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On the use of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine in the study of lipid polymorphism

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The change in the fluorescence properties of dioleoyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) as an indicator of the (liquid-crystalline) bilayer-to-non-bilayer hexagonal$_{II}$ (H_{II}) phase transition has been investigated. Lipid bilayer systems which are known to undergo the bilayer-to-H_{II} phase transition on addition of Ca^{2+} were compared with systems which can undergo aggregation and fusion but not H_{II} phase formation. The former included Ca^{2+}-triggered non-bilayer transitions in cardiolipin and in phosphatidylethanolamine mixed with phosphatidylserine. The latter type of system investigated included the addition of polylysine to cardiolipin and Ca^{2+} to phosphatidylserine. Freeze-fracture electron microscopy was used to confirm that under the experimental conditions used, the formation of H_{II} phase was occurring in the first type of system, but not in the second, which was stable in the bilayer state. It was found that the fluorescence intensity of *N*-NBD-PE (at 1 mol% of the phospholipids) increased in both types of system, irrespective of the formation of the H_{II} phase. A dehydration at the phospholipid head group is a common feature of the formation of the H_{II} phase, the interaction of divalent cations with phosphatidylserine and the interaction of polylysine with lipid bilayers, suggesting that this may be the feature which affects the fluorescence properties of the NBD. The finding of a fluorescence intensity increase in systems lacking H_{II} phase involvement clearly indicates that the effect is not unique to the formation of the H_{II} phase. Thus, while offering high sensitivity and the opportunity to follow kinetics of lipid structural changes, changes in the *N*-NBD-PE fluorescence properties should be interpreted with caution in the study of the bilayer-to-H_{II} phase transition.

Introduction

NBD-labeled lipids have been used to investigate a number of diverse membrane properties. For example, they have been used to study lipid trafficking between subcellular membranes [1], and are processed by the normal cellular metabolic systems such as phospholipases [2]. Further applications include fluorescence-energy transfer assays of vesicle-vesicle lipid mixing which occurs on lipid fusion [3], phase separation phenomena [4] and, most recently, in the study of lipid polymorphic transitions [5–9].

In the descriptions of fluorescence-energy transfer lipid mixing assays, which use *N*-NBD-PE as the fluorescence-energy donor and rhodamine-PE as the acceptor [3], it has been noted that the NBD fluorescence itself changes, even in the absence of the acceptor [4,5,10–13]. This change consists of a fast fluorescence intensity increase followed by a slow intensity decrease, termed the fast and slow artifacts, respectively, with respect to the fluorescence-energy transfer assay with which they interfere [13]. The slow effect is due to a phase separation of the NBD-lipid at it is gradually excluded from the bulk lipids, and this forms the basis of the lateral phase separation studies (for recent review, see Ref. 14). Recently, alternative fluorophores have been synthesized which lack these effects [13].

Abbreviations: *N*-NBD-PE, dioleoyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)PE; H_{II} phase, hexagonal$_{II}$ phase; PE, phosphatidylethanolamine; PS, phosphatidylserine; DOPE, dioleoyl-PE; DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; MLV, multilamellar vesicles; CL, cardiolipin.

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While the fast fluorescence intensity increase has been noted in a number of studies, its potential in the study of membrane lipid polymorphic changes was not realized until recently. In this regard it has been shown that an increase in the fluorescence intensity of NBD in *N*-NBD-PE-labeled vesicles is associated with the formation of the non-bilayer lipid phase [5–9]. This effect has been used as a method for the detection of the bilayer-H_{II} phase phase transition temperature [9]. In addition the fluorescence intensity has been shown to increase on bilayer-H_{II} phase transitions induced by protons [6,7,9].

In this work we have characterized the fluorescence properties of *N*-NBD-PE in the divalent cation induced bilayer-H_{II} transition of cardiolipin and PE/PS systems. Using freeze-fracture electron microscopy we were able to confirm the formation of the H_{II} phase under the same conditions as those employed for the fluorescence experiments, along with identifying the type of phase formed. We have also addressed the question of whether the NBD fluorescence intensity increase is unique to the transition from a bilayer to non-bilayer phase. Our results suggest that the NBD-response is not unique to the formation of the non-bilayer (H_{II}) phase, but is due to a more basic phenomenon, possibly a 'dehydration' at the surface of the membrane. It cannot therefore be used alone as the sole indicator of the formation of non-bilayer phase lipids, although in combination with other techniques, it appears to have potential for the study of the close bilayer-bilayer contact which may underly many membrane processes, including fusion and lipid polymorphic changes. It may also be useful for measuring the kinetics of the bilayer-to-non-bilayer transition.

