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Fluorescent Probes Alter Miscibility Phase Boundaries in Ternary Vesicles

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We use ^{2}H NMR to study the effects of probes on the miscibility transition in multilamellar vesicles of di(18:1) phosphatidylcholine (PC; DOPC), chain perdeuterated di(16:0)PC (DPPCd62), and cholesterol both with and without 0.5 mol % of the fluorescent probes DiIC12 and DiOC18. Both probes raise the miscibility transition temperature in dispersions of 1:1 DOPC/DPPCd62 + 30% cholesterol but to differing extents. In membranes containing the popular probe DiIC12, the fraction of DPPCd62 lipids in the liquid disordered phase is increased, and the ordering of that phase is reduced even at low temperatures. All findings are consistent with a probe-induced expansion of the entire miscibility phase boundary. We examine membranes with smaller DiIC12 fractions and find a significant increase in transition temperature for samples with 0.05 mol % DiIC12, demonstrating that trace components can dramatically alter membrane phase behavior.

Coexisting liquid ordered (L_o) and liquid disordered (L_d) phases are observed in a wide variety of three-component lipid membranes.[1,2] In part due to its potential application to lipid rafts,[3] liquid immiscibility has been an active area of research over the past several years.[4−12] Most experiments aimed at characterizing liquid immiscibility in bilayers have relied on the presence of probe molecules.[1,2,4−11] For example, ternary phase diagrams have been produced by monitoring the distribution of a fluorescent probe in giant unilamellar vesicles (GUVs) as a function of temperature and composition.[1] Others have used multiple fluorophores to detect submicron lipid organization by fluorescence resonance energy transfer (FRET),[10,11] or used spin labels in electron spin resonance (ESR) experiments.[9] It is often argued that probes do not alter membranes when used at trace concentrations. Recently, we[2] and others[13,14] have questioned this assumption.

In this Letter, we directly compare the phase behavior of ternary membranes with and without fluorescent probes by ^{2}H NMR. We find that trace quantities of commonly used fluorescent probe molecules dramatically increase miscibility transition temperatures (T_{mix}). In addition, probes alter the fraction and physical properties of coexisting phases, even at temperatures far from the transition. The magnitude of the effect depends on the type and concentration of probe used, and we propose that it is dependent on probe partitioning, as has been previously characterized in three-dimensional liquid mixtures. Perturbations due to the inclusion of probe molecules may explain seemingly conflicting findings from different laboratories,[10,15] and we propose that our results may be biologically relevant. It is appealing to speculate that small changes in biomembrane composition lead to large changes in membrane organization.

^{2}H NMR spectra were acquired for membranes of 1:1 DOPC/DPPCd62 + 30% Chol with varying amounts of the probes DiIC12 and DiOC18 between 10 and 60 °C.[16] ^{2}H NMR spectra of membranes with coexisting liquid phases are a superposition of spectra from DPPCd62 lipids in distinct lipid environments.[12] DPPCd62 acyl chains in L_d phases are more disordered and produce narrower quadrupolar splittings than those in L_o phases. T_{mix} is identified as the highest temperature where three distinct methyl splittings are present.[12] T_{mix} is determined qualitatively by examining ^{2}H NMR powder spectra as a function of temperature (Figure 1) and quantitatively by fitting methyl peaks from dePaked spectra.[17]

We measure a significant (+6 °C) shift in T_{mix} for membranes with 0.5 mol % of the popular probe DiIC12. Coexisting phases are easily visualized in GUVs by fluorescence microscopy at this probe concentration (Figure 2A). The increase in transition temperature due to probes depends on probe type. A larger temperature shift is found in membranes with 0.5% DiIC12 (6 °C) than in membranes containing 0.5% DiOC18 (4 °C). At 15 °C, the short chain DiIC12 probe strongly partitions into the L_d phase (Figure 2A), whereas the longer chain DiOC18 probe partitions more equally between phases (Figure 2B), although probe partition coefficients are temperature dependent (data not shown).

