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ORIGINAL PAPER

Fluorescence Origin of 6,P-toluidinyl-naphthalene-2-sulfonate (TNS) Bound to Proteins

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Abstract 6,P-toluidinylnaphthalene-2-sulfonate (TNS) is a highly fluorescent molecule when dissolved in a low polarity medium or when bound to proteins. The aim of the present work is to explain origin of this fluorescence, to find out how the medium (solvent, protein matrix) affects fluorescence observables such as lifetimes and spectra and finally to put into evidence possible relation that exists between these observables and fluorophore structure. To achieve our goal we performed studies on TNS dissolved in ethanol, at high concentrations in water (aggregated form) and bound to proteins. Our experiments allowed us to find out that TNS in the three environments has different structures. Presence of three lifetimes observed in proteins and in water instead of one lifetime found in ethanol can be assigned to the high contact between TNS molecules. Our results are discussed in terms of solvent polarity and interaction within fluorophore molecules bound to proteins.

Keywords 6,P-toluidinylnaphthalene-2-sulfonate (TNS) · Emission spectrum · Excitation spectrum · Fluorescence lifetime · Solvent polarity · Solvation dynamics · Interaction between fluorophore molecules

Introduction

6,P-toluidinyl-naphthalene-2-sulfonate (TNS) (Fig. 1) is a fluorescent probe widely used to study the interaction between two proteins, a protein and its ligands and to follow structure and dynamics modification within proteins and membranes [1–6]. TNS, such as many fluorophores, is characterized by fluorescence properties that are dependent on the surrounding environment. For example, a hydrophobic environment will yield a fluorescence emission spectrum whose peak is located at short wavelength compared to a hydrophilic environment. TNS fluorescence is very weak in water, almost non-existent, and increases when the fluorophore is bound to a protein or dissolved in a hydrophobic medium. The intense fluorescence of TNS observed in these media was always explained by the specific interaction between fluorophore, protein matrix and hydrophobicity increase of the fluorophore microenvironment. However, up to now, there are no studies describing origin of TNS fluorescence.

In the present work, we recorded TNS fluorescence spectra (excitation and emission) and we measured its fluorescence lifetimes dissolved in ethanol, in water or bound to proteins. Fluorescence excitation spectrum characterizes electron distribution of the molecule in the ground state and thus gives a clear indication of the state of the molecule before excitation. On the other side, emission spectrum will characterize electron distribution of the molecule in the excited state which is in general different from the ground state. Therefore, recording excitation and emission spectra of a fluorophore in different solvents would yield information on its structure within these solvents and would allow detecting structural modifications and differences.

Fluorescence lifetimes occur from the excited state and thus could be dependent on the fluorophore environment (solvent and nature of the macromolecule where the fluorophore is bound.) Finally, it is worth to indicate that the Jobin-Yvon fluorometer used allows measuring lifetimes with a very high precision and the intensity of the

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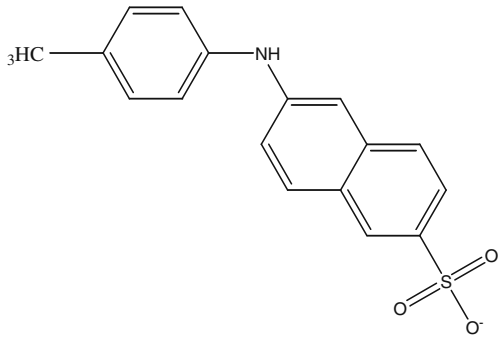

Fig. 1 Structural formula of 6,P-toluidinylnaphthalene-2-sulfonate (TNS)

excitation source, to the difference of a laser, does not induce photo degradation of the fluorophore.

Since the purpose of this work is to explain origin of fluorescence of TNS bound to proteins, we performed fluorescence measurements of TNS in different conditions: bound to proteins, dissolved in ethanol and in water. In ethanol, TNS is completely dissolved and thus fluorescence observables obtained would characterize specific structure (s). In water, at high concentrations, TNS is aggregated and fluorescence observables, if any, would characterize aggregated state. Bound to protein, TNS fluorescence will characterize interaction between fluorophore molecules and surrounding environment and/or specific TNS substructures observed only in presence of proteins. Therefore, comparison of the data obtained in the three different conditions would allow finding out the correlation between the fluorescence observables, the fluorophore structure (s) and the environment.

Materials and methods

TNS (purity >97%), bovine β-lactoglobulin (purity >90%), deoxyribonuclease I (purity >80%) and bovine serum albumin (purity >98%) were from Sigma and helicase (purity >97%) was from Pharindustrie, Clichy, France. Serotransferrin (purity >98%) and human α1-acid glycoprotein (purity >99%) were prepared by Professor H. Debray (actually retired) of University of Lille 1.

TNS concentration was determined spectrophotometrically using an extinction coefficient equal to 18.9 mM^{-1} cm^{-1} at 317 nm [7]. Proteins concentrations were determined at 278 nm with the following extinction coefficients: 17.6×10^3 M^{-1} cm^{-1} for bovine β-lactoglobulin, 4.2×10^4 M^{-1} cm^{-1} for bovine serum albumin, 10.65×10^4 M^{-1} cm^{-1} for helicase and 29.7 mM^{-1} cm^{-1} for human α_1-acid glycoprotein. Concentrations of deoxyribonuclease I and serotransferrin were measured using an $E^{1\%}_{1cm}$ equal to 11.1 and 14.0, respectively.

Absorbance data were obtained with a Shimadzu MPS-2000 spectrophotometer using 1-cm pathlength cuvettes. Fluorescence spectra were recorded with a Perkin–Elmer LS-5B spectrofluorometer. The bandwidths used for the excitation and the emission were 5 nm. The quartz cuvettes had optical pathlengths equal to 1 and 0.4 cm for the emission and excitation wavelengths, respectively. Fluorescence spectra were corrected for the background intensities of the buffer solution. Observed fluorescence intensities were first corrected for the dilution, and then corrections were made for the inner filter effect as described [8, 9].

