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Fluorescence resonance energy transfer from Fluorescein to Safranine T in solutions and in micellar medium

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Abstract

Energy transfer by dipole–dipole interaction between anionic dye Fluorescein and cationic dye Safranine T (3,6 diamino-2,7-dimethyl-5-phenyl phenazinium chloride) solubilised in aqueous solution and micellar solution of cetyl trimethyl ammonium bromide (CTAB) was studied where donor–acceptor Förster critical radius (R_0) is larger than the micellar diameter (d_m). The mechanism of quenching of fluorescence of Fluorescein by Safranine T in aqueous solution and in normal micellar medium has been related to the non-radiative energy transfer processes. The value of overlap integral and critical energy transfer distance is higher in CTAB micellar medium compared to that in the aqueous solution. The variation of quantum yield and efficiency of energy transfer between two dyes in micellar medium and aqueous solution has been compared. Effect of viscosity on the fluorescence resonance energy transfer (FRET) between the dye pairs has been determined from fluorescence anisotropy study.

Keywords: Fluorescence resonance energy transfer; Fluorescein; Safranine T; Micelle; Viscosity

1. Introduction

Excitation by light may initiate both intramolecular and intermolecular transformations of a molecule. The most conspicuous intermolecular events are electron, proton and energy transfer [1,2]. Fluorescence resonance energy transfer (FRET) is a photophysical process and occurs via intermolecular energy transfer mechanism where energy that is absorbed by fluorescencent molecule (donor) is transferred non-radiatively to a second fluorescent molecule (acceptor). Thus the process is experimentally manifested in simultaneous quenching of the donor fluorescence and electronic excitation of the acceptor. Förster energy transfer occurs for the very weak range of dipole–dipole interaction energies (10^{-1}–10^{1} cm^{-1}) and has a rate range of 10^{6}–10^{11} s^{-1} [3]. According to Förster's theory the rate of energy transfer depends mainly upon the following factors [2,4–6]: (1) the extent of spectral overlap between the donor emission and the acceptor absorption spectra; (2) the quantum yield of the donor (Φ_f^D); (3) the relative orientation of the donor and acceptor transition dipoles, and (4) the distance between the donor and acceptor transition dipoles.

The distance and orientation dependence of FRET makes it extremely useful to detect conformational change, irrespective of the donor and the nature of the donor–acceptor interaction. The probability of energy transfer will be increased if the donor–acceptor distance can be decreased or if both donor and acceptor are parts of the same molecule [2,4,7,8]. The single-molecule sensitive FRET have been successfully applied to monitor the conformational dynamics in biopolymer. Single-pair FRET experiments are ideally suited to study conformational dynamics occurring on the nanometer scale during protein folding and defolding. One consequence of energy transfer is photosensitization (e.g. photosynthesis) [5,6]. Photodynamic action, which is often used in the treatment of cancer, is also a consequence of energy transfer [9].

The kinetics of long range electronic energy transfer between molecular probes dispersed in or chemically bound to micro-heterogeneous systems, like micelles, polymer coils

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in solution, microemulsions, and nano-latex particles is recognized as an efficient tool to investigate the morphology of those as well as their interactions with solutes at molecular level [10–19]. The energy of an electron transfer process in various systems of restricted geometries such as micelles, silica gels, clays, polymer latexes and zeolites are known to be influenced significantly by the topology of these systems [20–23]. Energy transfer in micellar system is of current interest due to its similarity with biological membrane [24–26].

Measurement of fluorescence anisotropy plays an important role in biochemical research owing to the fact that any factor affecting size, shape or segment flexibility of a molecule will affect the parameter [4]. It directly reflects any motional restriction imposed on the fluorophore by the environment [27]. The steady state anisotropy, however, gives information about the rotational rate of the solute molecule. The viscosity of the microenvironments around the fluorophore has also been determined from the fluorescence anisotropy value.

Energy transfer reaction between the donor Fluorescein and the acceptor Rose Bengal adsorbed on the surface of the micelle has been reported by observing the quenching of fluorescence of Fluorescein [28]. Efficiency of energy transfer has been investigated for the donor (Fluorescein and Acridine Orange) and the acceptor (Nile Red) in organised media, i.e. micelles and reverse micelles of Triton X100 [29]. Electronic energy transfer efficiency of different donor–acceptor pairs has been reported in different alkanols, mixed solutions of different viscosity [30,31]. In the present work FRET has been observed when donor has motional restriction in viscous CTAB microenvironment. Viscosity dependent FRET has been explained from the data obtained in different alkanols.

