Photophysical Processes of Ethidium Bromide in Micelles and Reverse Micelles

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Photophysical processes of ethidium bromide (EB) in homogeneous solutions, micelles, and reverse micelles have been investigated. In acetonitrile, the fluorescence intensity and lifetime of EB are 6.3 ± 0.3 times those in water and 1.25 ± 0.1 times those in acetone. This is attributed to the weaker hydrogen-bond acceptor property of acetonitrile, compared to water and acetone. Addition of water to acetonitrile leads to a marked quenching of the EB emission, with a quenching constant of $(1.7 \pm 0.3) \times 10^7$ M^{-1} s^{-1}. In aqueous solution, hydroxyl ion quenches EB emission more dramatically with a quenching constant of $(4.4 \pm 0.4) \times 10^{10}$ M^{-1} s^{-1}. This is ascribed to the abstraction of the amino proton of the excited ethidium ion by the hydroxyl ion. Emission properties of EB are found to be good monitors for the micellization of an anionic micelle, sodium dodecyl sulfate (SDS). In AOT microemulsion, EB exhibits nearly (1.8 ± 0.1)-fold emission enhancement relative to water. The emission properties of EB are found to be independent of the water-to-surfactant ratio, w_0. In AOT microemulsion when instead of water D$_2$O is injected, a further 2.3 times emission enhancement is observed. However, in AOT microemulsion, the hydroxyl ion does not quench the EB emission, even when a highly alkaline aqueous solution of EB (pH = 12.6) is injected into the reverse micelle. It is proposed that the anionic surfactant, AOT, strongly attracts the ethidium cation to the AOT–water interface but expels the hydroxyl anion from the AOT–water interface to the water pool, and hence, the hydroxyl anion cannot access the ethidium cation.

Introduction

Ethidium bromide (EB, **I**; Scheme 1) is a well-known fluorescent probe for DNA and readily intercalates into the DNA double helix.[1,2] Compared to the case of bulk water, the emission intensity and lifetime of EB increase nearly 11 times when EB intercalates into the double helix of DNA. This remarkable fluorescence enhancement of EB is utilized to study the motion of DNA segments,[2a,b] significantly enhanced quenching of DNA bound EB by various agents,[2d–f] and the interaction of DNA with surfactants and drug.[2g,h] Though many aspects of DNA have been studied using EB as an emission probe, relatively less attention has been given to explain the remarkable fluorescence enhancement of EB on binding to DNA and also to explore the possibility of using EB to probe other organized environments.[3] To understand the nonradiative pathway of EB, Ohmstead and Kearns studied EB in a number of solvents.[3a] They demonstrated that the fluorescence enhancement of EB has nothing to do with the viscosity of the medium, as the emission quantum yield and lifetime of EB are very similar in methanol and glycerol, whose viscosity differ by a factor of 2000. The emission intensity of EB is low in highly polar, protic solvents, such as alcohol and water, compared to polar, aprotic solvents, e.g., acetone or pyridine. EB is insoluble in nonpolar, aprotic solvents like alkanes or dioxane. Compared to the case of water, the emission intensity and lifetime of EB increase nearly 5 times in acetone. Addition of water to acetone is found to quench fluorescence of EB, while deuterated solvents enhance emission of EB. On the basis of these results, Ohmstead and Kearns concluded that in the excited state EB donates one of the amino protons to the solvents studied, all of whom are good

SCHEME 1: Structure of Ethidium Ion
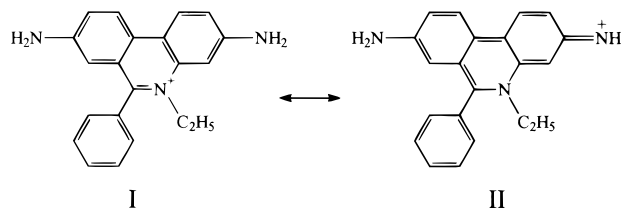
proton acceptors.[3a] This is quite reasonable, because in the quinonoid structure (**II**), the amino proton of the ethidium ion becomes quite acidic, even in the ground state, and its acidity is expected to increase further in the excited state, like other aromatic amines. If this conjecture is correct, the emission intensity of EB should depend on the hydrogen-bond acceptor (HBA) basicity of the solvent, β, instead of the polarity. The HBA basicity, β, introduced by Kamlet et al.[4a] and other polarity scales of various solvents are elaborately discussed in many reviews.[4] The polarity of acetone (dielectric constant, $\epsilon = 20.7$ and $E_T(30) = 42$) is less than that of another polar, aprotic solvent, acetonitrile ($\epsilon = 37.5$ and $E_T(30) = 46$).[4a] However, the HBA basicity, β, of acetone (0.48) is greater than that of acetonitrile (0.31), and thus, acetone is a better proton acceptor than acetonitrile.[4a] In this work, we have compared the emission properties of EB in acetone and acetonitrile to establish conclusively that the HBA basicity of the solvent plays a more important role in controlling the nonradiative rates of EB than the solvent polarity. We have also decided to throw further light on the abstraction of the amino protons of EB by studying, in detail, the effect of pH on the emission properties of EB in aqueous solutions. Finally, we have tested the sensitivity of EB, as a probe, to study micelles and reverse micelles. It is

