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Monitoring the looping up of acyl chain labeled NBD lipids in membranes as a function of membrane phase state

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Abstract

Lipids that are labeled with the NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) group are widely used as fluorescent analogues of native lipids in biological and model membranes to monitor a variety of processes. The NBD group of acyl chain labeled NBD lipids is known to loop up to the membrane interface in fluid phase membranes. However, the organization of these lipids in gel phase membranes is not resolved. In this paper, we monitored the influence of the membrane phase state on the looping up behavior of acyl chain labeled NBD lipids utilizing red edge excitation shift (REES) and other sensitive fluorescence approaches. Interestingly, our REES results indicate that NBD group of lipids, which are labeled at the fatty acyl region, resides in the more hydrophobic region in gel phase membranes, and complete looping of the NBD group occurs only in the fluid phase. This is supported by other fluorescence parameters such as polarization and lifetime. Taken together, our results demonstrate that membrane packing, which depends on temperature and the phase state of the membrane, significantly affects the localization of acyl chain labeled NBD lipids. In view of the wide ranging use of NBD-labeled lipids in cell and membrane biology, these results could have potentially important implications in future studies involving these lipids as tracers.

Keywords: NBD lipids; DPPC; Phase transition; REES; Gel phase; NBD looping

1. Introduction

Lipid probes have proved to be very useful in membrane biology due to their ability to monitor lipid molecules by a variety of physicochemical approaches at increasing spatiotemporal resolution [1]. Fluorescent lipids offer a powerful approach for monitoring organization and dynamics in membranes due to their high sensitivity, suitable time resolution, and multiplicity of measurable parameters. Lipids that are covalently linked to extrinsic fluorophores are commonly used to probe membrane dynamics. The advantage with this approach is that one has a choice of the fluorescent label to be used, and therefore, specific probes with appropriate characteristics can be designed for specific applications. A widely used extrinsic fluorophore in biophysical, biochemical, and cell biological studies of membranes is the NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) group (for a review, see Ref. [2]). NBD-labeled lipids are extensively used as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes [3–10]. The NBD moiety possesses some of the most desirable properties to serve as an excellent probe for both spectroscopic and microscopic applications. It is very weakly fluorescent in water. Upon transfer to a hydrophobic medium, it fluoresces brightly in the visible range and exhibits a high degree of environmental sensitivity [11–15]. More importantly, lipids labeled with the NBD group mimic endogenous lipids in studies of intracellular lipid transport [16,17].

We have earlier used NBD-labeled lipids as efficient probes to explore the organization and dynamics of molecular

Abbreviations: 6-NBD-PC, 1-palmitoyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl)-*sn*-glycero-3-phosphocholine; 12-NBD-PC, 1-palmitoyl-2-(12-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl)-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; NBD-cholesterol, 25-[-N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino]-27-norcholesterol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; REES, red edge excitation shift

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assemblies such as membranes, micelles and reverse micelles utilizing the wavelength-selective fluorescence approach in general, and red edge excitation shift (REES) in particular [4,5,9,15,18,19]. 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, is termed REES. This approach can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system [20–22]. We have earlier shown that REES varies as a function of probe penetration depth in membranes [23], and is a sensitive tool to monitor water penetration in the deep hydrocarbon region of the membrane, at least in the fluid phase [5], using two differentially localized NBD lipid probes, NBD-PE and NBD-cholesterol (see Fig. 1). In NBD-PE, the fluorescent NBD label is covalently attached to the headgroup of a phosphatidylethanolamine molecule. The precise orientation and location of the NBD group in this molecule in fluid phase membranes is known [9,11,24–26]. The NBD group has been found to be localized at the membrane interface which has unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the hydrocarbon-like interior of the membrane [20], thus making it an ideal probe for monitoring red edge effects. Further, previous electrophoretic measurements have shown that the NBD group in NBD-PE is uncharged at neutral pH in the membrane [11]. This would ensure that the NBD group does not project into the external aqueous phase.

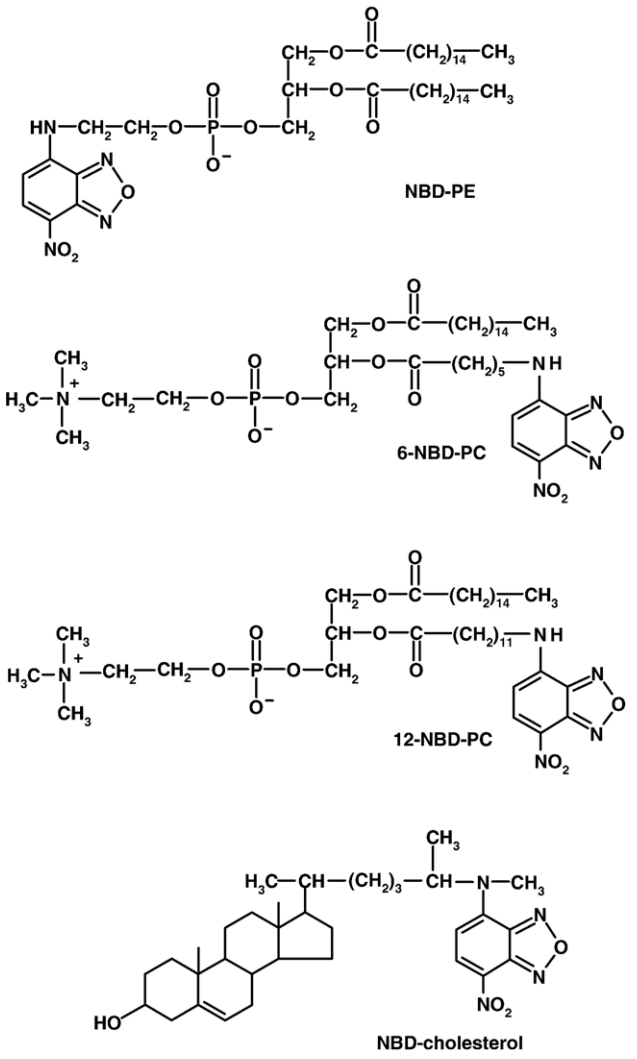

Fig. 1. Chemical structures of NBD-labeled lipids used.

