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Research article

The effect of immobilization in the PVA films on the fluorescence and phosphorescence lifetime of indole and its derivatives

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Abstract: *This work is devoted to study how immobilization in the PVA films affects the fluorescence and phosphorescence lifetime of indole and its derivatives. The obtained results indicated that immobilization of the studied indoles in the PVA matrix, which leads to the increased microrigidity of the environment around the indole moiety, results in the increase of singlet and triplet excited state lifetime of the studied compounds. Most probably, the enhancement of the rigidity of the environment near the chromophore reduces the rates of the non-radiative deactivation pathways, which leads to the increase of excited state lifetimes of the studied compounds.*

Keywords: *indole; N-acetyl-L-tryptophanamide (NATA); tryptophan; fluorescence and phosphorescence lifetime.*

Introduction

The room temperature tryptophan fluorescence and phosphorescence can be observed from proteins in aqueous solution, therefore fluorescence and phosphorescence techniques can be used in the study of protein structure and dynamics. Steady state luminescence studies provide the overall information as they contain intensity-weighted average information of the emitting chromophore, while time-resolved studies may provide information on the population distribution of the excited states of the emitting species. Therefore, a combination of steady state and time-resolved luminescence measurements of the tryptophan fluorescence in proteins can provide details of the local environment of the protein and its dynamics [1].

It is well known that fluorescence lifetime of tryptophan in proteins depends on the local environment of indole moiety and can vary from a few to tens of nanoseconds. Therefore, fluorescence techniques offers a possibility to study protein conformational changes in the above mentioned nanosecond time scale. Many studies have demonstrated that in almost all cases proteins containing a

single tryptophan residue exhibit multiexponential fluorescence decay kinetics [1]. Most probably, the heterogenous intensity decay of tryptophan in proteins is due to the existence of different rotameric conformations of the tryptophan side chain. It has been suggested that tryptophan can populate a number of low energy conformations, as a result of rotation around the Cα-Cβ and/or the Cβ-Cγ bonds, with each conformer exhibiting a distinct fluorescence lifetime. This model was firstly proposed by Szabo [2] to explain the biexponential fluorescence decay of pure tryptophan in aqueous solution and was subsequently shown to be applicable to small tryptophan containing peptides. The distinct decay times of different tryptophan rotamers most probably reflect interactions between the excited indole moiety and the unique surrounding amino acid residues and peptide groups of the protein conformation [3].

Recently, thanks to the rapid development of the instrumentation, which allows to register the low intensity phosphorescence at ambient temperature, the room temperature tryptophan phosphorescence (RTTP) methodology has been increasingly applied in the protein dynamics studies. The above mentioned RTTP techniques permits the monitoring of much slower processes, extending the observation time scale from the nanosecond range of fluorescence up to microsecond to second range. Additional potential of a triplet state as a structural probe comes from the drastic dependence of the tryptophan phosphorescence lifetime on the differences in the rigidity (microviscosity) of the indole environment [4, 5]. Therefore, the photophysics of triplet state of indole and its derivatives, tryptophan and NATA (N-acetyl-L-tryptophanamide); the latter considered as the standard reference compound for tryptophan in proteins by its mimicking of the amino acid's attachment in the backbone chain, in different media have been recently widely studied.

The results of a great number of previously performed studies have shown that the phosphorescence lifetime of unquenched tryptophan in proteins at ambient temperature ranges from 0.2 ms for a solvent exposed tryptophan residue to 4 s for that one which is deeply buried within protein interior [5, 6], while tryptophan luminescence from dry protein films was found to be of 10-15 seconds [4]. It has been observed that protein folding, which involves internalization of solvent exposed tryptophan residues, is accompanied with the increase in the lifetime of room temperature phosphorescence for tryptophan residues on the surface of protein.

Due to the fact, that the similarity is expected between room temperature tryptophan phosphorescence lifetime of the tryptophan residues which are exposed to solvent and the lifetime of free tryptophan in solution, it is of a great importance to determine the lifetime of the latter. Although many studies have been devoted to study photophysics of triplet state of aqueous indole and its derivatives, the mechanism of the triplet state decay is still unclear. The most significant ambiguity is that concerning the lifetimes of free indole and its derivatives in solution.

According to Fischer [7], the triplet-state lifetime of aqueous deoxygenated indole and several its derivatives, determined using sensitive photon-counting techniques, was approximately 40 μs, which is close to the previous reports by Bent and Hayon [8] (12 μs for indole, 14 μs for tryptophan) and Tsentalowich [9] (12.5 μs for tryptophan) based on flash photolysis, but much shorter than the millisecond long lifetime observed by Strambini [6] (about 5 ms for indole (5 μM)) using similar photon-counting techniques.

