Effects of Ethanol on the Organization of Phosphocholine Lipid Bilayers

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We have investigated the consequences of the addition of ethanol to aqueous solutions containing 100 nm diameter phosphocholine unilamellar vesicles. We have studied the effect of ethanol addition on both gel phase and fluid phase phospholipid bilayers of 1,2-dimyristoyl-*sn*-phosphatidylcholine (DMPC), using time-resolved fluorescence measurements of perylene incorporated into the vesicles. We observe an increase in the perylene rotational diffusion time constants for ethanol concentrations of ca. 0.6 M in both the gel phase (289 K) and the fluid phase (303 K), indicating a change in the bilayer interacyl chain spacing and/or organization. While the change in rotational diffusion behavior of perylene is seen for both phospholipid phases, the details of the change in chromophore dynamics are not the same for the two phases, likely due to the differing extents of disorder in the phospholipid acyl chain region at the two temperatures. These data provide insight into the effects of ethanol on the local environment of the probe in both gel phase and fluid phase lipid bilayers.

Introduction

Mammalian cell membranes are highly complex biological structures that function as regulators of cell life, allowing specific chemical species to pass into and out of the cell. Plasma membranes are heterogeneous structures composed primarily of glycerophosopholipids, sphingolipids, cholesterol, and proteins arranged in a bilayer structure.[1−3] Even in compositionally simple systems, phospholipids self-assemble in aqueous environments to form nominally spherical bilayer liposomes, a structure which serves to minimize interactions between hydrophobic acyl chains and the aqueous solvent. In such structures, the hydrophobic acyl chain regions of lipids comprise the interior of a double-leaflet arrangement, while the polar headgroups act to shield the acyl chains from the aqueous environment and, as such, are located at the surfaces of the bilayer in direct contact with the solution in which they are suspended. In this structural format, the acyl chain terminal methyl groups of the opposing lipid monolayers are oriented facing one another. This lipid packing arrangement is the most energetically favorable, where the number of *gauche* rotamers in the acyl chain region is minimized, and van der Waals interactions between chains are optimized. The process of optimizing van der Waals interactions represents a balance between attractive acyl chain interactions and the thermal energy present in the system. Lipid bilayer assemblies are known to undergo phase transitions from ordered to disordered acyl chains, with the transition temperature depending on the length of the acyl chain and the presence of any unsaturations.

A variety of cellular processes are mediated by both lipid bilayer composition and dynamic properties of cell membranes, including intercellular and intracellular signaling, protein and enzyme activity, and cellular response to environmental stresses.[4] One way in which cells can regulate these functions is by controlling or manipulating the physical properties of the membrane, such as membrane fluidity, which is mediated by viscous drag effects between the membrane constituents.[5] Such interactions, along with membrane composition and structural heterogeneity, determine the motional freedom of these constituents in the membrane.[6] Membrane fluidity as it relates to the motional freedom of lipids refers to translational diffusion, rotational diffusion, and lipid translocation.[5,6] One way of experimentally altering the membrane fluidity of a bilayer is by changing the temperature of the local environment of the membrane. Phospholipid vesicles exhibit a characteristic phase transition at T_m, where the dynamic properties of lipids are altered due to changes in the organization of the lipid acyl chains. At temperatures below T_m, lipids exist in the gel phase, characterized by highly ordered acyl chain packing, where the acyl chains are tilted slightly away from the bilayer normal.[7] The close-packed arrangement of lipids in the gel phase results from van der Waals interactions in the hydrophobic acyl chain region of the membrane. Interacyl chain van der Waals interactions limit lipid rotational and translational freedom. At temperatures above T_m, lipid bilayers exist in a disordered fluid phase, characterized by an increase in the number of *trans*−*gauche* conformers and, as a result, an increase in the average interfacial area subtended by the lipid headgroups.[8] Lipids in the fluid phase experience greater translational and rotational freedom than lipids in the gel phase, resulting in increased overall membrane fluidity. The fluid phase has been shown experimentally to support a number of protein functions.[9,10]

