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Spectroscopic Studies of Encapsulation of Dapoxyl Sodium Sulphonate [2-(4′-Sulfophenyl)-5-(4″-dimethylamino phenyl)oxazole, Sodium salt] in the Nanocavities of Cyclodextrins- A Review.

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ABSTRACT:

Dapoxyl sodium sulphonate [2-(4′-Sulfophenyl) -5- (4″- dimethylamino phenyl) oxazole, Sodium salt] (DAP), is a water soluble environment-sensitive fluorescent dye consists of electron rich (dimethylamino groups) and electron deficient (sulphonyl) groups undergoes intramolecular charge transfer (ICT) in the excited state. The enhanced solvatochromic effects of the Dapoxyl dyes than those of the readily used well known fluorescent dyes like Prodan, Dansyl derivative, Nile red, Anthradan etc. make this promising fluorescent probe for studying its photophysical characteristics in different microenvironments. The aims of the present study are to examine the change in photophysical properties of the DAP after its inclusion complex with α-, β- and γ-Cyclodextrins (CD) with different stoichiometries. The review highlights the change in quantum yield together with the binding constants as well as the stoichiometries of DAP inclusion in different CDs at different experimental conditions. Complexation also increases the fluorescence lifetime. Important photophysical properties like polarity dependence fluorescence, large Stokes shift and high quantum yield have also been summarized which helps to explore its photophysics in different micro-environments. Possible clarification of their photophysical properties has been explained as well.

KEYWORDS: Cyclodextrins, ICT, Solvatochromic dyes, Fluorescence quantum yield, Fluorescence lifetime.

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INTRODUCTION:

Numerous environment-sensitive fluorescent dyes have been developed to study the micropolarity in different heterogeneous media, organized media, biological media.[1, 2] Many solvatochromic fluorescent dyes including intramolecular charge transfer (ICT) dye are used at present as an important tool to probe the local polarity, polarizability, viscosity, and acidity[3]. Presence of electron rich and electron deficient groups within the dye facilitates the ICT. An increase in charge separation within the fluorophore following excitation occurs in the excited state from the donor part to the acceptor part of these dyes results a high dipole moment which leads to the alteration of the energy of the excited state through the interaction with surrounding solvent molecules[4]. Dapoxyl sodium sulphonate [2-(4/-Sulfophenyl)-5-(4//- dimethylaminophenyl) oxazole, Sodiumsalt] (DAP) contains a 'push-pull' electron transfer system having the electron rich (dimethylamino groups) and electron deficient (sulphophenyl) groups connected with a oxazole spacer (Fig.1) thus exhibits intramolecular charge transfer (ICT) in the excited state[5].

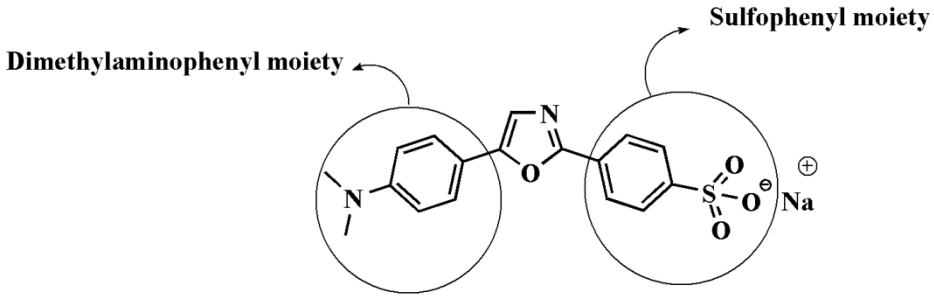


Fig.1 Dapoxyl sodium sulfonate (DAP)

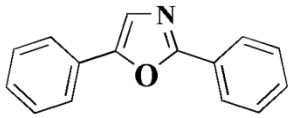


Fig. 2 2,5-di phenyl oxazole

The solvent polarity dependent fluorescence properties of the Dapoxyl dyes are much larger than those of the readily used well known solvatochromic dyes like Prodan, Dansyl derivative, Nile red, Anthradan etc. (Table 1) makes DAP a promising dye for photophysical studies of different microenvironments[11-20].

Table: 1 Comparative study of the spectral properties of Dapoxyl derivative with other conventional Solvatochromic fluorescent dye

Dye	λ_{ab} (nm) in MeOH	λ_{em} (nm) in MeOH	ε [a] (M^{-1} cm^{-1})	φ (%)
Dapoxyl derivative	373	584	28000	39 [b]
Prodan	361	498	18400	51[c]
Dansyl derivative	335	526	4600	49 [d]
Nile red	553	632	45000	38[e]
Anthradan	456	604	12100 [h]	41[f]
3MC-2	445	597	29000	1.4 [g]

λ_{ab}, λ_{em}, φ, ε are the absorption maximum, emission maximum, quantum yield of fluorescence, dielectric constant, respectively.
[a] values in MeOH
[b] From ref. [5]
[c] From ref. [6]
[d] From ref. [7]
[e] From ref. [8]
[f] From ref. [9]
[g] Fromref.[10]
[h] value in acetonitrile

The electronic excitation corresponds to the delocalization of electron density from the electron donor part to the electron acceptor part across a conjugated spacer (oxazole) of DAP significantly increase the permanent dipole moment of the excited state (S_1) relative to that of the ground state (S_0) this generates the environment-sensitive fluorescence[11,14,15,21]. DAP exhibits high extinction coefficients, large Stokes shift, high fluorescence quantum yields, solvent polarity sensitive lifetime and pH-dependent fluorescence [12, 22].

