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7-Hydroxy-4-methyl-8-(4′-methylpiperazine-1′-yl)methyl coumarin: An efficient probe for fluorescence resonance energy transfer to a bioactive indoloquinolizine system

Arabinda Mallick, Basudeb Haldar, Saumitra Sengupta, Nitin Chattopadhyay*

Department of Chemistry, Jadavpur University, Calcutta-700 032, India

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Abstract

Solvatochromic effects on the fluorescence behavior of 7-hydroxy-4-methyl-8-(4′-methyl-piperazine-1′ yl)methylcoumarin (HMMC) was studied in different solvents. The fluorescence of HMMC was found to be highly sensitive to both the polarity and the protic character of the solvent. Exploiting the polarity-sensitive fluorescence property of HMMC, its excited-state dipole moment has been determined. Fluorescence (Förster) resonance energy transfer (FRET) process from HMMC to a potent bioactive molecule 3-acetyl-4-oxo-6,7-dihydro-12 H indolo-[2,3-a] quinolizine (AODIQ) was studied. From the determined K_{SV} and R_0 values, it is argued that a long-range dipole–dipole interaction is operating for the energy transfer mechanism. The energy transfer efficiency (E) and the distance between the acceptor and the donor (r_0) have been determined.

Keywords: Fluorescence resonance energy transfer; Solvatochromic shift; Stokes shift; Excited-state dipole moment

1. Introduction

Solvents play a major role, both in vitro and in vivo, in the behavior of molecules in solution [1]. Solvents have their own intrinsic characteristics due to which they can alter the reaction pathways by altering the energies of the reactants and contributing to the final state of a reaction [2]. This has led to a widespread interest to study the effects of solvation and solvation dynamics of molecular systems in different solvents [3–8]. Solvent molecules can induce stabilization of a probe molecule either through dipolar relaxation between the probe and the solvent molecules or through some specific interactions such as hydrogen bonding.

Aromatic fluorophores bearing polar substituents on the aromatic ring are known to be particularly sensitive to the chemical and physical

*Corresponding author. Fax: 91 33 2414 6266.
E-mail address: pcnitin@yahoo.com (N. Chattopadhyay).

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properties of the solvents. Redistribution of the electron density in the excited state often leads to a large change in the dipole moment of the molecule. The solvent molecules, being unable to follow this sudden change, undergo a re-orientation around the probe leading to a shift in the fluorescence band of the fluorophore [9]. When the probe molecule does not disturb the solvent structure, the solvatochromic shift of a fluorescence or absorption band is linear with respect to the solvent polarity function. However, when a local enrichment of the solvent molecules around the probe occurs (preferential solvation), a deviation from linearity is obtained [10–13]. The latter may be due to various interactions, viz., electrostatic forces, hydrogen bonding, etc., between the probe and the two different solvent components.

Coumarins belong to an important class of fluorescent compounds having interesting photophysical properties and a wide range of applications as laser dyes [14], photosensitizers [15], pesticides [16], etc. Several coumarin derivatives are also biologically important (as anticoagulants, as fluorescent indicators for physiolgical pH measurements and as fluorescent probes to determine the rigidity and fluidity of living cells and the surrounding medium). Coumarin derivatives substituted with nitrogenous heterocycles are known to possess a broad range of biological activities. Given the fact that piperazine rings are commonly present in many synthetic drugs used for modulating the central nervous systems, we became interested in the biophysical properties of coumarin derivatives having a piperazine substituent.

The present article deals with the solvent-dependent fluorescence behavior of a newly designed piperazine-substituted coumarin derivative 7-hydroxy-4-methyl-8-(4′-methylpiperazine-1′-yl)methylcoumarin (HMMC). We have also looked at the fluorescence resonance energy transfer (FRET) from HMMC to the bioactive nitrogen heterocycle 3-acetyl-4-oxo-6,7-dihydro-12H-indolo-[2,3-a] quinolizine (AODIQ) [17,18]. The indole nucleus appears to be a promising basis for the design and synthesis of new derivatives that can effectively protect the nervous system [19]. Carbazoles and β-carbolines containing the indole nucleus are now well established as potent bioactive molecules [20]. β-carbolines are known to be efficient cancer cell photosensitizers and are used in photodynamic therapy (PDT). Beljansky et al. have found that some β-carbolines can destroy selectively and completely the proliferative capacity of various types of cancer cells which is enhanced upon excitation with UV radiation [21]. AODIQ, being essentially a β-carboline derivative, also shows promising biological activities [22].

