



Organization and dynamics of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labeled lipids: a fluorescence approach

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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. NBD-labeled lipids have previously been used to monitor the organization and dynamics of molecular assemblies such as membranes, micelles and reverse micelles utilizing the wavelength-selective fluorescence approach. In this paper, we have characterized the organization and dynamics of various NBD-labeled lipids using red edge excitation shift (REES) and other fluorescence approaches which include analysis of membrane penetration depths of the NBD group using the parallax method. We show here that the environment and location experienced by the NBD group of the NBD-labeled lipids could depend on the ionization state of the lipid. This could have potentially important implications in future studies involving NBD-labeled lipids as tracers in a cellular context.

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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 (Chattopadhyay, 2002). 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. A widely used extrinsic fluorophore in biophysical, biochemical, and cell biological studies

of membranes is the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group (for a review, see Chattopadhyay, 1990). NBD-labeled lipids are extensively used as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes (Chattopadhyay, 1990; Mazeris et al., 1996; Rukmini et al., 2001). The NBD moiety possesses some of the most desirable properties to serve as an excellent probe for both spectroscopic and microscopic applications (Chattopadhyay et al., 2002).

We have earlier used NBD-labeled lipids as efficient probes to explore the organization and dynamics of molecular assemblies such as membranes, micelles and reverse micelles utilizing the wavelength-selective fluorescence approach (Chattopadhyay and Mukherjee, 1993, 1999; Rawat et al., 1997;

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Chattopadhyay et al., 2002). A major reason for choosing NBD-labeled lipids for such studies is the environmental sensitivity of NBD fluorescence. Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy which can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system (Mukherjee and Chattopadhyay, 1995; Chattopadhyay, 2003; Raghuraman et al., 2003). 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 red edge excitation shift (REES) (Demchenko, 2002; Mukherjee and Chattopadhyay, 1995; Chattopadhyay, 2003; Raghuraman et al., 2003). 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.

We have previously shown that REES and related techniques (wavelength-selective fluorescence approach) serve as powerful tools to monitor the organization and dynamics of probes and peptides bound to membranes, micelles and reverse micelles (Chattopadhyay and Mukherjee, 1993, 1999; Kelkar et al., in press; Rawat et al., 1997; Chattopadhyay et al., 2002; Raghuraman and Chattopadhyay, in press). In our previous work, we used NBD-PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine) as a probe to explore organization and dynamics of membranes (Chattopadhyay and Mukherjee, 1993, 1999), micelles (Rawat et al., 1997) and reverse micelles (Chattopadhyay et al., 2002) utilizing the wavelength-selective fluorescence approach in general, and REES in particular. 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 of this molecule in the membrane is known (Chattopadhyay and London, 1987, 1988; Wolf et al., 1992; Abrams and London, 1993). 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 (Perochon et al., 1992), thus making

it an ideal probe for monitoring red edge effects. Furthermore, previous electrophoretic measurements have shown that the NBD group in NBD-PE is uncharged at neutral pH in the membrane (Chattopadhyay and London, 1988). This would ensure that the NBD group does not project into the external aqueous phase. This is advantageous since the ability of a fluorophore to exhibit red edge effects could very well be dependent on its precise location in the membrane (see later).

In addition, the change in dipole moment of the NBD group upon excitation, a necessary condition for a fluorophore to exhibit REES, has been found to be ~ 4 D (Mukherjee et al., 1994). This makes NBD-labeled probes suitable for monitoring REES in chosen systems. In another study, we reported REES of membrane-bound NBD-cholesterol, in which the NBD group is covalently attached to the flexible chain of the cholesterol molecule (Chattopadhyay and Mukherjee, 1999). 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. (2002) to monitor REES in monolayers at the air/water interface. In yet another study, REES of NBD-labeled peptide fragment of apolipoprotein C-II bound to membranes has been used to monitor the insertion of the peptide into the motionally restricted membrane interface (MacPhee et al., 1999).