Fluorescence lifetime measurements of TNS were obtained with a Horiba Jobin Yvon FluoroMax-4-P, using the time correlated single photon counting method. A Ludox solution was used as scatter. Excitation was performed at 296 nm with a nanoLED. Each fluorescence decay was analyzed with one, two and three lifetimes and then the values of χ^2 were compared in order to determine the best fit. Although TNS (around 5 μM) added to the protein solutions is not totally bound, observables (spectra and lifetimes) recorded in presence of proteins correspond to bound fluorophore molecules. In fact, it was not possible to measure any fluorescence lifetime or to record any significant fluorescence spectra for 5 μM TNS in water or in buffer. This observation was also mentioned by other authors in their studies on the interaction between TNS and micelles of poly (ethylene oxide)–poly (propylene oxide)–poly (ethylene oxide) [10].

Since lifetimes data are based on the value of χ^2, it is important to make a description of the meaning and importance of this value. In the single photon counting method, the detection system measures the time between the excited pulse and the arrival of the first photon. The distribution of arrival times represents the decay curve called also the impulse response function. In order to obtain a decay curve that characterizes the sample only, excitation pulse should be infinitely sharp or should have a pulse much shorter than the decay time of the sample. Nevertheless, even if these two conditions are met, this is not always the case; the presence of the light pulse does not allow identifying the zero time point of the fluorescence decay. This will induce errors in number of lifetimes determination and calculation. The time distribution of the lamp pulse L (t), called also the instrument response function, is measured in a separate experiment using scatter solution. The observed fluorescence decay is called R(t) and the final response function is called F(t).

R(t) is given by the convolution of the lamp pulse with the impulse response of the sample [8, 11, 12]:

$$R(t) = \int_0^t L(t')F(t-t')dt' \quad (1)$$

A method applied to estimate the impulse response function F(t) is the least-squares analysis. The method calculates the expected value of R(t) given assumed values of α_i and τ_i and the calculated value [$R_c(t)$] is compared with the observed value R(t). The α_i and τ_i values are varied until the best fit is obtained. The goodness of fit χ^2 is calculated from

$$\chi^2 = \sum_{i-1}^{n} w[R(t) - R_c(t)]^2 \quad (2)$$

where

$$w = \frac{1}{R(t)} \quad (3)$$

is a statistical weighting factor to account for the expected error in each value of R(t). A minimal value of χ^2 indicates the best fit. A χ^2 value that approaches 1 indicates a good fit.

Differences between R(t) and $R_c(t)$ is described by a function called the autocorrelation function of the differences and can be displayed by experimental curves. Close values of R(t) and $R_c(t)$ yield experimental autocorrelation curves that are randomly distributed around zero, indicating by that a good fit. Finally, to find out whether a decay curve should be best analysed with one, two or more lifetimes, values of χ^2 have to be compared. For example, let us consider the value of χ^2 equal to 1.054, 1.06 and 1.1 when analysis is done with 1, 2 and 3 lifetimes, respectively. One lifetime should be considered as the best description of the decay curve since there was no real improvement in χ^2 value when the experimental decay was fitted with two or three lifetimes.

The mean fluorescence lifetime calculated is the second order mean [8]:

$$\tau_o = \sum f_i \tau_I \quad (4)$$

and

$$f_i = \alpha_i \tau i \Big/ \sum \alpha_i \tau i \quad (5)$$

where α_i are the preexponential terms, τ_i are the fluorescence lifetime and f_i the fractional intensities.

All experiments on proteins were performed at 20 °C in 10 mM phosphate buffer and 0.143 M NaCl buffer (PBS buffer), pH 7.

Results and discussion

The purpose of this work is to find out origin of TNS fluorescence. For this, we compared fluorescence lifetime of TNS dissolved in water, in ethanol and bound to different proteins. Also, we compared emission and excitation spectra of TNS in these different environments. Fluorescence intensity decay of 5 μM TNS dissolved in distilled ethanol can be adequately described with one lifetime equal to 7.39 ns, the χ^2 is equal to 1.015 (λ_{em}= 425 nm) (Fig. 2). Analysing fluorescence intensity decay with two exponentials yields fluorescence lifetimes equal to 3.65 and 7.37 ns, the χ^2 is equal to 1.055. Contribution of the first lifetime to the decay represents 2.5%. The close values of the χ^2 indicate that we can fit fluorescence decay of TNS in ethanol with one fluorescence lifetime. We measured fluorescence lifetime of TNS along the emission wavelengths and the values of χ^2 obtained for one and two lifetimes were not significantly different (Table 1). Thus, absence of improvement in the value of χ^2 between one and two lifetimes allow us to consider that TNS dissolved in ethanol can be best described with one fluorescence lifetime. Nevertheless, considering that TNS fluorescence decay in ethanol can be fitted with two lifetimes is not necessarily wrong, although the 3.7 ns lifetime contributes to around 3% of the fluorescence decay. Thus, the 7.4 ns component is the major and main contributor to TNS emission decay in ethanol. Fluorescence spectra originate mainly from this longest component and thus from a dominant structure.

Measuring fluorescence decay of 130 μM TNS dissolved in ethanol yields the same fluorescence lifetime as 5 μM. Thus, TNS dissolved in ethanol is not aggregated; the measured fluorescence lifetime would characterize a structure having its specific fluorescence properties (emission and excitation spectra and lifetime).