Fluorescence (Förster's) resonance energy transfer (FRET) is an important technique for investigating a variety of biological phenomena including energy transfer processes [23]. One important consequence of energy transfer is photosensitization, a classic example of which is photosynthesis [24]. Moreover, FRET plays a key role in PDT of cancer [24,25] and is extensively used to study the structure, conformation, spatial distribution and assembly of complex proteins [26]. Since FRET causes a number of important biological phenomena [23], much interest lies in FRET studies between two potent bioactive molecules in vitro. Given the broad-range biological activities of coumarins and indoloquinolizines, we became interested in the FRET studies between HMMC and AODIQ. The present FRET study has been performed considering its future application in PDT.

2. Experimental

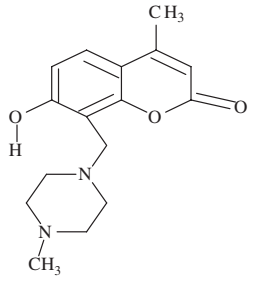
HMMC was easily synthesized via Mannich reaction of 7-hydroxy-4-methylcoumarin with 37% aqueous formaldehyde and *N*-methylpiperazine in refluxing ethanol (2 h). HMMC was obtained as a pale yellow solid which was recrystallized from ethanol; mp 128–130 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.32 (s, 3H), 2.38 (s, 3H), 2.36–2.98 (br m, 8H), 4.07 (s, 2H), 6.07 (s, 1H), 6.76 (d, 1H, J 8.4 Hz), 7.41 (d, 1H, J 8.4 Hz), 8.09 (br s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.7, 45.7, 52.4, 53.7, 54.6, 107.4, 110.5, 112.2, 113.3, 124.6, 152.4, 153.3, 161.2, 162.3.

The solvents *n*-heptane (HEP), 1,4-dioxane (Diox), dicholoromethane (DCM), acetonitrile (ACN), 2-propanol (PrOH), ethanol (EtOH) and

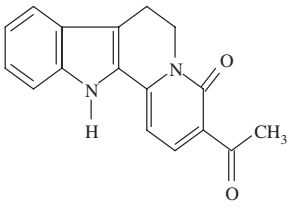
methanol (MeOH) were all of UV spectroscopic grade (Spectrochem India). Triple-distilled water was used wherever required.

The absorption and fluorescence studies were performed using a Shimadzu MPS 2000 spectrophotometer and a Spex Fluorolog II spectrofluorimeter, respectively. The fluorescence quantum yields were measured against quinine sulfate solution in 0.1 N sulfuric acid ($\phi = 0.54$) [27].

Optimization of the molecular structure of HMMC was performed adopting the semiempirical method (AM1-SCI) using the HyperChem 5.01 software, procured from Hypercube, Canada. For the dipolar solvent stabilization and determination of the difference in the dipole moments between the excited state and ground state ($\Delta\mu$), we have taken half the maximum molecular length (11.50 Å) of the optimized structure of HMMC as the Onsager's cavity radius ($a = 5.75$ Å). The structure of HMMC and AODIQ are given below.



Structure of HMMC



Structure of AODIQ

3. Results and discussion

The absorption spectra of HMMC in all the standard solvents consist of a single band peaking at around 320 nm. The excitation spectra of HMMC agree with the absorption spectra in all the solvents. The high molar absorption coefficients ($>10^4$) lead to correspond the absorption band to $\pi \rightarrow \pi^*$ transition in all the solvents.

The emission measurements of HMMC have been carried out in different non-polar aprotic, polar aprotic and polar protic solvents and the data along with some other photophysical parameters are collected in Table 1. The fluorescence spectra of HMMC are broad and structureless. Unlike the absorption spectra, the maximum of the fluorescence band shows a significant bathochromic shift with an increase in the solvent polarity. In alkane solvent, it shows fluorescence band at 414 nm. With an increase in the solvent polarity (in case of polar aprotic solvents), the fluorescence maximum gradually shifts towards the red (Fig. 1) and it reached at 440 nm in acetonitrile.

Plots of Stokes shift ($\bar{\nu}_a - \bar{\nu}_f$) and the energy corresponding to the fluorescence maximum (E_f) as a function of solvent polarity parameter E_T (30), has been shown in Fig. 2.

