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Fluorescence Properties of Dibenzofluorescein in Aqueous Solution

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Abstract The deprotonation of dibenzofluorescein (DBFL), a long wavelength fluorescence probe, results in the simultaneous occurrence of neutral form, monoanion and dianion under physiological conditions. The fluorescence properties of the former two cannot be measured directly because they are always coexistent with some other species. By measuring the fluorescence under various pHs we computed the fluorescence parameters for each species involved in the prototropic equilibria of DBFL, including each species' emission spectrum, excitation spectrum, emission and excitation maximum, fluorescence quantum yield and lifetime. It was found that the monoanion is the most fluorescent chromospheres (Φ_f=0.66, compared to Φ_f=0.25 for dianion, 0.18 for cation and 0.0 for the neutral form). Together with the computed pK_as, we are able to suggest that the monoanion plays a major role under physiological conditions when DBFL is used as a fluorescence probe, contrary to the assumption in literature.

Keywords Dibenzofluorescein · Fluorescence · Fluorescein · Fluorescence probe

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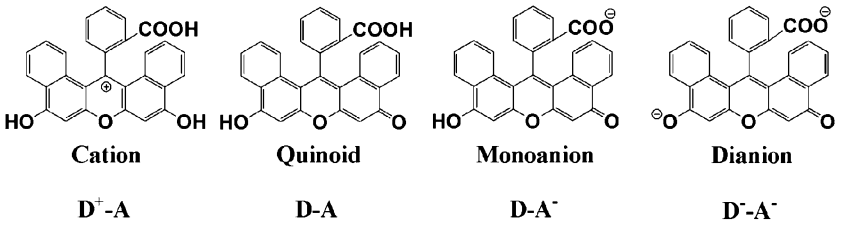
Introduction

1,2,7,8-Dibenzofluorescein (DBFL), a dibasic organic acid as shown in Scheme 1, is a member of oxyxanthene family and has found several applications, as a fluorescence probe, in sensing alkali metal ions [1], sequencing DNA [2], labeling oligonucleotide [3], flow cell cytometry [4], and assaying glutathione transferase [5] etc due to its remarkably red shifted emission maximum compared to that of fluorescein (FL). The advantages of such a dye are obvious which include: the much reduced autofluorescence from biological materials (e.g., DNA); the ease of finding portable light emitting diode (LED) lasers to match its absorption maximum, and hence simplified fiber optics and detectors which are small, low cost and robust [6–8]. Nevertheless, information about DBFL fluorescence properties is still rare except for a report on its dianion [9] in which the monoanion is assumed to be nonfluorescent. It is not known, however, which species plays the most important role because of the coexistence of several species as shown in Scheme 1. This is in sharp contrast to FL, which has been the attention of hundreds of reports on its prototropic equilibria and spectral properties under various conditions [10–17 and references cited therein].

Besides, the presence of the monoanion and neutral species can be significant when the chromophore is present in the media with lower polarity, at the lipid–water interface of micelles, bilayers or conjugated to macromolecules. In fact we have shown in a previous report that the molar ration of monoanion to dianion is about 1:1 under physiological conditions [18]. As a result, the monoanion or quinoid may contribute remarkably to the fluorescence signal in these instances because of the serious overlapping of their emission spectra. The fluorescence parameters for the involved species are therefore necessary in order to interpret



Scheme 1 Chemical structures for various prototropic forms of DBFL

properly the spectral properties of DBFL in biological systems. Unlike the dianion species, however, it is usually not possible to observe the quinoid or monoanion in the absence of other species. Therefore, this information has to be extracted from the mixture by some analytical methods. Continuing our previous study [18] on the prototropic equilibria of DBFL by absorption spectra, we wish to report herein the fluorescence properties of dianion, mono anion, neutral form and cation of DBFL in aqueous solutions. We found that the monoanion is highly fluorescent (Φ_f=0.66, compared to Φ_f=0.25 for dianion) and plays a major role at neutral pH when DBFL is used as a fluorescence probe, which is a correction to the assumption in literature.