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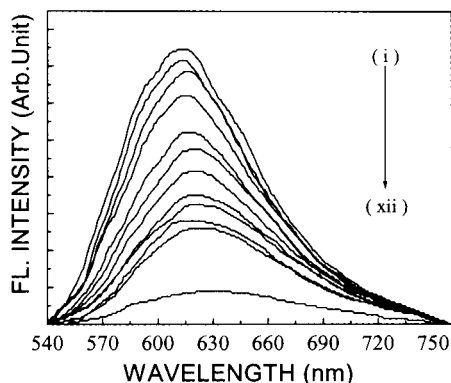
Figure 1. Emission spectra of ethidium bromide in acetonitrile−water mixtures containing (i−xii) 0, 0.5, 1, 2, 3.9, 5.7, 9.1, 16.7, 23.0, 33.3, 50, and 100% water (v/v) ($\lambda_{ex} = 300$ nm).

well-known that many surfactant molecules form aggregates when their concentration exceeds a certain critical value.[5] The aggregate, formed in water or other highly polar, protic solvents, with the polar headgroups pointing outward, is called a micelle.[5] On the other hand, the reverse micelles refer to the aggregates formed in a bulk nonpolar solvent (e.g., *n*-heptane), with the polar ends pointing inward.[6] When water is injected in such a reverse micelle, a microemulsion is formed, which is basically a nanometer-sized water droplet ("water pool"), surrounded by a layer of the surfactant. The most common surfactant, used in microemulsions, is sodium dioctyl sulfosuccinate (AOT). In *n*-heptane, the radius of the water pool of the AOT microemulsions is about $2w_0$ (in angstroms), where w_0 is the ratio of the number of water and the AOT molecules.[6d] Since the emission properties of EB are so sensitive to the presence of water, we have decided to study how the water molecules, confined in the Stern layer of the micelles or in the water pool of microemulsions, affect the emission of EB. We have also investigated whether the hydroxyl ion induced quenching of emission of EB is different, in the water pool of the microemulsions, from that in ordinary water. The last aspect has been addressed in a number of previous studies, and it has been concluded that the local pH and the acid−base property in such organized assemblies could be vastly different from those of bulk water and depend crucially on the location of the probe.[7]

Experimental Section

Ethidium bromide (Sigma) and sodium dodecyl sulfate (SDS, Aldrich) were used as received. Sodium dioctyl sulfosuccinate (AOT, Aldrich) was purified following a reported procedure.[6f] All the solvents were of spectroscopy grade and distilled just before use. Doubly distilled water was used. Preparation of microemulsions is described in our earlier publications.[8d] For lifetime measurements, the sample (EB) was excited at 300 nm with the second harmonic of a cavity dumped Rhodamine 6G dye laser (Coherent 702-1) pumped by a CW mode locked Nd: YAG laser (Coherent, Antares 76s). The emission was detected at magic angle polarization using a Hamamatsu MCP photomultiplier tube (2809U). Many authors have discussed the difficulty of extracting meaningful numbers from the multiexponential decays in the micellar and reverse micellar and other microheterogeneous media where the probe experiences different environments.[10] In the present work, we have fitted all the decays to single-exponential decays to get an average picture.

