Red Edge Excitation Shift of a Deeply Embedded Membrane Probe: Implications in Water Penetration in the Bilayer

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The biological membrane is a highly organized anisotropic molecular assembly. While the center of the bilayer is nearly isotropic, the upper portion, only a few angstroms away toward the membrane surface, is highly ordered. How this organization correlates with the degree of water penetration into the bilayer interior is not clear. In general, it is believed that there is not much water in the deeper hydrocarbon regions of the bilayer. In this study, we have utilized the phenomenon of wavelength-selective fluorescence to address this question. We show here that when the same fluorescent group (i.e., 7-nitrobenz-2-oxa-1,3-diazol-4-yl or NBD) is localized at different depths within the bilayer (viz., near the membrane interface in case of the headgroup-labeled NBD-phosphatidylethanolamine (NBD-PE) and near the center of the bilayer in NBD-cholesterol), the degrees to which their fluorescence properties exhibit solvent-induced effects are markedly different. For example, the headgroup-labeled NBD-PE exhibits a much stronger red edge excitation shift (REES) relative to that of NBD-cholesterol. This indicates lesser restriction to mobility in this region as compared to the polar/hydrocarbon interface. In the gel phase, however, REES of NBD-PE did not show any significant change while NBD-cholesterol exhibited no REES. In addition, NBD-cholesterol exhibits a stronger dependence of fluorescence polarization on excitation wavelength in fluid membranes. We attribute these results to the more compact arrangement of the lipid acyl chains in the gel phase which results in lesser water penetration. Since the hydrophobic core of the lipid bilayer is made up of methyl and methylene groups, the only solvent dipoles capable of any interaction with the dipole of the fluorophore giving rise to the REES effect in the fluid phase have to be water molecules that have penetrated deep into the bilayer close to the NBD moiety of NBD-cholesterol. Our results indicate that at least in the fluid phase of the membrane, penetration of water in the deep hydrocarbon region of the bilayer does indeed occur.

Introduction

The biological membrane is a highly organized molecular assembly, largely confined to two dimensions, and exhibits considerable degree of anisotropy along the axis perpendicular to the membrane plane. While the center of the bilayer is nearly isotropic, the upper portion, only a few angstroms away toward the membrane surface, is highly ordered.[1−8] As a result, properties such as polarity, fluidity, segmental motion, ability to form hydrogen bonds, and extent of solvent penetration would vary in a depth-dependent manner in the membrane. A direct consequence of such an anisotropic transmembrane environment will be the differential extents to which the mobility of water molecules will be retarded at different depths in the membrane relative to the water molecules in bulk aqueous phase. It is such retardation in the rate of solvent reorientation in the immediate vicinity of a fluorophore that is assessed by wavelength-selective fluorescence in general, and red edge excitation shift, in particular.

Red edge excitation shift (REES) is one of the effects that is observed when a polar fluorophore is placed in motionally restricted media such as very viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime.[9−12] REES is defined as a shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band. REES arises from slow rates of solvent relaxation (reorientation) around an excited state fluorophore which is a function of the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. Further, since the ubiquitous solvent for biological systems is water, the information obtained in such cases will come from the otherwise "optically silent" water molecules. This makes REES and related techniques extremely useful since hydration plays a crucial modulatory role in a large number of important cellular events,[13] including lipid−protein interactions[14] and ion transport.[15−17] We have previously shown that REES and related techniques (collectively termed as wavelength-selective fluorescence approach) can be used to study motional restriction experienced by membrane-bound molecules and serve as a powerful tool to monitor organization and dynamics of probes and peptides bound to membranes or micelles.[9,18−29]

The origin of the red edge effect lies in the change in fluorophore-solvent interactions in the ground and excited states, brought about by a change in the dipole moment of the fluorophore upon excitation, and the rate at which solvent

