Photophysical properties of dibenzofluorescein and the presence of its tautomers or prototropic forms in organic solvents

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The UV/Vis absorption and fluorescence properties of dibenzofluorescein (DBFL) in organic solvents were measured and used to shed light on the possible presence of its tautomers or various prototropic forms. DBFL in aprotic solvents mainly exists in two tautomeric forms, *viz.* quinoid and lactone, but neither are efficiently fluorescent. In protic solvents, such as methanol and ethanol, both the monoanion and neutral quinoid are present and showed the highest fluorescence quantum yield. In contrast, DBFL is fully dissociated to the monoanion and dianion in deionized water.

Introduction

1,2,7,8-Dibenzofluorescein (DBFL), a long-wavelength fluorescence probe, is an analogue of fluorescein (FL) with significantly extended π system. It has been used in sensing alkali metal ions,[1] sequencing DNA,[2] labeling oligonucleotides,[3] cell cytometry,[4] and assaying glutathiane transferase,[5] *etc.* This is attributed to its very high molar absorption coefficient at the wavelength of the portable diode laser (85 000 M^{-1} cm^{-1} at 532 nm), the much reduced background fluorescence from biological materials (*e.g.*, DNA),[6–8] good fluorescence quantum yield (0.66 for monoanion, 0.25 for dianion in water),[9,10] easy preparation, good solubility in water, a pK_a value close to physiological pH,[9,10] as well as its high photostability. These properties allow the probe to be highly sensitive, fast-responding, and to achieve good spatial resolution through microscopic imaging.

Nevertheless, little is known about DBFL, except for a report on its dianion in aqueous solution[10]. Similarly to fluorescein (FL), this type of oxyxanthene chromophore is expected to show complex protonation and tautomerization behavior in aqueous solutions,[11–17] and several species, including various prototropic forms and/or tautomers, may coexist (Fig. 1). In particular, the presence of the monoanion and neutral quinoid species can be significant when the chromophore is present (a) in media of lower polarity, (b) at the lipid–water interface of micelles or bilayers or (c) conjugated to macromolecules. The simultaneous occurrence of several species induced by this behavior has a profound effect on the fluorescence capability of the probe because the occurrence of non- or low-fluorescent species will decrease the observed fluorescence intensity. To evaluate the effect and interpret the probe spectra properly, we need to know each species' distinct spectral properties, the relative amount of each species, and how they are dependent on the properties of the surrounding environment, such as acidity, hydrogen-bonding capability, polarity *etc.* Herein we report the UV/Vis absorption and fluorescence properties of DBFL in organic solvents and discuss the possible presence of its tautomer and various prototropic forms.

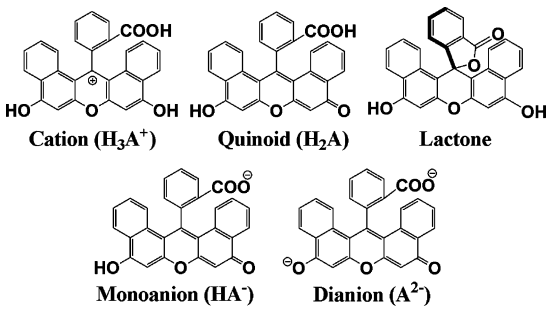

Fig. 1 Prototropic forms and tautomers of 1,2,7,8-dibenzofluorescein (DBFL).

Results and discussion

The dianion of DBFL can be prepared without the presence of the monoanion and other species (as in the case of FL) by adding sufficient NaOH to the alcohol. We therefore characterized the photophysical properties of DBFL dianion in methanol containing 0.01 M NaOH and compared it with that of FL dianion. The absorption and fluorescence spectra of DBFL dianion are shown in Fig. 2, and the photophysical parameters are collected in Table 1. The peak pattern for absorption and emission spectra of DBFL dianion are quite similar to that of FL dianion, except for a significant red shift of *ca.* 45 nm owing to the extension of the π system. The emission spectrum is good mirror image of the absorption spectrum, while the excitation spectrum is identical to its absorption spectrum, indicating that the fluorescence originates solely from its S_1 state. However, the Stokes shift for DBFL dianion is smaller, and as expected, the molar absorption coefficient is higher than that of FL because of the extended aromatic ring. The fluorescence quantum yield (Φ_f), on the other hand, is significantly lower than that of FL. The fluorescence decay is shown in the inset of Fig. 2; it can be well fitted by the monoexponential function, and the lifetime (τ_f) in MeOH is slightly shorter than that of FL.

