Solvent effect on fluorescence quenching of biologically active
6-methoxy-4-azidomethyl coumarin by aniline in different solvents

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Solvent effect of fluorescence quenching of biologically active studies of 6-methoxy-4-azidomethyl coumarin (6MAMC) by aniline in five different solvents namely dioxane, tetrahydrofuran, dichloroethane, dimethylformamide and acetonitrile has been carried out at room temperature with a view to understand the quenching mechanisms. The Stern-Volmer (S-V) plot has been found to be non-linear with a positive deviation for all the solvents studied. In order to interpret these results, we have invoked the ground state complex formation and sphere of action static quenching models. Using these models, various quenching rate parameters have been determined. The magnitudes of these parameters suggest that sphere of action static quenching model agrees well with the experimental results. Hence, the positive deviation is attributed to the static and dynamic quenching. Further, with the use of Finite Sink approximation model, it was possible to check these bimolecular reactions as diffusion-limited and to estimate independently distance parameter $R'$ and mutual diffusion coefficient $D$. Finally, an effort has been made to correlate the values of $R'$ and $D$ with the values of the encounter distance $R$ and the mutual coefficient $D$ determined using the Edward’s empirical relation and Stokes-Einstein relation.

Keywords: Fluorescence quenching, 6-methoxy-4azidomethyl coumarin, Diffusion limited, Static and dynamic quenching, Finite sink approximation model

1 Introduction

Fluorescence quenching of organic molecules in solution by various quenchers like carbon tetrachloride, bromobenzene, aniline, halide ions, metal ions etc. has been studied by several researchers1-10. The study has been mainly to understand the nature of bimolecular reactions taking place both under steady state and transient conditions. This study has not only been of importance in physical sciences but also in chemical, biological and medical sciences11-16. The fluorescence yield in bimolecular liquid systems is hindered due to several mechanisms such as static and dynamic quenching, excimer and exciplex formation, charge transfer processes, etc. One of the well known experimental techniques used to study the role of fluorescence quenching is to determine the quenching rate parameter using Stern–Volmer (S-V) plots. If the quenching mechanism is mainly due to dynamic process, then it will be largely due to diffusion in which case diffusion rate parameter $k_d$ equals the quenching rate parameter $k_q = K_{SV}/\tau$ where $K_{SV}$ is the slope of the linear $S$-$V$ plot and $\tau$ is the decay time of the solute in the absence of the quencher. On the other hand, if the experimental results do not simulate with the simple linear Stern-Volmer relationship, then it may be due to one of the above processes other than or along with diffusion process.

Coumarins establish a family of dyes5-7,10 that are applicable in different fields of science and technology. They exhibit strong fluorescence in the UV and VIS region that makes them suitable to use as a colorants, dye laser media and as nonlinear optical chromophores. In medicine, coumarin derivatives are used as anti-coagulants, as fluorescent indicators for the physiological pH region and fluorescent probes to determine the rigidity and fluidity of living cells and its surrounding medium. Coumarins and its
derivatives has been a subject of considerable interest in numerous fields. Due to the interesting properties associated with coumarins, we undertook the fluorescence studies of newly synthesized 6-methoxy-4-azidomethyl coumarin. In the present study, we have used steady state experimental set-up to investigate quenching of 6MAMC by aniline in five different solvents with a view to understand the nature of quenching mechanism involved in these systems.

2 Theory

The dynamic process in which quenching mechanism is mainly due to collision is governed by the linear Stern-Volmer (S-V) equation.

\[
\frac{I_0}{I} = 1 + K_{SV} [Q]
\]  

where \( I_0 \) is fluorescence intensity of solute in the absence of quencher and \( I \) is the fluorescence intensity in the presence of quencher, \( K_{SV} (= k_q \tau \) where \( k_q \) is quenching rate parameter) is the S-V constant and \( \tau \) is the lifetime of the solute molecule in the absence of quencher. Eq. (1) is applicable as long as the experimental results show linear variation. The departure from linearity in S-V plot, suggests that quenching mechanism is not purely collisional and this may be due to sphere of action static quenching model\(^{1,2} \).