Materials and methods

Chemicals

1,3-Dihydroxynaphthalene, acetic anhydride, ethanol, $ZnCl_2$ and phthalic anhydride were analytical grade and purchased from Beijing Chemical Company. HCl and NaOH were reagent grade and used without purification further.

1,2,7,8-Dibenzofluorescein preparation

In a round bottomed flask, 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 in an oil bath to 190 °C for 6 h. After cooling down, 100 ml aqueous 0.1 mol/l NaOH were added to dissolve the solid. The solution was then acidified with concentrated HCl to pH 3 and filtered. The dried solid was refluxed with acetic anhydride (30 ml) until homogeneous and filtered to remove insoluble solid. The solution was cooled overnight. The diacetate was filtered and 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 mobile phase. m. p.>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 spectra under various pH, our sample of dibenzofluorescein was purified chromatographically until the ε_{max} reached maximum. DBFL dissolved in 0.010 M NaOH, had an extinction coefficient at 532 nm of 0.85×10^5 $M^{-1}cm^{-1}$, slightly higher than that reported by Lee et al. [9]. Solutions were prepared by dissolving a weighed 0.0212 g (0.0049 mmol) of the purified dye in 100 ml volumetric flask containing 0.010 mol/l NaOH. The prepared solution then was diluted with 0.01 mol/l NaOH to obtain the concentration at 0.0049 mM. Taking 10.0 ml of above solution and adjusting pH by adding 10 M HCl with a micro liter syringe. Each time, only a few micro liter HCl was added and thus the total volume change is negligible. The total ion (from acid or base and salt) concentration was kept constant at 0.01 mol/l. The accurate pH was measured by a Beckman pH-meter. All solutions were air saturated. All measurements were performed at room temperature of 26 °C.

Apparatus

Absorption measurements were made with a HP 8451A spectrophotometer with 1 cm path length cuvettes. Fluorescence measurements were made with a F4500 spectrophotometer with a standard 1 cm path length cuvettes. Fluorescence quantum yields were measured relative to a fluorescein standard in 0.01 mol/l Na0H. The exciting light was of 450 nm with the absorbance less than 0.09 to get the full emission spectrum and avoid self-absorption.

Method to determine fluorescence quantum yield for each species

For the equilibria below, H_2A stands for the neutral quinoid form of DBFL. It is not difficult to get Eq. 2 by assuming that the total fluorescence F measured by the area under the observed emission spectrum is the addition of that from each species. Eq. 1 is obtained by summation of the

absorbance at a given wavelength for all present species, it is a more generalized version to that of Diehl [12].

$$HA^- \overset{Ka,1}{\rightleftharpoons} A^{2-} + H^+ \quad K_{a,1} = \frac{[A^{2-}]a_{H^+}}{[HA^-]} \frac{\gamma_{A^{2-}}}{\gamma_{HA^-}}$$

$$H_2A \overset{Ka,2}{\rightleftharpoons} HA^- + H^+ \quad K_{a,2} = \frac{[HA^-]a_{H^+}}{[H_2A]} \frac{\gamma_{HA^-}}{\gamma_{H_2A}}$$

$$H_3A^+ \overset{Ka,3}{\rightleftharpoons} H_2A + H^+ \quad K_{a,3} = \frac{[H_2A]a_{H^+}}{[H_3A^+]} \frac{\gamma_{H_2A}}{\gamma_{H_3A^+}}$$

under our conditions of ion strength I=0.01 and C_0= 4.9 μM, the activity coefficients γ_i for each species can be calculated by Debye–Hueckel equation to be essentially 1.0, except for H^+ whose activity is directly measured by the pH meter.

$$A = \frac{\sum_{i=0}^{3} \beta_i \varepsilon_{H_iA^{i-2}} a_{H^+}^i}{\sum_{i-0}^{3} \beta_i a_{H^+}^i} C_0 \tag{1}$$

$$\Phi A = \frac{\sum_{i=0}^{3} \beta_i \varepsilon_{H_iA^{i-2}} \phi_i a_{H^+}^i}{\sum_{i=0}^{3} \beta_i a_{H^+}^i} C_0 \tag{2}$$