Cyclodextrins (CD) is a naturally occurring oligomer, a toroid (hydrophilic outside and relatively less polar inside compared to water) biopolymer are a family of cyclic compounds composed of sugar units connected by 1,4-glycosidic linkages[23,24]. The commonly used Cyclodextrins are α-CD, β-CD and γ-CD which consist of 6, 7 and 8 sugar units, respectively (Fig 3).

Chemical structure of Cyclodextrin (CD)

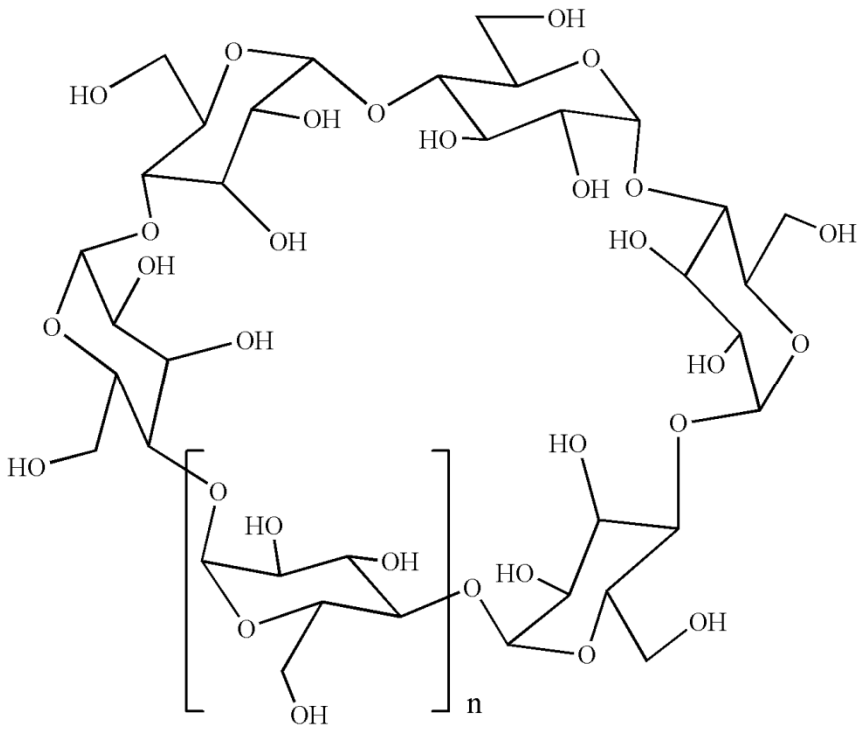


Fig.3

Hydrophobic cavity along with hydrogen bonding possibility makes the cyclodextrins to form an inclusion complex with a variety of hydrophobic or amphiphilic guest molecules of appropriate size. The non-toxic, water-soluble and biodegradable cyclodextrins (CDs) are capable of encapsulating a wide range of organic and inorganic compounds have been used as effective models to study various interactions in biological systems as well as drug delivery vehicles[25-27]. Fluorescent probes whose spectra or quantum yields are responsive to their environments can be used in the study of their encapsulation in CDs. The photophysical study of DAP in different CDs may be used as an effective tool to understand various interactions of the dye in biological system as CDs have been readily used as effective model system. The present article highlights the effects of CD cavity size on the stoichiometry, stability, and structure of the host-guest complexation of the synthetic organic probe DAP with three natural cyclodextrins (α-CD, β-CD, and γ-CD) using steady-state and time-resolved fluorescence techniques in a wide pH range.

MATERIALS AND METHOD:

2-(4′-Sulfophenyl) -5- (4″- dimethylamino phenyl) oxazole, Sodium salt (DAP) was purchased from Molecular Probes Inc. 2,5- diphenyl oxazole (DPO) was purchased from Sigma-Aldrich. All the solvents were of spectroscopic grade; Chloroform, Acetonitrile, Methanol were purchased from Sigma-Aldrich and were used without further purification. The stock solution of the dye was prepared using deionised water of Milli-Q grade. Steady state absorption measurements were recorded on Cary 4 spectrophotometer (Varian) using 1 cm optical path length quartz cuvette. Fluorescence spectra was recorded on FluoroMax 3.0 (Jobin Yvon, Horiba) spectrofluorometer using 1 cm optical path length quartz cuvette in the region from 200 nm -700 nm range by exciting DAP at 340 nm and DPO at 280 nm. The final concentration of DAP and DPO was taken 5µM for all the measurements. All the experiments are performed at 25^0c.

PHOTOPHYSICAL STUDY OF DAPOXYL SODIUM SULFONATE (DAP) IN SOLVENTS:

The red-shifted fluorescence spectra with large FWHM (Fluorescence width at half maxima) of DAP (Fig.4) compared to its parent compound 2, 5-diphenyl oxazole (DPO) may be attributed to the attachment of electron donor and electron acceptor group with the DPO (Fig 1 & Fig 2). Thus DAP exhibit ICT in the excited state and increase the dipole moment[15, 20].