It has been previously shown that the NBD group of acyl chain labeled NBD lipids such as 6- and 12-NBD-PC (Fig. 1) loops up to the membrane interface in fluid phase membranes due to its polarity [8,9,11,24,27]. Further, we have recently shown that 6- and 12-NBD-PC exhibit considerable REES in fluid phase membranes which supports the interfacial localization of the NBD group [9] since it is the membrane interface which is most likely to display red edge effects [20]. In addition, lipids that are labeled with the NBD group both at the headgroup and in the tail region have recently been used by Tsukanova et al. [28] to monitor REES in monolayers at the air/water interface. Interestingly, the looping up of the NBD group in acyl chain labeled NBD lipids has been utilized to monitor lipid–protein interactions in membranes [29]. However, all data showing looping up of the NBD group in acyl chain labeled NBD lipids are in the context of fluid phase membranes. Whether such looping up of the NBD group in acyl chain labeled NBD lipids takes place in gel phase membranes remains an unexplored issue. Interestingly, it has recently been suggested that the NBD group of acyl chain labeled NBD lipids could reside in the deeper hydrocarbon region of the membrane at temperatures below the main phase transition, i.e., in the gel phase [30,31].

In this paper, we monitored the influence of the membrane phase state on the looping up behavior of acyl chain labeled NBD lipids in DPPC membranes using REES and other sensitive fluorescence approaches. Interestingly, our REES results show that NBD group of acyl chain labeled lipids resides in a more hydrophobic region of the membrane in the gel phase. This is supported by other fluorescence parameters such as polarization and lifetime. Our results show that there is a vertical distribution of the NBD group of acyl chain labeled NBD lipids in gel phase DPPC membranes, and complete looping of the NBD group in acyl chain labeled NBD lipids takes place only in the fluid phase i.e., above the phase transition temperature.

2. Materials and methods

2.1. Materials

6-NBD-PC, 12-NBD-PC, DPPC and NBD-cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). NBD-PE was from Molecular Probes (Eugene, OR). DMPC and MOPS were obtained from Sigma (St. Louis, MO). Lipids were checked for purity by thin layer chromatography on silica gel precoated plates (Sigma) in chloroform/methanol/water (65:35:5, v/v/v), and were found to give only one spot in all cases with a phosphate-sensitive spray and on subsequent charring [32]. The concentration of stock solution of DPPC was determined by phosphate assay subsequent to total digestion by perchloric acid [33]. DMPC was used as an internal standard to assess lipid digestion. The purity of NBD-labeled lipids was checked by thin layer chromatography in chloroform/methanol/water (65:25:4, v/v/v) and were found to be pure when detected by their color or fluorescence. Concentrations of stock solutions of

NBD-labeled lipids in methanol were estimated using molar extinction coefficients [9,34] of 21,000 M^{-1} cm^{-1} for NBD-PE and 6-NBD-PC at 463 and 465 nm, 22,000 M^{-1} cm^{-1} for 12-NBD-PC and NBD-cholesterol at 465 and 484 nm. All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

2.2. Methods

2.2.1. Sample preparation

All experiments were done using large unilamellar vesicles (LUVs) of 100 nm diameter of DPPC containing 1 mol% NBD-labeled lipids (0.3 mol% in experiments using NBD-cholesterol). In general, 640 nmol of DPPC in methanol was mixed with 6.4 nmol (1.92 nmol of NBD-cholesterol) of the specific NBD-labeled lipid in methanol. The sample was mixed well and dried under a stream of nitrogen while being warmed gently (~35 °C). After further drying under a high vacuum for at least 6 h, 1.5 ml of 10 mM MOPS, 150 mM sodium chloride, pH 7.2 buffer was added, and each sample was vortexed for 3 min to disperse the lipid and form homogeneous multilamellar vesicles. The temperature of the buffer was maintained higher than the phase transition temperature of DPPC (i.e., >41 °C) while vortexing the samples. LUVs of diameter of 100 nm were prepared by the extrusion technique using an Avestin Liposofast Extruder (Ottawa, Ontario, Canada) as previously described [9]. Briefly, the multilamellar vesicles were freeze-thawed five times using liquid nitrogen to ensure solute equilibration between trapped and bulk solutions, and then extruded through polycarbonate filters (pore diameter 100 nm) mounted in the extruder fitted with Hamilton syringes (Hamilton Company, Reno, NV). The samples were subjected to 11 passes through polycarbonate filters to give the final LUV suspension. Samples were incubated in dark for 12 h at room temperature (~23 °C) for equilibration before measuring fluorescence. Background samples were prepared in the same way except that fluorophore (NBD-labeled lipid) was not added to them.

2.2.2. Steady state fluorescence measurements

Steady state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. This instrument is equipped with a magnetically stirred cuvette compartment thermostated with a circulating water bath. While heating, the sample temperature was continuously measured with a thermocouple. Excitation and emission slits with a nominal bandpass of 5 nm were used for all measurements. Background intensities of samples in which NBD-labeled lipids were omitted were negligible in most cases and were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. 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. Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from the equation [35]:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}.