In Eqs. 1 and 2, A is the total absorbance observed at a given excitation wavelength, Φ is the corresponding observed fluorescence quantum yield, ϕ_i is the fluorescence quantum yield for species H_iA^{i-2},a_{H^+} is the activity of H^+, C_0 is the known total concentration, and $\varepsilon(H_iA^{i-2})$ is the molar extinction coefficient for species H_iA^{i-2} at the same wavelength, while β_i is the accumulated protonation constant, i.e.

$\beta_i = \left(K_{a,0} \times K_{a,1} \times \ldots \times K_{a,i}\right)^{-1}$ with the definition of β_0 $= \left(K_{a,0}\right)^{-1} = 1$.

By selecting the wavelengths where all species can absorb light but minimizing the self absorption to fluorescence, recording the change of A and F to pH, we can then fit Eq. 1 to get β_i and $p_i = \beta_i \varepsilon_{H_iA^{i-2}}$, and fitting Eq. 2 to obtain $q_i = \beta_i \varepsilon_{H_iA^{i-2}} \phi_i$. ϕ_i is then obtained by q_i/p_i.

Method to obtain the fluorescence spectra for each species

The emission spectrum of dianion is easily obtained by recording at pH>8 where it is the only species. As we will see later that the neutral form of DBFL is actually nonfluorescent and therefore no emission spectrum to be measured. This fact also makes us easier to get the emission