Bound to proteins, TNS fluorescence decays with three lifetimes, the shortest one going from 0.5 to 1.3 ns, the second from 3 to 6 ns and the longest lifetime from 10 to 12 ns. Figure 3 displays the fluorescence intensity decay at 425 nm of 5 μM TNS in presence of 12 μM β-lactoglobulin. The value of χ^2 increases from 1.042 to 1.145 when we tried to fit the fluorescence decay with two fluorescence lifetimes instead of three. This increase in χ^2 value was observed for all the measurements performed in presence of different proteins (Tables 2 and 3). Thus, interaction between TNS and proteins can be characterized by the presence of three fluorescence lifetimes. Nevertheless, the shortest lifetime could be assigned to free TNS in solution. However, it was difficult to record any fluorescence decay of 5 μM TNS dissolved in water or in buffer and thus to measure a fluorescence lifetime that characterizes free TNS in water. Therefore, the shortest TNS lifetime measured in presence of proteins would characterize TNS interaction with the macromolecules. Since three lifetimes are always measured in presence of all studied proteins, the decay parameters can in no way characterize different protein sub-structures, as it was suggested [13], otherwise all proteins would have three sub-structures.

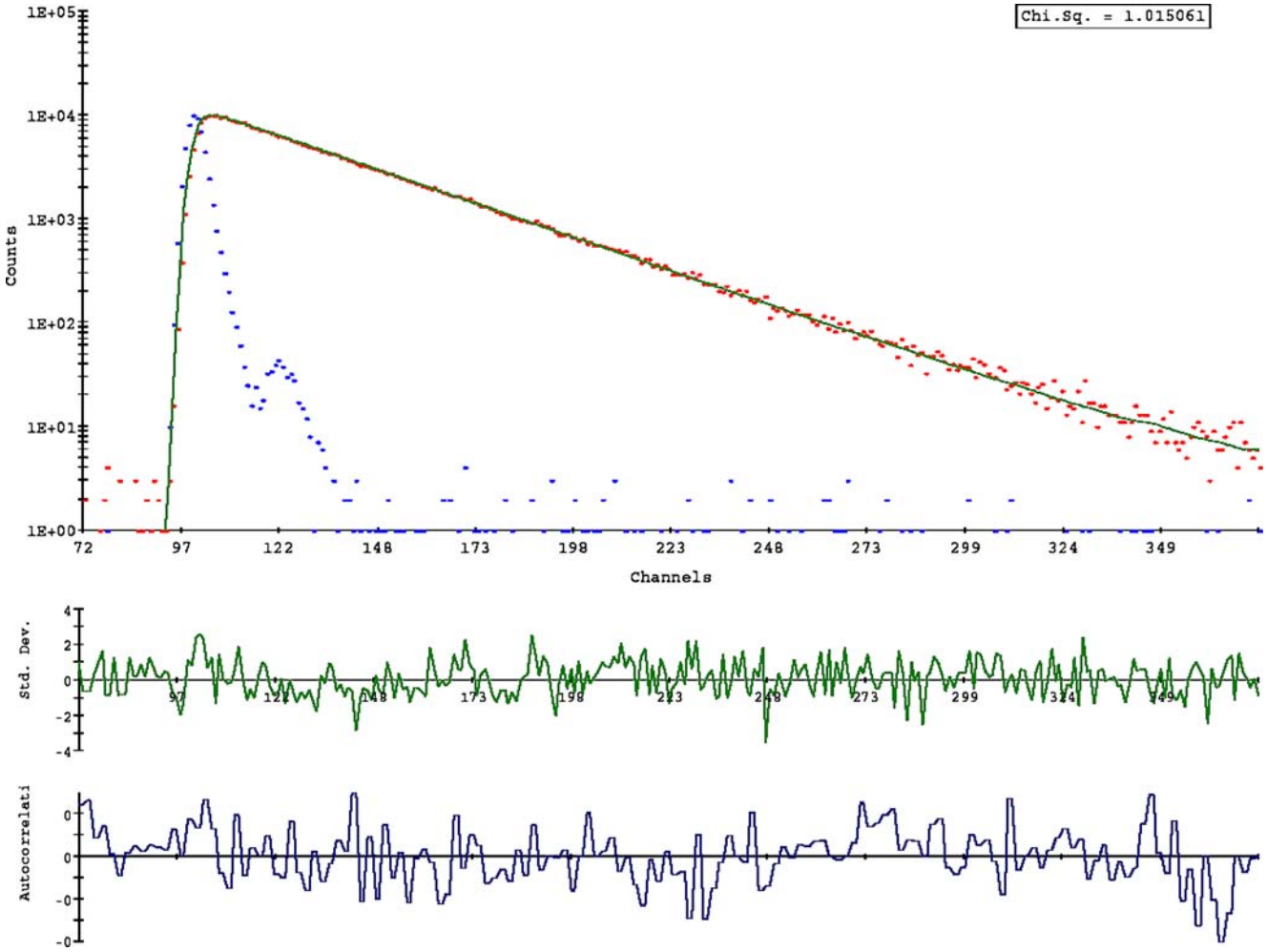

Fig. 2 Fluorescence intensity decay of TNS dissolved in distilled ethanol. λ_{ex}=296 nm and λ_{em}=425 nm. The curve displayed on the *left* side of the figure characterizes the instrument response function and was obtained with a scatter solution (ludox)

Also, the fact that three fluorescence lifetimes are measured in presence of all studied proteins clearly indicates that these lifetimes are independent of the structures of the protein and are simply the result of the interaction between the fluorophore and the proteins. This interaction does not vary from a protein to another since TNS is tightly bound to proteins [14–18]. Also, electrostatic interactions would interfere between the fluorophore and the binding site. Thus, although the three fluorescence lifetimes are the result of TNS binding to the proteins, their values and those of the pre-exponential terms characterize the specific interactions that exist between the TNS and each protein.