2.2.3. Time-resolved fluorescence measurements

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology International (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single photon counting mode. This machine uses a thyratron-gated nanosecond flash lamp filled with nitrogen as the plasma gas (16±1 in. of mercury vacuum) and is run at 19–22 kHz. This instrument is equipped with a magnetically stirred cuvette compartment thermostated with a circulating water bath. Lamp profiles were measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. To optimize the signal to noise ratio, 10000 photon counts were collected in the peak channel. The excitation wavelength used was 465 nm. Emission wavelength was set at 530 nm. All experiments were performed using excitation and emission slits with a bandpass of 6 nm or less. The sample and the scatterer were alternated after every 5% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. This arrangement also prevents any prolonged exposure of the sample to the excitation beam thereby avoiding any possible photodamage of the fluorophore. The data stored in a multichannel analyzer was routinely transferred to an IBM PC for analysis. Intensity decay curves so obtained were fitted as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (2)$$

where $F(t)$ is the fluorescence intensity at time t and α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i such that $\Sigma_i \alpha_i = 1$. The decay parameters were recovered using a nonlinear least squares iterative fitting procedure based on the Marquardt algorithm as described previously [6]. A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value not more than 1.4. Mean (average) lifetimes $<\tau>$ for biexponential decays of fluorescence were calculated from the decay times and preexponential factors using the following equation [35]:

$$<\tau> = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2}. \quad (3)$$

3. Results

The chemical structures of the NBD-labeled lipids used in this study are shown in Fig. 1. The NBD group is covalently attached to the *sn*-2 fatty acyl chain in 6- and 12-NBD-PC. In NBD-PE, the fluorescent NBD label is covalently attached to the headgroup of a phosphatidylethanolamine molecule. On the other hand, the NBD group is covalently attached to the flexible chain of the cholesterol molecule in case of NBD-cholesterol. It has earlier been shown that the NBD group of NBD-PE localizes in the membrane interface (~19–20 Å from the center of the fluid bilayer) [9,25]. In contrast, the NBD group of NBD-cholesterol has been shown to be localized in the hydrocarbon region of the membrane [4,5,11,34], i.e., ~5–6 Å from the center of the fluid bilayer [24]. In other words, the NBD group of NBD-cholesterol has been shown not to loop back either due to the rigidity of the sterol ring and/or the reduction of hydrophilicity due to the methyl group attached to NBD [2]. The NBD-cholesterol used here should not be confused with a closely related analogue of cholesterol, 22-NBD-cholesterol (22-[-N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3β-ol), in which the NBD group is attached to a shorter alkyl chain. 22-NBD-cholesterol has previously shown to be not a faithful mimic of cholesterol [36]. The differential localization of the NBD group in NBD-PE and NBD-cholesterol in membranes has been shown to influence the fluorescence properties due to difference in microenvironment [5]. We have therefore used these two probes as controls to monitor the looping up of the NBD group in acyl chain labeled NBD lipids (6- and 12-NBD-PC) in DPPC vesicles in conditions above and below the phase transition temperature.

3.1. Fluorescence of NBD-labeled lipids as a function of membrane phase state

The fluorescence emission maxima[1] of NBD-labeled lipids as a function of increasing temperature across the gel-to-fluid phase transition of DPPC vesicles are shown in Table 1. As seen in the table, the fluorescence emission maximum of NBD-PE (530 nm) remains invariant as a function of temperature and is independent of the phase state of the membrane. This result is not surprising in view of the fact that changes in membrane organization brought about by phase transition (or temperature) are largely restricted to the fatty acyl region of the membrane [37] and are not sensed by the NBD moiety, which is located at the membrane interface. In contrast, 6-NBD-PC, 12-NBD-PC, and NBD-cholesterol exhibit a change in the fluorescence emission maximum upon increasing the temperature in DPPC vesicles. This indicates a change in the immediate environment around the excited state NBD group under these conditions. The effect of increasing temperature across the gel-to-fluid phase transition of DPPC vesicles on the fluorescence intensity of NBD-labeled lipids is shown in Fig. 2. Fluorescence intensities of NBD-labeled lipids were normalized with respect to the value obtained at 20 °C (gel phase) in all cases. Fig. 2 shows that the fluorescence intensity of NBD-labeled lipids decreases significantly beyond the phase transition temperature of DPPC (~40 °C) in all cases which could be attributed to an increase in the non-radiative decay of the fluorophore upon increasing temperature. In case of NBD-PE and NBD-cholesterol, there is no appreciable change in fluorescence intensity when the temperature is increased from 20 to 40 °C. Interestingly, the fluorescence intensities of 6- and 12-NBD-PC increase dramatically (~2 fold) in this temperature range, which is in agreement with an earlier report [38]. It has been shown that quantum yield of the NBD group depends on the site of attachment and the phase state of host membranes with maximum values around the gel-to-fluid phase transition of lipids [38]. The increase in fluorescence intensity of 6- and 12-NBD-PC could therefore be due to an increase in quantum yield with temperature. An alternate explanation could be that the fluorescence of the NBD group is self quenched in the gel phase due to aggregation, and the self quenching is released as the transition temperature is approached [30]. Interestingly, since the change in fluorescence intensity below the phase transition temperature is significant only for 6- and 12-NBD-PC, it could indicate a change in the location of these probes induced by temperature and upon gel-to-fluid phase transition.

3.2. Red edge excitation shift (REES) of NBD-labeled lipids as a function of membrane phase state

Red edge excitation shift (REES) is a powerful approach which can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system [20–22]. This effect is mostly observed with polar fluorophores in motionally restricted environments such as 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. REES arises due to slow rates of solvent relaxation (reorientation) around an excited state fluorophore, which is dependent on 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 (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.

