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Phase diagram of a polyunsaturated lipid mixture: Brain sphingomyelin/1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine/cholesterol

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ABSTRACT

Phospholipids having a polyunsaturated acyl chain are abundant in biological membranes, but their behavior in lipid mixtures is difficult to study. Here we elucidate the nature of such mixtures with this report of the first ternary phase diagram containing the polyunsaturated lipid SDPC in mixtures of BSM/SDPC/Chol (brain sphingomyelin/1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine/cholesterol). These mixtures show coexisting macroscopic liquid-disordered (Ld) and liquid-ordered (Lo) phase separation, with phase boundaries determined by FRET and by fluorescence microscopy imaging of giant unilamellar vesicles (GUVs). Surprisingly, SDPC mixes with BSM/Chol similarly to how DOPC and POPC mix with BSM/Chol. Notably, intermediate states are produced within the Ld + Lo liquid–liquid immiscibility region upon addition of fourth component POPC. These mixtures of BSM/SDPC/POPC/Chol exhibit nanoscopic Ld + Lo domains over a very large volume of composition space, possibly because Ld/Lo line tension is not high.

1. Introduction

Eukaryotic plasma membranes comprise a complex chemical mixture of proteins and lipids [1]. These membranes are both protective barriers, and also participate in a variety of cellular processes including signaling, protein sorting, endocytosis, and virus entry and exit. The thermodynamics of mixing provides a powerful framework for describing such complex mixtures.

The mixtures studied here are ternary lipid bilayer mixtures containing cholesterol and exhibiting phase separation. They can be grouped into two categories [2]: Type II diagrams exhibit macroscopic regions of Ld + Lo and Ld + Lβ + Lo immiscibility, with domains resolved under the light microscope. Type I mixtures seem to have the same phase coexistence regions, but with nanoscopic domains [3]. These systems have liquid–liquid immiscibility regions that might resemble phase separation in real cells [4–9] where domains have been proposed to be in the nanoscopic range between 20 and 200 nm [8,9]. Nanoscopic domains are detected in vivo in plasma membranes by methods that are sensitive to small length scales, such as Forster resonance energy transfer (FRET) [10,11], fluorescence recovery after photobleaching (FRAP) [12], electron spin resonance (ESR) [13], and stimulated emission depletion (STED) far-field fluorescence nanoscopy [14]. Nanodomains that have Ld and Lo characteristics have been detected with FRET [10] and ESR [12] in live cells. Thus, there is experimental evidence that lipid raft domains, enriched in cholesterol and sphingolipids, together with non-raft domains, are present in plasma membranes [12,15–20].

To date, a number of lipid mixture phase diagrams have been solved [21–29], but none of these lipid mixtures have included polyunsaturated lipids, in large part because of the experimental difficulties in working with polyunsaturated fatty acids (PUFAs). In this study we solve the phase diagram of a ternary mixture containing a PUFA-containing lipid together with two other biologically abundant species, BSM/SDPC/Chol. We also discuss overcoming experimental difficulties in with working with PUFAs.

The predominant high-Tm components of the mammalian plasma membrane (PM) are sphingomyelins (SM). We chose to study the natural sphingomyelin derived from porcine brain, BSM. Cholesterol, present at 35–45 mol% in mammalian plasma membranes [30], is an indispensable component of any plasma membrane model mixture. SDPC contains a stearoyl chain and the omega-3 polyunsaturated fatty acyl chain docosahexaenoic acid (DHA), with 22-carbons and 6 double bonds [31,32].

Abbreviations: BSM, sphingomyelin derived from porcine brain; SM, sphingomyelin; PC, phosphatidylcholine; SDPC, 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; Chol, cholesterol; Ld, liquid-disordered phase; Lo, liquid-ordered phase; 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; FRET, Förster resonance energy transfer; DHE, ergosta-5,7,9(11),22-tetraen-3β-ol; C12:0 DiI, 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; BoDIPY-PC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine; C20:0-DiI, 1,1′-dieicosanyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; TOE, Trp-Oleoyl Ester, N-oleoyl-dl-tryptophan ethyl ester; TLC, thin-layer chromatography; GUV, giant unilamellar vesicle; RSE, rapid solvent exchange; 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_{BSM}; LHS, left hand side, refers to left side of phase diagram, i.e. at lower χ_{BSM}; T1–T2, trajectories 1–2.