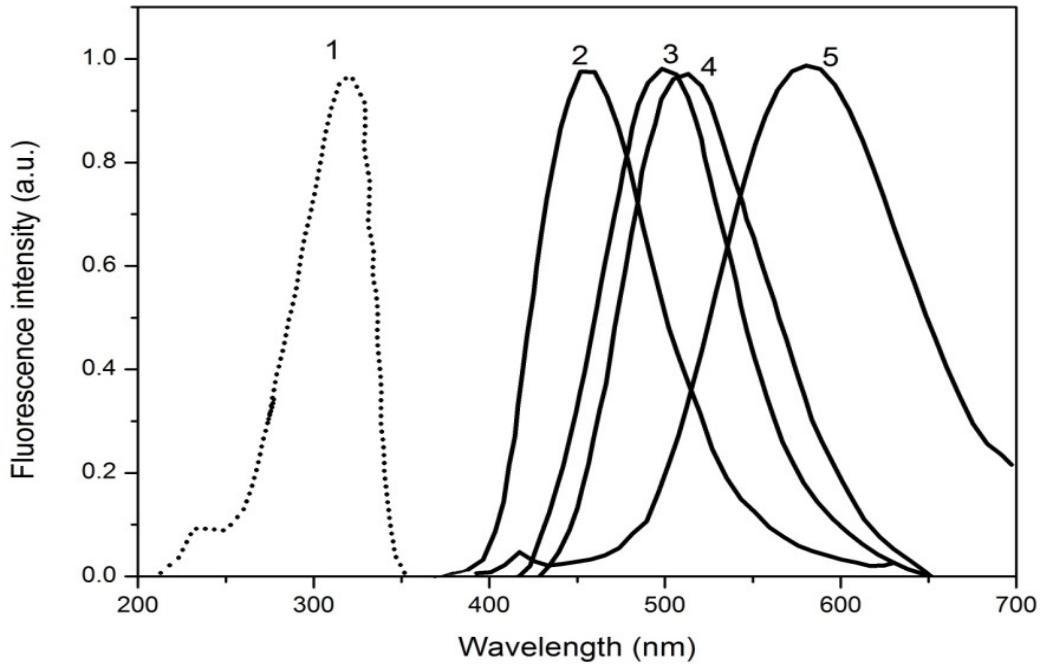
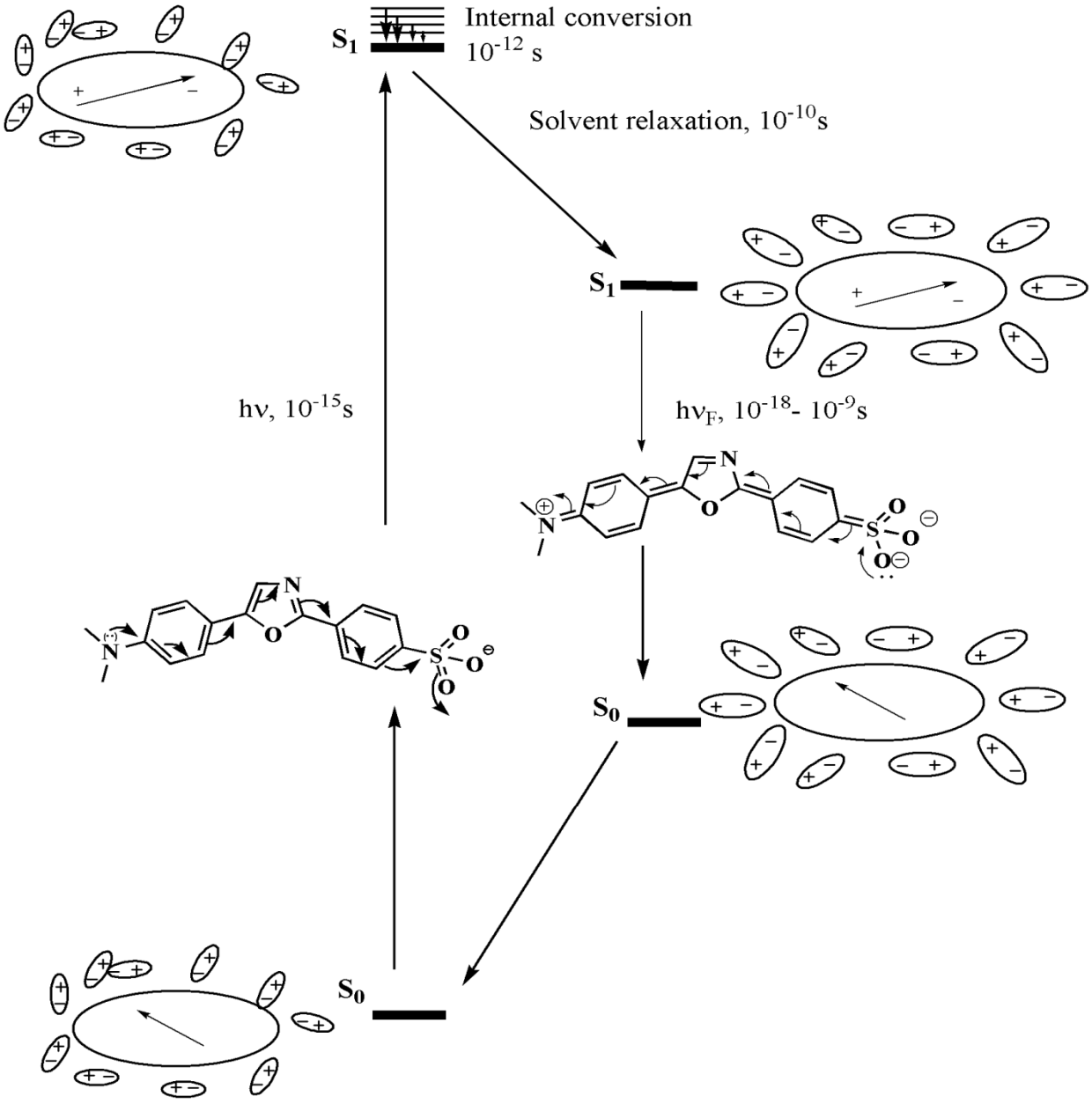