The absorbance of DBFL dianion is proportional to the concentration in the measured range from 1 μM to 100 μM, which indicates that no aggregation occurs. Therefore the decrease of Φ_f

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Table 1 Photophysical parameters of DBFL dianion in methanol

	λ_{abs}/nm	$\varepsilon/10^5$ M^{-1} cm^{-1}	λ_{em}/nm	Φ_f	τ_f/ns	E_{00}/eV [a]	$k_r/10^8$ s^{-1}	$k_{nr}/10^8$ s^{-1}
FL	491	0.77	530	0.92	4.25	2.44	2.2	0.19
DBFL	534	0.83	565	0.25	3.87	2.26	0.64	1.93

[a] E_{00} is the energy for the lowest singlet state.

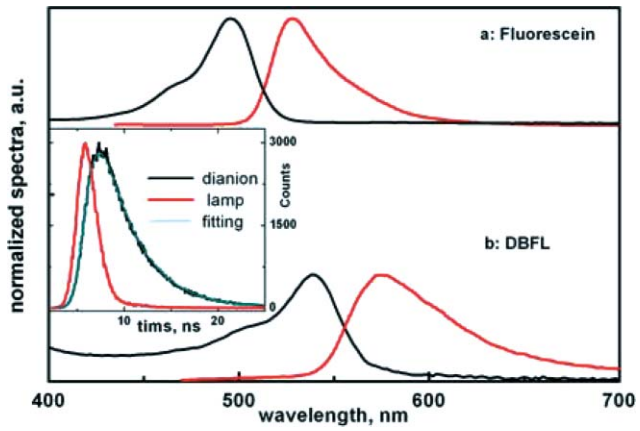

Fig. 2 Absorption and emission spectra for (a) FL and (b) DBFL (excitation at 370 nm). The inset is the decay trace (excited at 450 nm and monitored at 570 nm) for DBFL and its curve-fit.

is likely caused by the reduction of the radiation rate constant k_r according to $k_r = \Phi_f/\tau_f$, as shown in Table 1. The nonradiative rate constant of DBFL dianion (calculated by $k_{nr} = (1/\tau_f) - k_r$) is 10 times higher than that of FL. Since the rotation of the benzoic acid moiety in DBFL is even more restrained than that in FL, the internal conversion is not expected to increase. The larger value of k_{nr} is caused either by inter-system crossing or by another process, such as photo-induced intramolecular electron transfer. Such photo-induced intramolecular electron transfer could cause the reduction of Φ_f, this mechanism having been recently suggested to control the emission efficiency of FL.[17]

As for neutral DBFL, we recorded the UV/Vis spectrum of a solution in deionized water (pH 6.90) without any other additives, as shown in Fig. 3. Apparently, the spectrum, which is rather similar to that in Fig. 2, can mainly be attributed to the dianion

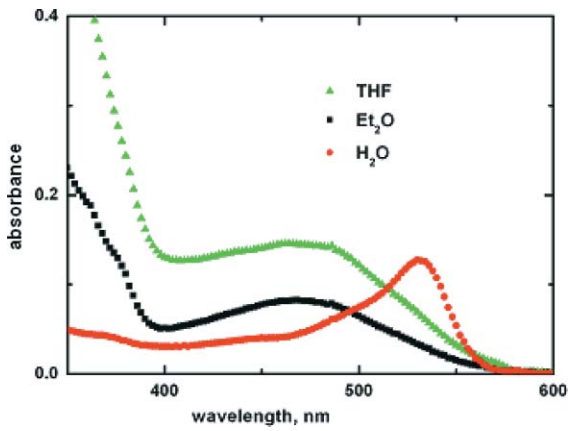

Fig. 3 Absorption spectra of DBFL in THF (*ca.* 11 μM), diethyl ether (*ca.* 6.0 μM) and deionized water (2.5 μM).

(A^{2-}). The water molecule is a good proton acceptor, facilitating the dissociation and dissolution of organic acids. The percentage of dianion can be rationalized by the pK_a values measured previously by our group[9] for the prototropic equilibria below (the structures are shown in Fig. 1). Based on the pK_a values, the molar ratio for A^{2-} at pH 6.90 is calculated to be 82%, and 18% for HA^-. In other words, DBFL (H_2A) is fully dissociated to $A^{2-}$and HA^- in deionized water.

