International Journal of Advanced Chemistry, 8 (1) (2020) 15-21

International Journal of Advanced Chemistry

Website: www.sciencepubco.com/index.php/IJAC

Research paper

Determination of fluorescence lifetimes of fluorescein from fluorescence quenching data

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Abstract

The fluorescence quenching of fluorescein by 1,4-benzoquinone (BQ) in five solvents – methanol, ethanol, phosphate buffer saline (PBS, pH 7.4), *N*,*N'*-dimethylformamide (DMF) and dimethylsulphoxide (DMSO) is hereby investigated. Fluorescein's fluorescence was effectively quenched by BQ, and the quenching was dynamic (purely collisional) within the BQ's concentration range (0.001 to 0.004 M) used in this work. Accordingly, the quenching data were in conformity with the Stern-Volmer's model. Stern-Volmer's constant (K_{SV}) values range between 17.4 in DMSO and 43.4 in methanol. K_{SV} values, together with the calculated bimolecular rate constants (k_D) in the respective solvents, were used to semi-empirically estimate the fluorescence lifetimes (τ_F) of fluorescein in the individual solvents. Just as for K_{SV} values, τ_F values are solvent-viscosity dependent, with the lowest values being obtained in methanol and the highest in DMSO, which are the least and most viscous solvents respectively. τ_F values obtained in this work are 3.55, 3.71, 3.78, 4.13 and 4.51 ns (in methanol, PBS 7.4, ethanol, DMF and DMSO, respectively).

Keywords: *Fluorescein; Benzoquinone; Fluorescence; Quenching; Lifetime.*

1. Introduction

Fluorescein, a yellowish-green emitting dye, possesses many attractive photophysicochemical properties (e.g., high molar absorptivity, high fluorescence quantum yield, and high photostability); in addition, it is water-soluble, biocompatible (Ha et al. 2009, Veres et al. 2017) and cheap. These attributes bestow on it considerable versatility, and make it a compound of choice for many industrial applications and in academia. Fluorescein has found application in the production of advanced molecular probes for biological, toxicological, biomedical, and environmental studies (Burchak et al. 2006, Oliveira et al. 2018, Xiong et al. 2014, Zhwng et al. 2013). Fluorescein has been used as a lasing medium (green, 530–560 nm) in laser dyes (Al-Aqmar et al. 2015, Govindanunny & Sivaram 1980). It is desirable for a dye laser gain medium to possess an appreciably long fluorescence lifetime, and as such, a thorough knowledge of its photophysical properties is worthwhile. Fluorescence lifetime refers to the average time a fluorophore molecule stays in the excited state before emitting a photon and returning to the ground state. Fluorescence lifetime is also an essential consideration for other practical applications of fluorescence such as Fluorescence Resonance Energy Transfer (FRET) and fluorescence lifetime assays, sensing, imaging and imaging microscopy [He et al. 2003, Al et al. 2003, Jose et al. 2007, Becker 2012, Ma et al. 2004); this has necessitated the development of techniques for the estimation of fluorescence lifetime. Fluorescence lifetime is an intrinsic property of a fluorophore, and can be measured in either the frequency domain or the time domain. While both approaches have different instrumentation and data acquisition procedures, they are mathematically equivalent and their data can be interconverted through Fourier transform. The most familiar technique for fluorescence lifetime measurement is time-correlated single photon counting (TCSPC), which simplifies data collection and enhanced quantitative photon counting. A relatively unfamiliar but cheaper and simpler method of fluorescence lifetime determination is hereby presented; this method is based on the diffusion-controlled collisional interaction between the fluorophore and quencher molecules, and data treatment via the Stern-Volmer model (Lakowicz 1999). In this work, 1, 4-benzoquinone is used as a quencher for the quenching of fluorescein's fluorescence; the data treatment ultimately leads to the estimation of the fluorescence lifetimes of fluorescein (Fig. 1) in different solvents.

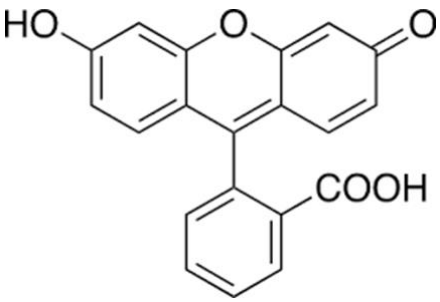


Fig. 1: Molecular Structure of Fluorescein.

