Fluorescent probes of membrane surface properties

Raquel F. Epand [a], Ruud Kraayenhof [b], Geert J. Sterk [c], Harro W. Wong Fong Sang [b], Richard M. Epand [a,*]

[a] *Department of Biochemistry, McMaster University, Health Sciences Centre, Hamilton, Ont. L8N 3Z5 Canada*
[b] *Institute of Molecular Biological Sciences, BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands*
[c] *Byk Nederland BV, P.O. Box 61, 1160 AB Zwanenburg, The Netherlands*

Abstract

We have studied the properties of two new fluorescent probes, 7-dimethylaminocoumarin derivatives, 4-[N,N-dimethyl-N-(n-tetradecyl)ammoniummethyl]-7-(N,N-dimethylamino)coumarin chloride (TAMAC) and 4-(n-dodecylthiomethyl)-7-(N,N-dimethylamino)coumarin (DTMAC) in model membrane systems. Both probes are sensitive to solvent polarity. The TAMAC probe has a quaternary ammonium function to position it at a fixed location with respect to the membrane interface. In membranes of dipalmitoleoylphosphatidylethanolamine (DiPoPE), both probes detect marked increases in surface hydrophobicity as the bilayer to hexagonal phase transition temperature is approached. This does not occur when the probes are embedded in dipalmitoleoylphosphatidylcholine (DiPoPC) in which case the fluorescence emission is found to be largely independent of temperature. A nitroxide quencher covalently linked to the 5 position of the *sn*-2 acyl chain of phosphatidylcholine quenches the fluorescence of DTMAC in DiPoPC more than in DiPoPE, indicating the deeper insertion of this probe in DiPoPC. As the temperature is increased the DTMAC fluorophore moves even further out of the membrane. These findings indicate that DTMAC, which does not contain a group to fix its location along the bilayer normal, adjusts its position to small changes in environment polarity, so as to maintain an environment of a fixed dielectric constant. However, with greater changes in membrane interfacial polarity the environment of the probe will be altered. Thus, in addition to the sensitivity of these probes to solvent polarity, the ability of a fixed nitroxide to quench DTMAC becomes another parameter with which to characterize membrane properties with these probes.

Keywords: Membrane surface polarity; Membrane/water interface; Intrinsic monolayer curvature; Bilayer-hexagonal transition

1. Introduction

Membrane surface properties affect many biological processes, including the partitioning of proteins and peptides into membranes, determining the rate of membrane fusion, the transport of substances through the membrane and the modulation of the activity of certain membrane-bound enzymes. It is important to develop methods to measure the physical properties of membranes that modulate specific membrane functions.

Inter-related to interfacial properties is the property of monolayer curvature strain [1]. This is because factors such as the extent of hydration of the membrane, interlipid H-bonding and steric repulsion are among those that affect intrinsic membrane curvature [2]. The phenomenon of curvature strain is attracting increased interest as a mechanism for the regulation of biological activity [3].

Fluorescent probes have been used to measure membrane surface hydrophobicity. These studies are beginning to provide an assessment of the nature of the differences in the interfaces 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 behaviour. Kimura and Ikegami [4] proposed the use of L-α-dansylphosphatidylethanolamine (DNS-PE) to monitor the polarity of the

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Abbreviations: DNS-PE, L-α-dansylphosphatidylethanolamine; DNS-Lys, N^{ε}-dansyl-L-Lys; TAMAC, 4-[N,N-dimethyl-N-(n-tetradecyl)ammoniummethyl]-7-(N,N-dimethylamino)coumarin chloride; DTMAC, 4-(n-dodecylthiomethyl)-7-(N,N-dimethylamino)coumarin; DiPoPC, dipalmitoleoylphosphatidylcholine; DiPoPE, dipalmitoleoylphosphatidylethanolamine; 5-doxyl-PC, 1-palmitoyl-2-(5-doxyl)stearoylphosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; T_H, bilayer to hexagonal phase transition temperature.

