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Steady-state and time-resolved emission studies of Thioflavin-T

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ABSTRACT

Steady-state absorption and emission properties of 3,6-dimethyl-2(4-dimethylaminophenyl)-benzothiazolium cation (ThT^+) were studied at room temperature in variety of solvents. The multifluorescence of this dye was observed in different environmental conditions. The predominant emission bands observed are around 400 nm (I band), 450 nm (II band) and 485 nm (III band). The fluorescence emission bands I and II observed in acidic, basic and aqueous solutions are for 350 nm excitation and band III for 390 nm excitation at low concentration (1×10^{-5} mol dm^{-3}). However, the emission band at 520 nm was observed in all solvents at high concentration (1×10^{-2} mol dm^{-3}). The emission band around 400 nm shifts from 400 nm to 470 nm (shift 70 nm) when the excitation wavelength is varied from 350 nm to 380 nm and the emission at 485 nm shifts from 485 nm to 520 nm (shift 35 nm) for excitation wavelength 390–420 nm. Fluorescence decays of this dye in various solvents monitored over each emissions showed a tri-exponential behavior and the analysis yielded three decay components, respectively, in the range 0.13–0.76 ns, 1.08–2.00 ns and 2.50–12 ns. Based on the steady-state and time-resolved emission measurements band I (400 nm) is assigned due to protonation, band II (450 nm) locally excited state (LE) and the band III (485 nm) due to dimerization via H-bonding. Time-resolved fluorescence anisotropy measurements as a function of viscosity were carried out over emissions 450 nm and 550 nm and were fitted to single exponential. The shorter reorientation times (56–332 ps) at emission maximum 450 nm were compared with the theoretical values estimated using the hydrodynamic model (SED model) and are attributed to free rotations of the molecule. However, longer reorientation times (188–726 ps) at emission maximum 550 nm were assigned to dimerization/higher aggregates of the solute molecule. The deviations of experimentally measured τ_r values at emission maxima 450 nm with those of theoretically estimated values were discussed.

1. Introduction

The ThT^+ belongs to an important class of fluorescent molecule which is widely used in biological and biophysical studies [1–4]. Due to its biological importance an extensive work on this molecule were carried out by several researchers [1–8]. The interaction of ThT^+ with DNA and clays has been reported [9]. The γ-CD induced dimer emission of ThT^+ and its "off–on" control in the presence of an acid have been reported in the literature [10,11]. It is believed that an increase in quantum yield upon binding to amyloid fibril results from the inhabitation of this free rotation of the rings. In free ThT^+, when the rotation is not hindered, excited ThT^+ molecule can undergo a torsional relaxation which effectively competes with the radiative transition. The theoretical work related to internal rotation of molecular groups/fragments were carried out by several researchers and they suggested that the internal rotations of molecular groups/fragments are associated with intra-molecular charge transfer processes [12–14]. However, not much experimental work was carried out by the experimental researchers to verify these theoretical findings. Therefore, a desire to get better understanding about the structure and molecular properties of ThT^+ in the ground and excited state has motivated us to study in detail about the mechanisms involved. In the present work, the photophysical properties of ThT^+ have been investigated in detail in a large number of organic solvents using steady-state absorption, fluorescence excitation and emission under varying conditions of concentration and excitation wavelength. The time-resolved fluorescence measurements were carried out to identify the emitting species.

2. Experimental

The ThT^+ was obtained from Merck and used without further purification. All solvents were of spectroscopic grade (Fluka) and were used as received. The purity of the solvents was checked before use by HPLC–MS method. The absorption spectra were

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recorded using Hitachi Model U-3010/U-3310 spectrophotometer and fluorescence measurements were made using Hitachi model F-7000 fluorescence spectrophotometer. Time-resolved fluorescence measurements were carried out using a picosecond operated time-correlated single photon counting unit, which is described elsewhere [15]. In the present work, a 373 nm Nano LED (373 nm, 1.2 ns, 1 MHz) and 408 nm diode laser (<100 ps, 1 MHz) were used as the excitation light source and a TBX4 detection module (IBH) coupled with a special Hamamatsu PMT was used for fluorescence detection. For the present setup, the instrument response was ~1.2 ns (~240 ps) at FWHM. Fluorescence decays were recorded with a vertically polarized excitation beam and fluorescence was collected at magic angle 54.7° and was analyzed by a deconvolution method.