$$HA^- \rightleftharpoons H^+ + A^{2-} \quad pK_{a,1} = 6.28$$

$$H_2A \rightleftharpoons H^+ + HA^- \quad pK_{a,2} = 4.14$$

$$H_3A^+ \rightleftharpoons H^+ + H_2A \quad pK_{a,3} = 3.07$$

Dichloromethane, chloroform, tetrahydrofuran and diethyl ether, on the other hand, are aprotic and low polarity solvents that are not expected to cause acid dissociation. The shape of the UV/Vis spectra of DBFL in these solvents is different from that in water, as shown in Fig. 3. In the visible region (above 400 nm), they show a single peak, and the peak maxima are blue-shifted over 50 nm compared to the dianion, and the corresponding molar absorption coefficient is only about 25% of that for the dianion. These spectra can therefore be attributed to the neutral quinoid form. The neutral lactone form does not absorb in this region because of its non-planar structure, which is also concluded from a quantum chemical calculation using Gaussian2003 at the DFT/6-311G level. However, the existence of the lactone cannot be excluded since its absorption falls into the UV region, which overlaps with that of the quinoid form.

Methanol and ethanol are highly polar and protic solvents, but weaker proton acceptors than water, and thus the degree of dissociation of DBFL in them is expected to be less intense. Comparing the spectra in ethanol (Fig. 4) with that of the quinoid in diethyl ether (Fig. 3), we can see that in these alcohols a new peak

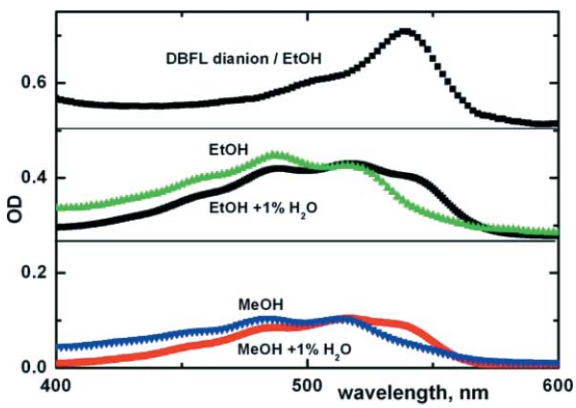

Fig. 4 Absorption spectra of DBFL and its dianion in alcohols.

appears, located on the red side of the quinoid band but on the blue side of the dianion peak. This is assigned to the absorption from the monoanion, and this can be further confirmed by the addition of 1% water, which causes the appearance of the dianion band because of the further dissociation of the monoanion. It is therefore concluded that the neutral form and the monoanion coexist in these alcohols.

In highly polar and aprotic solvents, such as acetone, acetonitrile and DMSO, the absorption spectra showed that the quinoid form is predominant (Fig. 5). The peaks for anions are either absent or much less significant than those in alcohols, which results from the intermediate capability of such solvents to accept protons (stronger than dichloromethane but much weaker than methanol). With the addition of sufficient water, however, these spectra all can be converted into those of the anions.

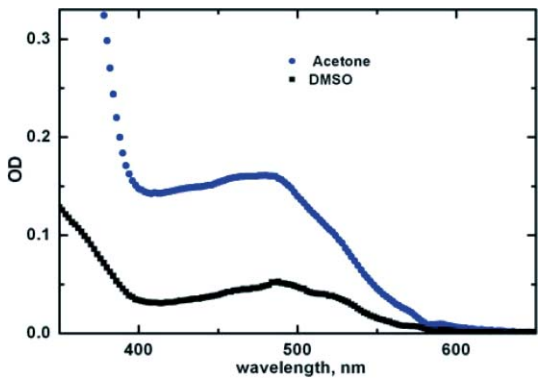

Fig. 5 Absorption spectra of DBFL in acetone and DMSO.

The typical fluorescence emission and excitation spectra in different types of solvents are shown in Fig. 6. Comparing the excitation spectrum with that of the corresponding absorption in aprotic solvents, such as THF, a significant difference is seen in the UV region below 400 nm. The large decline of the intensity in its excitation spectrum, relative to the absorption band in the region, indicates that there is an additional species that absorbs strongly in the UV region but does not emit at the measured emission wavelengths; this is the lactone.

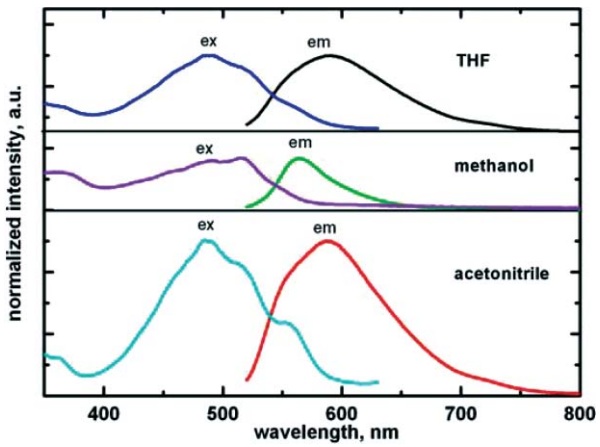

Fig. 6 Emission spectra (450 nm excitation) and excitation spectra (emission at 650 nm) of DBFL in THF, methanol and acetonitrile.