In view of the wide ranging use of NBD-labeled lipids in membrane biology and the suitability of the NBD group in studies utilizing the wavelength-selective fluorescence approach, it would be of interest to monitor the organization and dynamics of various lipids labeled with the NBD group both at the headgroup and the tail region. For this purpose, we used NBD-PE and NBD-PS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)) which are labeled at the headgroup with the NBD group. The other lipids used include 6-NBD-PC (1-palmitoyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl)-*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), 6-NBD-SM (6-([*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl)sphingosylphosphocholine), and 6-NBD-CM (6-([*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl)sphingosine). The chemical structures of these lipids used are shown in Fig. 1. In this paper, we have characterized the

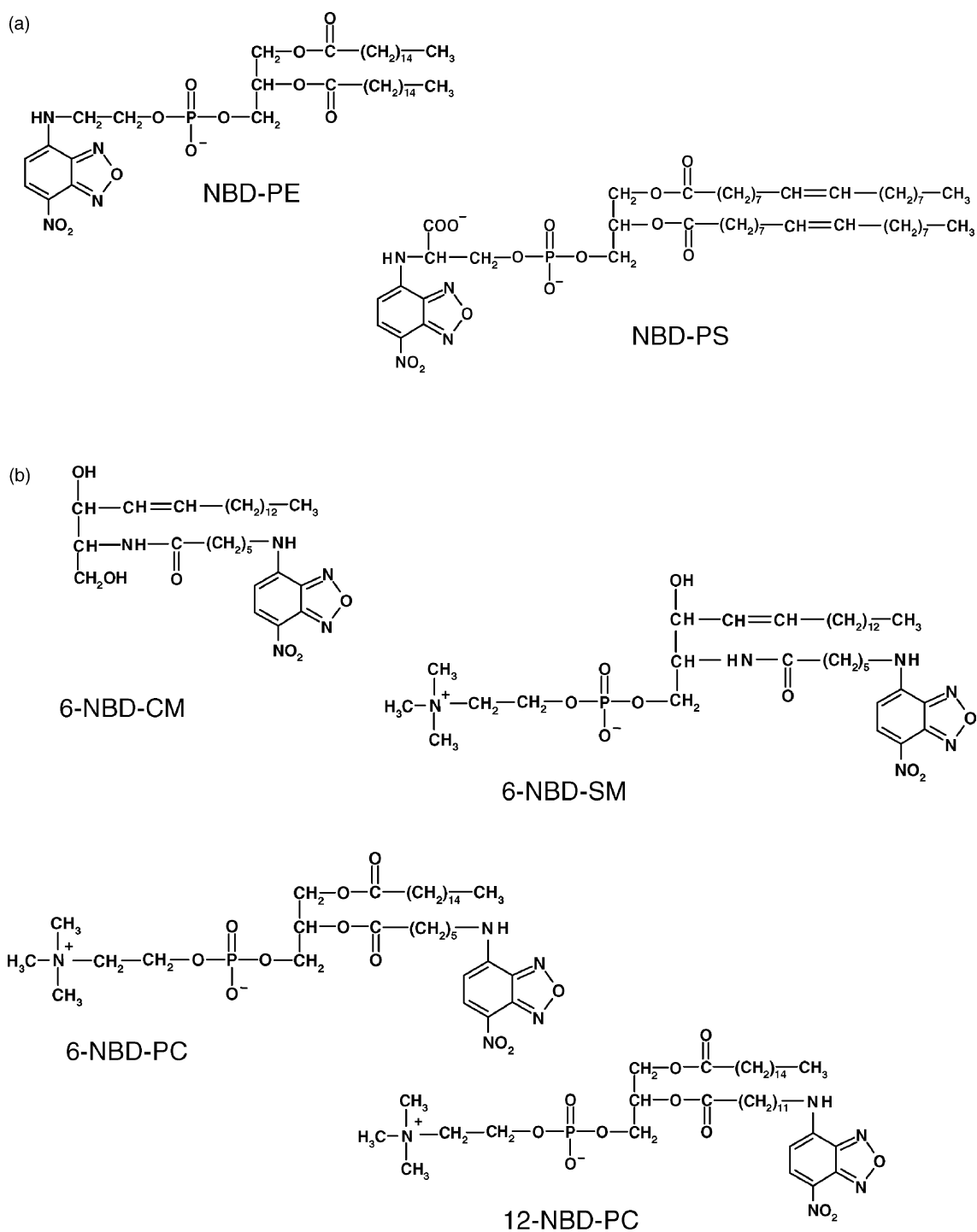


Fig. 1. Chemical structures of NBD-labeled lipids used: (a) the NBD group is attached to the polar lipid headgroup, and (b) the NBD group is attached to the *sn*-2 fatty acyl chain of the lipid.

organization and dynamics of various NBD-labeled lipids using REES and other fluorescence approaches. In addition, we have analyzed the depths of penetration of the NBD group in the membrane using the parallax method (Chattopadhyay and London, 1987). Our results show that the environment and location of the NBD group in NBD-labeled lipids could depend on the net charge of the lipid under experimental conditions.