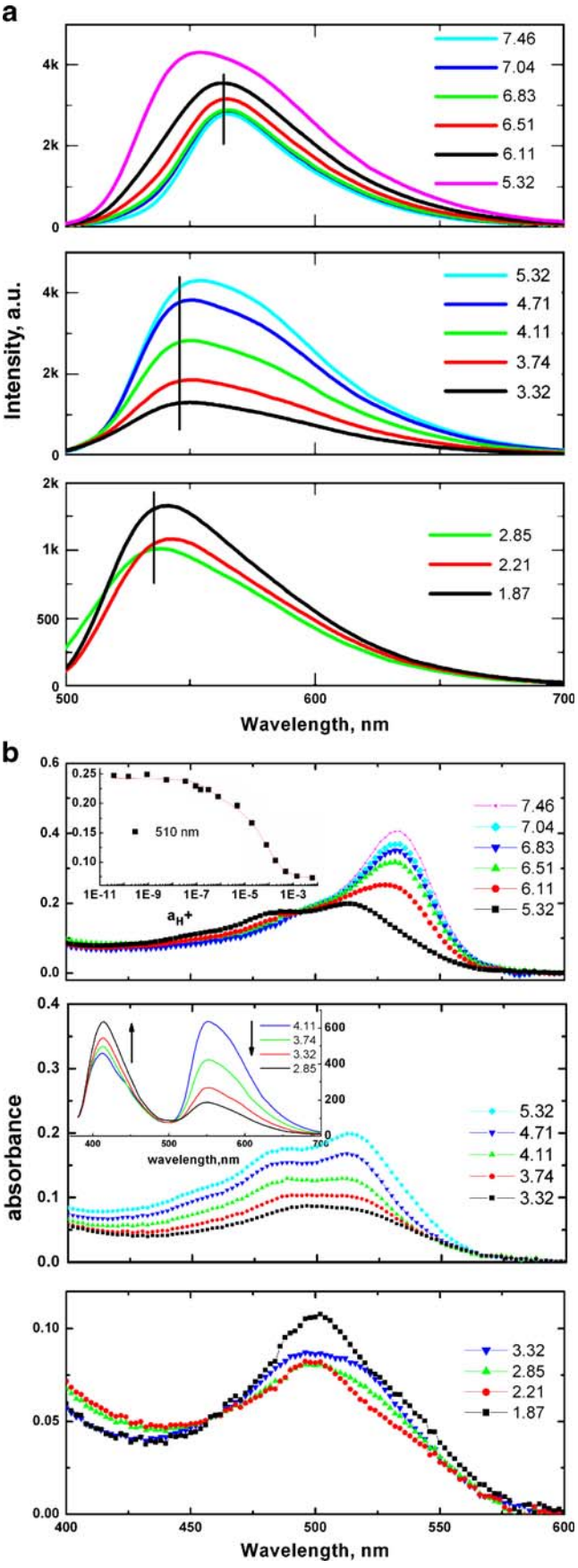


Fig. 1 **a** Emission spectra of DBFL at different pH, excitation at 450 nm (*top*). **b** pH effect on absorption spectra (*bottom*). *Inset* of **b**: emission spectra excited at 360 nm. [DBFL]=4.9 μM in aqueous solution

spectrum for the cation. In the case pH<3 where both cation and the neutral form coexist, but only the cation is fluorescent, the observed emission spectrum is therefore that from the cation.

The difficulty is to gain the emission spectrum for the monoanion because it always coexists with some other species with overlapped emission. The following quantitative procedure is developed to solve the problem. It is based on the fact that only the dianion and monoanion are in the equilibrium for pH between 6 and 8 according to the measured pK_as by Eq. 1 and data shown in Fig. 3. During the acid titration, the total dye concentration C_0 and ion strength I are constants. The detailed procedure is as following:

(a) Measuring emission spectrum at pH>8.5 where dianion is the only form of DBFL, we get $k_{\lambda,D} = F_{\lambda,D}/C_0$ for dianion A^{2-} at any given wavelength λ. $F_{\lambda,D}$ is the fluorescence intensity of A^{2-} at λ. $k_{\lambda,D}$ is independent of $[A^{2-}]$ but changes with wavelength, because fluorescence intensity is proportional to the concentration of a fluorophore when the concentration is sufficiently low.

(b) At a pH, for example 6, where only monoanion and dianion are present, we record the emission spectrum. At that pH the fluorescence intensity F_λ contains the contribution from both monoanion($F_{\lambda,M}$) and dianion ($F_{\lambda,D}$) at any wavelength λ.

(c) Fitting the data by Eq. 2, p$K_{a,1}$ can be obtained.

(d) Calculate $[A^{2-}]$ at the pH using the known p$K_{a,1}$ and the total concentration.

(e) Calculate the fluorescence intensity of dianion $F_{\lambda,D}$ at given wavelength λ by using $k_{\lambda,D}$ from (a) and $[A^{2-}]$ from (c), i.e. $F_{\lambda,D} = k_{\lambda,D}[A^{2-}]$.

(f) Compute $F_{\lambda,M}$ by subtracting F_λ measured in (b) with $F_{\lambda,D}$ calculated in (d), i.e. $F_{\lambda,M} = F_\lambda - F_{\lambda,D}$.

(g) Plot $F_{\lambda,M}$ against λ.

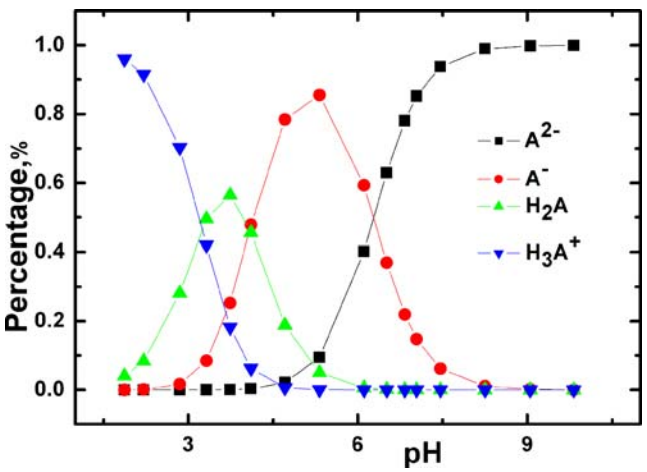


Fig. 2 pH effect on the molar ratio of each species in aqueous solution

Method to get excitation spectrum for each species

Based on the same reason as mentioned above, the excitation spectrum of dianion is easily obtained by recording at pH>8, while the observed excitation spectrum at pH<3 is that from the cation.