Materials and Methods

Materials. *N*-NBD-PE, cardiolipin, DOPC, DOPE, DOPS, DPPE and PS (from brain) were from Avanti Lipids (Birmingham, AL). All lipids were monitored for purity by thin layer chromatography and were stored under nitrogen at −20 °C. Other chemicals were from Sigma Chemical Co. (St. Louis, MO), and general reagents and solvents were otherwise of the highest quality available. The phospholipids were quantified by the determination of lipid phosphorus [15].

Preparation of vesicles. Aliquots of lipids and *N*-NBD-PE (both in chloroform) were placed together in a test-tube, the solvent removed by a stream of nitrogen, and the lipid dispersed to form multilamellar vesicles by addition of 0.05 M Tris-HCl buffer (pH 7.4) followed by vortexing with glass beads added to aid dispersion. Sonication, when required, was accomplished using an Ultrasonic Disrupter at 50% power for 10 min with the test-tube kept at 0–4 °C, which resulted in a clear vesicle suspension.

Fluorescence measurements. Fluorescence intensity measurements were made using an SLM 4800 or 48000 spectrofluorimeter. Excitation was at 475 nm (4-nm slit width) and emission at 530 nm using a monochromator (4-nm slit width) or a 530-nm red pass (Schott) filter. Glan-Thompson polarizers were placed vertically (excitation and at 55° (emission). Fluorescence anisotropy measurements were made using two emission channels (A and B), and the ratio (R) of the emission signals was converted to anisotropy according to $(1 - RG)/(1 + 2RG)$, where $G = A/B$ for horizontally polarized excitation and $R = A/B$ for vertically polarized excitation. The emission polarizers for channels A and B were oriented horizontally and vertically, respectively. Fluorescence lifetime measurements were made as described elsewhere [16]. The temperature was controlled at 37 °C using a Neslab water circulator, and samples were stirred continuously during measurements.

Freeze-fracture electron microscopy. Samples for freeze-fracture were removed from the cuvettes containing samples which were being continuously monitored for fluorescence intensity at specified time points. These were sandwiched between a pair of Balzer (Nashua, N.H.) copper support plates and rapidly plunged into liquid propane. No cryoprotectants were used, and freezing was obtained within 10 s of removal of the sample from the cuvette. The specimens were fractured and replicated in a Balzer's BAF 400 freeze-fracture unit at $3 \cdot 10^{-7}$ mBar and at −115 °C. Replicas were viewed on a Philips 300 electron microscope at a magnification of 27000.

Results

Cardiolipin systems

When Ca^{2+} was added to cardiolipin MLV, containing 1 mol% *N*-NBD-PE, there was an immediate increase in the fluorescence intensity (see Fig. 1). The magnitude and stability of the increase depended on the Ca^{2+} concentration. For concentrations of Ca^{2+} up to 0.5 mM, the increase was stable for prolonged periods. For higher concentrations, in particular above 10 mM, the fluorescence intensity peaked after about 1–5 min, then began to decrease. The decrease was due to the formation of large aggregates which precipitated in the cuvette and occurred in spite of continuous stirring, with some material adhering to the walls of the cuvette and the Teflon stir bar. A similar fluorescence intensity increase was obtained upon addition of the charged anesthetic dibucaine (Fig. 1d).