Results and Discussion

Ethidium Bromide in Acetonitrile: Emission Enhancement and Quenching by Water. Figure 1 depicts the emission spectra of EB in acetonitrile, water, and their mixtures. It is readily seen that the emission intensity of EB in acetonitrile is 6.3 ± 0.3 times that in water and is 1.25 ± 0.1 times that in acetone. If polarity were the governing factor to accelerate the nonradiative decay rate of EB, one would expect that the emission intensity of EB should be lower in the more polar solvent, acetonitrile, compared to acetone. The stronger emission intensity in acetonitrile compared to acetone suggests that polarity is not the governing factor to control the nonradiative pathways of EB. According to Kamlet et al., acetonitrile is a weaker HBA base compared to acetone.[4a] Thus, it appears that the higher is the ability of the solvent to accept a proton or a hydrogen bond, the faster is the nonradiative process of EB. Since compared to acetonitrile acetone is a better proton/hydrogen-bond acceptor, the nonradiative rate of EB is faster, and consequently, the emission intensity is lower in acetone than those in acetonitrile. This is consistent with the earlier model proposed by Ohmstead and Kearns that the main nonradiative pathway of EB is abstraction of proton by the solvent.[3a]
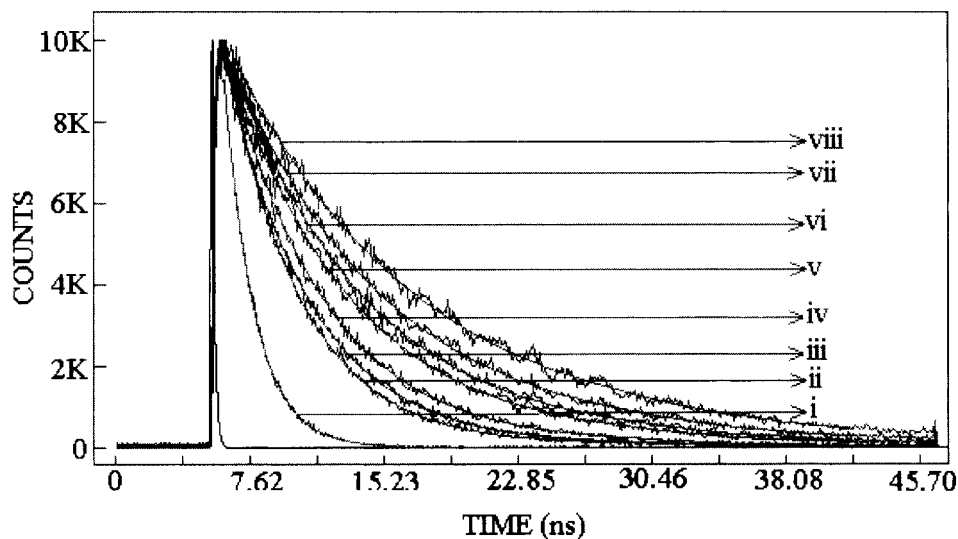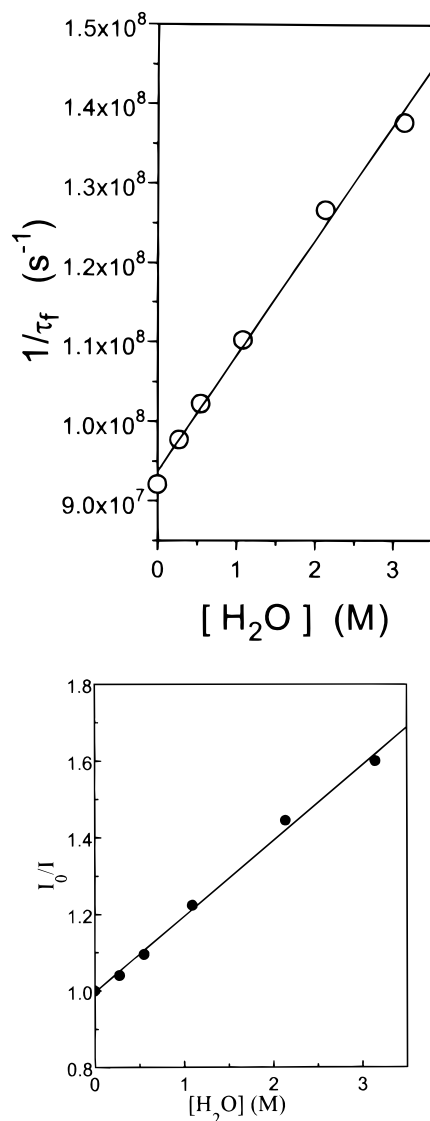

Figure 2. Fluorescence decays of ethidium bromide ($\lambda_{em} = 620$ nm) in acetonitrile−water mixtures containing (i−viii) 100, 50, 33.3, 16.7, 5.7, 3.9, 2, and 0% water (v/v).

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TABLE 1: Emission Quantum Yield of Ethidium Bromide, ϕ_f, Relative to That in Water at pH = 7 and Lifetimes (τ_f) in Acetonitrile–Water Mixture

| % of water (v/v)	$\phi_f{}^a$	τ_f (ns)^b	% of water (v/v)	$\phi_f{}^a$	τ_f (ns)^b
0	6.3	10.9	9.1	3.4	6.4
0.5	5.8	10.2	16.7	2.9	5.6
1.0	5.6	9.8	23.0	2.7	5.2
2.0	5.1	9.1	33.3	2.3	4.9
3.9	4.3	7.9	50.0	2.1	4.3
5.7	3.9	7.25	100.0	1.0	1.7

^{a} ±5%. ^{b} ±0.05 ns.

Figure 3. (a, top) Plot of reciprocal of τ_f of ethidium bromide vs concentration of water in acetonitrile–water mixture. (b, bottom) Plot of I_0/I of ethidium bromide vs water concentration in acetonitrile–water mixtures.

The fluorescence decays also indicate suppression of the nonradiative process of EB in acetonitrile, compared to water and acetone. The fluorescence lifetime (τ_f) of EB in acetonitrile (10.9 ± 0.05 ns, Figure 2) is 6.4 times that in water (1.7 ± 0.05 ns) and 1.17 times that in acetone (9.3 ns).[3a] It is evident that the increase in τ_f in acetonitrile, in comparison to water and acetone, is commensurate with the increase in ϕ_f.