2. Experimental procedures

2.1. Materials

DOPC (dioleoyl-*sn*-glycero-3-phosphocholine), Tempo-PC (dioleoyl-*sn*-glycero-3-phosphotempocholeline), 5-PC (1-palmitoyl-2-(5-doxy)stearoyl-*sn*-glycero-3-phosphocholine), NBD-PS, 6-NBD-PC and 12-NBD-PC were obtained from Avanti Polar Lipids (Alabaster, AL, USA). NBD-AHA (6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid), NBD-PE, 6-NBD-CM, 6-NBD-SM, 2-AS (2-(9-anthroyloxy)stearic acid), and 12-AS (12-(9-anthroyloxy)stearic acid) were from Molecular Probes (Eugene, OR, USA). The purity of lipids and concentration of stock solution of DOPC were checked as described previously (Rukmini et al., 2001). 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 (Haugland, 1996) of $21,000 \text{ M}^{-1} \text{ cm}^{-1}$ for NBD-PE and 6-NBD PC at 463 and 465 nm, $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ for 6-NBD-CM and 6-NBD-SM (at 466 nm), NBD-PS (at 463 nm), and 12-NBD-PC (at 465 nm). Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout.

2.2. Methods

2.2.1. Sample preparation

All experiments were done using large unilamellar vesicles (LUVs) of 1000 nm diameter of DOPC containing 1 mol% NBD-labeled lipids. In general,

320 nmol (640 nmol for time-resolved fluorescence measurements) of DOPC in methanol was mixed with 3.2 nmol (6.4 nmol for time-resolved fluorescence measurements) of the specific NBD-labeled lipid in methanol. The sample was mixed well and dried under a stream of nitrogen while being warmed gently ($\sim 35^\circ\text{C}$). After further drying under a high vacuum for at least 6 h, 1.5 ml of 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2 buffer (or, 10 mM sodium acetate, 150 mM sodium chloride, pH 5.0 buffer) was added, and each sample was vortexed for three minutes to disperse the lipid and form homogeneous multilamellar vesicles. LUVs of diameter of 1000 nm were prepared by the extrusion technique using an Avestin Liposofast Extruder (Ottawa, Ontario, Canada) as previously described (Rukmini et al., 2001). Samples were incubated in dark for 12 h at room temperature ($\sim 23^\circ\text{C}$) 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. Depth measurements using the parallax method

The actual spin (nitroxide) content of the spin-labeled phospholipids (Tempo- and 5-PC) was assayed using fluorescence quenching of anthroyloxy-labeled fatty acids (2- and 12-AS) as described earlier (Abrams and London, 1993). For depth measurements, multilamellar vesicles were made by co-drying 160 nmol of total lipid (DOPC) containing 10 mol% spin-labeled phospholipid (Tempo- and 5-PC) and the specific NBD-labeled lipid (1 mol%) under a stream of nitrogen while being warmed gently ($\sim 35^\circ\text{C}$) followed by further drying under high vacuum for 3 h. The dried lipid film was hydrated with 1.5 ml of 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2 buffer (or, 10 mM sodium acetate, 150 mM sodium chloride, pH 5.0 buffer). Duplicate samples were prepared in each case except for samples lacking the quencher (Tempo- and 5-PC) for which triplicates were prepared. Background samples lacking the fluorophore (NBD-labeled lipids) were prepared in all cases, and their fluorescence intensity was subtracted from the respective sample fluorescence intensity. Samples were kept in dark for 12 h before measuring fluorescence.

2.2.3. Steady state fluorescence measurements

Steady state fluorescence measurements were performed with a Hitachi F-010 spectrofluorometer using 1 cm path length quartz cuvettes. 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 and polarization values were calculated as described previously (Chattopadhyay et al., 2002). All experiments were done with multiple sets of samples and average values of polarization are shown in Table 1.

For depth measurements, samples were excited at 471 nm and emission was collected at 533 nm. Excitation and emission slits with nominal bandpass of 5 nm were used. Fluorescence was measured at room temperature ($\sim 3^\circ\text{C}$) and averaged over two 5-s readings. Samples were kept in dark for 30 s in the fluorimeter between two readings to avoid any photodamage. Intensities were found to be stable over time. In all cases, intensities from background samples without fluorophore were subtracted. Membrane penetration depths were calculated using Eq. (4) (see Section 3).