The presence of probes also alters the ordering and quantity of coexisting phases (Figure 3). In membranes containing 0.5 mol % DiIC12, the L_d phase is more disordered, and more DPPCd62 is found in an L_d state over a wide range of temperatures. Interestingly, the L_o phase is also more ordered in membranes with DiIC12, even though the probe lipid partitions almost completely away from this phase (Figure 2A). These results suggest that probes alter the entire phase boundary, not simply the character of a single phase. Indeed, we find that

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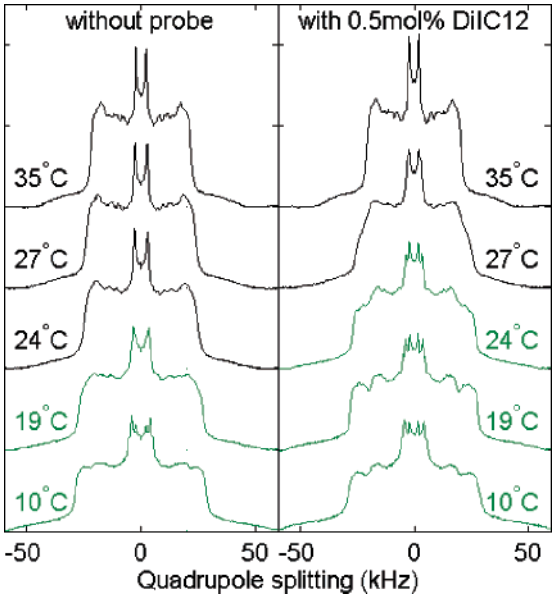

Figure 1. Representative ^{2}H NMR spectra for membranes of 1:1 DOPC/DPPCd62 + 30% cholesterol (left) without and (right) with 0.5 mol % of the fluorescent probe DiIC12. Spectra of membranes with coexisting phases are drawn in green.

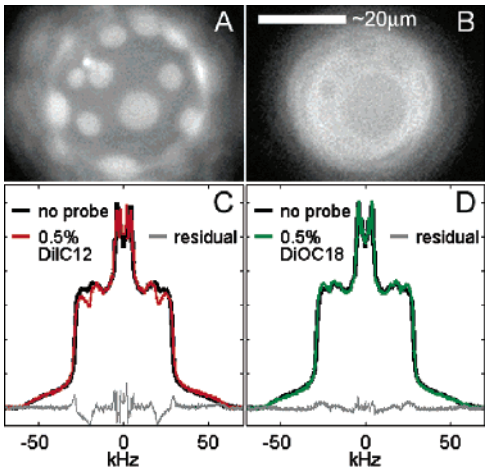

Figure 2. (A and B) Phase separation is observed at 15 °C in GUVs of 1:1 DOPC/DPPC + 30% Chol doped with 0.5 mol % (A) DiIC12 or (B) DiOC18 probes by fluorescence microscopy.[18] The probe DiIC12 partitions strongly into the L_d phase, whereas DiOC18 partitions roughly equally between phases at this temperature. (C and D) ^{2}H NMR spectra differ in membranes with and without probe at 13 °C. Larger perturbations are observed in membranes with DiIC12 than in membranes with DiOC18.

membranes containing 0.5% DiIC12 are much closer to a critical point at T_{mix} than membranes without a probe. In membranes with 0.5% DiIC12, we find that DPPCd62 partitions nearly equally between phases just below T_{mix} (Figure 3C), and we observe broadened spectra above T_{mix} (Figure 1). Spectral broadening of ^{2}H resonances occurs near known critical points in DOPC/DPPCd62/Chol membranes and is likely a consequence of critical fluctuations (manuscript in preparation). In 3D systems, impurities have been used to modulate the location of critical points.[19]

We also examined how T_{mix} varies with DiIC12 concentration (Figure 4). Remarkably, we detect modulated spectra and an increased T_{mix} value in membranes with as little as 0.01% fluorescent probe (1:10 000 probe/lipids) and find a significant shift in T_{mix} for membranes with 0.05% probe (1:2000 probe/lipid). In addition, the shift in T_{mix} due to probes in these