3. Results and discussion

3.1. Electronic absorption, fluorescence excitation and emission studies

The absorption spectrums of ThT^+ in various solvents including water, acidic and basic solutions were measured at low concentration (1×10^{-5} mol dm^{-3}). The absorption spectrum consists of two band systems in the region 250–340 nm and 350–450 nm with peak positions at about 310 nm (weak) and 412 nm, respectively, in acidic, basic and water solutions, whereas the peak positions of absorption maxima in all other solvents is around 418 nm. The absorption band has the shortest wavelength maxima (~412 nm) in water solution and the longest wavelength maxima (421–430 nm)

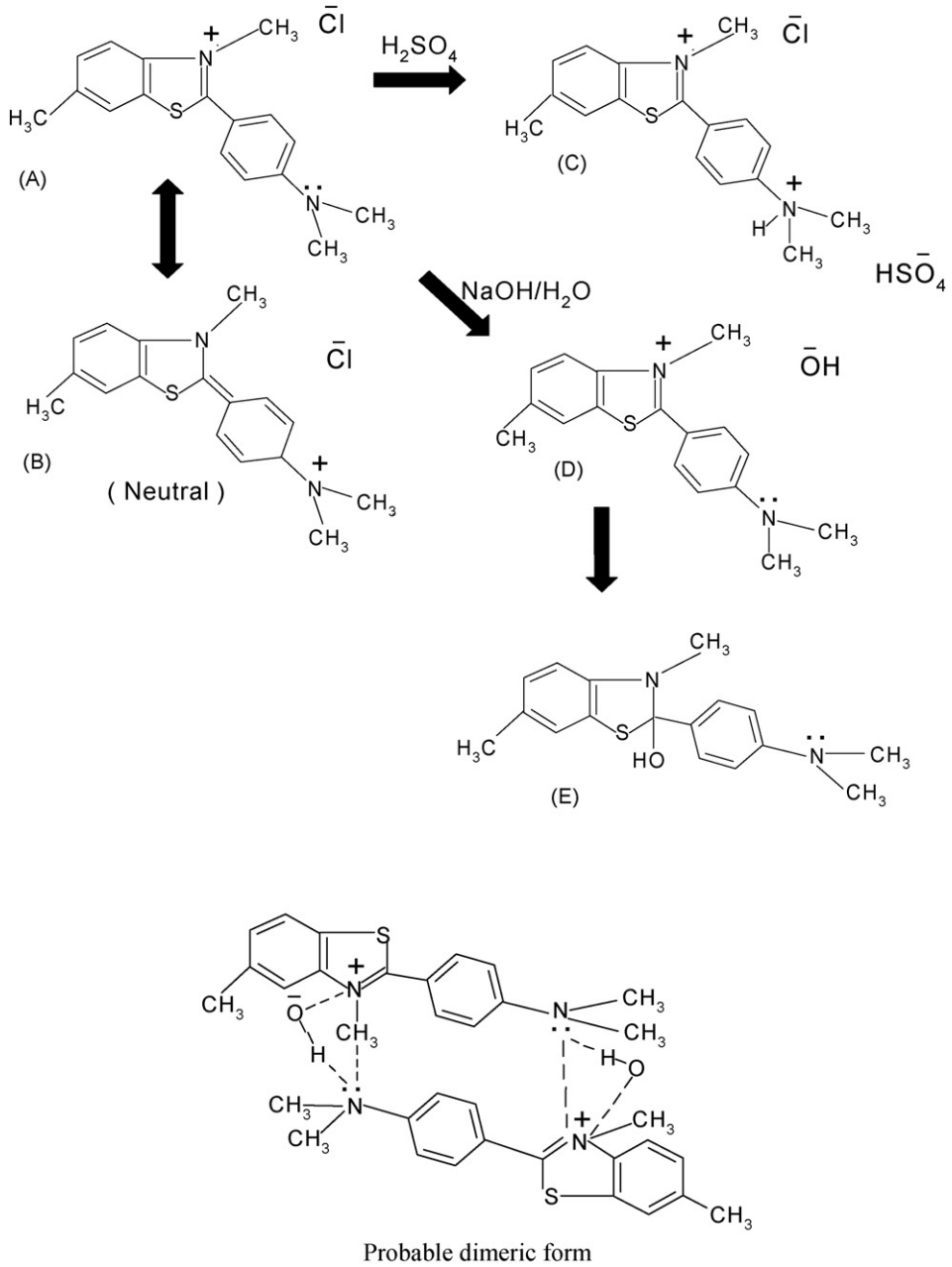

Fig. 1. The possible resonance structure of the ThT^+.

