

Fluorescence Properties of Twenty Fluorescein Derivatives: Lifetime, Quantum Yield, Absorption and Emission Spectra

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Abstract The fluorescence lifetime (τ_f), emission quantum yield (Φ_f), absorption and emission spectral data of 20 fluorescein derivatives were measured under the same conditions by using time-correlated single photon counting, steady state fluorescence and absorption methods to get comparable data. Based on the results, the factors and mechanism that control the fluorescence properties of the fluorescein dyes are discussed. Both Φ_f and τ_f are remarkably dependent on the substitution on either xanthene or phenyl rings, but their ratio (Φ_f/τ_f), i.e. rate constant of radiation process, is a constant value ($0.20 \times 10^9 \text{ s}^{-1}$). The rate constant of nonradiation process, on the other hand, is varied with both the structure and the solvent used.

Keywords Fluorescence lifetime · Fluorescence quantum yield · Fluorescein · Eosin · Rose Bengal

Introduction

Fluorescein and its derivatives form a family of versatile functional dyes (structures shown in Fig. 1), which have been widely used due to their excellent fluorescence and other photophysical properties [1, 2]. Interests in designing and synthesizing new fluorescein derivatives are still enormous to meet needs in various areas [3–5]. The basic fluorescence properties of some well known fluorescein dyes have been reported [6–15], but the experiments for different dyes were performed under varied conditions by different authors and

different techniques or instruments. Therefore it is difficult to compare the data between the different dyes and correlate their structures to emission properties and mechanisms. Consistent data are not only important for choosing a dye with a specific property in an application, but also helpful for comparing the quantum chemical computation with the experimental values.

In this study, we have collected various fluorescein derivatives as many as possible and measured the fluorescence lifetime (τ_f) values for the often-used 20 commercial dyes (Fig. 1) under the same conditions. For some of them, the τ_f data have not been reported. For others, the τ_f values were scattered in literatures and measured under different conditions. To be consistent on the data, hereby we also report their fluorescence quantum yield (Φ_f) and spectral parameters, so that the emission mechanism of fluorescein and its derivatives can be discussed. The study is an extension of our previous reports for various fluorescein and its analogs [7, 16–21].

Materials and Methods

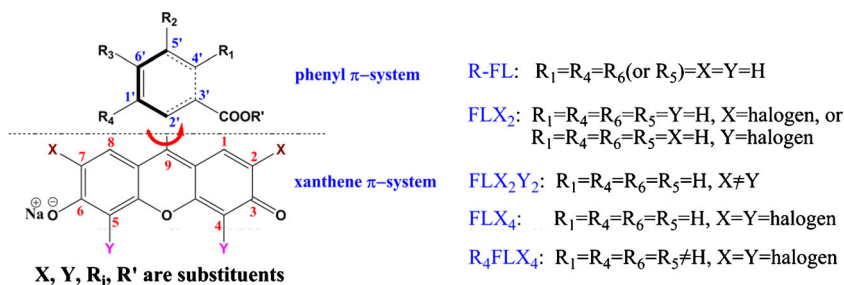
Materials

The dyes in the study (structures are shown in Fig. 1) include Fluorescein (FL), 2,7-dichlorofluorescein (2,7-FLCl₂), 4,5-dichlorofluorescein (4,5-FLCl₂), 4,5-dibromofluorescein (4,5-FLBr₂), 4,5-diiodo fluorescein (4,5-FLI₂), calcein, diiodofluorescein, eosin Y (EY), eosin B (EB), ethyl eosin (EE), 2,4,5,7-tetrachloro fluorescein (FLCl₄), 3',4',5',6'-tetrachlorofluorescein (Cl₄FL), 3',4',5',6'-tetrabromo-2,4,5,7-tetrachlorofluorescein (Br₄FLCl₄), Rose Bengal (RB, i.e. 3',4',5',6'-tetrachloro tetrachloro-2,4,5,7-tetraiodofluorescein), erythrosine (2,4,5,7-tetraiodofluorescein), 5'-aminofluorescein (5-NH₂-FL), 5'(6')-tert-butylfluorescein (*t*-butyl-FL), FITC (fluorescein isothiocyanate), 5-FITC (5'-fluorescein isothiocyanate), 5'-carboxylfluorescein (5-COOH-FL), 6'-carboxylfluorescein (6-

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Fig. 1 Chemical structure and abbreviation of fluorescein dyes



COOH-FL), 5'(6')-nitrofluorescein (NO₂-FL). All dyes were purchased with analytical grade or better and used as received. The purity was stated to be better than 97 % and only one spot was detectable by TLC. Ethanol of analytical grade was dried and redistilled immediately before use. Deionized water was purified by Milli-Q system (Millipore, USA).

