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On the Diffusion of Hypericin in Dimethylsulfoxide/Water Mixtures—The Effect of Aggregation

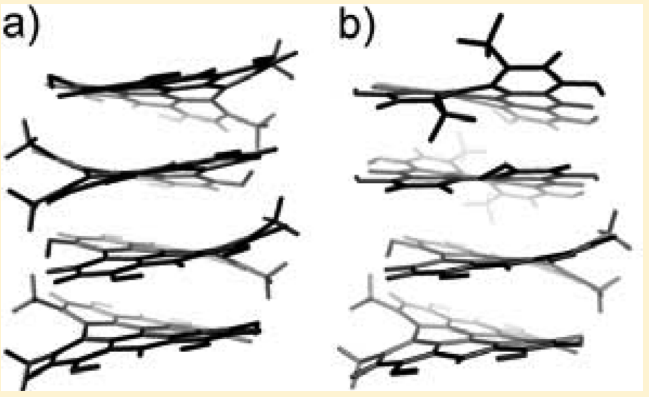
Gregor Bánó,*[,†] Jana Staničová,[‡] Daniel Jancura,[†] Jozef Marek,[§] Mikuláš Bánó,[§] Jozef Uličný,[†] Alena Strejčková,[†] and Pavol Miškovský[†]

[†]Department of Biophysics, P. J. Šafárik University, Košice, Slovakia

[‡]Institute of Biophysics and Biomathematics, University of Veterinary Medicine and Pharmacy, Košice, Slovakia

[§]Institute of Experimental Physics, Slovak Academy of Sciences, Košice, Slovakia

ABSTRACT: Hypericin (Hyp) is a natural photosensitizing pigment with a possible application in the photodynamic therapy of cancer. Hyp is readily dissolved in dimethylsulfoxide (DMSO) but forms nonsoluble aggregates in an aqueous environment. Fluorescence spectroscopy and diffusion coefficient measurements are used to investigate the self-association of Hyp molecules in DMSO/water mixtures. Fluorescence measurements reveal that Hyp remains in its monomeric form in DMSO/water mixtures containing up to ~20−30 wt % water. At higher water concentration, Hyp starts to form nonfluorescent aggregates. To determine the size of the aggregates, the diffusion coefficient of Hyp is determined for different DMSO/water mixtures both experimentally and theoretically. Our data indicate that the size of the aggregates increases as more water is added into DMSO. At 50 wt % water content, the effective diffusion coefficient is about 30% smaller than the calculated value for the stacked Hyp tetramer. The results indicate that in an aqueous environment, Hyp presumably produces large molecular weight stacked H-aggregates. We have also confirmed that in an aqueous environment at alkaline pH, molecules of Hyp remain in the monomeric state.



INTRODUCTION

Hypericin (Hyp) (Figure 1)—a natural photosensitizing pigment from plants of the genus *Hypericum*—has been extensively studied because of its antiviral and antibacterial properties[1,2] and its possible application in cancer photodynamic therapy.[3−7] Hyp is readily dissolved in polar organic solvents, like dimethylsulfoxide (DMSO), acetone, ethyl acetate, and alcohols. Hyp dissolved in these solvents remains in its monomeric form up to concentration of ~10^{-3} M and possesses relatively high quantum yield of fluorescence (~0.2). Hyp is sparingly soluble in nonpolar organic solvents and oil. In an aqueous environment, Hyp is almost insoluble and forms nonfluorescent aggregates,[8−10] which significantly suppress its photodynamic activity.[9,11] Hyp molecules remain in neutral form in acidic environments, while they are predominantly monoanions at neutral pH.[10] However, Hyp is partially soluble in alkaline aqueous solutions, where it appears as a dianion.

Two different mechanisms for neutral and/or monoanionic Hyp self-association have been proposed: (i) stacked (face-to-face) association forming H-aggregates and (ii) planar association forming J-aggregates. Planar association of Hyp involves H-bonding between the hydroxyl and carbonyl groups of Hyp molecules. This form of aggregation was suggested by Sevenants[12] and was further corroborated by Yamazaki et al.[13] In this model, H-bonded planar oligomeric structure involves additional stacking of Hyp molecules. Likely formation of Hyp J-aggregates has been also reported for the case of Hyp 1,6-tautomer dissolved in tetrahydrofuran or butyl acetate.[14] On the other hand, comprehensive studies of Falk and Meyer[15] have confirmed stacked association of Hyp dissolved in DMSO/water mixtures. This stacking is due to the hydrophobic effect of the aromatic core of Hyp molecules. They proposed a model in which the neighboring molecules within the stack are rotated against each other by about 180°. The corresponding ball and stick model of the aggregates was published[9,15] and is also reproduced by our present simulations (Figure 2).