2.2.4. 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 as described previously (Rukmini et al., 2001). The excitation wavelength used was 337 nm which corresponds to a peak in the spectral output of the nitrogen lamp. Emission wavelength was set at 531 nm. All experiments were performed using excitation and emission slits with a nominal bandpass of 4 nm or less. Intensity decay curves obtained were fitted as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp\left(-\frac{t}{\tau_i}\right) \quad (1)$$

where α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i . The decay parameters were recovered as described previously (Rukmini et al., 2001). Mean (average) lifetimes ($\langle\tau\rangle$) for biexponential decays of fluorescence were calculated from the decay times and preexponential factors using the following equation (Lakowicz, 1999):

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \quad (2)$$

2.2.5. Measurement of apparent rotational correlation times

The apparent limiting anisotropy (r_o), defined as the anisotropy in a rigid medium at very low temperatures, of the NBD group was determined by measuring the anisotropy of NBD-AHA in glycerol in the temperature range of $15\text{--}30^\circ\text{C}$ using the excitation and emission wavelengths of 465 and 531 nm, respectively. The inverse of the measured anisotropy ($1/r$) was plotted versus T/η (the ratio of temperature and viscosity), and the data were fitted by linear regression. According to the modified Perrin equation (Lakowicz, 1999), the value of the intercept on the ordinate (y-axis) gives the value of the inverse of apparent limiting anisotropy ($1/r_o$). These measurements gave an apparent limiting anisotropy of 0.354 for the NBD group of NBD-AHA. The apparent rotational correlation times (τ_c) were calculated using Perrin's equation (Lakowicz, 1999):

$$\tau_c = \frac{\langle\tau\rangle r}{r_o - r} \quad (3)$$

Table 1
Fluorescence polarization of NBD-labeled lipids^a

NBD-labeled lipid	Fluorescence polarization
NBD-PE	0.117 ± 0.003
6-NBD-PC	0.157 ± 0.002
12-NBD-PC	0.174 ± 0.002
6-NBD-CM	0.158 ± 0.003
6-NBD-SM	0.160 ± 0.003
NBD-PS	0.162 ± 0.003
NBD-PS (pH 5.0)	0.171 ± 0.004

^a The excitation wavelength was 465 nm; emission was monitored at 531 nm. All experiments were done at pH 7.2 unless mentioned otherwise. See Section 2 for other details.

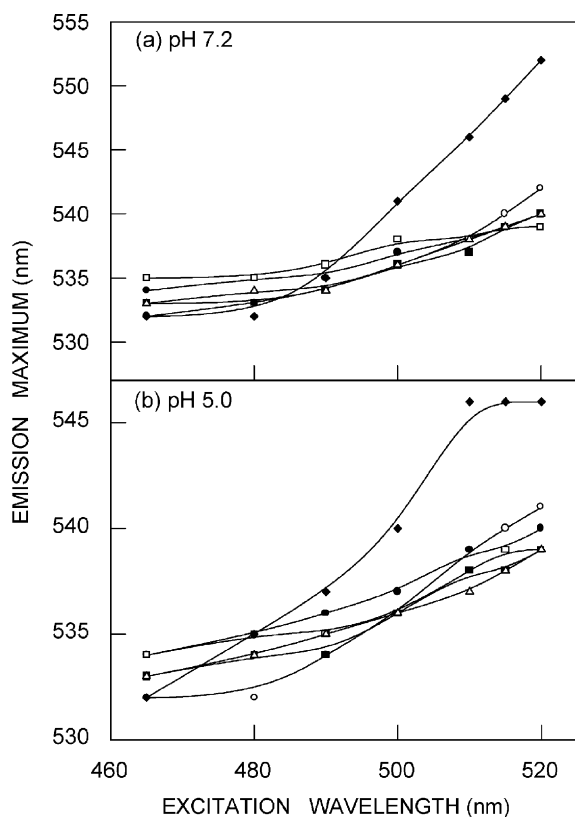


Fig. 2. Effect of changing excitation wavelength on the wavelength of maximum emission for NBD-labeled lipids in DOPC large unilamellar vesicles at (a) pH 7.2 and (b) pH 5.0. The plots correspond to NBD-PE (O), 6-NBD-PC (●), 12-NBD-PC (□), 6-NBD-SM (■), 6-NBD-CM (△) and NBD-PS (◆). The concentration of DOPC was 0.21 mM and fluorophore to lipid ratio was 1:100 (mol/mol). See Section 2 for other details.

where r_o is the apparent limiting anisotropy, 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 calculated from Eq. (2).

