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4-Aminophthalimide Derivatives as Environment-Sensitive Probes[1]

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The potential of 4-aminophthalimide (AP) and its derivatives as fluorescence probes for organized media is highlighted. The fluorescence response of AP, as measured from the position of the fluorescence maximum, fluorescence intensity and lifetime, is highly sensitive to the polarity of the medium. The sensitivity of the fluorescence parameters is further enhanced due to the involvement of the emitting intramolecular charge transfer state in hydrogen bonding interaction with the solvent molecules containing hydroxyl groups. It is shown that the microheterogeneous environments of organized media such as cyclodextrins and micelles can be very conveniently monitored using this probe. The results of the investigations carried out employing AP and its derivatives as fluorescence probe molecules in these media clearly suggest that a combination of the hydrophobic interaction with the host media and hydrogen bonding interaction with the solvent molecules determine the location of the fluorophore, which in all cases is found to be the interfacial region separating the nonpolar core of the micelle or the cyclodextrin cavity and the polar aqueous environment. Guidelines for the design of probes of this class of systems for the nonpolar core region of the micelles are provided and possible ways to increase the sensitivity of the fluorescence response of the systems are suggested.

KEY WORDS: 4-Aminophthalimide; organized media; fluorescence probe; donor–acceptor systems.

INTRODUCTION

Because of their biological relevance, organized media have become the focus of extensive investigations in recent years [1–27]. While a variety of techniques currently in use for the study of the structure and dynamics of a number of complex chemical and biological organized systems, the most commonly employed technique is one involving the use of fluorescent systems whose fluorescence response or output is sensitive to the surrounding microenvironment [23,25–27]. The microheterogeneous environments in the biological systems such as membranes, proteins, enzymes, or simpler model systems such as cyclodextrins, micelles, reverse micelles, or vesicles have been investigated along with some complex chemical systems such as polymers, polyelectrolytes, zeolites, clays, etc., using various fluorescence probes or reporter molecules [23,25–27].

Since the polarities and viscosities of different regions of an organized medium are quite different, one needs to employ probes which would respond differently depending on the surrounding microenvironment. A variety of probes is being employed at present to extract details on the structural information of the organized media and the dynamics therein [23,25–27]. One of the most extensively used fluorescence probes that forms a class by itself is pyrene [28–30]. The vibronic band intensities in the fluorescence spectra of pyrene are rather sensitive to the polarity of the surrounding environment. The ratio of the intensity of the third and the first vibronic band is 1.65 in nonpolar hexane, while that in polar acetonitrile is only 0.54 [29]. This polarity-depen-

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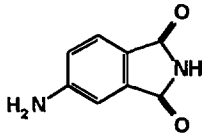
dent property has been made use of in the study of several organized systems. A few systems possessing close-lying n, π^* and π, π^* states also display solvent-sensitive fluorescence properties because changes in the polarity of the medium bring a change in the ordering of the two states. Pyrenecarboxaldehyde, Nile red, and alkoxycoumarins are notable examples of this class of probes [31–37]. A third category of systems whose excited states are involved in hydrogen bonding interaction in the excited state often shows solvent polarity-dependent fluorescence properties and hence serves as useful fluorescent probes. Xanthene dyes belong to this class of systems [38,39]. The most attractive systems, however, for probing the microheterogeneity of organized media are perhaps electron donor–acceptor systems [23–27]. The lowest excited state from which the fluorescence originates is of intramolecular charge transfer (ICT) nature and is sensitive to even small changes in the polarity around the fluorophore. Quite a few systems where the electron donor and the acceptor groups are connected by a single bond with the possibility of conjugation display a second emission band resulting from a state in which the donor and the acceptor moieties are orthogonal to each other [40–42]. This state, commonly termed the twisted intramolecular charge transfer (TICT) state [40], is highly dipolar because of complete separation of the charge in the orthogonal conformation and hence displays solvatochromic shift of the fluorescence band of magnitude that can hardly be found in other systems. 4-Dimethylaminobenzonitrile represents the most extensively studied probe of this category [25]. It should be noted that when the locally excited ICT state is coupled to a low-lying highly polar TICT state, the solvent sensitivity of the fluorescence properties of the ICT state is enhanced considerably. In other words, whether emitting or not, a low-lying TICT state increases the sensitivity of the emitting ICT state. Another possible way to increase the sensitivity of the fluorescence response of ICT systems is to engage an already sensitive ICT state in hydrogen bonding interaction with the solvent. While investigating electron donor–acceptor systems that may be useful for the study of organized media, we found that 4-aminophthalimide (AP), a highly fluorescent dye, displays quite interesting solvent-sensitive properties [43–45].