As far as NATA is concerned, the average phosphorescence lifetime of aqueous NATA at 20°C determined by Fischer [7] was about 39 μs. In contrast to the result obtained by Fischer, the study of Strambini [10] using similar photon counting technique reported that in 10 mM Tris the phosphorescence lifetime of NATA was around 2 ms. In 2004 Strambini reexamined the lifetime of NATA and estimated the value of the NATA (5 μM) triplet state lifetime in low-purity water to be about 5 ms at 20°C. Strambini [6] explained that the latter higher value is the result of lowered sample contamination, which was obtained applying purer fresh water supply and more effective degassing procedures. Banks and Kerwin [11] also have found millisecond long (2 ms) phosphorescence lifetime of NATA in 1 mM Tris solution of pH = 7.

The above mentioned considerations show that the reported phosphorescence lifetime of aqueous indole derivatives vary from μs to ms. It seems that the main sources of this discrepancy are, on one hand, the effect of triplet state quenching by residual oxygen, impurities or photoproducts in solution, and on the other hand, the formation of triplet state in non-radiative reactions of radical species, which lead to the increase in the observed radiative lifetime [6, 7, 9].

Previous studies have shown that the increased rigidity and viscosity of the environment surrounding the indole moiety results in the increase of phosphorescence lifetime of indole and its derivatives. For example, the lifetime of the triplet states of indole and its derivatives earlier measured in rigid media (frozen solutions) was found to be several seconds [12]. At low temperature, about 12 K, the phosphorescence lifetime of NATA and L-tryptophan in 60% glycerol/H_20 mixtures and in the trehalose/sucrose film at pH = 7 was determined to be about 5 seconds [13].

As far as we know there is no study on the photophysics of indole, tryptophan and NATA immobilized in the PVA matrix. Poly(vinyl alcohol) (PVA) is a polymer in which hydroxyl groups of carbone chain backbone are attached to methane carbons. These OH groups can be a source of hydrogen bonding and hence assist the formation of polymer complexes. Over recent years polyvinyl alcohol (PVA) polymers have attracted attention due to their variety of applications. Poly(vinyl alcohol) (PVA) has been developed for various biomedical applications such as artificial pancreas, hemodialysis and implantable medical materials [14].

While the lifetime of aqueous indole derivatives (indole, NATA, tryptophan) is still under debate, the main aim of this study is to examine how the changes in microrigidity caused by immobilization of the studied indoles in the PVA matrix

affects the fluorescence and phosphorescence lifetime of indole and its derivatives (L-tryptophan, NATA).

Experimental

Materials

Indole, NATA (N-acetyl-L-tryptophanamide) and L-tryptophan were of the highest purity grade available from commercial sources. Indole, NATA and L-tryptophan were from Sigma-Aldrich and were used without further purification. All absorption and fluorescence measurements in solutions were performed in water at 20°C.

Poly(vinyl alcohol) purchased from Sigma-Aldrich was used as is. PVA powder of 10 g was dissolved in distilled water of 100 ml at 90°C. Immobilization of the indoles were obtained simply by mixing 1ml of stock solutions of the studied indoles (10^{-3} M) with 9 ml of room temperature PVA solution to obtain 10^{-4} M concentration of the studied indoles in the PVA solution. 5 ml of the solution was poured on a horizontal plastic circular plate of the radius 3 cm. After it was dried at room temperature in 10 days, a PVA film about 0.1 cm thick was obtained.

Methods

Absorption measurements

Absorption spectra were recorded in a Nicolet Evolution 300 (Thermo Electron Corporation) UV-Vis spectrophotometer. The measurements were performed at room temperature (20°C).

Steady-state fluorescence measurements

Steady-state fluorescence measurements were performed with a FluoroMax2 (Jobin Yvon Spex) spectrofluorimeter using an excitation wavelength of 290 nm. All measurements were performed in a standard quartz cuvette at 20°C.

Time-resolved fluorescence measurements

Fluorescence lifetime measurements were carried out with a FL900CDT time-correlated single photon counting fluorimeter from Edinburgh Analytical Instruments. The excitation and emission wavelengths were set to 290 and 360 nm, respectively. The fluorescence emission decays were monitored at a 90° angle to excitation at 20°C. The instrument response function was recorded by collecting scattered light from a LUDOX silica suspension.