Acid–base equilibrium exists for fluorescein dyes in protic solvents, which gives different chemical forms with different fluorescence properties. To simplify the question, we use either the freshly prepared alcohol containing 1 mM NaOH or water (10 mM PBS buffer pH 7.3) as the solvent, respectively. In each case a constant pH was maintained. Under the basic conditions, the OH on the xanthene ring and the COOH on the phenyl ring are deprotonated, while the protonated, zwitter ion and lactone form were excluded.

The fluorescence quantum yield is very sensitive to the sample concentration and excitation wavelength due to the self-absorption effect. Therefore the dye concentrations were held low enough to a value yielding an absorbance of ~ 0.020 in a 1 cm path to avoid self-absorption effect. The accurate absorbencies of all the dye solutions were then measured by a 5 cm path length cuvettes on a Hitachi 2450 UV–Vis spectrophotometer at the excitation wavelength with a value 0.100 (± 0.005).

Under the condition in the solvents, the aggregation is negligible. For example, reported values for the dimerization constant of fluorescein in this solvents lie in the range of 3.6–250 M⁻¹ [22]. Accordingly, in our concentration range 0.1 μ M to 1 μ M for the quantum yield measurements, less than 0.1 % of dyes could form aggregates.

Also the presence of 1 mM NaOH in ethanol consumed protons so that only one emitting species (deprotonated form of OH on the xanthene ring and COOH on the phenyl) is present in ethanol. So is the case in PBS buffer.

Neither spectral shape nor intensity was changed over time within a week in either ethanol or PBS buffer.

Methods

Fluorescence measurements were performed by using a FLS 920 fluorospectrometer of Edinburgh Instruments with

cuvettes of 1 cm and excitation at 500 nm (20 °C). The emission slit was 1.0 nm while the excitation slit was 0.5 nm. The fluorescence was measured at 90° to the incident excitation beam. The fluorescence intensity at a wavelength was calibrated against the detector response and the excitation light intensity. The fluorescence quantum yield was computed by using

$$\Phi_f = \Phi_f^0 \frac{F_s A_0 n_s^2}{F_0 A_s n_0^2},$$

in which **F** is the integrated fluorescence intensity, **A** is the absorbance at excitation wavelength, **n** is the refractive index of the solvent used, the subscript 0 stands for a reference compound and **s** represents samples. Fluorescein in basic ethanol was used as the reference ($\Phi_f^0 = 0.92$) [10]. Φ_f values of Eosin Y, Eosin B, and RB thus obtained in ethanol also match the reported values. Several measurements for a Φ_f value were performed and averaged by choosing different concentrations and excitation wavelengths.

Measurements of the fluorescence lifetimes were performed with standard time-correlated single-photon counting method. The exciting light was a portable diode laser (EPL-375, Edinburgh Instruments), the 379 nm (69 ps, 0.10 mW) laser beam was guided into the samples, and fluorescence (the wavelength at the emission maximum of a dye) was detected with a PMT (Hamamatsu R928) cooled to -21° . The repetition rate is 10 MHz whilst the count rate did not exceed 20 kHz (0.2 %) in order to avoid pile-up effects. The bandwidth for excitation as well as for emission was < 2 nm. The prompt response function of the system had an fwhm between 500 and 700 ps. The deconvolution method was used to fit the $I(t) = A + B \times e^{(-t/\tau_f)}$ to obtain the fluorescence lifetime τ_f . If the chi-squared value is larger than 1.25, $I(t) = A + B_1 \times e^{(-t/\tau_{f1})} + B_2 \times e^{(-t/\tau_{f2})}$ was used to fit data.