AP

The hydrogen bond donating solvents have tremendous influence on the position of the emission maximum, fluorescence quantum yield, and lifetime. This observation prompted us to employ this system as fluorescence probe for the study of some simple organized systems such as cyclodextrins and micelles, which represent model systems for more complex biological counterparts, enzyme–substrate binding, and membranes [44–47]. In this article, we point out the salient features of our observation and discuss the possible scope of improvement of the sensory behavior of the probes based on AP derivatives.

FLUORESCENCE RESPONSE AS A FUNCTION OF POLARITY

AP displays solvatochromic absorption and fluorescence bands typical of a charge transfer transition [43]. While the solvatochromic shift of the absorption band is small, that of the fluorescence band is quite significant. This behavior has to be rationalized in terms of an increase in the dipole moment (by 3.7 D) of the molecule on excitation. Since the fluorescent ICT state is not very polar (estimated μ_e of 8.3–9.0 D), one cannot expect a dramatic shift of the fluorescence band with polarity. A shift of 33 nm can be observed on changing the solvent from nonpolar diethyl ether (ε = 4.2) to polar acetonitrile (ε = 35.94). Interestingly, what dramatically increases the sensitivity of the fluorescence band position of AP is the specific interaction of the excited state of the molecule with hydrogen bond donating solvents such as alcohols and water. This is clearly evident from the fact that in alcoholic solvents such as methanol (ε = 32.66), the fluorescence maximum appears at 518 nm, whereas in aprotic acetonitrile (a solvent with higher ε) the maximum is observed at 458 nm.

AP is highly fluorescent in nonpolar media. With an increase in the polarity of the medium, the fluorescence quantum yield of the system does not change significantly as long as aprotic solvents are used. The yield remains more or less constant in the range of 0.73 to 0.63. However, a drastic fall in the yield takes place in protic solvents due to hydrogen bonding interaction of the emitting state of the molecule with the solvents, which presumably enhances the intersystem crossing process in the system. The yields are measured to be only 0.1 and 0.01 in methanol and water, respectively.

The emitting state of AP is quite long-lived in aprotic solvents. Lifetimes in the range of 14–15 ns can be observed in these solvents. However, in accordance with the observation of the variation of the fluorescence

yield, the lifetime gets shortened significantly in protic media; in water, the lifetime is as low as ~1 ns.

The fact that AP displays a 115-nm shift of the fluorescence maximum, a 70-fold change in the fluorescence yield, and a 15-fold change in the fluorescence lifetime with the change of solvent clearly points to its potential as an ideal fluorescence polarity indicator for microheterogeneous media.

GUEST–HOST COMPLEXATION WITH CYCLODEXTRIN

Cyclodextrins are cyclic oligosaccharides containing six, seven, or eight glucose units, called α, β, and γ cyclodextrins (CD), respectively. These are cone-shaped molecules with cavities (diameter ranging from 4.7 to 8.3 Å) which can encapsulate molecules. An aqueous solution of CD represents one of the simplest microheterogeneous media. The large number of studies on aqueous solutions of CDs are motivated by the realization that an understanding of CD–guest binding may lead to a better understanding of enzyme–substrate binding, a process that is of tremendous biological importance.