There may also have been some slow, time-dependent, phase separation of the *N*-NBD-PE, which would have cause a *decrease* in the fluorescence intensity due to fluorescence quenching, an effect which was definitely seen in the PS systems (see below and Fig. 7). Other effects, such as apparent increases in emission

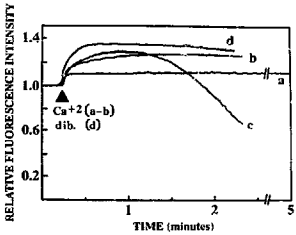

Fig. 1. Effect of the addition of Ca^{2+} and dibucaine on the fluorescence intensity of *N*-NBD-PE (1 mol% of the lipid) at 530 nm on excitation at 470 nm in cardiolipin MLV (50 μM in 50 mM Tris-HCl, pH 7.4). (a) 0.5 mM Ca^{2+}, (b) 10 mM Ca^{2+}; (c) 20 mM Ca^{2+}; (d) 1 mM dibucaine. Details are described in Materials and Methods.

due to light-scattering changes as vesicles aggregate, were examined using unlabeled vesicles, but the contribution was found to amount to only about 1% of the fluorescence intensity increase (results not shown). To discount the possibility that the Ca^{2+} was interacting directly with the *N*-NBD-PE, we examined DOPC vesicles, but found no changes in fluorescence on the addition of Ca^{2+} (data not shown). When the effect of different *N*-NBD-PE : lipid molar ratios was also examined, it was found that the effects were similar within the range of 1–0.01 mol% *N*-NBD-PE. When the dependence of the *N*-NBD-PE fluorescence changes on the Ca^{2+} : cardiolipin ratio was examined for three different cardiolipin concentrations (5, 50 and 500 μM), no difference in the relative fluorescence intensity increase was found (data not shown).

In order to characterize the lipid structures formed on Ca^{2+} addition, samples were taken for freeze-fracture electron microscopy. This technique has been extensively used in the identification of non-bilayer lipid phases with the H_{II} phase, lipidic particles and bilayer phases being easily distinguished (for review, see Ref. 17). Samples for freeze-fracture were taken from a cuvette in which the fluorescence intensity was being monitored continuously. Before the addition of Ca^{2+} only bilayer structures were present (Fig. 2a). These were not highly multilayered, but rather consisted of a few widely spaced lamellae, as would be expected for a charged lipid system. At the point at which the fluorescence intensity had reached a maximum, after the addition of 10 mM Ca^{2+}, H_{II} phase lipids were apparent (Fig. 2b), and after 18 min, much larger fusion aggregates exhibiting the typical H_{II} phase were seen (Fig. 2c), by which time the fluorescence intensity had decreased considerably from the maximum value. Lipidic particles were not found as either intermediate or stable fusion products.

In previous ^{31}P-NMR studies [18], the importance of the Ca^{2+} : cardiolipin ratio being equal or greater than unity for the formation of the H_{II} phase has been stressed. We note that in the present study, which uses much more dilute vesicles, the ratio was always greater

Fig. 2. Freeze fracture electron micrographs of cardiolipin vesicles prepared as in Fig. 1 (details in Experimental Procedures). (a) Before Ca^{2+} addition showing a typical bilayer structure; (b) 5 min after the addition of 10 mM Ca^{2+}; (c) 18 min after Ca^{2+} addition. Bar, 100 nm.

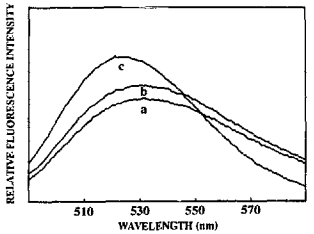

Fig. 3. Fluorescence emission spectra (uncorrected) of *N*-NBD-PE (1 mol%) in cardiolipin vesicles (as for Fig. 1). (a) Before the addition of Ca^{2+}; (b) taken 1 min after the addition of 0.5 mM Ca^{2+}; and (c) for 10 mM Ca^{2+}, also 1 min after addition. Details are described in Materials and Methods.

than one, while the Ca^{2+} concentrations were comparable to those used for ^{31}P-NMR.

The fluorescence emission maxima of the *N*-NBD-PE also changed after Ca^{2+} addition. Fig. 3 shows that coupled with the fluorescence intensity increase there is a marked blue shift in the emission. It should be pointed out that the spectrum (for 10 mM Ca^{2+}) was obtained during a period in which the fluorescence intensity was changing, since the spectra took about 2 min to collect.

It has previously been pointed out that there is a difference in adding Ca^{2+} to cardiolipin vesicles in a concentrated solution compared to a gradual addition using a dialysis procedure [18,19]. In contrast, in our system with lower lipid concentrations, the relative fluorescence intensity increase was the same whether a small aliquot of Ca^{2+} was added to the vesicle suspension in a concentrated solution or if a concentrated vesicle suspension was added to buffer already containing the Ca^{2+} (data not shown).

