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Fluorescence quenching of fluorescein with molecular oxygen in solution

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Abstract

The fluorescence quenching of fluorescein (FL) with molecular oxygen in alkali solution and in different solvents was investigated by steady-state absorption and fluorescence spectroscopy techniques. Fluorescence quantum yield (Φ_f), radiative rate constant (k_r), fluorescence lifetime (τ_f), and non-radiative rate constant (k_{nr}) values were determined. Stern–Volmer equation was employed to calculate bimolecular quenching rate constants (k_q) and, then, Smoluchowski equation was used for the determination of diffusion-controlled rate constants (k_{diff}) to compare with the experimental k_q values. The temperature, quencher concentration, and solvent viscosity were experimental parameters to understand fluorescence quenching process of fluorescein with molecular oxygen in solution. Experimental findings indicated that the mechanism of fluorescence quenching is diffusive in nature.
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1. Introduction

Fluorescein (FL) that is a highly fluorescent molecule and a type of xanthene dyes was firstly synthesized by Von Bayer in 1871 [1]. Fluorescein is widely used as a photosensitizer compound for the chemical reactions and non-linear optical media (this property of FL has been observed in boric acid glass thin films and gelatin) [2], as a quantum counter in dye lasers [3], and in the conversion and storage of solar energy [4–6]. Another use of FL is to label proteins in fluorescence microscopy [7]. All these applications require that how much of fluorescence intensity of FL is affected by the solvating environment. Therefore, the basic understanding of photophysical features of these dyes depending on solvent properties, for instance; solvent viscosity, solvent polarity, and pH of media etc., is very important for the use of these dyes in various applications [8].

Fluorescence quenching has been widely studied in both as a fundamental phenomenon and in the application of fluo-

rescence to biochemical problems [7]. Quenching process is frequently used in protein and membrane systems to examine their structural and functional properties. Molecular oxygen is an important quencher for many fluorophores because of its high solubility in aqueous solutions and organic solvents [9–12]. Fluorescence quenching by molecular oxygen is usually a diffusion-controlled mechanism as mentioned by previous studies in the literature [10,13–16].

In general, the mechanism of fluorescence quenching can be followed by either dynamic or static but in some cases both. The dynamic quenching mechanism results from diffusive encounters between fluorophore and quencher during the lifetime of the excited state. This type of fluorescence quenching is described by Stern–Volmer equation [7]:

$$\frac{I_o}{I} = 1 + \tau_o k_q [Q] \tag{1}$$

where I_o and I are the fluorescence intensities in the absence and presence of quencher, respectively, τ_o is the fluorescence lifetime of the fluorophore in the absence of quencher, k_q is the bimolecular quenching rate constant and $[Q]$ is the molar concentration of quencher. If k_q occurred through a diffusion-

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controlled path, theoretically diffusion-controlled bimolecular rate constants must be calculated as a function of temperature by using the Smoluchowski equation and then theoretical values of bimolecular rate constants can be compared with experimental k_q values. The Smoluchowski equation is given as:

$$k_{diff} = \frac{4\pi RND}{1000} \tag{2}$$

where R is the collision radius which is generally assumed to be the sum of the molecular radii of the fluorophore and quencher, N is the Avogadro's number and D is the sum of the diffusion coefficients of the fluorophore and quencher [7]. Diffusion coefficients can be obtained from the Stokes–Einstein equation:

$$D = \frac{kT}{6\pi\eta R} \tag{3}$$

where k is the Boltzmann constant and η is the solvent viscosity. Frequently, the Stokes–Einstein equation underestimates the diffusion coefficients of small molecules like molecular oxygen. Therefore, the nomograms [17] must be used based upon the thermodynamic and physical properties of the system to calculate the diffusion coefficients. In addition, the viscosity dependence of k_q for diffusion-controlled bimolecular reaction is described by the following equation [18]:

$$k_q = A\eta^{-\alpha} \tag{4}$$

where the parameters A and α are assumed to be constant that are invariant with viscosity. Values of α ranging from zero to unity have been reported for a number of bimolecular reactions [19].

The other quenching mechanism is the static quenching. In this case, fluorescence quenching occurs as a result of the formation of a nonfluorescent ground state complex between fluorophore and quencher [7]. This kind of quenching causes sharp changes in the absorption spectra of the fluorophore. Therefore, Stern–Volmer equation and absorption spectra studies would be helpful to examine which quenching mechanism is valid for the system of interest.