The gel-to-fluid phase transition described above is dominated by acyl chain interactions, with the lipid headgroups playing a secondary role. Membrane fluidity and organization can also be controlled by the introduction of short-chain alkanols to the interfacial region of the bilayer. Alcohols such as ethanol are amphipathic and can displace some of the water molecules associated with the gel phase lipid bilayer interfacial region, penetrating to some extent into the nonpolar region of the bilayer.[11] The displacement of water molecules in the vicinity of the lipid headgroups weakens the interactions between the headgroups, allowing the bilayer structure to dilate, thus increasing the average surface area per headgroup. The addition of a sufficient amount of alcohol can perturb the bilayer interfacial region to the extent that a phase transition to an

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interdigitated structure occurs.[12−15] Ethanol has been used widely to induce the formation of the interdigitated phase, with much of the early work being done by Rowe et al.[16−18] and Simon et al.[19,20] The interdigitated phase occurs when the acyl chains of lipids from one leaflet interpenetrate with the chains from the opposing leaflet, eliminating the bilayer midplane, and leading to a consequent decrease in bilayer thickness. The decrease in bilayer thickness can have a dramatic effect on the function of proteins imbedded within biological cell membranes as well.[11] This explanation does not resolve whether the interdigitation process is spatially homogeneous or heterogeneous, or whether there are any molecular-scale changes in the acyl chain region as a consequence of interdigitation. It is the purpose of this work to address the issue of ethanol-concentration-dependent changes in the molecular-scale organization of these bilayers.

Several analytical techniques have been used in the past to elucidate the packing arrangement and properties of phospholipids in interdigitated bilayer arrangements, including differential scanning calorimetry,[21,22] fluorescence,[23−25] NMR,[26,27] and X-ray diffraction.[28,29] A significant limitation of conventional diffraction techniques for examining the structure and organization of unilamellar vesicles is their relatively low sensitivity, and calorimetric data provide limited molecular-scale insight. Fluorescence and NMR methods provide the requisite molecular-scale information, and fluorescence is easily capable of detecting low chromophore concentrations. For time-resolved fluorescence measurements, a chromophore can be selected to probe a specific region of the bilayer (i.e., the nonpolar acyl chain region or the polar headgroup region). We are interested in examining the interior acyl chain region of phospholipid vesicles because this region undergoes substantial structural and dynamic changes as a result of temperature- or alcohol-induced phase transitions.

In this work, we use 100 nm diameter unilamellar vesicles comprised of 1,2-dimyristoyl-*sn*-phosphatidylcholine (14:0 DMPC, $T_m = 297$ K)[30] and time-resolved fluorescence techniques to investigate the properties of a chromophore incorporated into the vesicle bilayer. We measured the anisotropy decay dynamics of the fluorescent probe perylene in vesicle-containing solutions at 289 K (gel phase DMPC) and 303 K (fluid phase DMPC) with controlled amounts of ethanol to gauge the extent to which the lipid phase and ethanol concentration mediate the properties of the bilayer structure, as sensed by perylene rotational motion. We have used perylene for time-resolved fluorescence measurements because of its well characterized anisotropy decay dynamics[31−33] and because its fluorescence lifetime and anisotropy decay time constants are similar, allowing for efficient collection of the requisite data. We measured the reorientation dynamics of perylene using time-correlated single photon counting (TCSPC), and found that this chromophore appears to be sensitive to the ethanol-induced phase transition from the gel phase to the interdigitated phase in DMPC at 289 K. We also observed a similar, more pronounced change in the reorientation behavior of perylene as a function of ethanol concentration at 303 K, a temperature at which DMPC is in the fluid phase. The interactions between lipid bilayers and ethanol produce measurable changes in lipid bilayer organization in both the gel and fluid phases.

Experimental Section

Vesicle Preparation. The phospholipid 1,2-dimyristoyl-*sn*-phosphatidylcholine (14:0 DMPC) in chloroform was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), and used as received. Perylene (99+ %) was purchased from Sigma-Aldrich (Milwaukee, WI) and used without further purification. For each sample, 10 mg of lipid was mixed with 30.5 μg of chromophore, and the chloroform solvent was evaporated. Tris buffer (Sigma-Aldrich) was then added to each sample to make the lipid concentration 1 mg/mL. Tris buffer (10 mM, pH 8.0) was prepared with purified water from a Milli-Q Plus water purification system (Millipore, Bedford, MA), and was purged with Ar prior to use. The lipid and chromophore mixtures were processed five times through a freeze−thaw−vortex cycle to ensure complete mixing of the constituents. Each cycle was comprised of first freezing the solution by immersion in $N_2(l)$ (5 min), then thawing in a 60 °C water bath (5 min), followed by vortexing the mixture (2 min). After completion of the freeze−thaw−vortex process, sample solutions were extruded 11 times through a polycarbonate membrane (Whatman) with a nominal pore diameter of 100 nm using a mini-extruder apparatus (Avanti Polar Lipids, Inc.).[34,35] Once the vesicle solutions were extruded, controlled amounts of 95% ethanol (Sigma-Aldrich) were added to the bulk mixtures, and the samples were allowed to equilibrate under ambient conditions prior to fluorescence acquisition. A time study (results not shown) indicated that the amount of lipid−ethanol equilibration time needed to achieve reproducible fluorescence results was ca. 24 h.