Addition of water to acetonitrile leads to reduction in both the τ_f and ϕ_f of EB (Figures 1 and 2). On addition of water to acetonitrile, the lifetime of EB decreases gradually from 10.9

ns in acetonitrile to 1.7 ns in pure water (Table 1). At low water concentration (Figure 3a), the plot of $1/\tau_f$ against water concentration is a straight line, and from the slope of this straight line, the rate constant for the quenching (k_Q) of EB emission by water is calculated to be (1.7 ± 0.3) × 10^7 M^{-1} s^{-1}. At high concentrations of water, the curve deviates from linearity, presumably due to the large scale change of the local structure around the charged probe, EB, involving strong hydrogen bonds between the ethidium ion and water. The plot of I_0/I against water concentration is also a straight line (Figure 3b) with the slope, $t_0 k_Q$, equal to (1.8 ± 0.25) × 10^{-1} M^{-1}, from which using the lifetime of EB in acetonitrile ($\tau_o = 10.9 ± 0.05$ ns) k_Q is calculated to be (1.7 ± 0.25) × 10^7 M^{-1} s^{-1}. The nice agreement of the k_Q values in the two plots conclusively establishes that the water-induced quenching is purely dynamic in nature and is due to the interaction of water with EB in the excited state. The lifetime (τ_f) and relative quantum yield (ϕ_f) of EB in acetonitrile–water mixtures are summarized in Table 1.

Effect of Micelle. Since the emission properties of EB are so sensitive to the presence of water, one would expect that in aqueous solutions when a micellar aggregate is formed and the probe EB binds to the micellar aggregate, it will be partially shielded from bulk water, and as a result, its emission intensity would increase. We studied absorption and emission properties of EB in neutral (triton X-100, TX), anionic (sodium dodecyl sulfate, SDS), and cationic (cetyltrimethylammonium bromide, CTAB) micelles. It is observed that the emission properties of EB do not change detectably from those in bulk water in the case of CTAB and TX. This indicates that the positively charged ethidium ion does not bind well with the cationic CTAB and neutral TX micelles. For the anionic SDS micelle, appreciable changes in the absorption and emission properties of EB are observed. In aqueous solutions EB exhibits two absorption bands at 480 and 285 nm (Figure 4a) and an emission band at 620 nm. On addition of SDS, below the cmc of SDS, the absorbance of EB decreases and the emission intensity also decreases by a similar factor so that the emission quantum yield of EB, relative to that in water, remains unchanged (Figure 5a,b). The decrease in the absorbance is presumably because of the formation of association complexes involving the ethidium cation and dodecyl sulfate anion and precipitation of the complex out of the solution onto the walls of the container. Above the cmc of SDS (8 mM), the absorbance of EB gradually increases, indicating increased solubilization of EB in water, once the micellar aggregates are formed and the two absorption bands exhibit a distinct red shift to 292 and 512 nm (Figure 4a). The excitation spectra of EB remain identical to the absorption spectra of EB both above and below the cmc and exhibit a red shift above the cmc (Figure 4b). Above the cmc of SDS, the relative emission quantum yield of EB, after correction for changes in absorbance, exhibits a 2.4 ± 0.2 times enhancement (Figure 5a,b). The emission lifetime of EB increases nearly 3 times from 1.7 ± 0.05 ns in water to 5.0 ± 0.05 ns in SDS above the cmc (Figure 5c). This indicates that, due to its inherent positive charge, the ethidium ion binds strongly with the anionic SDS micelles, and its emission is enhanced in the micellar environment. In fact, as shown in Figure 5b, the change in ϕ_f is sharp enough about the cmc of SDS (8 mM) to use EB as a probe for the micellization process. Table 2 summarizes emission intensity and lifetime of EB in different organized media. It should be noted that the ϕ_f and τ_f of EB, bound to SDS, are intermediate between those in water and in alcohol.[3a]
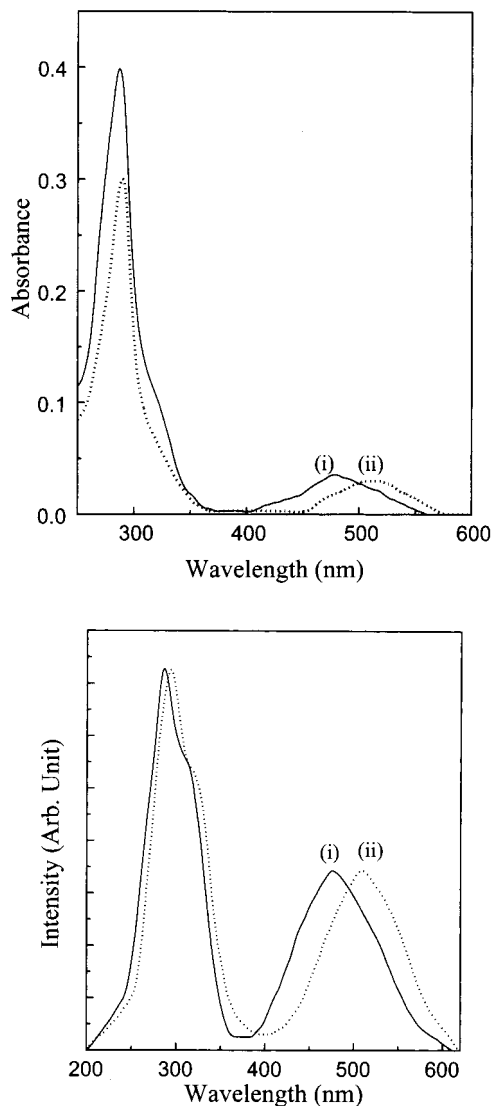
Figure 4. (a, top) Absorption spectra of EB in (i) 0 and (ii) 100 mM SDS. (b, bottom) Excitation spectra of EB in (i) 0 and (ii) 100 mM SDS.