Attempts were made to assess the freedom of motion of the *N*-NBD-PE by performing fluorescence anisotropy measurements. The fluorescence anisotropy was found to increase in a time-dependent manner, as shown in Fig. 4 for both MLV and sonicated vesicles. The increase persisted, even during the period when the fluorescence intensity had begun to decrease. The fluorescence anisotropy data present some problems in interpretation. The anisotropy should not be appreciably affected by a decrease in the signal intensity due to the precipitation since the anisotropy is not dependent on the intensity (within limits). A more important interference might arise from the increase in light scattering as the vesicles aggregate. This would tend to cause a decrease in the anisotropy, opposite to the effect obtained. An additional problem would arise if the *N*-NBD-PE phase separates from the bulk lipid phase and self-quenches, causing a fluorescence intensity decrease. This would tend to increase the anisotropy, since the fluorescence lifetime would decrease.

We also attempted to obtain lifetime data using the phase and modulation option of the fluorimeter. The light scattering was too severe to recover accurate lifetime data, but the phase and modulation lifetimes were found to increase over the first 15 min after the addition of Ca^{2+} (10 mM) to cardiolipin vesicles (data not shown). This suggests that self-quenching was not occurring to any great extent. If this is so, then the fluorescence anisotropy increase seen may have been a true reflection of a restriction of the freedom of the NBD motion. The initial rate of increase of the anisotropy was greater for vesicles which had been sonicated. The fluorescence intensity also increased more rapidly for sonicated vesicles (data not shown).

Effect of polylysine and spermine

Polylysine, a highly charged polypeptide, has been shown by ^{31}P-NMR to stabilize the bilayer phase [20]. In the present study, at a concentration of 20 $\mu g \cdot ml^{-1}$, it was found to block the 10 mM Ca^{2+}-mediated increase in the fluorescence intensity of *N*-NBD-PE in cardiolipin MLV (Fig. 5a). In contrast, at a lower concentration (e.g. 2 $\mu g \cdot ml^{-1}$, Fig. 5b) it did not have this effect. These results are largely in keeping with the ^{31}P-NMR data. A more important observation, however, was that the polylysine itself was found to cause a modest increase in the fluorescence intensity. Freeze-fracture electron microscopy revealed aggregated vesicles but no H_{II} phase (Fig. 6).

Spermine is known to modulate lipid bilayer structure (see for example Ref. 21 and references therein) and has been implicated in mitochondrial function [22]. We therefore investigated whether its effect might be similar to that of polylysine. The results in Fig. 5c show that spermine is also capable of preventing the increase

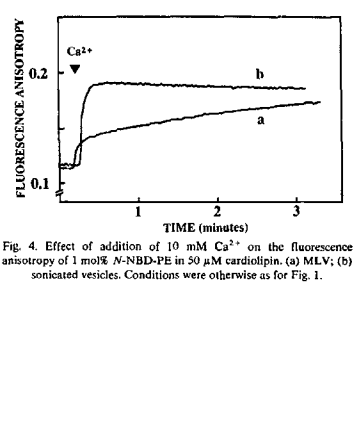

Fig. 4. Effect of addition of 10 mM Ca^{2+} on the fluorescence anisotropy of 1 mol% *N*-NBD-PE in 50 μM cardiolipin. (a) MLV; (b) sonicated vesicles. Conditions were otherwise as for Fig. 1.

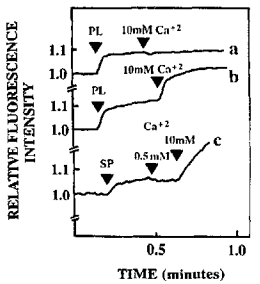

Fig. 5. Effect of polylysine and spermine on the fluorescence intensity of *N*-NBD-PE in 50 μM cardiolipin, before and after the addition of Ca^{2+}: (a) 20 $\mu g \cdot ml^{-1}$ polylysine followed by 10 mM Ca^{2+}; (b) 2 $\mu g \cdot ml^{-1}$ polylysine followed by 10 mM Ca^{2+}; (c) 20 $\mu g \cdot ml^{-1}$ spermine followed by Ca^{2+} (0.5 mM then 10 mM).