The unique feature about REES is that while all other fluorescence techniques (such as fluorescence quenching, energy transfer, and polarization measurements) yield information about the fluorophore (either intrinsic or extrinsic) itself, REES provides information about the relative rates of solvent (water in biological systems) relaxation dynamics which is not possible to obtain by other techniques. This makes REES extremely useful since hydration plays a crucial modulatory role in a large number of important cellular events including protein folding, lipid–protein interactions and ion transport [39]. Results from our group [5,9,15,18–20,22,23,34,40,41] and other groups [28,42,43] have previously shown that REES serves as a powerful tool to monitor the organization and dynamics of fluorescent probes, peptides and proteins bound to membranes and membrane-mimetic media such as micelles and reverse micelles. In particular, REES has been shown to be a powerful tool to monitor the 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 in membranes [5,23]. For example, the magnitude of REES obtained for membrane-bound NBD-PE and NBD-cholesterol has been shown to vary in direct correlation with the locations of the NBD group in the membrane [5], i.e., the magnitude of REES was more pronounced when the NBD group was localized at the membrane interface. Localization of the NBD group at the deeper regions of the membrane resulted in reduced REES.

Table 1
Fluorescence emission maximum of NBD-labeled lipids in DPPC membranes with increasing temperature[a]

Temperature (°C)	NBD-PE	NBD-cholesterol	6-NBD-PC	12-NBD-PC
20	530	522	535	535
30	530	522	534	534
35	530	521	532	533
40	530	520	532	533
45	530	518	532	532
50	530	517	532	532
60	530	517	531	532

The concentration of DPPC was 0.43 mM and the concentration of NBD lipids used was 1 mol% of the total lipid in all cases except for NBD-cholesterol (0.3 mol%).

[a] The excitation wavelength was 465 nm in all cases.

[1] We have used the term maximum of fluorescence emission in a somewhat wider sense here. In every case, we have monitored the wavelength corresponding to maximum fluorescence intensity, as well as the center of mass of the fluorescence emission. In most cases, both these methods yielded the same wavelength. In cases where minor discrepancies were found, the center of mass of emission has been reported as the fluorescence maximum.

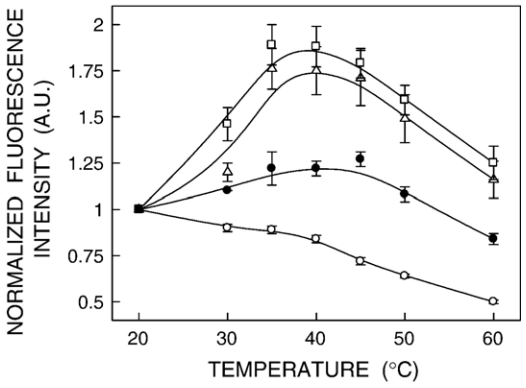

Fig. 2. Effect of increasing temperature across the gel-to-fluid phase transition of DPPC large unilamellar vesicles on fluorescence intensities of NBD-PE (○), 6-NBD-PC (Δ), 12-NBD-PC (□), and NBD-cholesterol (●). Fluorescence intensities of NBD-labeled lipids were normalized with respect to the value obtained at 20 °C in all cases. Data shown are the means ± S.E. of at least three independent measurements. The concentration of DPPC was 0.43 mM and the concentration of NBD lipids used was 1 mol% of the total lipid in all cases except for NBD-cholesterol (0.3 mol%).

Fig. 3 shows the effect of changing excitation wavelength on the wavelength of maximum emission for membrane-bound NBD lipids in representative gel (20 °C) and fluid (50 °C) phase DPPC vesicles. Fig. 3a shows that the fluorescence emission maximum of NBD-PE is shifted from 530 to 536 nm upon changing the excitation wavelength from 465 to 515 nm, which corresponds to a REES of 6 nm, irrespective of the physical state of the membrane. In contrast, the emission maximum of NBD-cholesterol in the gel phase (20 °C) DPPC membranes is at 522 nm and does not exhibit any red shift upon changing the excitation wavelength from 465 to 507 nm (Fig. 3b). In other words, NBD-cholesterol does not exhibit any REES under these conditions. We chose to use 0.3 mol% of NBD-cholesterol in our experiments to avoid any complication due to the formation of NBD-cholesterol dimers in membranes (4,6,34). It should be mentioned here that the fluorescence of NBD-cholesterol is relatively weak [11], and we found it difficult to work in excitation wavelengths longer than 507 nm due to low signal-to-noise ratio and artifacts due to the Raman peak that remained even after background subtraction. Moreover, it was not possible to excite NBD-cholesterol beyond 507 nm due to smaller Stokes' shift.

Interestingly, the fluorescence emission maximum of 6- and 12-NBD-PC is shifted from 535 to 536 nm upon changing the excitation wavelength from 465 to 515 nm, which corresponds to a REES of 1 nm at 20 °C (see Figs. 3c and d). The magnitude of REES of acyl chain labeled NBD lipids is therefore negligible and comparable to that of NBD-cholesterol at 20 °C. This result suggests that the NBD group in 6- and 12-NBD-PC is located in a more hydrophobic environment, similar to that of NBD-cholesterol under these conditions (see above). This is surprising since it has been shown that NBD group in 6- and 12-NBD-PC is localized at a shallow interfacial region in fluid phase membranes [8,9,11,24,27]. The reduced REES for 6- and 12-NBD-PC may therefore suggest that, unlike in fluid

