}/dT_{crit} = \alpha_{ij}/k_B.
\]

Experimental values for \( \alpha_{ij} \) for cholesterol-phospholipid pairs are large, of magnitude \(-10 \) to \(-40 \; \text{Å}^2 \) (17), which corresponds to a large effect of surface pressure on monolayer critical temperature, \( d\Pi_{crit}/dT_{crit} \sim -5 \) to \(-10^\circ \text{C}/(\text{mN/m}) \); or a corresponding small effect of temperature on monolayer critical pressure (\( d\Pi_{crit}/dT_{crit} \sim 0 \)). Hence, similar to red blood cell lipids, \( d\Pi_{crit}/dT_{crit} \) is small for the CYT monolayers, which also consist primarily of phospholipids and cholesterol (Table 2). However, cholesterol-saturated cerebrosides interactions are more ideal and the condensation is smaller, of \(-1 \) to \(-5 \; \text{Å}^2 \) (18), so we expect that \( d\Pi_{crit}/dT_{crit} \sim -1^\circ \text{C}/(\text{mN/m}) \) for EXT monolayers, compared to the \(-0.7^\circ \text{C}/(\text{mN/m}) \) we measure for both control and EAE EXT monolayers.

It is difficult to predict over what range of temperature we would expect phase separation once these two monolayers are assembled into an asymmetric myelin bilayer. Demel et al. (33) estimates that the equivalent surface pressure in red blood cell bilayers is \(-30 \; \text{mN/m} \) based on phospholipase activity, marked by the X on Fig. 6. However, Veatch and Keller (23) show phase separation occurs for symmetric

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{miscibility_diagram.png}
\caption{Miscibility pressure versus temperature phase diagrams for the (a) EXT and (b) CYT monolayers. The \( X \) is an estimate of the approximate surface pressure (~30 mN/m) of a bilayer in vivo according to Demel et al. (33). Both control and EAE EXT monolayers should be single phase at 37°C, although EAE EXT monolayers are much closer to phase separation than control EXT monolayers under physiological conditions. On the other hand, both control and EAE CYT monolayers are close to their phase-separation pressure at 37°C and 30 mN/m; the mixing-denixing boundaries for CYT monolayers have less temperature dependence and also show less variation between EAE and control lipid compositions.}
\end{figure}
bilayers made up of monolayers with miscibility transitions <15 mN/m. The coupling between monolayers with different temperature-miscibility pressure relationships (34) likely complicates things further. However, phase separation in bilayers has not been observed in systems in which both monolayers do not show phase separation (23,34).

**Effect of myelin basic protein on CYT monolayer miscibility**

While the miscibility transition of the CYT monolayers is relatively independent of temperature, myelin basic protein (MBP) has a significant, concentration-dependent influence on monolayer mixing (Fig. 7). MBP is a polycationic protein normally located between the opposed cytoplasmic faces of the myelin sheath. Each MBP has ~20 positive charges due to an excess of lysine and arginine residues (6). On adding increasing amounts of MBP to the subphase, the mixing-demixing surface pressure decreases from ~22 mN/m to ~5 mN/m at room temperature (Fig. 7). If electrostatic repulsion (i.e., the dipole density difference) within the phases drives phase separation, we expect that as the MBP neutralizes the charged lipids in the \( L_d \) phase of the monolayer (in which we expect the anionic lipids to reside), there is a decreasing driving force for phase separation. Given the relative charge of the MBP (+20) to PS (−1), the minimum critical surface pressure (\( \Pi_{\text{crit}} \)) might be expected to occur when the mole ratio of MBP/PS (the only anionic lipid in the CYT mixture) is ~0.05. However, the actual minimum \( \Pi_{\text{crit}} \) occurs when the mole ratio of MBP/PS (−) is ~0.12 (PS/MBP ~8), corresponding to a bulk MBP concentration of ~0.023 μg/mL. This may be because the locations of the positive charges on the MBP are constrained by the secondary structure of the protein—all of the positive charges may not be available to bind to the anionic lipids in the monolayer simultaneously.

However, for MBP/PS ratios >0.12, the miscibility pressure increases again, suggesting that there is an increase in the electrostatic interactions, possibly induced by an excess of positively charged MBP relative to PS. The \( L_d \) domains may undergo charge reversal and develop a net positive charge due to the excess MBP. This effect is similar to what we see in the interbilayer forces between opposed cytoplasmic leaflets in the presence of MBP. At a similar overall concentration, a maximum in adhesion was observed (presumably near charge neutrality), where all the positive charges on MBP can be matched with a negatively charged PS on one or the other opposed bilayers (5). The maximum adhesion, like the minimum in miscibility transition, is bounded on either side by decreased adhesion, or increased miscibility pressures, likely due to uncompensated charges on the lipids for low MBP concentrations, or on MBP, for higher concentrations. As far as we are aware, this is the first demonstration of protein-induced miscibility in lipid monolayers and the first indication of the possible effects of charge reversal on the miscibility transition. In addition, it suggests that proteins can inhibit, as well as promote, the formation of raft structures in membranes by suppressing the lipid-lipid interactions that lead to phase separation.