Time-Resolved Fluorescence Measurements. All fluorescence lifetime and anisotropy decay data were acquired using a time-correlated single photon counting (TCPSC) instrument. The light source is a CW passively mode-locked, diode-pumped Nd:YVO_4 laser (Spectra Physics Vanguard) that produces 2.5 W of average power at 355 nm and 2.5 W of average power at 532 nm at a 80 MHz repetition rate, with 13 ps pulses at both wavelengths. The output of the Nd:YVO_4 laser pumps a cavity-dumped dye laser (Coherent 702-2), which operates in the range 430−850 nm, producing 5 ps pulses. The repetition rate of the dye laser is adjustable between 80 MHz and 80 kHz by means of the cavity dumping electronics (Gooch & Housego). The dye laser was operated with Stilbene 420 dye (Exciton) to produce an excitation wavelength of ca. 430 nm. The fundamental excitation pulse from the dye laser is divided, with one portion of the pulse directed to a reference photodiode (Becker & Hickl PHD-400-N) and the other portion directed to the sample. Emission is collected using a 40× reflecting microscope objective. The collected emission is separated into polarization components parallel (0°) and perpendicular (90°) to the vertically polarized excitation pulse using a polarizing cube beam splitter. The parallel and perpendicular polarized signal components are detected simultaneously using microchannel plate photomultiplier tubes (MCP-PMT, Hamamatsu R3809U-50), each equipped with a subtractive double monochromator (Spectral Products CM-112) for wavelength selection. The detection electronics (Becker & Hickl SPC-132) resolve the parallel and perpendicular transients separately, yielding ca. 30 ps response functions for each detection channel. The detection electronics include a time-to-amplitude converter (TAC) and a constant fraction discriminator (CFD) that temporally resolves the fluorescence signal for each polarization component. Data are collected using multichannel analyzers (MCAs), which are integral components of the SPC-132 electronics. Data acquisition, detector bias, and collection wavelength are all controlled using an in-house written LabVIEW (National Instruments) program on a PC. The sample temperature was regulated to ±0.1 °C with a water-circulating bath (Neslab RTE-110) connected to a temperature-controlled

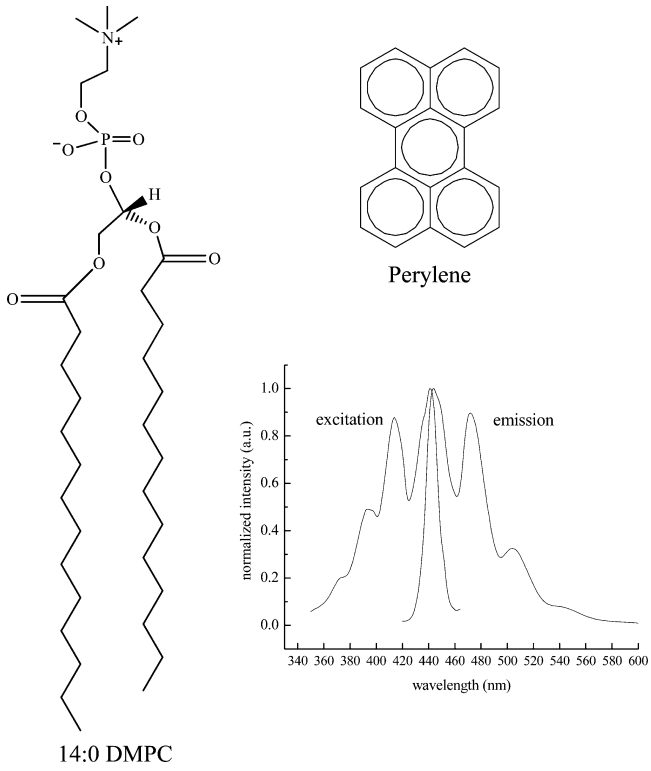

Figure 1. Structures of 14:0 DMPC and perylene and normalized absorption and emission spectra of perylene in DMPC vesicles.

brass cell jacket which held the sample cuvette. All samples were allowed to thermally equilibrate for 10 min prior to data acquisition.