According to “Sphere of action static quenching model” the instantaneous or static quenching occurs if the quencher molecule is very near to, or in contact with the fluorescent molecule at the exact moment it happens to be excited. This was explained by the fact that only a certain fraction ‘\( W \)’ of the excited state is actually quenched by the collisional mechanism. Some molecules in the excited state, the fraction of which is (1–W), are de-activated almost instantaneously after being formed because a quencher molecule happens to be randomly positioned in the proximity at the time the molecules are excited and interacts very strongly with them. Several models were employed (Smoluchowski model) to describe this static quenching process, all leading to the following modified form of the S-V Eqs (1 and 2).

\[
\frac{I_0}{I} = 1 + K_{SV} [Q] \quad \text{and} \quad \frac{I_0}{I} = \frac{1 + K_{SV} [Q]}{W}
\]  

where \( K_{SV} \) have its usual meanings as explained earlier and \([Q]\) is the quencher concentration. The Smoluchowski diffusion controlled equation containing transient term is written as:

\[
K_0 = 4\pi N' DR + 4R^2 N (\pi D)^{1/2} t^{-1/2}
\]  

where \( N' \) is the Avogadro’s number per millimole, \( R \) is the encounter distance i.e. the sum of the radii of the diffusion coefficients of solute and quencher molecules and \( t \) is the time. The retention of the later term of Eq. (3) leads to an additional factor, \( W \) in Eq. (2). This additional factor\(^{1,2} \) \( W \) is given by:

\[
W = \exp^{-V/Q} \quad \text{and} \quad \ln(1/W) = V[Q]
\]  

where \( V \) is the static quenching constant and it represents an active volume element surrounding the excited solute molecule.

Instantaneous (static) quenching occurs in a randomly distributed system when a quencher happens to reside within a sphere of action with a volume \( VN \) and radius \( r \) i.e.

\[
V/N = (4\pi r^3)/3
\]  

surrounding a solute molecule at the time of excitation.

As \( W \) depends on the quencher concentration \([Q]\) the S-V plots for a quencher with a high quenching ability generally deviate from linearity. Thus, it is worth rewriting Eq. (2) as:

\[
\frac{[I - (I_0)/I_0]}{[Q]} = K_{SV} \left[ \frac{I}{I_0} \right] + \frac{(1-W)}{[Q]}
\]  

From Eq. (6), one can easily calculate \( K_{SV} \) and the values of \( W \) by least square fit method. The static quenching constant \( V \) and the values of ‘\( r \)’ of sphere of action can also be calculated using the Eqs (4-6). According to Andre et al\(^{11} \) and Zeng et al\(^{16} \) if the distance between the quencher molecules and the excited molecule lies between the encounter distance and the kinetic distance, the static effect takes place especially in the case of steady state experiments irrespective of the ground state complex formation provided the reactions are limited by diffusion. In order to find whether the reactions are diffusion limited one can invoke the finite sink approximation model.
2.1 Finite Sink Approximation model

Keizer\textsuperscript{17-19} has proposed a non-equilibrium statistical modification of the Smoluckowski – Collins – Kimball (SCK) expression to fit the $I_0/I$ ratio in fluorescence quenching.

In the case of SCK model time-dependent rate coefficient $k(t)$ for diffusion – limited reaction of initially randomly distributed reactants\textsuperscript{20} is given by:

$$k(t) = a + b \exp(c^2 t) \text{erfc}(ct^{1/2}) \quad \ldots (7)$$

where

$$a = k_a \left[ 1 + \frac{k_a}{4\pi N'RD} \right]^{-1} \quad \ldots (8)$$

$$b = k_a \left[ 1 + \frac{4\pi N'RD}{k_a} \right]^{-1} \quad \ldots (9)$$

$$c = \left[ 1 + \frac{k_a}{4\pi N'RD} \right] D^{1/2} \frac{1}{R} \quad \ldots (10)$$