R6G in ethanol was used as the reference to test the lifetime measurement ($\tau_f = 3.99$ ns), which is in excellent agreement with the measured value of 3.96 ns [12].

The fluorescence lifetimes of the dyes showed no dependence on the emission wavelength and sample concentration

Table 1 Fluorescence properties for the dyes in EtOH and PBS buffer

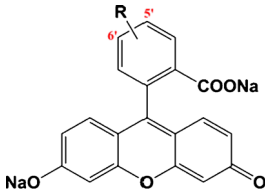
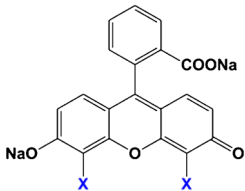
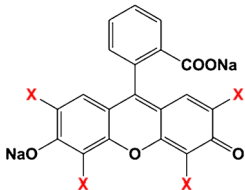
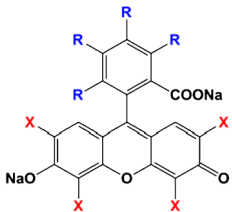
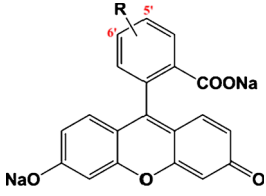
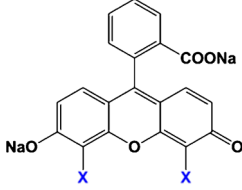

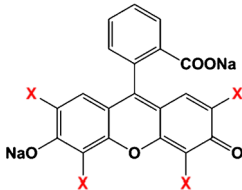
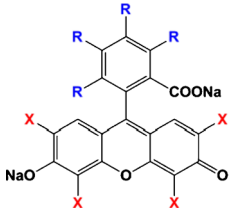
		EtOH/ 1 mM NaOH					
		substitution	τ_f , ns	Φ_f	λ_{abs} , nm	λ_{em} , nm	k_f , 10^9 s^{-1}
 <p style="text-align: center;">R-FL</p>	5-NH₂	0.24,3.68	0.044	496	517	n.a.	
	5(6)-butyl	4.23	0.99	500	521	0.23	
	5-H	4.16	0.92	500	522	0.22	
	5-COOH	4.34	0.92	500	524	0.21	
	6-COOH	4.27	0.91	500	521	0.21	
	5-SCN	4.18	0.84	503	530	0.20	
	5(6)-SCN	4.32	0.87	504	530	0.20	
	5-NO₂	1.28,3.93	0.08	507	523	n.a.	
 <p style="text-align: center;">FLX₂</p>	X=H	4.16	0.92	500	522	0.22	
	X=Cl	4.72	0.96	512	535	0.20	
	X=Br	3.50	0.69	511	534	0.20	
	X=I	0.80	0.16	524	546	0.20	
	Y=H, X=Cl	4.03	0.86	511	532	0.21	
<p style="text-align: center;">FLX₂Y₂</p>	calcein	1.99	n.a.	507	535	n.a.	
	Y=Br, X=NO₂	3.02	0.32	522	546	0.10	
	<hr/>						
 <p style="text-align: center;">FLX₄</p>	X=H	4.16	0.92	500	522	0.22	
	X=Cl	4.84	0.96	522	540	0.19	
	X=Br	3.47	0.68	525	547	0.20	
	X=Br, Na→Et	3.56	0.72	535	557	0.20	
	X=I	0.70	0.14	527	551	0.20	
	<hr/>						
 <p style="text-align: center;">R₄FLX₄</p>	R=X=H	4.16	0.92	500	522	0.22	
	R=Cl, X=H	4.76	1.00	522	540	0.21	
	R=Cl, X=I	0.65	0.12	543	562	0.19	
	R=Br, X=Cl	4.19	0.94	548	570	0.22	
	R=H, X=Cl	4.84	0.97	522	540	0.20	

Table 1 (continued)