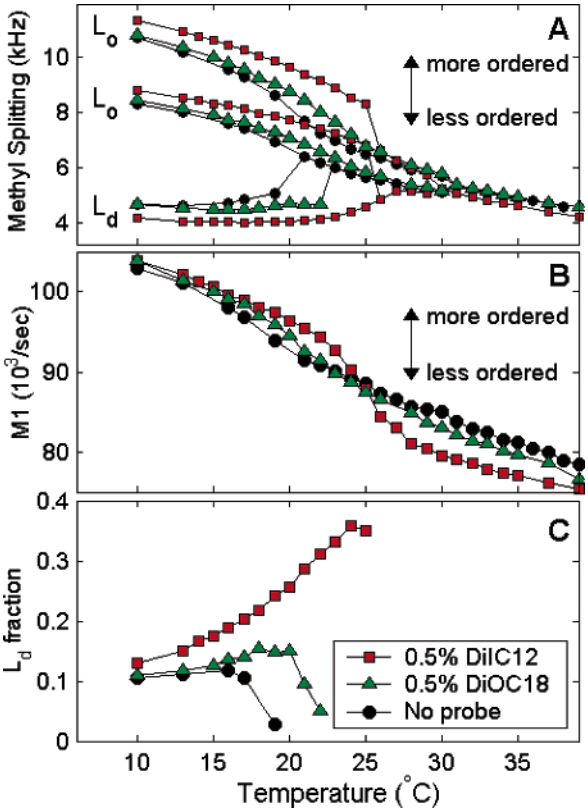

Figure 3. DPPCd62 methyl group ordering, first moment, and partitioning are altered by the addition of fluorescent probes. (A) The DPPCd62 methyl group splits into three distinct signals when L_d and L_o phases coexist.[12] Membranes with probes have a more disordered L_d phase and a more ordered L_o phase. (B) The average quadrupolar splitting, M1, is altered in the presence of probes. M1 is calculated as described previously.[12] (C) The fractional intensity of the narrow (L_d) methyl peak gives a lower limit on the fraction of DPPCd62 lipids in the L_d phase.[20] More DPPCd62 lipids are in the L_d phase in membranes with DiIC12 than in membranes without probes.

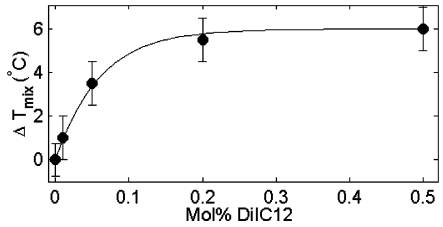

Figure 4. The shift in the miscibility transition temperature of 1:1 DOPC/DPPCd62 + 30% Chol varies with the concentration of DiIC12. A significant shift in transition temperature is found for membranes containing 0.05% DiIC12.

membranes is saturable. This is consistent with our previous observation that transition temperatures do not differ in GUVs with 0.2−2% of the fluorescent probe Texas Red DPPE when viewed by fluorescence microscopy.[12]

While it is surprising that trace concentrations of probe molecules can have a dramatic effect on T_{mix} in bilayers, sensitivity to impurities has been well characterized in three-dimensional liquid mixtures.[21−23] For example, transition temperatures are altered in hexane−nitrobenzene binary mixtures in the presence of <0.05% of a range of impurities.[23] In addition, the magnitude and sign of transition temperature shift depends on the partitioning of the impurity, with impurities that partition strongly into one phase leading to increased transition temperatures. The effects of impurities on the miscibility transition in

3D systems is attributed to a modulation of the interfacial energy between phases.[22]

Our observation that the miscibility transition is highly sensitive to the presence of impurities may have biological relevance. In biological processes, trace quantities of specific target molecules can initiate signaling. Here, we describe a mechanism by which small perturbations can lead to large changes in membrane structure. In model systems, it has been demonstrated that changes in the structure of minor components can have a large impact on lipid organization (e.g., cross-linking of GM1[6] or oxidation of phospholipids[24]). In this study, we are unable to directly demonstrate that the observed effect on T_{mix} is due to the probes themselves or due to indirect effects caused by probe inclusion (e.g., increased lipid oxidation in the presence of fluorophores).

In conclusion, we find that the fluorescent probes DiIC12 and DiOC18 raise miscibility transition temperatures in membranes of 1:1 DOPC/DPPCd62 + 30% cholesterol when viewed using ^{2}H NMR. Probes alter the composition and physical properties of coexisting phases, and low probe concentrations (e.g., 1:2000 probe/lipid) lead to a significant increase in the miscibility transition temperature. We conclude that the miscibility transition in lipid membranes is exquisitely sensitive to the presence of trace components, a property that may be utilized in biological processes.

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