There is no work done in the literature related with quenching mechanism of FL with molecular oxygen in solution. Therefore, this study covers fluorescence quenching of FL in 0.1N NaOH aqueous solutions and in different solvent systems by molecular oxygen. The main goal of this study is to see which mechanism explains the fluorescence quenching of FL with oxygen in solution. To shed a light on this matter, the quencher concentration, the temperature and solvent viscosity were chosen as experimental parameters and interpretations were done accordingly.

2. Experimental

Fluorescein and NaOH were purchased from Sigma. Acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), *n*-propanol (PrOH), and *n*-buthanol (BuOH) solvents were available from Merck and were in spectroscopic grade. FL was stored in the dark as a concentrated stock solution of 5.0×10^{-3} M in MeOH. For all measurements, Fluorescein of 1.0×10^{-6} M was prepared from stock solution to avoid self-absorption effect. Absorption spectra of the samples were recorded with Shimadzu UV-3101PC UV-VIS-NIR spectrophotometer. Fluorescence spectra were taken with Shimadzu RF-5301PC spectrofluorophotometer by using a cuvette of 1.0 cm optical path length for the dye solutions and then these spectra were converted to the corrected fluorescence spectra. For this purpose, rhodamine B in ethylene glycol was employed to observe corrected fluorescence spectra for the machine response according to the method reported earlier [7]. The temperature of the samples was controlled by using Grant W14 circulating water bath during the absorption and fluorescence spectra measurements.

Fluorescence quantum yields were determined by comparing the spectrally corrected emission intensity of the sample to that of a fluorescence standard using the following equation [3]:

$$\Phi_S = \Phi_R \left(\frac{D_S}{D_R}\right) \left(\frac{n_S^2}{n_R^2}\right) \left(\frac{1 - 10^{-OD_R}}{1 - 10^{-OD_S}}\right) \tag{5}$$

where D_S and D_R are the integrated area under the corrected fluorescence spectra for the sample and reference, n_S and n_R are the refractive indexes of the sample and reference, respectively. OD_S and OD_R are the optical densities for the sample and reference at the excitation wavelength, respectively. The quantum yield reference used for this study is the rhodamine 101 of 1.0×10^{-6} M in ethanol, which has a known fluorescence quantum yield of 1.00 [6,20].

Fluorescence lifetime values of the dye samples were obtained after using Strickler–Berg equation [21]. This equation is a modification of Einstein's fundamental relationship between the probability of absorption and emission, which is applicable to polyatomic molecules in solution. Through this equation, k_r can be obtained from the absorption and fluorescence spectra by using equation:

$$k_r = 2.88 \times 10^{-9} n^2 \langle \bar{\upsilon}_f^{-3} \rangle_{Av}^{-1} (g_l/g_u) \int \frac{\varepsilon_a(\bar{\upsilon}_a) d\bar{\upsilon}_a}{\bar{\upsilon}_a} \tag{6}$$

where ε_a (in units of $M^{-1} cm^{-1}$) is the decadic molar absorption coefficient at wavenumber $\bar{\upsilon}_a$ (in units of cm^{-1}), n is the refractive index of the solution, g_l and g_u are the electronic degeneracies of the lower and upper electronic states, and the term in the angular brackets is the weighted wavenumber function that contains fluorescence intensity. Then, fluorescence lifetime values (τ_f) were calculated by using the photophysical relationship between fluorescence lifetime and k_r

Table 1
Some photophysical parameters of fluorescein in 0.1N NaOH aqueous solutions at different temperatures

Temperature (°C)	Φ_f	τ_f (ns)[a]	k_{nr} (s^{-1}/10^7)	k_r (s^{-1}/10^9)
5	0.98 ± 0.01	3.80	0.52 ± 0.05	0.26 ± 0.02
15	0.93 ± 0.01	3.70	1.92 ± 0.07	0.25 ± 0.03
25	0.88 ± 0.02	3.60	3.38 ± 0.05	0.24 ± 0.02
35	0.85 ± 0.03	3.55	4.30 ± 0.05	0.24 ± 0.02
50	0.74 ± 0.03	3.20	8.25 ± 0.06	0.23 ± 0.03

[a] Uncertainty: ±10%.

values given as [22]:

$$\tau_f = \frac{\Phi_f}{k_r} \qquad (7)$$

The refraction indexes were determined by using a refractometer. The solvent viscosities were obtained from literature [23] or measured with Ostwald viscosimeter. The oxygen concentrations in solutions were determined by using Origin Model 835 dissolved oxygen meter.