2. Experimental

Fluorescein (Fl) was carefully purified by passing its alkaline solution through alumina column followed by precipitation with HCl [32]. The process of dissolution in alkali and precipitation with HCl was repeated thrice. Safranine T (ST) (E. Merck) was recrystallised twice from ethanol–water mixture. The alkanol (methanol, MeOH; ethanol EtOH; propanol, PrOH and *n*-butanol, *n*-BuOH) used were spectroscopic grade products of E. Merck, Germany. They were dried following the standard procedure [33] and purified by fractional distillation. The presence of photoactive impurities was checked by emission measurements and was found to be absent. The surfactant CTAB was of BDH product and used as received.

Absoption spectra were recorded using a Shimadzu (Japan) UV–vis 1700 spectrophotometer with a matched pair of silica cuvettes. Fluorescence spectra were taken in a F-III A spectrofluorimeter (Spex, INC, NJ, USA) with a slit width of 1.25 nm. All the measurements were done thrice. Doubly distilled water was used for solution preparation. The excitation wavelength was 490 nm and absolute quantum yields (Φ_f^D) were determined using Rhodamine 6G in ethanol [34] as a standard for 490 nm excitation.

The steady state fluorescence anisotropy were measured with a Hitachi spectrofluorimeter (F-4500) at 298 K and for anisotropy measurements the excitation and emission bandwidths were 2.5 nm each. The steady-state fluorescence anisotropies (r) were calculated using the following equation:

$$r = \frac{[I_{VV}(\lambda) - G(\lambda)I_{VH}(\lambda)]}{[I_{VV}(\lambda) + 2G(\lambda)I_{VH}(\lambda)]} \quad (1)$$

where I_{VV} and I_{VH} are the intensities obtained with the excitation polarizer oriented vertically and the emission polarizer oriented in vertical and horizontal orientation, respectively [4]. $G(\lambda)$ is an instrumental factor representing the polarisation characteristics of the photometric system and is given by

$$G(\lambda) = \frac{I_{HV}(\lambda)}{I_{HH}(\lambda)} \quad (2)$$

I terms refer to parameters similar to those mentioned above for the horizontal position of the excitation polarizer.

For lifetime measurements, the sample solutions were excited at 380 nm of a picosecond light emitting laser diode of pulse duration 200 ps in a spectrofluorimeter of Fluorocube model (IBH, U.K.). The fluorescence was detected at magic angle polarization. The detector was a Hamamatsu MCP photomultipier (2809U). The time resolution of the setup is ≈100 ps. The overlap integrals for the donor–acceptor systems were determined using numerical integration of the normalized spectroscopic data.

3. Results and discussion

The donor (Fl) and acceptor (ST) pair has been chosen so that the extent of overlap between the fluorescence spectra of donor and absorption spectra of acceptor is satisfied with large overlap zone. The donor Fl has high Φ_f^D and the value is 0.91. Regarding the orientation of the donor and acceptor transition dipoles in aqueous solution as well as in CTAB micellar medium, a random orientation has been assumed for FRET study. In the energy transfer process, the critical energy transfer distance (R_0) is 8.52 nm which is greater than the micellar diameter of CTAB (4.57 nm). Maintaining the concentration of Fl (3.3×10^{-6} mol dm^{-3}) and concentration of ST (3.2×10^{-6} mol dm^{-3}) constant, the effect of CTAB on energy transfer efficiency has been observed. It is observed that energy transfer efficiency increases linearly with increasing concentration of CTAB in the premicellar region and it approaches constant after CMC of CTAB, i.e. in micellar solution of CTAB. As the viscosity of the microenvironment surrounding Fl increases with increasing CTAB concentration, so the CTAB concentration has been maintained at higher than the CMC in order to maintain a highly viscous medium. The concentration of CTAB in the