3. Results and discussion

Fig. 2(a) shows that upon excitation at 465 nm, the fluorescence emission maxima¹ of NBD-labeled lipids

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

are found to be between 532 and 535 nm at pH 7.2. The position of these emission maxima indicates that the NBD moiety in these lipids is localized at the membrane interface region (Chattopadhyay and London, 1987, 1988; also see later). Among the three major regions in the membrane, the interfacial region is characterized by unique motional and dielectric characteristics (Epand and Kraayenhof, 1999) different from the bulk aqueous phase (experienced by charged aqueous probes such as ANS and TNS) and the more isotropic hydrocarbon-like deeper regions of the membrane. This specific region of the membrane exhibits slow rates of solvent relaxation and is also known to participate in intermolecular charge interactions and hydrogen bonding through the polar headgroup (Boggs, 1987). These structural features which slow down the rate of solvent reorientation have previously been recognized as typical features of environments giving rise to significant red edge effects. It is therefore the membrane interface which is most likely to display red edge effects and is sensitive to wavelength-selective fluorescence measurements (Chattopadhyay, 2003).

As mentioned above, we have previously shown that NBD-PE exhibits REES in membranes (Chattopadhyay and Mukherjee, 1993, 1999). This is also seen in Fig. 2 which shows the shift in the maxima of fluorescence emission of NBD-labeled lipids as a function of excitation wavelength in DOPC vesicles. The emission maximum of NBD-PE in DOPC vesicles shifts from 532 to 542 nm, as the excitation wavelength is changed from 465 to 520 nm. This amounts to a REES of 10 nm. Interestingly, Figs. 2 and 3 show that all the other NBD-lipids display REES of comparable magnitude when incorporated in DOPC vesicles. Thus, as the excitation wavelength is changed from 465 to 520 nm, the emission maxima are shifted from 534 to 540 nm for 6-NBD-PC, from 535 to 539 nm for 12-NBD-PC, from 533 to 540 nm for 6-NBD-SM and 6-NBD-CM. The largest shift is obtained with NBD-PS at pH 7.2. The emission maximum of NBD-PS shows a shift from 532 nm (when excited at 465 nm), to 552 nm (when excited at 520 nm). This corresponds to a REES of 20 nm, highest observed among all the NBD-labeled lipids.

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.

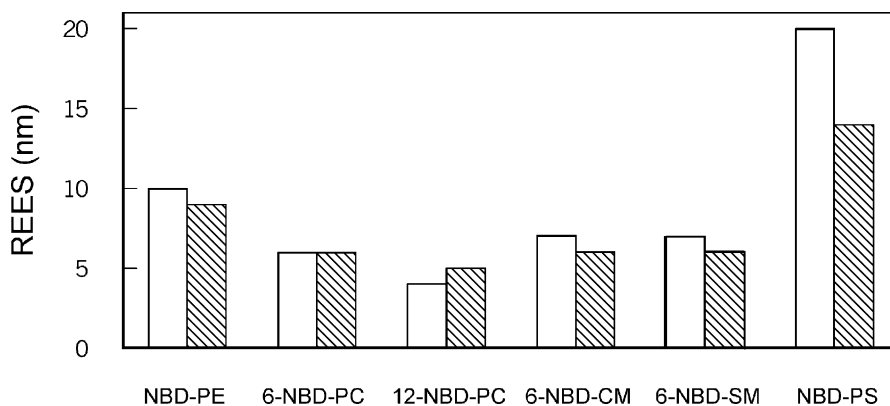


Fig. 3. A comprehensive representation of the magnitude of red edge excitation shift (REES) obtained with NBD-labeled lipids in DOPC large unilamellar vesicles at pH 7.2 (white bar), and pH 5.0 (shaded bar). All other conditions are as in Fig. 2. See Section 2 for other details.

It is noteworthy that the net charge of membrane-bound NBD-PS at pH 7.2 is -2 (electrophoretic mobility measurements have earlier shown that the NBD group itself is uncharged at this pH (Chattopadhyay and London, 1988)). All the other NBD-labeled lipids, on the other hand, are zwitterionic under the experimental conditions except NBD-PE which has a net charge of -1 (see Fig. 1). The greater sensitivity of NBD-PS to REES could be related to its charge since a charged interface can promote the formation of a stable hydration shell of oriented water molecules (Kraayenhof et al., 1996) characterized by a time scale that would be sensitive to REES measurements (also see later).

