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Exploring membrane organization and dynamics by the wavelength-selective fluorescence approach

Amitabha Chattopadhyay *

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Abstract

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. 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). This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. REES arises from slow rates of solvent relaxation (reorientation) around an excited state fluorophore which is a function of the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. Further, since the ubiquitous solvent for biological systems is water, the information obtained in such cases will come from the otherwise 'optically silent' water molecules. This makes REES and related techniques extremely useful since hydration plays a crucial modulatory role in a large number of important cellular events, including lipid–protein interactions and ion transport. The interfacial region in membranes, characterized by unique motional and dielectric characteristics, represents an appropriate environment for displaying wavelength-selective fluorescence effects. The application of REES and related techniques (wavelength-selective fluorescence approach) as a powerful tool to monitor the organization and dynamics of probes and peptides bound to membranes, micelles, and reverse micelles is discussed.

Keywords: Wavelength-selective fluorescence; Red edge excitation shift; Membrane organization and dynamics; Membrane interface; Membrane hydration; Membrane probes

1. Introduction

Biological membranes are complex assemblies of lipids and proteins that allow cellular compartmentalization and act as the interface through which cells communicate with each other and with the external milieu. The biological membrane constitutes the site of many important cellular functions including transfer of information from outside to the interior of the cell. However, our understanding of these processes at the molecular level is limited by the lack of high resolution three-

* Tel.: +91-40-2719-2578; fax: +91-40-2716-0311.
E-mail address: amit@ccmb.res.in (A. Chattopadhyay).

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dimensional structures of membrane-bound molecules. It is extremely difficult to crystallize membrane-bound molecules for diffraction studies. Only a few years back was the first complete X-ray crystallographic analysis of an integral membrane protein successfully carried out (Deisenhofer et al., 1985). Even high resolution NMR methods have limited applications for membrane-bound molecules because of slow reorientation times in membranes (Opella, 1997).

Due to the inherent difficulty in crystallizing membrane-bound molecules, most structural analyses of membranes have utilized other biophysical techniques with an emphasis on spectroscopic approaches. Fluorescence spectroscopy has been one of the principal techniques to study organization and dynamics of biological and model membranes because of its suitable time scale, minimal perturbation, non-invasive nature and intrinsic sensitivity (Radda, 1975; Lakowicz, 1981; Chattopadhyay, 1992; Stubbs and Williams, 1992; Mukherjee and Chattopadhyay, 1995). This review is focused on the application of a novel approach, the *wavelength-selective fluorescence approach*, as a powerful tool to monitor the organization and dynamics of probes and peptides bound to membranes and membrane-mimetic systems such as micelles and reverse micelles.

2. Red edge excitation shift

In general, fluorescence emission is governed by Kasha's rule which states that fluorescence normally occurs from the zero vibrational level of the first excited electronic state of a molecule (Birks, 1970; Rohatgi-Mukherjee, 1978). It is obvious from this rule that fluorescence should be independent of wavelength of excitation. In fact, such a lack of dependence of fluorescence emission parameters on excitation wavelength is often taken as a criterion for purity and homogeneity of a molecule. Thus, for a fluorophore in a bulk non-viscous solvent, the fluorescence decay rates and the wavelength of maximum emission are usually independent of the excitation wavelength.

However, this generalization breaks down in case of polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases, that is, when the mobility of the surrounding matrix relative to the fluorophore is considerably reduced. This situation arises because of the importance of the solvent shell and its dynamics around the fluorophore during the process of absorption of a photon and its subsequent emission as fluorescence. Under such conditions, when the excitation wavelength is gradually shifted to the red edge of the absorption band, the maximum of fluorescence emission exhibits a concomitant shift toward higher wavelengths. Such a shift in the wavelength of maximum emission toward higher wavelengths, caused by a corresponding shift in the excitation wavelength toward the red edge of the absorption band, is termed the red edge excitation shift (REES) (Chen, 1967; Fletcher, 1968; Galley and Purkey, 1970; Rubinov and Tomin, 1970; Castelli and Forster, 1973; Itoh and Azumi, 1975; Demchenko, 1982; Lakowicz and Keating-Nakamoto, 1984; Macgregor and Weber, 1981; Mukherjee and Chattopadhyay, 1995). Since REES is observed only under conditions of restricted mobility, it serves as a reliable indicator of the dynamics of fluorophore environment.