2. Experimental

2.1. Materials and equipment

Fluorescein was purchased from Sigma-Aldrich. N,N′-dimethylformamide (DMF), dimethylsulphoxide (DMSO), methanol, ethanol and ether (diethylether) were obtained from SAARCHEM; water was freshly distilled. Chlorophyll a and benzoquinone (BQ) were purchased from Aldrich.
UV-Vis absorption spectra were recorded on a Varian 500 UV/visible/NIR spectrophotometer, and fluorescence spectra on a Varian Eclipse spectrofluoremeter.

2.2. Fluorescence quantum yields

Fluorescence quantum yields (Φ_F), were determined using the comparative method, as described before (Fery-Forgues and Lavabre 1999, Maree et al. 2002, Ogunsipe & Nyokong 2005a), Eq. 1, using chlorophyll a in ether ($\Phi_F = 0.32$ (Montalban 1999), as the reference.

$$\Phi_F = \Phi_F\,(\mathrm{Std})\frac{F_x\,.\,A_{Std}.\,n_x^2}{F_{Std}.\,A\,.\,n_{Std}^2} \quad (1)$$

F_x and F_{std} are the areas under the emission curves of the sample and standard, respectively. A_x and A_{std} are the absorbances of the sample and standard, respectively, and n_x and n_{Std} are the refractive indices of the solvents used for sample and standard, respectively. Both the sample and reference were excited at the same wavelength. The absorbance of the solutions at the excitation wavelength ranged between 0.04 and 0.05.

2.3. Fluorescence quenching by benzoquinone

Fluorescence quenching experiments on fluorescein in methanol, PBS 7.4, ethanol, DMF and DMSO were carried out by the addition of varying (increasing) concentrations of BQ to fluorescein (fixed concentration), and the concentrations of BQ in the resulting mixtures were 0, 0.001, 0.002, 0.003 and 0.004 M. The fluorescence spectra of fluorescein at each BQ concentration were recorded, and the changes in fluorescence intensity at various BQ concentrations were related by the Stern-Volmer (S-V) equation (Eq. 2):

$$\frac{I_0}{I} = 1 + K_{SV}\,[BQ] \quad (2)$$

Where I_0 and I are the fluorescence intensities of fluorescein in the absence and presence of BQ respectively; [BQ], the concentration of the BQ, and K_{SV}, the Stern-Volmer constant; which is the product of the bimoleculear quenching constant (k_Q) and the fluorescence life-time τ_F (Eq. 3):

$$K_{SV} = k_Q.\tau_F \quad (3)$$

The ratios $\frac{I_0}{I}$ were calculated and plotted against [BQ] according to Eq. 2, and K_{SV} determined from the slope.
The bimolecular rate constant for diffusion-controlled reactions (k_D) is related to the apparent bimolecular quenching constant (k_Q) by Eq. 4 (Maree et al. 2002):

$$k_Q = f.k_D \quad (4)$$

Where f is the quenching efficiency.
The bimolecular rate constant (k_D) can be obtained from the Einsten-Smoluchowski relationship (Eq. 5):

$$k_D = \frac{4\pi\,N_A(D_{Flrc} + D_{BQ})(r_{Flrc} + r_{BQ})}{1000} \quad (5)$$

Where N_A is the Avogadro's number; D_{Flrc} and D_{BQ}, the diffusion coefficients of the fluorophore and quencher respectively, while r_{Flrc} and r_{BQ} are the molecular radii of fluorescein and BQ respectively.
The diffusion coefficient D is given by the Stokes' equation (Eq. 6).

$$D = \frac{kT}{6\pi\eta r} \quad (6)$$

Where k is the Boltzmann constant; T, the absolute temperature; η, the solvent's viscosity and r, the fluorescein or BQ radius.
R_{Flrc} and r_{BQ} is assumed to be equal to the respective molecule's Onsager cavity radius (a), which is obtained from molecular volume, as given by Suppan's equation:

$$a = \sqrt[3]{\frac{3M}{4\pi\rho N}} \quad (7)$$

M is the molecular weight of fluorescein (332.31 g mol^{-1}) or BQ (108.10 g mol^{-1}); ρ, the density (1.602 g cm^{-3} for fluorescein and 1.32 g cm^{-3} BQ); and N, the Avogadro's number.
k_Q values may be determined from Eq. 4 using the calculated k_D value, provided that f is known. From the values of k_Q, the values of τ_F can then be calculated using Eq. 3.