If the enhanced REES observed with NBD-PS is related to its charge, altering the charge should, in principle, change its REES. Since the pK_a of membrane-bound carboxylic acids is ~ 7 (Abrams et al., 1992), at pH 5 the charged carboxyl group of NBD-PS (Fig. 1) should be in its protonated form which will reduce the net charge to -1 . We therefore tested this out by performing the REES measurements at pH 5 (see Figs. 2b and 3). The emission maximum of NBD-PS remains invariant (at 532 nm when excited at 465 nm) when the pH is changed to 5. Interestingly, as the excitation wavelength is changed from 465 to 520 nm, the wavelength of maximum emission shifts from 532 to 546 nm which corresponds to a REES of 14 nm. This shows that the observed REES for membrane-bound NBD-PS is dependent on its net charge in the membrane which is related to the stability and maintenance of a hydration shell

of oriented water molecules characterized by optimal time scale for REES to be displayed (Mukherjee and Chattopadhyay, 1995; Raghuraman et al., 2003). It is worth noting here that REES of other NBD-labeled lipids show no significant change at pH 5. This reinforces our earlier proposal that it is the change in net charge of NBD-PS that brings about a corresponding change in REES upon lowering pH of the medium.

The steady state fluorescence polarization of NBD-labeled lipids in DOPC vesicles is shown in Table 1. The polarization values indicate that the NBD groups of the lipids experience motional restriction when bound to DOPC vesicles. The polarization value of NBD-PE (0.117) is in agreement with previously reported value (Chattopadhyay and Mukherjee, 1993). It is interesting to note that the other NBD-labeled lipids show higher polarization values than NBD-PE. It has previously been shown that the NBD group of the tail-labeled 6- and 12-NBD-PC loops up to the membrane interface due to its polarity (Chattopadhyay and London, 1987, 1988; Huster et al., 2001, 2003). This will reduce the local segmental flexibility of the NBD group in these lipids which could make it more constrained resulting in higher polarization. For lipids where the NBD group is attached to the tail of the fatty acyl region (6-NBD-CM and 6-NBD-SM), this packing constraint could also be present since depth measurements show that the NBD group loops up in these cases (see Table 3). This could account for their high polarization. In case of NBD-PS, the higher polarization (compared to NBD-PE) could be related to its charge which facilitates in organizing and orienting

water molecules in its immediate vicinity in a manner so as to constitute an ordered environment which gives rise to higher polarization. Fluorescence polarization, unlike REES, appears to be less sensitive to the charge effect since lowering the pH to 5 does not seem to have any appreciable effect on polarization.

The fluorescence lifetimes of various NBD-labeled lipids in DOPC vesicles are shown in Table 2. A typical decay profile of NBD-PE incorporated in DOPC LUVs with its biexponential fitting and the various statistical parameters used to check the goodness of the fit is shown in Fig. 4. As seen from Table 2, all fluorescence decays could be fitted well with a biexponential function. The mean fluorescence lifetimes of NBD-labeled

Table 2

Fluorescence lifetimes^a and apparent rotational correlation times^b of NBD-labeled lipids

NBD-labeled lipid	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)	τ_c (ns)
NBD-PE	0.71	8.43	0.29	3.58	7.71	2.29
6-NBD-PC	0.37	7.63	0.63	5.01	6.25	2.82
12-NBD-PC	0.23	7.53	0.77	4.88	5.72	3.05
6-NBD-CM	0.61	7.59	0.39	4.23	6.71	3.07
6-NBD-SM	0.74	7.36	0.26	3.48	6.81	3.19
NBD-PS	0.63	7.12	0.37	3.72	6.32	3.00
NBD-PS (pH 5.0)	0.57	7.64	0.43	3.94	6.60	3.43

^a The excitation wavelength was 337 nm; emission was monitored at 531 nm. All experiments were done at pH 7.2 unless mentioned otherwise. See Section 2 for other details.

^b Calculated from Eq. (3). See Section 2 for other details.

