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New method for determining tie-lines in coexisting membrane phases using spin-label ESR

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Abstract

A full description of coexisting phases includes their respective compositions, which are provided by the thermodynamic tie-lines. Fluorescence microscopy enables visualization of coexisting lipid phases in giant unilamellar phases, but the composition information is missing. For cholesterol-containing lipid mixtures, knowledge of the compositions of the coexisting phases is important for understanding the nature of "membrane rafts". We propose and demonstrate a new method, based on ESR spectroscopy, for determining tie-lines in regions of two-phase coexistence in a ternary lipid mixture. Over 100 different lipid compositions containing the spin-labeled phospholipid 16-PC in or near the two-phase coexistence region of the liquid-disordered and the gel phases of dipalmitoyl-PC/dilauroyl-PC/cholesterol (DPPC/DLPC/Chol) were studied to determine five tie-lines, spread over virtually the full range of this coexistence region. The method is based on the facts that (1) along a tie-line the ESR spectrum must be a superposition of the two ESR spectra from the respective single phases at the phase boundaries (connected by the tie-line) in a ratio given by the lever rule; (2) along a tie-line the partition coefficient, K_p, for the spin-label, which is also determined in our method, must be constant. We do find that K_p for 16-PC is close to unity, but its value depends on the particular tie-line. The coexisting phases in equilibrium are characterized by the K_p of the spin-label and its respective dynamic parameters obtained from fitting the ESR spectra to dynamical models.

Keywords: Tie-line; Partition coefficient; Membrane phase coexistence; ESR; Ternary lipid mixture

1. Introduction

Nonrandom mixing of lipid bilayer components is especially interesting when domains of different composition coexist. In particular, so-called lipid rafts seem to play important roles in functions of cell membranes such as signaling, protein transport, endocytosis and adhesion [1]. One aspect of raft formation in cell membranes is whether there is actual lateral separation of lipids into coexisting liquid phases, i.e., liquid-ordered (L_o) and liquid-disordered (L_α) phases. Detection of coexisting lipid bilayer phases in vesicles has been accomplished by a variety of methods, such as fluorescence techniques [2–4] and ESR methods [5]. However, the compositions of the coexisting phases have been exceedingly difficult to determine [6]. The present study does not examine the region of coexistence of L_o+L_α phases, which might be a good model of lipid rafts, and instead focuses on method development in the most tractable region of the phase diagram.

Previous investigations by fluorescence and ESR techniques showed the coexistence of phases in model membrane vesicles, but did not quantitatively analyze the composition

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of each coexisting phase in order to determine tie-lines in phase coexistence regions. The goal of the present investigation was to develop a method for determining tie-lines in two-phase coexistence regions in a three-component lipid mixture. Methods for tie-line determination in ordinary three-component mixtures include the method of analysis and the method of titration. Such methods can be traced back to early work [7–10]. A series of investigations on the liquid–liquid equilibrium system that gives an excellent review of experimental analysis and prediction of phases in equilibrium can be found in the literature [11–13]. An important characteristic that all of these systems have in common is the relative ease of adding or removing the phases that are in equilibrium, making possible the determination of tie-lines in ternary equilibrium mixtures. However, for the lipid mixture of current interest, dipalmitoyl-PC/dilauroyl-PC/cholesterol (DPPC/DLPC/Chol), it is impossible to employ the above methods to separate the conjugate phases in equilibrium, precluding direct analysis of the respective coexisting phases. In earlier work, we reported the ternary phase diagram of DPPC/DLPC/Chol from measurements of FRET, confocal fluorescence microscopy, and dipyrene-PC excimer/monomer ratios [14]. We then mapped out the dynamic structure of the phase diagram using ESR spectral simulations [15]. In this report, we present a new method of tie-line determination using spin-labeling ESR, and we have determined the tie-lines in the liquid-disordered phase and gel phase coexistence region. This new method should be useful in finding tie-lines in other two-phase coexistence regions of lipid membrane systems. Potentially, characterization of "lipid raft mixtures" could be taken to a much higher level than has been previously achieved, such that the compositions of both coexisting phases are precisely established.