The genesis of REES lies in the change in fluorophore-solvent interactions in the ground and excited states brought about by a change in the dipole moment of the fluorophore upon excitation, and the rate at which solvent molecules reorient around the excited state fluorophore (Galley and Purkey, 1970; Itoh and Azumi, 1975; Demchenko, 1982, 1986, 1988; Lakowicz and Keating-Nakamoto, 1984; Macgregor and Weber, 1981; Demchenko and Ladokhin, 1988). For a polar fluorophore, there exists a statistical distribution of solvation states based on their dipolar interactions with the solvent molecules both in the ground and excited states. Since the dipole moment of a molecule changes upon excitation, the solvent dipoles have to reorient around this new excited state dipole moment of the fluorophore, so as to attain an energetically favorable orientation. This readjustment of the dipolar interaction of the solvent molecules with the fluorophore essentially consists of two components. First, the redistribution of electrons in the surrounding solvent

molecules because of the altered dipole moment of the excited state fluorophore, and then, the physical reorientation of the solvent molecules around the excited state fluorophore. The former process is almost instantaneous, i.e., electron redistribution in solvent molecules occurs at about the same time scale as the process of excitation of the fluorophore itself (10^{-15} s). The reorientation of the solvent dipoles, however, requires a net physical displacement. It is thus a much slower process and is dependent on the restriction to their mobility as offered by the surrounding matrix. More precisely, for a polar fluorophore in a bulk non-viscous solvent, this reorientation occurs at a time scale of the order of 10^{-12} s, so that all the solvent molecules completely reorient around the excited state dipole of the fluorophore well within its excited state lifetime, which is typically of the order of 10^{-9} s. Hence, irrespective of the excitation wavelength used, all emission is observed only from the solvent-relaxed state. However, if the same fluorophore is now placed in a viscous medium, this reorientation process is slowed down to 10^{-9} s or longer. Under these conditions, excitation by progressively lower energy quanta, i.e., excitation wavelength being gradually shifted towards the red edge of the absorption band, selectively excites those fluorophores which interact more strongly with the solvent molecules in the excited state. These are the fluorophores around which the solvent molecules are oriented in such a way as to be more similar to that found in the solvent-relaxed state. Thus, the necessary condition for giving rise to REES is that a different average population is excited at each excitation wavelength and, more importantly, that the difference is maintained in the time scale of fluorescence lifetime. As discussed above, this requires that the dipolar relaxation time for the solvent shell be comparable to or longer than the fluorescence lifetime, so that fluorescence occurs from various partially relaxed states. This implies a reduced mobility of the surrounding matrix with respect to the fluorophore.

The essential criteria for the observation of the red edge effect can thus be summarized as follows: (i) The fluorophore should normally be polar so as to be able to suitably orient the neighboring solvent molecules in the ground state; (ii) The solvent molecules surrounding the fluorophore should be polar; (iii) The solvent reorientation time around the excited state dipole moment of the fluorophore should be comparable to or longer than the fluorescence lifetime; and (iv) There should be a relatively large change in the dipole moment of the fluorophore upon excitation. The observed spectral shifts thus depend both on the properties of the fluorophore itself (i.e., the vectorial difference between the dipole moments in the ground and excited states), and also on properties of the environment interacting with it (which is a function of the solvent reorientation time). It has previously been shown for 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled phospholipids incorporated into model membranes, that a dipole moment change of ~ 4 D upon excitation is enough to give rise to significant red edge effects (Mukherjee et al., 1994). A recent comprehensive review on the red edge effect is provided by Demchenko (2002).

3. The wavelength-selective fluorescence approach

In addition to the dependence of fluorescence emission maxima on the excitation wavelength (REES), fluorescence polarization and lifetime are also known to depend on the excitation and emission wavelengths in viscous solutions and in otherwise motionally restricted media. Taken together, these constitute the wavelength-selective fluorescence approach which consists of 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).

Early applications of REES and wavelength-selective fluorescence to systems of biological relevance has been restricted mainly to indole, tryptophan, and other fluorescent probes in viscous solvents, and when present in proteins. The application of REES and related techniques to elucidate organization and dynamics in proteins have previously been reviewed (Demchenko, 1988, 1992; Lakowicz, 2000) and will be addressed

elsewhere (Raghuraman et al., in press) since it is beyond the scope of this review. The application of the wavelength-selective fluorescence approach as a powerful yet sensitive tool to monitor the organization and dynamics of probes and peptides bound to membranes and micelles constitutes the subject matter of this article.

4. The wavelength-selective fluorescence approach: a novel tool to monitor organization and dynamics of the membrane interfacial region

Organized molecular assemblies such as membranes can be considered as large cooperative units with characteristics very different from the individual structural units which constitute them. A direct consequence of such highly organized systems is the restriction imposed on the mobility of their constituent structural units. It is well known that interiors of biological membranes are viscous, with the effective viscosity comparable to that of light oil (Cone, 1972; Poo and Cone, 1974). The biological membrane, with its viscous interior, and distinct motional gradient along its vertical axis, thus provides an ideal system for the utilization of REES in particular and wavelength-selective fluorescence in general to study various membrane phenomena. The use of this technique becomes all the more relevant in view of the fact that no crystallographic database for membrane-bound probes and proteins exists to date, due to the inherent difficulty in crystallizing such molecules.