Both the plots reflect linear relationships with the E_T (30) parameter of the solvent. The good correlation of Stokes shift with E_T (30) scale is indicative of the fact that the dielectric solute–solvent interactions are responsible for

Table 1
Some photophysical parameters of HMMC in different solvents

Solvent	E_T (30)	Molar extinction coefficient (ε) ($L\,mol^{-1}\,cm^{-1}$)	Absorption maximum (nm)	Fluorescence maximum (nm)	Fluorescence quantum yield (ϕ)
Heptane	31.1	9915	322	414	0.02
Dioxane	36.0	10216	320	422	0.56
DCM	40.7	11237	320	424	0.71
ACN	45.6	10216	318	440	0.68
PrOH	48.4	12019	320	440	0.59
EtOH	51.9	10637	320	440	0.59
MeOH	55.4	11418	320	440	0.56

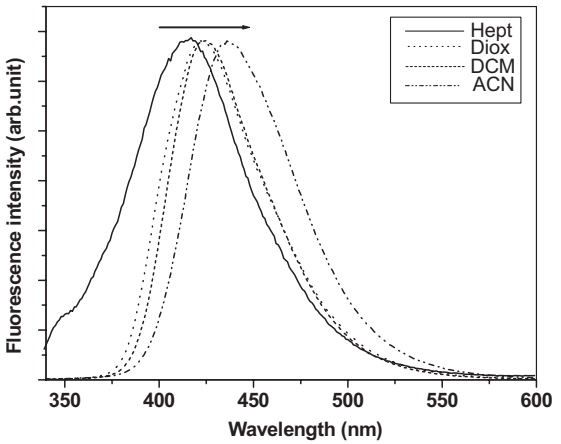


Fig. 1. Normalized fluorescence spectra of HMMC in different pure aprotic solvents ($\lambda_{exc} = 320$ nm). The correspondences of the curves with different solvents are given in the inset.

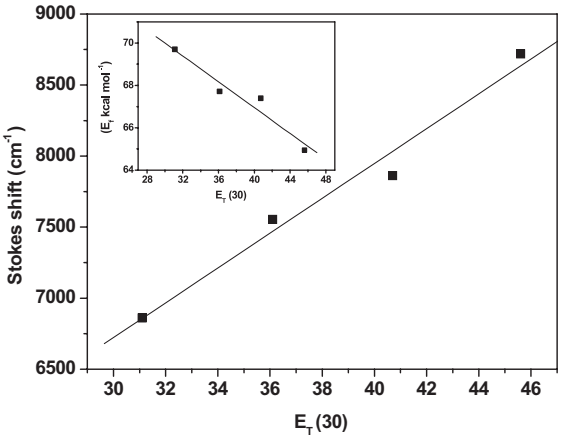


Fig. 2. Variation of Stokes shift with E_T (30). Inset shows the variation of fluorescence energy as a function of E_T (30).

the observed solvatochromic shift for the present molecule [28].

On the other hand, for the protic solvents with varying polarity, the fluorescence maxima are more or less in the same position as that is in acetonitrile. To check whether the solvent polarity alone or the polarity along with the protic factor is responsible for the observed red shift, we investigated the emission profile of HMMC in dioxane–water mixture with varying composition. The emission maximum was found at 422 nm in pure dioxane, but addition of a very small amount of water (only 0.5%) shifted the fluorescence band maximum to 440 nm similar to that in acetonitrile. It is obvious that the above composition (dioxane:water = 99.5:0.5) does not correspond to a polarity as high as that of acetonitrile. This proves that not only the polarity factor but also the protic factor plays an important role for the observed shift in the emission maximum in case of protic solvents.

3.1. Excited state dipole moment

For the determination of the difference in dipole moment of the excited state and ground state ($\Delta\mu$), we followed the method as described by Ravi et al. [4,6]. According to this method, we have plotted the stokes shift ($\bar{\nu}_a - \bar{\nu}_f$) of the fluorescence maximum against E_T^N of different polar aprotic solvents (Fig. 3).

From the slope of the above plot and Onsager's radius (a) for the probe (HMMC), we have determined the $\Delta\mu$ taking the literature values of $\Delta\mu$ and 'a' for some reference dye systems as used by Ravi et al. [4]. The excited-state dipole moment has been estimated to be 9.37 D taking the value of the ground-state dipole moment as 3.82 D (corresponding to the optimized geometry of HMMC through AM1 calculation).