Effect of Microemulsions: Deuterium Isotope Effect. In recent years, microemulsions have been shown to be a good model of the water molecules confined in small regions of nanometer dimensions.[6,7,8d] EB is insoluble in *n*-heptane with and without AOT but readily dissolves in AOT in *n*-heptane, in the presence of water. This indicates that the EB remains inside the water pool of the microemulsions. It is observed that the emission behavior of EB in AOT microemulsions is markedly different from that in bulk water. Compared to bulk water, in microemulsions the steady-state emission intensity increases 1.8 ± 0.1 times (Figure 6a) and the lifetime (Figure 6b and Table 2) increases 2.3 times to 3.8 ns. The emission intensity and lifetime of EB in the AOT microemulsions are found to be independent of the water-to-surfactant ratio, w_0, and hence the size of the water pool. This suggests that the positively charged ethidium ions are held very strongly by the negatively charged AOT ions and are, thus, located near the surfactants and remain more or less inaccessible to the water pool. The lifetime of EB in AOT microemulsion, which is higher than that in water, is less than that in methanol (6 ns[3a]). Thus, the polarity of the microenvironment of EB in AOT microemulsions is intermediate between those of water and
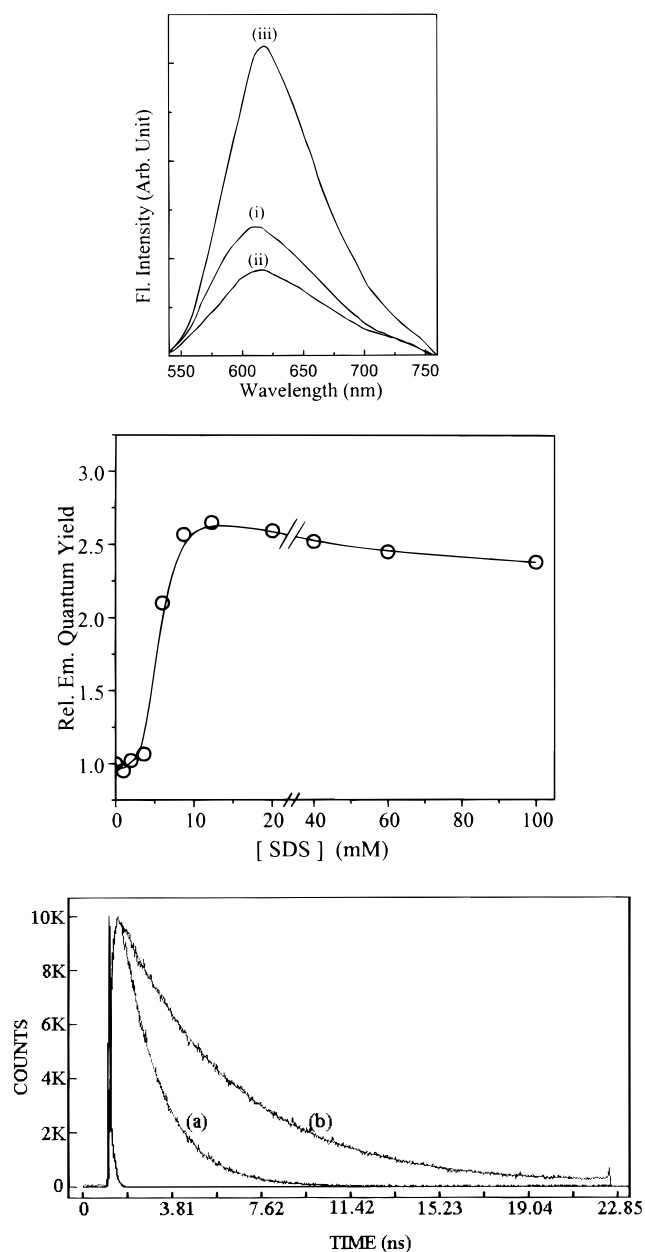
Figure 5. (a, top) Emission spectra of EB in (i) 0, (ii) 1, and (iii) 10 mM SDS. (b, middle) Plot of emission quantum yield of ethidium bromide, relative to that in water vs concentration of SDS. (c, bottom) Fluorescence decays of ethidium bromide (a) in water and (b) in 100 mM SDS.