Among the three major regions in the membrane, the interfacial region is characterized by unique motional and dielectric characteristics (Ashcroft et al., 1981) 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 and plays an important role in functional aspects such as substrate recognition and activity of lipolytic enzymes (El-Sayed et al., 1985). This specific region of the membrane exhibits slow rates of solvent relaxation and is also known to participate in intermolecular charge interactions (Yeagle, 1987) and hydrogen bonding through the polar headgroup (Boggs, 1987; Gennis, 1989; Shin et al., 1991). These structural features which slow down the rate of solvent reorientation have previously been recognized as typical features of solvents giving rise to significant red edge effects (Itoh and Azumi, 1975). It is therefore the membrane interface which is most likely to display red edge effects and is sensitive to wavelength-selective fluorescence measurements (Fig. 1).

Initial reports, in which excitation wavelength dependence of emission maxima of the fluorescence probes TNS or ANS in model lecithin membranes were investigated, indicated no appreciable red shift (Lakowicz and Keating-Nakamoto, 1984; Demchenko and Shcherbatska, 1985). However, REES was reported in case of dioleoyl-sn-glycero-3-phosphocholine (DOPC) vesicles labeled with Patman, an amphiphilic phase-sensitive probe (Lakowicz et al., 1983). The above results can be rationalized on the basis of the location of these probes in the membrane. On one hand, both TNS and ANS are charged at neutral pH and, therefore, access the phospholipid headgroup from the external aqueous phase. This implies that, for these probes, the immediate environment will be the aqueous phase adjacent to the headgroup, where solvent relaxation is extremely fast, and as such, no red edge effect can be expected. On the other hand, because of the fatty acyl chain in Patman, it partitions well into the membrane, and therefore, experiences a motionally restricted environment. In a later report (Gakamsky et al., 1992), REES of membrane-bound 1-phenylnaphthylamine (1-AN) was monitored. However, this probe has the limitation of not having a unique location in the membrane—rather, it has a distribution of locations in the membrane. This makes the interpretation of REES data more difficult since the spectral information obtained can no longer be attributed to a unique environment in the membrane.

The choice of a suitable probe is thus of utmost importance in designing membrane-active molecules capable of exhibiting REES. It is desirable that the probe be polar and be able to strongly partition into the membrane and intercalate with its normal components, i.e., the lipids. Further, the fluorescent portion of the molecule should be