Data acquisition and analysis were performed using the software provided by Edinburgh Analytical Instrumentation. Time-resolved data results were analyzed according to the multi-exponential decay law:

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp(-\frac{t}{\tau_i}) \quad (equation\ 1)$$

where α_i and τ_i are preexponential factor and decay lifetime of component I, respectively. The parameters describing the decay function were extracted from experimental data by a non-linear least squares convolution process. The goodness of the fit of fluorescence curves was judged by the reduced χ^2 value.

Phosphorescence measurements

Phosphorescence measurements were made on homemade system. The heart of this system was 800 MHz gated photon counters with 32 bit counter resolution and down to 250 ns time per channel. (PMS-400A, Becker&Hickl GmbH). Emission was excited by UV xenon flash lamp with 400 ns pulses, light output stability 1.9% p-p and repetition rate up to 100 Hz. Intensity of light drops down 1000 times after 30 microseconds. (L9455-01, Hamamatsu). There were two moods of wave-length selection by monochromator or interference filters. Due to the low energy passing through the sample acquisition was stopped at 100 000 sweeps. The system was calibrated on aqueous solution of $TbCl_3$. The lifetime was 426.5 microsecond and very well agreed with literature data (427 microsecond).

Since molecular oxygen is known to be a strong quenching agent as energy is easily transferred from the excited triplet state of the molecule to the triplet ground state of O_2, molecular oxygen has to be efficiently removed from the sample. For phosphorescence measurements in PVA films, O_2 removal was achieved by the application of moderate vacuum and inlet of ultrapure nitrogen. The pre-purified nitrogen gas was further purified by passing through an oxygen-trapping filter. For the measurements in solutions this degassing procedure was additionally accompanied by the addition of 0.3 ml of 0.1 M Na_2SO_3 as an O_2 scavenger [15]. The sample (indole solution (10^{-4} M) with Na_2SO_3 or PVA film) was placed in quartz cuvette, which was connected to the N_2/vacuum line by tubing. Five cycles of deoxygenation were performed. After deoxygenation, the cuvette was moved into the phosphorimeter, while remaining attached to the tubing and allowed to equilibrate to 30°C before taking the measurements. The background emission was determined by measurements carried out before deoxygenation of the sample and was normally subtracted from the phosphorescence decay.

All phosphorescence decays, after subtraction of the background, were analyzed in terms of a sum of exponential components by a nonlinear least squares fitting algorithm using the software provided by Edinburgh Analytical Instrumentation.

Results and Discussion

Determination of quantum yield of indole and NATA

The fluorescence quantum yield is defined as the ratio of photons absorbed to photons emitted through fluorescence. The quantum yield of indole and NATA in water was determined using the comparative method of Williams et al. [16], in which tryptophan with known quantum yield value of 0.14 [2] was applied as a standard sample.

It is known that the lowest energy absorption band of indoles originates from two close-lying electronic states with perpendicularly oriented transition dipole moments. These transitions, labeled as $1L^a$ (4.77eV) and $1L^b$ (4.37 eV), by analogy with the Platt scheme, differ substantially by their permanent dipole moments [17]. The $1L^a$ state has a larger static dipole moment than its ground state and thus it is more sensitive to solvation [18]. At longer wavelengths (>250 nm) both the $1L^a$ and $1L^b$ states contribute to the absorption spectrum of indoles in water, and around 260 nm, the contribution of the $1L^a$ state is dominant [18].

Absorption spectra of indole, NATA and tryptophan in water are shown in Figure 1. In the wavelength range 260-290 nm, the ground-state absorption spectrum of indole in water is characterized by the wide band with two peaks at about 270 and 280 and with the shoulder at about 290 nm. The ground-state absorption spectra of NATA and tryptophan in the wavelength range 260-290 nm consist of the broad band with the maximum at 280 nm and two shoulders at 270 and 290 nm. This characteristic of the fluorescence spectra of indole, NATA and tryptophan in water is consistent with previously reported data [19].