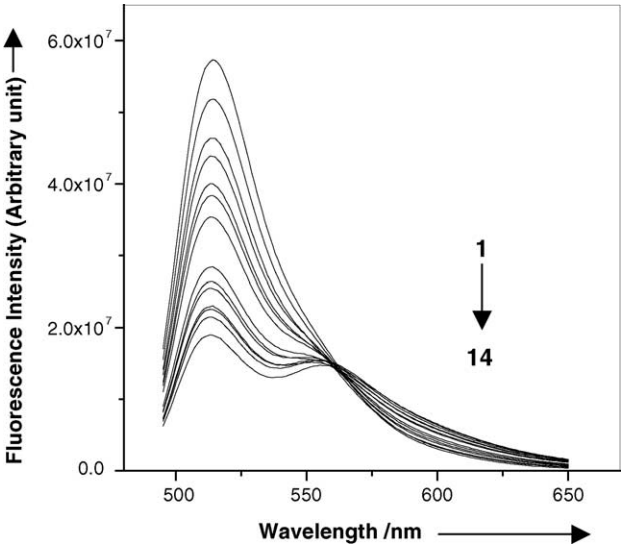

Fig. 1. Fluorescence spectra of aqueous solution of Fluorescein in presence of Safranine T. [Safranine T] $\times 10^7$/mol dm^{-3}: (1) 0.17; (2) 0.52; (3) 0.69; (4) 0.86; (5) 1.03; (6) 1.20; (7) 1.37; (8) 1.54; (9) 1.70; (10) 1.87; (11) 2.04; (12) 2.20; (13) 2.36; and (14) 2.50. [Fluorescein] = 3.3×10^{-6} mol dm^{-3}.

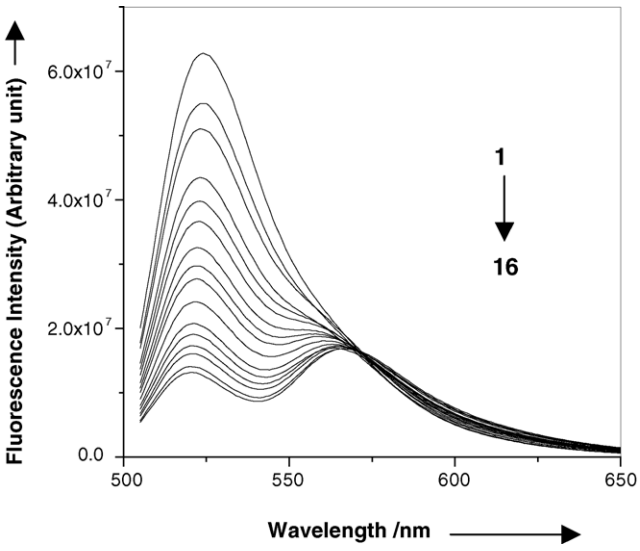

Fig. 2. Fluorescence spectra of Fluorescein in micellar solution of CTAB (0.025 mol dm^{-3}) in presence of Safranine T. [Safranine T] $\times 10^7$/mol dm^{-3}: (1) 0.17; (2) 0.35; (3) 0.52; (4) 0.68; (5) 0.86; (6) 0.95; (7) 1.03; (8) 1.20; (9) 1.37; (10) 1.54; (11) 1.70; (12) 1.87; (13) 2.04; (14) 2.20; (15) 2.36; and (16) 2.50. [Fluorescein] = 3.3×10^{-6} mol dm^{-3}.

solution was maintained at 0.025 mol dm^{-3} for the entire study.

3.1. Steady state fluorescence quenching and time-resolved studies

In the aqueous solution of Fl having concentration 3.3×10^{-6} mol dm^{-3}, ST was gradually added from 4.5×10^{-8} mol dm^{-3} to 3.2×10^{-6} mol dm^{-3} and the electronic absorption spectra of Fl in the aqueous solution are not affected in presence of ST. On gradual addition of ST in the aqueous solution and micellar solution of Fl, fluorescence intensity and quantum yield of Fl decreases and hence ST plays the role of quencher. An isoemmissive point has been observed at 561 nm (Fig. 1) and at 570 nm (Fig. 2) in aqueous solution and micellar solution, respectively. The linearity of Stern–Volmer plot indicates only one type of quenching occurs [4]. Förster had shown that the energy transfer can be regarded as a bimolecular process [35]. The quenching rate constant K_{SV} and the energy transfer rate constant k_{ET} can be determined from the Stern–Volmer relation [36,37]

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_{ET}\,\tau_0[Q] \tag{3}$$

where F_0 and F represent the fluorescence intensity of the donor in absence and in the presence of acceptor, respectively; $[Q]$ is the acceptor concentration and τ_0 is the fluorescence lifetime of donor.

