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Phase diagram of a 4-component lipid mixture: DSPC/DOPC/POPC/chol

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ABSTRACT

We report the first 4-component phase diagram for the lipid bilayer mixture, DSPC/DOPC/POPC/chol (distearoylphosphatidylcholine/dioleoylphosphatidylcholine/1-palmitoyl, 2-oleoylphosphatidylcholine/cholesterol). This phase diagram, which has macroscopic Ld + Lo phase domains, clearly shows that all phase boundaries determined for the 3-component mixture containing DOPC transition smoothly into the boundaries for the 3-component mixture containing POPC, which has nanoscopic phase domains of Ld + Lo. Our studies start from two published ternary phase diagrams, and show how these can be combined into a quaternary phase diagram by study of a few hundred samples of intermediate compositions.

1. Introduction

Phase diagrams of chemically well-defined lipid mixtures help to clarify the behavior of biological membranes. In particular, mixtures of a high-melting phosphatidylcholine or sphingomyelin, a low-melting PC, and cholesterol have proven to be especially useful. These 3-component mixtures show coexisting bilayer phases over much of the composition space. A standard format for showing the phase behavior of all possible combinations of a 3-component mixture is the triangular phase diagram, or "Gibbs Triangle". The particular phase behavior where liquid-disordered (Ld) and liquid-ordered (Lo) phases coexist has been termed the "raft region", and might provide a useful model for understanding behaviors of the outer leaflet of animal cell plasma membranes.

Lipid compositional phase diagrams describe the occurrence and location in composition space of phase types (e.g., solid gel Lβ, liquid-ordered Lo, and liquid-disordered Ld) and their coexistence regions at equilibrium. For 3-component lipid mixtures, many fluorescence imaging-based studies have focused on either DOPC or else diphytanoyl-PC as the low-melting lipid, because the region of coexisting Ld + Lo phases shows easily identifiable macroscopic phase domains in giant unilamellar vesicles (GUVs), whether the high-melting lipid is a SM, DPPC, or DSPC [1–4]. In contrast, Ld + Lo phase domains are not visible with standard light microscopy when (i) the low-melting lipid component has one saturated acyl chain and one monounsaturated chain, e.g., POPC [3,5] or SOPC [5]; or (ii) the low-melting lipid is DLPC [6, M. Doktorova unpublished results]. In this case, domains can be detected by some spectroscopic methods, for example, by fluorescent or spin-label probes that partition between the domains [7,8], or by neutron scattering without probes [9]. In brief, methods sensitive to submicron length scales consistently imply liquid phase heterogeneity in POPC- and SOPC-containing ternary mixtures [7–12]. The size scale of phase domains found with POPC or SOPC is therefore below the optical diffraction limit, or "nanoscopic". Such small phase domains might be a good model for the outer leaflet of animal cell plasma membrane, which also seems to have nanoscopic coexisting Ld + Lo phase domains [13–15]: Liquid–liquid phase separation has been detected in vivo in cell membranes using techniques including FRET [16], FRAP [17], ESR [18], and super resolution optical methods [19–21].

Abbreviations: DLPC, 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-Distearoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SOPC, 1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PC, Phosphatidylcholine; SM, Sphingomyelin; DHE, Ergosta-5,7,9(11),22-tetraen-3β-ol; BoDIPY-PC, 2-(4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; C12:0-DiI, 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; C20:0-DiI, 1,1′-dieicosanyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; TOE, Trp-Oleoyl Ester, N-oleoyl-dl-tryptophan ethyl ester; Chol, Cholesterol; TLC, Thin-layer chromatography; GUV, Giant unilamellar vesicle; RSE, Rapid solvent exchange; FRET, Förster resonance energy transfer; SAE, Sensitized acceptor emission; RRE, Region of reduced efficiency; REE, Region of enhanced efficiency; RHS, Right hand side, refers to right side of phase diagram, i.e.,, at higher χ_{DSPC}; LHS, Left hand side refers to left side of phase diagram, i.e. at lower χ_{DSPC}; T1–T6, Trajectories 1–6. bSM, sphingomyelin derived from porcine brain

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In cells, the range of size scales of any phase domains, as well as domain shape and connectivity, are not known. Furthermore, it is quite possible that membranes of living cells use spatial organization on multiple length scales, rather than one special size. We would benefit from chemically simple model systems that also have phase coexistence that spans the range from nanoscopic to macroscopic, thus enabling study of a range of domain sizes [22]. Here we explore the complexity of such a lipid mixture. We find that four components are the minimum to exhibit the range of size scales of coexisting phase domains from nanometers to microns as a function of mixture lipid composition. Unexpectedly, we discovered that the transition from small to large Ld + Lo domains is not at all linear, but instead passes through a compositional range in which phases show a variety of shapes and sizes [23,24]. The compositional location of this range might be significant, and by solving the entire quaternary mixture phase behavior, we are able to place this region of "modulated phases" in composition space.

A challenge for constructing a phase diagram is to determine phase boundaries with sufficient precision to establish (i) every phase region; (ii) whether any stoichiometric compositions appear; (iii) whether any phase boundaries intersect the binary axes; and (iv) comparisons of phase diagrams when, for example, lipid chain lengths differ. Such precision requires examination of a large number of samples. For example, with 20 samples binary mixtures can yield ~+/−5 mol% compositional resolution. This much uncertainty would be acceptable for certain studies, but not in cases where the uncertainty is comparable to the compositional range of interest. Ternary mixtures would require approximately 400 samples of different compositions to achieve +/−5 mol% resolution, and quaternary mixtures would require nearly 8000 samples to evenly cover the entire composition space. A second problem is that the location of phase boundaries is not always well marked by changes in fluorescence or other phase-sensitive measurements. In order to solve this problem, we introduce here a new method to locate phase boundaries with higher accuracy. Our studies, at 2 mol% compositional resolution for most boundaries, start from two published ternary phase diagrams [4,7], and show how these can be combined into a quaternary phase diagram by study of just a few hundred samples of intermediate compositions.