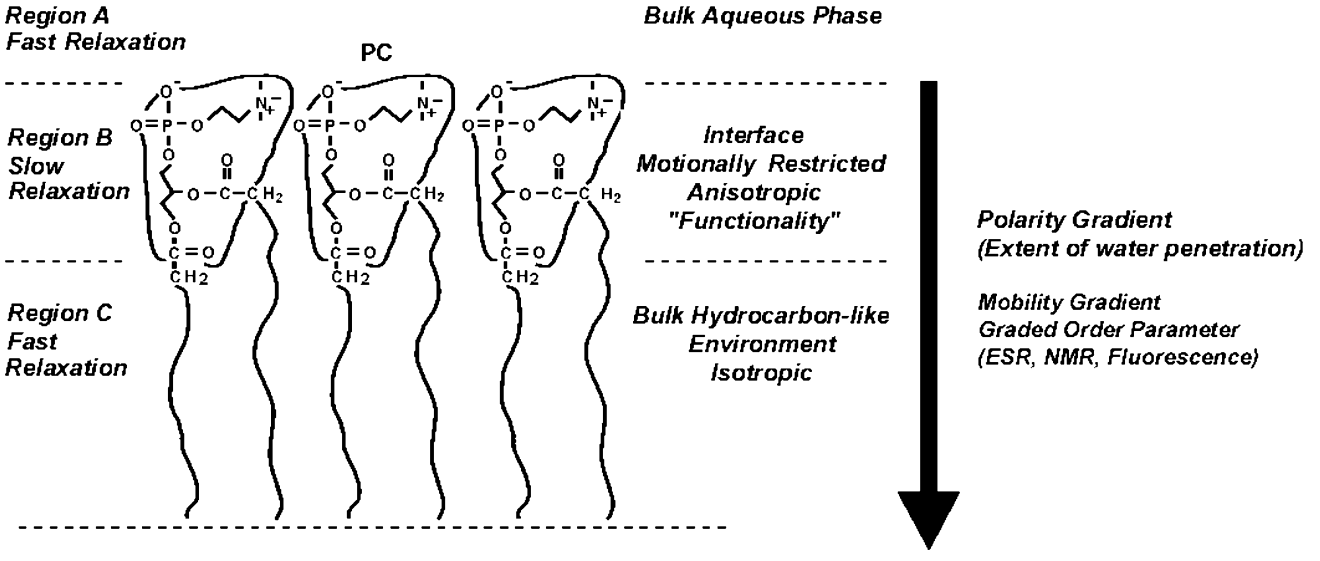

Fig. 1. A schematic diagram of half of the membrane bilayer showing the motional anisotropy of the bilayer. The dotted horizontal line at the bottom indicates the center of the bilayer. The membrane anisotropy along the axis perpendicular to the plane of the bilayer divides the membrane leaflet into three broad regions exhibiting very different dynamics (as revealed by various spectroscopic techniques such as ESR, NMR and fluorescence measurements). *Region A:* bulk aqueous phase, fast solvent relaxation; *Region B:* slow (restricted) solvent relaxation, hydrogen bonding (important for functionality), water penetration (interfacial water), highly anisotropic medium; *Region C:* bulk hydrocarbon-like environment, isotropic, fast solvent relaxation. A polarity gradient is also set up along this axis. Fluorescent probes and peptides localized in the interfacial region B are most likely to display red edge effects and exhibit sensitivity to wavelength-selective fluorescence measurements.

suitably embedded in the membrane. REES is indeed observed when the above criteria are satisfied (Ladokhin et al., 1991; Chattopadhyay and Mukherjee, 1993, 1999a,b; Chattopadhyay and Rukmini, 1993; Mukherjee and Chattopadhyay, 1994; Hutterer et al., 1996; Chattopadhyay et al., 1997; Ghosh et al., 1997; Santos et al., 1998; MacPhee et al., 1999; Raja et al., 1999; Granjon et al., 2001). In addition, it is preferable that the membrane-embedded molecule has only one fluorescent group which has a unique location in the membrane and not a distribution of locations. Such probes can be used to correlate the extent of REES with a specific fluorophore environment which in case of such uniquely localized probes, translates to a specific region of the membrane.

One such probe that has been employed to study the phenomenon of REES and related effects in membranes (Chattopadhyay and Mukherjee, 1993, 1999b) and membrane-mimetic systems (Rawat et al., 1997; Rawat and Chattopadhyay, 1999) is the widely used lipid probe *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (NBD-PE). The NBD group is an extensively used fluorophore in biophysical, biochemical, and cell biological studies. NBD-labeled lipids are extensively used as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes (for a review on NBD-labeled lipids, see Chattopadhyay, 1990). The NBD moiety possesses some of the most desirable properties for serving as an excellent probe for both spectroscopic and microscopic applications. It is very weakly fluorescent in water. Upon transfer to a hydrophobic medium, it fluoresces brightly in the visible range and exhibits a high degree of environmental sensitivity (Chattopadhyay and London, 1988; Lin and Struve, 1991; Chattopadhyay and Mukherjee, 1993; Fery-Forgues et al., 1993; Mukherjee et al., 1994; Mazeres et al., 1996). Fluorescence lifetime of the NBD group is extremely sensitive to the environmental polarity (Lin and Struve, 1991; Rawat and Chattopadhyay, 1999). It is relatively photostable, and lipids labeled with the NBD group mimic endogenous lipids in studies of intracellular lipid transport (Van Meer et al., 1987; Koval and Pagano, 1990).

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; Mitra and Hammes, 1990; Wolf et al., 1992; Abrams and London, 1993). This 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 (Seelig, 1977; Ashcroft et al., 1981; Stubbs et al., 1985; Perochon et al., 1992; Slater et al., 1993; Venable et al., 1993; White and Wimley, 1994; Gawrisch et al., 1995) 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). NBD-PE exhibits REES in model membranes of DOPC (Chattopadhyay and Mukherjee, 1993, 1999b). Since the precise localization of the fluorescent NBD group in membrane-bound NBD-PE is known to be interfacial (Chattopadhyay and London, 1987, 1988; Mitra and Hammes, 1990; Wolf et al., 1992; Abrams and London, 1993), this result directly implies that the interfacial region of the membrane offers considerable restriction to the reorientational motion of the solvent dipoles around the excited state fluorophore. In another study, REES of membrane-bound NBD–cholesterol, in which the NBD group is covalently attached to the flexible chain of the cholesterol molecule, was reported (Chattopadhyay and Mukherjee, 1999b).