The diffusion of self-associated Hyp molecules is affected by the aggregates' size and structure. Consequently, diffusion coefficient measurements can provide information on the level of Hyp aggregation under different conditions. The diffusion of Hyp aggregates in an aqueous environment may be—under certain circumstances—the driving force of Hyp transport within the

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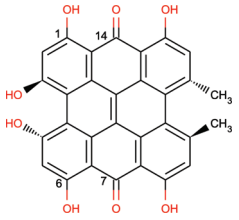

Figure 1. Chemical structure of hypericin. The propeller conformation of the 7,14-dioxo Hyp tautomer is shown in the (*P*) enantiomeric form.[9]

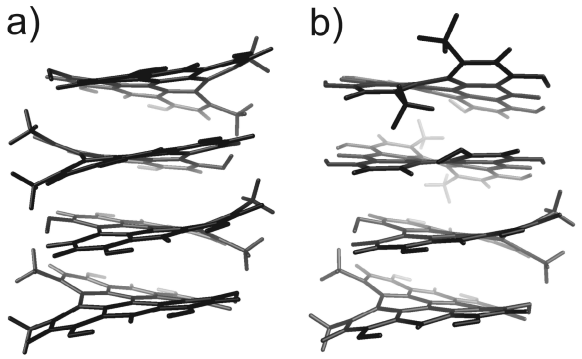

Figure 2. Ball-and-stick model of two different stacked Hyp^- tetramers calculated by the Autodock 4 software package. (a) Homochiral (*P*)(*P*)(*P*)(*P*) tetramer with the neighboring molecules rotated by about 180° against each other. (b) Racemic (*P*)(*P*)(*M*)(*M*) tetramer with the neighboring (*P*) and (*M*) enantiomers rotated by about 90°.

living tissue, which is highly important for biological and medical applications of Hyp.

The aim of this work is to provide and analyze new experimental data on the formation of Hyp aggregates in an aqueous environment. Fluorescence spectroscopy and diffusion coefficient measurements are used to accomplish this goal. Fluorescence spectroscopy can easily discriminate between the monomeric and the aggregated states of Hyp. By means of this technique, the relative concentration of the monomeric form of Hyp is determined in various DMSO/water mixtures. There are several experimental methods by which the diffusion coefficient of solutes can be determined in liquid solvents. In most cases (exceptions are fluorescence correlation spectroscopy or dynamic light scattering), time-dependent concentration changes of the solute are detected in the presence of spatial concentration gradients. In the present work, a steplike spatial gradient of the solute is created in a diffusion-capillary tube by connecting two capillaries (with and without the solute) at the beginning of the measurement. The time dependence of the diffusing species' concentration is detected at the distance of about 1 mm from the joint of the two capillaries by measuring the absorption of Hyp molecules.

THEORETICAL CONSIDERATIONS

The diffusion coefficients of Hyp^- molecules were calculated following the method given in ref 16. The diffusion coefficient was derived from the Stokes−Einstein equation

$$D = \frac{kT}{\xi} \qquad (1)$$

with k and T being the Boltzmann constant and temperature, respectively. The friction coefficient ξ was calculated based on hydrodynamic−electrostatic analogy[17,18] as

$$\xi = 6\pi\eta C \qquad (2)$$

where η is the viscosity of the solvent and C represents the electrostatic capacitance of a fictive conductive surface of the molecule. For this purpose, the molecules were replaced with a system of atomic spheres with van der Waals radii. The capacitance was calculated by the Monte Carlo method described in our previous work.[16]

The molecular geometry and the charge distribution of the hypericinate monomer anion in the propeller conformation[9] were evaluated at the B3LYP/6-31G* level, and effective point charges were obtained by the Merz−Kollman scheme. Here, we used our previous results of quantum-mechanical studies on Hyp properties.[19] The structure of stacked Hyp^- aggregates was calculated in consecutive steps by the Autodock4 software.[23] In each step, the next (higher) aggregate was calculated by docking a Hyp^- monomer to the previous (smaller) aggregate. The Hyp^- tetramer was the highest aggregate studied in this work.