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molecules reorient around the excited-state fluorophore.[9−12] For a polar fluorophore, a dipolar interaction with the solvent molecules occurs in the ground state in order to minimize the energy of the given state. Since the dipole moment (magnitude as well as direction) of a molecule changes upon excitation, the solvent dipoles have to reorient around this new excited state dipole moment of the fluorophore so as to attain an energetically favorable orientation. This readjustment of the dipolar interaction of the solvent molecules with the fluorophore essentially consists of two components: first, the redistribution of electrons in the surrounding solvent molecules because of the altered dipole moment of the excited-state fluorophore, and second, the physical reorientation of the solvent molecules around the excited-state fluorophore. The former process is almost instantaneous, i.e., electron redistribution in solvent molecules occurs at about the same time scale as the process of excitation of the fluorophore itself (10^{-15} s). The reorientation of the solvent dipoles, however, requires a net physical displacement. It is thus a much slower process and depends on the restriction offered by the surrounding matrix to their mobility. More precisely, for a polar fluorophore in a bulk nonviscous solvent, this reorientation time (τ_s) is on the order of 10^{-12} s, so that all the solvent molecules completely reorient around the excited-state dipole of the fluorophore well within its excited-state lifetime (τ_F), which is typically on the order of 10^{-9} s.[30] Hence, irrespective of the excitation wavelength used, all emission is observed only from the solvent-relaxed state. However, if the same fluorophore is now placed is a viscous medium, this reorientation process is slowed such that the solvent reorientation time is now on the order of 10^{-9} s or longer. Under these conditions, excitation at the red edge of the absorption band selectively excites those fluorophores which interact more strongly with the solvent molecules in the excited state. These are the fluorophores around which the solvent molecules are oriented in a way similar to that found in the solvent-relaxed state. Thus, the necessary condition for REES is that different fluorophore populations are excited at the maximal and the red edge excitation and, more importantly, this difference is maintained in the time scale of fluorescence lifetime. As discussed above, this requires that the dipolar relaxation time for the solvent shell be comparable to or longer than the fluorescence lifetime. This implies a reduced mobility of the fluorophore with respect to the surrounding matrix.

Using anthroyloxy probes that are localized at different depths in the membrane, we have recently shown that for a given fluorophore, REES varies as a function of probe penetration depth.[28] In this paper, we demonstrate that this is also true for membrane-bound (7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD) probes and that REES exhibited by these probes depend on their precise location in the membrane. For this purpose, we have employed two probes, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) and 25-[*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino]-27-norcholesterol (NBD-cholesterol), for monitoring two different regions of the membrane bilayer (see Figure 1). NBD-labeled lipids are widely used as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes.[31,32] In NBD-PE, the NBD group is covalently attached to the headgroup of a phosphatidylethanolamine molecule (Figure 1). The NBD group in NBD-PE has earlier been shown to be localized in the interfacial region of the membrane.[33−38] In contrast to this, the NBD group in NBD-cholesterol is attached to the flexible acyl chain of the cholesterol molecule. The NBD group of this molecule has been found to be localized deep in the hydrocarbon region of the membrane.[33,34,39] Our results obtained with these probes are relevant in terms of depth dependence of REES effects as well as water penetration in the deep hydrocarbon region of the bilayer and its modulation by phase properties of the membrane.