Time resolved emission spectra of TNS bound to BSA indicate that the three lifetimes contribute to the global emission spectrum of the fluorophore (Fig. 4). Contribution of each fluorescence lifetime to the global emission spectrum is 0.5%, 14% and 85.5% for the short, middle and long lifetimes, respectively. The emission peak of each of the three spectra is equal to 428 nm, indicating that the structures responsible of the fluorescence lifetimes are surrounded by the same environment. Figures 5 and 6 display respectively lifetimes and pre-exponential variation of TNS-BSA complex with emission wavelength. One can notice that the three lifetimes do not vary in the same way along the emission wavelengths. Also, the pre-exponential profile variations are completely different. These results indicate that fluorescence lifetimes originate from different TNS sub-structures. Nevertheless, it is interesting to find out the origin of the three lifetimes. In order to answer to this question, we have measured fluorescence lifetime of aggregated TNS in water. At high concentration (>126 μM) (aggregated form) TNS emits with three fluorescence lifetimes equal to 0.3, 4 and 8 ns, values within the same range of those found for TNS bound to proteins. Figure 7 displays TNS lifetimes variations with emission wave-

Table 1 Fluorescence decay data (in ns) of TNS dissolved in ethanol analyzed with one and two fluorescence lifetimes and measured along the emission spectrum

Wavelength	τ_1	α_1	τ_2	α_2	τ_o	χ^2
395 nm	7.41	100			7.41	1.234
	7.56	96.13	3.70	3.87		1.143
400 nm	7.42	100			7.42	1.085
	7.46	98.91	3.75	1.09		1.115
405 nm	7.41	100			7.41	1.162
	7.47	98.46	3.77	1.54		1.272
410 nm	7.41	100			7.41	1.358
	7.52	96.99	3.76	3.01		1.314
415 nm	7.41	100			7.41	1.177
	7.52	97.06	3.75	2.94		1.126
420 nm	7.46	100			7.46	1.033
	7.56	97.16	3.72	2.85		0.992
425 nm	7.39	100			7.39	1.015
	7.50	97.02	3.69	2.98		0.966
430 nm	7.47	100			7.47	1.132
	7.56	96.52	3.75	3.48		1.154
440 nm	7.41	100			7.41	1.342
	7.54	96.46	3.75	3.54		1.268
450 nm	7.40	100			7.40	1.300
	7.51	97.06	3.74	2.94		1.249
460 nm	7.42	100			7.42	1.336
	7.50	97.7	3.81	2.30		1.306
470 nm	7.42	100			7.42	1.025
	7.53	96.84	3.77	3.16		1.061

The fluorescence lifetimes relative amplitudes (α_i) are expressed in %

length. The values of the longest lifetime and its pre-exponential term (see Table 2) are very low compared to the values observed for TNS bound to proteins. Also, pre-exponential term of the middle lifetime is much higher in water than when TNS is bound to a protein, while the fluorescence lifetime does not vary. Therefore, the mean lifetime (τ_o=6.1 ns) of aggregated TNS dissolved in water, is much lower than the mean value (8 to 12 ns) calculated for TNS bound to proteins.

Time-resolved-emission spectra of aggregated TNS indicate that the 0.3, 4 and 8 ns lifetime components participate to the global emission spectrum by 2%, 61% and 37%, respectively (not shown). Also, the three spectra obtained emit at a peak equal to 415 nm. The contribution of each of the fluorescence lifetimes of TNS dissolved in water differ from that observed for TNS bound to proteins.

Mean fluorescence lifetime of TNS dissolved in ethanol does not change with the emission wavelength, which is not the case for TNS dissolved in water at high concentration or for TNS bound to a protein (Fig. 8). This means that fluorescence lifetime characterizes a specific fluorophore structure within a specific environment.

Fluorescence spectra (excitation and emission) are also dependent on the fluorophore states or sub-structures. In fact, emission spectrum of at least 126 μM TNS dissolved in water is equal to 415 nm, a blue-shift peak in comparison to the maximum (430 or 423 nm) observed for TNS in ethanol or bound to proteins (Fig. 9). At high concentrations in water, TNS is aggregated. Energy transfer occurs between the different fluorophore molecules inducing an emission peak located at short wavelengths (415 nm). Similar results have been already reported for 1-anilinonaphthalene 8-sulfonate at high concentrations. Increasing fluorophore concentrations induced an enhancement in the quantum yield and a blue shift of the emission maximum compared to ANS dissolved at low concentration. This fluorescence modification was attributed to aggregates formation and thus to partial exclusion of water [19]. Excitation spectra of TNS recorded in water, ethanol and bound to BSA, are different indicating clearly that TNS, in each medium, is within specific structure(s) (Fig. 10). Although, the shapes of TNS excitation spectra recorded in ethanol and bound to BSA are close, the positions of the peaks are not the same. Binding of TNS to BSA induces a shift of the 273 nm excitation peak to 280 nm, a position already observed for TNS bound to other proteins (not shown).

