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Membrane order parameters for interdigitated lipid bilayers measured via polarized total-internal-reflection fluorescence microscopy

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ABSTRACT

Incorporating ethanol in lipid membranes leads to changes in bilayer structure, including the formation of an interdigitated phase. We have used polarized total-internal-reflection fluorescence microscopy (pTIRFM) to measure the order parameter for Texas Red DHPE incorporated in the ethanol-induced interdigitated phase ($L_{\beta}I$) formed from ternary lipid mixtures comprising dioleoylphosphatidylcholine, cholesterol and egg sphingomyelin or dipalmitoylphosphatidylcholine. These lipid mixtures have 3 co-existing phases in the presence of ethanol: liquid-ordered, liquid-disordered and $L_{\beta}I$. pTIRFM using Texas Red DHPE shows a reversal in fluorescence contrast between the $L_{\beta}I$ phase and the surrounding disordered phase with changes in the polarization angle. The contrast reversal is due to changes in the orientation of the dye, and provides a rapid method to identify the $L_{\beta}I$ phase. The measured order parameters for the $L_{\beta}I$ phase are consistent with a highly ordered membrane environment, similar to a gel phase. An acyl-chain labeled BODIPY-FL-PC was also tested for pTIRFM studies of ethanol-treated bilayers; however, this probe is less useful since the order parameters of the interdigitated phase are consistent with orientations that are close to random, either due to local membrane disorder or to a mixture of extended and looping conformations in which the fluorophore is localized in the polar headgroup region of the bilayer. In summary, we demonstrate that order parameter measurements via pTIRFM using Texas Red-DHPE can rapidly identify the interdigitated phase in supported bilayers. We anticipate that this technique will aid further research in the effects of alcohols and other additives on membranes.

1. Introduction

The effects of alcohols on membranes continue to attract interest due to their implications for human health in areas such as alcohol intoxication, anesthetics and the use of ethanol as a cosolvent for drug delivery [1–3]. Alcohol effects are also relevant to the production of biofuels and specialty chemicals by yeast and bacteria. Many of the consequences of alcohol incorporation are related to changes in the properties and organization of lipid membranes and their embedded proteins [4–6].

Alcohols embedded in lipid membranes orient their hydroxyl moieties near the lipid headgroups [7,8], thus increasing the inter-headgroup spacing in the bilayer. Alcohols with shorter hydrocarbon chains may also create voids between the lipid tails, while those with longer chains may increase the lateral packing of the membrane [9]. At a threshold concentration that depends on the chain length of the alcohol [10], the increased inter-headgroup spacing causes the lipid tails to interdigitate [7]. Light-scattering [11], X-ray diffraction [7,11,12], and differential scanning calorimetry [11] of vesicle solutions have shown that alcohol-induced interdigitation changes the molecular tilt angle of the lipids, reduces the bilayer thickness, and increases the phase transition temperature. Fluorescence spectroscopy with polarity-sensitive probes has also been used to report on membrane order in the presence of alcohols [13–15].

Atomic force microscopy (AFM) has been used to examine the impact of alcohols on the thickness and mechanical properties of supported lipid bilayers prepared from lipids in either the gel or fluid phase [16,17]. Adding alcohol to the membrane produces a thinner interdigitated phase of reduced mechanical strength, as evidenced by the force required to break though the bilayer. Recent AFM studies have examined alcohol-treated supported lipid bilayers prepared from ternary lipid mixtures with coexisting liquid-ordered (L_o) and liquid-disordered (L_d) phases [18–20]. Such bilayers provide a model for cellular membranes [21,22]. The AFM work demonstrated the coexistence of interdigitated ($L_{\beta}I$), L_o and L_d phases for some sample compositions, and provided information on the morphology and area fraction of

Abbreviations: AFM, atomic force microscopy; BODIPY-FL-PC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; Chol, cholesterol; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; ESM, egg sphingomyelin; $L_{\beta}I$, interdigitated; L_d, liquid-disordered; L_o, liquid-ordered; PBS, phosphate buffered saline; pTIRFM, polarized total internal reflection fluorescence microscopy

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interdigitated phase at a given alcohol concentration. Despite the utility of AFM for monitoring formation of the $L_{\beta}I$ phase, it is challenging to resolve the small height differences between the L_d and $L_{\beta}I$ phases in aqueous ethanol solutions which have higher viscosity than water [23] and require the use of stiffer cantilevers with which it is difficult to image at sufficiently low force to avoid compressing the membrane [18].

As an alternative or complementary technique for rapid identification of the interdigitated phase, Longo and coworkers have demonstrated its labeling by Texas Red DHPE [18]. Fluorescence imaging showed that the $L_{\beta}I$ phase had slightly higher fluorescence intensity than the L_d phase but substantially higher intensity than the L_o phase. The authors hypothesized that the large area expansion caused by interdigitation allows the $L_{\beta}I$ phase to accommodate the bulky Texas Red fluorophore in the polar headgroup region of the bilayer.

To further probe the characteristics of the interdigitated phase, we turned to polarized total-internal-reflection fluorescence microscopy (pTIRFM). The fluorescence intensity of a membrane-embedded dye is measured upon excitation by light of different polarization angles, and an order parameter is calculated which is related to the tilt of the dye with respect to the bilayer normal [24–26]. Recently this technique was used in conjunction with AFM [27] to investigate effects of membrane-active peptides on lipid bilayer reorganization [28] and the restructuring of lipid bilayers in response to enzymatic ceramide generation [29]. It is possible to obtain order parameters with other techniques such as NMR, infrared spectroscopy, small angle X-ray scattering, linear dichroism, and fluorescence anisotropy [30–33]. However, because pTIRFM is an imaging technique, it allows the correlation of order parameters with specific features in a sample. Nevertheless, it is important to note that pTIRFM measures an order parameter of the fluorophore, and so the information on the lipid membrane order is indirect.