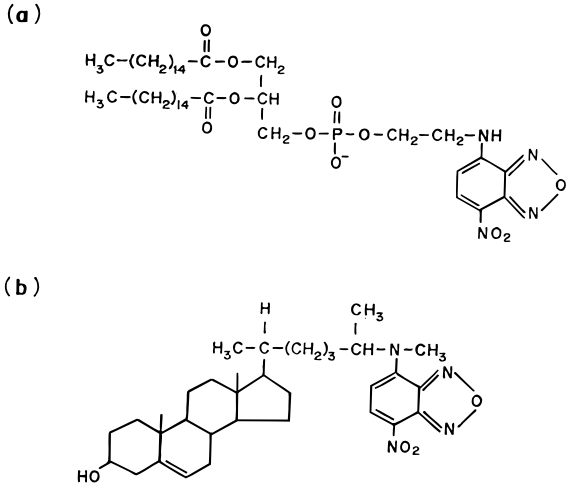

Figure 1. Chemical structures of (a) NBD-PE and (b) NBD-cholesterol

Experimental Section

Materials. Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were purchased from Sigma Chemical Co. (St. Louis, Missouri). NBD-PE and NBD-cholesterol were from Molecular Probes (Eugene, Oregon). Lipids were checked for purity by thin-layer chromatography (TLC) on silica gel precoated plates (Sigma) in chloroform/methanol/water (65:35:5, v/v/v) and were found to give one spot with a phosphate-sensitive spray and on subsequent charring.[40] NBD-PE was found to be pure when detected by its color or fluorescence. TLC of NBD-cholesterol was done using the same solvents but in a slightly different proportion (65:35:4, v/v/v), and it was found to be pure when detected by its color or fluorescence.[39] Concentration of DPPC was determined by phosphate assay after total digestion by perchloric acid.[41] DMPC was used as a standard to assess lipid digestion. Concentrations of stock solutions of NBD-PE and NBD-cholesterol in methanol were estimated using their molar absorption coefficients (ϵ) of 21 000 and 22 000 M^{-1} cm^{-1} at 463 and 484 nm, respectively. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, Massachusetts) Milli-Q system and used throughout.

Preparation of Vesicles. Unilamellar vesicles (ULV) of DPPC labeled with 0.1% (mol/mol) NBD-cholesterol were prepared by the ethanol injection method.[42] For this, 640 nmol of DPPC and 0.64 nmol of NBD-cholesterol were dried together. The dried lipids were then dissolved in ethanol to give a final concentration of about 40 mM lipid. This ethanolic lipid solution was then injected into 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.0 buffer while vortexing to give a final concentration of 0.43 mM lipid in the buffer. The temperature of the buffer was maintained higher than the phase transition temperature of DPPC (i.e., >41 °C) while the injections were made. Background samples were prepared the same way except that the probe was omitted.

Fluorescence Measurements. Steady-state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal band-pass of 5 nm were used for

all measurements. Experiments involving the gel phase were done at 23 °C, whereas the experiments with fluid phase membranes were carried out at 54 °C. Background intensities of samples in which fluorophores were omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from the equation:[43]

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}. All experiments were done with multiple sets of samples and average values of fluorescence and polarization are shown in the figures. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ±1 nm of the ones reported.

Results

The Red Edge Effect. In general, for a fluorophore in a bulk nonviscous solvent, the fluorescence decay rates and the wavelength of maximum emission are independent of the excitation wavelength. This is because of Kasha's rule which states that fluorescence normally occurs from the zero vibrational level of the first excited electronic state of a molecule.[44] However, this generalization breaks down in case of polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases, that is, when the mobility of the surrounding matrix relative to the fluorophore is considerably reduced. Under such conditions, when the excitation wavelength is gradually shifted to the red edge of the absorption band, the maximum of fluorescence emission exhibits a concomitant shift toward higher wavelengths. Such a shift in the wavelength of maximum emission toward higher wavelengths, caused by a corresponding shift in the excitation wavelength toward the red edge of the absorption band, is termed red edge excitation shift or REES.[9] Since REES is observed only under conditions of restricted mobility, it serves as a faithful indicator of the dynamics of the fluorophore environment.

Red Edge Excitation Shifts of Membrane-Bound NBD Probes: The Dipstick Effect. The emission maxima of NBD-labeled lipids are sensitive to the polarity of the probe microenvironment.[22,31−34,45−47] The fluorescence emission maxima for NBD-PE and NBD-cholesterol in fluid phase DPPC vesicles are at 529 and 518 nm, respectively (see Figure 2). The blue shift of the emission maximum for NBD-cholesterol (compared to NBD-PE) is indicative of its deeper location in the nonpolar region of the membrane, as reported earlier by one of us.[33,34] The shifts in the maxima of fluorescence emission[48] of NBD-PE and NBD-cholesterol in fluid DPPC vesicles as a function of excitation wavelength are shown in Figure 2. For NBD-PE, as the excitation wavelength is changed from 465 to 515 nm, the emission maximum shifts from 529 to 538 nm, which corresponds to a REES of 9 nm. Such shift in the wavelength of emission maximum with change in the excitation wavelength is characteristic of the red edge effect and indicates that the NBD moiety in NBD-PE is localized in a motionally restricted region of the membrane that offers considerable resistance to solvent reorientation in the excited state. Figure 2 also shows