5. Wavelength-selective fluorescence as a membrane dipstick

The biological membrane is a highly organized molecular assembly, largely confined to two-dimensions, and exhibits considerable degree of anisotropy along the axis perpendicular to the membrane. This not only results in the anisotropic behavior of the constituent lipid molecules, but more importantly, the environment of a probe molecule becomes very much dependent on its precise localization in the membrane. While the center of the bilayer is nearly isotropic, the upper portion, only a few angstroms away toward the membrane surface, is highly ordered (Seelig, 1977; Ashcroft et al., 1981; Stubbs et al., 1985; Perochon et al., 1992; Slater et al., 1993; Venable et al., 1993; White and Wimley, 1994; Gawrisch et al., 1995). As a result, properties such as polarity, fluidity, segmental motion, ability to form hydrogen bonds and extent of solvent (water) penetration would vary in a depth-dependent manner in the membrane (Fig. 1). A direct consequence of such an anisotropic transmembrane environment will be the differential extents to which the mobility of water molecules will be retarded at different depths in the membrane relative to the water molecules in bulk aqueous phase. This offers the possibility of using wavelength-selective fluorescence as a novel approach to investigate the depth of membrane penetration of a reporter fluorophore i.e., as a membrane dipstick. This was tested by demonstrating that chemically identical fluorophores, varying solely in terms of their localization at different depths in the membrane, experience very different local environments, as judged by wavelength-selective fluorescence parameters (Chattopadhyay and Mukherjee, 1999b). Two anthroyloxy stearic acid derivatives where the anthroyloxy group has previously been found to be either shallow (2-AS) or deep (12-AS) were used. It was shown that the anthroyloxy moiety of 2- and 12-AS experiences different local membrane microenvironments, as reflected by depth-dependent variation of REES as well as varying degrees of wavelength dependence of fluorescence polarization and lifetime, and rotational correlation times. These results were attributed to differential rates of solvent reorientation in the immediate vicinity of the anthroyloxy group as a function of its membrane penetration depth. Wavelength-selective fluorescence therefore constitutes a novel approach to probe defined depths in the membrane and can be conveniently used as a dipstick to characterize the depth of penetration of membrane-embedded fluorophores.

6. Application of the wavelength-selective fluorescence approach to membrane peptides and proteins

The presence of tryptophan residues as intrinsic fluorophores in most peptides and proteins makes them an obvious choice for fluorescence spectroscopic analyses of such systems. The role of tryptophan residues in the structure and function of membrane proteins has recently attracted a lot of attention (Chattopadhyay et al., 1997; Reithmeier, 1995). Membrane proteins have been reported to have a significantly higher tryptophan content than soluble proteins (Schiffer et al., 1992). In addition, it is becoming increasingly evident that tryptophan residues in integral membrane proteins and peptides are not uniformly distributed and that they tend to be localized toward the membrane interface, possibly because they are involved in hydrogen bonding (Ippolito et al., 1990) with the lipid carbonyl groups or interfacial water molecules. As mentioned earlier, the interfacial region in membranes is characterized by unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the hydrocarbon-like interior of the membrane (Seelig, 1977; Ashcroft et al., 1981; Stubbs et al., 1985; Perochon et al., 1992; Slater et al., 1993; Venable et al., 1993; White and Wimley, 1994; Gawrisch et al., 1995). The tryptophan residue has a large indole side chain that consists of two fused aromatic rings. In molecular terms, tryptophan is a unique amino acid since it is capable of both hydrophobic and polar interactions. In fact, the hydrophobicity of tryptophan, measured by partitioning into bulk solvents, has previously been shown to be dependent on the scale chosen (Fauchere, 1985). Tryptophan ranks as one of

the most hydrophobic amino acids on the basis of its partitioning into polar solvents such as octanol (Fauchere and Pliska, 1983) while scales based on partitioning into non-polar solvents like cyclohexane (Radzicka and Wolfenden, 1988) rank it as only intermediate in hydrophobicity. This ambiguity results from the fact that while tryptophan has the polar –NH group which is capable of forming hydrogen bonds, it also has the largest non-polar accessible surface area among the naturally occurring amino acids (Wimley and White, 1992). Wimley and White (1996) have recently shown from partitioning of model peptides to membrane interfaces that the experimentally determined interfacial hydrophobicity of tryptophan is highest among the naturally occurring amino acid residues, thus accounting for its specific interfacial localization in membrane-bound peptides and proteins. Due to its aromaticity, the tryptophan residue is capable of π–π interactions and of weakly polar interactions (Burley and Petsko, 1988). The amphipathic character of tryptophan gives rise to its hydrogen bonding ability which could account for its orientation in membrane proteins and its function through long-range electrostatic interaction (Fonseca et al., 1992). The amphipathic character of tryptophan also explains its interfacial localization in membranes due to its tendency to be solubilized in this region of the membrane, besides favorable electrostatic interactions and hydrogen bonding. It has already been mentioned (see above) that the membrane interface is most likely to display red edge effects and is sensitive to wavelength-selective fluorescence measurements. This makes study of membrane peptides and proteins by the wavelength-selective fluorescence approach very appropriate. Indeed, tryptophan octyl ester, often used as a simple model for membrane-bound tryptophan residues, exhibits pH-dependent REES when bound to membranes (Chattopadhyay et al., 1997).