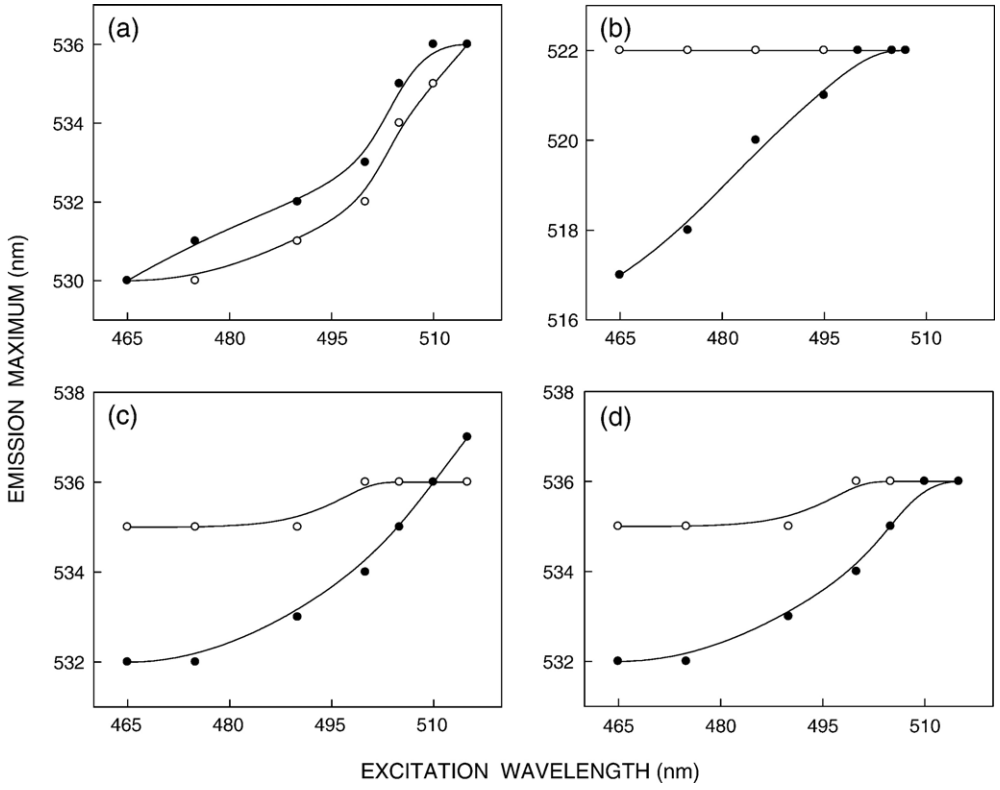

Fig. 3. Effect of changing excitation wavelength on the wavelength of maximum emission for NBD-labeled lipids in DPPC vesicles. The plots correspond to (a) NBD-PE, (b) NBD-cholesterol, (c) 6-NBD-PC, and (d) 12-NBD-PC at 20 (○) and 50 (●) °C. All other conditions are as in Fig. 2. See Materials and methods for other details.

phase membranes, the NBD group in these lipids is localized at a deeper hydrophobic environment in the membrane at 20 °C where the membrane is in the gel phase. On the other hand, we observe changes in fluorescence emission maximum of NBD-cholesterol, and 6- and 12-NBD-PC with increasing excitation wavelength in the fluid phase (50 °C) DPPC membranes. Figs. 3b–d shows that the emission maxima are shifted from 517 to 522, 532 to 537, and 532 to 536 nm for NBD-cholesterol, and 6- and 12-NBD-PC, respectively, upon increasing the excitation wavelength. This corresponds to REES of 4–5 nm in these cases. The observation of REES for these lipid probes in fluid phase (50 °C) DPPC membranes could be attributed either to increase in water penetration or a change in probe location (vertical distribution) upon gel-to-fluid phase transition. To distinguish between these possibilities, we performed REES experiments at graded temperatures across the gel-to-fluid phase transition of DPPC vesicles.

Fig. 4 shows the effect of increasing temperature across the gel-to-fluid phase transition of DPPC vesicles on the magnitude of REES of NBD-labeled lipids. As expected, the magnitude of REES of NBD-PE remains invariant over the entire temperature range (20 to 60 °C) and is independent of the phase state of the membrane. As mentioned earlier, this is not surprising since changes in membrane organization brought about by phase transition are largely restricted to the fatty acyl region of the membrane [37]. In addition, it has been previously reported that the rates of solvent relaxation for probes that are not deeply buried in the hydrocarbon interior of the membrane are more or less independent of temperature [44]. In case of NBD-cholesterol, the magnitude of REES does not change appreciably when the temperature is raised from 20 to 40 °C, beyond which there is a considerable increase in REES (up to 5 nm). The fact that NBD-cholesterol exhibits negligible REES up to 40 °C could be due to the more compact arrangement of lipid acyl chains in the gel phase which results in reduced 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 fluorophore dipole giving rise to REES effects in the fluid phase have to be water molecules that have penetrated deep into the bilayer close to the NBD group of NBD-cholesterol.

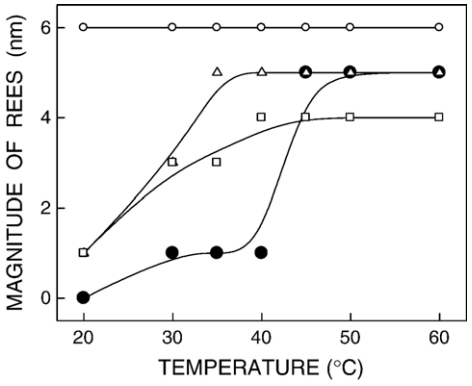

Fig. 4. Effect of increasing temperature across the gel-to-fluid phase transition of DPPC large unilamellar vesicles on the magnitude of REES of NBD-PE (○), 6-NBD-PC (Δ), 12-NBD-PC (□), and NBD-cholesterol (●). All other conditions are as in Fig. 2. See Materials and methods for other details.