3. Results and discussion

3.1. Fluorescein structure

Fluorescein contains the hydroxy and the carboxy groups (Fig. 1), which are active sites that can link electron donors and acceptors to form multicomponent compounds (Zhang et al 1997), hence the multi-functionability of the molecule. The linking of aromatic ring with oxygen makes fluorescein rather rigid, and this has desirable effects on its photophysical properties. For example, fluorescein is known as one of the most fluorescent organic fluorophores.

3.2. UV-Visible and fluorescence spectra

The ground state electronic absorption spectra of fluorescein in methanol, water and DMSO is shown in Fig. 2. The absorption bands arise from π-π* transitions and can be explained in terms of linear combination of HOMO-LUMO transitions. There are multiple absorption bands in the spectra, which imply the occurrence of numerous non-degenerate electronic transitions upon UV-Visible irradiation.

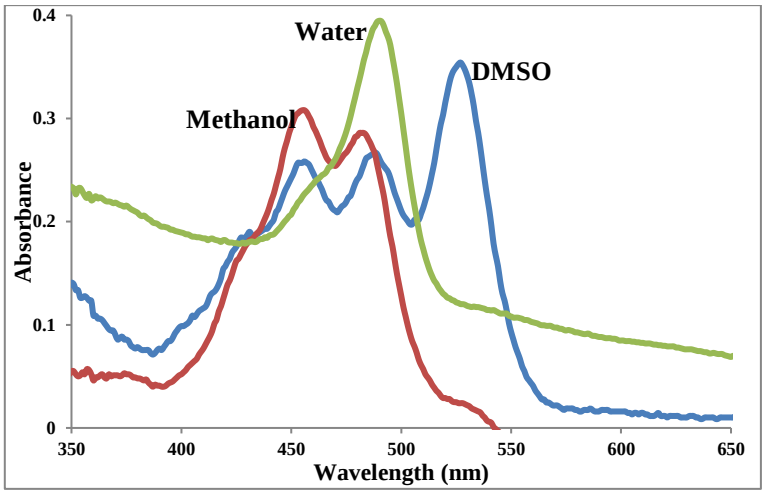


Fig. 2: Absorption Spectra of Fluorescein in Methanol, Water and DMSO.

The UV-vis spectrum of fluorescein has intense bands in the 450 – 550 nm spectral expanses, with extinction coefficients greater than 10^4 L mol^{-1} cm^{-1}, accompanied by a series of vibrational bands. Fig. 3 (and Table 1) shows the absorption and emission spectra (normalized and overlaid) of fluorescein in DMSO. These spectra are typical of xanthene derivatives.

Table 1: Spectral and Fluorescence Data for Fluorescein in Various Solvents

	n	λ_Q (Abs) /nm (Log ε)	λ_Q (Ems) /nm	E_S /eV	Φ_F
Methanol	1.326	478 (72406) 452 (77790)	520	2.39	0.90
PBS 7.4	1.332	488 (77500)	525	2.37	0.91
Ethanol	1.362	483 (92300) 453 (85847)	518 537	2.40 2.31	0.91
DMF	1.430	490 (93000) 525 (95700)	515	2.41	0.93
DMSO	1.633	487 (73825) 453 (70271)	545	2.28	0.95

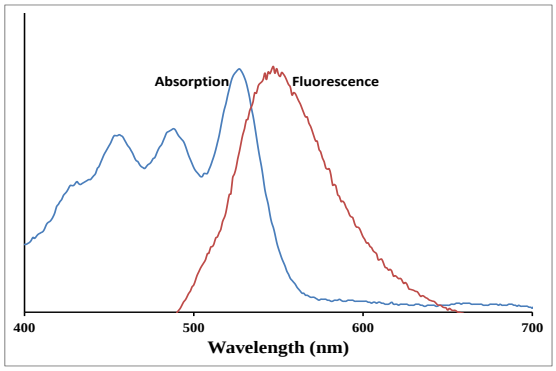


Fig. 3: Normalized Absorption and Fluorescence Spectra of Fluorescein in DMSO.

3.3. Fluorescein fluorescence quenching by benzoquinone (BQ)

The effect of benzoquinone (BQ) on the fluorescence of fluorescein in water in demonstrated in Fig. 4 (A similar trend was observed in other solvents used in this work). The tendency of BQ to quench the fluorescence of organic macrocycles is well documented in the literature (Ogunsipe & Nyokong 2005b, Ogunsipe & Nyokong 2011, Ogunsipe 2018, Gazdaru 2001). The sustained reduction in fluorescein's fluorescence intensity with increase in BQ concentration is an attestation to the fluorescence quenching ability of BQ on xanthene derivatives. A plot of I_0/I versus [BQ] (Eq. 2) is linear (Fig. 4 inset), which implies an exclusively dynamic quenching. There was no alteration to the absorption spectra upon addition of BQ; hence the existence of static quenching is ruled out.