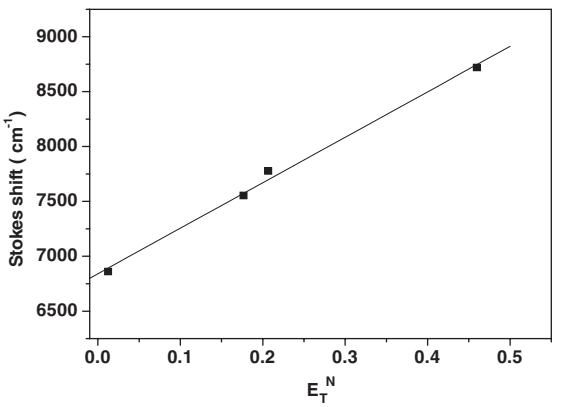


Fig. 3. Plot of Stokes shift of HMMC in pure solvents against E_T^N.

3.2. Fluorescence (Förster's) resonance energy transfer from HMMC to AODIQ

FRET [9,24] is a distance-dependent interaction between the different electronic excited states of dye molecules in which excitation energy is transferred from one molecule (donor) to the other (acceptor) without emission of a photon from the former molecular system. According to Förster's theory, the efficiency of FRET depends mainly on the following factors: (i) the extent of overlap between donor emission and the acceptor absorption, (ii) the orientation of the transition dipole of donor and acceptor and (iii) the distance between the donor and the acceptor [9,24]. In case of the present work, the origin of idea of studying the FRET from HMMC to AODIQ stemmed from the consideration of condition (i), i.e., the absorption and fluorescence band positions of the two fluorophores. In dioxane solvent, HMMC absorbs appreciably at 320 nm giving fluorescence at around 420 nm. The molecule AODIQ does not absorb significantly at 320 nm, but absorbs appreciably at 420 nm. The fluorescence coming out of AODIQ has a band at 455 nm [6]. Considering the fluorescence band of HMMC and the absorption band of AODIQ, the pair was judged as an excellent pair of donor–acceptor system for the FRET study. Gradual addition of AODIQ to the HMMC solution in dioxane associated with bathochromic shift of the emission maximum along with a decrease in the fluorescence intensity at 420 nm (Fig 4a) and finally a new band at 455 nm appears to correspond to the acceptor molecules [6]. Since the emission band position of the donor (420 nm) and the acceptor (455 nm) are very close, it was not possible to get any isoemissive point, which is an interesting characteristic of FRET process.

It is important to mention here that we did not detect any additional absorption band for the mixture of HMMC and AODIQ, instead the absorption spectrum showed two distinct bands at the donor and acceptor absorption maximum. It points to the absence of any detectable ground-state complex of the donor–acceptor pair in the solution [29–31]. The fluorescence emission spectrum of the mixture of donor and acceptor did not show any additional new broad peak at longer wavelengths which is an evidence of the absence of exciplex formation between the excited donor and the acceptor molecules. Thus, the decrease in fluorescence intensity of the donor with increasing acceptor concentration indicates non-radiative energy transfer between the excited donor and the acceptor [30,31]. Furthermore, the fluorescence excitation profile monitoring at 455 nm (emission maximum in the presence of donor and acceptor molecules) shows that besides the $S_0 \rightarrow S_1$ transition of AODIQ ($\lambda_{max} \sim 420$ nm), a prominent band with $\lambda_{max} \sim 320$ nm corresponding to the HMMC absorption occurs (Fig. 4b). Consistent with the

