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Comments on Fluorescence Methods for Probing Local Deviations from Lamellar Packing

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Fluorescence probes have been used to monitor the conversion from the lamellar to nonlamellar phases. In addition there is current interest in understanding the nature of the changes in the physical properties of phospholipid bilayers that are correlated with their propensity for forming nonlamellar phases. Fluorescent probes have been used to demonstrate changes in surface hydrophobicity, quenching efficiency, and rates of interbilayer transfer of fluorescently labeled analogs. These studies are beginning to provide an assessment of the nature of the differences between stable bilayers and those that readily convert into inverted phases. Such studies are important because these differences in physical properties often lead to altered membrane functional behavior.

KEY WORDS: Pretransition defects; nonlamellar phases; inverted phases; hexagonal phase; membrane surface hydrophobicity; membrane curvature strain; membrane bilayers.

FLUORESCENT PROBES MONITOR LAMELLAR TO NONLAMELLAR PHASE CHANGES

There has been continued interest on the possible functional relevance of the fact that several of the major lipid components of biological membranes form nonbilayer phases under physiological conditions when in purified form [1]. One of these nonbilayer phases is the inverted hexagonal phase. The molecular arrangement of phospholipids in this phase is shown in Fig. 1, in comparison to the bilayer phase. The hexagonal phase is formed by water-filled cylinders packed with hexagonal symmetry. The figure shows the cross section of one such cylinder. The conversion of phospholipid bilayers to nonlamellar structures can be monitored by X-ray diffraction, freeze fracture electron microscopy, and ^{31}P NMR [2]. In addition, once the nature of the phase transition is known, it can be conveniently monitored using differential scanning calorimetry (DSC) or fluorescence spectroscopy. These methods allow the facile determination of the effect of changes in conditions or the presence of other substances in the membrane on the temperature and in the case of DSC, the enthalpy of this transition. It has been demonstrated that the fluorescent probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) undergoes an increase in fluorescence emission intensity at the bilayer to hexagonal phase transition temperature (T_H) [3]. It was suggested that the change in fluorescence intensity is a consequence of changes in the hydrophobicity at the membrane surface. With fluorescence methods, the phase transition can be measured in dilute suspensions of liposomes and using low mole fractions of the fluorescent probe. It is found that changes in the fluorescence intensity using membranes containing 1% of the fluorescent probe NBD-PE are in good agreement with the phase transition measured by DSC of membrane samples also containing 1% NBD-PE. This probe does raise T_H compared with that of the pure lipids by a few degrees even at these low mole fractions. It is known that T_H is very sensitive to the presence of small mole fractions of certain additives [4]. An alternative fluorescent method for

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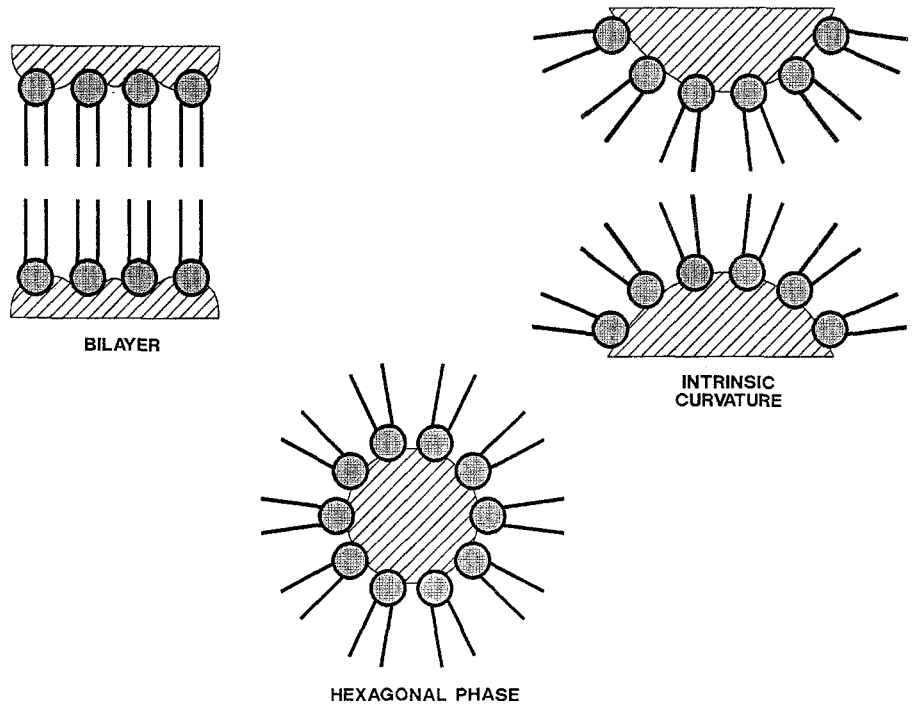