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In the inner leaflet of some animal cell plasma membranes PUFAs comprise up to ~50% of sn-2 fatty acyl chains (http://lipidlibrary.aocs.org/Lipids/complex.html; [19]). In retinal rod outer segment disk membranes DHA comprises ~50% of the total acyl chains, with this high percentage required for optimal rhodopsin function [33,34]. DHA is also found at high concentrations in certain other membranes, including synaptosomes [35] and sperm [36]. The importance of DHA and PUFAs for human health has been well-studied [37–39]. Spectroscopic, computational, and other biophysical methods [40–54] have established significant PUFA effects on membrane properties. PUFAs seem to have a weaker interaction with cholesterol compared with saturated or monounsaturated acyl chains [55]. A relatively low solubility of cholesterol in PUFA-containing membranes was measured using both X-ray diffraction and solid-state ^{2}H NMR [56–64]. It has been proposed [57–59] that DHA could be directly involved in inducing lateral phase separations into DHA-rich/cholesterol-poor and DHA-poor/cholesterol-rich lipid domains.

Despite the abundance and importance of DHA-containing lipids such as SDPC, SDPE (1-stearoyl-2-docosahexaenoyl-sn-glycero-3- phosphatidylethanolamine), PDPC (1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine), and PDPE (1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphatidylethanolamine), very few different PUFA-containing lipid compositions have been examined. The vast majority of studies conducted on DHA-containing lipids have focused on only a few sample compositions, most commonly 1/1/1 = DHA-containing lipid/SM/Chol [52,53–65]. Here, we show that mixing behavior over all possible compositions of these three-component mixtures, including the key regions of immiscibility, can be described by use of a triangular phase diagram.

Since 1999, GUVs have proven to be useful tools for the visualization of bilayer phases and have been widely used to study phase behavior in model membranes [21–25,66–68]. However, GUVs have their own characteristic limitations. One such limitation is the relatively low compositional resolution of ~5% that can be achieved with this method (but see [69] for higher compositional precision). Also, GUVs are not useful for finding phase boundaries of mixtures with coexisting domains that are much smaller than the wavelength of light, due to the limits of optical resolution. Other GUV limitations are the potential for electrolysis-induced artifacts associated with the electroswelling method, and oxygen- and free radical-induced artifacts [67,68]. These particular problems can be mitigated by forming GUVs by use of "gentle hydration" [9]. Cuvette-based spectroscopic methods such as FRET can be used to establish phase boundaries with few artifacts and with the higher compositional resolution of ~2%, but a large number of samples is required. FRET experiments are cuvette-based, enabling sample preparation by use of rapid solvent exchange (RSE), which minimizes potential lipid demixing, especially of cholesterol, that can occur when lipid mixtures are dried [70]. RSE also has the benefit of convenient maintenance of an inert atmosphere at all stages of sample preparation, minimizing oxygen-induced artifacts that are a significant problem in mixtures with PUFA-containing lipids.

Biological membrane phase behavior can be modeled rather well by mixtures of four lipids, enabling study of Ld + Lo domain size from a few nanometers to many microns, controlled by composition. Why do we care about the switch of domain size from nano to macro? The tens of nanometer scale seems to describe the phase-separated domains in animal cell plasma membranes better than does the micron scale [71–73]. Silvius [74] proposed the presence of nanodomains in a lipid bilayer with compositions mimicking the outer leaflet bilayer. Lipids such as SOPC or POPC, having one saturated sn-1 chain and one monounsaturated sn-2 chain, naturally occur as the most abundant phospholipid species in animal cell membranes [19]. Optical microscopy studies on such lipids in ternary mixtures with cholesterol and a high-melting lipid show uniform membranes [21–75], yet other methods indicate the presence of lateral heterogeneity [27,74,75] invisible to optical microscopy imaging. We have previously reported the presence of intermediate states that have so-called modulated phase morphology in the 4-component mixture DSPC/DOPC/POPC/Chol [28,77,78]. Modulated phase morphology occurs when two liquid phases coexist, if the line tension that drives the minimization of domain perimeter is opposed by a long-range interaction such as bending energy of a curved membrane that acts to break up domains [78,79].