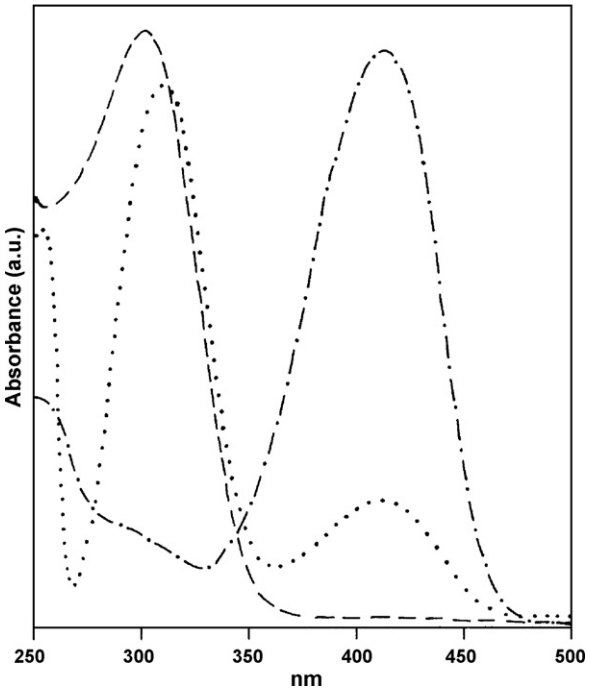

Fig. 2. Absorption spectra of ThT in (a) water (–·–), (b) 2N H_2SO_4 (···) and (c) 2N NaOH (---) at 1×10^{-5} mol dm^{-3}.

in ethylene glycol, chloroform and dichloromethane solutions. The dominant band at 412 nm in aqueous solution and polar solvents is assigned to monomer. The ThT^+ molecule undergoes protonation in acidic solution and an absorption band 310 nm was observed for the protonated ThT^+ molecule ($ThTH^{2+}$) [9]:

$$ThT^+ + H^+ = ThTH^{2+} \quad (1)$$

In basic solution the intensity of absorption band at 410 nm decreases whereas intensity of the absorption band at 300 nm increases enormously. In almost all solvents no change in absorption band is observed except broadening of the spectra as a function of concentration (10^{-6} to 10^{-4} mol dm^{-3}). The above results are explained in terms of the possible resonance structure of the molecule as shown in Fig. 1. The predominant form of ThT^+ (Fig. 1A) is the quinonoid structure (Fig. 1B) which accounts for the longest wavelength band at 412 nm (Fig. 2a). In 2N H_2SO_4 the dimethyl amino group gets protonated preventing the delocalization of the nitrogen lone pair which is shown in structure (Fig. 1C). This accounts the weak band at 412 nm (Fig. 2b). In 2N NaOH the corresponding hydroxide partially prevents the delocalization of the nitrogen lone pair by a probable attack on the C-2 cation of benzothiazole (Fig. 1E) which hinders the delocalization. This accounts for the reduced intensity of the 412 nm band (Fig. 2c). Absorption maxima, excitation maxima and emission are listed in Table 1 for all solvents. It is to be noted that ThT^+ exhibited large red shift in the emission maxima compared to the shift in absorption which indicates solvation is large in S_1 state as compared to ground state. The excitation spectra recorded with the emissions monitored at the emission maximum (450 nm and 485 nm) show two distinct bands at 340 nm and 360 nm, confirming the presence of two distinctly different species in all solvents used. Fluorescence quantum yields (ϕ_f) in different solvents were estimated at room temperature and listed in Table 1. It is seen that the ϕ_f values in water and acidic solutions drastically lower in comparison to those in other solvents and still maximum in basic solutions. We attribute this reduction of ϕ_f values in water and acidic solutions to the intermolecular H-bonding effect/protonation. The molecule is more fluorescent in basic solution as compared to in acidic solutions is due to deprotonation of the molecule. The dual emission was observed in different environmental conditions in all solvents used. In order to understand the existence of the dual emissions of ThT^+ in different solutions the effect of concentration and excitation wavelength has been investigated and the result shows concentration and excitation wavelength dependence. The predominant emission bands observed are around 400 nm (I band), 450 nm (II band) and 485 nm (III band). The fluorescence band I was observed in n-butanol, chloroform, dichloromethane and acidic solutions, whereas band II was observed in basic and other solutions at low concentration for 350 nm excitation (1×10^{-5} mol dm^{-3}). However, band III was observed in all solvents for 418 nm excitation. The emission band around 400 nm shifts from 400 nm to 470 nm (shift 70 nm) when the excitation wavelength is varied from 350 nm to 380 nm and the emission at 485 nm shifts from 485 nm to 520 nm (shift 35 nm) for excitation wavelength 390–420 nm in all solutions (Fig. 3a). These findings clearly indicate that these two emissions originate from different species originally formed in the ground/excited states. When the concentration is increased from 10^{-6} mol dm^{-3} to 10^{-2} mol dm^{-3}, there is a continuous shift of emission maxima from 400 nm to 520 nm (Fig. 3b). In alcoholic, aqueous, acidic and basic solutions the emission band observed around 400 nm and 450 nm is assigned to protonation and deprotonation/LE state of the molecule; however, the band around 485 nm is ascribed to dimerization via H-bonding. In addition, overlapping of absorption spectra with emission spectra is in favor of charge transfer character of the electronically excited state. With increasing concentration emission bands are red shifted and the intensity of the bands is enhanced. However, with the further increase in the concentration, intensity is reduced and the emission bands are considerably red shifted. The continuous shift of emissions as a function of concentration is due to the formation dimer/higher aggregates [16,17].