		Water/ 10 mM PBS					
		substitution	τ_f , ns	Φ_f	λ_{abs} , nm	λ_{em} , nm	k_f , $10^9 s^{-1}$
 <p style="text-align: center;">R-FL</p>	5-NH₂	0.22,2.39	0.016	486	519	n.a.	
	5(6)-butyl	4.10	0.85	488	514	0.21	
	5-H	4.11	0.85	489	515	0.21	
	5-COOH	4.10	0.75	491	520	0.18	
	6-COOH	4.15	0.75	490	516	0.18	
	5-SCN	4.08	0.76	494	522	0.19	
	5(6)-SCN	4.09	0.75	492	521	0.18	
	5-NO₂	1.69,4.08	0.030	495	517	n.a.	
 <p style="text-align: center;">FLX₂</p>	X=H	4.11	0.85	489	515	0.21	
	X=Cl	4.35	0.77	504	529	0.18	
	X=Br	1.93	0.36	501	526	0.19	
	X=I	0.3	0.058	518	542	0.19	
	Y=H, X=Cl	3.89	0.83	500	524	0.21	
 <p style="text-align: center;">FLX₂Y₂</p>	calcein	4.2	0.75	492	517	0.18	
	Y=Br, X=NO₂	1.16	0.15	515	539	0.13	
 <p style="text-align: center;">FLX₄</p>	X=H	4.11	0.85	489	515	0.21	
	X=Cl	4.49	1	508	530	0.23	
	X=Br	1.21	0.24	516	540	0.20	
	X=Br, Na→Et	1.39	0.27	520	545	0.19	
	X=I	0.24	0.047	521	544	0.20	
 <p style="text-align: center;">R₄FLX₄</p>	R=X=H	4.11	0.85	489	515	0.21	
	R=Cl, X=H	4.35	1	510	529	0.23	
	R=Cl, X=I	0.10	0.019	526	530	0.19	
	R=Br, X=Cl	1.23	0.25	537	561	0.20	
	R=H, X=Cl	4.49	1	508	530	0.23	