We report that the phase diagram of BSM/SDPC/Chol at constant temperature is remarkably similar to phase diagrams having DOPC or POPC instead of SDPC. We also report the existence of modulated phases upon the addition of POPC as a fourth component to BSM/SDPC/Chol, finding that with SDPC-containing mixtures, only a small fraction of POPC results in nanodomains, compared to the much larger fractions of POPC required for nanodomains in DOPC-containing mixtures.

2. Materials and methods

2.1. Materials

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL), and cholesterol from Nu Chek Prep (Elysian, MN). Fluorescent dyes C12:0 DiI and BoDIPY-PC were from Invitrogen (Carlsbad, CA), DHE was from Sigma-Aldrich (St. Louis, MO). Lipid stock solutions were prepared in HPLC grade chloroform with lipid concentration determined to better than 1% precision by inorganic phosphate assay [79]. BSM stocks included 0.5% methanol by volume to prevent precipitation during humid weather. Phospholipid purity was verified to be >99% by thin-layer chromatography (TLC) of ~20 μg of lipid on washed and activated Adsorbosil TLC plates (Alltech, Deerfield, IL), developed in the solvent system chloroform/methanol/water = 65/25/4. SDPC stocks were frequently checked for purity using a spectroscopic absorbance method (see Section 2.2 for more details). Cholesterol stocks were prepared analytically and purity checked with TLC in petroleum ether/diethyl ether/chloroform = 7/3/3. Probe extinction coefficients were obtained from lot certificates of analysis: 91,800 M^{-1} cm^{-1} at 504 nm for BoDIPY-PC and 12,900 M^{-1} cm^{-1} at 324 nm for DHE. 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. Concentrations of fluorescent dyes were measured by absorption spectroscopy using an HP 8452A spectrophotometer (Hewlett-Packard, Palo Alto, CA).

2.2. Control for PUFA lipid breakdown

PUFA breakdown is readily initiated by free-radical formation and subsequent propagation of a chain-reaction that proceeds autocatalytically [81–84]. Therefore, it is crucial to eliminate or greatly minimize factors such as oxygen, multivalent cations especially iron, and light, which initiate or propagate this breakdown pathway [81–85]. Our experimental protocol produces less than 2% breakdown after all experimental steps, as measured by TLC and a UV spectroscopic assay for lipid breakdown. All physical handling of lipids is performed in an inert atmosphere under low light conditions. All solvents and buffers that come into contact with SDPC were deoxygenated by bubbling with inert gas to carry away dissolved oxygen. A low oxygen atmosphere was achieved by handling SDPC inside a glove box with O_2 levels reduced from the atmospheric reading of 18.2% down to the glove box reading of ~0.02%, as measured by an oxygen sensor (OXY-sen oxygen monitor, Alpha Omega Instruments). Minimization of iron ions was achieved by the addition of 50 μm DTPA, efficient in chelating iron [85], to all aqueous solutions in contact with SDPC. SDPC was purchased from Avanti packaged into small aliquots of 2.5 mg per vial, enabling use of a fresh vial for every week of experimental work. Purity of lipid stocks was checked by TLC and UV spectroscopic assay for lipid breakdown. Samples for UV spectroscopic assay were prepared by extracting aqueous lipid suspensions from GUVs using Bligh-Dyer conditions [86]. A mixture of cyclohexane/EtOH = 10/1 was used to re-dissolve the dry