3.2. Determination of excited state dipole moment

Lippert-Mataga's formula [18] has been used in our investigations to determine the dipole moments of the excited states by the

Table 1
Absorption maxima, excitation maxima, fluorescence emission maxima and relative quantum yield of ThT in various solvents at 1×10^{-5} mol dm^{-3}.

Solvents	Absorption maxima (nm)	Excitation maxima (nm) at emissions 400 nm and 485 nm		Emission maxima (nm) for excitation 350 nm and 418 nm		Relative quantum yield (ϕ_f)
n-Propanol	417	357	363	438	501	0.28
Methanol	416	358	361	434	487	0.25
Acetonitrile	415	351	360	434	487	0.20
DMSO	418	361	363	438	501	0.31
n-Butanol	415	354	360	410	478	0.26
Chloroform	424	360	371	400	481	0.33
Dichloromethane	430	354	360	400	484	0.32
Water	412	345	352	444	489	0.05
Water + 2N H_2SO_4	310 and 410	315	315	410	487	0.06
Water + 2N NaOH	300 and 418	353	360	436	487	0.50

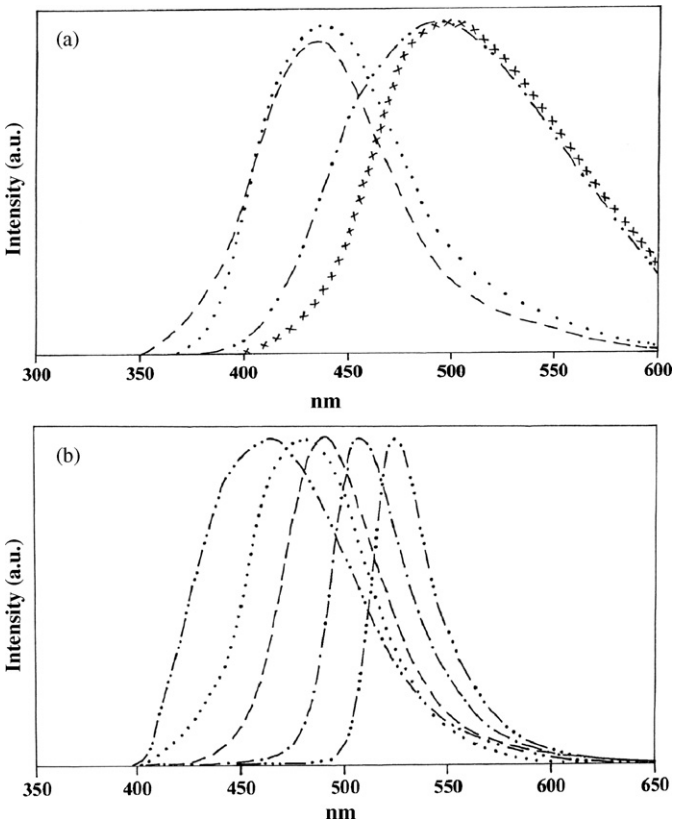

Fig. 3. (a) Fluorescence emission spectra of ThT in methanol for excitation (1) 350 nm (---), (2) 380 nm (···), (3) 400 nm (–··–) and (4) 420 nm (× × ×) (at 1×10^{-5} mol dm^{-3}). (b) Fluorescence emission spectra of ThT in methanol in various concentrations for λ_{ex} 350 nm (1) 1×10^{-6} (–··–), (2) 1×10^{-5} (···), (3) 1×10^{-4} (---), (4) 1×10^{-3} (–·–) (5) 1×10^{-2} (–···–) (mol dm^{-3}).

solvatochromic shift method which is

$$\Delta\bar{\nu} = \bar{\nu}_a - \bar{\nu}_f = \frac{2F_1(\mu_e - \mu_g)^2}{hca^3} \quad (2)$$

where $\bar{\nu}_a$ and $\bar{\nu}_f$ are the absorption and emission maxima wave numbers in cm^{-1}, respectively, μ_g and μ_e are the dipole moments of the ground and excited states, respectively, and

$$F_1(\varepsilon, n) = \left[\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right]$$