TABLE 2: ϕ_f and τ_f Ethidium Bromide in Different Organized Environments

medium	$\phi_f{}^a$	τ_f (ns)^b
water	1	1.7
100 mM SDS	2.4	5.0
AOT/*n*-heptane/water (pH =7)	1.8	3.8
AOT/*n*-heptane/water (pH =12.9)	1.8	3.8
AOT/*n*-heptane/D$_2$O (pH =7)	4.1	9.0

$^a \pm 5\%$; relative to ϕ_f of ethidium bromide in water at pH $= 7$.
$^b \pm 0.05$ ns.

methanol. This is consistent with the previous measurements on the micropolarities of AOT microemulsions.[6,8c,d]

If D$_2$O is injected into AOT reverse micelles, instead of H$_2$O, the fluorescence intensity of EB is increased by a factor of 2.3 ± 0.1 and the lifetime by a factor of 2.4, relative to the pool containing H$_2$O (Table 2), at the same w_0. This 2.3 ± 0.1 times
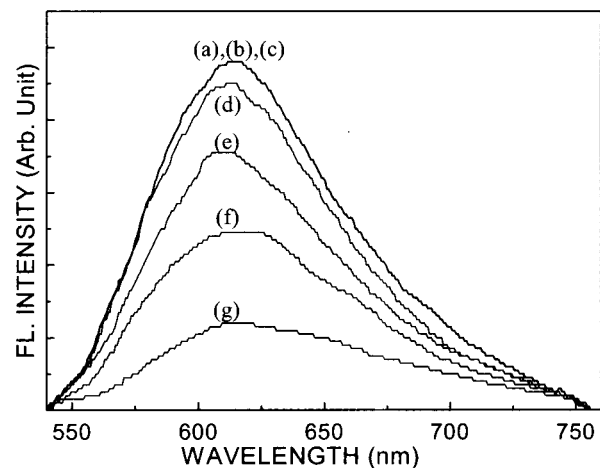
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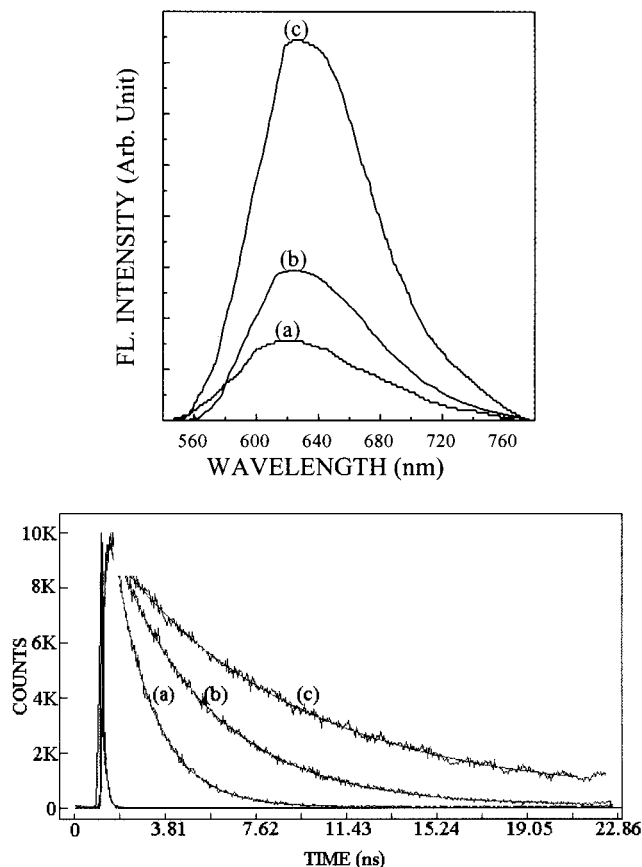
Figure 6. (a, top) Emission spectra of ethidium bromide in (a) water; (b) 0.09 M AOT/ *n*-heptane/H_2O, w_0= 20; and (c) 0.09 M AOT/*n*-heptane/D_2O, w_0 = 20. (b, bottom) Fluorescence decays of ethidium bromide in (a) water; (b) 0.09 M AOT/*n*-heptane/H_2O, w_0 = 20; and (c) 0.09 M AOT/*n*-heptane/D_2O, w_0 = 20.