Fig. 1. Schematic diagrams of the usual planar packing of phospholipids in a bilayer, a hypothetical state in which each monolayer can attain its intrinsic radius of curvature free of packing constraints (in this diagram the terminal methyl ends of the acyl chains are in contact with a nonpolar solvent or are in a hypothetical vacuum), and the cross section of a single hexagonal phase cylinder. In all the figures, diagonal lines represent water. Reprinted from Ref. 26.

observing lipid polymorphism is through the use of time-resolved fluorescence anisotropy. Just as the chemical shift anisotropy of ^{31}P NMR becomes narrower when the bilayer phase is converted to the inverted hexagonal (H_{II}) phase, the anisotropy of the fluorescence emission also becomes smaller at T_H. Chen *et al.* [5] used the fluorescence anisotropy of the probe 1-palmitoyl-2-[2-[4-(6-phenyl*trans*-1,3,5-hexatrienyl)phenylethyl]carbonyl] 3-*sn*-phosphatidylcholine (DPH-PC) to monitor T_H. The decreased anisotropy at T_H correlated well with the known phase transition behavior of the systems studied. Chen *et al.* [5] also used DPH-PC and 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylcholine to study the rate of diffusion of phospholipids around hexagonal phase cylinders.

FLUORESCENT PROBES TO DETECT LOCAL DEVIATIONS AWAY FROM LAMELLAR PACKING

The studies described above focus primarily on the use of fluorescent probes to monitor the interconversion between lamellar and inverted phases. In recent years, however, the focus of interest in the functional role of hexagonal phase-forming lipids has shifted from an interest in the question of whether nonlamellar lipid structures exist in small domains in bilayers to how they influence function through changes in bilayer properties. Currently, the functional role of non-lamellar-forming lipids in biological systems is thought to arise from their ability to alter the physical properties of the lamellar phase, rather than actually forming nonlamellar phases. One such property is the intrinsic curvature of a membrane monolayer (Fig. 1). In a bilayer arrangement each monolayer has to lie flat, joining the opposing monolayer. However, if each monolayer has a tendency to curve, this will result in a destabilization of the bilayer as a consequence of curvature strain. The bilayer will thus be in a less stable arrangement which will lead to changes in hydration and head-group packing density and, therefore, changes in surface hydrophobicity. In addition, such curvature strain is also likely to lead to transient irregularities in the packing of phospholipid molecules which could result in increased water penetration into the bilayer. Such changes in bilayer properties induced by the presence of non-lamellar-forming lipids may help to explain the observed phenomenolog-

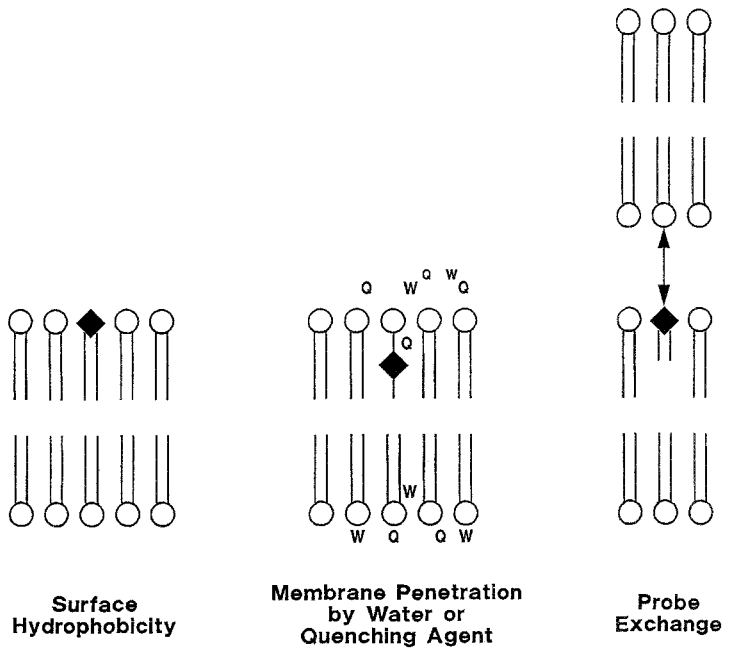

Fig. 2. General methods for monitoring membrane physical properties related to deviations from lamellar packing with the use of fluorescent probes. Fluorescent chromaphore is indicated by the filled diamond.