Integration of Eq. (7) between the limits ($Q$) at $r \to \infty$ and $[Q]$ ($R$) at $r = R$ provides the well known expression:

$$1/k_q = (1/k_d) + (1/k_o) \quad \ldots (11)$$

where $k_o = 4\pi N'RD$ and $k_o$ is the activation energy controlled rate constant describing the reaction of encountered pairs at a reactive distance $R$. $D$ is the sum of the diffusion coefficients of solute and quencher molecules. Following Eq. (11), $k_q$ is independent of $[Q]$. But for efficient quenching process in liquids, $k_q$ is often observed to increase with $[Q]$. This might be attributed to static quenching of solute molecule in the vicinity of $[Q]$ and transient effects arising from an initial time dependence of the concentration gradient or combination of them\textsuperscript{16}.

But if one assumes that only the first encounter is of interest in the case of efficient fluorescence quenching an initial average separation distance $r_0$ can be defined (sink radius) so that the diffusive region of interest for a first encounter is in the range $R \leq r \leq r_0$ such that all subsequent encounters are eliminated. Integration of the flux equation between the limits ($Q$) ($r_0$) at $r_0$ and $[Q]$ ($R$) at $R$ leads to modification of expression given in Eq. (11) as:

$$\frac{1}{k_q} = \frac{1}{k_d} \left( \frac{R}{r_o} \right) + \frac{1}{k_o} \quad \ldots (12)$$

Eq. (12) reduces to the reaction limited form ($k_o = k_d$) both for inefficient quenching ($k_o \ll k_d$) and for quenching in pure quenching solvents where $R = r_o$. In the diffusion controlled limit ($k_o \gg k_d$), Eq. (12) reduces to:

$$k_q = \frac{k_d}{1 - R/r_o} \quad \ldots (13)$$

and $k_q$ depends on the quenching concentration through $r_o$. Since the sink radius ($r_o$) is identified with the most probable nearest neighbour initial separation, the appropriate distribution\textsuperscript{18} requires that $r_o = (2\pi N'[Q]^{-1/3})$. Replacing $r_o$ in Eq. (12) by $(2\pi N'[Q]^{-1/3})$ and $k_d$ by its value (i.e. $4\pi N'RD$) and dividing everywhere by the fluorescence lifetime of solute in the absence of quencher ($\tau$), one obtains the modified $S-V$ relationship\textsuperscript{17} as:

$$K_s^{-1} = (K_o)^{-1} \left( \frac{2\pi N'}{4\pi N'D\tau} \right)^{1/3} \left[ Q \right]^{1/3} \quad \ldots (14)$$

where $K_o^{-1} = 4\pi N'RD/\tau k_o (4\pi N'RD + k_o)$. A plot of $K_s^{-1}$ against $[Q]^{1/3}$ becomes linear with negative slope. Mutual diffusion coefficient $D$ becomes directly accessible from the slope of the graph exemplified in Eq. (14) and $K^{-1}_{o,SV}$ is obtained at $[Q] = 0$ regardless of the relative magnitudes of $k_o$ and $k_d = 4\pi N'RD$, whether quenching is diffusion limited or not. $K^{-1}_{o,SV}$ can also be written as:

$$K_{o,SV}^{-1} = 4\pi N'D\tau$$

where $R'$ is the distance parameter and has the same meaning as in the long time SCK model\textsuperscript{20,32} and is given as:

$$R' = R \left[ 1 + 4\pi N'D\tau/k_o \right]^{1/3} \quad \ldots (15)$$

Then according to the theory if $ka$ is greater than $k_d$ [i.e. Eq. (11)] then the reactions are said to be diffusion limited\textsuperscript{16} (i.e. for $K' < R$). But for $K' > R$, the bimolecular reactions of fluorescence quenching are said to be diffusion limited\textsuperscript{32}, if the values of $k_q$ determined from Eq. (6) are greater than $4\pi N'R'D$.