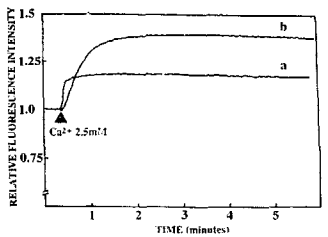

Fig. 7. Effect of 2.5 mM Ca^{2+} on the fluorescence intensity of *N*-NBD-PE (1 mol%) in sonicated vesicles (50 μM) of (a) DOPS; and (b) DOPS/DOPE (1 : 3); in 50 mM Tris-HCl.

in fluorescence intensity caused by the addition of Ca^{2+}, suggesting that it can also stabilize the bilayer configuration as well as itself increasing the fluorescence intensity, as with polylysine. In contrast to polylysine, the stabilizing effect of spermine could be overcome by higher Ca^{2+} concentrations even if higher spermine concentrations were used. Both polylysine and spermine addition also caused a blue shift in the fluorescence emission maxima, as well as a small increase in fluorescence anisotropy.

The finding of the fluorescence intensity increase with these systems indicated that the fluorescence intensity increase is not unique to the formation of the H_{II} phase.

PS systems

In previous studies it had been noted that there was a fluorescence intensity increase with *N*-NBD-PE in PS systems [4,11,3]. These studies were more concerned with the use of *N*-NBD-PE in fusion or lipid phase separation assays, and the study of this effect was not of prime concern. However, in the present study this would be of some importance, since PS does not normally form non-bilayer structures except at low pH [23]. We also investigated PE in mixtures with PS, since it has been shown that the Ca^{2+}-induced lateral phase separation of the PS leads to the formation of the H_{II} phase of the PE [24].

For sonicated vesicles of DOPS, the addition of Ca^{2+} resulted in a rapid increase in the fluorescence intensity similar to that found for the cardiolipin system (see Fig. 7). Brain PS gave a similar response (data not shown; and see Ref. 12). Freeze-fracture electron microscopy of the effect of Ca^{2+} on vesicles of brain PS showed aggregated vesicles, and as expected, non-bilayer phases were absent (see Fig. 8).

We also examined sonicated vesicles consisting of a DOPE/DOPS 3 : 1 mixture. This experiment was modeled on that of Tilcock and Cullis [24], the idea being

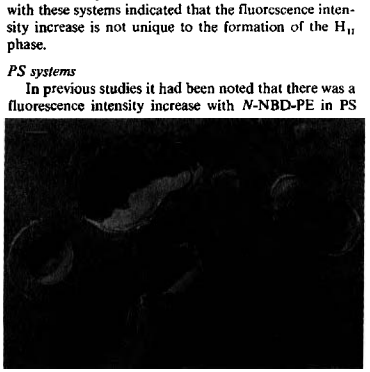

Fig. 6. Freeze-fracture electron micrograph of 50 μM cardiolipin after 30 s in the presence of 20 $\mu g \cdot ml^{-1}$ polylysine (details in Materials and Methods). Bar, 100 nm.

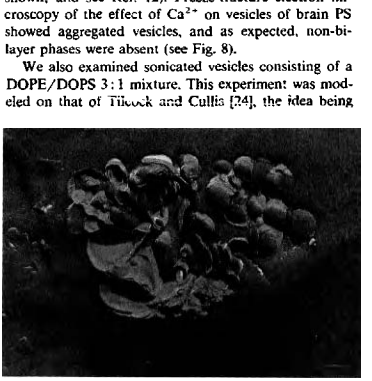

Fig. 8. Freeze-fracture of brain PS taken 1 min after the addition of 2.5 mM Ca^{2+} (details in Materials and Methods). Bar, 100 nm.

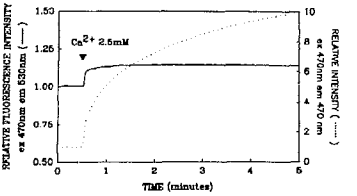

Fig. 9. Comparison of the rates of the *N*-NBD-PE fluorescence intensity increase and the light scattering for 50 μM DOPS sonicated vesicles in 10 mM Tris-HCl (pH 7.4).

that the DOPE should be forced to remain in the bilayer state when mixed with DOPS. DOPE is exclusively in the H_{II} phase above about 10 °C. After the addition of Ca^{2+}, the DOPS should become cross-linked and phase-separated from the DOPE, which would then be free to adopt the H_{II} phase. The increase in the fluorescence intensity of *N*-NBD-PE after the addition of Ca^{2+} to DOPE/DOPS 3:1 vesicles was in fact greater than for DOPS alone (Fig. 7).