3. Results and discussion

The photophysical parameters (τ_f, Φ_f, k_r and k_{nr}) for fluorescein in 0.1N NaOH aqueous solutions are listed in Table 1 as a function of temperature. The non-radiative rate constants (k_{nr}) were obtained from τ_f and k_r values by using the equations [22]:

$$k_{nr} = \left(\frac{1}{\tau_f}\right) - k_r \qquad (8)$$

The activation energy value (E_{nr}) associated with k_{nr} for FL in alkali aqueous solution was calculated from Arrhenius plot. The value of E_{nr} was found to be 10.3 ± 1.6 kcal mol^{-1}. The discussions of fluorescence quenching of FL with molecular oxygen follows as:

3.1. Quenching effect on absorption and fluorescence spectra

The absorption spectra of FL in alkali aqueous solution were taken at 20 °C as a function of quencher concentration given in Fig. 1. There is no any sharp difference between each spectrum of the FL with varying concentration of quencher. This implies that there is no any formation of ground state complex between the fluorophore and quencher molecules. On the other hand, the dynamic quenching affects only excited states of the fluorophores and thus no changes in the absorption spectra are predicted. Our observation regarding absorption spectra indicates that this system has interactions between fluorophore and quencher molecules at the excited state level, therefore, fluorescence quenching mechanism should be discussed in terms of this perspective. In addition, fluorescence spectra measurements were carried out at different temperatures with varying concentrations of molecular oxygen. The fluorescence spectra of FL in alkali aqueous solution with varying concentrations of oxygen obtained at 20 °C are shown in Fig. 2. These spectra show that fluorescence intensity of FL in aqueous solution decreases with increasing quencher concentration while there is no any change of spectral shape and no any shift at maxima during the quenching process. Furthermore, fluorescence quantum yield and excited state lifetime values were also de-
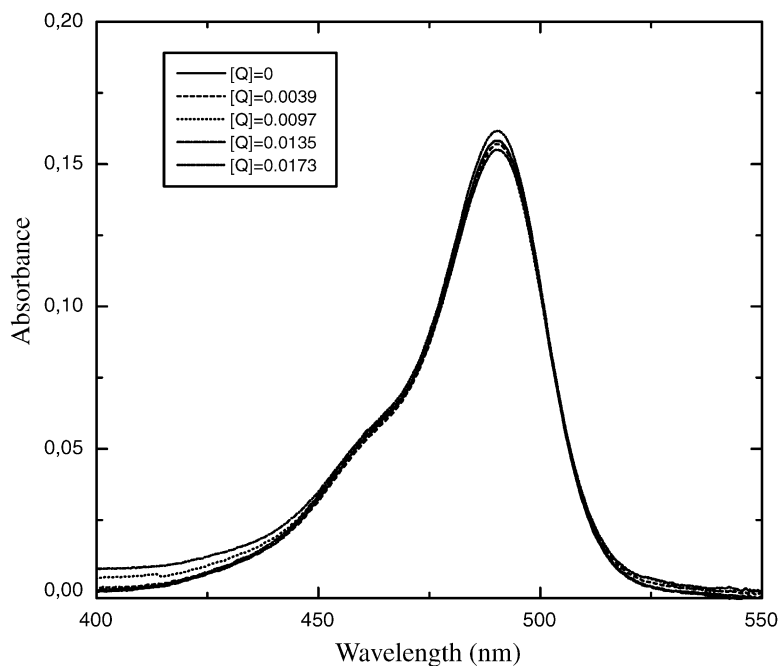