Interestingly, the magnitude of REES of 6- and 12-NBD-PC increases continuously as a function of increasing temperature from 20 to 40 °C, i.e., even in gel phase membranes. However, there is no further change in the magnitude of REES of acyl chain labeled NBD lipids at higher temperatures (>40 °C) in DPPC vesicles. We therefore interpret the observation of REES in acyl chain labeled NBD lipids in gel phase membranes to be suggestive of a change in the vertical location of the fluorophore induced by the change in membrane phase state. Taken together, these novel results point out the tendency of the NBD group in acyl chain labeled NBD lipids to loop up as a function of phase transition. This would indicate that the average location of the NBD group in these lipids will significantly differ in fluid and gel phase membranes. While looping up in fluid phase membranes is well documented [8,9,11,24,27], our results indicate that the NBD group in these lipids resides in deeper regions of the membranes in the gel state. It is envisioned that at any given condition, a distribution of these two locations exists and the balance shifts as the phase state of the membrane changes from gel to fluid. In a control experiment, we monitored REES at 50 °C (fluid phase) followed by measurement at 20 °C (gel phase) and we found that the results are independent of the order in which the experiments were carried out.

3.3. Fluorescence polarization and lifetimes of NBD-labeled lipids as a function of membrane phase state

Fluorescence polarization is a useful parameter in providing information about the rotational mobility of a fluorophore [35] which in turn is sensitive to the lipid acyl chain packing in membranes. The effect of gel-to-fluid phase transition of DPPC vesicles on steady state fluorescence polarization of NBD-labeled lipids is shown in Fig. 5. All NBD-labeled lipids exhibit a reduction in fluorescence polarization upon gel-to-fluid phase transition indicating a change in the membrane order and packing. It is interesting to note that the difference in polarization values (ΔP) from 20 to 60 °C is much higher (~2-fold) for NBD-PE (0.17) and NBD-cholesterol (0.14) compared to 6-(0.09) and 12-NBD-PC (0.07). The higher values of ΔP in case of NBD-PE and NBD-cholesterol could be due to the precise localization of these probes in DPPC membranes so that the polarization change is reflective only of the change in membrane packing induced by phase transition. On the other hand, smaller ΔP values of acyl chain labeled NBD lipids could be attributed to their looping up behavior (i.e., a change in location) when DPPC membranes undergo phase transition. This could be rationalized as follows: the change in polarization values due to the general membrane packing difference induced by phase transition would be counteracted by the change in polarization values induced by looping up of the NBD group. The simultaneous change in both the membrane order as well as the localization of the NBD group could therefore result in lesser ΔP for acyl chain labeled NBD lipids. Since membranes exhibit a mobility gradient along their vertical axis [20], these results support the looping up of acyl chain labeled NBD lipids

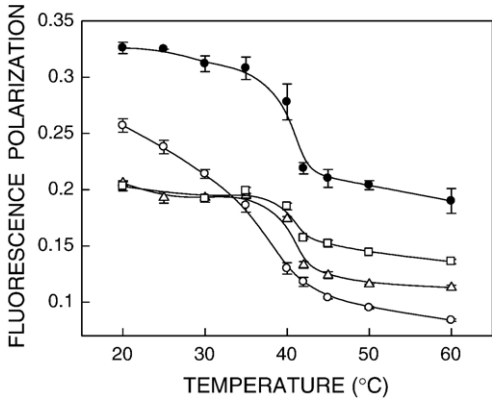

Fig. 5. Effect of increasing temperature across the gel-to-fluid phase transition of DPPC vesicles on the fluorescence polarization of NBD-PE (○), 6-NBD-PC (Δ), 12-NBD-PC (□), and NBD-cholesterol (●). Polarization values were recorded at 530 nm in all cases except for NBD-cholesterol (522 nm), and the excitation wavelength used was 465 nm. Data shown are the means±S.E. of at least three independent measurements. All other conditions are as in Fig. 2. See Materials and methods for other details.

when DPPC membranes undergo gel-to-fluid phase transition, and are in agreement with REES results (see Fig. 4).

Fluorescence lifetime serves as a faithful indicator of the local environment in which a given fluorophore is placed [45]. In addition, it is well known that the fluorescence lifetime of the NBD group is sensitive to the environment in which it is placed [12,15]. The lifetimes of NBD-PE incorporated in various membranes have previously been reported to be ~8 ns [9,46,47]. In pure water, NBD lifetime reduces to ~1.5 ns, which has been attributed to hydrogen bonding interactions between the fluorophore and the solvent [12] which is accompanied by an increase in the rate of nonradiative decay [38]. The fluorescence lifetimes of NBD-labeled lipids incorporated in DPPC membranes as a function of temperature are shown in Table 2. The mean fluorescence lifetimes were calculated using Eq. (3) and are shown in Fig. 6. As shown in the figure, there is a considerable decrease in mean fluorescence lifetimes of NBD-PE (~40%) and NBD-cholesterol (~54%) with increasing temperature indicating a change in polarity around the excited state of the NBD group in DPPC vesicles. The change in polarity could be due to increased water penetration in DPPC membranes undergoing phase transition from gel to fluid phase. In case of acyl chain labeled NBD-lipids, the corresponding change in mean fluorescence lifetime is significantly less (<15%). These results suggest that the environment around the NBD group of 6- and 12-NBD-PC is quite different. The relative insensitivity of fluorescence lifetimes of acyl chain labeled NBD lipids with temperature further reinforces the possibility of vertical distribution of fluorophores in membranes at different temperatures while undergoing phase transition.