Excited-state reactivity of a molecule depends to some extent on its lifetime. However, k_{ET} can be calculated by the following relation [1,2]. R_0 and r represent the critical energy transfer distance and distance between donor and acceptor, respectively.

$$k_{ET} = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \tag{4}$$

the average lifetime of the donor were used to determine the k_{ET} (Table 1) and the k_{ET} values obtained from Eqs. (3) and (4) agree well.

Lifetime (τ) measurements of Fl were performed by exciting the samples at 380 nm to excite the donor (Fl) only. The lifetime of Fl in aqueous solution and CTAB micellar medium is 3.76 ns (Fig. 3a) and 4.40 ns (Fig. 3b), respectively. In CTAB micellar medium the lifetime of Fl is higher than that in aqueous medium, which is due to restricted movement of Fl in micellar medium as corroborated from anisotropy value (Table 1) also [4]. On gradual addition of ST in the aqueous solution of Fl, lifetime remains unaltered (3.76 ns) and $\tau_0/\tau = 1$. In the CTAB micellar medium of Fl τ_0/τ is also equal to unity on gradual addition of ST and hence in aqueous

Table 1
Photophysical and energy transfer parameters of Fluorescein in presence of Safranine T in aqueous and CTAB micellar medium

Medium	$K_{SV} \times 10^{-6}$ (dm^3 mol^{-1})	ζ (ns)	$k_{ET} \times 10^{-15}$ (dm^3 mol^{-1} s^{-1})	$K \times 10^{-5}$ (dm^3 mol^{-1})	n	$J \times 10^{12}$ (dm^3 cm^3 mol^{-1})	R_0 (nm)	r_0 (nm)	Φ_f^D	r
Aqueous	6.58	3.76	1.75	5.29	1.29	2.14	8.52	7.57	0.91	0.01
CTAB	7.49	4.40	1.7	6.76	1.27	2.96	9.31	6.87	0.98	0.14

Microviscosity in aqueous solution and CTAB micellar solution and 0.2 cp and 23.0 cp, respectively.

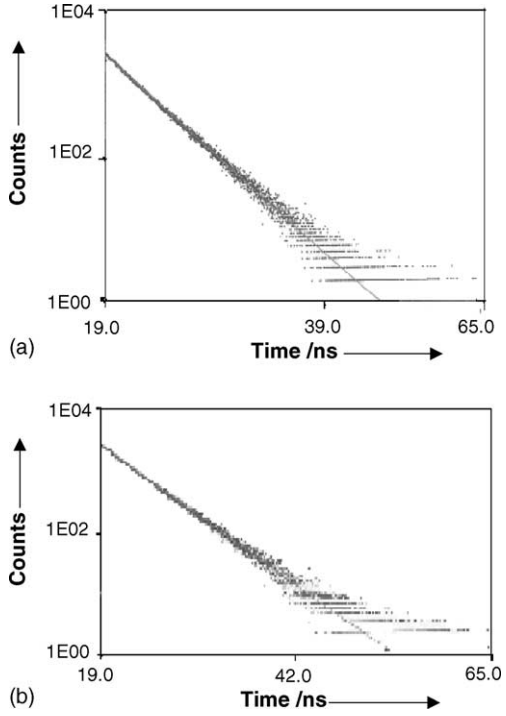

Fig. 3. (a) Fluorescence decay of Fluorescein in aqueous solution, [Fluorescein] = 3.3×10^{-6} mol dm^{-3}. Best-fit line has been considered ($\chi^2 = 1.01$). (b) Fluorescence decay of Fluorescein in CTAB micellar medium (0.025 mol dm^{-3}), [Fluorescein] = 3.3×10^{-6} mol dm^{-3}. Best-fit line has been considered ($\chi^2 = 1.00$).

solution and aqueous CTAB micellar solution the quenching is static in nature [4].