The above calculation procedure of diffusion coefficients does not take into account the possible solvation, which may increase the hydrodynamic size of the aggregate. Thus, the calculated values overestimate the diffusion coefficient and can be considered an upper limit value.

The propeller conformation of Hyp (and Hyp^-) is a racemate with two possible enantiomeric forms, denoted as (*P*) and (*M*).[9] The enantiomers of Hyp were investigated theoretically and experimentally by other authors.[9,20−22] In the present work, the structure of the homochiral (*P*)(*P*)(*P*)(*P*) Hyp^- tetramer was compared to that of the (*P*)(*P*)(*M*)(*M*) and (*P*)(*M*)(*P*)(*M*) racemic aggregates. The illustrative ball-and-stick models of the (*P*)(*P*)(*P*)(*P*) and (*P*)(*P*)(*M*)(*M*) Hyp^- tetramers are shown in Figure 2. In agreement with Falk et al.,[15] the binding energy for formation of the homochiral tetramer (18.5 kcal/mol—as calculated by the Autodock4 software[23]) was found to be the highest among all investigated structures. However, the obtained binding energies for formation of (*P*)(*P*)(*M*)(*M*) and (*P*)(*M*)(*P*)(*M*) aggregates were lower only by 1.5% and 5%, respectively. The neighboring molecules within the homochiral tetramer were found to be rotated with respect to each other by about 180°, while the neighboring (*P*) and (*M*) enantiomers within the racemic tetramers were found to be rotated by about 90° (see Figure 2). It is obvious that a system containing only (*P*)(*M*)(*P*)(*M*) aggregates would contradict the experimental observation of Falk and co-workers,[15] where 180° rotation of neighboring molecules was detected by NMR measurements. However, because of the small binding energy differences between the formation of homochiral and racemic aggregates, we cannot exclude the presence of racemic structures in aqueous environment. The diffusion coefficients calculated for the (*P*)(*M*), (*M*)(*P*)(*M*), (*P*)(*P*)(*M*)(*M*), and (*P*)(*M*)(*P*)(*M*) Hyp^- aggregates are only slightly higher than the diffusion coefficients of corresponding homochiral aggregates, the differences being within 0.8%. Thus, possible different types of Hyp^- aggregates cannot be distinguished by the present diffusion coefficient measurements.

EXPERIMENTAL SECTION

Hypericin and spectrometric grade DMSO (99.99%) were purchased from Sigma-Aldrich. Stock solution of Hyp in DMSO with a final concentration of 10^{-3} M was prepared. Deionized water (ISO 3696) was used to prepare DMSO/water mixtures.

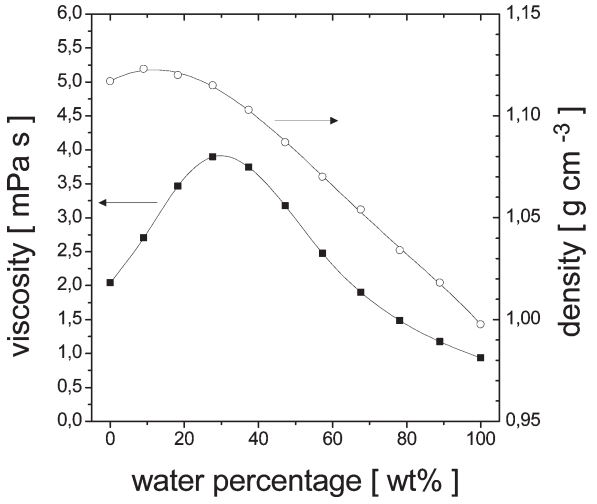

Figure 3. Viscosity and density of DMSO/water mixtures as a function of the water content.

Absorption and Fluorescence Measurements. The absorption of Hyp in DMSO/water mixtures was measured for Hyp molar concentrations of 1×10^{-4} and 5×10^{-6} M at the wavelength range of 400−700 nm by a Shimadzu UV-2401PC UV/vis and a Varian Cary 100 Bio UV−visible spectrophotometer using 1 mm and 10 mm fused quartz cuvettes, respectively. The fluorescence of Hyp at 5×10^{-6} M was measured by a Varian Cary Eclipse fluorescence spectrophotometer using an excitation wavelength of 550 nm.