With DOPS MLV, there was no significant effect for concentrations less than approximately 5 mM Ca^{2+}, and although at 10 mM Ca^{2+} there was a very small initial increase in the fluorescence intensity, the main effect was that the fluorescence intensity slowly decreased. This which would be compatible with the *N*-NBD-PE phase separating and undergoing self-quenching as it was phase-separated out of the PS-Ca^{2+} complexes (results not shown).

The *N*-NBD-PE fluorescence intensity increase for DOPS vesicles was found to be unaffected by the presence of 150 mM NaCl (for 10 mM Tris-HCl) and was similar for 10 mM and 50 mM Tris-HCl. For 10 mM Tris-HCl at low Ca^{2+} concentrations, the rate of the increase in light scattering, observed on excitation and emission at 470 nm, was much slower than the fluorescence intensity increase (Fig. 9). This clearly demonstrates that the aggregation process continued long after the fluorescence changes had occurred, and point to the fluorescence intensity increase reflecting very early events.

Discussion

For the purposes of the discussion it may be useful to depict the effect of Ca^{2+} on cardiolipin vesicles as follows:

$$CL_{bil} \rightleftharpoons (CL\text{-}Ca^{2+})_{bil} \rightleftharpoons (CL\text{-}Ca^{2+})_{int} \rightleftharpoons (CL\text{-}Ca^{2+})_{H_{II}}$$

$$\rightleftharpoons [(CL\text{-}Ca^{2+})_{H_{II}}]_{agg}$$

Three intermediate states are shown. The first, $(CL\text{-}Ca^{2+})_{bil}$, represents a transitory step of Ca^{2+} binding to the bilayer surface. The second step, $(CL\text{-}Ca^{2+})_{int}$ formation, would be found when two bilayer surfaces come into close juxtaposition due to the cross-linking action of Ca^{2+}. $(CL\text{-}Ca^{2+})_{int}$ may represent non-bilayer intermediate forms such as lipidic particles, which have been found in a number of systems including cardiolipin (for review, see Ref. 17). Whether the lipidic particle or inverted micelle forms as an intermediate stage of vesicle fusion has been the subject of some discussion [26–29], which is not entirely resolved. Previously, lipidic particles were found for cardiolipin/DOPC and PS/PE vesicles, although this was only well after initial fusion events [26]. At Ca^{2+} : cardiolipin ratios of less than 1 (mol/mol) an intermediate stage in the formation of the H_{II} phase was observed [26]. However, for the dilute vesicle systems of the present study, the freeze-fracture data would suggest that the only stable intermediate state is $(CL\text{-}Ca^{2+})_{H_{II}}$, since lipidic particles were not found in the cardiolipin system. Here we distinguish between $(CL\text{-}Ca^{2+})_{H_{II}}$ and $[(CL\text{-}Ca^{2+})_{H_{II}}]_{agg}$ on the basis of the freeze-fracture electron microscopy, the former remaining in suspension, with the latter as large aggregates precipitating in the cuvette. Thus, although it is not possible to completely exclude the formation of intermediate structure, the individual vesicles may well be converted directly to $(CL\text{-}Ca^{2+})_{H_{II}}$. Finally, if the Ca^{2+} concentration is sufficiently high, the small H_{II} areas coalesce to form the final large aggregates $[(CL\text{-}Ca^{2+})_{H_{II}}]_{agg}$, which then precipitate. For lower Ca^{2+} concentration (< 5 mM) with cardiolipin (50 μM) MLV, the last step appears to be extremely slow or absent, and the fluorescence intensity increase stabilizes. In addition, there is an immediate and full reversibility by EGTA only for the low Ca^{2+} concentration. Similar Ca^{2+} concentration-dependent effects have been previously noted [30–32]. This suggests that the H_{II} phase areas are very small or are perhaps incompletely converted to the H_{II} phase at the low Ca^{2+} concentration, and thus are more easily reversible. With the higher Ca^{2+} concentration, there was only a partial immediate reversibility, and it has in fact been shown that it takes several hours to fully return to a bilayer form [18,19].