In order to ensure that the observed change in steady state polarization of NBD lipids in DPPC membranes as a function of increasing temperature is not due to any change in lifetime with increasing temperature (see above and Table 2), the apparent (average) rotational correlation times for NBD lipids in DPPC membranes at increasing temperatures were calculated using Perrin's equation [35]:

$$\tau_c = \frac{\langle\tau\rangle r}{r_o - r} \quad (4)$$

where r_o is the limiting anisotropy of the NBD group, r is the steady state anisotropy (derived from the polarization values using $r=2P/(3-P)$, and $\langle\tau\rangle$ is the mean fluorescence lifetime as taken from Table 2. Although Perrin's equation is not strictly applicable to this system, it is assumed that this equation will apply to a first approximation, especially because we have used mean fluorescence lifetimes for the analysis of multiple component lifetimes. The values of the apparent rotational correlation times, calculated this way using a value of 0.354 [9] are shown in Table 2. It is clear that the change in rotational correlation times for NBD-PE (10.23 to 1.25 ns) and NBD-cholesterol (9.27 to 1.34 ns) is significantly higher when compared to acyl chain labeled NBD lipids. In addition, the change in the rotational correlation times for NBD-PE and NBD-cholesterol is drastic when the temperature is increased

Table 2
Fluorescence lifetimes[a] and apparent rotational correlation times[b] of NBD-labeled lipids in DPPC membranes as a function of temperature

Temperature (°C)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)	χ^2	τ_c (ns)
(a) NBD-PE							
20	0.40	11.71	0.60	4.49	9.07	1.21	10.23
30	0.47	10.94	0.53	4.47	8.90	1.16	6.82
35	0.47	10.62	0.53	4.93	8.66	1.32	5.16
40	0.56	8.76	0.44	4.29	7.52	1.28	2.60
45	0.55	7.37	0.45	3.85	6.32	1.18	1.69
50	0.46	6.69	0.54	3.77	5.53	1.22	1.25
(b) 6-NBD-PC							
20	0.19	9.41	0.81	4.10	5.96	1.09	4.24
30	0.14	9.83	0.86	4.48	5.89	1.24	3.74
35	0.19	9.29	0.81	4.59	6.10	1.19	3.93
40	0.14	10.02	0.86	4.87	6.16	1.37	3.30
45	0.14	8.87	0.86	5.41	6.14	1.21	1.98
50	0.11	7.52	0.89	4.86	5.29	1.30	1.55
(c) 12-NBD-PC							
20	0.18	8.21	0.82	3.31	5.04	1.22	3.50
30	0.24	7.61	0.76	3.94	5.33	1.09	3.36
35	0.20	7.71	0.80	4.13	5.27	1.12	3.53
40	0.10	8.85	0.90	4.32	5.16	1.29	3.05
45	0.04	9.20	0.96	4.57	4.93	1.18	2.13
50	0.01	11.63	0.99	4.07	4.28	1.35	1.70
(d) NBD-cholesterol							
20	0.08	9.19	0.92	2.71	4.19	1.15	9.27
30	0.10	8.08	0.90	2.59	4.00	1.22	7.58
35	0.07	7.01	0.93	2.04	3.06	1.19	5.59
40	0.06	6.10	0.94	1.89	2.61	1.32	3.54
45	0.05	4.54	0.95	1.69	2.04	1.37	1.50
50	0.05	4.46	0.95	1.53	1.92	1.28	1.34

[a] The average values of mean fluorescence lifetime were calculated using Eq. (3). The excitation wavelength was 465 nm in all cases. The concentration of DPPC was 0.43 mM and the concentration of NBD lipids used was 1 mol% of the total lipid in all cases except for NBD-cholesterol (0.3 mol%).

[b] Calculated from Eq. (4). See Results for other details.

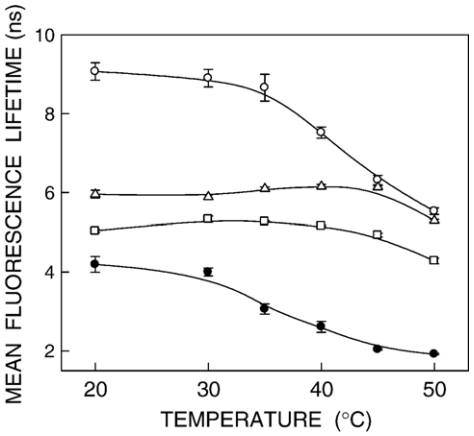

Fig. 6. Effect of increasing temperature across the gel-to-fluid phase transition of DPPC vesicles on the mean fluorescence lifetime of NBD-PE (○), 6-NBD-PC (Δ), 12-NBD-PC (□), and NBD-cholesterol (●). Data shown are the means± S.E. of at least three independent measurements. The average values of mean fluorescence lifetime were calculated using the recovered parameters of the fluorescence intensity decay shown in Table 2 and Eq. (3). The excitation wavelength used was 465 nm, and the emission was set at 530 nm in all cases except for NBD-cholesterol (522 nm). All other conditions are as in Fig. 2. See Materials and methods for other details.

from 20 to 40 °C. It is interesting to note that this change is similar to what we had observed with steady state fluorescence polarization values. Overall, the rotational correlation times of NBD lipids in DPPC membranes as a function of increasing temperature indicate that the observed changes in polarization values (Fig. 5) are not due to any lifetime-induced artifacts.