Fig. 1. Absorption spectra of fluorescein in alkali aqueous solutions with and without quencher at 20 °C. [FL] = 1.0×10^{-6} M in 0.1N NaOH solution.
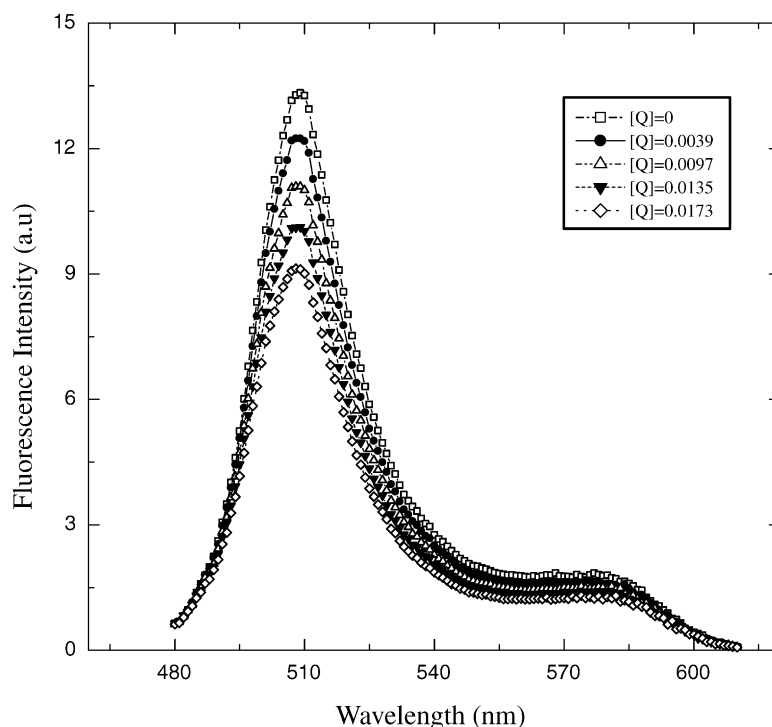
Fig. 2. Corrected fluorescence spectra of fluorescein in alkali aqueous solutions with and without quencher at 20 °C. [FL] = 1.0×10^{-6} M in 0.1N NaOH solution.

termined for all the temperatures and quencher concentrations. For example, when the quencher concentration varied from 0 to 0.0173 mol/L, fluorescence lifetime and quantum yield values varied from $\tau_f = 3.70$ ns and $\Phi_f = 0.93$ to $\tau_f = 2.64$ ns and $\Phi_f = 0.82$ at 15 °C, respectively. The same behavior was also observed for the other experimental temperatures. This observation indicates that the higher the quencher concentration the lower the fluorescence lifetime and quantum yield values. This kind of dependence of the excited state lifetime and fluorescence spectra on quencher concentration implies that the quenching phenomena mainly occur at the excited state level through a dynamical path for FL [24,25].

3.2. The dependence of the k_q value on temperature and viscosity

For this purpose, the temperature studies were carried out and k_q values were determined from the slopes of Stern–Volmer plots given in Fig. 3. One can see that k_q values in Table 2 are about two orders of magnitude bigger than k_{nr} values given in Table 1 for FL in alkali solution. The activation energy value (E_q) associated with k_q was calculated by using Arrhenius equation and was found to be 4.87 ± 0.42 kcal mol^{-1}. When E_q value is compared with the E_{nr} value (10.3 ± 1.6 kcal mol^{-1}), the dynamic quenching path has the lower barrier energy than the nonradiative energy transfer step. Thus, the fluorescence quenching of FL through bimolecular interaction is more operative than the nonradia-

Table 2
Quenching rate constants, diffusion rate constants and solvent viscosity values of fluorescein in 0.1N NaOH aqueous solutions at different temperatures

Temperature (°C)	η (cP)	k_q ((M^{-1}s^{-1})/10^9)	k_{diff} ((M^{-1}s^{-1})/10^9)
5	1.701 ± 0.046	3.84 ± 0.42	3.85
15	1.385 ± 0.020	5.21 ± 0.48	7.91
25	0.996 ± 0.030	7.64 ± 0.39	11.00
35	0.754 ± 0.005	11.53 ± 0.53	14.30
50	0.581 ± 0.001	14.56 ± 0.40	19.50

tive processes which can be an internal conversion process of FL in alkali aqueous solution. Moreover, the activation energy value (E_q) of bimolecular quenching mechanism suggests that the rate of the reaction is limited only by the diffusion of the reactants in alkali aqueous solution. The reason is that the activation barrier energy according to the solvent viscosity of alkali solution was calculated as $E_\eta = 4.46 \pm 0.34$ kcal mol^{-1}. This value of E_η is much closer to the value of E_q for FL in alkali solution.