2. Materials and methods

2.1. Materials

Phospholipids (DPPC and DLPC), cholesterol and the spin label 1-palmitoyl-2-(16-doxyl stearoyl) phosphatidylcholine (16-PC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Purity>99.5% was determined by thin-layer chromatography for phospholipids in chloroform/methanol/water=65:25:4 (by volume) and hexane/diethyl ether/chloroform=7:3:3 for cholesterol. All materials were used without further purification.

Measured stock solutions of the lipids and 16-PC in chloroform were mixed in a glass tube. The concentration of spin label was 0.2 mol% of the lipids. Multibilayer samples were prepared by the method of Rapid Solvent Exchange [16] in a buffer of PIPES/KCl/EDTA=5 mM:200 mM:1 mM at pH 7.0. Samples were then pelleted using a desktop centrifuge, and transferred to 1.5-mm I.D. capillaries with excess buffer. Samples were deoxygenated in a glove bag by alternately pumping and adding N_2 gas over 3 h. Each capillary was flame-sealed.

2.2. ESR spectra measurement and simulation methods

ESR spectra were obtained on a Bruker Instruments EMX ESR spectrometer at a frequency of 9.55 GHz at room temperature (~24 °C).

Nonlinear least-squares (NLLS) fitting based on the stochastic Liouville equation [17,18] was performed for analyzing the spectra from 16-PC. The most relevant dynamic parameters used in the fitting program are the rotational diffusion rates ($R_\perp$) and the order parameter (S_0). Molecular axis systems and definitions have been well defined for spectral simulations of membranes [19]. The MOMD model, which stands for microscopic order and macroscopic disorder [20], was used in the simulations.

2.3. Tie-line determination

The tie-line determination method using spin-label ESR is based on linear combinations of experimental ESR spectra, which was originally used to determinate the partition coefficient (K_p) of 16-PC spin label in the two-component liquid-disordered (L_α) and gel (L_β) coexistence phase [15]. K_p was found to be invariant along the tie-line, as required by thermodynamics, by both spectral simulation and investigations using linear combinations of experimental data (see below for details). Here, we extend this approach to find the tie-lines in a two-phase coexistence region of a ternary mixture with the criterion that K_p is invariant along a tie-line.

In a two-phase coexistence region our tie-line method consists of two steps: First, a trial pair of boundary samples is selected. This trial pair defines a trial tie-line. Second, samples are prepared that fall on this proposed tie-line. The k^{th} experimental spectrum of these samples along the hypothetical tie-line is expressed in terms of a vector, $\mathbf{C}_{i,k}$. For analyzing spectra collected from samples on the trial tie-lines, each i^{th} spectrum vector is fit with a vector $\mathbf{\Phi}_{i,k}$, which is composed of a pair of boundary spectral vectors, $\mathbf{A}_i$ and $\mathbf{B}_i$, with component fraction $\gamma_{i,k}$ for $\mathbf{A}_i$ and $(1-\gamma_{i,k})$ for $\mathbf{B}_i$ (cf. Eq (1)). The partition coefficient $(K_p)_{i,k}$ for the k^{th} spectrum on the i^{th} hypothetical tie-line is given in Eq. (2) below in terms of the lever rule prediction for the fraction $\mu_{i,k}$ of component $\mathbf{A}_i$ and the estimated component fraction $\gamma_{i,k}$. A partition coefficient $(K_p)_{i,k}$ would be unity if the spin label partitions into each coexisting phase with equal concentration. A $(K_p)_{i,k}$ that is greater than unity would indicate the spin label favors the phase associated with spectral component $\mathbf{A}$. Eq. (3), derived from Eqs. (1) and (2) (by solving Eq. (2) for $\gamma_{i,k}$ in terms of $(K_p)_{i,k}$ and then substituting into Eq. (1)), shows the spectrum vector $\mathbf{\Phi}_{i,k}$ expressed in terms of $(K_p)_{i,k}$, which must be constant for a true tie-line (i.e.,$(K_p)_{i,k}$ is independent of k). The analysis would then be to find the single $(K_p)_i$ that is independent of