Results and Discussion

The goal of the work we report here is to gain a better understanding of the effects of ethanol concentration and system temperature on the fluidity and molecular-scale organization of the lipid acyl chain region of 100 nm diameter DMPC vesicles. As noted above, we have used perylene for time-resolved measurements because its anisotropy decay dynamics are well understood and are sensitive to changes in its local environment. Perylene (Figure 1) partitions into the hydrophobic region of DMPC vesicles. The reorientation dynamics of perylene thus provide information useful for understanding the behavior of the bilayer acyl chain region as it experiences structural changes associated with temperature and exposure to solution phase ethanol. The results of our time-resolved fluorescence measurements show that the organization of DMPC in both the gel and fluid phases is influenced by the presence of ethanol.

When incorporating a chromophore into the lipid bilayer structure, there is always a chance that the fluorescent probe can perturb the bilayer to the extent that it could alter the phase transitions sensed by the probe. Previous work has shown, however, that perylene does not change the observed gel-to-fluid phase transition temperature (297 K)[30] measurably in DMPC bilayers.[35] We have used an even smaller probe-to-lipid ratio in this work than we have in the past, and for this reason, we do not expect to perturb the gel-to-fluid phase transition temperature from its reported value.[30] Another important characteristic of perylene to be considered is where, within the bilayer, the chromophore localizes. Perylene is thought to locate among the acyl chains of the bilayer leaflets. Some recent work has indicated that perylene moves within the bilayer to be in closest proximity to the acyl chain terminal methyl groups for vesicles of ca. 1 μm diameter and larger.[36] Those changes in perylene local environment were sensed by anisotropy decay measurements. We consider next the time-resolved emission behavior of perylene in both the gel and fluid phases of 100 nm diameter lipid vesicles as a function of solution phase ethanol concentration to gain insight into the alcohol-mediated changes in the organization of DMPC bilayer structures.

It is useful to consider at this point the nature of the interactions between the lipid bilayers and the solution phase ethanol. Because the lipid bilayers are present in the form of unilamellar vesicles, and ethanol is introduced to the vesicle-containing solutions, at least the initial interactions between the vesicles and ethanol must of necessity be at the outer vesicle leaflet. The asymmetric perturbation to the structure of the outer leaflet must therefore be the first step in the ethanol-induced changes we observe. If the chromophore is sequestered within the acyl chain region in a location that is asymmetric with respect to the interleaflet gallery, as our previous data on small vesicles suggest,[35,36] then we would expect more pronounced dynamical effects for the chromophore residing in the outer leaflet than in the inner leaflet.

For fluorescence anisotropy decay measurements, polarized fluorescence transients are collected for polarizations parallel and perpendicular to the vertically polarized excitation pulse. These quantities, $I_{||}(t)$ and $I_{\perp}(t)$, respectively, are combined to produce the induced orientational anisotropy function, $R(t)$ (eq 1),

$$R(t) = \frac{I_{||}(t) - I_{\perp}(t)}{I_{||}(t) + 2I_{\perp}(t)} \quad (1)$$

The chemical information available from these measurements is contained in the functional form of the $R(t)$ decay. There are several theoretical treatments that relate the anisotropy decay to the Cartesian components of the rotational diffusion constant and the transition dipole moment orientation(s) of the reorienting chromophore.[37−39] We treat the reorientation dynamics of perylene in DMPC bilayers in the context of a free rotor rather than a hindered rotor[40,41] because perylene is not tethered to the lipid acyl chains, and the planar shape and aspect ratio of perylene is such that, when incorporated into the bilayer structure of DMPC, there is no intrinsic limit on the rotational motion the chromophore can execute. We note that the nonpolar chromophore 1,6-diphenyl-1,3,5-hexatriene (DPH) has been used previously in the examination of lipid bilayer structures, and in that work, it was treated as a hindered rotor.[42] We assert that perylene rotational motion cannot be treated in the same context as that of DPH in lipid bilayers because of the substantially different structures and aspect ratios of these two molecules.