3 Materials and Methods

The solute 6-methoxy-4-azidomethyl coumarin (6MAMC) was synthesized using standard methods\textsuperscript{21,23} and the molecular structure of the same is shown in Fig 1. The quencher aniline was obtained from S d fine Chemicals Ltd and tested for its purity before
use. The HPLC grades of all solvents (Sd fine Chemicals Ltd) were used without further purification to prepare the solution. However, the purity of the solvents was checked by the background fluorescence. The solutions were prepared by keeping the concentration of $1 \times 10^{-4}$ M/L and varying the quencher concentration (0.02 M to 0.10 M) in all the solvents. Fluorescence intensities of the solutions were measured on Hitachi F2000 spectrofluorimeter at room temperature with perpendicular geometry. Fluorescence measurements were made by taking fresh solution each time in a rectangular quartz cell having an airtight stopper. The solute has maximum absorption at 350 nm. The solute was excited at 350 nm and the fluorescence spectra were recorded. The maximum fluorescence corresponds to 440 nm. First the fluorescence intensity $I_0$ was measured without the quencher and then at different quencher concentrations at a fixed solute concentration. The experimental values are reproducible within 5% of the experimental error.

### 4 Results and Discussion

The S-V plots obtained using the experimentally determined values of $I_0$ and $I$ are found to be non-linear in all the solvents showing positive deviation as shown in Fig. 2. Similar experimental results were also observed by researchers. Thus, positive deviation from linearity suggests that quenching mechanism is not purely collisional and this may be attributed either to the ground state complex formation or sphere of action static quenching models.

The role of ground state complex formation is ruled out in the present case. This is also confirmed by the fact that there is no shift in the peak position in the emission spectrum of 6MAMC in dichloroethane with different quencher concentrations and the emission spectra of the same are shown in Fig. 3. The analysis of the data for positive deviation in S-V plots was made using “Sphere of action static quenching model”. Fig. 4 shows the plots of $[1-(I/I_0)]/[Q]$ against $I/I_0$ for 6MAMC with aniline as quencher for different solvents and these plots are linear according to Eq. (6).

The dynamic quenching constant $K_{SV}$ is determined in all the cases by least square fit method using Eq. (6) and the values are given in Table 1. Fluorescence lifetime $\tau$ of the solute was measured using Photophysics model of TCSPC nanosecond fluorescence spectrometer and data are given at the

![Molecular structure of 6-methoxy-4-azidomethyl coumarin (6MAMC)](image)
determine the values of $m$ and $Z$. The values of $V$ are given in Table 1. Bimolecular quenching parameter $k_q$ was determined from the experimentally determined values of $K_{SV}$ and $r$ according to the relation $k_q = K_{SV} \tau$ and these values are given in Table 1.

In order to support static and dynamic (transient) effects, we have determined the magnitudes of static quenching constant $V$ and radii $r$ of sphere of action (or kinetic distance) using the above-mentioned theory for sphere of action model. With the use of Eqs (4-8), the values of $V$ and $r$ are determined by least square fit method in all the systems and the values of $V$ and $r$ are given in Table 1. Similar results were also obtained by researchers$^{5,8}$.

The radii of the solute ($R_y$) and the quencher ($R_Q$) molecules were determined by adding the atomic volumes of all the atoms constituting the molecule as suggested by Edwar$^{16}$ and are given at the bottom of Table 1. From these values of $R_y$ and $R_Q$ the sum of the molecular radii ($R$) of the solute 6MAMC and the quencher aniline is determined. This sum of the molecular radii $R$ is referred as encounter distance or contact distance or reactive distance. This value of $R$ is then compared with the values of ‘$r$’ to verify whether the reaction is due to “sphere of action model” or not. Since according to Andre et al$^{11}$, and Zeng et al$^{16}$, the static effect takes place especially in the case of steady state experiments irrespective of the ground state complex formation provided the reactions are limited by diffusion. From Table 1, we see that the values of kinetic distance ($r$) are larger than the encounter distance ($R$) indicating that the sphere of action model holds good in our case also. Further, it may also be noted that a positive deviation in the S-V plot is expected when both static and dynamic quenching occurs simultaneously$^{5,8}$.