Figure 7. Emission spectra of ethidium bromide in water at pH (a−g) 6.1, 9.2, 10.4, 10.8, 11.7, 12.2, and 12.6.

Figure 8. Variation of emission lifetime (●) and relative quantum yield (○) of ethidium bromide with pH in aqueous solution.
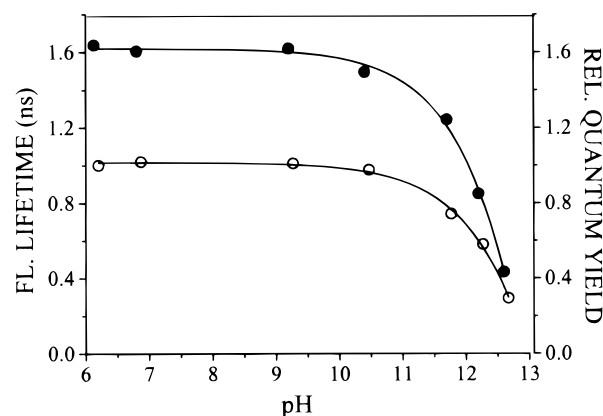
fluorescence enhancement is consistent with a similar deuterium isotope effect of EB fluorescence in bulk water.[3a] It appears that in D_2O the amino protons of EB get readily exchanged. Since the N−H bonds have the highest vibrational frequency in EB, they act as an acceptor mode for the nonradiative transitions. Replacement of the N−H bonds by N−D bonds reduces the vibrational frequencies, and this causes reduction in the nonradiative rates. Similar models have been used earlier to explain the D_2O-induced fluorescence enhancement in neat water and microemulsions for 7-azaindole[8a−c] and 4-aminophthalimide.[8d]

Effect of Hydroxyl Ion in Neat Water and in Microemulsions. In aqueous solution τ_f and ϕ_f of EB remain unchanged over a wide pH range from 4 to about 10 (Figures 7 and 8). Above pH = 10.4, the τ_f and ϕ_f of EB decrease very rapidly from 1.5 ns at pH = 10.4 to 0.45 ns at pH = 12.6, and EB becomes almost nonfluorescent above pH = 13 (Figure 9a). From the plot of $1/\tau_f$ against hydroxyl concentration (Figure 9a) and of I_0/I against [OH^-] (Figure 9b), the emission quenching constant of EB by OH^- is calculated to be (4.4 ± 0.4) × 10^{10} M^{-1} s^{-1}. Obviously, the quenching of EB emission by OH^- ion may be attributed to the abstraction of the amino proton from the quinonoid form of EB (**II**) in the excited state. In this case, it is found that the deprotonated form is nonemissive and the process is irreversible. The emission intensities and lifetimes of EB at different pH are summarized in Table 3.

We finally investigated how the hydroxyl ion affects emission properties of EB in the confined water pool of the reverse micelles. For this purpose, aqueous solutions of EB at different pH were injected into AOT reverse micelles. It is observed, while in bulk water, on increasing pH from 6 to 12.6 the τ_f and ϕ_f of EB decrease by a factor of more than 3; in AOT microemulsions, the τ_f and ϕ_f at these two pH's are remarkably similar. We could not go to higher pH since at pH >12.9, the microemulsions become unstable and the solution becomes turbid.

Before explaining the remarkable suppression of the hydroxyl ion quenching of the EB fluorescence in the microemulsions, it may be recalled that Menger and Saito earlier reported that the acid−base property of *p*-nitrophenol (PNP) gets substantially changed in AOT microemulsions.[7a] While in bulk water, at pH = 11.5, 95% of the PNP molecules remain in the anionic form; when an alkaline aqueous PNP solution is injected in the AOT microemulsion, no *p*-nitrophenolate anion is detected until the pH of the injected solution exceeds 11.5. On the basis of this result, Menger and Saito concluded that the pK_a of PNP, in the AOT microemulsion, is greater than that in bulk water (7.14) by more than 4 units. However, subsequent workers ascribed this phenomenon to the fact that the local pH near the negatively charged AOT headgroup could be substantially different from that in bulk water. Oldfield et al. demonstrated that if a negatively charged group is attached to PNP, the probe remains in the water pool, and its acid−base properties are similar to that in bulk water.[7b] Politi et al. studied excited-state deprotonation of a trinegatively charged probe, hydroxy-pyrene-trisulfonate in AOT microemulsions.[7c] They observed that while in the large pools the prototropic behavior is similar to that in bulk water, in the small water pools (w_0 < 7) the