**CONCLUSIONS**

Although previous studies have examined the behavior of complete myelin extracts in monolayers at the air-water interface (15,16), the phase behavior and morphology of monolayers based on the compositions of the extracellular or cytoplasmic leaflets of the myelin bilayer are quite different from each other and from that of the overall myelin composition. The phase separation in both the model EXT and CYT monolayers is consistent with an unequal distribution of cholesterol between a phase likely enriched in saturated lipids, and one enriched in unsaturated and charged lipids in analogy to simpler cholesterol/phospholipid and cholesterol/sphingomyelin mixtures (17,22,26) in monolayers and bilayer vesicles (23–25,31,35–37).

Typically, in monolayers containing a saturated phosphatidylcholine (or sphingomyelin), an unsaturated phosphatidylcholine, and cholesterol, coexisting micron-sized domains of a liquid-ordered (\( L_o \), cholesterol and saturated lipids...
lipid-rich) phase and liquid-disordered ($L_d$, cholesterol and saturated lipid-poor) phase form over a range of temperatures and surface pressures (23). Fluorescent lipids are excluded from the $L_o$ domains in favor of the $L_d$ domains (17,20,22,26), enabling direct visualization with fluorescence microscopy (Figs. 1–5), as is the case here.

As the surface pressure is increased, the two liquid phases mix to form a single homogeneous liquid phase. However, the miscibility pressure and the nature of the transition is quite different for the EXT compared to the CYT monolayers. The inner and outer leaflet compositions of red blood cell membranes also form critical mixtures (20). One reason for this is that, even in the homogeneous phase, the energy associated with lateral compositional fluctuations is low, which can stabilize nanometer scale “rafts” enriched in cholesterol, saturated, long-chain lipids and certain proteins (17,31) even above the miscibility transition. Such membrane rafts may be important to a variety of cell functions.

The miscibility transition of the EXT monolayers is strongly temperature-dependent, consistent with a relatively small condensing effect of cholesterol on cerebrosides (18) compared to phospholipids (17). Over the entire temperature range, the miscibility pressure of monolayers based on EAE EXT is significantly greater than that of control EXT monolayers; at 37°C, the miscibility pressure of EAE EXT monolayers is ~20 mN/m compared to ~8 mN/m for control EXT monolayers. This suggests that phase separation is more likely in the EAE EXT than in the control, which could influence the composition fluctuations in the extracellular monolayer, which in turn could influence bilayer-bilayer interactions and adhesion (31). Differences in the bending rigidity between the phases coupled to variations in the line tension at the domain boundaries can result in one phase bulging relative to the other (31,35). Hence, the EAE outer monolayers may be more prone to forming bulges and delaminating than the control monolayers, which may correlate with the changes observed in the myelin sheath in MS.

On the other hand, CYT monolayers are not near a critical composition; the miscibility transition is abrupt and not accompanied by significant pretransitional effects. Only within ~1 mN/m of the transition do the domains show polygonal instabilities (19) consistent with a decrease in the ratio of line tension to dipole density difference ($\lambda / (\Delta m)^2$) near the transition. The miscibility transition pressure is insensitive to temperature over the range of 15–37°C, similar to simple phospholipid/cholesterol mixtures. This can be explained by the strong nonideality of the cholesterol-saturated phospholipid interaction that causes a strong condensation of the phospholipid area per molecule at the interface in the presence of cholesterol (17). There are minimal differences between CYT monolayers with the composition based on healthy marmoset white matter (control) and those based on the lipid composition corresponding to marmoset white matter with experimental allergic encephalomyelitis (EAE), an accepted animal model for MS (10).

While neither temperature nor the lipid changes accompanying EAE alter the miscibility transition of the CYT monolayers, which range from ~20 to 30 mN/m, MBP significantly modulates phase separations in the CYT monolayers, suppressing the transition from ~23 mN/m to ~5 mN/m with increasing MBP concentration at 23°C. Equally surprising is that the miscibility transition increases again for further increases in the MBP concentration. This suggests that the suppression of the miscibility transition is due to charge neutralization, as the cationic MBP binds to the anionic lipids in the CYT monolayer. The increase in the miscibility transition also suggests that charge reversal might occur for excess MBP, leading to positively charged domains that begin to experience the electrostatic interactions that drive phase separation.

Our previous work has shown that the MBP concentration that promotes the greatest adhesion between CYT monolayers (5) is similar to that which produces the greatest decrease in the miscibility surface pressure; more or less amounts of MBP causes the adhesion to decrease (5). This suggests that rather subtle changes in lipid and protein concentrations may lead to dramatic changes in the organization of the myelin membranes, and such changes may contribute to bilayer-bilayer adhesion which can lead to the alterations in the myelin sheath that contribute to disease. In addition, it suggests that proteins can inhibit, as well as promote, the formation of raft structures in membranes by altering the lipid-lipid interactions that lead to phase separation.

**SUPPORTING MATERIAL**

Two movies are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)00197-4](http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)00197-4).

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13. Reference deleted in proof.