Fig.4. The normalised fluorescence emission spectra of (1) DPO in Methanol (dotted line) and (2-5) DAP in different solvents with increasing polarity (solid line): 2-Chloroform, 3-Acetonitrile, 4-Methanol, 5-Water. As the solvent polarity sensitive fluorescence of DAP originates from ICT [15], the red shifted fluorescence maxima of DAP (2-5) relative to DPO indicates that DAP contains a "push-pull" electron transfer system which differs the fluorescence spectra from its parent compound DPO.

As illustrated in the Scheme-1 the dipoles of the solvent molecules are randomly oriented around the ground state (S_0) of DAP. Following absorption of a photon due to delocalization of electron density from the amino group of dimethylamino phenyl moiety towards the electronegative sulfonic acid group of sulphophenyl moiety via the oxazole spacer, the dipole moment (μ_E) of the 1st excited state (S_1) of DAP is much larger than the dipole moment (μ_G) of the ground state (S_0). K.Pal *et al.* measured (15.5 ± 2) D change of dipole moment of DAP using Stokes shift and solvent polarizability values of different solvents[18]. Initially, orientations of the solvent molecules around the 1st excited state (S_1) remains the same as the reorganization of the atomic nuclei requires more time (10^{-10} s) than the electronic excitation (10^{-15} s) from S_0- S_1[29,30]. Then the solvent dipoles can reorient around μ_E and stabilizing the excited state. The energy of the fluorescence transition $h\gamma_F$ influenced by the polarity of the solvent molecules as the dipole moment (μ_E) of the excited state (S_1) lowered again through the transfer of electron density back across the conjugated spacer [31.] This effect becomes more prominent as the polarity of the solvent increases [32.]



Scheme 1

Solvent sensitivity of DAP can be known from the Lippert plot (Fig.5). The linearity of this plot is often regarded as an evidence of general solvent effect[20]. This is also in good agreement with the earlier report by K.Pal *et al.* where Stokes shift of DAP correlates linearly with the E_T (30) of different solvents[18]. In the Lippert plot, some solvents are apart from the fitted line presumably due to the hydrogen bonding nature of the solvents.

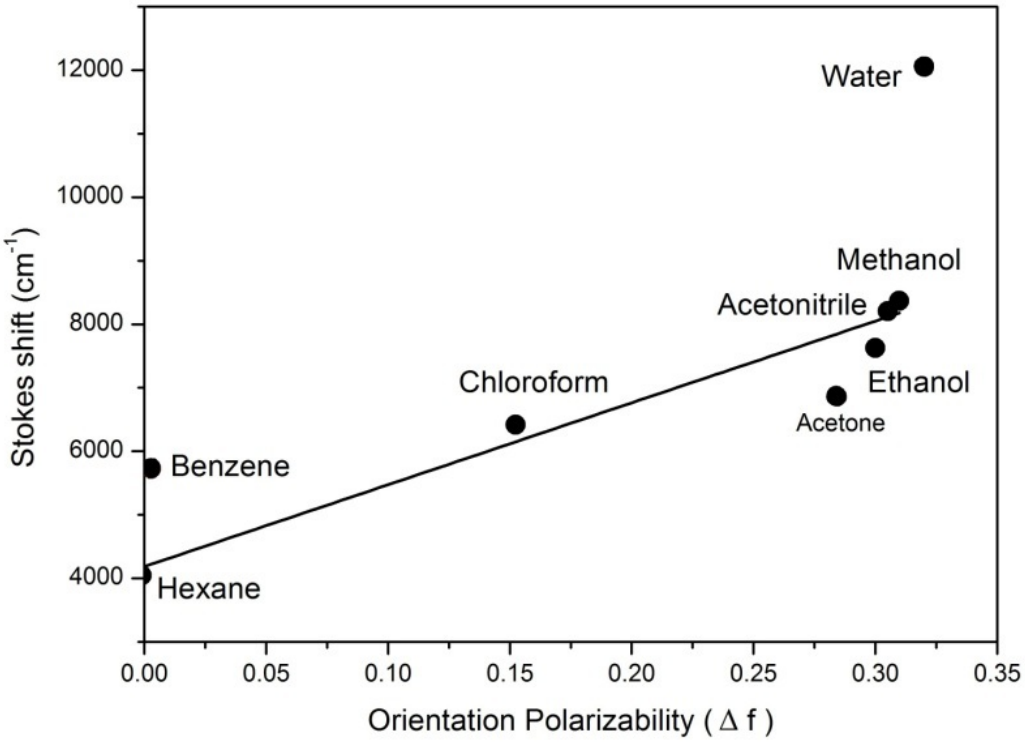


Fig.5 The plot of the Stokes shift of DAP against Orientation polarizability in different solvents [33,18]

Interestingly the average lifetime of DAP gradually increases with increase in solvent polarity except for water presumably due to the presence of other competing non-radiative decay processes (Fig.6).