To apply this according to Eq. (14), we need to determine the values of $K^{-1}_{SV}$ and $[Q]^{1/3}$, where $K_{SV} = [(H_l)/V]_t$ and $[Q]$ the quencher concentration from 0.02 to 0.10M. For efficient quenching processes (concentration dependent) the value of $K_{SV}$ is often observed to increase with $[Q]$. Hence, the values of $K_{SV}$ were determined at each quencher concentration in all the solvents and the values of $K^{-1}_{SV}$ and $[Q]^{1/3}$ are also determined. Fig. 5 shows the plots of $K^{-1}_{SV}$ against $[Q]^{1/3}$ in different solvents, are almost linear and small deviations may be due to experimental uncertainties. Hence, the linear dependence of $K^{-1}_{SV}$ on the one-third power of quencher concentration within the error limits is confirmed$^{16}$. Then from the least square fit value of $K_0^{SV}$ (Stern-Volmer constant at $[Q] = 0$) was obtained from the intercept of the plot of $K^{-1}_{SV}$ against $[Q]^{1/3}$ according to Eq. (14). Similarly, the mutual diffusion coefficient $D$ was determined from the slope of Eq. (14) by least square fit method and the values of $K_{SV}$ and $D$ are given in Table 2. Using these values of $K_{SV}$ and $D$, the distance parameter $R'$ was determined according to Eq. (15) and the values of $R'$ are given in Table 2. From the values of distance parameter $R'$ and encounter distance $R$ activation energy controlled rate constant $k_a$ was also determined according to Eq. (16). This value$^{16}$ of $k_a$ can only be determined for $R'$ less than $R$. In the case of dichloroethane, dimethylformamide and acetonitrile,

### Table 1 — Dynamic quenching constant $K_{SV}$, quenching rate parameter $k_q$, intercept ($1-\alpha$)/$Q$, range of $W$, static quenching constant $V$ and Kinetic distance $r$ for different solvents

<table>
<thead>
<tr>
<th>Solvents</th>
<th>$K_{SV}$ (m$^{-1}$)</th>
<th>$K_q \times 10^{-9}$ (m$^{-1}$s$^{-1}$)</th>
<th>Intercept</th>
<th>Range of $W$</th>
<th>$V$ (mole$^{-1}$ dm$^{-3}$)</th>
<th>$r$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioxane</td>
<td>0.877</td>
<td>0.0169</td>
<td>5.463</td>
<td>0.454-0.891</td>
<td>8.385</td>
<td>14.924</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>3.490</td>
<td>0.0673</td>
<td>4.593</td>
<td>0.541-0.909</td>
<td>6.465</td>
<td>13.685</td>
</tr>
<tr>
<td>Dichloroethene</td>
<td>13.058</td>
<td>0.2520</td>
<td>2.120</td>
<td>0.788-0.958</td>
<td>2.455</td>
<td>9.909</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>13.649</td>
<td>0.2634</td>
<td>3.129</td>
<td>0.688-0.938</td>
<td>3.860</td>
<td>11.522</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>14.682</td>
<td>0.2834</td>
<td>2.118</td>
<td>0.789-0.958</td>
<td>2.430</td>
<td>9.690</td>
</tr>
</tbody>
</table>