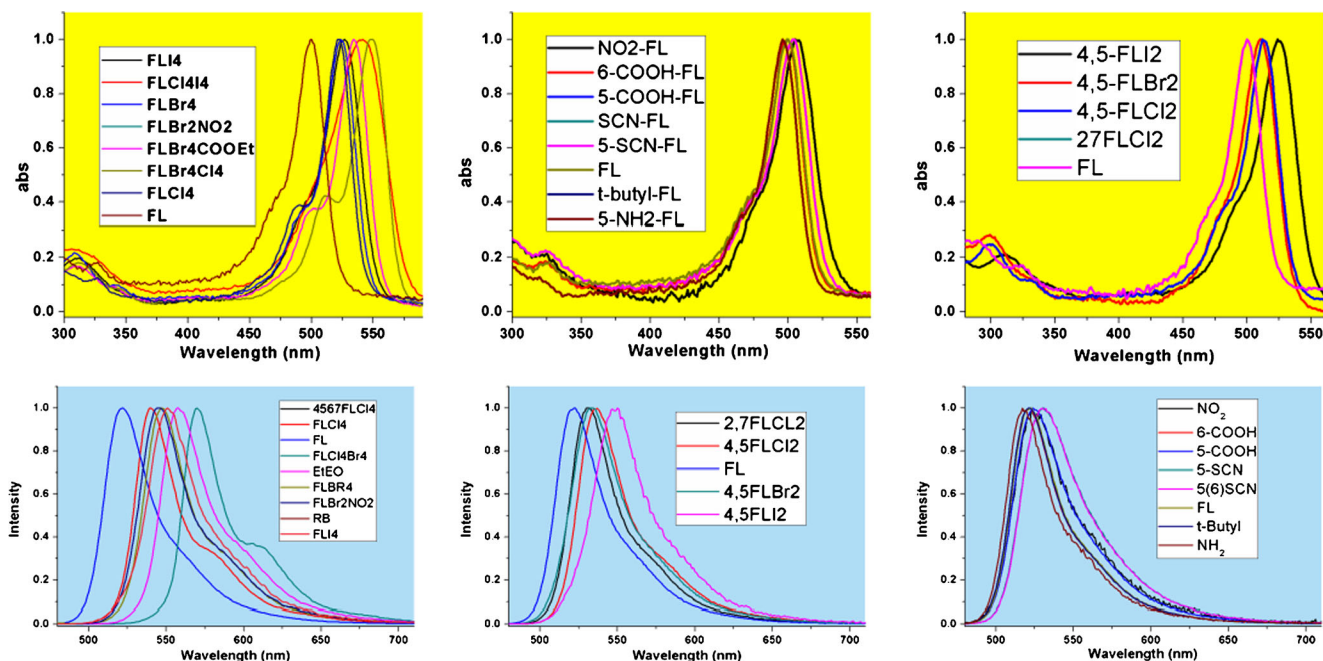


Fig. 2 Normalized absorption (*top*) and emission (*bottom*) spectra in ethanol containing 1 mM NaOH. The excitation wavelength was 470 nm for emission spectra

in the test range from 0.1 to 10 $\mu\text{mol/L}$. The lifetime value of a fluorescein dye was easily reproducible regardless of the change on emission wavelength and the dye concentration.

Results and Discussion

Fluorescein and its derivatives exist in solution in a number of ionic and neutral forms [23, 24], but we limit our attention to the dianion form, which is the most emissive and usable form in diverse areas. There are two independent π systems in these dyes: the phenyl and the xanthene rings. It is the xanthene π -ring that acts as the chromophore and fluorophore. The crystal structure showed that the two π -systems are mutually perpendicular and therefore independent of each other due to the very weak interaction between the two electron-clouds

[25]. In liquid solutions, however, the thermal rotation of the phenyl will lead to the deviation from the orthogonal position and hence the overlapping of the electron clouds of the two π -systems.

The chemical modification of anyone of the two π -systems can affect the fluorescence properties through different mechanisms. The dyes in this study can be grouped into five types (Fig. 1 and Table 1) according to the structural change on either the phenyl or the xanthene moiety: R-FL, FLX₂, FLX₂Y₂, FLX₄, and R₄FLX₄.

- 1) Dyes in which only the phenyl ring is mono substituted (R-FL, R=5-NO₂, 5-COOH, 6-COOH, 5-SCN, SCN, *t*-butyl, 5-NH₂). Such a dye could be a single isomer, such as 5-COOH-FL, or a mixture of two isomers, such as 5(6)-*t*-butyl-FL.

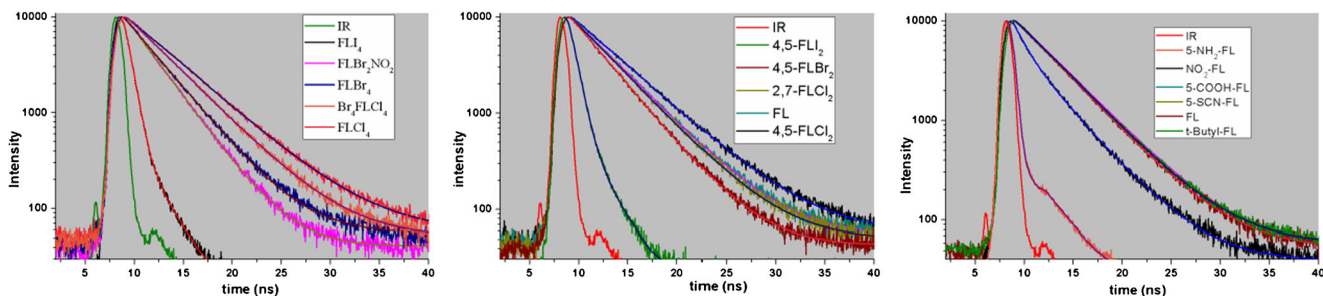


Fig. 3 Fluorescence decay curves, instrument response (IR), and curve fitting line of fluoresceins in EtOH containing 1 mM NaOH. Excitation: 379 nm laser (0.1 mW, 69 ps); Emission: 520 nm. Chi squared values are between 1.00 and 1.25

- 2) Compounds in which a xanthene ring is bi-substituted (FLX₂) at position (4,5) or (2,7), including 4,5-FLX₂ (X = Cl, Br, I, CH₂NCH₂COONa) and 2,7-FLCl₂.
- 3) Dyes in which a xanthene ring is tetra-substituted at position (2,4,5,7), including FLX₄ (X = Cl, Br, I), FLX₂Y₂ (X = Br, Y = NO₂). One exception is ethyl eosin, in which the COOH on the phenyl ring is changed to COOCH₂CH₃ compared to eosin.
- 4) Dyes in which the phenyl ring is tetra-substituted, while the xanthene may also be tetra-substituted: R₄FLX₄. This type compounds include Cl₄FL, Br₄FLCl₄, and Cl₄FLL₄ (RB).