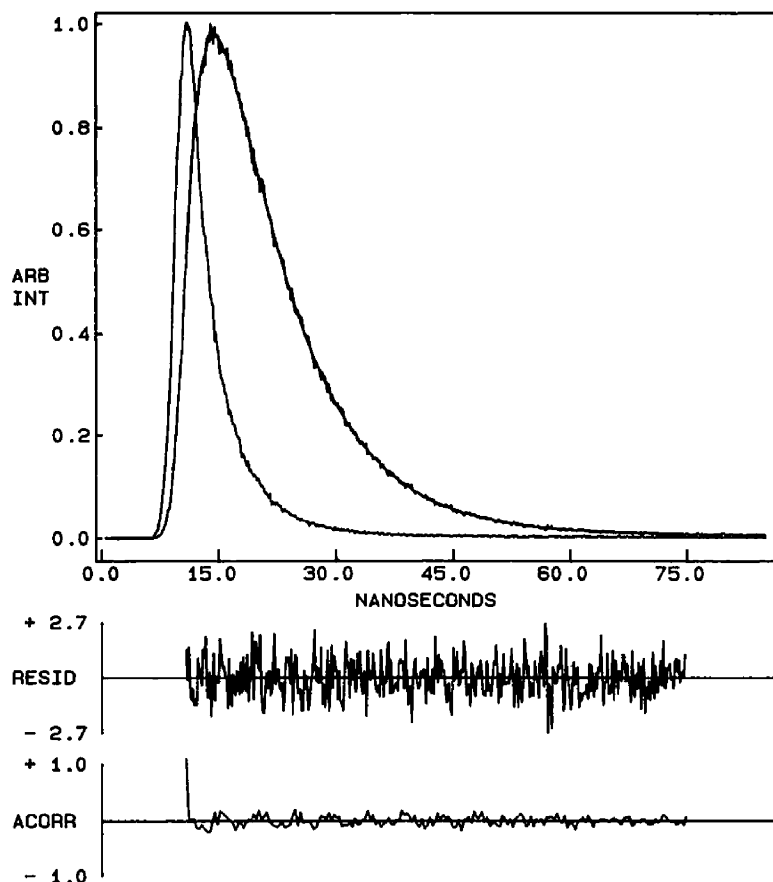


Fig. 4. Time-resolved fluorescence intensity decay of NBD-PE in DOPC large unilamellar vesicles. Excitation was at 337 nm which corresponds to a peak in the spectral output of the nitrogen lamp. Emission was monitored at 531 nm. The sharp peak on the left is the lamp profile. The relatively broad peak on the right is the decay profile, fitted to a biexponential function. The two lower plots show the weighted residuals and the autocorrelation function of the weighted residuals. The concentration of DOPC was 0.43 mM and probe to lipid ratio was 1:100 (mol/mol). See Section 2 for other details.

lipids in DOPC LUVs were calculated using Eq. (2) and shown in Table 2. The lifetime of NBD-PE is found to be 7.71 ns. This is consistent with earlier literature values since the lifetime of NBD-PE incorporated in various membranes have previously been reported to be ~ 7 ns (Chattopadhyay and Mukherjee, 1993). Table 2 shows that the lifetimes of NBD-labeled lipids incorporated in DOPC LUVs is ~ 6 ns except for NBD-PE which shows a somewhat longer lifetime.

In order to ensure that the observed steady state polarization values of NBD-labeled lipids (see Table 1) are not influenced by lifetime-induced artifacts, the apparent rotational correlation times were calculated using Eq. (3). The values of the apparent rotational correlation times, calculated this way using a value of r_o of 0.354 (see Section 2), are shown in Table 2. These values of apparent rotational correlation times show that the observed polarization values are free from lifetime-induced artifacts.

Knowledge of the precise depth of a membrane embedded group or molecule often helps define the conformation and topology of membrane probes and proteins (Chattopadhyay, 1992; London and Ladokhin, 2002). In order to gain a better understanding of the orientation and organization of NBD-labeled lipids in DOPC LUVs, penetration depths of the NBD group were determined by the parallax method (Chattopadhyay and London, 1987) using the equation:

$$Z_{CF} = L_{c1} + \left\{ \frac{[(-1/\pi C)\ln(F_1/F_2) - L_{21}^2]}{2L_{21}} \right\} \quad (4)$$

where Z_{CF} is the depth of the fluorophore from the center of the bilayer, L_{c1} is the distance of the center of the bilayer from the shallow quencher (Tempo-PC in this case), L_{21} is the difference in depth between the two quenchers (i.e. the transverse distance between the shallow and the deep quencher), and C is the two-dimensional quencher concentration in the plane of the membrane (molecules/ \AA^2). Here F_1/F_2 is the ratio of F_1/F_o and F_2/F_o in which F_1 and F_2 are fluorescence intensities in the presence of the shallow and deep quencher (5-PC), respectively, both at the same quencher concentration C ; F_o is the fluorescence intensity in the absence of any quencher. All the bilayer parameters used were the same as described