We note that a different view of the DSPC/POPC/chol mixture is that no Ld + Lo "nanodomain phase separation" occurs, and instead, fluctuations in the vicinity of a critical point give rise to transient changes of bilayer physical properties [25–27]. As we make clear below, the findings described here of continuous phase boundaries between DSPC/DOPC/chol and DSPC/POPC/chol over the entire composition space, support the view that Ld + Lo phase coexistence occurs whether phase domains are small or large, with all regions throughout the tetrahedron in accord with the Gibbs Phase Rule.

2. Materials and methods

2.1. Materials

Phospholipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL), and cholesterol from Nu-Chek Prep, Inc. (Elysian, MN). Fluorescent dyes C20:0-DiI, C12:0-DiI, and BoDIPY-PC were from Invitrogen Corporation (Carlsbad, CA), DHE was from Sigma-Aldrich (St. Louis, MO), and TOE (tryptophan oleoyl ester) was a gift from Erwin London. Phospholipid stocks were quantitated by phosphate assay, and purity verified to be >99% by thin-layer chromatography (TLC) of ~20 μg of lipid on washed and activated Adsorbosil TLC plates (Alltech Associates Inc., Deerfield, IL), developed in a solvent system chloroform/methanol/water (65/25/4). Cholesterol stocks were prepared analytically and purity checked with TLC in petroleum ether/diethyl ether/chloroform (7/3/3). Fluorescent dyes were checked for purity with the following solvent systems: BoDIPY-PC in chloroform/methanol/water (65/25/4); C12:0-DiI in chloroform/methanol (10/1); C20:0-DiI in petroleum ether/diethyl ether/chloroform (7/3/3); and TOE in hexane/ethyl acetate (3/1). Concentrations of fluorescent dyes were measured by absorption spectroscopy using an HP 8452A spectrophotometer (Hewlett-Packard Company, Palo Alto, CA).

2.2. Terminology

Whereas at constant temperature the ternary mixture phase behavior is conveniently represented in the plane of a Gibbs Triangle, the quaternary mixture phase behavior is best shown in the volume of a tetrahedron. Our construction of the quaternary phase diagram begins with the two ternary mixtures DSPC/DOPC/chol [4] and DSPC/POPC/chol [7] that form two triangular faces of the tetrahedron for DSPC/DOPC/POPC/chol. The tetrahedral phase diagram can be thought of as the locus of all phase observations traveling through composition space from one ternary mixture to the other, replacing POPC by DOPC. We find it convenient to refer to compositions within the tetrahedron between DOPC-containing and POPC-containing triangular faces in terms of the fractional replacement of POPC by DOPC, $\rho \equiv [DOPC] / ([DOPC] + [POPC])$. Thus, the POPC-containing face of the tetrahedron has $\rho = 0$, whereas the DOPC-containing face has $\rho = 1$.

We use the term "trajectory" to mean a series of samples along a specified path of compositions. Trajectories are used to examine the compositional dependence of a measurement such as fluorescence; in this study, trajectories were used to find the dependence of phase boundaries on ρ, that is, to connect the 3-component phase diagrams previously determined at $\rho = 0$ and 1 [4,7]. Concentrations of mixture components are specified as a mole fraction (e.g., χ_{DSPC}). We note that the mixtures studied here should be considered pseudo-quaternary because we neglect all components of the aqueous buffer. Most important, we neglect water on the basis that its chemical potential is constant at every lipid composition examined because of the presence of excess water. Even so, the water composition of each phase varies over the phase diagram, and we do not measure its fraction in any of the phases.

Fluorescent dyes can provide a unique signal of their local environment, for example, by a change in wavelength or intensity in different phase environments. Here we make use of changes in FRET as donor and acceptor dyes partition between coexisting phases. When dyes concentrate within the same phase, their average separation distance decreases, resulting in a compositional region of enhanced FRET efficiency (REE). When dyes prefer different phases, the increase in average dye separation distance leads to a region of reduced FRET efficiency (RRE). We used the donor/acceptor FRET dye pair DHE/BoDIPY-PC (which partition into Lo and Ld phase, respectively) to generate RREs when Ld and Lo phases coexist. In a second type of experiment, described below in more detail, we used the FRET pair TOE/BoDIPY-PC (both of which partition strongly into the Ld phase) to generate REEs, but with an additional dye, C12:0-DiI, added to the energy transfer chain. C12:0-DiI also partitions strongly into the Ld phase, and accepts energy selectively from BoDIPY-PC. This "3-dye method" can be especially sensitive, in at least some regions of the phase diagram, to the formation of a small fraction of Ld phase.

2.3. FRET

We used FRET to find many of the phase boundaries in the quaternary phase diagram. All samples for FRET measurement were prepared by use of rapid solvent exchange (RSE) to minimize cholesterol de-mixing [28]. Lipids and dyes were dispensed into glass tubes with a 25 μL Hamilton syringe attached to a repeating dispenser (Hamilton USA, Reno, NV). RSE buffer (0.500 mL, 200 mM KCl, 5 mM PIPES, 1 mM EDTA, pH 7.0) was added to the chloroform solution of lipids and dyes. Samples were vortexed while vacuum pumping for 1 min, sealed under argon and placed in a water bath at 60 °C. Samples were cooled at 2 °C/h to 23 °C and equilibrated at room temperature for 48 h before measurement.