* Corresponding author. Fax: +1 (905) 5229033; e-mail: epand@fhs.csu.mcmaster.ca.

head group region of a phospholipid bilayer. Subsequently Ohki and Arnold [5] showed that increased hydrophobicity in the region of the membrane surface, monitored by DNS-PE, was associated with increased rates of membrane fusion. This probe has also been used in another recent study to monitor surface polarity by comparing fluorescence emission intensity in the presence of ^2H_2O vs. H_2O [6]. Another dansylated probe, N^ε-dansyl-L-lysine (DNS-Lys), has also been used to study membrane surface properties [7]. For many of the fluorescent probes that have been employed to study membrane surface characteristics, the exact location of the probe in the bilayer structure is not known. Recently 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 [8]. The present paper describes fluorescent probes that measure membrane surface hydrophobicity at different locations with respect to the membrane surface. In the case of DNS-Lys, the probe may partition between aqueous and membrane phases [7]. However, the probes used in the present study are anchored in the membrane. The aminonaphthalene-containing probes PRODAN, LAURDAN [9] and PATMAN [10] have been successfully employed as polarity-sensitive fluorophores. Two 7-dimethylaminocoumarin derivatives, i.e., 4-[N,N-dimethyl-N-(n-tetradecyl)ammoniummethyl]-7-(N,N-dimethylamino)coumarin chloride (TAMAC) and 4-(n-dodecylthiomethyl)-7-(N,N-dimethylamino)coumarin (DTMAC) have been synthesized. The structures of these compounds are given in Fig. 1. The synthesis of these probes and preliminary model experiments have been reported elsewhere [11].

2. Experimental procedures

2.1. Materials

The diacyl lipids dipalmitoleoylphosphatidylcholine (DiPoPC), dipalmitoleoylphosphatidylethanolamine (DiPoPE) and 1-palmitoyl-2-(5-doxyl)stearoyl-phosphatidylcholine (5-doxyl-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). The synthesis of TAMAC and DTMAC was described previously [11]. All other chemicals were of the purest grade commercially available.
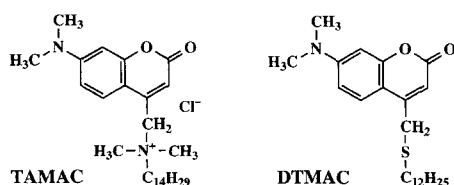

Fig. 1. Structures of the probes TAMAC and DTMAC.

2.2. Sample preparation

Lipids and fluorescent probe (as well as 5-doxyl-PC, when employed) were codissolved in chloroform/methanol (2:1, v/v). The solvent was evaporated with a stream of nitrogen and the last traces of solvent removed in a vacuum desiccator for at least 1 h using a liquid nitrogen trap. The lipid films were then hydrated at room temperature with 20 mM Pipes, 1 mM EDTA, 150 mM NaCl, 0.02 mg/ml NaN_3 (pH 7.4) by vigorous vortexing.

2.3. Steady-state fluorescence measurements

Lipid suspensions were placed in 1 cm^2 fluorescence cuvettes with constant stirring. The concentration of lipid generally used was 3.5 mM. The temperature was regulated with a constant temperature bath which circulated fluid through the cell holder. The temperature was monitored with a thermistor probe in a blank cuvette. The temperature was allowed to equilibrate for at least 5 min before doing a spectral scan and the contents vortexed vigorously for a brief period before the start of each scan. This was particularly important with DiPoPE in order to disperse the sample. In experiments in which temperature was varied, measurements were always begun at the lowest temperature. Fluorescence measurements were made with an SLM Aminco Series II spectrofluorimeter. For both TAMAC and DTMAC, an excitation wavelength of 397 nm was used. A 420 nm cut off filter was used between the sample and photomultiplier.