In view of the fact that the interior of the CD cavities is relatively nonpolar, encapsulation of the guest molecules from the aqueous solution leads to changes in the immediate environment of the molecule. In the case of AP, the encapsulation is associated with a blue shift of the spectral maxima and an increase in the fluorescence yield and lifetime [44,45]. This observation is consistent with the results obtained in homogeneous media [43]. The formation of a 1:1 complex between AP and β-CD (clearly indicated by the isosbestic point in the absorption spectrum) leads to a four-fold enhancement of the fluorescence yield and a nearly a 27-nm blue shift of the fluorescence maximum (observed in the presence of 2 m*M* β-CD). α-CD also forms a 1:1 complex with AP. However, both the shift and the enhancement are considerably lower in this case. The maximum shift and intensity enhancement observed in the presence of α-CD (10 m*M*) are 17 nm and 1.7-fold, respectively. With γ-CD, the changes in the fluorescence properties were found too small. This behavior is in agreement with the fact that guest-binding in the CD cavity is determined largely by the size of the guest molecule and the cavity diameter. The binding constants, estimated from the analysis of the fluorescence intensity data as a function of the concentration of CD on the basis of 1:1 complex formation, are 208 M^{-1} for the β-CD complex and 92 M^{-1} for the α-CD complex. While the lifetime of AP in water, as stated earlier, is ~1 ns, the decay curves are clearly biexponential in the presence of CD with a long-lived component (7.98 and 8.9 ns in the presence of α-CD and β-CD, respectively) representing the CD complex and a short-lived component with lifetime very similar to that of AP in the aqueous solution representing the unbound fluorophore. Since in this case it is possible to resolve the two components clearly, the complex and the free fluorophore, through temporal resolution of fluorescence, the preexponential factors contributing to the signal at zero time, have been used to estimate the formation constant of the complex. Such an analysis yielded binding constants of 53 and 185 M^{-1} for complexes with α-CD and β-CD, respectively. These values are in good agreement with those obtained from the analysis of the fluorescence intensity data.

It should be noted that since the interior of the CD cavity is believed to be nonpolar, the changes in the fluorescence properties of AP on encapsulation were expected to be much more pronounced than what was observed here. Obviously, the results indicate that a portion of the fluorophore is protruded outside the cavity. A consideration of the molecular dimensions of AP (AM1 calculated structure) and the cavity size suggests that, in the case of the α-CD complex, the fluorophore cannot completely go inside the cavity, as the width of the molecule (5.06 Å), measured from the distance between the two hydrogen atoms at positions 3 and 6, is larger than the cavity diameter (4.9 Å) of α-CD. Therefore, a part of the molecule has to remain outside. Interestingly, even though such an analysis suggests that AP could go completely into the cavity of β-CD (as the cavity diameter is 6.2 Å), the fluorescence data clearly suggest that a portion of the molecule still remains outside the cavity and the effective polarity experienced by the fluorophore is quite high. This particular arrangement of the probe molecule within the CD cavity can be rationalized only considering the strong tendency of the carbonyl groups of the molecule to form hydrogen bonds with water molecules or with the hydroxyl groups of CD at the rim. Thus, a combination of the hydrophobic interaction (responsible for binding) and the hydrogen bonding interaction determines the structure of the AP–CD complex in which the carbonyl groups of the molecule remain exposed to the aqueous phase.

FLUORESCENCE RESPONSE IN MICELLAR MEDIA

Surfactant molecules possessing a long alkyl chain and polar head group readily form aggregates in aqueous solution. While the size and shape of the aggregates de-

pend on various factors such as the length of the hydrocarbon chain, nature of the head group, temperature, presence of electrolytes, etc., the most commonly observed aggregate is spherical, in which hydrocarbon chains form the inner nonpolar core, while the polar head groups are located at the hydrocarbon–water interface. Obviously, micelles represent another simple class of organized medium. Depending on the nature of the polar head group, the aggregates can be cationic, anionic, or neutral. The formation of micellar aggregates takes place over a narrow concentration range of the surfactants around the critical micelle concentration (CMC). It is possible to follow the aggregation of surfactant molecules into micelles using fluorescent systems that respond differently on media of different polarities.