The procedure to obtain the excitation spectrum for the monoanion is simplified by the fact that the neutral form is nonfluorescent in the region above 450 nm. At pH 4.7 the presence of dianion and cation are negligible, while monoanion and the nonfluorescent neutral form are coexistent, the observed excitation spectra at the pH is assigned to the monoanion.

Results and discussion

Figure 1a illustrates the fluorescence emission spectra at various pH with excitation at 450 nm. Figure 1b shows the corresponding absorption spectra. These spectra can be classified into four groups as following.

1. 8.0≤pH≤12, in this range the band shape of all measured spectra are nearly identical (not shown) which are mainly attributed to the emission from the dianion. So is the case for the UV/Vis spectra.

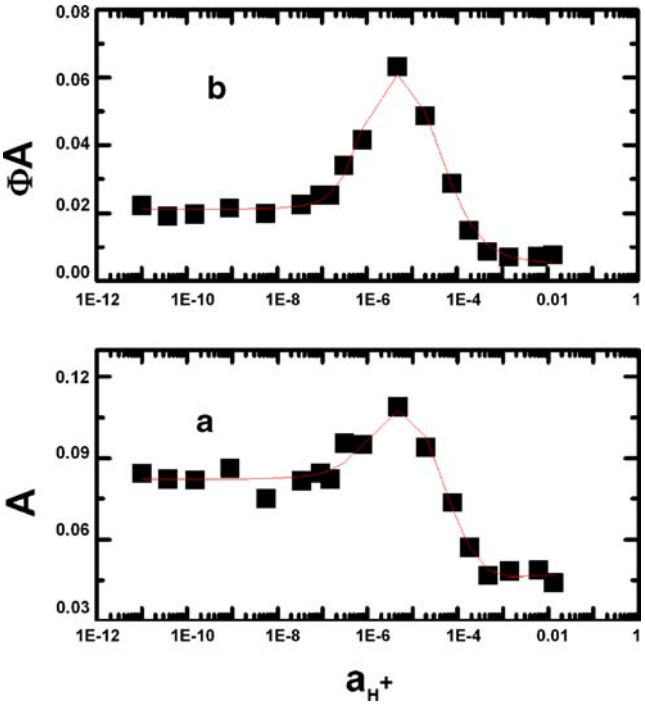


Fig. 3 Observed A and ΦA data upon pH change, fitted line by Eqs. 1 and 2. Total [DBFL] is 4.9 μM and λ_{ex}=450 nm

Table 1 Photophysical parameters for the species

	λ_{max}(abs), nm	λ_{max}(ex), nm	λ_{max}(em), nm	E_{00}, ev	Φ_f	τ_f, ns
Cation	494	502	543	2.40	0.18	n.d.
Quinoid	500	N/A	N/A	N/A	0	N/A
Monoanion	510	524	558	2.28	0.66	3.24
Dianion	534	537	566	2.25	0.25	3.93