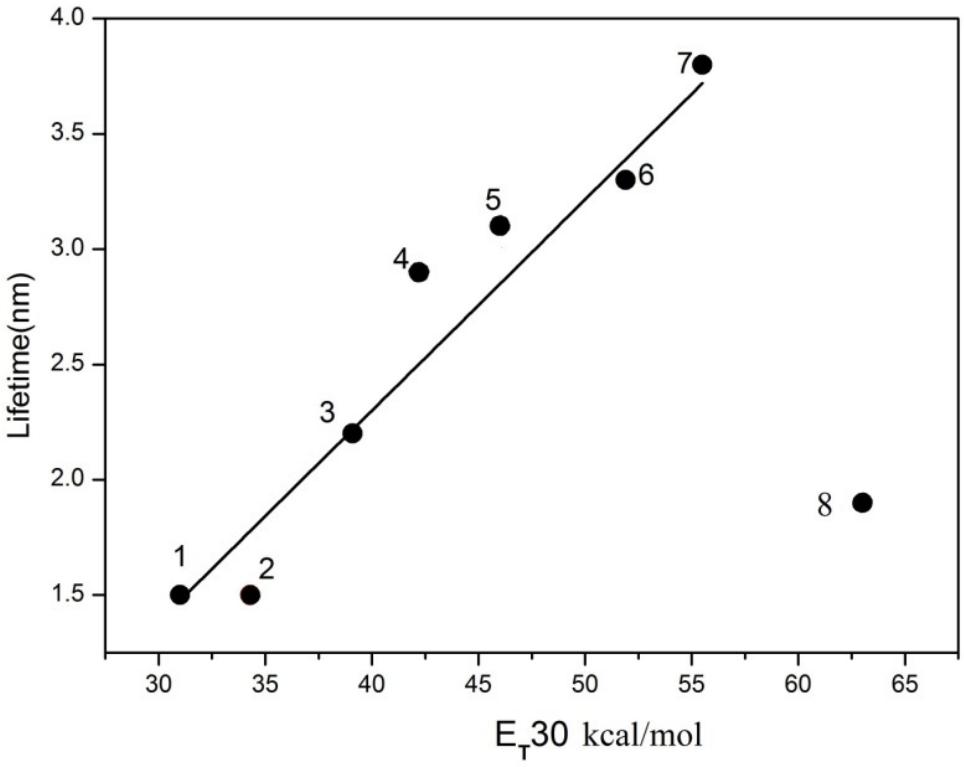


Fig.6 The plot of the average lifetime of DAP against solvent polarity parameter (E_T30) in (1) n-Hexane, (2) Benzene, (3) Chloroform, (4)Acetone, (5) Acetonitrile, (6) Ethanol, (7) Methanol, (8) Water [15,18]. The E_T (30) values are taken from references[33,34.]

Fluorescent probes having nonlinear molecular structure distorts in the excited state to form twisted intermolecular charge transfer (TICT) state[13, 36, 37]. Diew *et al.* reported the broad featureless spectra of DAP in water/acetonitrile mixture might be assigned to twisted intermolecular charge transfer in the excited state (TICT), confirming its flexible structure which was supported by MOPAC and MM2 derived force field calculations[15, 38].

SPECTRAL STUDY OF DAPOXYL SODIUM SULFONATE DYE IN DIFFERENT CYCLODEXTRINS

Several studies have established the encapsulation of the guest DAP with different host α-, β- and γ-cyclodextrins using steady-state and time-resolved fluorescence spectroscopy[15,17,18,39]. Host-guest complexation of DAP in different CDs changes not only the stoichiometry of the complex but also the photophysical properties of DAP. Use of change in emission maxima, fluorescence quantum yield and fluorescence lifetime of DAP upon CD inclusion have been extensively examined in the present study. DAP is a water-soluble fluorophore, its fluorescence intensity, as well as quantum yield in water, is very low. The steady-state emission spectra of DAP is sensitive to the nature of the CD. Large blue shift with fluorescent enhancement in the emission spectra is observed as the dye

enters into the hydrophobic cyclodextrin nanocavity from water[15]. In comparison to water, a significantly large blue shift ~ 43 nm and ~ 29 nm in the emission is observed in the 1:1 inclusion complex of DAP with α- and β-CD, respectively (Table 2). The results indicate that incorporation of the dye in the 1:1 inclusion complex with β-CD experiences much higher cavity polarity in comparison to α-CD. This may be attributed to the higher cavity volume of β-CD (262 $Å^3$) compared to the α-CD (174 $Å^3$)[39]. In comparison to water, the steady state emission spectra is strongly blue shifted (~ 94 nm) in the inclusion complex of DAP with γ-CD presumably due to the larger cavity volume of γ-CD (427 $Å^3$) and higher order complex i.e. 1:2 (γ-CD:DAP) complex [19, 39].