At high Hyp concentrations (above 1×10^{-5} M), self-absorption of the fluorescent light causes a significant distortion of the measured spectra. To suppress self-absorption, the thickness of the sample has to be sufficiently small. We used our own optical setup to measure the fluorescence of Hyp at 1×10^{-4} M. The excitation beam of a 488 nm argon ion laser (∼5 mW, ∼3 mm beam diameter) passed through a special cuvette made from two glass plates. The distance between the two plates was kept constant by means of two (0.17 mm thick) glass spacers. The narrow gap formed this way was filled with the solution. The fluorescent light was collected and focused onto the entrance slit of an Acton Research Corporation SpectraPro-300i spectrograph equipped with a Princeton Instruments Spec-10:400 TE cooled CCD camera. The maximal depletion of the fluorescence intensity due to self-absorption near the absorption maximum of Hyp around 600 nm was estimated to be below 4%.

Viscosity Measurements. The theoretical calculations of the diffusion coefficient of Hyp monomers and its aggregates rely on viscosity values of the DMSO/water mixture. Precise viscosity data were obtained using the viscosimeter described in ref 24. The viscosimeter operates with a magnetically suspended rotor submerged in the sample. Rotating permanent magnets are used to induce the spinning of the rotor, and the viscosity is calculated from the measured angular velocity of the rotor. The density of the mixture was also determined (even if not directly needed in this study) by the same apparatus. All the viscosity and diffusion coefficient measurements presented in this paper were carried out at a temperature of 296 K.

The measured viscosity of the DMSO/water mixture is shown in Figure 3 together with corresponding density values. Viscosity reaches a maximum (η = 3.9 mPas) at about 30 wt % water in DMSO. The highest density (1.12 g cm^{-3}) is attained at 12 wt % water in DMSO.

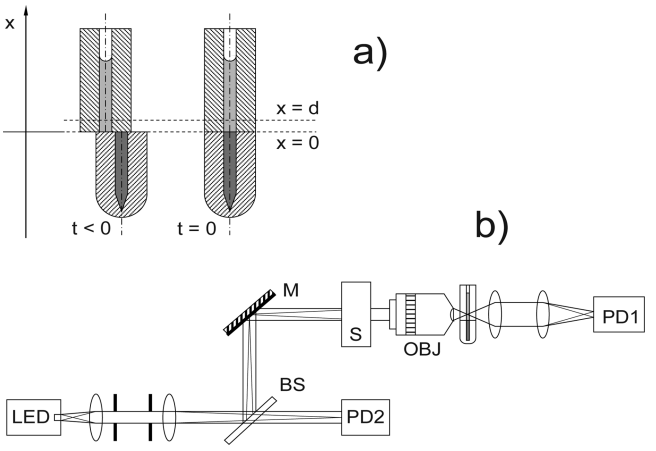

Figure 4. Experimental setup for diffusion coefficient measurements. (a) The two half capillaries before the measurement (misaligned) and at the beginning of the measurement at t = 0. (b) The optical setup. PD1 and PD2, photodiodes; BS, beam splitter; M, mirror; OBJ, microscope objective; S, shutter.

Diffusion Coefficient Measurements. *The Diffusion-CapillaryApparatus.* The schematic view of the experimental setup for diffusion measurements is shown in Figure 4. There were two capillary tubes (inner diameter = 0.9 mm, outer diameter = 4 mm) placed above each other. These capillaries were made out of a single piece by cutting it in two and polishing the opposite faces along the cut. The lower capillary was closed at the bottom. The upper capillary was kept open. The length of the lower and upper capillaries was about 10 and 15 mm, respectively.

Prior to the measurement, the bottom and top capillaries were filled with the solvent containing the solute and the pure solvent, respectively. As a next step, the two capillaries were placed above each other—having their axis misaligned—as shown in Figure 4a. At the start of the experiment, the bottom capillary was horizontally shifted so that it became aligned with the upper one. As a consequence, the solute diffused into the upper part. The shifting took about 5 s. To leave the upper capillary open was advantageous in order to ensure smooth contact of the two volumes, and it also resulted in a sharp initial division between the region with and without the solute.