2.4. Fluorescence lifetime measurements

Fluorescence lifetime measurements were performed with the LS-100 multi-purpose instrument from Photon Technology International (London, Ontario, Canada), in 1 cm^2 cuvettes at 25°C. This instrument uses a pulse sampling stroboscopic technique. The flash lamp typically operates at 20–27 kHz and produces light pulses of about 1.3 ns (in hydrogen gas). Lifetimes were calculated using a iterative curve fitting procedure based on the Marquardt algorithm which was supplied by the instrument manufacturer. Optimal excitation and emission settings were chosen.

3. Results

The two fluorescent probes, TAMAC and DTMAC, exhibit a marked sensitivity to the dielectric constant of the solvent in which they are dissolved. The emission spectra shift by about 100 nm between solutions in hexane vs. water [11]. The fluorescence lifetime of these probes is also sensitive to solvent polarity Table 1. This is more evident for TAMAC than for DTMAC, indicating different solubility and/or aggregation states of the two probes in

Table 1

Fluorescence lifetimes (τ) of TAMAC and DTMAC in solvents and liposomes

Medium	τ(ns) [c]	
	TAMAC	DTMAC
Hexane	7.2	1.1
Ethanol	5.8	2.6
DMF	2.6	1.7
Glycerol	3.2	2.3
PC/PS liposomes [a]	1.7	3.6
Buffer [b]	1.0	1.4

[a] PC/PS (9:1, molar ratio) liposomes were prepared by extrusion through 400 nm pore-polycarbonate membranes in buffer. The probe:lipid molar ratio was about 150.

[b] 10 mM KCl, 2 mM Hepes (pH 7.4).

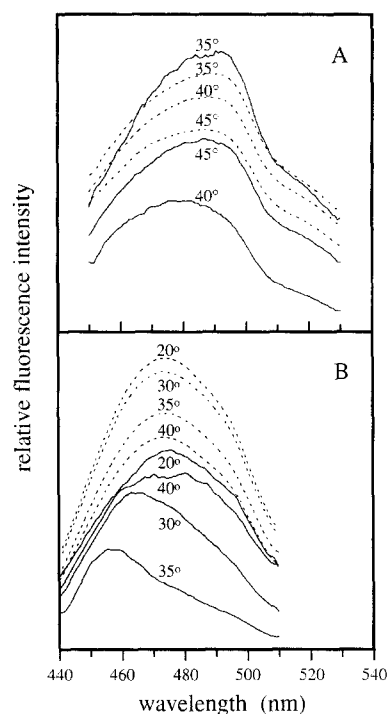[c] The concentration of probe was 5 μM for both cases.

Fig. 2. Emission spectra of TAMAC (A) and of DTMAC (B) at different temperatures. Solid lines correspond to DiPoPE and dashed lines to DiPoPC. TAMAC used at 0.17 mol% and DTMAC at 0.5 mol%.

the different solvents. Both probes show a marked increase of lifetime after insertion into liposomes as compared to buffer.

We have been particularly interested in the behavior of these probes when incorporated into membranes. In our earlier report we have shown that in liposomes of phosphatidylcholine (PC)/phosphatidylserine (PS) (9:1, molar ratio), the TAMAC and DTMAC fluorophores are in environments with dielectric constants of about 21 and 12, respectively, and that TAMAC was more accessible to hydrophilic quenching agents [11]. In the present work we have focussed on the relationship between membrane curvature and the polarity of the membrane interface.

Phosphatidylcholine and phosphatidylethanolamine are both zwitterionic lipids with similar chemical structures. However, phosphatidylethanolamine lacks the three methyl groups on its head group moiety and as a consequence it has a much greater propensity of forming inverted phases. The emission spectra of the two fluorescent probes in DiPoPC and DiPoPE show that in DiPoPE liposomes the probes show a marked sensitivity to changes in temperature (Fig. 2). This is particularly the case for DTMAC. The fluorescence emission maxima, taken from smoothed curves, are plotted against temperature for the two probes in each of the two lipids (Fig. 3). This clearly shows that the environment of the two probes in DiPoPC does not change with temperature. This is in contrast to that of DiPoPE which shows a marked shift toward lower wavelengths for both probes at temperatures just below the bilayer to hexagonal phase transition temperature (T_H). At and above T_H, the emission maximum again shifts back toward the red. Using differential scanning calorimetry, we have separately measured the shifts in T_H in the presence of the 0.5 mol% probe, as used for the data of Fig. 3. The T_H for DiPoPE alone was 42°C which shifted to 40.2°C with 0.5 mol% DTMAC and to 43.9°C with TAMAC.