Melittin, the major toxic component in the venom of the European honey bee, *Apis mellifera*, was one of the earlier membrane peptides studied using the wavelength-selective fluorescence. Results from these studies showed that when bound to zwitterionic membranes, the microenvironment of the sole functionally active tryptophan of melittin was motionally restricted as evident from REES and other results (Chattopadhyay and Rukmini, 1993). However, when bound to negatively charged membranes, studies using the wavelength-selective fluorescence approach indicate that the microenvironment of the tryptophan gets modulated and this could be related to the functional difference in the lytic activity of the peptide observed in the two cases (Ghosh et al., 1997).

In yet another study, the phenomenon of REES, in conjunction with time-resolved fluorescence spectroscopic parameters such as wavelength-dependent fluorescence lifetimes and time-resolved emission spectra were utilized to study the localization and dynamics of the functionally important tryptophan residues in the gramicidin channel (Mukherjee and Chattopadhyay, 1994). Gramicidin belongs to a family of prototypical channel formers which are naturally fluorescent due to the presence of four tryptophan residues. These interfacially localized tryptophans are known to play a crucial role in the organization and function of the channel. The results from the above study point out the motional restriction experienced by the tryptophans at the peptide–lipid interface of the gramicidin channel. This is consistent with other studies (Becker et al., 1991; Fonseca et al., 1992) in which such restrictions are thought to be imposed due to hydrogen bonding between the indole rings of the tryptophan residues in the channel conformation and the neighboring lipid carbonyls. The significance of such organization in terms of functioning of the channel is brought out by the fact that substitution, photodamage, or chemical modification of these tryptophans are known to give rise to channels with altered conformation and reduced conductivity. Tryptophans in another pore-forming toxin, *Staphylococcus aureus* α-toxin, also exhibit REES indicating a restricted and buried environment for these residues (Raja et al., 1999).

In addition, the red edge effect has also been utilized to study the microconformational heterogeneity of the membrane-binding domain of cytochrome b_5 by comparing the information obtained from the native protein and its mutant

which has a single tryptophan residue in this domain (Ladokhin et al., 1991). Both these proteins show a red shift in the emission spectrum when excited at the long wavelength edge of the excitation spectrum, indicating thereby that the tryptophan residue(s) in both cases are localized in a region of motional constraint. Very recently, REES of tryptophans in membrane-bound mitochondrial creatine kinase has been reported (Granjon et al., 2001).

7. Wavelength-selective fluorescence in micelles

Micelles represent yet another type of organized molecular assembly formed by the hydrophobic effect and are highly cooperative, dynamic assemblies of soluble amphiphiles (detergents). They offer certain inherent advantages in fluorescence studies over membranes since micelles are smaller and optically transparent, have well defined sizes, and are relatively scatter-free. Further, micelles can be of any desired charge type and can adopt different shapes and internal packing, depending on the chemical structures of the constituent monomers and the ionic strength of the medium. A direct consequence of such organized systems is the restriction imposed on the dynamics and mobility of the constituent structural units. The studies on micellar organization and dynamics assume special significance in light of the fact that the general principle underlying the formation of micelles is common to other related assemblies such as reverse micelles, bilayers, liposomes and biological membranes. Micelles are extensively used as membrane mimetics in studies of membrane proteins (Mattice et al., 1995) and peptides and as a model for the anesthetic action of pharmacological compounds (Desai et al., 1994).

The organization and dynamics of micellar environments, namely, the core, the interface, and the immediate layers of water on the interface, have been investigated using experimental (Shinitzky et al., 1971; Kalyanasundaram and Thomas, 1977; Mukerjee and Cardinal, 1978; Leung and Shah, 1986; Nery et al., 1986; Maiti et al., 1995; Saroja and Samanta, 1995) and theoretical (Gruen, 1985) approaches. It is fairly well established that practically all types of molecules have a surface-seeking tendency in micelles (due to very large surface area to volume ratio) and that the interfacial region is the preferred site for solubilization, even for hydrophobic molecules (Mukerjee and Cardinal, 1978; Ganesh et al., 1982; Shobha and Balasubramanian, 1986; Shobha et al., 1989). The suitability of micellar systems for studies employing wavelength-selective fluorescence was therefore tested using the interfacial fluorescence probe NBD-PE (Rawat et al., 1997). NBD-PE exhibits REES when incorporated in micelles formed by a variety of detergents (SDS, Triton X-100, CTAB, and CHAPS) which differ in their charge, aggregation number and shape (Rawat et al., 1997; Rawat and Chattopadhyay, 1999). These results clearly demonstrate that the relaxation rates of micellar interfacial hydration are very different from that of the bulk water and this feature may play an important role in the reactions catalyzed by micelles.