The fluorescence quantum yields (Φ_f) in different solvents are 0.36 (methanol), 0.21 (ethanol), 0.045 (acetonitrile), 0.041 (acetone), 0.040 (THF), and 0.045 (dichloromethane). This result matches the conclusion from absorption studies. The emission efficiency in alcohols is much higher than that in other solvents, which reflects the presence of the monoanion in alcohols. In water, Φ_f is 0.66 for the monoanion, 0.25 for the dianion, and almost zero for the quinoid.[9] This helps us to understand why Φ_f is much lower in aprotic organic solvents, where the quinoid is predominant. The higher value of Φ_f in methanol than in ethanol is attributed to the higher ratio of monoanion to quinoid, which can also be concluded from the relative height of absorption peaks in the two solvents illustrated in Fig. 4. The Φ_f values in other solvents show no remarkable differences from each other within experimental error, since the quinoid form is predominant in these solvents.

Conclusion

It has been shown that the photophysical properties of DBFL are strongly affected by the surrounding medium, in particular the capability of a solvent to accept protons, the polarity of the solvent and the amount of water added to the solvent. DBFL in aprotic solvents mainly exists in two tautomeric forms, *viz.* quinoid and lactone, which are not efficiently fluorescent. In contrast, in the protic solvents methanol and ethanol, both monoanion and neutral quinoid are present, and show the highest fluorescence quantum yield. In water, however, DBFL is dissociated to the monoanion and dianion.

Experimental

Chemicals

1,3-Dihydroxynaphthalene, acetic anhydride, ethanol, $ZnCl_2$ and phthalic anhydride were analytical grade and purchased from Beijing Chemical Company. All solvents were highest grade available and redistilled before use.

Preparation of 1,2,7,8-dibenzofluorescein

In a round-bottomed flask immersed in an oil bath, 1,3-dihydroxynaphthalene (8.0 g, 50 mmol), $ZnCl_2$ (0.50 g, 3.7 mmol) and phthalic anhydride (3.7 g, 25 mmol) were heated and stirred at 190 °C for 6 hours. After cooling, 0.1 M aqueous NaOH (100 mL) was added to dissolve the solid. The solution was then acidified with concentrated HCl to pH 3 and filtered. The dried solid was refluxed in acetic anhydride (30 mL) until homogeneous and filtered to remove insoluble solid. The solution was cooled overnight, filtered, and the diacetate collected as a pale orange solid. Sodium hydroxide (1 g) and ethanol (10 mL) were added to the diacetate in a round-bottomed flask and the mixture was concentrated to dryness under reduced pressure. Water (20 mL) and concentrated HCl were added to adjust pH to 3; the solid was filtered and dried. Yield: 5.8 g (54%). The product was purified by column chromatography using MeOH–ethyl acetate (1 : 4) as the mobile phase. Mp >300 °C. MS: 433.2 (M + H), ^{1}H NMR (CD_3OD + NaOD) δ: 8.32–8.36 (d, 2H), 8.23–8.26 (d, 1H), 7.65–7.75 (t, 1H), 7.57–7.64 (t, 1H), 7.15–7.25 (m, 4H), 6.85–6.96 (m, 5H).

Preparation of solutions

Before measuring UV/Vis and fluorescence spectra, the sample of dibenzofluorescein was purified chromatographically until the ε_{max} remained constant. DBFL dissolved in 0.010 M NaOH had an absorption coefficient at 532 nm of 0.85×10^5 M^{-1} cm^{-1}, slightly higher than that reported by Lee.[9] Solutions were prepared by dissolving a weighed amount of the purified dye in organic solvents and then diluted to the desired absorbance. All solutions were air-saturated. All measurements were performed at room temperature (26 °C).

Apparatus

Absorption measurements were made with a HP 8451A spectrophotometer. Fluorescence measurements were made with a F4500 spectrophotometer. Fluorescence lifetime measurements were made with an Edinburgh FL920 time-correlated single photon counting spectrophotometer. Fluorescence quantum yields were measured relative to a fluorescein standard[18] in 0.1 M NaOH ($\Phi_f = 0.91$). The excitation light was of 370 nm or 450 nm with the absorbance less than 0.09 to obtain the full emission spectrum and avoid self-absorption.

Acknowledgements

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