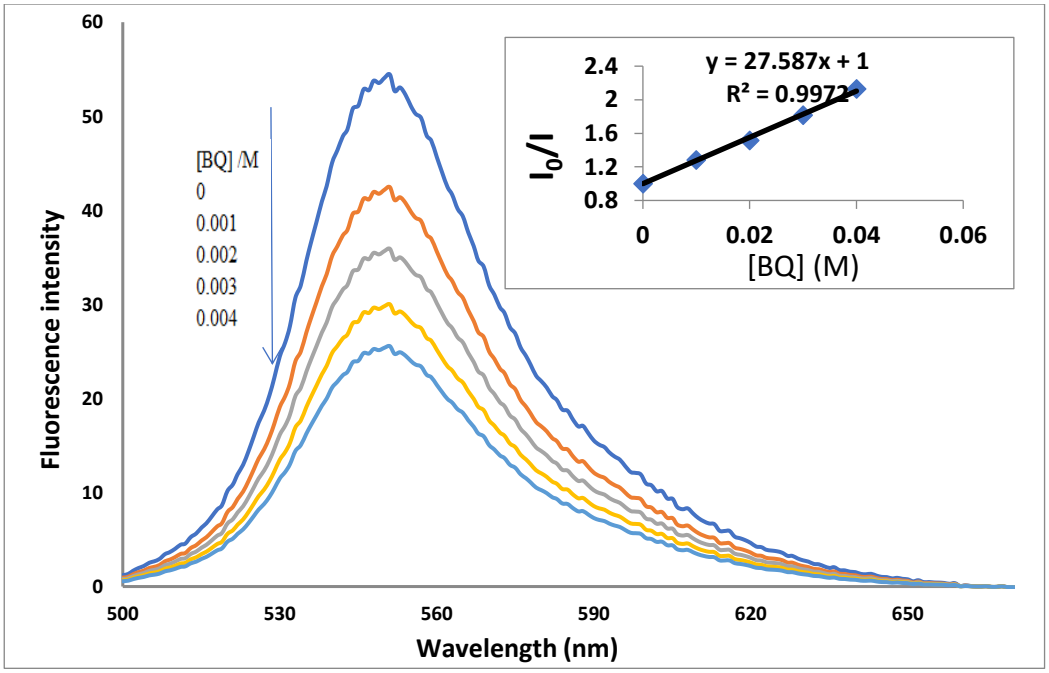


Fig. 4: Fluorescein Fluorescence Quenching by Benzoquinone in Water (Inset: Stern-Volmer Plot for the Quenching Process).

In effect, the operational quenching mechanism involves a diffusion-controlled collisional interaction between fluorescein and the BQ molecules; and under this situation, f, the quenching efficiency (Eq. 4) is assumed to be ≅ 1. Diffusion coefficients of fluorescein (D_{Flrc}) and BQ (D_{BQ}) (Table 2), in the various solvents were calculated using Eq. 6; and these were in turn used to calculate the diffusion-controlled bimolecular rate constant (k_d) for the quenching of fluorescein by BQ. The Stern-Volmer quenching constant (K_{SV}, Table 2), determined as the slope of the plot in Fig. 4, ranged between 17.4 M^{-1} (in DMSO) and 43.4 M^{-1} (in methanol).

Table 2: Data for Fluorescein Fluorescence Quenching by Benzoquinone in Various Solvents

	η /10^{-4} kg m^{-1}	D_{Flrc} /10^{-10} m^2 s^{-1}	D_{BQ} /10^{-10} m^2 s^{-1}	$^{\dagger}k_d$ /10^9 M^{-1} s^{-1}	K_{SV} /M^{-1}	τ_F /ns	Φ_F
Methanol	5.43	9.23	10.3	12.2	43.4	3.55	0.90
PBS 7.4	8.90	5.63	6.28	7.45	27.6	3.71	0.91
Ethanol	10.74	4.67	5.22	6.18	23.4	3.78	0.91
DMF	7.80	6.42	7.16	8.56	35.3	4.13	0.93
DMSO	19.9	2.52	2.81	3.33	17.4	4.51	0.95

†It is assumed that f (quenching efficiency) unity; in which case $k_d = k_Q$ (Eq. 4).