For the majority of anisotropy decay data, a single exponential decay is observed, and the chemical information contained in such data is limited. Perylene is well suited as a probe of local organization because it can exhibit either a one- or two-component anisotropy decay, depending on its immediate environment. We observe a two-component anisotropy decay in this work, a condition which yields information on each of the Cartesian components of the chromophore rotational diffusion constant, D. It is the relative values of D_x, D_y, and D_z that are useful in probing the ethanol- and temperature-induced lipid bilayer organizational changes of interest.

Relating the functional form of the anisotropy decay to the components of the rotational diffusion constant requires the

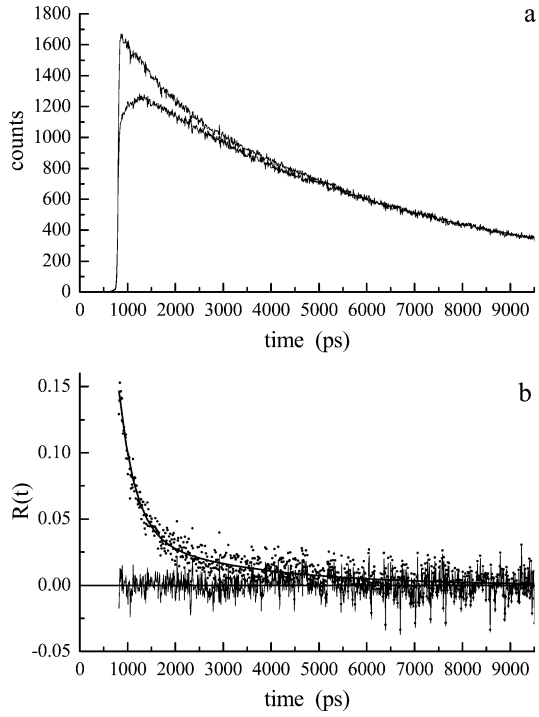

Figure 2. (a) Experimental data for perylene reorientation in a 100 nm diameter DMPC vesicle, with 0.25 M ethanol concentration at 289 K. (b) Induced orientational anisotropy decay function for the data shown in panel a, along with the best-fit function to the data. For this fit, the function is $f(t) = (0.11 \pm 0.004)\exp(-t/(339 \pm 24\text{ ps})) + (0.04 \pm 0.003)\exp(-t/(2483 \pm 185\text{ ps}))$.

assignment of axes and the assumption of a rotor shape, i.e., the volume swept out by the reorienting molecule. The excited and emitting transition dipole moments of perylene accessed in our experiments ($S_1 \leftrightarrow S_0$) lie along the chromophore long in-plane axis, which we designate as the *x*-axis. The short in-plane axis is the *y*-axis, and the *z*-axis is perpendicular to the chromophore π-system plane. Given these assignments, a single-exponential anisotropy decay indicates the probe reorients as a prolate rotor ($D_x > D_y = D_z$)[37]

$$R(t) = 0.4\exp(-6D_z t) \tag{2}$$

and a two-component exponential decay indicates the probe reorients as an oblate rotor ($D_z > D_x = D_y$), and is described by[37]

$$R(t) = 0.1\exp(-(2D_x + 4D_z)t) + 0.3\exp(-6D_x t) \tag{3}$$

For the data we report here, the decay of $R(t)$ was fit best by a two-component exponential decay (Figure 2), consistent with eq 3. We have reported previously on the reorientation dynamics of perylene in DMPC vesicles.[35,36] For vesicles ranging in size from 100 nm to 5 μm in diameter, a two-component exponential decay was found. The data we report here, both above and below the known T_m value of DMPC, show perylene to reorient as an oblate rotor. From the experimental anisotropy decay data, we extract the Cartesian components of the rotational diffusion constant, *D*. As noted above, eq 3 assumes $D_z > D_x = D_y$. We present in Table 1 the experimental values of D_z, D_x ($= D_y$) and the ratio D_z/D_x, as a function of system temperature and solution phase ethanol concentration. These data contain several interesting features.

At the outset of this discussion, it is important to consider the extent to which subtle trends in reorientation data can be relied upon. The rotational diffusion constants we report here are the average and standard deviation (1σ) for at least four individual determinations. We understand that the data we report reveal subtle ethanol concentration dependencies, a fact that results from the nature of the physicochemical phenomena we are examining.