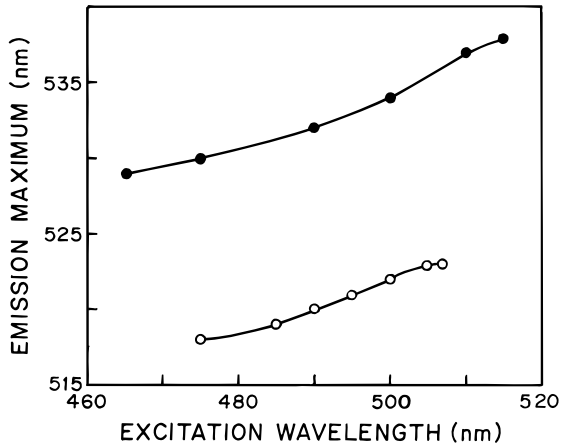

Figure 2. Effect of changing excitation wavelength on the wavelength of maximum emission for 1% (mol/mol) NBD-PE (l) and 0.1% (mol/mol) NBD-cholesterol (O) in fluid phase DPPC vesicles. The data for NBD-PE is plotted from Table 3 of ref 19. See Experimental Section for other details.

that, for NBD-cholesterol, as the excitation wavelength is changed from 475 to 507 nm, the emission maximum shifts from 518 to 523 nm, which amounts to a REES of 5 nm. We chose to use 0.1% (mol/mol) of NBD-cholesterol in our experiments to avoid any artifacts due to NBD-cholesterol aggregation in the membrane.[39] It should be noted here that the fluorescence of NBD-cholesterol is relatively weak,[34] and we found it difficult to work in excitation wavelengths longer than 507 nm because of the very low signal-to-noise ratio and artifacts due to the Raman peak that remained even after background subtraction.

An interesting feature of this result is that the magnitude of REES obtained for membrane-bound NBD probes varies in direct correlation with their penetration depths. In other words, whereas NBD-PE, which is a shallow probe present in the membrane interfacial region exhibits a REES of 9 nm, the deep probe NBD-cholesterol, present in the inner hydrocarbon-like region of the membrane, shows a REES of 5 nm under identical conditions. We attribute this to differential rates of solvent reorientation (which is a function of different degrees of motional restriction experienced by the solvent molecules) as a function of probe depth. These results are in good agreement with our previous report in which we showed that such depth-dependent REES is exhibited by membrane-bound anthroyloxy probes localized at various depths in the membrane.[28] Furthermore, the present results obtained using NBD probes localized at different depths in the membrane indicate that such "dipstick effect" is independent of the probe used.

Red Edge Excitation Shifts of Membrane-Bound NBD Probes in the Gel Phase. Figure 3 shows the effect of changing excitation wavelength on the wavelength of maximum emission for membrane-bound NBD probes in gel phase DPPC vesicles. The fluorescence emission maximum of NBD-PE was found to be at 530 nm when excited at 465 nm (see Figure 3). As the excitation wavelength of NBD-PE in gel phase DPPC vesicles is changed from 465 to 515 nm, the emission maximum is shifted from 530 to 538 nm, which corresponds to a REES of 8 nm. Thus, no appreciable change either in the emission maximum or REES was observed with the variation in the physical state of the membrane. These results indicate that the immediate environment of the NBD moiety in NBD-PE, as measured either by the absolute position of its emission maximum at a particular excitation wavelength or by REES, is not affected when the membrane undergoes the phase transition

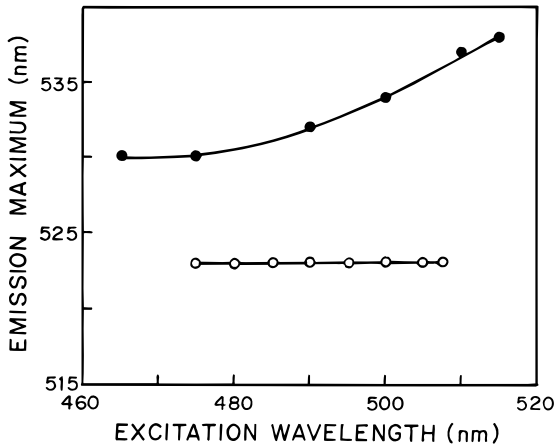

Figure 3. Effect of changing excitation wavelength on the wavelength of maximum emission for 1% (mol/mol) NBD-PE (●) and 0.1% (mol/mol) NBD-cholesterol (○) in gel phase DPPC vesicles. All other conditions are the same as those in Figure 2. See Experimental Section for other details.