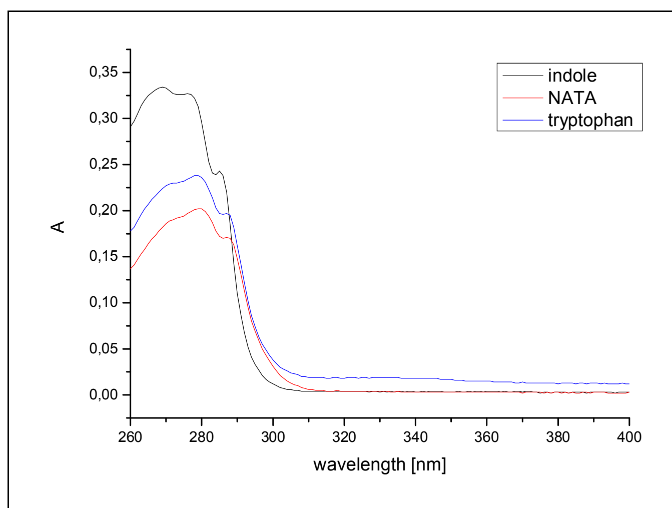

Figure 1. Absorption spectra of indole, NATA and tryptophan ($4*10^{-5}$ M) in water

Most probably, the above mentioned two electronic excited states, $1L^a$ and $1L^b$, are both involved not only in the absorption, but also in fluorescence emission.

The main characteristic of indoles fluorescence spectra is the large Stokes shift in polar solvents. The origin of the large Stokes shift is the result of a rapid reorientation of the solvent molecules, which are in the closest surrounding of the excited state forming a new solvent equilibrated excited $1L^a$ state, which is of lower energy than the initially excited $1L^a$ state. Due to a large change in the dipole moment of the excited $1L^a$ state compared to the ground state, in polar solvents the equilibrated $1L^a$ state falls below that of a close lying $1L^b$ excited

state. It has also been proposed that the large Stokes shift in polar hydrogen-bonding solvents is due to the formation of a solvent-indole(s) exciplexes. A third alternative for the large Stokes shift is that it may be due to emission from a solvated Rydberg state of indoles [20].

The normalized fluorescence spectra of indole, NATA and tryptophan in water, excited at 290 nm is shown in Figure 2. The maximum of emission is located at about 345, 360 and 355 nm for indole, NATA and tryptophan, respectively. This is consistent with previously reported data [20, 21].

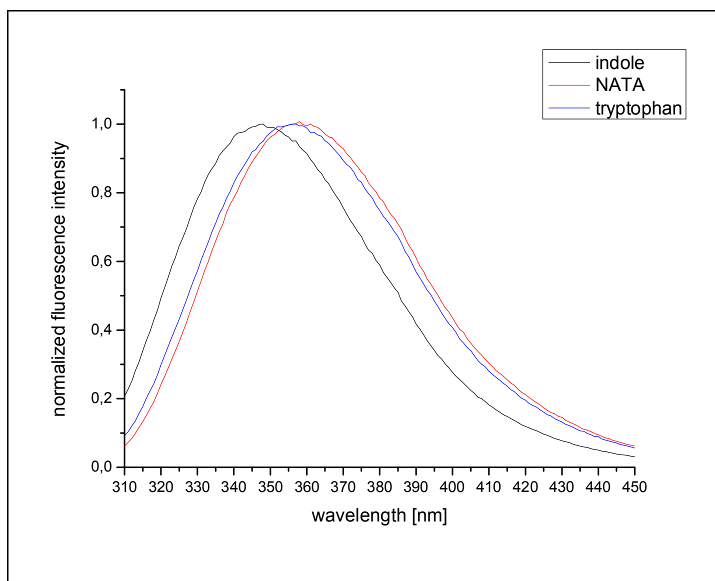

Figure 2. Normalized fluorescence spectra of indole, NATA and tryptophan ($4*10^{-5}$ M) in water, excitation at 290 nm

The determined quantum yield of indole and NATA at 290 nm is equal to 0.252 and 0.168, respectively. The calculated values of quantum yield are in good agreement with the literature (0.25 for indole; 0.14 (0.15) for NATA (pH = 7, 20°C, λ_{ex} = 280 nm, λ_{em} = 330 nm) [2, 19, 22].

Fluorescence characteristic of the studied indoles in PVA matrix

The emission spectrum of PVA in the region 315-450 nm, (not presented) showed very low fluorescence intensity. The normalized fluorescence spectra of indole, NATA and tryptophan in PVA film are depicted in Figure 3.