There exists a correlation between the Stern-Volmer quenching constant (K_{SV}) and solvent viscosity; the more viscous the solvent, the lower the K_{SV} value. Fig. 5 demonstrates the dependence of K_{SV} values on solvent viscosity.

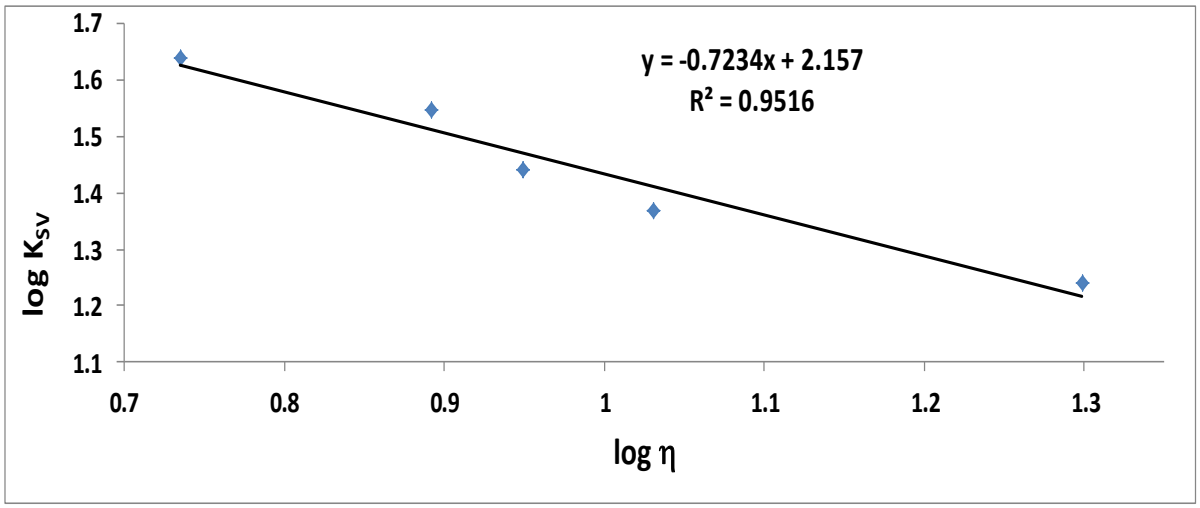


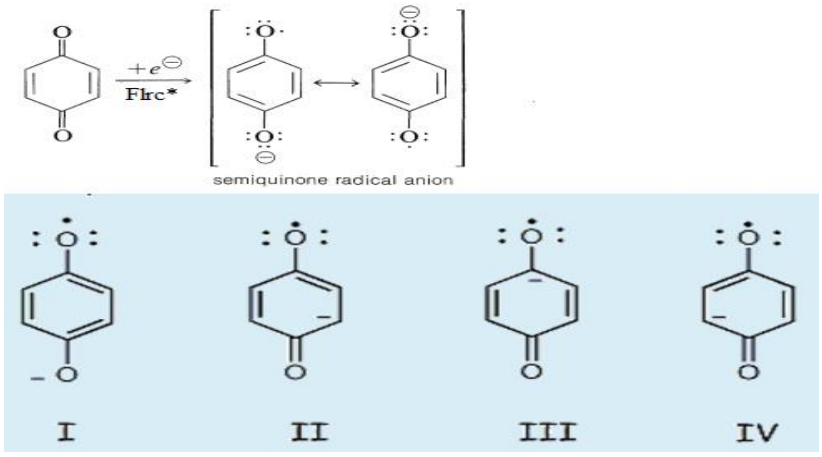
Fig. 5: Dependence of Stern-Volmer's Quenching Constant on Solvent Viscosity.

In DMSO, the motion of both the fluorophore (fluorescein) and quencher (BQ) are slowed down, thereby hindering the frequency of their collision. We propose a phenomenological relation for the variation of K_{SV} with solvent viscosity (Eq. 8a, b).