from the gel to the fluid (liquid crystal) phase. This is not particularly surprising since changes in membrane organization brought about by phase transition are largely restricted to the fatty acyl region of the membrane[49] and are not sensed by the NBD moiety of NBD-PE which is attached to the headgroup and is located at the membrane interface.[33−38] In addition, small molecular motions of the headgroup region of membranes, which are responsible for spectral relaxation, have previously been shown not to be affected in a major way by phase transition.[50,51]

Figure 3 also shows that the emission maximum of NBD-cholesterol in gel phase DPPC membranes is at 523 nm. Interestingly, while the emission maximum of NBD-PE shows no significant shift upon phase transition (Figures 2 and 3), that of NBD-cholesterol exhibits a red shift of 5 nm when taken from fluid to gel phase membranes. The emission maximum of NBD-cholesterol in gel phase DPPC vesicles, however, does not change at all when the excitation wavelength is changed from 475 to 507 nm, i.e., NBD-cholesterol shows no REES in the gel phase membrane. This is in sharp contrast to a REES of 5 nm observed in case of NBD-cholesterol in fluid phase DPPC membranes as shown in Figure 2.

It should be mentioned here that the difference in REES observed for NBD-cholesterol in the fluid (experiments done at 54 °C) and the gel (experiments done at 23 °C) phase cannot be attributed to temperature effects. This is because in control experiments with dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)[52] vesicles at 23 °C, NBD-cholesterol exhibits a REES of 5 nm.[53]

Polarization Changes with Excitation Wavelength for Membrane-Bound NBD-Probes. In addition to the dependence of fluorescence emission maxima on the excitation wavelength, fluorescence polarization is also known to depend on the excitation wavelength in viscous solutions or in otherwise motionally restricted media (ref 9 and references therein). Due to strong dipolar interactions with the surrounding solvent molecules, there is a decreased rotational rate of the fluorophore in the relaxed state. On red edge excitation, a selective excitation of this subclass of fluorophore occurs. Because of strong interaction with the polar solvent molecules in the excited state, one may expect these "solvent relaxed" fluorophores to rotate more slowly, thereby increasing the polarization.

The excitation polarization spectra (i.e., a plot of steady-state polarization vs excitation wavelength) of NBD-cholesterol in fluid and gel phase DPPC vesicles are shown in Figure 4. When excited at 475 nm, the polarization of NBD-cholesterol in fluid phase vesicles is significanltly lower than in gel phase reflecting the difference in dynamics in the two phases. However, the polarization of NBD-cholesterol in the fluid phase undergoes considerable change (increases by 81%) upon increasing the excitation wavelength from 475 to 507 nm, with a sharp increase occurring toward the red edge of the absorption band (Figure 4a). Such an increase in polarization upon red edge excitation has been previously reported for fluorophores in media of reduced mobility.[9] The polarization of NBD-cholesterol in gel phase membranes, on the other hand, although starts at a higher initial value (i.e., when excited at 475 nm), shows a much weaker dependence (8% increase) upon excitation wavelength (see Figure 4b). Thus, NBD-cholesterol in the fluid phase exhibits a much stronger dependence of its fluorescence polarization (on excitation wavelength when compared to the gel phase.

Discussion

Our results in fluid DPPC membranes show that while NBD-PE, a shallow probe present in the membrane interfacial region, exhibits a REES of 9 nm, the deep probe NBD-cholesterol, present in the hydrocarbon-like interior of the membrane, shows a significantly reduced REES of 5 nm. Thus, the magnitude of REES can be correlated with membrane penetration depths of these two NBD probes. This result correlates very well with