4. Discussion

The phospholipid main phase transition (gel-to-fluid) is accompanied by a change in lipid headgroup area, lipid acyl chain order and segmental motion, membrane thickness, membrane permeability, extent of water penetration and membrane packing [48,49]. Interestingly, the change in membrane packing has been shown to affect the localization of probes incorporated into membranes [27]. It is well known that acyl chain labeled NBD lipids loop up to the membrane interface in fluid phase membranes [8,9,11,24,27,50]. In addition, we recently showed, by analyses of membrane penetration depths and REES data, that NBD groups covalently attached to the fatty acyl chain region of a number of lipids loop up to the membrane interface, irrespective of the nature of the headgroup [9]. An important consequence of the looping up of the NBD group is an increase in the headgroup area. For example, it has been estimated that in POPC membranes, looping up of the NBD group results in a ~3% increase in the headgroup area [8]. The nature of molecular interaction responsible for looping up of acyl chain labeled NBD group could be hydrogen bonding of the NBD group at the membrane interface. The polar imino group and the oxygen atoms of the NBD group may form hydrogen bonds with the lipid carbonyls, interfacial water molecules, and the lipid headgroup. This is similar to the well-known tendency of tryptophan residues in membrane proteins and peptides to be localized interfacially due to favorable interaction of the aromatic tryptophan group with the membrane interface [22,51].

Although the location of the NBD group in acyl chain labeled NBD lipids (6- and 12-NBD-PC) is well characterized in fluid phase membranes, the organization of these lipids in gel phase membranes is still not resolved. In this paper, using REES and other sensitive fluorescence approaches, we monitored the influence of the membrane phase state on the looping up of the NBD group in acyl chain labeled NBD lipids. In these experiments, we used the previously characterized NBD lipids [5], NBD-PE and NBD-cholesterol, as control probes in which the NBD groups are differentially localized in the membrane. Our results show that the NBD group of NBD-PE, which localizes in the membrane interface, shows a phase-independent REES of 6 nm in both gel and fluid phase DPPC vesicles. Interestingly, 6- and 12-NBD-PC exhibits a REES of 1 nm in gel phase (at 20 °C). This correlates with the localization of these probes in a hydrophobic environment since NBD-cholesterol (in

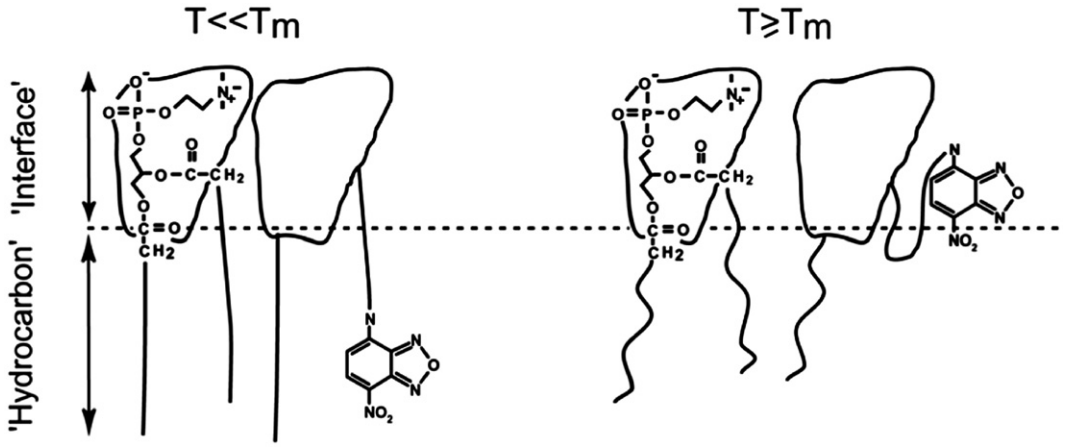

Fig. 7. A schematic representation of the organization (looping up) of the NBD group of acyl chain labeled NBD lipids as a function of phase state of the membrane. While the NBD group completely loops up to the membrane interface in fluid phase ($T \geq T_m$) membranes, it may localize in deeper regions of the membranes in the gel phase ($T << T_m$). It should be noted that although the NBD group is shown to localize in a single location in gel phase membranes, it actually represents a vertical distribution along the membrane axis.

which the NBD group is known to be localized at a deeper hydrophobic region of the membrane) at this temperature exhibits no REES (see Figs. 3 and 4). Interestingly, these results are in agreement with Alakoskela and Kinnunen [30] who suggested two populations of NBD-PC below the main phase transition [30,31] using wavelength-dependent anisotropy measurements. Upon increasing the temperature from 20 to 40 °C, the magnitude of REES of 6- and 12-NBD-PC increases by 3–4 nm, compared to 1 nm in case of NBD-cholesterol at this temperature range (Fig. 4). We attribute these results to the looping up of acyl chain labeled NBD lipids as a function of increasing temperature below the phase transition temperature, and complete looping up to the membrane interface occurs only at the fluid phase (see Fig. 7). This is supported by fluorescence polarization and time-resolved fluorescence measurements (Figs. 5 and 6). These results could be explained by the fact that increased membrane packing in gel phase DPPC vesicles would decrease the propensity of the NBD group to loop up completely to the membrane interface since an interfacial location of the NBD group would require a significant reduction in membrane packing [27]. The looping up of the NBD group of acyl chain labeled NBD lipids is therefore favorable only in fluid phase membranes.

In summary, we demonstrate that membrane packing, which depends on temperature and the phase state of the membrane, significantly affects the localization of acyl chain labeled NBD lipids. In view of the wide ranging use of NBD-labeled lipids in membrane biology, our results could have potentially important implications in future studies involving these lipids as tracers.

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