Structural transition can be induced in charged micelles by increasing ionic strength of the medium or amphiphile concentration. Thus, spherical micelles of sodium dodecyl sulfate that exist in water at concentrations higher than critical micelle concentration assume an elongated rod-like structure in presence of increased electrolyte (salt) concentration when interactions among the charged headgroups are attenuated due to the added salt (Fig. 2). This is known as sphere-to-rod transition (Missel et al., 1982). The change in organization and dynamics that is accompanied with the salt-induced sphere-to-rod transition in SDS micelles was monitored using NBD-labeled lipids utilizing the wavelength-selective fluorescence approach. It thus appears that REES and related parameters are sensitive indicators of the structural transition in micelles induced by salt.

8. Wavelength-selective fluorescence in reverse micelles

Amphiphilic surfactants self assemble to form reverse micelles in non-polar solvents in which the polar head groups of the surfactant monomers cluster to form a micellar core and are directed

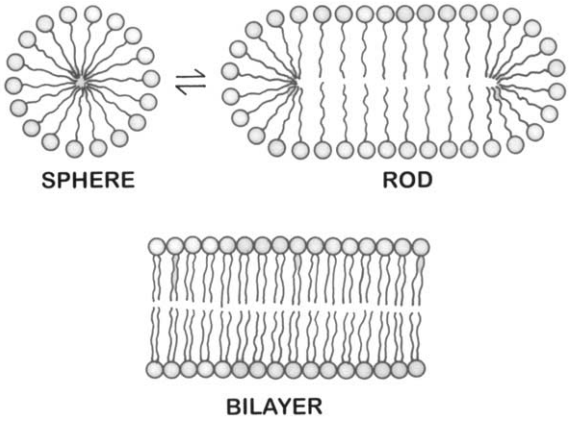

Fig. 2. A schematic representation of the sphere-to-rod transition in charged micelles induced by salt (adapted from Rawat and Chattopadhyay, 1999). Note that the headgroup spacing is reduced in rod-shaped micelles due to attenuation of electrostatic interactions among the charged headgroups by the added salt (electrolyte). For comparison, the bilayer arrangement is also shown at the bottom.

toward the center of the assembly and the hydrophobic tails extend outward into the bulk organic phase (Luisi and Magid, 1986; Luisi et al., 1988). Reverse micelles are optically transparent nanometer-sized water droplets of various size surrounded by a layer of surfactant molecules dispersed in non-polar solvents. Studies on reverse micellar organization and dynamics are relevant since the general principle underlying their formation (the hydrophobic effect) is common to other related organized assemblies such as micelles, bilayers, liposomes and biological membranes. In addition, reverse micelles offer the unique advantage of monitoring dynamics of molecules with varying states of hydration (see later) which is difficult to achieve with complex systems such as membranes. The double chain anionic surfactant AOT (sodium bis(2-ethylhexyl) sulfosuccinate) has been extensively used to form reverse micelles in non-polar solvents. The water pools entrapped in reverse micelles have been extensively used as micro-media for chemical and biochemical reactions. The nature of water in reverse micelles, especially at low water content, has been studied extensively and is believed to be different from that of bulk water. The various types of water pools in reverse micelles, characterized by graded dynamics, represent interesting models for water present in biological systems such as membranes (Fig. 3). The physical and chemical properties of the entrapped water are markedly different from the properties of bulk water but similar in several aspects to those of biological interfacial water as found in membrane or protein interfaces. Both experimental (Jain et al., 1989; Ikushima et al., 1997; Brubach et al., 2001; Venables et al., 2001) and theoretical approaches (Faeder and Ladanyi, 2000) have shown that the crucial structural parameter of reverse micelles is the (water/surfactant) molar ratio (w_o) which determines their size as well as the extent of deviation of the properties of the entrapped water from those of normal bulk water. Reverse micelles thus represent a type of organized molecular assembly which offer the unique advantage of monitoring dynamics of molecules with varying degrees of hydration.