where ε is the dielectric constant of the solvent, n is the refractive index of the solvent. According to the Suppan equation [19] the value of the solute cavity radius a was calculated from the molecular volume of ThT$^+$ and was found to be 4.11 Å. The solute–solvent properties and solvent polarity function F_1 are collected in Table 2. The dielectric constants and the refractive indices of the solvents at 25 °C are taken from the literature [20]. Fig. 4 shows correlation between Stoke's shift and solvent polarity function. The poor

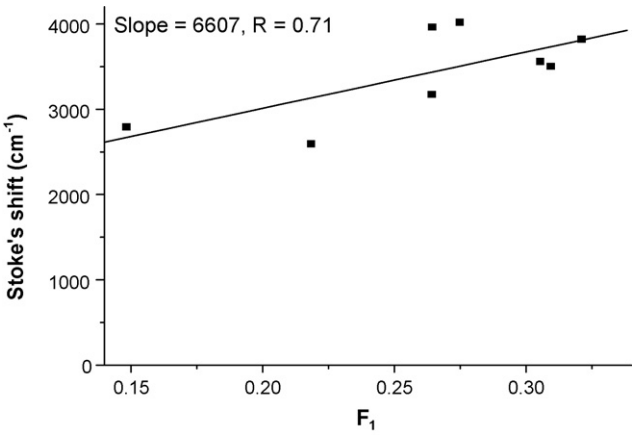

Fig. 4. Plot of Stokes shift versus F_1, according to Lippert-Mataga relation.

correlation ($R = 0.71$) and scattering points suggest specific interactions between solute and solvent molecule (in general, H-bonding). The change in dipole moment $\Delta\mu$ ($\mu_e - \mu_g$) = 6.75 D estimated using Lippert-Mataga's equation is greater than the theoretically estimated values ($\Delta\mu = 4.8 \pm 0.5$ D) [12]. Substituting the value of ground state dipole moment μ_g from Ref. [12] in Eq. (2), the excited dipole moment was obtained to be about 10.55 D. This higher value of estimated excited state dipole moment provides the evidence for the formation of dimers via H-bonding.

3.3. Fluorescence decays

In order to understand the mechanism involved in ThT$^+$ molecule responsible for unusual behavior of florescence emission, the fluorescence decays in all solvents has been studied for each emission maxima. Fig. 5 shows fluorescence decay of ThT$^+$ in n-propanol at room temperature monitored at emission 450 nm. The fluorescence decays measured at three emission wavelengths, 400 nm, 450 nm and 500 nm keeping the excitation wavelength at 373 nm was fitted to a tri-exponential function:

$$I(t) = a_1 \exp\left(\frac{-t}{\tau_1}\right) + a_2 \exp\left(\frac{-t}{\tau_2}\right) + a_3 \exp\left(\frac{-t}{\tau_3}\right) \quad (3)$$