The experimental results indicate that the higher the temperature the higher the k_q values as well. Moreover, we are unable to detect any significant upward curvature in Stern–Volmer plots. This indicates that in the present case ground state complexation or static quenching may be absent for the system studied here. In some cases, static quenching may also result in a linear behavior for Stern–Volmer plot [7,24]. To distinguish dynamic quenching from static quenching, one must investigate the changes in k_q with experimental temperature. Since dynamic quenching depends
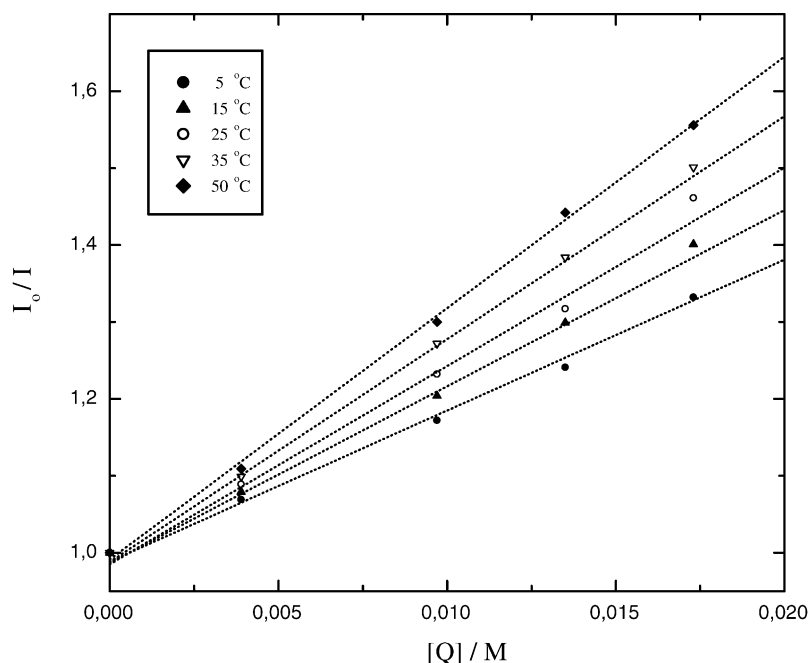
Fig. 3. Stern–Volmer plot for fluorescein with varying quencher concentration at different temperatures. [FL] = 1.0×10^{-6} M in 0.1N NaOH solution.

on the diffusion of probe and quencher molecules, the bimolecular quenching rate constants are expected to increase with increasing temperature. Therefore, higher temperatures result in larger diffusion coefficients. This means that k_q values have to increase with increasing temperature for the dynamic quenching [7,19]. Our observations on k_q values agree well with the dynamic quenching mechanism instead of the static quenching mechanism in which increased temperature results in decreased stability of ground state complex formation and thus lower values of the static quenching constants. So far, our experimental findings and argument support that the quenching phenomenon of FL with molecular oxygen in solution is due to a diffusive process. For this reason, diffusion-controlled bimolecular rate constants (k_{diff}) were calculated as a function of temperature by using Eq. (2) and then they were compared with k_q values. Calculated k_{diff} values are given in Table 2. When we compare the experimental k_q values with the calculated k_{diff} values as shown in Fig. 4, one can see that these values are in good agreement with each other and indicate a diffusive process of the quenching. Moreover, the quenching rate constant values (k_q) decrease with increasing the medium viscosity and get much closer

to the k_{diff} values given in Table 2. To observe viscosity effect on quenching process, experiments were carried out with FL in different solvents having different viscosities at constant temperature of 20 °C as shown in Table 3. In this table, one can see that the higher the viscosity of solvent the closer k_q values to the k_{diff} values for the fluorophore. Therefore, the viscosity dependence of k_q also implies that diffusion-controlled quenching is valid. Furthermore, k_q has an inverse dependence on T/η of FL in alkali solution, as expected for a diffusion-controlled reaction [26]. When we studied this relation by using the data from Table 2, we observed the linearity for k_q versus T/η that is a good evidence for the diffusive nature of the fluorescence quenching and that supports the validity of Fig. 4 for our system. Moreover, the slope of Fig. 4 is lower than 1 and, hence, this kind of dependence directly justifies Eq. (4). Therefore, a plot of ln k_q versus ln η yields a straight line by using the data from Table 3. The slope of plot was found to be $\alpha = 0.62$ with a correlation coefficient of 0.97 for alcoholic solutions indicating the strong power dependence of k_q on the viscosity for our system. This observation is consistent with some other studies done in the literature. For instances, Pandey et al. have reported α