We also studied fluorescence quenching of DTMAC in DiPoPC and DiPoPE using the 5-doxyl-PC quencher at a 15 mol% concentration (Fig. 4). The quencher has a

nitroxide moiety at the 5 position of the sn-2 acyl chain. Hence for the type of interfacial fluorophore we are evaluating, greater quenching is expected to be observed in cases where the fluorophore is more deeply embedded in the membrane. In the case of TAMAC, the quenching by
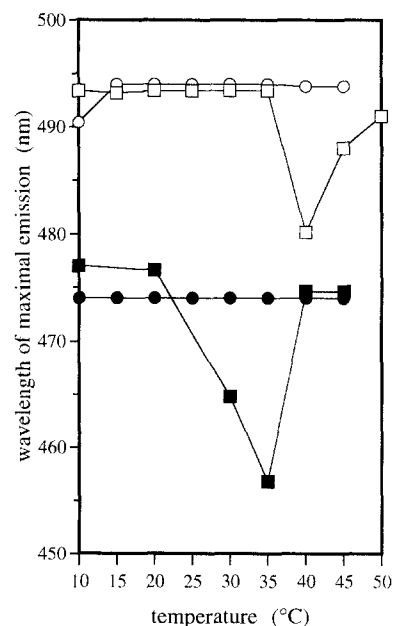

Fig. 3. Plot of fluorescent emission maxima vs. temperature. Conditions as for Fig. 2 but over a wider range of temperatures. Open symbols, TAMAC; filled symbols, DTMAC. DiPoPC, circles; DiPoPE, squares.
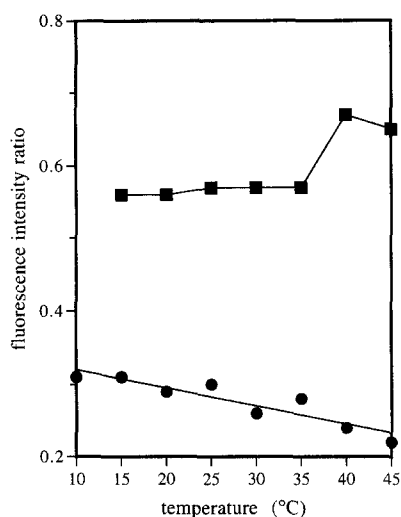
Fig. 4. Temperature dependence of fluorescence quenching of 0.5 mol% DTMAC by 15 mol% 5-doxyl-PC. The intensity ratio is calculated as the ratio of the fluorescence intensity at the wavelength of maximal emission in the presence vs. the absence of 5-doxyl-PC. DiPoPC, circles; DiPoPE, squares.

this spin-labelled lipid was smaller but the results were quantitatively less reproducible and are not included here. DTMAC, however, gave consistent results. The degree of quenching by 5-doxyl-PC was sensitive to differences between DiPoPC and DiPoPE as well as to temperature (Fig. 4).

4. Discussion

The two fluorescent probes evaluated in this study are markedly sensitive to changes which accompany the approach of T_H. These changes are observed at temperatures below those in which this transition is detected by other methods. This indicates that the nature of the bilayer is altered as T_H is approached even before inverted structures are formed. In fact at higher temperatures in which the H_{II} phase would form, the shifts in emission wavelength are reversed, again indicating that it is not the presence of the H_{II} phase itself that causes these changes. These spectral shifts are not observed with these probes in DiPoPC. It is interesting that a similar study with these lipids using DNS-Lys as the fluorescent probe also showed this sensitivity. However, that probe indicated a less hydrophobic environment as T_H was approached [7]. This is opposite to the shifts observed with both TAMAC and DTMAC (Fig. 3). The difference in the properties of these probes from that of DNS-Lys can be explained by the fact that TAMAC and DTMAC have a long hydrocarbon chain which would anchor their fluorophores at close proximity to or inside the membrane/water interface. Both probes are very sparsely soluble in water. DNS-Lys on the other hand readily partitions into water. In the hexagonal phase the lipid head groups become more closely packed. This is