n.d.: not determined, *N/A*: not available

2. 6.1<pH<8.0, with the decrease of acidity, the spectra became slimmer and the intensity was lowered. The absorption spectra in the pH range, however, showed an isosbestic point at 514 nm (top of Fig. 1b). The presence of the isosbestic point in absorption spectra is resulted from the following facts: (a) only two absorbing species are in equilibrium (dianion+H^+⇆ monoanion), (b) total concentration is kept at constant, (c) there exists an overlapping area, and (d) there exists a cross point where two species have the same molar absorptivity, i.e. $\varepsilon_1=\varepsilon_2$ at the wavelength of 514 nm, so that the total absorbance A ($=A_1+A_2=\varepsilon_1 C_0$) shows no change upon the movement of the equilibrium. In the case of fluorescence spectra, however, the forth condition changes to $\Phi_1\varepsilon_1=\Phi_2\varepsilon_2$ to satisfy that F ($=F_1+F_2=\Phi_1\varepsilon_1 C_0$) is constant when the equilibrium is moved, in which Φ_1, Φ_2 represent fluorescence quantum yields while ε_1 and ε_2 are the molar absorptivity for two species at the excitation wavelength. Only at the excitation wavelength where $\Phi_1\varepsilon_1=\Phi_2\varepsilon_2$ is met, an isostilbic point (equal emission point) will be observed, this is not the case for 450 nm. But with excitation at 360 nm in low pH solutions, such a point is observed as shown in the inset of Fig. 1b because of the equilibrium between the cation and the neutral form.
3. 3.32<pH≤6.1, the spectra show a broad band shape which obviously contains at least two separate emission bands. For the absorption spectra, on the other hand, the isosbestic point was disappeared. This reflects the transformation of monoanion (HA^-) to the neutral form upon protonation by decreasing pH. What is also noted is the blue shift of emission maximum owing to the major contribution from monoanion.
4. pH<3.32, the band shape became narrow again and the emission maximum is further blue shifted which suggests the formation of a new species of cation.

Plotting emission maxima and emission intensity against pH respectively, it shows that upon the increase of acidity, the fluorescence intensity grows rapidly with the formation of monoanion and reaches maximum at pH 5.32, but then falls upon the production of neutral DBFL and gets to the minimum when the cation is predominant. Treatment of the absorbance data (inset in the top of Fig. 1b) by Eq. 1 at wavelengths close to absorption maximum, pK_as were obtained as 3.14, 4.07 and 6.28. While fitting the fluorescence data with excitation at 450 nm by Eq. 2 (shown in

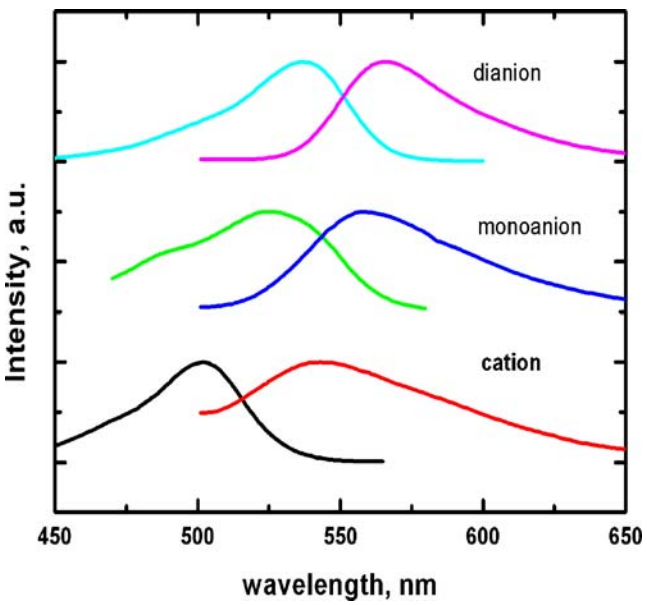


Fig. 4 Excitation (*left*) and emission (*right*) spectra for fluorescent species

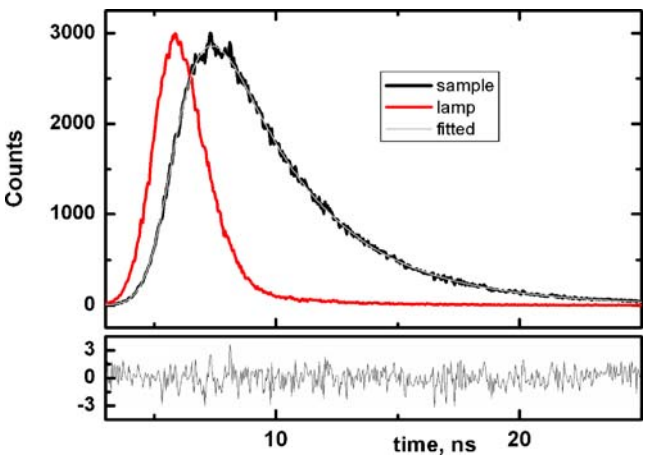


Fig. 5 Fluorescence decay curve for DBFL in aqueous solution, pH 7.0, excitation wavelength 450 nm