Table: 2 Steady state and time resolved photophysical parameters of DAP in water and in different Cyclodextrin

DAP in	Stoichiometry (Host: Guest)	λ_{ab} (nm)	λ^a_{em} (nm)	Stokes shift (cm^{-1})	Φ^b	τ_{av} (ns)
Water	-	342	582	12,058	0.19	1.9[c]
α-CD	1:1	350	539	10,018	0.70	3.8[d]
β-CD	1:1	353	553	10,245	0.61	2.5[d]
γ-CD	1:2	369	488	6608	0.68	6.1[c]

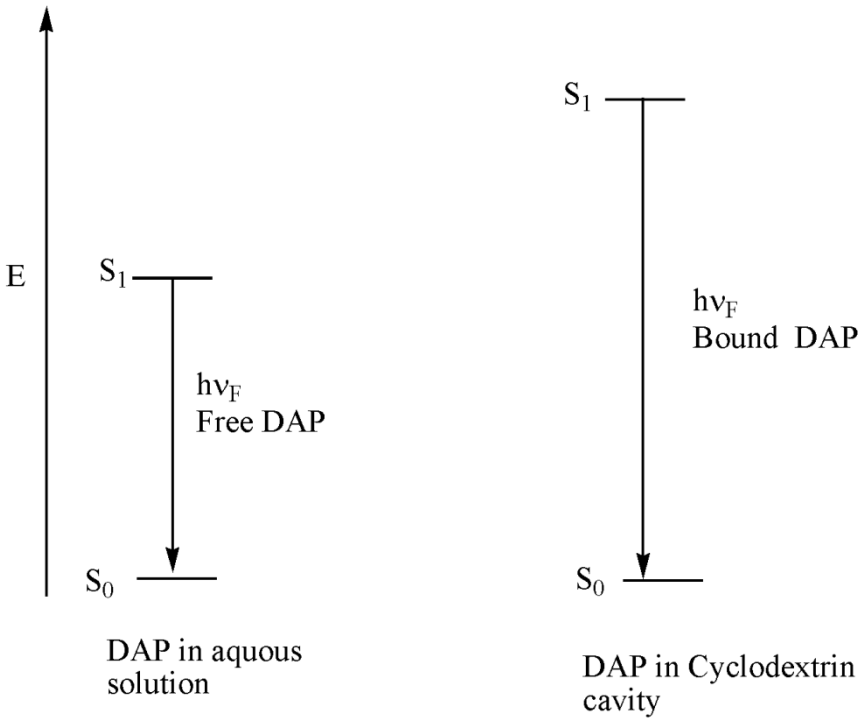
$\lambda_{ab,}$ and λ^a_{em} are wave length of absorption maximum and wave length of emission maximum, respectively.
Φ^b Relative quantum yield of fluorescence.
τ_{av} is the average lifetime at pH 7.
[a] emission maximum of charge transfer state,
[b] Quantum yield was determined with respect to 8-anilinonapthalene-1-sulphonic acid in methanol (QY = 0.21) as a reference standard [33]
[c] From ref.

A wide range of reports suggesting the polarity inside the β-CD cavity in the range of that of an alcohol solvent e.g. Hamai estimated the polarity inside the cavity as similar to 1-propanol [40], Cox *et al* and Heredial *et al*. reported the polarity inside the β-CD cavity is similar to ethanol [41,42]. Diew *et al.* reported that the polarity of DAP in its 1:1 inclusion complex with saturated β-CD solution are similar to 4:1 water-ethanol solution[15] whereas K. Pal *et al.* determined the micropolarity inside the β-CD cavity of 1:1 inclusion complex is similar to 1:1 water: methanol mixture[18]. Actually the hydrophobic environment destabilizes the polar excited state of DAP which results blue shift emission. The hydrophobic cavity of CD also reduced the flexibility of DAP and protects it from the collisional quenching, this facilitates the electron transfer from the donor to the accepter part of DAP and results in a huge enhancement of fluorescence intensity[18, 43].

Furthermore, the fluorescence quantum yield (Φ) of DAP increases significantly by 3.7 and 3.2 times upon 1:1 complexation with α- and β-CD, respectively compared to water, whereas by 3.6 times upon 1:2 (γ-CD:DAP) complex formation (Table 2). The reduced polarity inside the cyclodextrin cavity destabilizes the excited state of DAP which is polar in nature due to an intramolecular charge transfer[15] and hence energy gap between the ground state and 1st excited state (S_1 - S_0) of DAP increases on encapsulation (Scheme 2). As a consequence, significantly reduced rate

of internal conversion (k_{IC})[44] and/or the prevention of specific hydrogen bonding interaction of DAP with the surrounding water molecules results upon inclusion in CD which leads to the increase in fluorescence quantum yield[39, 43-45]. As the dye experience much higher polarity in the β-CD cavity than in the α-CD, the non-radiative deactivation rate increases in β-CD: DAP complex ($1.56 \times 10^8\ s^{-1}$) in comparison to α-CD: DAP complex ($0.78 \times 10^8\ s^{-1}$)[18]. Hence quantum yield of DAP in the 1:1 inclusion complexes with α-CD is greater than that in the β-CD (Table 2). Significant increase of quantum yield of DAP results with the γCD in 1:2 (γCD: DAP) inclusion complex in comparison to water is presumably due to the positioning of DAP in the hydrophobic environment which suppress the non-radiative rate ($0.52 \times 10^8\ s^{-1}$) in the higher order inclusion complex [19]. In addition, upon cyclodextrin encapsulation the dye DAP is screened from perturbation of the water molecules, which quenches its fluorescence due to radiation less transition induced by specific solute-solvent hydrogen bonding interactions [46].