In this study, we employ pTIRFM to measure the order parameter of two different dye-labeled lipids (Texas Red DHPE and BODIPY-FL-PC) incorporated into lipid bilayers deposited from ethanol/buffer solution onto mica substrates. Bilayers are prepared from ternary lipid mixtures of dioleoylphosphatidylcholine (DOPC), cholesterol (Chol) and either egg sphingomyelin (ESM) or dipalmitoylphosphatidylcholine (DPPC); similar mixtures are frequently used as a model for the plasma membrane of mammalian cells. The two probes differ in the location of the fluorophore and its orientation with respect to the bilayer normal. The tail-labeled BODIPY-FL-PC adopts a position along the bilayer normal in non-interdigitated lipid bilayers [34]; its orientation has previously been characterized by pTIRFM and by molecular dynamics simulations [27,35,36]. By contrast, the headgroup-labeled Texas Red DHPE is expected to orient with its dipole moment nearly perpendicular to the bilayer normal. We demonstrate that measurement of order parameters via pTIRFM can be used to identify the interdigitated phase in supported lipid bilayers, and that the $L_{\beta}I$ phase is significantly more ordered than either the L_o or L_d phase of bilayers formed from ternary lipid mixtures in the presence of alcohol.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), egg sphingomyelin (ESM), and cholesterol (Chol) were obtained in powder form and dioleoylphosphatidylcholine (DOPC) as a chloroform solution from Avanti Polar Lipids (Alabaster, AL, USA). Texas Red (1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) and BODIPY-FL-PC ((2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine) were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Solutions were maintained at pH 7.3–7.5 with phosphate buffered saline, PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).

2.2. Bilayer preparation

The lipids were dissolved in chloroform or 3:1 chloroform/methanol (in the case of ESM) and combined in the following proportions: DOPC/ESM/Chol (2:6:1 molar ratio) and DPPC/DOPC (4:1 molar ratio) + 20 mol% Chol. The fluorescent dyes were dissolved in methanol and added to the lipid mixtures at 0.5 mol%. The solvent was evaporated by blown argon, and the lipid films were dried under vacuum overnight. Films were stored at −20 °C for up to 1 week.

The lipids were deposited onto mica-on-glass substrates (see details on substrate preparation in Section 2.4) by vesicle fusion. Fresh vesicles were prepared on the day of each experiment. The dry lipid films were hydrated with 20 vol.% ethanol in PBS to a lipid concentration of 0.5 mg/mL, vortexed for ~5 s to dissolve the lipids, and sonicated for 60 min at 50–65 °C in a closed container to prevent evaporation. Meanwhile, 20-mL aliquots of ethanol/PBS (1 per sample) were warmed to 45.5 °C.

Into each cell was placed 680 μL warm ethanol/PBS + 120 μL of 0.5 mg/mL lipids, in that order. To minimize evaporation, the cells were placed in closed plastic boxes with a wet Kimwipe and incubated for 5 min at 45.5 °C, and then rinsed with warm ethanol/PBS (about 20 mL) to remove excess vesicles. The cells were returned to the oven and subjected to the following heating/cooling cycle: Heat to 45.5 °C @ 70 °C/h. Hold at 45.5 °C for 15 min. Cool to 39.3 °C @ 51.6 °C/h. Cool to 36.2 °C @ 34.4 °C/h. Cool to 32.7 °C @ 22.4 °C/h. Cool to 29.2 °C @ 15.9 °C/h. Cool to 26.8 °C @ 12.5 °C/h. Cool to 19.5 °C @ 8.0 °C/h. Larger and more reproducible domains are typically formed when vesicle fusion is carried out above the melting temperature for the lipid mixture, and followed by slowly cooling the sample [37,38]. The cells were then washed with 19.5 °C ethanol/PBS, and sealed with a glass coverslip that had been cleaned with piranha solution and dried with argon.

2.3. Ethanol rinse experiment

DOPC/ESM/Chol bilayers were prepared as above in 20 vol.% ethanol/PBS and imaged. Each sample was then rinsed with at least 20 mL PBS to remove ethanol, incubated 90 min at 20 °C, and imaged again. To replace the ethanol, each sample was rinsed with at least 20 mL of ethanol/PBS, incubated 90 min at 20 °C, and imaged. Finally, to approximately mimic the temperature cycle used in direct deposition of vesicles from ethanol solution, each sample was heated to 45.5 °C at 90 °C/min, held at this temperature for 5 min, cooled to 19.5 °C at 60 °C/min, and imaged.

2.4. Polarized total internal reflection fluorescence microscopy

Polarized light can be used to determine the orientational order of membrane components because fluorophore excitation is dependent on the reporter's molecular orientation with respect to the polarization of the exciting light [24,27]. In TIRF microscopy fluorophores are excited by an evanescent field generated by total internal reflection of the illuminating light at the substrate/water interface. Because the polarization state of the evanescent field depends, among other factors, on the polarization of the illuminating beam, the orientational order of fluorophores embedded in a supported membrane can be probed by measuring the intensity of fluorescence as a function of the illuminating beam polarization angle. Fluorescent molecules are excited most efficiently when the electric field of the evanescent wave is aligned with their absorption dipole moments. Fluorophores oriented at different tilt angles relative to the substrate normal θ_c will therefore be preferentially excited at different polarization angles. For a planar solid-supported membrane the possible probe tilt angles range from 0° to 90° meaning that the order parameter $\langle P_2 \rangle$ ranges from −0.5 to 1.0, where

$$\langle P_2 \rangle = \frac{1}{2}\left(3\left[\cos^2\langle\theta_c\rangle\right]-1\right)$$