ical relationship between the presence of membrane components that promote H_{II} phase formation and altered rates of membrane fusion [6], or of the activity of membrane-bound enzymes [7], or the transbilayer diffusion of phospholipids [8]. Fluorescent probes have begun to be used to monitor these changes in bilayer properties. Three classes of fluorescence experiments have been used for this purpose. These methods will be reviewed below, particularly in relation to how they can provide information about alterations of bilayer properties. The general basis of these methods is outlined in Fig. 2. The first method utilizes fluorescent probes to monitor the hydrophobicity of the surface of the membrane. This property may be directly related to alterations in membrane fusion [9] or to the translocation of amphitropic enzymes, such as protein kinase C, to the membrane surface [10]. The nature of the probe is not specified in this general scheme. In general, the probe will have interactions with neighboring lipid molecules as well as with the surrounding solvent and therefore it will not exclusively monitor the membrane interface. Furthermore, the location of the probe with respect to the membrane surface may also change with the type of lipid and/or the physical conditions such as temperature. This is particularly likely if the probe does not have a long hydrocarbon membrane anchor. The second type of measurement involves penetration of the membrane by water or by a quenching agent. In the case of water penetration into the bilayer, this will result in a change in the polarity of the environment of a fluorophore internal to the membrane. Water penetration can often be demonstrated by comparing the effects of H_2O and ^2H_2O on the fluorescence properties (see below). In addition, the accessibility of internal fluorescent probes to quenching by polar, water-soluble quenching agents also provides a method for detecting packing defects in lamellar membranes. Less stable bilayers will allow greater penetration of quenching agents. Another property that may increase with decreased bilayer stability is the ease of extraction of fluorescently labeled amphiphiles from the membrane. The rate of transfer of amphiphiles between membrane bilayers provides a third type of measurement which may reflect deviations from stable lamellar structures.

None of these fluorescence methods directly monitors local deviations from lamellar packing which are unique to instabilities related to the tendency to form nonlamellar phases. At the present state of knowledge, the association of these alterations in membrane properties with the tendency to form nonlamellar phases is largely correlative. Nevertheless, these types of studies will establish the conditions in which the properties of the membrane bilayer are altered as well as suggest the nature of these alterations. More specific details about

the molecular nature of these changes will be obtained by future application of a combination of fluorescence methods and other physical measurements.

Surface Hydrophobicity

Kimura and Ikegami [11] proposed the use of L-α-dansylphosphatidylethanolamine (DNS-PE) to monitor the polarity of the polar region of a phospholipid bilayer. Subsequently, Ohki and Arnold [12] showed that increased hydrophobicity in the region of the membrane surface, monitored by DNS-PE, was associated with increased rates of membrane fusion. Another dansylated probe, N^{ε}-dansyl-L-Lys (DNS-Lys), has also been used to study membrane surface properties [13]. The fluorescence emission wavelength of the dansyl group is particularly sensitive to changes in the dielectric constant of the surrounding medium, undergoing shifts of more than 100 nm between organic and aqueous solvents, which are fluid systems with rapid relaxation rates. Of course, correlating these spectral shifts with changes in the surrounding solvent polarity requires that the relaxation rate of solvent reorientation be rapid compared with the fluorescent lifetime. This may not be the case in the condensed medium of a membrane. In the case of NBD-PE, it has recently been demonstrated that the fluorophore is motionally restricted, leading to a red edge excitation shift [14]. This phenomenon is sensitive to changes in temperature and may provide an additional tool for probing membrane organization and dynamics. Another difficulty with measuring the polarity of specific locations in the membranes is that the exact position of the probe in the bilayer structure is not known. Recently, to solve this type of problem, a series of fluorescent probes of membrane surface potential have been prepared with a charged group to position the fluorophore at a specific distance from the membrane surface [15]. No such probes are yet available for measuring membrane surface hydrophobicity. In the case of DNS-Lys, the probe may even partition between aqueous and membrane phases. Nevertheless, the results with NBD-PE do clearly show that surface hydrophobicity, as monitored by this fluorescent probe, is related to the functional property of membrane fusion. In addition, the DNS-Lys results demonstrate that the surface properties of the membrane undergo marked changes as T_H is approached, although the membrane is still in a bilayer arrangement.

Other fluorescent probes have also been used to monitor surface hydrophobicity. One of these is Laurdan, which is sensitive to the phase behavior of the membrane [16]. Laurdan contains a C-12 acyl group to sequester the probe in the membrane. Its fluorescence emission is highly sensitive to the polarity of the environment. The fluorescent properties of this probe are not sensitive to changes in the nature of the lipid head group but rather may be determined by the extent of water penetration into the lipid bilayer. This would then make Laurdan a very useful probe for monitoring changes in lamellar packing. The properties of Laurdan are reviewed in this issue [17]. The properties of Laurdan are also sensitive to the approach of T_H [13]. There are also fluorophores more internal to the bilayer whose properties have been suggested to be sensitive to the ability of water to penetrate the membrane structure. Such probes include DPH-PC, DPH and TMA-DPH, which undergo changes in fluorescent lifetimes, the latter showing sensitivity to substitution of H_2O with ^2H_2O [18]. Penetration of water into bilayer structures has also been evaluated by analyzing the fluorescence decay curves of phospholipids labeled in the acyl chain with an anthroyl group [19]. The position of the anthroyl group is determined with the use of quenching agents. The emission spectra from this probe exhibit three distinct components with different emission maxima and different excited-state lifetimes. The relative intensity of these components is shown to be sensitive to the solvent environment in isotropic solvents. The behavior of these probes in membranes is interpreted on the basis of a change in solvent polarity caused by different extents of penetration of water. Cholesterol was shown to alter the relative intensity of the three components. This would be consistent with cholesterol stabilizing the lamellar phase and inhibiting water penetration. This method should prove of great value in assessing the polarity of the interior of the membrane and in evaluating its sensitivity to deviations in bilayer packing.