$$\text{Log } K_{SV} = k + \text{Log } \eta. \quad (8a)$$

$$\text{Log } K_{SV} = 2.157 - 0.7234\text{Log } \eta. \quad (8b)$$

The mechanism of fluorescence quenching could be via energy transfer (from the excited fluorescein to BQ) or charge transfer (between excited fluorescein and BQ). The highest energy excited singlet state energy of fluorescein in Table 1 is 2.41 eV, which is even lower than the lowest excited state $^1(n, \pi^*)$ energy in BQ (2.6 eV) (Losev et al. 1999). It follows that the Flrc* → BQ energy transfer is 'uphill' and therefore not practicable; and one can safely conclude that the quenching process is bimolecular photo-induced electron transfer (BPET) from the excited singlet state of fluorescein to BQ. An electronically excited fluorescein molecule has a great propensity to give away an electron nearby BQ molecules, and also to replace the removed electron to its original position in the molecule (Rabnowich 1945). As opined before, the quenching occurred via an ion-radical pair intermediate (Ogunsipe & Nyokong 2011). The thrust behind the oxidizing ability of BQ is its high electron affinity (1.89 eV (Cooper et al. 1975)) in addition to the development of a resonance-stabilized aromatic system of the semiquinone anion radical (Scheme 1).



Scheme 1: Flrc*→BQ Electron Transfer and Resonance Structures of the Resulting Semiquinone Anion Radical.

The high electron affinity and stability of the resonance structures account for the formation of charge-transfer complexes between BQ and electron donors (Laird 1979).

The transferred electron can go back to fluorescein via a process known as back electron transfer (BET), which is an 'energy-wasting' process as it regenerates the fluorescein and BQ molecules in their ground states (Scheme 2).

$$\text{Flrc} \xrightarrow{h\nu} \text{Flrc}^*$$

$$\text{Flrc}^* + \text{BQ} \xrightarrow{\text{ET}} \text{Flrc}^{+\cdot} \ldots\ldots \text{BQ}^{\cdot -} \text{ (Charge separation)}$$

$$\text{Flrc}^{+\cdot} \ldots\ldots \text{BQ}^{\cdot -} \xrightarrow{\text{BET}} \text{Flrc} + \text{BQ}$$

Scheme 2: Fluorescence quenching of fluorescein via charge transfer to benzoquinone (ET = electron transfer; BET = Back electron transfer.

3.4. Fluorescence lifetimes and quantum yields

Values of Fluorescence lifetimes (τ_F) and quantum yields (ϕ_F) for fluorescein in the five solvents are listed in Table 2. τ_F values were estimated according to using K_{SV} and k_Q, Eq. 3. A trend of increasing τ_F and ϕ_F with increasing solvent viscosity is clear from the table (though not much pronounced in ϕ_F values) . A highly viscous solvent would impede translational and rotational motions in the fluorecein molecules, and may also inhibit the deformation of its structure through viscous damping by the solvent (Ogunsipe & Nyokong 2011, Georghiou & Stacy Gerke 1999). This ultimately results is a lowering of the excited singlet state decay rate constant (k_S). In low-viscosity solvent however, free rotational diffusions about σ-bonds (the benzoic acid addendum about a C-C bond, hydroxyl group about a C-O bond, and hydrogen atom about an O-H bond) result in a new equilibrium state where the rate of internal conversion will undoubtedly be high and the excited molecule fast deactivated. The fluorescence lifetimes determined in this work might be expected to be shorter than those obtained by direct instrumentation. For example in this work, the fluorescence lifetime of fluorescein in PBS 7.4 is 3.71 ns, which is shorter than a literature value of 4.0 ns (Magde et al. 1999). This discrepancy could be attributed to the crucial assumption made in this work, that the quenching efficiency in Eq. 4 is unity; in which case the bimolecular rate constant for diffusion-controlled reactions (k_D) is assumed to be equal to the apparent bimolecular quenching constant (k_Q). It is very unlikely that k_D will be equal to k_Q (it is expected that k_Q should be less than k_D (in which case f is less than 1).

4. Conclusion

The fluorescence lifetimes of fluorescein in different solvents have been estimated via a relatively unfamiliar procedure, which involves the application of fluorescence quenching data. Fluorescein's fluorescence was effectively quenched by benzoquinone within the latter's concentration range of 0.001 – 0.004 M. The quenching process is dynamic (diffusion-controlled collisional interaction between the fluorophore and quencher) as judged by the linearity of the Stern-Volmer's plot of the quenching data. Fluorescein's fluorescence quenching by 1,4-benzoquinone is believed to have been via charge transfer, and not energy transfer, since the latter is 'uphill' and therefore not plausible. Fluorescence lifetimes exhibit a well-pronounced dependence on solvent viscosity, with the longest lifetime being obtained in DMSO, the most viscous of the solvents used in this work. However, fluorescence quantum yield values only exhibit a slight dependence on solvent viscosity.

Acknowledgement

Thanks to Professor Tebello Nyokong of Rhodes University for the use of her laboratory.

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