The applicability of the wavelength-selective fluorescence approach to reverse micellar systems

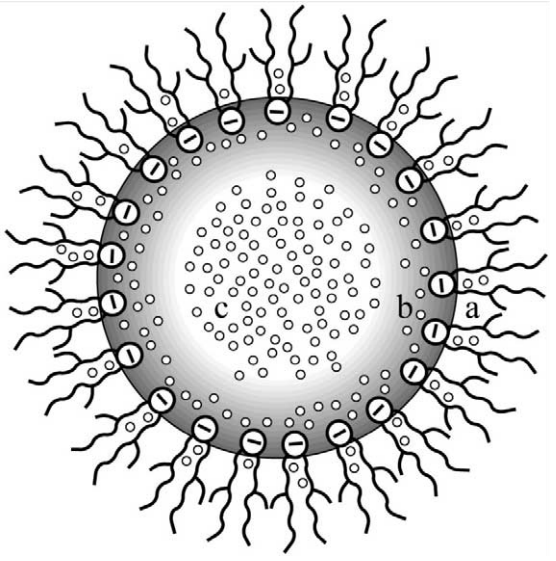

Fig. 3. A schematic diagram of a reverse micelle showing highly structured yet heterogeneous water pools of graded dynamics: (a) trapped water, (b) bound water, and (c) free water molecules. The crucial parameter is the [water]/[surfactant] molar ratio (w_o) which determines the relative proportions of these three types of water pools and the micellar size.

has been recently tested using the interfacial fluorescence probe NBD-PE incorporated in AOT/heptane reverse micelles. NBD-PE was found to exhibit REES when incorporated in AOT/heptane reverse micelles (Chattopadhyay et al., submitted for publication). Interestingly, the extent of REES was found to decrease with increasing w_o indicating that REES is sensitive to changing hydration dynamics and it is possible to detect differences in water dynamics that is accompanied with increasing water content.

It is known that the dynamics of liquids in confined spaces is different than that of their bulk counterparts (Granick, 1991; Brubach et al., 2001) and this constitutes one of the main reasons for the popularity that reverse micelles enjoy as a model system in studies of water dynamics. The highly structured yet heterogeneous water molecules in reverse micelles represent interesting models for water molecules present in biological systems such as membranes which are more difficult to analyze experimentally. Moreover, the dimension, shape, and overall charge of reverse micelles can be conveniently modulated which make them particularly useful for monitoring the dynamics of confined liquids. The properties of water in reverse micelles of AOT at low w_o values are rather different from those of bulk water (Jain et al., 1989; Ikushima et al., 1997; Brubach et al., 2001; Venables et al., 2001). Even at higher water content ($w_o = 50$), the apparent microviscosity is 6–9 times greater than that of free aqueous solutions (Andrade et al., 2000). Three types of water populations (pools) have been shown to coexist in reverse micelles (Fig. 3). These are bound water, trapped water, and free water (Jain et al., 1989; Ikushima et al., 1997; Hazra and Sarkar, 2001). The crucial parameter w_o determines the relative proportions of these three types of water pools. The above results with NBD–PE incorporated in AOT reverse micelles show that wavelength-selective fluorescence in general, and REES in particular, is sensitive to the changing dynamic hydration profile and can be conveniently used to probe dynamics of molecules in various states of hydration.

9. Conclusion

Water plays a crucial role in the formation and maintenance of both folded protein and membrane architecture in a cellular environment. Knowledge of dynamics of hydration at a molecular level is thus of considerable importance in understanding the cellular structure and function (Crowe and Crowe, 1984; Rand and Parsegian, 1989; Ho and Stubbs, 1992; Ho et al., 1994; Fischer et al., 1994; Kandori et al., 1995; Sankararamakrishnan and Sansom, 1995; Häussinger, 1996; Israelachvili and Wennerström, 1996; Nishimura et al., 1997; Hummer et al., 2001; Mentré, 2001). As mentioned earlier, REES is based on the change in fluorophore-solvent interactions in the ground and excited states brought about by a change in the dipole moment of the fluorophore upon excitation, and the rate at which solvent molecules reorient around the excited state fluorophore. Since for biological systems, the ubiquitous solvent is water, the information obtained in such cases will come from the otherwise 'optically silent' water molecules. The unique feature about REES is that while all other fluorescence techniques (such as fluorescence quenching, energy transfer, polarization measurements) yield information about the fluorophore (either intrinsic or extrinsic) itself, REES provides information about the relative rates of solvent (water in biological systems) relaxation dynamics which is not possible to obtain by other techniques. This makes the use of REES and the wavelength-selective fluorescence approach extremely useful in membrane biology since hydration plays a crucial modulatory role in a large number of important cellular events involving the membrane such as lipid–protein interactions (Ho and Stubbs, 1992) and ion transport (Crowe and Crowe, 1984; Rand and Parsegian, 1989; Ho and Stubbs, 1992; Ho et al., 1994).

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