In this study the primary concern was to investigate the *N*-NBD-PE fluorescence intensity change and their correlation with H_{II} phase formation. Nevertheless, some comments on the possible mechanism of its formation may be useful, since little is known on this subject. The fluorescence intensity increase of *N*-NBD-PE in the cardiolipin MLV system was independent of the cardiolipin concentration (5–500 μM). This suggests that the Ca^{2+} may first interact with the outer edge of the vesicle, transforming it to localized non-lamellar structures (possibly H_{II}) and thereby allowing access to the next or deeper bilayers, and so on, until it is

eventually transformed into the H_{II} phase. While cross-linking by Ca^{2+} of the surfaces of different vesicles did not appear to be important in MLV, for single unilamellar vesicles this may have been more important, and indeed, we found the fluorescence intensity increase to be more rapid (results not shown). In contrast, for PS systems, although single unilamellar vesicles showed the fluorescence intensity increase, MLV showed no appreciable fluorescence intensity increase on Ca^{2+} addition. This would be expected, since the H_{II} phase was not formed and thus Ca^{2+} could not gain access to most of the the bilayer surface, which would be inside the MLV. Although these observations are preliminary, we feel that *N*-NBD-PE fluorescence intensity properties may provide more useful information concerning, e.g., the mechanisms of bilayer-bilayer contact, and further studies along these lines are in progress.

From the above considerations it would appear that, since the first semi-stable state after Ca^{2+} addition is $(CL\text{-}Ca^{2+})_{H_{II}}$, then the *N*-NBD-PE must experience a different environment in this phase. This was also concluded in the study of PE systems [5,9]. This leaves the important question as to what characteristics of the altered environment are being sensed by the NBD, and whether they are unique to the formation of H_{II} phase.

Apart from the fluorescence intensity increase, we also observed a marked blue shift in the emission maxima and an increase in the fluorescence anisotropy of the NBD fluorophore. This would be compatible with a more hydrophobic and restricted environment. Previously, it has been shown that dehydration of the lipid head groups at the membrane surface is an important component of the H_{II} phase [33–35]. This dehydration effect may be responsible for the altered, more hydrophobic, NBD environment.

Evidence in support of the *N*-NBD-PE detecting a dehydration at the membrane surface are our results with PS and polylysine. The aggregation and fusion process in such systems is well documented [36]; more importantly, it has been shown that with PS vesicles the divalent ions cross-link two apposing bilayers, resulting in the membrane lipid head group region becoming dehydrated [4,10,37–41]. We have also considered the additional possible contribution of aggregation itself to the fluorescence intensity increase. However, aggregation kinetics did not exactly follow the NBD fluorescence intensity increase (Fig. 9). Although we were able to induce aggregation in PS and even with phosphatidylcholine using high Na^+ concentrations, we did not observe a correlation of aggregation with NBD fluorescence properties (data not shown). We therefore suggest that dehydration may be the basic change underlying the fluorescence changes of NBD.

Polylysine [42–45] and spermine [20,46] have been shown to initiate membrane fusion in a number of systems. Furthermore, on the basis of X-ray data, it has been suggested that polylysine acts as a bridge between bilayers and causes aggregation and also dehydration [47]. Therefore, our finding that the *N*-NBD-PE fluorescence intensity again increases on interaction of polylysine and spermine with the cardiolipin system would provide additional support for the dehydration hypothesis.

This leaves the question of the uniqueness of the fluorescence intensity increase and its potential in the study of lipid polymorphism. It is readily apparent that the increase in *N*-NBD-PE in lipid vesicles cannot of itself be used to indicate the formation of the H_{II} phase. Confirmation by ^{31}P-NMR, X-ray diffraction or freeze-fracture electron microscopy is required. Balanced against this is the extreme sensitivity of the NBD method and the fact that kinetics can be easily followed. In conclusion, we would suggest that fluorescence intensity changes of the NBD chromophore attached to PE or other lipids be used for the study of lipid polymorphism with some caution. However, it would appear that the sensitivity of the NBD to its environment may have some potential for the study of membrane structural transformations in general, for example with bilayer fusion processes.

Acknowledgements

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