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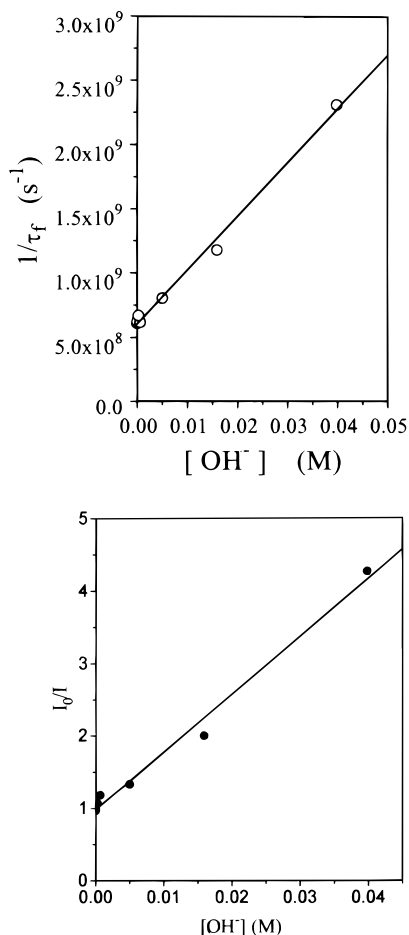
Figure 9. (a, top) Plot of reciprocal of τ_f of ethidium bromide vs concentration of hydroxyl ion, [OH$^-$], in aqueous media. (b, bottom) Plot of I_0/I of ethidium bromide vs concentration of hydroxyl ion, [OH$^-$], in aqueous media.

TABLE 3: Effect of pH on the ϕ_f and τ_f of Ethidium Bromide in Water

pH	$\phi_f{}^a$	τ_f (ns)^b	pH	$\phi_f{}^a$	τ_f (ns)^b
7.0	1	1.7	12.2	0.60	0.85
10.4	0.95	1.50	12.6	0.30	0.45
11.7	0.75	1.25			

^{a} ±5%; relative to ϕ_f of ethidium bromide in water at pH = 7.
^{b} ±0.05 ns.

proton-transfer process is quite different. They proposed that in the large water pool, due to the electrostatic repulsion from the negatively charged AOT ions, the negatively charged probe remains in the large water pools, far from the AOT anion, and experiences an almost bulk water-like microenvironment. But in the small water pool, the very different local pH, near the AOT anions, renders the deprotonation/reprotonation behavior quite different. Okazaki and Toriyama studied the location of an organic acid, at different pH, in AOT microemulsions, using ESR spectroscopy.[7d] They showed that at low pH, when the molecule remains in the neutral form, it stays close to the AOT−water interface, while at high pH when the carboxyl group becomes ionized, the anion formed is expelled from the AOT−water interface to the water pool. Similar changes in the pK_a and the local pH, at the air−water interface, are also reported in the more recent surface second harmonic generation[9a−c] and neutron reflection studies.[9d]

In light of all these observations, the complete suppression of the quenching of the emission of EB by the hydroxyl ion, in

the water pool of AOT microemulsions, can be understood as follows. Due to its inherent negative charge, the AOT anions very strongly attract the positively charged ethidium ion and repel the negatively charged hydroxyl ion. Thus in the microemulsions, the ethidium cation and the hydroxyl anion remain in drastically different locations. The ethidium ions remain strongly attached to the AOT anion at the AOT−water interface, while the hydroxyl ions are expelled from the AOT−water interface and reside in the water pool. As a result, the hydroxyl ion cannot access the ethidium ion, and no quenching is observed. This observation coupled with the result that emission properties of EB are independent of w_0 indicates that the ethidium cation does not reside in the water pool and remains firmly attached, by electrostatic attraction, to the AOT anion.

Conclusion

The major findings of the present work are as follows:

1. The nonradiative pathway of EB depends strongly on the HBA basicity of the solvent rather than the polarity of the solvent. Thus, the emission intensity and lifetime of EB in acetonitrile, which is more polar than acetone but weaker in HBA basicity, are 1.25 ± 0.1 times those in acetone.

2. The quenching constants of EB by water in acetonitrile−water mixture and by hydroxyl ion in bulk water have been determined to be $(1.7 \pm 0.3) \times 10^7$ and $(4.4 \pm 0.4) \times 10^{10}$ M^{-1} s^{-1}. This indicates that the hydroxyl ion quenches EB emission nearly 2500 times more efficiently compared to water. This is consistent with the proton abstraction model, proposed earlier by Ohmstead and Kearns.[3a]

3. The emission of EB is found to be a good monitor for the microenvironment of SDS micelles and AOT reverse micelles. In AOT/*n*-heptane/water microemulsions, the emission properties of EB are found to be independent of the water-to-surfactant ratio, w_0. This indicates that ethidium cation is not exposed to the water pool and resides near the oppositely charged AOT anion.

4. Though the hydroxyl ion quenches EB emission markedly in bulk water, in the water pool of the AOT microemulsions, *no quenching is observed even when the pH of the injected aqueous solution of EB is as high as 12.6.* It is proposed that the ethidium cation remains strongly bound to the AOT anion, while the hydroxyl anion, being strongly repelled by the AOT anion, resides in the water pool and hence cannot access the ethidium cation.

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