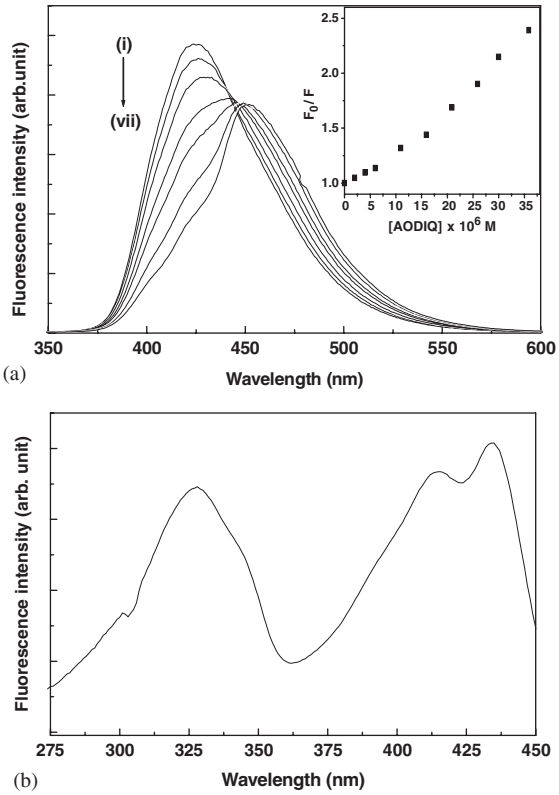


Fig. 4. (a) Fluorescence spectra of HMMC in dioxane as a function of AODIQ concentrations ($\lambda_{exc} = 320$ nm). Curves (i) → (vii) correspond to 0, 2, 6, 10.9, 15.9, 25.9 and 35.8 μM AODIQ concentrations. (b) Fluorescence excitation spectra in presence of donor (1.6 μM) and acceptor (2.6 μM) monitoring at 465 nm.

discussions with Lakowicz [9] and the recent reports of Sengupta et al. [32,33] and De et al. [31], this is a clear indication of an efficient Förster's type resonance energy transfer from HMMC to AODIQ system. De et al., while reporting the FRET from fluorescein and acridine orange (donors) to the nile red (acceptor) in different organized environments, observed the excitation spectrum of the donor [31]. In their works, monitoring the emission of quercetin (acceptor, 15 μM), Sengupta et al. [32,33] found the excitation spectrum of the donor tryptophan present in HSA (18 μM). Thus, their works suggest that the excitation band can be visible even when the concentration of acceptor is less than that of the donor. However, in our case, the acceptor (2.6 μM) concentration is higher than that of the donor concentration (1.6 μM).

The quenching of the fluorescence of the donor (HMMC) with the addition of acceptor (AODIQ) was followed by Stern–Volmer plot (inset of Fig. 4a). From this figure, it is evident that at high concentration of acceptor it undergoes a positive deviation from the linearity. In spite of the fact that the SV plot is non-linear, formation of the ground-state complex formation was ruled out since there was no appreciable change in the absorption spectrum of the fluorophore. The non-linearity was attributed to the fact that at high quencher concentration, a fraction of the fluorophore is just adjacent to the acceptor at the moment of excitation, and thus immediately deactivated [9,24] upon excitation. From the linear portion of the curve, we have determined the quenching rate constant (K_{SV}) value to be $3.5 \times 10^4\,M L^{-1}$. This determined K_{SV} value falls in the normal range reported earlier for such type of FRET study and is order of magnitude higher than that observed for a normal diffusion-controlled quenching process [29,30]. In tune with the earlier studies [29,30], this observation suggests that the dominant mechanism of fluorescence quenching is the resonance energy transfer through long-range dipole–dipole interaction rather than the simple diffusion-limited process between the excited donor and the ground–state acceptor molecule.

3.3. Energy transfer efficiency and distance

In this section, our principal objective is to see the energy transfer efficiency between HMMC and AODIQ. Since AODIQ is a potential PDT agent, determination of this efficiency is very much useful in PDT application through energy transfer. As an additional outcome of the experiment, we have determined the probable distance between the reacting species for the maximum energy transfer. According to the Förster non-radiative energy transfer theory [34,35], the energy transfer efficiency E depends not only on the distance (r_0) between the donor and the acceptor, but also on the critical energy transfer distance (R_0) expressed by the following relation:

$$E = R_0^6/(R_0^6 + r_0^6), \tag{1}$$

where R_0 is the characteristic distance, called the Förster's distance or critical distance, at which the efficiency of transfer is 50%. The magnitude of R_0 is dependent on the spectral properties of the donor and acceptor molecules. R_0 is expressed as follows:

$$R_0^6 = 8.8 \times 10^{23}[\kappa^2 n^{-4} \phi_D J(\lambda)] \text{ in } \mathring{A}^6, \tag{2}$$

where κ^2 is the factor expressing the relative orientation of the donor and acceptor molecule, n is the refractive index of the medium, ϕ_D is the quantum yield of the donor in absence of the acceptor and $J(\lambda)$ is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor in units of $M^{-1}\,cm^3$. The overlap spectrum ([HMMC] : [AODIQ] = 1:1) is shown in Fig. 5.