What are the physical insights of these results? In general, fluorescence spectra data (peak and width) are

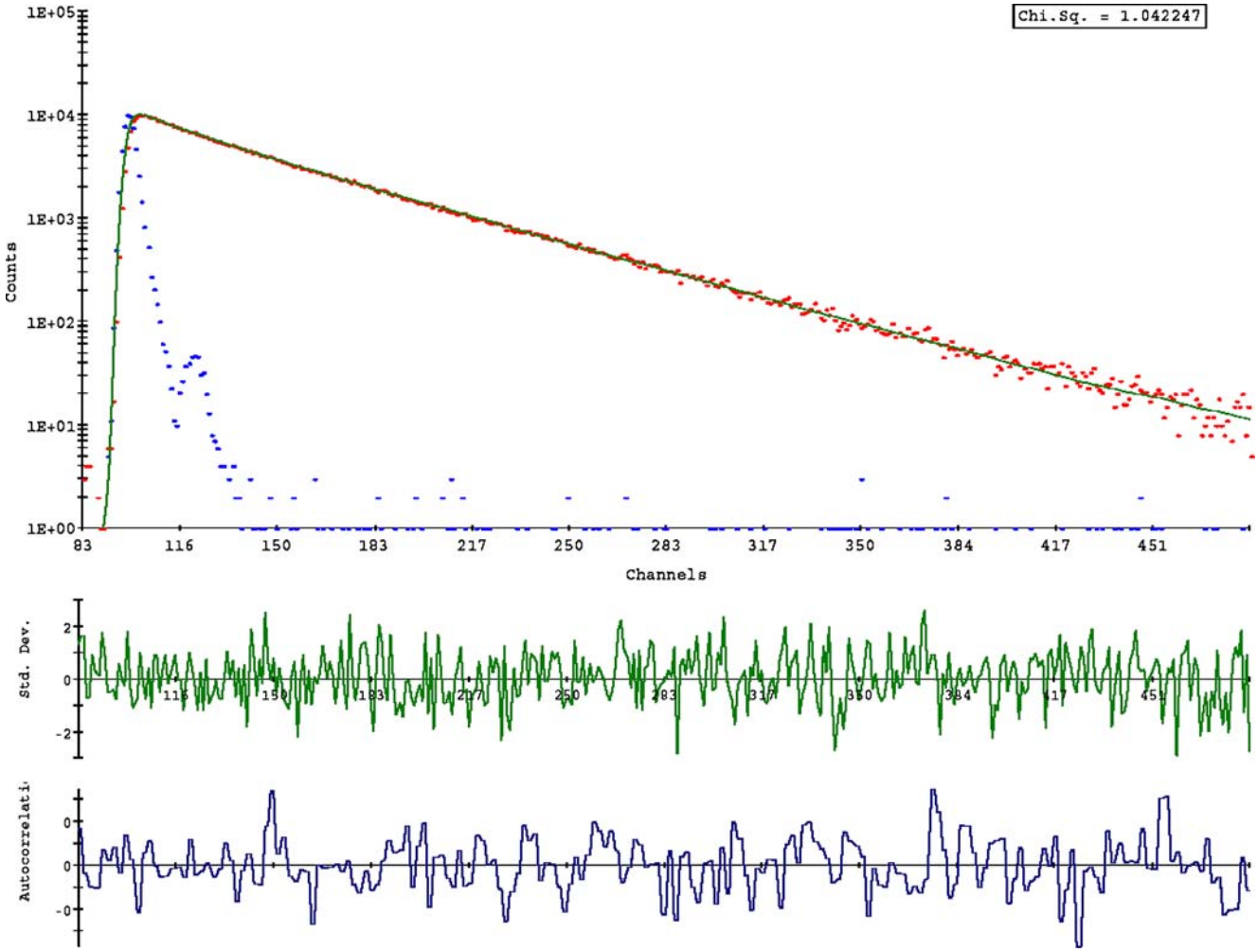

Fig. 3 Fluorescence intensity decay of 5 μM in presence of 12 μM β-lactoglobulin. λ_{ex}=296 nm and λ_{em}=425 nm. The curve displayed on the *left* side of the figure characterizes the instrument response function and was obtained with a scatter solution (ludox)

analysed as the result of an emission from a fluorophore surrounded by a hydrophobic or a hydrophilic environment. In this case, the term solvent-sensitivity is commonly used. Also, rigidity or flexibility of the surrounding environment would influence the global shape of the spectrum. Thus, we often use the term of solvent relaxation. Measured fluorescence lifetimes are also interpreted as the consequence of the chemical and physical properties of the surrounding environment. The present work shows for the first time that origin of fluorescence lifetimes of a

Table 2 Fluorescence decay data of TNS at 425 nm in different media analyzed with three fluorescence lifetimes

Sample	τ_1	α_1	τ_2	α_2	τ_3	α_3	τ_o	χ^2
5 μM TNS in ethanol	7.39	100					7.39	1.015
126 μM TNS in water	0.32	5.98	4.05	67.91	8.65	26.12	6.10	1.311
5.66 μM TNS-7 μM helicase	0.51	20.11	3.19	44.74	10.02	35.15	7.89	1.297
5.82 μM TNS-7.3 μM α_1-Acid glycoprotein	0.74	14.22	4.12	40.63	11.5	45.16	9.56	1.318
6 μM TNS-5 μM serotransferrin	0.92	14.59	4.85	40.43	11.64	44.98	9.27	1.412
6 μM TNS-8 μM deoxyribonuclease I	1.30	7.48	5.84	39.7	11.79	52.82	10.07	1.045
5.6 μM TNS-1 μM BSA	1.27	3.84	6.48	33.54	12.64	62.62	11.26	1.147
5.8 μM TNS on 12 μM bovine β-lactoglobulin	0.85	1.47	4.91	11.35	12.33	87.19	11.95	1.0422

Only TNS in ethanol was analyzed with one lifetime

Table 3 Fluorescence decay data of TNS at 425 nm in different media analyzed with two fluorescence lifetimes