In previous work, the ratio of D_z/D_x was shown to be a useful gauge of changes in the immediate environment of the chromophore,[31] but such a permutation of our data provides limited insight into the systems we report on here. Of more immediate benefit, however, is a direct examination of the ethanol concentration dependence of the D_z and D_x values at both 289 and 303 K. We present these data in Figure 3, revealing measurable differences in the effect of solution phase ethanol on the local environment of perylene for the two temperatures. We ascribe the differences in the ethanol concentration dependencies of D_z and D_x at the two different temperatures to be the result of the lipid bilayer existing in the gel phase at 289 K and the fluid phase at 303 K, at least for modest ethanol concentrations.[35] The D_z values reported at 289 K over all ethanol concentrations (Figure 3a) were found to be statistically the same using an ANOVA analysis at the 99% ($\alpha = 0.01$) confidence level, while the D_x values were found to be statistically different for ethanol concentrations in the proximity of 0.6 M. It should be noted that all ANOVA analyses were tested at both a 95% ($\alpha = 0.05$) and 99% ($\alpha = 0.01$) confidence level. In all cases, the same conclusion was reached in both instances. The value of D_x at 0.58 M is anomalously low and the value at 0.67 M appears to be slightly high relative to the corresponding values at other ethanol concentrations, albeit within the uncertainty of the D_x values at 0.50 and 0.75 M. These results suggest that, near 0.6 M ethanol, there is some perturbation to the lipid bilayer structure that makes rotation of perylene about its *x*-axis somewhat more restricted than for other ethanol concentrations and that, for 0.67 M ethanol, the value of D_x has at least recovered, if not increased slightly. It is thought that, for small diameter vesicles, perylene resides in the bilayer between acyl chains, and if this is the case, the chromophore may be sensing the onset of an interdigitation process. Such a process would be most pronounced in the region between leaflets, and perylene is likely in close proximity to this portion of the bilayer. The fact that there is no observable change in D_z over this ethanol concentration range suggests that the confining, quasi-lamellar structure created within the bilayer acyl chain region is not disrupted significantly by the structural events responsible for the changes in D_x.

The D_x ($= D_y$) and D_z data for perylene in DMPC vesicles at 303 K provide a qualitatively different picture of ethanol-dependent changes in bilayer organization (Figure 3b). For these data, we also find, through the use of an ANOVA analysis, that values of D_x in the vicinity of 0.6 M ethanol are statistically different, while the values of D_z are statistically not distinguishable. Interestingly, as the ethanol concentration increases at 303 K, the value of D_x decreases. If the ethanol-dependent change in behavior we observe is associated with an interdigitation process, it appears that the onset of interdigitation causes the acyl chain region of the bilayer to become more restrictive to perylene out-of-plane rotation. The same general trend is seen for D_z, despite the fact that ANOVA analysis shows that D_z is statistically independent of ethanol concentration. However, the

TABLE 1: Cartesian Components of the Rotational Diffusion Constant and Aspect Ratio D_z/D_x as a Function of Solution Phase Ethanol Concentration at 289 and 303 K[a]

	$T = 289$ K			$T = 303$ K		
[ethanol], M	D_z (MHz)	D_x (MHz)	D_z/D_x	D_z (MHz)	D_x (MHz)	D_z/D_x
0	600 ± 50	55 ± 2	10.9 ± 1.2	888 ± 80	70 ± 5	12.7 ± 1.1
0.25	703 ± 117	59 ± 3	11.8 ± 1.9	851 ± 65	65 ± 5	13.1 ± 1.7
0.50	689 ± 51	54 ± 3	12.8 ± 1.0	879 ± 92	73 ± 4	12.1 ± 1.5
0.58	665 ± 35	49 ± 2	13.6 ± 0.8	923 ± 73	58 ± 5	16.0 ± 1.8
0.67	737 ± 89	61 ± 7	12.1 ± 2.0	762 ± 50	52 ± 3	14.8 ± 0.5
0.75	651 ± 72	56 ± 3	11.5 ± 1.0	816 ± 89	57 ± 3	14.3 ± 1.8
1.00	676 ± 36	52 ± 2	12.9 ± 0.4	882 ± 76	68 ± 8	13.0 ± 1.2

[a] Errors are $\pm 1\sigma$ for at least four individual determinations at each ethanol concentration.