3.2. Binding of Fluorescein and Safranine T in aqueous and CTAB micelle

For the quenching interaction, if it is assumed that there is interaction between two dyes, and one Fl molecule can bind to n number of ST molecules, the relationship between the Fluorescence intensity and the quencher concentration can be deduced from the following formulae:

$$nQ + B = (Q_n + B) \tag{5}$$

where B is the donor (Fl), Q is the quenchable molecule (ST), $(Q_n + B)$ is the complex formed between donor and quenchable molecule whose resultant constant is K_a. Here,

$$K_a = \frac{[Q_n + B]}{[Q]^n[B]} \tag{6}$$

If the overall amount of donor (bound or unbound with the quenchable molecule) is B_0, then $[B_0] = [Q_n + B] + [B]$, here $[B]$ is the concentration of unbound molecule, then the relationship between fluorescence intensity and the unbound molecule is

$$\frac{[B]}{[B_0]} = \frac{F}{F_0}$$

From this relation one may deduce

$$\log \frac{(F_0 - F)}{F} = \log K + n \log[Q] \tag{7}$$

With Eq. (7), the binding constant K and n can be found. The fluorescence quenching of Fl with varying concentration of ST is shown in Figs. 1 and 2. The best fit to the fluorescence data using Eq.7 was found by setting $n = 1.29$, $K = 5.29 \times 10^5$ dm^3 mol^{-1} and $n = 1.27$, $K = 6.76 \times 10^5$ dm^3 mol^{-1} (±10%) in aqueous and CTAB micellar medium, respectively. From the value of n, one may say that the complex is almost 1:1 in nature both in aqueous and CTAB solution. The higher value of Fl–ST binding constant in CTAB micelles compared to aqueous medium implies that binding is much preferable in CTAB micellar medium.

3.3. Energy transfer between Fluorescein and Safranine T

The distance between Fl and ST can be evaluated according to Förster mechanism of non-radiative energy transfer. According to Förster's theory [2–4], the energy transfer effect is related not only to the distance (r_0) between acceptor and donor, but also to the critical energy transfer distance (R_0), that is:

$$E = \frac{R_0^6}{R_0^6 + r_0^6} \tag{8}$$

where R_0 is the critical distance when the transfer efficiency [4,5] is 50%

$$R_0^6 = 8.8 \times 10^{-25} \chi^2 N^{-4} \Phi J \tag{9}$$

In Eq. (9), χ^2 is the spatial orientation factor of the dipole, N is the index of refraction of the medium, Φ is the fluorescence quantum yield of the donor, J is the overlap integral of the fluorescence spectra of the donor and the absorption spectra of the acceptor, therefore

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 \, \Delta\lambda \sum F(\lambda) \, \Delta\lambda \tag{10}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ.

From the overlapping of the absorption spectra of the acceptor and the fluorescence spectra of donor, J can be evaluated by integrating the spectra for $\lambda = 500$–530 nm and $J = 2.14 \times 10^{-12}$ dm^3 cm^3 mol^{-1}. Under these experimental calculations, we found $R_0 = 8.52$ nm from Eq. (9) using $\chi^2 = 2/3$, $N = 1.336$ and $\Phi = 0.91$ for the aqueous solution of Fl. The distance between Fl and ST can be evaluated from Eq. (8) where $r_0 = 7.57$ nm. In CTAB micellar medium $J = 2.96 \times 10^{-12}$ dm^3 cm^3 mol^{-1}, $R_0 = 9.31$ nm and $r_0 = 6.87$ nm. In CTAB micellar medium the overlap integral and critical energy transfer distance are higher, whereas the distance between donor and acceptor decreases relative to those in aqueous solution.

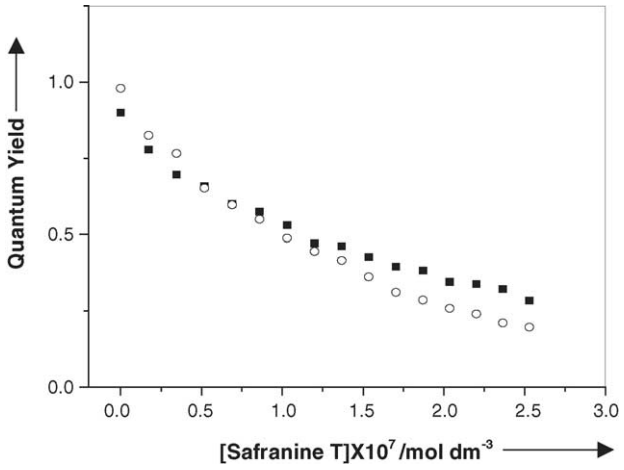

Fig. 4. Plot of Fluorescence quantum yield of Fluorescein vs. [Safranine T] in aqueous solution (◯) and in CTAB micellar (0.025 mol dm^{-3}) solution (■).

