Supplement 2 - Sample preparation, equilibration, and hydration considerations

Supplementary information for:

Liquid-liquid domains in bilayers detected by wide angle x-ray scattering

Thalia T. Mills, Stephanie Tristram-Nagle, Frederick A. Heberle, Nelson F. Morales, Jiang Zhao, Jing Wu, Gilman E. S. Toombes, John F. Nagle, and Gerald W. Feigenson

Because hydration and equilibration are frequently slow in lipid systems, especially those containing a solid phase, observed phase behavior can depend on sample preparation methods. Thus, when comparing our x-ray data on oriented multilayers (WAXS experiments) and MLVs (lamellar repeat experiments) with published data from MLVs (NMR) and GUVs (fluorescence microscopy), it is important to consider differences in sample preparation, hydration, and equilibration time in addition to differences in sensitivity for each technique. Although supported single bilayer systems have different boundary conditions than MLVs (1), our oriented multilayer stacks contain ~1800 bilayers, and so substrate interactions are not a major consideration. Preparation of oriented samples involves deposition of lipid from organic solvent, and following the evaporation of solvent the lipids are completely dehydrated. For samples with high cholesterol concentrations, sample preparation methods involving film deposition can lead to cholesterol crystals below the true cholesterol solubility limit (2). Also, demixing of lipid components in the dry film during preparation of GUVs has been suggested (3) to affect Ld/Lo phase boundaries as determined by fluorescence microscopy in DOPC/DPPC/cholesterol samples with much lower cholesterol concentrations. However, for our x-ray samples, we observed no diffraction characteristic of cholesterol crystallites. Furthermore, all samples were annealed in a hydrated state above the apparent demixing temperature for a period much longer than the observed rates of phase separation and coalescence. Thus, any phase separation induced during film deposition should have been able to re-equilibrate.

Finally, the degree of hydration can dramatically affect phase behavior. In recent x-ray studies of DPPC/cholesterol oriented samples (4,5), experiments at 98% RH (4) revealed a new phase (and novel phase diagram) that was not observed in a subsequent study by the same group conducted at higher hydration (5). Thus, two recent reports of double lamellar repeat spacings for ternary DOPC/DPPC/cholesterol mixtures (5,6) may have been affected by partial hydration, as in both cases, the reported *D* spacings are significantly smaller than the values we obtained (see Table S2.1).

For our experiments on ternary mixtures, two lamellar repeat spacings are present in both oriented samples and MLV samples prepared in a large excess of water. (Note that the small *D* values obtained for MLVs in (6) indicate that hydration and equilibration are issues even for MLVs in excess water.) Furthermore, all compositions showed only a single *D* spacing at 45˚C and the appearance of two lamellar repeat spacings at lower temperatures was independent of the thermal history of the sample. Thus, our observed double lamellar repeat spacings are unlikely to reflect incomplete sample hydration. Although the effect of hydration on liquid-liquid coexistence in ternary mixtures containing cholesterol has not been reported, severely dehydrated samples should be avoided. In dehydrated samples, the system can no longer be considered

pseudo-ternary and the Gibbs phase rule should then explicitly count water as a fourth component.

Table S2.1. Comparison of lamellar repeat (*D*) spacings for MLVs in excess water with some recent literature results.

Mixture	Our result *D* (Å)	Literature *D* (Å)
1:1 DOPC/DPPC + 30% Chol (15˚C)	65.3 Å and 68.5 Å	56.4 Å and 61.3 Å for 1:1:1 DOPC/DPPC/Chol at 10˚C*
1:1 DOPC/DPPC + 20% Chol (20˚C)	69.7 Å and 64.0 Å	54.4 Å and 62.1 Å†
1:1 DOPC/DPPC (25˚C)	63.2 Å (single *D*)	52.5 Å and 61.1 Å for the same mixture at 30˚C†

*Oriented samples at nominal 100% RH conditions (5). The sample is similar in composition and temperature to our mixture.
†MLVs with 4:1 buffer:lipid weight ratio (6).

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