Fig. 3), we get pK_as as 3.16, 4.34 and 5.85. The first two are reasonably agreement with the results from absorption data, but the third one is lowered by 8%, because 450 nm is far from the peak maximum and showed larger error.

The decrease of emission maximum upon acidity increase clearly indicates the significant formation of monoanion at pH 6.51, neutral form at 3.74 and the cation at 2.87, the three inflexions are close to p$K_{a,1}$, p$K_{a,2}$ and p$K_{a,3}$ respectively. Comparing to the molar ratio change in Fig. 2, which is computed by pK_as, the change of emission maximum indeed reflects the transformation of predominant species in solutions.

In summary, the observation made above is consistent with the description of the system by three equilibria and four species. It also shows that each species indeed has different fluorescent capabilities, which can be quantitatively described by its fluorescence quantum yield (Φ_f).

Before applying the procedure to our data, the reliability was tested by using the data for fluorescein reported by Diehl [12]. The pK_as calculated by this procedure for fluorescein are 2.26, 4.23 and 6.29, which is in good agreement with 2.18, 4.40 and 6.36 reported by Diehl [13]. And fitting the data in Fig. 3 by Eq. 2, the values of Φ_fs are obtained and listed in Table 1. As seen from Table 1, the neutral form of DBFL is essentially nonfluorescent (Φ_f= 0.00), while the monoanion is the most efficient fluorophore among the four species (Φ_f=0.66).

The emission spectra for each species are shown in Fig. 4, which are obtained by the procedure described previously. The neutral form is essentially non-fluorescent according to the calculated value 0.00 for its Φ_f by Eq. 2, hence no emission spectra should be deduced. Also included in Fig. 4 are the excitation spectra for three emitting species. As expected, they all show mirror symmetry in the normalized excitation and emission spectra. From the cross point, the energy for lowest excited singlet state can be calculated as shown in Table 1.

Figure 5 shows the decay of transient fluorescence of DBFL in pH 7 upon excitation by a nanosecond flash hydrogen lamp. The signal can be fit well by a biexponential decay with lifetimes of 3.93 (89%) and 3.24 ns (11%). The former is assigned to the lowest excited singlet state of dianion, whereas the second is for that of the monoanion.

The chemical structures for DBFL protolytic forms are shown in Scheme 1. According to Tanaka and Fukuzumi, the mechanism that controls the fluorescence quantum yields of fluorescein derivatives has been suggested to be photo-induced intra-molecular electron transfer [19]. This should also be applied to DBFL which consists of two moieties. For the quinoid of the neutral form, the benzoic acid moiety is the electron acceptor (A), while the xanthene moiety is the electron donor (D). We can then denote it by D–A. The other three species can then be written as D^+–A for cation, D–A^- for monoanion and D^-–A^- for dianion. This fact can help us understand the relative value of Φ_f for each species qualitatively. The emission process is quenched by the photo-induced electron transfer with a rate constant k_{et}, which depends on the excitation energy and the difference between a donor's oxidation potential and the corresponding acceptor's reduction potential. All species show very close excitation energy E_{00} and quite similar chemical structures, we therefore consider only the redox potential for the donor and acceptor in the species. Moreover, the redox potential is strongly influenced by the charge on it. We can therefore conclude k_{et} (D^-–A^-)>k_{et} (D–A^-)<k_{et} (D–A)>k_{et} (D^+–A) if none of them is located in the inverted region. Correspondingly, we have Φ_f (D^-–A^-)<Φ_f (D–A^-)>Φ_f (D–A)<Φ_f (D^+–A). This is agreement with the fact that the monoanion has the highest Φ_f while the quinoid shows the lowest Φ_f.