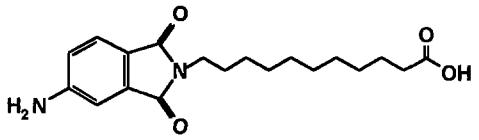
AP is found to be an excellent reporter molecule for the micellar environment [46]. At low surfactant concentrations, neither the fluorescence spectrum nor the fluorescence intensity displays any significant change. However, it is found that beyond a certain surfactant concentration, the changes, both the blue shift of the spectrum and the enhancement of fluorescence intensity, are quite significant. A sharp change in the slope of the plot of the relative fluorescence intensity versus the surfactant concentration can be noted around the CMC of the micelles. This allows one to measure the CMC values of the micelles. The CMC values for three well-studied micellar systems, anionic sodium dodecyl sulfate (SDS), cationic cetyltrimethylammonium bromide (CTAB), and neutral Triton-X, as measured using AP as probe, are found to be in excellent agreement with the literature values. The ability of AP to follow the micellar aggregation irrespective of the micellar charge makes this probe clearly superior to ionic ICT probes such as *p*-toluidinonaphthalenesulfonate (TNS) or 1-anilino-8-naphthalenesulfonate (ANS), which fail to follow micellization in some cases involving charged micelles because of electrostatic interactions [48]. The binding constants of AP with micelles have been estimated from the fluorescence intensity data using a method suggested by Almgren *et al.* [49]. The binding constants are 3400, 4500, and 5600 M^{-1} with SDS, CTAB, and Triton X, respectively. Since the binding constants are of the same order of magnitude, one can conclude that AP does not show any preference to any particular micelle; instead, the binding is independent of the micellar head group.

The maximum enhancement of fluorescence and the blue shift of the spectrum in the micellar environment are found to be 2.6–3.0 and 14–21 nm, respectively. These data indicate that micelle-bound AP is quite exposed to the polar environment. This conclusion is validated by the fluorescence lifetime data. The decay curves in the micellar media were biexponential, with the long-lived component displaying a lifetime in the range of 3.1 to 3.6 ns. It is to be noted that since the fluorescence lifetime of AP in methanol is approximately seven times longer than that in water, if the molecule sees an environment which is as polar as methanol, then one expects a much greater enhancement of lifetime. Further, the expected enhancement of fluorescence for a methanol-like environment, taking into consideration the binding constant values, is nine fold, much higher than the observed enhancement. Clearly, the strong affinity of AP toward hydrogen bond donating solvents does not allow it to penetrate into the deeper core region of the micelle; instead, the molecule resides at the interface, where it can form hydrogen bonds with the water molecules and sense an environment which is more polar than methanol. A detailed analysis of the spectral shift data allows precise determination of the polarity of the interfacial regions of the three micelles. Assuming the wavenumber of the fluorescence maximum, $\bar{\nu}_{max}$ to change linearly with the addition of the surfactant and with the microscopic polarity parameter $E_T(30)$ [50], the interface polarities of SDS, CTAB, and Triton X in the $E_T(30)$ scale are measured to be 57.9, 56.3, and 55.6, respectively. The selection of the probe molecule for the determination of the polarity of the interface is rather crucial. When the hydrophobic portion of the probe molecule is large, it can penetrate into the deeper core region of the micelle and report from therein. Similarly, if a probe is relatively hydrophilic, then one expects it to report higher polarity values. AP, with a small hydrophobic region and two active centers for hydrogen bonding, is an excellent probe of the micellar interface. With this polarity sensor, three parameters can be monitored (position of the maximum, fluorescence intensity, and lifetime) to arrive at a more definite estimate of the polarity values of the surrounding microenvironments. Further, the electroneutrality of the molecule and its small size make it superior to frequently used ionic probes such as ANS or TNS and larger probes such as betaine dye (which may perturb the micellar structure).

DERIVATIVES OF AP AS PROBES

AP reports exclusively from the micelle–water interfacial region. Hence it is not possible to extract any information from the interior core region of the micelle using this probe. In an attempt to develop new AP derivatives that could be used as probes for the nonpolar core region of the micelle, the fluorophore has been covalently attached to the nonpolar terminal of a fatty acid (undecanoic acid) and the fluorescence properties have

been investigated in a homogeneous and micellar environment [47]. It was thought that this particular attachment of the fluorophore would help its incorporation into the core region. This is because it is a commonly used practice to covalently attach the probe at different positions of a detergent molecule or lipid so as to sense microenvironments from various depths of the micelles or biological membranes [16,23,26,51].