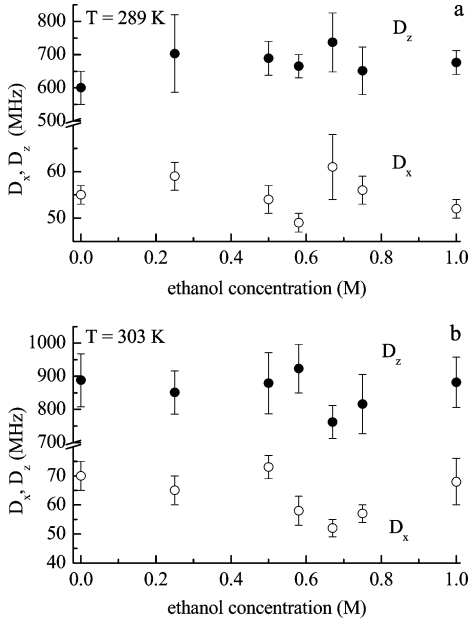

Figure 3. (a) Dependence of perylene D_x and D_z on ethanol concentration for 100 nm diameter DMPC vesicles at 289 K. (b) Dependence of perylene D_x and D_z on ethanol concentration for 100 nm diameter DMPC vesicles at 303 K. Errors are $\pm 1\sigma$ for at least four individual determinations at each ethanol concentration.

ethanol concentration at which the apparently anomalous behavior in D_z occurs is not commensurate with that observed for D_x (Figure 3b). This finding may be related to modulation of the acyl chain density by the interdigitation process. The ethanol-dependent reorientation dynamics of perylene at 289 and 303 K are measurably different from one another, and we correlate this difference with DMPC being in the gel phase at 289 K and the fluid phase at 303 K, at least for modest ethanol concentrations.[35]

In comparing the data taken at 289 K to those taken at 303 K, we note that the rotational diffusion constants at 303 K are uniformly faster than those at 289 K. This is not surprising, for two reasons. The first is that the local frictional (viscous) interactions experienced by perylene are expected on theoretical grounds to be temperature dependent,[43−45] and the second is that, for pure DMPC, there is a phase transition between the two temperatures at which we recorded data. The difference in phases is seen in the different D_z/D_x ratios for perylene at the two temperatures. ANOVA analysis showed that the value of D_z/D_x for perylene at 289 K is independent of ethanol concentration to within the experimental uncertainty, at a value of 12.2 ± 0.5. The same analysis applied to the D_z/D_x ratio for perylene at 303 K is ethanol-dependent. The acyl chain environment for perylene is slightly more anisotropic at 303 K (Table 1) than it is at 289 K. Such behavior may reflect either a difference in the relative location of the chromophore in the acyl chain region at the two temperatures or a change in the environment at a specific location within the bilayer acyl chain region. Regardless, the local environment sensed by perylene appears to be different at the two temperatures.

It is possible to gauge the frictional intermolecular interactions experienced by perylene in the bilayer structures. We can calculate the rotational diffusion constant, D, using the Cartesian components D_x ($= D_y$) and D_z given in Table 1.

$$D = \frac{D_x + D_y + D_z}{3} \tag{4}$$

The rotational diffusion constant is related to the viscosity of the local environment sensed by the probe according to the modified Debye−Stokes−Einstein equation[43−45]

$$\frac{1}{6D} = \frac{\eta V f}{k_B T S} \tag{5}$$

where η is the viscosity of the surrounding medium of the reorienting chromophore, V is the hydrodynamic volume of the chromophore, f is a frictional term to compensate for solvent−solute interactions, k_B is the Boltzmann constant, T is the temperature, and S is a shape factor determined from Perrin's equations to account for the nonspherical shape of the chromophore.[44] We used the rotational diffusion constants calculated from eq 4, the shape factor, $S = 0.70$,[46] and the hydrodynamic volume of perylene, $V = 225$ Å^3,[47] to determine the viscosity, η, of the perylene local environment as a function of ethanol concentration. We find that there is no resolvable ethanol concentration dependence of the acyl chain microviscosity sensed by perylene using ANOVA analysis, indicating that the ethanol is not penetrating fully into the acyl chain region but is instead acting primarily on the lipid headgroup region. For data taken at 289 K, we calculate $\eta = 7.9 \pm 0.3$ cP and, at 303 K, $\eta = 6.6 \pm 0.2$ cP. These values are consistent with those we have measured previously, indicative of an acyl chain region that does not contain constituents other than phospholipids.[35] We attribute the difference in calculated viscosity to the temperature difference between the two measurements and the fact that DMPC is initially in the gel phase at 289 K and the more disordered fluid phase at 303 K. It is significant that these values are consistent with those reported for DMPC without the addition of ethanol,[35] suggesting that ethanol penetration of the DMPC bilayer does not proceed to the extent

of perturbing the acyl chain region of the structure, at least prior to the point of causing interdigitation.