Table 1 summarizes τ_f and Φ_f values in ethanol and PBS buffer found for each fluorescein dye, together with the corresponding emission and absorption maximum (λ_{em} and λ_{abs}).

Apparently the fluorescent properties are very dependent on the structural modifications. There has been no report which measured and compared the τ_f and Φ_f of so many fluorescein dyes under the same condition, although the data for fluorescein, eosin, erythrosine, and rose Bengal were reported in either alcohols or water previously by different authors [10, 12, 14, 15].

Figure 2 shows the absorption and emission spectra for these dyes. In comparison to fluorescein, all other derivative dyes still maintain the similar spectral shape but have different band positions. For R-FL, the substituent R is located on the phenyl rather than directly attached on the xanthene chromophore; therefore, it exhibits only a minor effect on the absorption or emission maxima. The presence of NH₂ and NO₂ on the phenyl, however, does change the band maxima. The electron-donating group, 5-NH₂, leads to the blue shift of both absorption and emission maxima; the electron-withdrawing group, 5-NO₂, causes an opposite consequence. This effect is obviously contrary to the usual substitution effect in organic chemistry. Note that the xanthene ring bearing an ONa is negatively charged. The presence of 5-amino also makes the phenyl more negatively charged, so that an electric repulsion force exists between the two π -systems which destabilizes the xanthene and increases the energy gap of HOMO to LUMO (blue shift). On the other hand, 5-nitro renders the positive charge of the phenyl and an electric attraction to the xanthene, which stabilizes the xanthene and decreases the energy gap of HOMO to LUMO (red shift).

For FLX₂ and FLX₄, the increase in both the number and size of the atom X makes the red shift larger, which is consistent with the normal substitution effect in organic chemistry, since an X atom is directly attached to the xanthene chromophore.

Unexpectedly, the largest red shift occurs in Br₄FLCl₄ (48 nm) and Cl₄FLL₄ (43 nm) in which both the phenyl and the xanthene are fully halogenated. This is likely due to the electronic push-pull effect that strengthens the electronic overlapping between the two π -systems and lowers the energy of

the S₁ excited state. In Cl₄FLL₄, the iodine atoms make the xanthene more electron rich while the chlorine atoms cause the phenyl much more electron poor, leading to the electron push-pull effect.

The time profiles of the fluorescence decays after the laser excitation (69 ps) are shown in Fig. 3. Monoexponential function can be well fit to the experimental curves with the chi-squared values within 1.00 to 1.25, except 5-amino- and 5-nitro-fluorescein for which the biexponential fitting are needed. For fluorescein, eosin, erythrosine, and rose Bengal, the measured values for τ_f and Φ_f are consistent with that reported [10, 12, 14, 15].

τ_f varied with the change of chemical structure, as did Φ_f which follows the same trend. The bromination or iodination do cause the decrease of both τ_f and Φ_f . The more the bromine or iodine atoms, the less the value of both τ_f and Φ_f is. This is consistent with the expected heavy atom effect.

For all the halogenated dyes, however, chlorination on both the phenyl or xanthene ring increased the value of both τ_f and Φ_f . This result is different from the general heavy atom effect on photophysics in organic photochemistry. Chlorination also increases the oxidation potential of the attached chromophore, in addition to the heavy atom effect [7, 16]. It was suggested that intramolecular photoinduced electron transfer (PET) was also involved in the deactivation of the lowest excited singlet state (S₁) of the halogenated fluorescein dyes [7, 16], so $\tau_f = (k_{ic} + k_f + k_{isc} + k_{pet})^{-1}$. k_{ic} , k_f , k_{isc} , and k_{pet} are the rate constant of internal conversion, fluorescence, intersystem crossing, and PET, respectively.