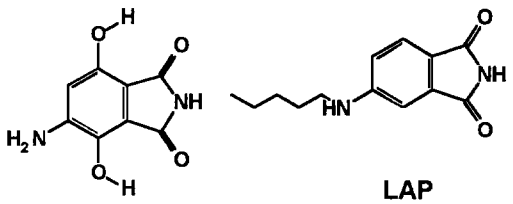


APL

The photophysical behavior of this detergent molecule (APL) in homogeneous media is quite similar to that of AP. Surprisingly, APL displays very similar properties in micellar media also. The CMC values of different micelles measured from the fluorescence intensity data at various surfactant concentrations are in agreement with the literature values. The binding constants of APL with micelles are 4200, 8000, and 8500 M^{-1} for SDS, CTAB, and Triton-X, respectively. Since these values are comparatively larger than those observed with AP, the attachment of the fatty acid chain clearly helps better binding of the probe molecule. The most interesting observation that has been noted with this fluorophore is that in the micellar environment both the blue shift and the enhancement of fluorescence are not significantly different from those observed with AP. This implies that covalent attachment is not helpful in the incorporation of the fluorescing moiety into the core region. That the fluorophore still localizes itself at the micelle–water interface is also supported by the quenching data. The ionic quenchers I^- and Cu^{2+}, which are expected to interact with the probe molecules residing either in the aqueous solution or in the interface, are found to quench the fluorescence of APL rather efficiently even when it is micellized. In fact, the quenching constants for micellized AP and micellized APL are quite similar. Quite obviously, in the micellized condition, the location of the fluorescing moiety in APL is similar to that of AP. This is possible only if the long hydrocarbon chain of APL gets folded in the micellar medium.

SCOPE OF DEVELOPMENT OF AP-BASED PROBES

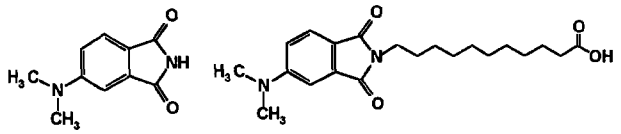
That AP is an excellent fluorescence probe for organized media is clearly supported by the above results. A combination of hydrogen bonding and hydrophobic interactions forces the fluorophore at the interface. While it was thought that if connected to the nonpolar end of the fatty acid, it would be possible to incorporate the fluorescing moiety into the core region and derive information from that region, the results on APL suggest that the hydrogen bonding interaction of the fluorophore is too strong to force it into the core region. Since the two carbonyl oxygens of the fluorophore are involved in the hydrogen bonding interaction, we can expect that if the two oxygen atoms are engaged in intramolecular hydrogen bond formation by introducing two hydroxyl groups at the 3 and 6 positions, as in derivative APH, the tendency of the oxygen atoms to form the hydrogen bonds with the water molecules will be significantly reduced and this may help incorporation of the fluorophore into the core. While reducing the hydrogen bonding interaction with the solvent molecules is one possible approach to push the fluorophore into the deeper core region, one can also attempt to increase the hydrophobic interaction instead by attaching an alkyl chain at the 4 position (as in LAP) to achieve the same goal. Studies along this line are necessary.



APH LAP

It has been reported that the fluorescence properties of 4-dimethylaminophthalimide (DAP) are even more sensitive to polarity than AP [43]. This is because of the fact that the emitting ICT state in DAP is coupled to a low-lying nonfluorescent TICT state in polar solvents. Obviously, DAP is expected to be superior to AP as a probe. Since the rotary decay process in the emitting state of DAP can be modulated by the viscosity of the medium, the emission intensity of this molecule, unlike that of AP, is dependent on the viscosity of the surrounding environment. Hence, DAP can also be used as a probe for the viscosity of the solubilizing site in microheterogeneous media. Further, one can expect that dimethyl functionalization would increase the hydrophobicity of the molecule, leading to relatively deeper penetration of the fluorophore into the micelle. It is therefore necessary to examine the fluorescence response of DAP and its derivatives such as DAPL in organized media. One can expect both DAP and DAPL to sense

and report details from a deeper region of the micellar aggregates. Finally, no studies have been carried out so far where AP or its derivatives have been employed to examine complex biological systems. In view of the fact that the photophysical behavior of most of these derivatives are fairly well understood in homogeneous or simple microheterogeneous media, it should not be difficult to extract meaningful information on the structure and dynamics of complex media using these efficient fluorophores.



DAP DAPL

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