Table 3
Photophysical parameters of fluorescein in different solvents at 20 °C

Solvent	Φ_f	τ_f (ns)[a]	η (cP)[b]	k_{nr} ((s^{-1})/10^7)	k_q (($M^{-1}s^{-1}$)/10^9)	k_{diff} (($M^{-1}s^{-1}$)/10^9)
ACN	0.81 ± 0.01	3.95	0,345	4.32 ± 0.02	1.65 ± 0.22	2.72
MeOH	0.83 ± 0.01	3.70	0.545	4.32 ± 0.05	1.30 ± 0.18	2.19
EtOH	0.80 ± 0.02	3.46	1.078	5.90 ± 0.04	0.72 ± 0.05	1.01
n-PrOH	0.79 ± 0.03	3.27	2.258	6.58 ± 0.05	0.65 ± 0.03	0.72
n-BuOH	0.72 ± 0.03	2.85	2.948	10.01 ± 0.06	0.38 ± 0.02	0.39

[a] Uncertainty: ±10%.
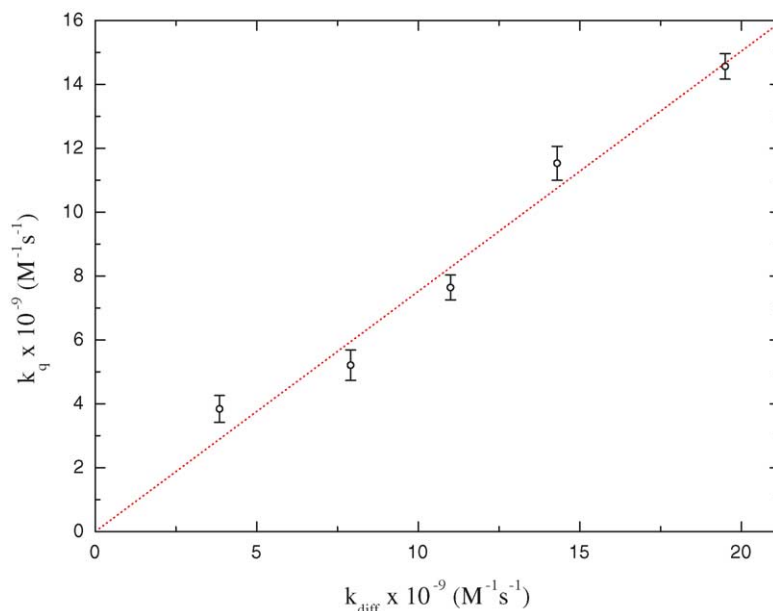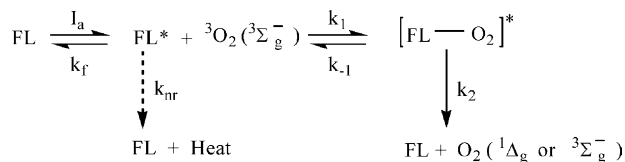
[b] Values η taken from [19].

Fig. 4. Plot of experimentally determined k_q versus theoretically calculated k_{diff}.

value as 0.62 for the viscosity dependence of fluorescence quenching of 9,10-dimethylanthracene in methylcyclohexane by molecular oxygen [14]. Moreover, El-Daly et al. have also reported another α value regarding viscosity depended fluorescence quenching of *N,N'*-bis(2,5-di-tert-butylphenyl)-3,4:9,10-perylenebis(dicarboximide) (DBPI) by molecular oxygen in alcoholic solutions [27]. In this case α has been found as 0.48 for DBPI.