Fluorescence Quenching

It has been shown that membrane defects that occur at the temperature of the phase transition between gel and liquid crystalline phases can result in increased penetration of iodide into the membrane [20]. However, there are complexities in interpreting quenching rates solely in terms of the ability of the quenching agent to penetrate the membrane. There appear to also be specific effects, probably related to the nature of the interaction between the quencher and membrane components [21], which affect quenching efficiency. While having some qualitative or comparative usefulness, fluorescence quenching probably will not provide a very accurate assessment of the extent or nature of defects of membranes. Quenching rates will be dependent on the concentration and location of the quenching agent within

the membrane as well as on the extent of molecular motion, determining the frequency of collisions. These various factors will be difficult to sort out.

Amphiphilic Probe Transfer

The transfer of amphiphiles from one bilayer to another has been studied using the fluorescent amphiphiles of the type 1-palmitoyl-2-(7-nitrobenzo-2-oxa-1,3-diazole-4-yl)amino-hexanoyl-phospholipid [22]. It was found that the rate of exchange of such probes was sensitive to the nature of the phospholipid head group. Evidence was presented to suggest that interlipid hydrogen bonding lowered the rate of amphiphile transfer. However, when using lipid mixtures [23] it was found that the exchange rate from phosphatidylcholine bilayers increased with the presence of phospholipids with poorly hydrated head groups such as phosphatidylethanolamine but decreased with the addition of well-hydrated phospholipids such as phosphatidylglycerol. Increased unsaturation of the acyl chain increased the rate of lipid exchange, but cholesterol had little effect. This suggests that bilayer structure and stability, rather than lipid "fluidity," modulate the rate of exchange of these labeled phospholipids. Silvius and Leventis [24] found that a homologous series of fluorescently labeled phospholipid derivatives had decreased phospholipid exchange rates that could be quantitatively correlated with increased hydrophobic interactions. These workers also found an increased exchange rate with greater unsaturation, with the largest effect resulting from the introduction of the first double bond.

A study of amphiphile probe transfer is of interest in its own right. It also has biological implications in providing models for lipid movement among organelles in cells and between plasma membranes and lipoprotein particles. However, from the findings thus far, the molecular and physical features determining transfer rates appear complex. Thus, our understanding of the factors determining the rates of amphiphile transfer are not yet at a stage where this phenomenon can be used to monitor deviations from lamellar packing.

CONCLUSIONS

Fluorescent probes provide sensitive indicators of changes in their local environment. Fluorescent probes can clearly be used to monitor the conversion from lamellar to inverted phases that have been established by other methods. Use of these substances, of course, carries the caveats that the probe is not disturbing the system and that it is monitoring an intrinsic property of the membrane such as surface polarity, rather than something particular to the environment of the probe. Fluorescent probes have also been used for many years to monitor changes in the properties of membranes. The difficulty arises in trying to relate these changes in the spectroscopic properties of fluorescence probes to changes in specific physical or structural properties of the membrane. There is currently no definitive way to use fluorescent probes to monitor local deviations from lamellar packing. Such deviations can be described in a variety of ways, such as pretransition defects, monolayer curvature strain defects, lipidic particles, and bilayer instability. Many of these terms cannot be correlated with a specific structural arrangement. In addition, fluorescence methods do not assess structure very directly, except perhaps for distance measurements by resonance energy transfer. Therefore, for the question of the presence of local deviations from lamellar packing, fluorescent methods have been used largely to observe changes in the environment of the fluorescent probe which can be correlated with the formation of nonlamellar phases. However, these methods do not provide a description of the nature of the structural rearrangements which are responsible for these changes. Nevertheless, they can assess changes in specific physical properties such as the polarity of the environment at a particular location in the membrane. Such changes in polarity, especially if caused by changes in hydration, may be very important to membrane function, and it will be useful to continue this type of approach to further advance our understanding of the role of hexagonal-phase-forming tendency in biological systems [25].

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