where τ_1, τ_2 and τ_3 are the lifetimes of the three decay components and a_1, a_2 and a_3 are their respective amplitudes such that $a_1 + a_2 + a_3 = 1$. The fluorescence lifetimes thus estimated in different solvents are listed in Table 3. It is seen that in all these solvents, the decay parameters depend on the emission wavelength and nature of the solvent. The fluorescence lifetime of the first component is shorter and varies between 0.13 ns and 0.76 ns (a_1 varies 3–20%) in the solvents used at all emissions; however, the fluorescence lifetime of the second component at all emissions is predominant and varies from 1.08 ns to 2.00 ns (a_2 varies 40–98%) in almost all solvents. The fluorescence lifetimes of the third component is longer and vary between 2.50 ns and 12 ns (a_3 varies 6–40%). In n-propanol and acetonitrile solutions first decay component has

Table 2
Solute–solvent properties, polarity functions and Stoke's shift of ThT$^+$.

Solvents	Dielectric constant (ε) at 25 °C	Refractive index (n) at 25 °C	Polarity function, F_1	$\bar{\nu}_a$ (cm^{-1})	$\bar{\nu}_f$ (cm^{-1})	$\bar{\nu}_a - \bar{\nu}_f$ (cm^{-1})
Chloroform	4.81	1.444	0.1482	23585	20790	2795
Dichloromethane	8.93	1.424	0.2184	23255	20661	2594
n-Butanol	17.51	1.397	0.2642	24096	20921	3175
n-Propanol	20.10	1.383	0.2748	23981	19960	4021
Methanol	32.63	1.326	0.3094	24038	20534	3504
Acetonitrile	35.94	1.342	0.3054	24096	20534	3562
Dimethyl sulfoxide	48.89	1.477	0.2644	23923	19960	3963
Water	78.54	1.331	0.3211	24272	20449	3823

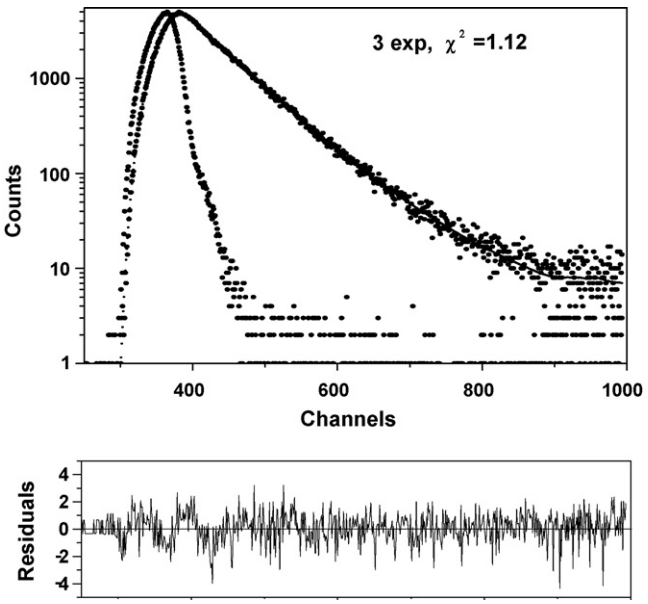

Fig. 5. Fluorescence decay of ThT⁺ in n-propanol at 373 nm excitation monitored at 450 nm emission (at 1×10^{-5} mol dm^{-3}) [τ_1 = 0.13E−9 s (−6.07), τ_2 = 1.66E−9 s (97.59) and τ_3 = 3.98E−9 s (8.48)].

negative pre-exponential term, second and third decay components have positive pre-exponential terms for emissions 400 nm and 450 nm. Pre-exponential factors are generally positive but can be negative whenever there is an excited state kinetics. A negative pre-exponential factor indicates the formation of an emissive species in the excited state reactions [21]. As can be seen from the table, the contribution of three decay components for each emission maxima indicates the overlapping of the states. The shorter lifetime of first decay component is assigned to protonation, second component was assigned to monomer state and the longer third decay components were assigned to dimerization via H-bonding but not due to twisted intra-molecular charge transfer state (TICT) as is favorable for ortho substitution. Whereas the present compound has a donor site at para position, hence a dimeric form obtained by 180° rotation is favored, since it allows columbic force of attraction as well as intermolecular H-bonding in aqueous and aqueous alkaline media (Fig. 1). For each emission maxima the decay components with their relative amplitudes in all solvents indicates their relative probability of contribution to the total lifetime. The contribution of emission band II at 450 nm due to monomer is more in dichloromethane, butanol, chloroform, DMSO and distilled water as compared to the emission at 485 nm due to dimerization. In the present investigation the fluorescence lifetimes of the bands observed around 485–500 nm are ascribed to dimerization via H-bonding.