Effects on Cyclodextrin encapsulation on guest (DAP) fluorescence



Scheme 2

STUDY OF BINDING CONSTANTS OF DAP WITH DIFFERENT (α-, β- AND γ-) CYCLODEXTRINS

DAP forms different inclusion complexes with different (α-, β- and γ-) CDs with varying stoichiometry. Effect of the cavity sizes in the association processes as well as in the strength of the host-guest interactions can be known from the binding constant values of DAP for three different

CDs. Number of contributing fluorescent component in the encapsulation of DAP with different CDs are two (in case of α-CD) and three (in case of both β- & γ-CD) reported by Granadero *et al.* from the combination of Principal component analysis (PCA) and Global analysis (GA) (i.e. PCGA) of a series of absorption and emission spectra of DAP in three different CDs. They also reported that the binding constant (K_1 in units of M^{-1}) of DAP with α-CD is 66.3±0.1, compared to 3488±14 with β-CD and 1865±250 with γ-CD[17]. K.Pal *et al.* determined the pK_a values of the DAP and its different inclusion complexes in CD (α-, β- and γ-) from the pH-dependent absorbance values using a two-state and a four-state model, respectively. They also reported K_1 values (in the units of M^{-1}) of DAP complexes with α- and β- CD are 70 and 2970, respectively at pH 7 which are obtained from 1:1 fitting equation (Table 3) and is maximum at this pH . The cavity size of γ-CD is large enough that it can form inclusion complex with DAP of higher-order stoichiometry. The binding constants (K_1 and K_2 in the units of M^{-1}) of DAP were determined to be 185 and 25, respectively at pH 7 of 1:2 (γ-CD:DAP) inclusion complex from 1:2 fitting equation[19, 47]. From PCGA analysis Granadero *et al.* reported γ-CD forms 2:1 (γ-CD: DAP) with binding constants (K_1 and K_2 in the units of M^{-1}) are 1865 ± 250 and 113.7 ± 0.8, respectively[17]. Diwu *et al.* reported the binding constant of DAP in its 1:1 β-CD inclusion complex is 5488 M^{-1} from the steady state fluorescence titration method using the excitation wavelength (365 nm) is the isobestic point [15]. This indicates the binding affinity of DAP is higher with β-CD compared to α- and γ- CD in the 1:1 complex, which implies that the dimension of DAP matches well with the apolar cavity diameter of β-CD. Sulfonate group of DAP have the strong tendency to form hydrogen bond with the hydroxyl groups of CD at the rim. Molecular modelling studies confirmed that the two wings of 'V' shaped DAP molecule having 4.4 x 10.1 Å dimension with an angle 118^0 fits well with the β-CD cavity as its cavity diameters of the narrow and wide sides are 6.0 Å and 6.5 Å respectively[15]. This implies that the dimethyl amino group rather than the sulphophenyl group of DAP fits best into the β-CD cavity. Smaller cavity diameter of α-CD (cavity diameters of narrow and wide sides are 4.7 Å and 5.3 Å respectively) cannot form strong inclusion complex with DAP whereas cavity diameter of γ-CD (cavity diameters of narrow and wide sides are 7.5 Å and 8.3 Å respectively) is large enough to forms 1:1 as well as 2:1 and 1:2 host-guest inclusion complexes with DAP[17, 19]. Thus a combination of hydrogen bonding along with hydrophobic interaction between the apolar cavity and aromatic moiety determines the structure of the DAP-CD inclusion complex.

Table: 3 Comparative study of binding constants of DAP in different Cyclodextrins

CD	Stoichiometry (CD:DAP)	Binding const. x 10^{-2} (M^{-1})
α-CD	1:1	0.70 [a,d]
β-CD	1:1	54.88 [b]
	1:1	29.70 [a,d]
γ-CD	1:2	1.85 [c,d], 0.25 [c,e]

[a] From ref. [18]
[b] From ref. [15]
[c] From ref. [19]
[d] Binding constants measured at pH 7
[e] Second order binding constant of 1:2 (CD:DAP) complex at pH 7

TIME RESOLVED FLUORESCENCE STUDIES:

Excited state fluorescence lifetime of the dye DAP serves as a sensitive parameter to probe the local environment in the cyclodextrin. DAP exhibits biexponential decays in water and in CD. Existences of several hydrogen bonded species formed with the surrounding water molecules are responsible for the biexponential decay in aqueous solution[48]. The lifetime values of DAP in different α-, β- and γ-CDs are longer than those measured in its aqueous solution (Table 2). The average lifetime values of DAP increases nearly 2 and 1.3 times upon 1:1 complexation with α- and β-CD, respectively whereas 3.6 times increase upon γ-CD in the 1:2 (γCD: DAP) inclusion complex compared to its aqueous solution[18,19]. Computational study reveals that inclusion of DAP inside β-CD is more pronounced than in α-CD where two hydrogen bond were present between the heteroatom (N and O) of oxazole ring and hydroxyl group of β-CD, in the contrary only one hydrogen bond is present between the oxygen atom of oxazole ring and one hydroxyl group of α-CD[18]. Thus the number of hydrogen bonded interaction of DAP substantially increases with the β-CD than that with the α-CD, this presumably results decrease in excited state lifetime of DAP in β-CD than in α-CD. This is also in agreement with the non-radiative deactivation rates[18]. The high value of lifetime of DAP in the 1:2 (γCD: DAP) inclusion complex indicates that the dye is tightly encapsulated and well shielded from the aqueous environment and stabilised by the hydrogen bonding which decrease the non-radiative deactivation rate.

pH DEPENDENT FLUORESCENCE PROPERTIES AND BINDING AFFINITY OF DAP WITH DIFFERENT CYCLODEXTRINS

Cavity size of the cyclodextrin as well as pH of the medium also plays an important role on the binding strength, fluorescence intensity and lifetime of DAP with different host CDs. DAP exhibits biexponential decay in aqueous solution and in different inclusion complexes with α-, β- and γ-CD which may be attributed to the existence of the two emissive states of the probe: locally excited

state (LE) and charge transfer state (CT)[18, 19]. Intensity of these bands changes on complexation of DAP with CD in different Ph[18,15]. Fluorescence lifetime which enhance upon encapsulation of DAP is also changes on the pH of the medium (Table 4) and is maximum at pH 7. Lifetime associated with the CT band is higher compared to the LE band in the same pH[18] presumably due to the strong quenching of the non-radiative deactivation process promoted by the water molecules in case of CT state.

Table: 4 Average lifetime of DAP[a] in water and in different Cyclodextrins

DAP in	Host: Guest Stoichiometry	τ_{av} (ns)			
		pH 2	pH 4	pH 7	pH 9
Water	-	2 [b]	1.9 [b]	1.9 [b]	2.1 [b]
α-CD	1:1	3.8 [c]	3.8 [c]	3.8 [c]	3.8 [c]
β-CD	1:1	2.2 [c]	2.5 [c]	2.5 [c]	2.5 [c]
γ-CD	1:2	5.5 [b]	5.9 [b]	6.1 [b]	5.7 [b]

[a] 4μM DAP was used for lifetime measurement with 40 mM α-CD [c], 15 mM β-CD [c] and 35 mM γ-CD [b]. τ_{av} is the average lifetime of CT state (λ_{ex} = 375 nm). [b] From ref. [19] [c] From ref. [18]

The binding constants of DAP in different CDs are also highest at neutral pH (Table 5). The pK_a value of DAP in water is about 4.2[11]. So at lower pH (i.e, below the pK_a value of DAP in water) the lone pair of N atom of dimethyl aniline moiety of the guest DAP is partially or fully protonated. But at neutral or basic medium (where pH greater than the pK_a value) the guest DAP is not protonated and facile charge delocalization between the dimethylamino moiety and the sulfonyl group are possible. Thus at higher pH only one fluorescence band due to the CT state was observed whereas at lower pH two emission bands (one band corresponds to the LE state, and the other band which corresponds to the CT state) was observed. At higher pH the host CD likely to encapsulate the neutral DAP due to hydrophobic interaction. Thus the binding constants of DAP in different CDs (α-CD, β-CD and γ-CD) are higher at neutral or basic medium than the acidic medium [18,19].

Table: 5 pH dependent binding constant of DAP in different Cyclodextrins

CD	Host: Guest Stoichiometry	Binding constants x 10^{-2} (M^{-1})[a]			
		pH 2	pH 4	pH 7	pH 9
α-CD	1:1	0.15 [b]	0.35 [b]	0.70 [b]	0.65 [b]
β-CD	1:1	4.00 [b]	9.25 [b]	29.70 [b]	29.10 [b]
γ-CD	1:2	0.12 [c] 0.05 [c,d]	0.55 [c] 0.20 [c,d]	1.85 [c] 0.25 [c,d]	1.55 [c] 0.15 [c,d]

[a] range of CD concentrations used are 0-5 mM with constant DAP (5 µM) for binding titration. [b] From ref. [18] [c] From ref.[1]
[d] indicates 2nd order binding constant values.

CONCLUTION AND FUTURE SCOPE OF STUDY OF THE DAPOXYL DYE

The present study represents the perspectives exemplifying novel uses of the steady state and time resolved intrinsic fluorescence of Dapoxyl dyes for probing and characterizing their interactions with cyclodextrins. Attention is focussed on novel uses of fluorescent probes as their own 'reporters' for probing and characterizing their interactions with cyclodextrins One can envision that expanding applications of this promising new approach would open the door for the study of Dapoxyl dyes with macromolecules like proteins and DNA. Complementary use of other experimental biophysical (spectroscopic as well as calorimetric) techniques and theoretical (molecular modelling) studies would permit detailed assessment of the role of structure and substitution patterns of these probes on their affinities and binding modes to their targets. Kinetic studies using fluorescent displacement assay may also be perform to analyze different drug binding, various enzyme activities monitoring the change of fluorescence which results from the competition of the drugs, enzymetic product and the Dapoxyl dye for forming a complex with different macrocylic receptors.

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