Sample	τ_1	α_1	τ_2	α_2	τ_o	χ^2
5 μM TNS in ethanol	3.65	2.46	7.37	97.54	7.32	1.055
126 μM TNS in water	2.99	44.17	6.77	55.83	5.79	2.048
5.66 μM TNS-7 μM helicase	1.52	44.6	7.96	55.40	7.10	4.068
5.82 μM TNS-7.3 μM α_1-Acid glycoprotein	1.96	35.2	9.62	64.80	8.86	2.977
6 μM TNS-5 μM serotransferrin	1.86	29.63	9.52	70.37	8.94	2.844
6 μM TNS-8 μM deoxyribonuclease I	2.87	22.85	10.52	77.15	9.95	1.580
5.6 μM TNS-1 μM BSA	4.00	20.31	11.69	79.69	11.07	1.428
5.8 μM TNS on 12 μM bovine β-lactoglobulin	3.61	9.73	12.19	90.27	11.92	1.117

fluorophore such as TNS is not the same as that of its fluorescence spectra. In fact, excitation spectra of TNS clearly indicate the state of the fluorophore in solution (aggregated at high concentrations in water, dissolved in ethanol at low and high concentrations and bound to a protein at low concentration). Although the spectra of TNS in water and bound to proteins are completely different, fluorescence lifetimes values in both conditions are very close. Aggregation induces a mobility decrease. Thus, the three fluorescence lifetimes of TNS in water can be explained by the important contact between the different molecules (aggregation) and the low mobility of these molecules. Bound to proteins, TNS is tightly rigid to the binding sites, a rigidity that could induce non-negligible contacts between the different fluorophore molecules. The position of TNS emission peak would reflect the importance of the different TNS molecules contacts present at the binding site of the protein. Also, interaction between TNS molecules and proteins modifies the value of the pre-exponential terms. Therefore, one should not exclude the possibility of having local structure of TNS that varies slightly with the nature of the interacting macromolecules.

Local structural modification of TNS was already suggested by Radda [20]. In fact, X-ray diffraction studies on TNS anhydrous crystals showed that TNS is planar while the hydrated molecule is bent [21]. Also, the fact that phenolphthalein is not fluorescent while the structurally similar but planar fluorescein shows important fluorescence, as it has been reported by Radda [20], would be in favor of the importance of planarity in fluorescence.

Our fluorescence lifetimes results indicate that TNS structure is not the same when the fluorophore is dissolved

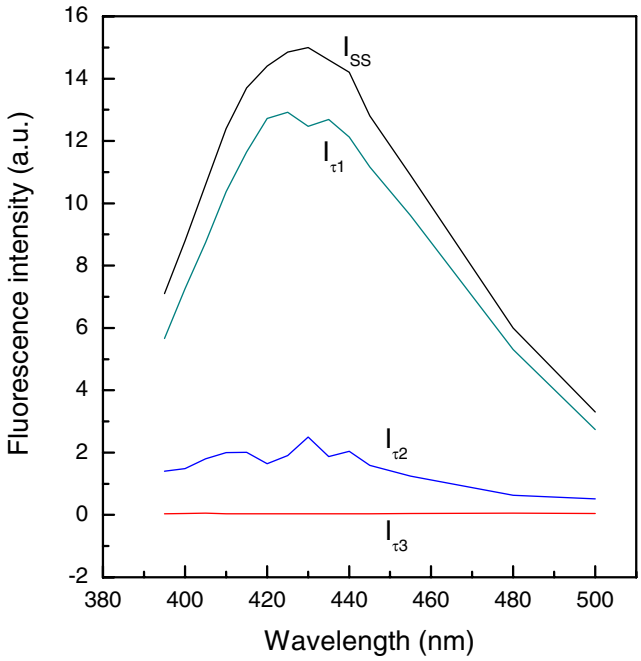

Fig. 4 Fluorescence emission spectra of the three lifetimes of TNS in presence of BSA. λ_{ex}=296 nm. I_{SS} is the steady state emission spectrum of TNS in presence of BSA obtained with λ_{ex}=295 nm

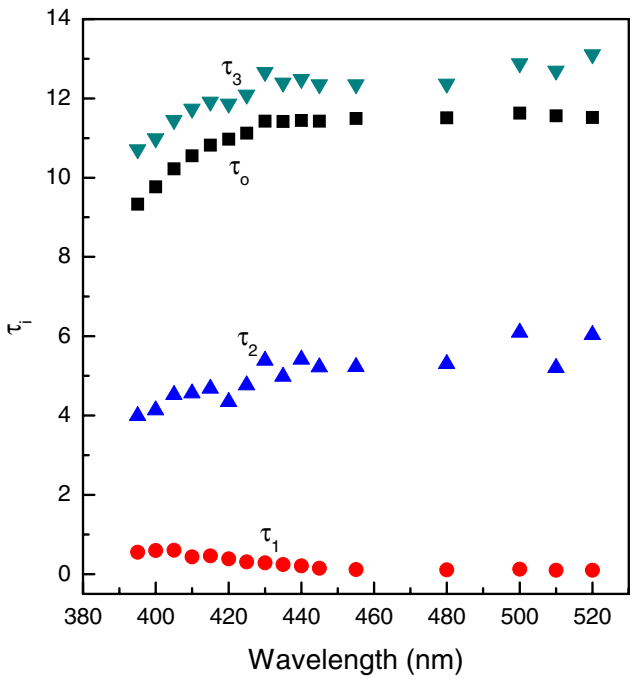

Fig. 5 Lifetimes variation of TNS in presence of BSA with emission wavelength. λ_{ex}=296 nm

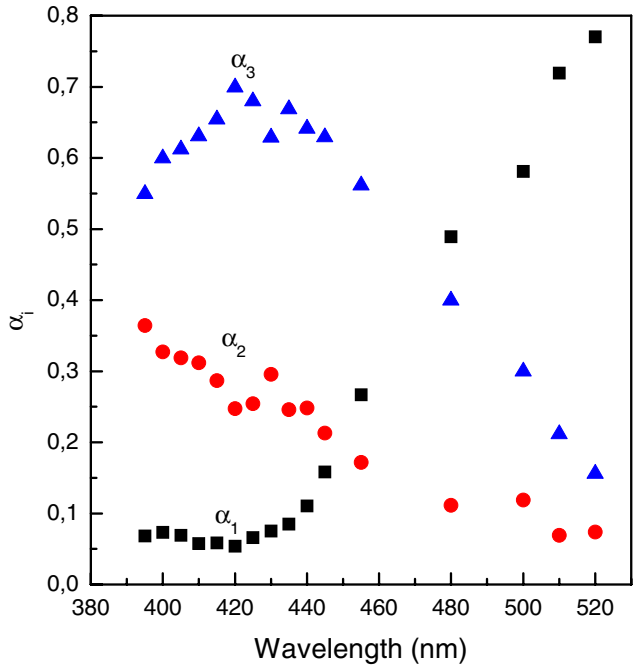

Fig. 6 Pre-exponential variations of TNS-BSA lifetimes with emission wavelength. λ_{ex}=296 nm