Conclusion

By measuring the dependence of fluorescence on pH we computed the fluorescence parameters for each species involved in the prototropic equilibria of DBFL, including each species' emission spectrum, excitation spectrum, emission and excitation maximum, fluorescence quantum yield and lifetime. The important finding is that the monoanion is the most efficient fluorophore (Φ_f=0.66, compared to Φ_f=0.25 for dianion, 0.18 for cation and 0.0 for quinoid) and may play a major role under physiological conditions when DBFL is used as a fluorescence probe.

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References

1. Kuhn MA, Haugland RP (1993) U. S. Patent 5516864
2. Lee LG, Connell CR, Woo SL, Cheng RD, McArdle BF, Fuller CW, Halloran ND, Wilson RK (1992) DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. Nucleic Acids Res 20:2471–2483
3. Kempe T (2003) U. S. Patent 6534646
4. Lee LG (1990) U. S. Patent 4933471
5. Diwu Z, Haugland RP (1997) U. S. Patent 5773236
6. Liu J, Diwu Z, Leung W-Y (2001) Synthesis and photophysical properties of new fluorinated benzo[c]xanthene dyes as intracellular pH indicators. Bioorg Med Chem Lett 11:2903–2905
7. Gómez-Hens A, Aguilar-Caballos MP (2006) Long-wavelength fluorophores—new trends in their analytical use. TrAC Trends Anal Chem 23:127–136
8. Thomas KJ, Sherman DB, Amiss TJ, Andaluz SA, Pitner JB (2006) A Long-Wavelength Fluorescent Glucose Biosensor Based on Bioconjugates of Galactose/Glucose Binding Protein and Nile Red Derivatives. Diabetes Technol Ther 8:261–268

9. Lee LG, Berry GM, Chen C-H (1989) Vita blue: a new 633-nm excitable fluorescent dye for cell analysis. Cytometry 10:151–164
10. Zhang H, Zhou Y, Zhang M, Shen T, Li Y, Zhu D (2003) Photoinduced interaction between fluorescein ester derivatives and CdS colloid. J Colloid Interface Sci 264(1):290–295
11. Zhang H, Zhou Y, Zhang M, Shen T, Li Y, Zhu D (2002) Photoinduced charge separation across colloidal TiO2 and fluorescein derivatives. J Phys Chem B 106(37):9597–9603
12. Diehl H (1989) absorbance of the various prototropic forms of yellow fluorescein in aqueous solution. Talanta 36:413–415
13. Diehl H, Horchak-Morris N (1987) The absorbance of fluorescein in the ultraviolet, as a function of pH. Talanta 34:739–741
14. Song A, Zhang J, Zhang M, Shen T, Tang JA (2000) Spectral properties and structure of fluorescein and its alkyl derivatives in micelles. Colloids Surf A 167:253–262
15. Jang YH, Hwang S, Chung FS (2001) Tautomeric equilibrium of fluorescein in solution: ab initio calculations. Chem Lett 12:1316–1317
16. Hungerford G, Benesch J, Mano JF, Reis RL (2007) Effect of the labelling ratio on the photophysics of fluorescein isothiocyanate (FITC) conjugated to bovine serum albumin. Photochem Photobiol Sci 7:152–158
17. Rodriguez HB, Lagorio MG, Román ES (2004) Rose Bengal adsorbed on microgranular cellulose: evidence on fluorescent dimers. Photochem Photobiol Sci 2:674–680
18. Zhang X-F, Liu Q, Song A (2008) Tautomeric and prototropic equilibria of dibenzofluorescein in solution. Photochem Photobiol Sci (in press)
19. Tanaka K, Miura T, Umezawa N, Urano Y, Kikuchi K, Higuchi T, Nagano T (2001) Rational design of fluorescein-based fluorescence probes. Mechanism-based design of a maximum fluorescence probe for singlet oxygen. J Am Chem Soc 123:2530–2536