3.4. Quantum yield and energy transfer efficiency

The values of quantum yield are 0.91 and 0.98 for Fl in aqueous solution and in CTAB micellar medium, respectively. As the value of Φ_f^D in CTAB micelle is higher, so, Fl acquires higher donating property in CTAB micellar medium. From Fig. 4 it is evident that the variations of quantum yield of FL in CTAB medium is higher compared to the aqueous solution. This may be due to various reasons. One is the higher value of overlap integral $J(\lambda)$. Another important reason of high variation in Φ_f^D is due to slow diffusion of dye in CTAB micellar medium.

The energy transfer efficiency [2–4] is

$$E = 1 - \frac{F}{F_0} \tag{11}$$

From Fig. 5 it is evident that efficiency of energy transfer is 10% higher when Fl is in CTAB micellar medium.

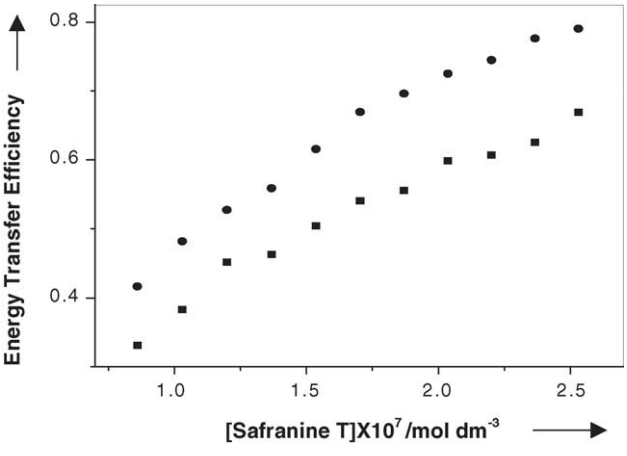

Fig. 5. Plot of energy transfer efficiency vs. [Safranine T] in aqueous solution (■) and in CTAB micellar (0.025 mol dm^{-3}) solution (●).

3.5. Location of Fluorescein and Safranine T in micellar medium

From the previous study it had been reported that the donor Fl has a strong interaction to the CTAB micelle [38] whereas the acceptor ST has less tendency of binding to the CTAB micelle [39]. Fl has been adsorbed on the CTAB micelle and the binding constant had been reported 3.68×10^3 dm^3 mol^{-1}. The $E_T(30)$ [40,41] value of the medium surrounding Fl in the CTAB micelle is 48 and from the $E_T(30)$ value it is clear that the polarity of the microenvironment surrounding Fl is less in CTAB micellar medium compared to the aqueous solution. From the $E_T(30)$ value it is clear that the donor resides on the micelle/water interface. From fluorescence anisotropy study, it is clear that in CTAB micellar medium r is high compared to the aqueous solution and hence Fl is in slightly motional restriction environment and higher viscous medium compared to water.

On gradual addition of CTAB in the aqueous solution of Fl ([Fl] = 3.3×10^{-6} mol dm^{-3}), fluorescence anisotropy (r = 0.01) gradually increases and after CMC of CTAB, i.e. in micellar solution a high value of r (=0.14) has been obtained which remains unaltered with increasing concentration of CTAB. The high value of r in CTAB micellar medium compared to the aqueous solution of Fl point to a slightly motional restricted environment of Fl in the micellar medium, there has no enhancement of anisotropy value. So, the energy transfer process occurs from the Fl present on the micelle/water interface to the acceptor (ST) solubilised in the aqueous solution. Fluorescence anisotropy is very much dependent upon the viscosity of the environment of the fluorophore. Thus, microviscosity, at a definite temperature, is often estimated by comparing the fluorescence anisotropy of a fluorophore in an environment with those of the probe in solvents of known viscosity. To have a relative measure of the microviscosity in CTAB micellar medium, fluorescence anisotropy of Fl in the micellar solutions were compared with the calibration curve based on available data in glycerol–water mixtures of different compositions at 298 K [42,43] and the estimated (error ±15%) microviscosity in the present system has been presented in Table 1.