The spectral overlap integral $J(\lambda)$ was calculated by numerical integration. The overlap integral $J(\lambda)$ for a donor–acceptor pair is defined as [9,31]

$$J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4\,d\lambda, \tag{3}$$

where $F_D(\lambda)$ is the corrected fluorescence intensity of the donor at wavelength λ to $(\lambda + \Delta\lambda)$, with the total intensity normalized to unity and $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor at wave length λ.

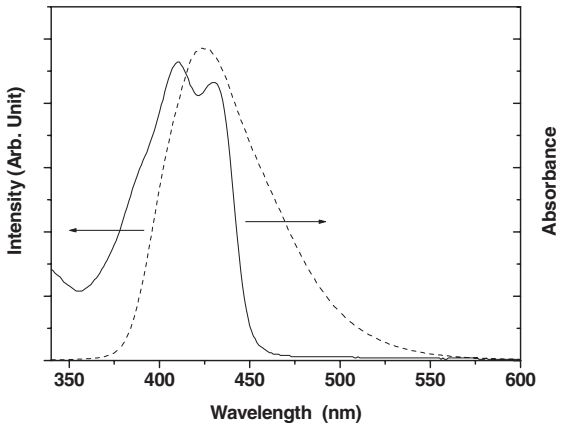


Fig. 5. Overlap of fluorescence spectrum of HMMC (broken line) and absorption spectrum of AODIQ (solid line). In both the cases, the solvent is dioxane.

The Förster distance (R_0) has been calculated assuming random orientation of the donor and acceptor molecules. Taking $\kappa^2 = 2/3$, $n = 1.422$, $\phi_D = 0.56$ and with the help of the relation (2), we have calculated this parameter and it was found to be 24.9 Å. The sum of collision radii of donor (HMMC) and acceptor (AODIQ) is about 12–13 Å [6]. The significant difference between the values of the collision radii (12–13 Å) and the critical radius of energy transfer (24.9 Å) suggests that for HMMC–AODIQ pair a long-range dipole–dipole interaction is responsible for the energy transfer mechanism consistent with the earlier proposition [9,24,29,30]. According to the Förster's non-radiative energy transfer theory, the energy transfer efficiency (E) is given by the following relation:

$$E = 1 - F/F_0, \tag{4}$$

where F is the fluorescence intensity of the donor in the presence of the acceptor and F_0 is the fluorescence intensity in the absence of the acceptor molecule. The energy transfer efficiency (E) for the pair of HMMC and AODIQ in case of 1:1 composition has been calculated and is found to be 0.33. With a knowledge of the energy transfer efficiency (E) and the critical energy transfer distance (R_0) for 1:1 composition, the distance (r_0) between the donor and acceptor molecule has been calculated to be 28 Å using relation (1). With an energy transfer efficiency 0.33 (<50%), one should expect a value of r_0 greater than the critical radius. Thus, our determined value of r_0 is rationally acceptable. Considering the statistical distribution of the molecules (donor and acceptor) within the liquid phase, there has to be a most probable distribution and our FRET study corresponds to the distance associated to this only. This distance can also be computed theoretically from the dimensions and concentrations of the acceptor molecules using the nearest-neighbor distribution [36]. As mentioned above, as per our prime interest we have not done this calculation and the program will be undertaken shortly to see the correspondence between the calculated and the experimental values. Since both the molecular systems are polarity sensitive, the FRET study can be extended to microheterogeneous environments including proteins before their uses in vivo [37].

4. Conclusion

The present study reports the solvatochromic effect on the fluorometric behavior of a piperazine substituted coumarin fluorophore in different solvents of varying polarity. The study reveals that fluorescence property of the molecule is very much sensitive to the polarity and the protic character of the solvent. The excited-state dipole moment of the probe has been estimated following the solvatochromic behavior of the fluorophore with solvent polarity. Steady-state FRET from the fluorophore to a bioactive indoloquinolizine molecular system (AODIQ) has been studied in some detail. K_{SV} and R_0 values suggest that a long-range dipole–dipole interaction is responsible for the energy transfer mechanism.

Acknowledgments

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