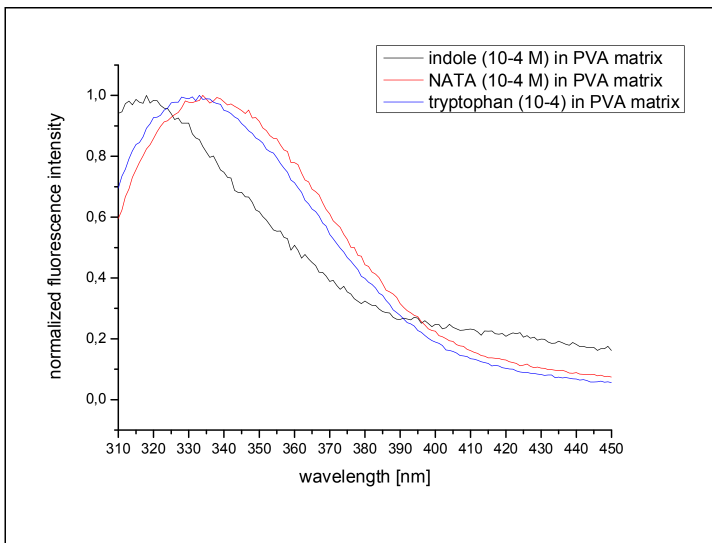

Figure 3. Normalized fluorescence spectra of indole, NATA and tryptophan (10^{-4} M) in the PVA film

From Figure 3 it can be seen, that the fluorescence maximum of indole, NATA and tryptophan in PVA film are located at about 318, 334 and 333 nm, respectively. Thus, these maxima are blue-shifted (about 30 nm) as compared to the fluorescence maxima of the studied compounds in water (Figure 2). Singh A.K. and Aruna R.V. [23] also found a blue shift (maximum at around 340 nm) of the emission spectrum of tryptophan in AOT (aerosol sodium bis(2-ethyl-hexyl) sulphosuccinate) reverse micelles as compared to the tryptophan emission maximum in water (363 nm).

In order to examine how the fluorescence lifetime of the studied indoles are influenced by immobilization in the PVA film, the time-resolved fluorescence measurements have been performed for the compounds dissolved in water and immobilized in the PVA matrices. The results of the above mentioned measurements are gathered in Table 1.

Table 1. Fluorescence decay times for aqueous and immobilized in the PVA film indole, NATA and tryptophan (10^{-4} M)

	Aqueous solutions			Immobilized in PVA film			
	τ_1 [ns]	τ_2 [ns]	χ^2	τ_1 [ns]	τ_2 [ns]	τ_3 [ns]	χ^2
indole	4.32	-	1.288	0.80	4.03	6.60	1.017
NATA	3.09	-	1.467	0.23	2.39	5.30	1.159
tryptophan	2.93	1.16	1.290	0.35	2.92	5.29	1.490

The values reported are those for measurements made at 20°C, with λ_{ex} = 290 nm, λ_{em} = 360 nm

The fluorescence decay of indole and NATA in water are described by single exponential kinetics with the lifetime of 4.32 ns (indole) and 3.09 ns (NATA).

The obtained results are in reasonable agreement with those (4.8 ns (indole), pH = 7, 20°C, λ_{ex} = 280 nm, λ_{em} = 330 nm) and 3.0 ns ((NATA), pH = 7, 20°C, λ_{ex} = 280 nm, λ_{em} = 330 nm) measured by Szabo and coworkers [2] and Qiang and Seeger (2.85 ± 0.05 ns for NATA) [24].

Our results indicated that the fluorescence of tryptophan in water obeys double exponential decay kinetics with the two components about 3 ns and 1 ns. Szabo and Rayner [2] also demonstrated that the fluorescent decay of aqueous solutions of tryptophan could be resolved into two exponentially decaying components, 3.25±0.03 ns and 0.65±0.14 ns (pH = 7, 20°C, λ_{ex} = 280 nm, λ_{em} = 360 nm). The authors demonstrated that the dual exponential decay of fluorescence of aqueous tryptophan originates from two or more rotamers along Cα-Cβ bond of L-tryptophan which has different configurations of alanyl side chain in reference to the indole nucleus. The rotamer with the ammonium group closest to the indole ring has been assigned as the 3 ns component, while the rotamer with the carboxylate group closest to the indole ring (the α-ammonium group is farthest away from the indole ring) is the short-lived (0.6 ns) component.

The fluorescence decay of indole, NATA and tryptophan immobilized in PVA film may be described by three exponential kinetics with the longer-lived component of about 5 ns (NATA, tryptophan) or 6.60 ns (indole), which may be attributed to the studied indoles immobilized in the PVA matrices. It may be concluded that the immobilization of the studied indoles in the PVA matrices leads to the increase of fluorescence lifetime. Zelent and coworkers [25] also reported that the changes in microviscosity of NATA leads to the increase of singlet state lifetime (the fluorescence lifetime of NATA in propylene glycol at 20°C has been determined to be about 5.3 ns).