3.6. Study of viscosity dependent FRET in the alkanol solution of Fluorescein

The effect of viscosity on the FRET of the donor Fl and acceptor ST has been studied by taking Fl in different alkanol solutions of different viscosity. The overlap integral (J) of the fluorescence spectra of donor and absorption spectra of acceptor increases from MeOH to n-BuOH. By using Eq. (7) the values of n and K have been evaluated for Fl and ST solution in MeOH, EtOH, PrOH and n-BuOH. The n value implies that there is a 1:1 complex formation between Fl and ST and the binding constant value in the solvent follows the order n-BuOH > PrOH > EtOH > MeOH (Table 2). So, with

Table 2
Photophysical and energy transfer parameters of Fluorescein in presence of Safranine T in different solvents

Medium	$K_{SV} \times 10^{-6}$ ($dm^3\,mol^{-1}$)	$K \times 10^{-5}$ ($dm^3\,mol^{-1}$)	n	$J \times 10^{12}$ ($dm^3\,cm^3\,mol^{-1}$)	R_0 (nm)	r_0 (nm)
Methanol	7.23	6.52	1.28	2.88	8.72	7.51
Ethanol	8.14	6.73	1.26	2.99	8.87	7.43
Propanol	8.83	7.03	1.27	3.05	8.96	7.35
n-Butanol	9.56	7.18	1.26	3.28	9.03	7.28

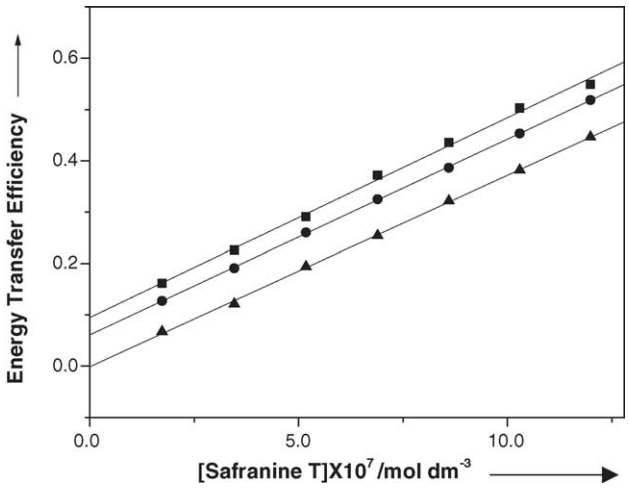

Fig. 6. Plot of energy transfer efficiency vs. [Safranine T] in methanol (◄), ethanol (●) and *n*-propanol (■).

increasing viscosity of the solvent binding constant value increases.

In Fig. 6 energy transfer efficiency has been plotted against the concentration of ST in different alkanol. From this figure it has been observed that the energy transfer efficiency decreases from *n*-BuOH to MeOH, i.e. with increasing viscosity of the medium energy transfer efficiency increases. The rate of enhancement of energy transfer efficiency with [ST] (dE/d[ST]) remains same in all the alkanols and its value is $8 \times 10^3\,mol^{-1}\,dm^3$. So, with increasing viscosity of the medium binding constant and energy transfer efficiency increases. This also supports the increase in efficiency of energy transfer in micellar medium due to the viscosity.

4. Conclusion

Summarizing the results reported in this work, this can be said that energy transfer efficiency and binding constant increases when donor (Fl) is in CTAB micellar interface. Overlap integral and critical energy transfer distance between Fl and ST also increases in CTAB micellar medium. From the study of viscosity dependent FRET in alkanols it may be concluded that the enhancement of energy transfer efficiency of FL increases with increasing viscosity of the microenvironment surrounding Fl. The enhancement in fluorescence anisotropy of Fl in CTAB micellar medium indicates motional restriction of the donor and enhanced viscosity of the medium. The enhancements of energy transfer efficiency and higher variation of quantum yield of Fl in CTAB micellar medium may be due to enhancement of viscosity of the microenvironment surrounding Fluorescein.

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