$R_Y = 3.622 \AA, R_Q = 2.840 \AA, t_0 = 0.518$ ns

Fig. 5 — Plots of $K^{-1}_{SV}$ versus $[Q]^{1/3}$ for 6MAMC + aniline system in different solvents
experimentally determined value of the distance parameter $R'$ are less than the encounter distance $R$. Hence, the value of $k_d$ is determined only for dichloroethane, dimethylformamide and acetonitrile and is given in Table 2. From Table 2, we see that $k_q$ approximately equals to $4\pi N R D$ and $k_d > k_q$ or alternatively $k_d > k_q$, may suggest that quenching is less efficient in dichloroethane, dimethylformamide and acetonitrile than in the remaining solvents. This may be due to the fact that the activation process is more predominant in the quenching mechanism than the diffusion process. However, according to Zeng et al. if $k_q$ is greater than $k_d (=4\pi N R D)$ [i.e. Eq. (11)] then the reactions are said to be diffusion limited. Here, the values of $k_q$ and $k_d$ are expressed in terms of $D$ (mutual diffusion coefficient, determined by Stokes Einstein relation) because $D$ is same in both cases and the $k_d$ value is given at the bottom of the Table 2, which is true in our case for dichloroethane, dimethylformamide and acetonitrile. However, for remaining solvents the values of $k_d$ cannot be determined because the values of $R'$ in these solvents are greater than the values of $R$. But according to Joshi et al., the bimolecular quenching reactions are said to be diffusion limited if the values of $k_q$ [determined from Eq. (6)] are greater than $4\pi N R D$. Hence, the values of $4\pi N R D$ are calculated using the experimentally determined values of $R'$ and $D$ of Eq. (14) and are given in Table 2. We see from Table 2 that in dichloroethane, dimethylformamide and acetonitrile, the values of $k_d$ are greater than $4\pi N R D$ (i.e. $k_q >> 4\pi N R D$), which is an expected result for diffusion limited reaction. But for the remaining solvents dioxane and tetrahydrofuran, values of $k_q$ are not greater than $4\pi N R D$, such low value of $k_q$ is due to low value of diffusion coefficient ($D$). It appears that in the dioxane and tetrahydrofuran solvents, efficiency of quenching is less when compared with other solvents. Hence, reduced quenching efficiency is due to decrease in diffusion coefficients of given bimolecular quenching reactions. However, it may also be noted that the values of $R'$ and 'r' kinetic distance do not change drastically.

It is important to note that, when an attempt was made to correlate the experimentally determined values of mutual diffusion coefficient $D$ and distance parameter $R'$ using finite sink approximation model with the corresponding values of $D$ and $R$ calculated from Stokes’s-Einstein and Edward’s empirical relations respectively in all the systems, the values of $D$ and $R'$ (or $R$) comparable only in some solvents. From Table 3, it is clear that correlation fails in some others solvents. Similar discrepancies have also been observed by researchers in some bimolecular quenching reactions. This may be due to the uncertainties in the values of adjustable parameter ‘$a$’ in the Stokes’s-Einstein relation and the approximation in the values of the atomic volume in the Edward’s
empirical relation. Hence, we may conclude that finite sink approximation model is valid in recovering the parameters $D$ and $R'$ (or $R$).

5 Conclusions

It is observed that: (1) the Stern-Volmer plots show positive deviation, indicating efficient fluorescence quenching. (2) Static quenching constant $V$ and kinetic distance $r'$ are found to be in agreement with the corresponding literature values. (3) The values of $k_q$ are greater than $4\pi NR'D$ in dichloroethane, dimethylformamide and acetonitrile. The values of $k_q$ are greater than $4\pi NR'D$ in dichloroethane, dimethylformamide and acetonitrile, which is an expected result for diffusion limited reaction. But for remaining solvents dioxane and tetrahydrofuran, values of $k_q$ are not greater than $4\pi NR'D$, such low value of $k_q$ is due to low value of diffusion coefficients ($D$). It appears that in the dioxane and tetrahydrofuran solvent, efficiency of quenching is less when compared with other solvents. Hence, reduced quenching efficiency is due to decrease in diffusion coefficients of given bimolecular quenching reactions. However, it may also be noted that the values of $R'$ and $r$ kinetic distance do not change drastically. (4) Finite sink approximation model is valid in recovering the parameters $D$ and $R'(R)$. Hence, it may concluded that quenching reaction is diffusion limited and both static and dynamic (transient) quenching processes are partly playing a role in these systems.

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