It is known that the ethanol-induced interdigitated phase results when amphipathic molecules disrupt the lipid headgroup interactions to the extent that the bilayers dilate and eventually interpenetrate.[13] When lipid interdigitation takes place, it is characterized by a closely packed gel phase structure, and a consequent decrease in membrane fluidity.[48] At a concentration of ca. 0.6 M ethanol in our systems, perylene experiences a decrease in its motional freedom for D_x at 289 and 303 K. These data are consistent with the DMPC lipid bilayers becoming interdigitated at ca. 0.6 M ethanol. One might expect there to be a requisite increase in D_x and/or D_z with the onset of lipid headgroup dilation preceding interdigitation, but this is not seen. We believe that this is due to the fact that perylene resides in the lipid acyl chain region, relatively well isolated from the phospholipid headgroups, and that up to the point of interdigitation the van der Waals interactions between acyl chains dominate the local environment experienced by perylene. For ethanol concentrations above 0.6 M, it is likely that the lipid headgroup interactions become further disrupted, eventually giving rise to the loss of bilayer integrity. Up to the point of bilayer destruction, however, it is likely that the perturbation to the headgroup organization is resisted by the van der Waals attractive interactions between lipid acyl chains. For ethanol concentrations of ca. 1 M, we observe dynamics that differ little from those measured for aqueous vesicle solutions, underscoring the importance of dispersion forces in mediating lipid local organization.

Our data on 100 nm diameter DMPC lipid vesicles at 303 K, a temperature above the known gel-to-fluid phase transition temperature (Figure 3b), show an ethanol concentration dependence that is qualitatively similar, albeit different in detail, to that seen for the 289 K data (Figure 3a). Much work has been conducted in the last two decades on ethanol-induced interdigitation of gel phase lipid bilayers, all of which have demonstrated through a variety of techniques that ethanol does promote lipid interdigitation.[14,16,25,28,29,49] While there are no data we are aware of that have reported on the existence of an ethanol-induced interdigitation in fluid phase lipid bilayers, our data reveal fluid phase behavior that is commensurate with gel phase behavior. Our data reveal ethanol concentration-dependent changes in D_x in the vicinity of 0.6 M ethanol. While the details of changes in D_x at 303 K differ from those seen at 289 K, they do show a change in lipid acyl chain environment, consistent with an interdigitation phase transition seen for gel phase DMPC. Further experiments will be required to resolve the details of this anomalous organizational change in fluid phase DMPC, and those experiments are underway in our laboratory.

Conclusions

We have found that the presence of solution phase ethanol has an effect on the organization of DMPC lipid bilayer structures, both below and above the gel-to-fluid phase transition temperature. At 289 K, we observe that the rotational diffusion dynamics of perylene incorporated in the bilayer acyl chain region experience a perturbation about their long in-plane rotational axis for ethanol concentrations in the vicinity of 0.6 M. Interestingly, there is no measurable effect on the rotation of perylene about the axis normal to its molecular plane. For the same measurements performed at 303 K, we similarly observe a perturbation to out-of-plane (D_x, D_y) perylene rotational motion when the solution phase ethanol concentration is in the vicinity of 0.6 M, although the onset of the perturbation occurs at slightly different ethanol concentrations, which is likely the result of the extent of structural perturbation experienced by the lipid acyl chain region. Again, there is no measurable perturbation to the in-plane (D_z) rotational motion of perylene. Our findings are consistent with the known phases of the lipid vesicles. Phospholipids are known to undergo interdigitation phase transitions in their gel phases at ethanol concentrations in the vicinity of 0.6 M.[28] Our findings point to the influence of ethanol in mediating a structural perturbation for DMPC in the fluid phase as well, although the structural details of this perturbation remain to be resolved fully. Further work is underway to understand this effect more thoroughly.

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