Phosphorescence lifetime of the studied indoles in PVA matrix

In order to verify how the immobilization of the studied indoles affects the phosphorescence lifetime, we have determined the triplet state lifetime of indole, NATA and tryptophan both in aqueous solutions as well as immobilized in the PVA matrices. A typical phosphorescence decay curve for tryptophan in PVA is shown in Figure 4. The triplet lifetime of indole, NATA and tryptophan in aqueous solutions as well as immobilized in PVA film are gathered in Table 2.

Table 2. Phosphorescence lifetimes of indole, NATA and tryptophan in aqueous solutions and immobilized in PVA film (concentration of indole, NATA and tryptophan 10^{-4} M)

	Aqueous solutions	Immobilized in PVA film		
	τ [μs]	τ_1 [μs]	τ_2 [μs]	χ^2
indole	39.7 (41)	185.4	46.7	1.104
NATA	137.4 (31)	159.2	37.6	1.205
tryptophan	53.8 (56)	148.4	39.3	1.305

The values in parentheses correspond to those measured by flash photolysis studies using triplet-triplet absorption, all measurements have been performed at 20°C

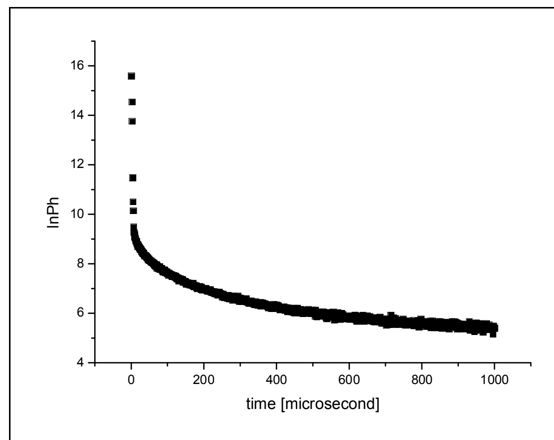

Figure 4. Representative single exponential decay of tryptophan phosphorescence

Although Strambini claims that the method of flash photolysis is unsuitable for studying the photophysics of the triplet state of indole chromophores [5], the phosphorescence lifetime of aqueous indole and tryptophan determined using both triplet-triplet absorption flash photolysis as well as applying photon counting phosphorescence technique are in reasonable agreement (Table 2). However, the triplet state lifetime of NATA determined using the above mentioned techniques are not consistent. The true lifetime of NATA is still under debate, one hypothesis is that it is milliseconds long (>5 ms) [6], while the another states that it is microseconds long (39 μs) [7]. Nevertheless, when comparing the triplet state lifetime of the indoles in aqueous solutions with that in PVA matrix, the increase in the lifetime can be seen. Most probably, the enhancement of the rigidity of the environment near the chromophore reduces the rates of the non-radiative deactivation pathways, as it suppresses diffusion of triplet quenchers such as molecular oxygen and restricts the internal motions of the molecule. In this way the increased rigidity of the environment leads to the increase of phosphorescence lifetimes of the studied compounds.

It should be emphasized that the phosphorescence measurements were performed using the PVA films, which have been dried only for 10 days. The triplet state lifetimes of the studied indoles in the PVA films measured after longer time of incubation are expected to have greater values.

Additionally, when comparing the decay kinetics of the studied indoles in water and in PVA matrix, it can be observed that the decay became more heterogeneous in the solid state. The requirement for at least a biexponential function for the kinetic description of processes in polymer matrices may be interpreted by the presence of at least two sorption sites for the guest molecules in the polymer matrices.

Conclusions

In this work the fluorescence and phosphorescence techniques have been applied in order to study the influence immobilization in PVA matrixes on the singlet and triplet excited state lifetime of indole and its derivatives. The results indicated that the maximum of fluorescence for all the studied indoles in the PVA matrix is approximately 30 nm blue shifted in comparison to the fluorescence maxima of the studied compounds in aqueous solutions.

Moreover, the obtained results showed that the immobilization in PVA matrix leads to the increase of both fluorescence and phosphorescence lifetime of the studied indoles, which may suggest that exploiting protective screening effect of organized media restricts the internal motion of the molecules (which minimizes self-quenching) and suppresses diffusion of triplet quenchers, which all together result in the increase of phosphorescence lifetime of the studied indoles.

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