likely to be the cause of the reversal of the spectral shifts seen at temperatures at and above T_H with TAMAC and DTMAC. A similar effect likely causes the more soluble DNS-Lys to completely dissociate from the membrane, even below T_H.

Although the TAMAC and DTMAC probes behave similarly with the two lipids studied, there are important differences between them which may be a consequence of their relative location in the membrane. DTMAC exhibits a spectral shift to shorter wavelengths at lower temperatures than does TAMAC. Thus the destabilization of the bilayer is sensed by DTMAC before TAMAC because it is reporting on changes in a different location in the membrane. Also this spectral shift persists to higher temperatures for TAMAC than for DTMAC, probably because the closer packing of the head groups in the hexagonal phase will first 'squeeze out' the fluorophore that is deeper in the membrane, i.e. DTMAC.

It is also interesting to note that despite the sensitivity of TAMAC and DTMAC to membrane surface properties, these probes have rather similar fluorescence emission properties in different lipid mixtures, i.e. the DiPoPC and DiPoPE used in this study as well as the phosphatidylcholine/phosphatidylserine vesicles previously studied [11]. In the case of TAMAC, the fluorophore may be too far from the membrane surface to detect interfacial polarity differences among membranes that form stable bilayers. Only in the case of an unstable bilayer with surface defects, will the fluorophore of TAMAC be sensitive to changes in membrane surface polarity. In the case of DTMAC, in order to place the fluorophore closer to the membrane interface, we had to forgo the use of a charged positioning group. Hence the DTMAC is more free to move along the bilayer normal. Thus it may partition to different locations in the membrane, depending on the nature of the membrane. The 5-doxyl-PC quencher will be largely fixed in its depth of burial in the membrane as a consequence of the fixed location of the phospholipid head group. From quenching experiments (Fig. 4), it appears that DTMAC is further from the fixed position of the nitroxide in DiPoPE than it is in DiPoPC. This is the expected direction based on the tighter packing of DiPoPE membranes because of the hydrogen-bonded network among head groups. Thus, although the surface polarity of DiPoPE is likely to be lower than DiPoPC, DTMAC reports the same polarity for both (Fig. 3) because it is deeper within DiPoPC membrane (Fig. 4). It is interesting that as T_H is approached with DiPoPE, DTMAC senses a *more* hydrophobic environment (Fig. 3), although the probe moves slightly to the surface of the membrane as indicated by the lower quenching with the 5-doxyl-PC as temperature is increased (Fig. 4). We suggest that DTMAC generally partitions into a membrane so as to position the fluorophore at a region of a particular dielectric constant. However, because of the alkyl chain anchor, there are limits to the movement of this probe along the bilayer

normal. Hence when the surface polarity is markedly changed this can be detected by DTMAC.

We have thus studied how the properties of the two fluorescent probes, TAMAC and DTMAC, are affected by membrane properties. Both probes are sensitive to the alterations which occur in the bilayer as T_H is approached. These results indicate that the region near the membrane interface becomes more hydrophobic at temperatures just below T_H. This increased hydrophobicity could be caused by both a combination of surface dehydration as well as rearrangement of the acyl chains so that the methyl terminus of the chain has more tendency to migrate toward the interface. We also suggest that the degree of quenching of DTMAC by 5-doxyl-PC is indicative of the depth to which substances added to the membrane can penetrate. Finally, we are continuing to design new fluorescent probes that will be at a fixed position in the membrane closer to the interface than TAMAC is.

Acknowledgements

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