3.4. Fluorescence anisotropy

In order to study more detail about dimer/aggregate formation and to understand whether the rotational motion of ThT⁺ could play a significant role in the fluorescence spectroscopy, reorientation times of this molecule were measured in a number of polar solvents. In all the solvents the anisotropy could be fitted to a single exponential. The fluorescence anisotropy $r(t)$ is defined as

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I(t) + 2I_{\perp}(t)} \quad (4)$$

where $I_{\parallel}$ and $I_{\perp}$ are parallel and perpendicular decays prior to deconvolution. The simultaneous analysis of the parallel and perpendicular components in which $r(t)$ the deconvoluted anisotropy decay was fit to a single exponential form:

$$r(t) = r_0 \exp\left(\frac{-t}{\tau_r}\right) \quad (5)$$

where r_0 is the amplitude at zero time, and based on the hydrodynamic model (SED Model), which works quite well for many similar dye molecules, one would anticipate the rotational anisotropy of ThT⁺ to decay as a single exponential. According to Stoke–Einstein–Debye (SED Model) the rotational diffusion time is

Table 3
Fluorescence lifetimes of ThT⁺ at room temperature in various solvents at 1×10^{-5} mol–dm^{-3} at emissions 400 nm, 450 nm and 500 nm.

Solvents	At emissions (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{av} (ns)	χ^2
	400	0.14 [−9.68]	1.61 [96.85]	3.24 [12.83]	1.96	1.10
n-Propanol	450	0.13 [−6.07]	1.66 [97.59]	3.98 [8.48]	1.95	1.23
	500	0.76 [18.52]	1.99 [81.48]		1.76	1.29
	400		1.72 [94.01]	5.15 [5.99]	1.93	1.27
Methanol	450		1.68 [92.66]	3.87 [7.34]	2.51	1.17
	500	0.39 [2.37]	1.88 [89.43]	5.84 [8.20]	1.68	0.98
	400		1.65 [60.41]	6.07 [39.59]	3.39	1.19
Dichloromethane	450		1.61 [60.61]	6.86 [39.39]	3.68	1.09
	500	0.22 [21.47]	1.78 [40.58]	8.62 [37.95]	4.04	1.04
	400		1.56 [80.60]	2.58 [19.40]	1.76	1.14
n-Butanol	450		1.55 [82.44]	2.77 [17.56]	1.76	1.23
	500	0.36 [15.11]	1.86 [84.89]		1.63	1.16
	400		1.17 [94.55]	4.01 [5.45]	1.32	1.38
Chloroform	450		1.08 [89.59]	4.48 [10.41]	1.43	1.42
	500	0.24 [13.24]	1.39 [42.61]	5.63 [44.15]	3.10	1.14
	400		1.68 [62.42]	11.73 [37.58]	5.45	1.51
Dimethyl sulfoxide	450		1.65 [75.26]	11.50 [24.74]	4.08	1.11
	500	0.42 [3.53]	1.91 [85.98]	10.45 [10.49]	2.75	1.05
	400	0.22 [−7.57]	1.58 [90.34]	6.23 [17.23]	2.48	1.44
Acetonitrile	450	0.16 [−10.72]	1.59 [96.82]	6.18 [13.91]	2.41	1.03
	500		1.94 [78.15]	7.39 [21.85]	3.13	1.21
	400	0.17 [41.22]	1.32 [44.76]	6.54 [14.02]	1.58	1.21
Distilled water	450		1.25 [70.69]	6.69 [29.31]	2.84	1.28
	500	0.46 [19.48]	1.86 [38.54]	7.03 [41.98]	3.76	1.07

Table 4
Reorientation time (τ_r) and calculated relaxation times (τ_{SED}) of ThT$^+$ in various solvents for two emissions (at 1×10^{-5} mol dm^{-3}).