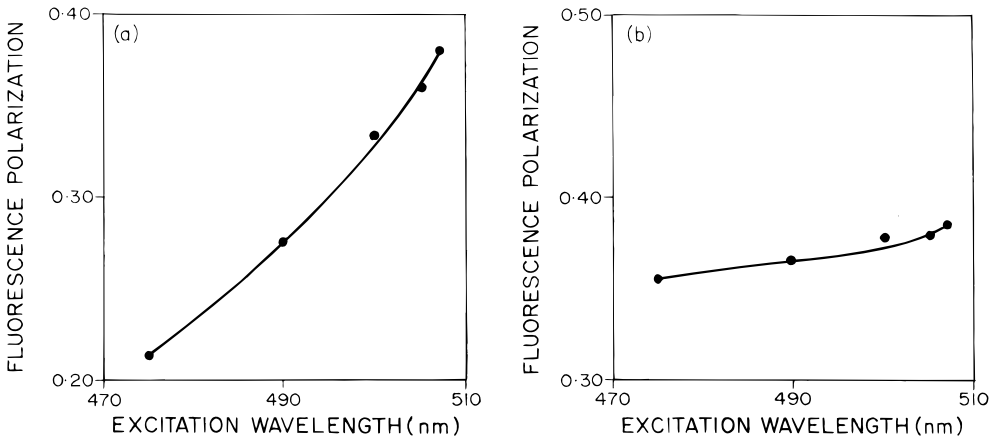

Figure 4. Fluorescence polarization of NBD-cholesterol in (a) fluid phase and (b) gel phase DPPC vesicles as a function of excitation wavelength. All other conditions are the same as those as in Figure 2. See Experimental Section for other details.

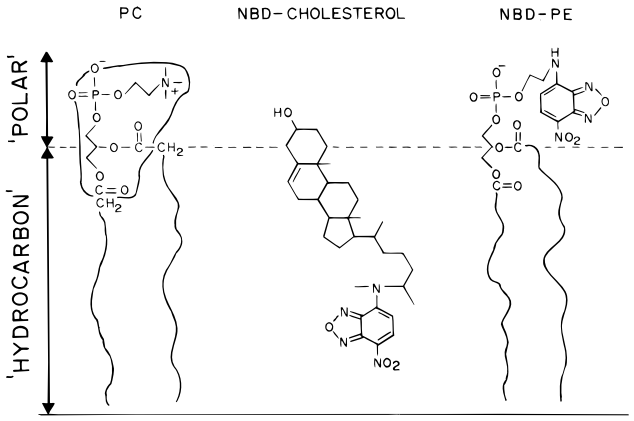

Figure 5. A schematic representation of half of the membrane bilayer showing the localizations of the NBD groups of NBD-PE and NBD-cholesterol in DPPC vesicles. The horizontal line at the bottom indicates the center of the bilayer.

differential rates of solvent relaxation at different depths in the membrane and is consistent with a motional gradient along the membrane axis as a function of depth of penetration. This reinforces our earlier observation[28] that the magnitude of REES could be used as a dipstick to characterize the depth of penetration of a membrane embedded fluorophore.

Relevance in Membrane Structure and Organization: Implications in Water Penetration in the Deeper Hydrophobic Regions of the Bilayer. Interestingly, NBD-cholesterol shows no REES in gel phase membranes although NBD-PE exhibits REES similar to what is observed in the fluid phase. In addition, NBD-cholesterol exhibits much stronger dependence of fluorescence polarization on excitation wavelength in fluid membranes when compared to gel phase membranes. We attribute these results to a more compact arrangement of the lipid acyl chains in the gel phase which results in lesser water penetration relative to the fluid phase.[54−58] Since the hydrophobic core of the lipid bilayer is made up of methyl and methylene groups, the only solvent dipoles capable of any interaction with the dipoles of the fluorophore giving rise to the REES effect (in the fluid phase) have to be water molecules that have penetrated deep into the bilayer close to the NBD moiety of NBD-cholesterol. The NBD group in NBD-cholesterol has previously been shown to be localized at ~6 Å from the center of the bilayer[33] which approximately corresponds to carbon position 12 in the acyl chain (see Figure 5). Our results thus address a very important issue in membrane biology, that of whether water penetration occurs in the inner hydrophobic region of the lipid bilayers. Approaches based on fluorescence techniques have proved to be effective in monitoring membrane hydration.[6] Very little data exists in the literature convincingly demonstrating the presence of water in the deeper hydrocarbon region of the membrane.[2] Our result of REES of NBD-cholesterol in fluid phase DPPC membranes thus indicates the presence of water ~6 Å from the bilayer center. We thus show penetration of water in the deeper regions of the membrane, at least in the fluid phase.

A minor concern arises from the artifactual possibility that such water penetration could be a consequence of the polarity of the NBD group. However, this is rather unlikely since the magnitude of REES was found to be independent of probe concentration (in the range of 0.1−2 mol % NBD-cholesterol). Moreover, even in natural membranes, polar residues of integral membrane proteins are often present in membrane hydrophobic regions.[59]

In summary, our results demonstrate the presence of water in the deep hydrocarbon region of the membrane and its modulation by the phase properties of the membrane.

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