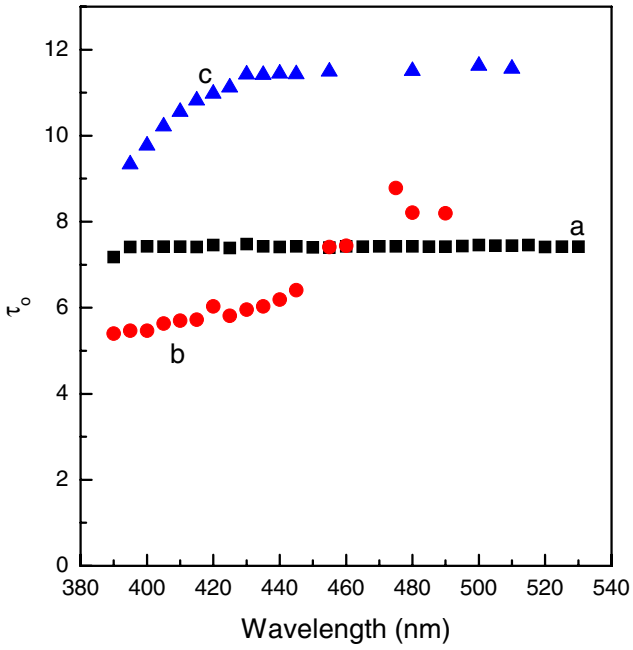

Fig. 8 Mean fluorescence lifetime of 5 μM TNS dissolved in ethanol (**a**), of 5 μM TNS in presence of BSA (**c**) and of 130 μM TNS dissolved in water (**b**), with emission wavelength. λ_{ex}=296 nm

in ethanol or when bound to proteins. In ethanol, one major lifetime was found indicating the presence of a major structure for TNS. In presence of proteins, three lifetimes were recorded revealing that the probe does have different structure and/or behavior than in ethanol. Our data and conclusions are complementary to the solvation effect related by many authors. Importance of hydrogen exchange between the fluorophore and its solvent modifies the

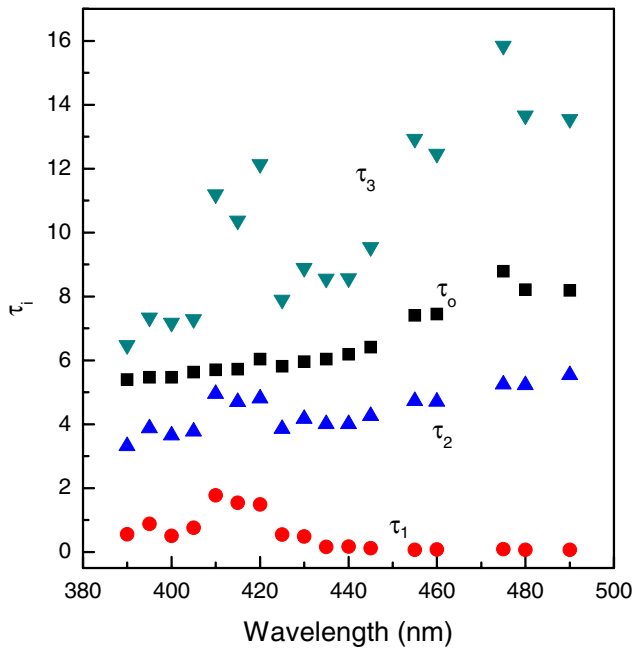

Fig. 7 Lifetimes variation of 130 μM TNS in water with emission wavelength. λ_{ex}=296 nm

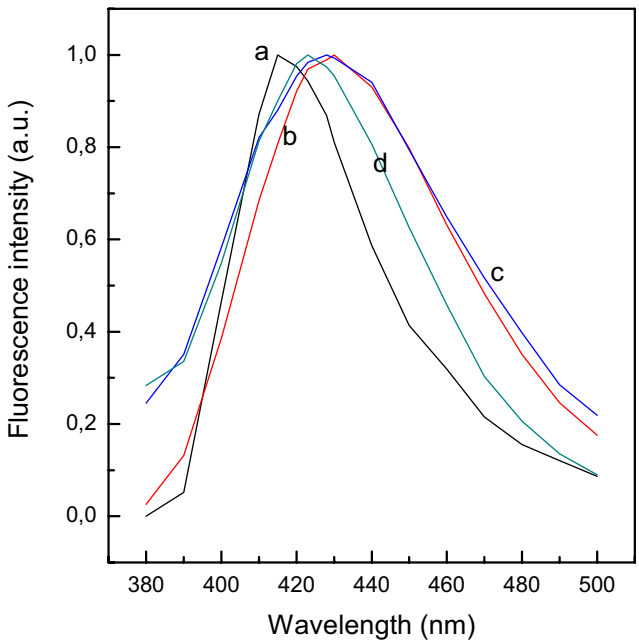

Fig. 9 Normalized fluorescence emission spectra of 126 μM TNS in water (λ_{em}=415 nm) (**a**), of 5 μM TNS in distilled ethanol (λ_{em}=430 nm) (**b**), of 6 μM TNS in presence of BSA (λ_{em}=428 nm (**c**) and of 6 μM TNS in presence of β-lactoglobulin (λ_{em}=423 nm) (**d**), λ_{ex}=295 nm. Proteins are dissolved in bis–Tris buffer, pH 7.5