Solvents	Viscosity, η (mPa s)	τ_r (ps) at emission 450 nm	τ_r (ps) at emission 550 nm	r_0	τ_{SED} (ps)
Acetonitrile	0.335	56	188	0.224	47
Dichloromethane	0.393	61	226	0.209	55
Chloroform	0.542	77	245	0.332	76
Methanol	0.547	79	262	0.266	77
Water	1.030	142	342	0.326	145
Dimethyl sulfoxide	1.078	149	547	0.354	152
n-Propanol	2.250	297	705	0.276	316
n-Butanol	2.300	332	726	0.287	324

given by

$$\tau_D = \frac{\eta V}{kT} \quad (6)$$

where η is the bulk viscosity of the solvent, V is the molecular volume of the solute calculated from the method of van der Waals increments, k is the Boltzmann constant and T is the absolute temperature. This relation is considered to be valid when the rotating molecule is spherical in shape and very large compared to the solvent molecule, which is treated as a structureless continuum.

Since in reality, molecules do not have spherical shapes, they are modeled as prolate and oblate ellipsoids [22], the modified relation is

$$\tau_D = \frac{\eta V f C}{kT} \quad (7)$$

where f is a molecular shape factor ($f > 1$) and C is a factor describing the boundary condition. Both f and C are functions of the axial ratio $\rho = b/a$ ($2a$ = length of shorter axis, $2b$ = length of longer axis). This condition imposed is generally referred to as the "stick" boundary condition. Under this condition it is assumed that the first layer of the solvent molecule enclosing the solute 'sticks' to the solute such that the relative velocity between the boundaries vanishes. In other words, as the molecule moves through the solvent, it must overcome both the forces involved in the displacement of the solvent and those involved in the viscosity shear near the surface. Thus size of the solute could be larger due to the binding of the solute to its nearest neighbors on a time scale comparable to the reorientation time. The specific solute–solvent interaction-like hydrogen bonding can result in an enhancement of the solute volume with an attendant increase in friction to molecular rotation. In view of this the ThT$^+$ molecule can be approximated as rectangular volume of dimensions 16.6 Å × 7 Å × 5 Å = 577.5 (Å)^3 [3]. And due to the increased effective size of the cation, its behavior would be different from that of neutral form. As reported earlier, if a sphere is deformed into a cylinder, because of solvent attachment via H-bonding/dimerization reorientation time becomes too slow [23,24] that is what we have observed for 550 nm emission. The results of rotational reorientation times, which are the average of at least three measurements in each case, for various solvents are listed in Table 4. From the table it is seen that the reorientation time of ThT$^+$ at 450 nm emission is parallel to the values obtained from SED model, whereas the reorientation time for 550 nm emission is more than two times slower. Thus when the ThT$^+$ molecule is excited to S_1 state one can distinguish two relaxation processes in the excited state. The shortest and longest relaxation time, the shortest relaxation time is ascribed due to LE state and the longest relaxation time is ascribed due to the formation of molecular aggregates. Thus this process results the increase in the volume of the ThT$^+$ molecule and is known to be accompanied by the spectral shift towards the longer wavelength. In contrast to the earlier report [14], our experimental results on steady-state measurements, fluorescence decays and reorientation times the fluorescence bands at 400 nm, 450 nm and at 480–500 nm regions are assigned due to protonated, LE and dimerized via H-bonding, respectively. However, the emission band around 550 nm at high concentration is ascribed to higher aggregates.

4. Conclusion

The multifluorescence of ThT$^+$ shows solvent polarity, concentration and excitation wavelength dependence. Emission bands I (400 nm), II (450 nm) and III (485 nm) are observed at different environmental conditions. Based on the steady-state and lifetime measurements, three different types of electronic states are inferred and those are due to protonated, the LE state and the dimerized state of the molecule. Longer decay components ($\tau_f \sim 2.50$–12 ns) were assigned to dimerization via H-bonding but not due to twisted intra-molecular charge transfer state (TICT) as is favorable for ortho substitution. Whereas the present compound has a donor site at para position, hence a dimeric form obtained by 180° rotation is favored, since it allows Coulomb force of attraction as well as intermolecular H-bonding in aqueous and aqueous alkaline media. Fluorescence anisotropy measurements monitored over 450 nm emission confirms that ThT$^+$ behaves as molecular rotor and also an increase in fluorescence anisotropy when monitored over the emission 550 nm favors to the formation of dimer/higher aggregates. Thus the longer reorientation time at 550 nm emissions as compared to shorter reorientation time at emission 450 nm due to monomer favors aggregate formation at high concentration.

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