The increase of the oxidation potential by chlorination makes ΔG of PET more positive, so that PET does not or hardly contribute to the deactivation of S₁ state of xanthene moiety; the smaller k_{pet} results in the increase of both τ_f and Φ_f . The bromination or iodination, however, decreases the oxidation potential of the attached chromophore, so that PET contributes more to the deactivation of S₁ state, resulting in the decrease of both τ_f and Φ_f , in addition to the heavy atom effect.

Among R-FL dyes, the biexponential decay of 5-amino- and 5-nitro-fluorescein indicates their different photophysical behavior from that of other fluoresceins. They lead to a much larger decrease in both τ_f and Φ_f , whereas *t*-butyl, SCN, and COOH showed only a slight effect.

The amino substitution makes the phenyl a strong electron donor (D), which renders the occurrence of PET from D to the xanthene ring [16]. In the case of nitro substitution, the attached phenyl becomes a strong electron acceptor (A) so that PET occurs from the xanthene to A [16]. Therefore an additional decay process, i.e. PET, exists in the deactivation of the S₁ excited state for the two dyes, which strongly quenches the fluorescence emission and leads to the decreased value of both Φ_f and τ_f .

The rate constant of PET (k_{pet}) depends on the electron overlapping between D and A. The rotation of the phenyl in liquids changes the relative orientation between the xanthenone and the phenyl, so that it changes the electron overlapping between D and A. Therefore k_{pet} varies with the rotational motion, since $\tau_f = (k_{\text{ic}} + k_f + k_{\text{isc}} + k_{\text{pet}})^{-1}$, so τ_f is not a constant which makes the associated fluorescence decay non monoexponential.

The solvent water shows a remarkable effect, which causes the decrease of both Φ_f and τ_f for all the dyes. This result is consistent with the previous reports for fluorescein, eosin, and erythrosine measured in a number of solvents. The solvent effect is due to the differential solvation of the S_1 and T_1 states, which results in a decreased S_1 – T_1 energy gap ΔE_{ST} and hence more efficient intersystem crossing in the more polar solvents [14].

The esterification of COOH in the phenyl of eosin also has effect on the fluorescence properties. Examining the data for ethyl eosin and eosin Y, it shows that the esterification increased the value of τ_f and Φ_f and enhanced the red shift in λ_{abs} and λ_{em} .

Although Φ_f and τ_f are influenced by the chemical structure of a fluorescein dye and the solvent used, their ratio (Φ_f/τ_f) is a constant and independent of both a dye structure and a solvent used. This ratio stands for the rate constant of radiation process ($k_f = \Phi_f/\tau_f$). The average k_f value for all fluorescein dyes is 0.20×10^9 , and $0.20 \times 10^9 \text{ s}^{-1}$ for ethanol, PBS respectively.

The rate constant of nonradiative process (k_{nr}) is calculated by $k_{\text{nr}} = (1 - \Phi_f)/\tau_f$. In contrast to k_f , the k_{nr} value is varied with both the structure and the solvent.

Conclusions

We have measured the fluorescence lifetimes, quantum yields and emission maxima of 20 fluorescein dyes under the same conditions in ethanol and PBS buffer. Based on the data we have discussed the mechanism that correlates the structures and their fluorescence properties. Both Φ_f and τ_f are dependent on the chemical modifications. The rate constant of radiation process (Φ_f/τ_f), however, shows no dependence on either the chemical structure or solvents but a constant value ($0.20 \times 10^9 \text{ s}^{-1}$). The rate constant of nonradiation process, on the other hand, is varied with both the structure and the solvent used. The results support the occurrence of intramolecular PET in fluorescein derivatives, in addition to the heavy atom effect in brominated and iodinated fluoresceins.

In this study we have provided a set of consistent fluorescence data for the widely used fluorescein dyes, some of them have not been reported before. We also advance the mechanism study on the fluorescence of fluorescein dyes. The consistent fluorescence data and related emission mechanism are useful for those who want to choose the fluorescent material in

different applications. It also provides the basis for comparing the quantum chemical computation with the experimental values.

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