According to our experimental findings, we consider the following scheme for the quenching of FL by molecular oxygen in solution. The similar mechanism has been offered for the quenching of 9,10-dicyanoanthracene, 9,10-dimethylanthracene, and *N,N'*-bis(2,5-di-tert-butylphenyl)-3,4:9,10-perylenebis(dicarboximide) by molecular oxygen in different solvents [14,27]. Our consideration for the quenching of FL by molecular oxygen in solution is given as:

$$FL \underset{k_f}{\overset{I_a}{\rightleftharpoons}} FL^* + {}^3O_2({}^3\Sigma_g^-) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \left[FL{-}O_2\right]^*$$

$$FL + Heat \qquad FL + O_2({}^1\Delta_g \ or \ {}^3\Sigma_g^-)$$

In this mechanism, the interaction of FL with quencher takes place in the second step. Therefore, the fate of fluorescence quenching is strongly dependent on the nature of this second step. FL* interacts with ${}^3O_2({}^3\Sigma_g^-)$ in the second step with the rate constant of k_1 to form [FL–O_2]*. This species either dissociates into the reactants with the k_{-1} rate constant or results in quenching with the k_2 reaction rate constant. Moreover, if the excited state complex formation ([FL–O_2]*) reaction is a diffusion-controlled step, then k_1 must be equal to k_{diff} diffusion rate constant. In this mechanism, if we compare experimental k_{nr} values in Table 1 with k_q and calculated

k_{diff} values given in Table 2 or experimental k_{nr} values with k_q and calculated k_{diff} values given in Table 3, one can see that k_q (and k_{diff}) values are about two orders of magnitude bigger than k_{nr} values. Therefore, we omit the nonradiative deactivation step of FL* with the k_{nr} rate constant in the mechanism. The experimental E_{nr} value (10.3 ± 1.6 kcal mol^{-1}) was also in good agreement with this assumption because this deactivation barrier requires more energy to overcome than that of the other diffusive path, which has the k_1 rate constant. By taking into account these in the mechanism under the photostationary condition, the observed quenching rate constant k_q is given by

$$k_q = \frac{k_1 k_2}{k_{-1} + k_2} \qquad (9)$$

If we assume that quenching reaction step is much faster than dissociation of excited state complex reaction step, that is $k_2 \gg k_{-1}$, k_q is approximated by the following equation

$$k_q = k_1 = k_{diff} \qquad (10)$$

This case is for the high viscosity limit of the solvent. One can see that our experimental k_q value at high viscosity ($\eta = 2.948$ cP for *n*-BuOH) is almost the same with the calculated k_{diff} value given in Table 3. In the limit of low viscosity, where $k_{-1} \gg k_2$, k_q is given by

$$k_q = k_1 \frac{k_2}{k_{-1}} \qquad (11)$$

In this case, k_q will depend on the ratio of k_2/k_{-1}. Since $k_{-1} \gg k_2$ is the case, the ratio of k_2/k_{-1} will be less than unity. Multiplication of this ratio with k_1 in Eq. (11) will result k_q value lower than $k_{diff} = k_1$ value. As a result, we obtain that the experimental k_q values are lower than calculated k_{diff} val-

ues at low viscosity case ($\eta = 0.345$ cP for MeOH) as shown in Table 3. At higher viscosities in which Eq. (10) holds, the observed quenching rate constant k_q is equal to k_1 and the quenching reaction becomes diffusion-controlled and, hence, k_q decreases with increasing solvent viscosity. Our experimental k_q values for FL decrease with increasing viscosity of the medium and get closer to the k_{diff} values as shown in Table 3. As a result, one can see that our experimental findings for FL in solution support offered quenching mechanism based on diffusion-controlled path with this frame of discussion.

4. Conclusion

The steady-state interpretation of Stern–Volmer equation shows a linear relationship between I_o/I and quencher concentration values for the studied system. No any changes of spectral shape in the absorption spectra but intensity decrease in the fluorescence spectra and decrease in fluorescence lifetime values with increasing quencher amount imply that fluorescence quenching of fluorescein occurs at the excited state level through diffusion-controlled bimolecular interactions. Experimental findings related with k_q and calculated k_{diff} values are discussed by considering a quenching mechanism in terms of viscosity limits. Therefore, the temperature and viscosity related experiments supported the validity of dynamic quenching mechanism for the fluorescence quenching of fluorescein with molecular oxygen in solution.

Acknowledgement

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