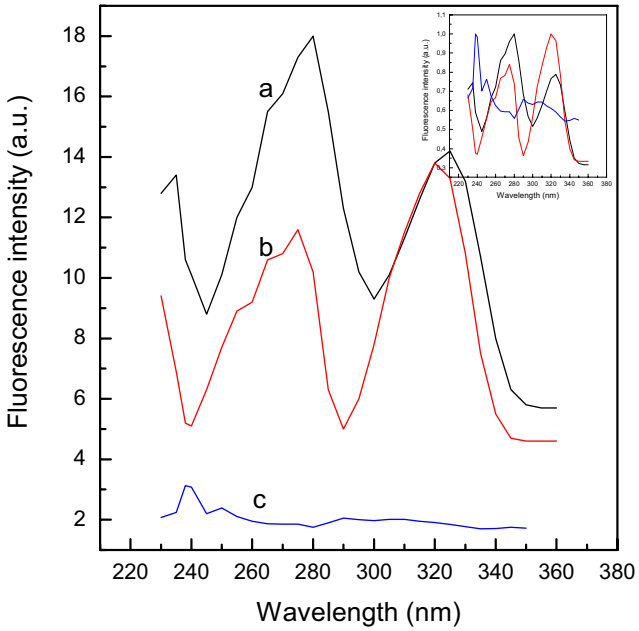

Fig. 10 Fluorescence excitation spectra of TNS (5 μM) in presence of BSA (**a**), of 5 μM TNS dissolved in ethanol (**b**) and of 130 μM TNS dissolved in water (**c**). λ_{em}=450 nm. Inset: Normalized excitation spectra

position of the emission peak and the values of quantum yield of the fluorophore and probably its fluorescence lifetime(s). The solvent effect is called solvation effect [20] and was explained as follow: Upon excitation, TNS decreases its potential energy while water molecules solvate it. In water, solvation occurs within the femtosecond range (≈700 fs). As solvation proceeds, twisting motion of TNS from an initial locally excited state to a final intramolecular charge transfer state occurs within the range of the picosecond (60 ps) [22]. This final state is known as the twisted intramolecular charge transfer (TICT) [23]. In presence of histone 1, solvation dynamics of TNS occur within the femtosecond range such it was observed for TNS in water. Thus, protein water "layer" is dominant in the hydrophobic globular domain and is used to conserve protein folded structures [22]. The results obtained by Zhong et al [22] are in good agreement with those already found by others and indicating that while ordinary water molecules relax in the subpicosecond time scale, inside organized assemblies solvation dynamics become several thousand times slower and occurs in the nanosecond timescale [24–30]. In fact, in micelles, where TNS is surrounded by a much higher hydrophobic and low-viscous environment than in histone 1 solution, solvation dynamics occur in hundred of picoseconds as the consequence of the rigid structure of water molecules in the peripheral Stern layer [31]. Significant binding of TNS to micelles or to polymers increases its fluorescence intensity by at least 100 to 200 times. This increase was attributed to "the suppression of the nonradiative pathway in the relatively less polar microenvironment and where the polymer chain wrap around TNS molecules, shielding it from bulk water". Solvation dynamics of water molecules around bound TNS is much longer than that of bulk water [32].

Das et al (1992) [23] indicated that influence polarity on fluorescence lifetime of TNS differs from that observed on 1-8-ANS. In fact, the authors show that while lifetime behavior of 1-8-ANS TICT process decreases linearly with increase in solvent polarity parameter, this behavior does not change monotonically for TNS. However, emission peaks of TNS and of ANS shift to the red with polarity increase. Thus, solvation effect cannot be analyzed similarly whether we are monitoring fluorescence lifetimes or emission peak of TNS. Also, the results have shown that solvation effect differs from a fluorophore to another. Finally, measuring fluorescence parameters of 1-anilino-8-naphthalene sulfonate in water and D_2O, Drew et al (1983) [33] found that while emission peaks are the same in both solvents (335 nm), fluorescence lifetimes differ (1.86 ns in water and 8.75 ns in D_2O). Thus, although both emission peak positions and fluorescence lifetime are associated to the relaxation from the Franck-Condon to a solvent stabilized intramolecular charge transfer (ICT) state) [33], the data described by the authors clearly show that emission peak position does not necessarily yield the same information as fluorescence lifetime. Also, the same authors showed that results obtained on 2-amino-1-naphthalene sulfonate differ completely from those obtained with 1-anilino-8-naphthalene sulfonate indicating that rules are not the same for all fluorophores.

All the work published on the solvation dynamics of water around the fluorophore TNS confirm the earlier work of McClure and Edelman [7], where the authors indicate that emission peak position and spectrum are very dependent on the solvation of the excited state of TNS. Nevertheless, solvation effects should be interpreted to include the fact that the fluorophore, in our case TNS, would adopt specific conformation(s) in each solvent as it has been already suggested [19]. Our present data confirm effectively that TNS adopts conformations that differ whether it is dissolved in water, in ethanol, bound to proteins or aggregated.

In conclusion, TNS fluorescence decay observables (lifetimes and pre-exponentials) and excitation and emission spectra would be specific to (1) sub-structures of the fluorophore within a studied environment, (2) interaction that exists between the fluorophore molecules themselves and (3) their interaction with their environment. Our data show that analysing fluorescence data of TNS in terms of solvent polarity or solvent relaxation is not sufficient; sub-structures of the fluorophore are additional physical parameters that should be taken into consideration.

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