Table 3

Membrane penetration depth of the NBD group in various NBD-labeled lipids by the parallax method^a

NBD-labeled lipid	Distance from the center of the bilayer z_{CF} (\AA)
NBD-PE	20.3
6-NBD-PC	20.7
12-NBD-PC	20.7
6-NBD-CM	20.8
6-NBD-SM	20.5
NBD-PS	18.8
NBD-PS (pH 5.0)	14.1

^a Depths were calculated from fluorescence quenches obtained with samples containing 10 mol% of Tempo-PC and 5-PC and using Eq. (4). Samples were excited at 471 nm, and emission was collected at 533 nm. All experiments were done at pH 7.2 unless mentioned otherwise. See Section 2 for other details.

previously (Chattopadhyay and London, 1987). The depths of penetration of the NBD groups for various NBD-labeled lipids are shown in Table 3.

It is worth mentioning here that several lines of evidence indicate that the spin label groups in spin-labeled phospholipids lie close to the corresponding position expected for an unlabeled phospholipid in membrane bilayers (summarized in Chattopadhyay and London, 1987). In addition, this question has been addressed by studying the positions of the spin label groups in vesicles by ^{13}C nuclear spin lattice relaxation (Ellena et al., 1988). This study showed that even if some amount of deviation is present in the positions of the spin labels, it is not enough to create a significant problem for depth measurements. The accuracy of such measurements has been checked by comparing the depths obtained by spin label quenching to that obtained from quenching by lipids labeled with bromine atoms at different positions of the fatty acyl chain (Abrams and London, 1992). Since the positions of the bromines in membranes are known (McIntosh and Holloway, 1987), the depth of the spin labels could be calibrated. This analysis shows that the depths obtained are accurate to around 2 \AA , thus justifying the assumed positions of the spin labels in membranes.

Table 3 shows that the NBD groups in all NBD-labeled lipids are localized at a distance of 18.8–20.8 \AA from the center of the bilayer at pH 7.2. These depth values indicate that the NBD group is located at a shallow interfacial region of the membrane in all

these cases as has been previously observed with the tail-labeled NBD lipids, 6- and 12-NBD-PC (Chattopadhyay and London, 1987, 1988; Huster et al., 2001, 2003). These results indicate that the NBD groups of the tail-labeled NBD lipids, i.e. 6-NBD-PC, 12-NBD-PC, 6-NBD-CM and 6-NBD-SM, loop up to the interface due to their polarity. More importantly, the depth of penetration of the NBD group of NBD-PS changes to 14.1 Å when pH is lowered to 5 in response to the protonation of the carboxyl group of NBD-PS. The depth of the NBD group in NBD-PS therefore increases by 4.7 Å when its net charge is changed in response to change in pH. This is similar to what was earlier observed with anthroyloxy- and carbazole-labeled fatty acid probes where an increase in probe depth was reported upon protonation of the fatty acid carboxyl group (Abrams et al., 1992). The shallower depth of the ionized NBD-PS at pH 7.2 is consistent with the tendency of ionized carboxyl groups to be localized at a more polar region of the membrane due to energetically favorable interaction (Abrams et al., 1992). Thus, the observed change in REES of the NBD group of NBD-PS as a function of pH (Figs. 2 and 3) could be correlated to the accompanying change in depth of the fluorophore when pH is lowered.

An important consequence of the looping back of the NBD group is an increase in the headgroup area. For example, it has been estimated that in membranes of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), looping back of the NBD group results in a ~3% increase in the headgroup area (Huster et al., 2001). The nature of molecular interaction responsible for looping up of tail-labeled NBD group deserves comment. A particularly energetically favorable interaction could be hydrogen bonding of the NBD group at the membrane interface. The polar imino group and the oxygens of the NBD moiety 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 residue with the membrane interface (Chattopadhyay et al., 1997; Yau et al., 1998; Raghuraman et al., 2003).

In summary, we have characterized the organization and dynamics of various NBD-labeled lipids us-

ing REES and analysis of membrane penetration depth using the parallax approach. It was earlier shown that the ionization state of the NBD-labeled lipids plays an important role in their spectroscopic properties and membrane location and orientation (Chattopadhyay and London, 1988). Our results presented here show that the environment and location experienced by the NBD group as monitored by REES and depth analysis of the NBD-labeled lipids could depend on the ionization state of the lipid. This could be important in studies where NBD-labeled lipids are used as tracers for monitoring sorting and trafficking of acidic vesicles such as endosomes and lysosomes.

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