The presence of the solute in the upper capillary was detected at a distance d above the joint by light absorption. The light emitted from a LED (type, Seoul Semiconductor A10292; central wavelength, 595 nm) was directed by lenses and diaphragms to form a 2 mm diameter beam. A microscope objective (10×, NA: 1.3) focused this beam into the capillary tube. The beam width inside the capillary was estimated to be approximately 0.1 mm. The light passing through the capillary was detected by a Hamamatsu S1336-5BQ photodiode (PD1). The signal was measured repetitively with a frequency of 0.1 Hz. Each measurement represented an average value taken over a 1 s period with a sampling rate of 20 kHz. A shutter blocked the light between the measurements to minimize the effect of photobleaching. The overall power entering the absorption region was about 20 μW. The photodiode PD2 (Thorlabs DET36A, see Figure 4) detected the slow power fluctuation of the LED during the measurement. The signal of PD2 was used to correct the measured data for these fluctuations.

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Data Analysis. The duration of all the diffusion measurements presented here was much shorter than the time of the solute diffusion through the overall length of the two half-capillaries. Thus, one can assume the length of the capillaries to be infinite in the evaluation. It is also assumed that there was no adsorption of the solute onto the capillary walls. In the case of single-type diffusing species, the one-dimensional diffusion equation can be solved in an infinite space with a step-function initial spatial profile of the solute concentration

$$c(x, t = 0) = c_0 \quad \text{for} \quad x < 0$$
$$c(x, t = 0) = 0 \quad \text{for} \quad x > 0 \tag{3}$$

where $c(x, t)$ is the molar concentration of the solute as a function of the position x and time t and c_0 is the initial concentration of Hyp in one of the two half-capillaries (see Figure 4). Under these conditions, the solution of the diffusion equation obeys the following form:

$$c(x, t) = \frac{c_0}{2}\left(1 - \mathrm{erf}\left(\frac{x}{2\sqrt{Dt}}\right)\right) \tag{4}$$

D represents the diffusion coefficient of Hyp. The intensity $I(t)$ of the light passing through the capillary at a distance d above the joint and detected with the photodiode PD1 is directly related to the concentration of the solute through the absorbance $A(t)$

$$A(t) = -\log\left(\frac{I(t)}{I_0}\right)$$
$$= \frac{A_0}{c_0}\, c(d, t) = \frac{A_0}{2}\left(1 - \mathrm{erf}\left(\frac{d}{2\sqrt{Dt}}\right)\right) \tag{5}$$

where A_0 denotes the absorbance that would correspond to the initial concentration c_0 and I_0 is the light intensity in the absence of the solute. The experimental absorbance curve was fitted by eq 5, and both A_0 and D were determined.

The above evaluation method applies for single-type diffusing molecules, e.g., Hyp monomers dissolved in pure DMSO in our case. Under conditions when there are different aggregates of Hyp present in the solution, the used method will not distinguish between them. Different spatial distribution of Hyp aggregates along the diffusion-capillary tube may arise because of two different reasons. First, smaller aggregates diffuse faster. Second, as indicated by our fluorescence measurements, the equilibrium (steady-state) abundance of the different aggregates (and the monomers) depends on the concentration of Hyp molecules, which varies both in time and in space in the diffusion-capillary tube. It was observed in our experiments that even in this case the measured absorbance can be fitted well with equation eq 5. However, one has to keep in mind that the corresponding diffusion coefficient, determined by this method, represents a weighted effective value, regarding all the different aggregates present in the solution. Becuase of this fact, the measured diffusion coefficient values are denoted as D_{eff} throughout this paper.

RESULTS AND DISCUSSION

Absorption and Fluorescence Measurements. Figure 5 shows the absorption (a) and fluorescence spectra (b) of Hyp at 1×10^{-4} M concentration in DMSO/water mixtures. Both the absorbance and the fluorescence intensities of Hyp decrease with the increase of water content. There is a slight blue shift of the two strongest Hyp absorbance peaks at 555 and 600 nm as more water is added to DMSO. This shift to shorter wavelengths appears when the water content is within the range 0−40 wt %. We suggest that this blue shift corresponds to slightly different absorption spectra of monomers of Hyp in pure DMSO and DMSO/water mixture. On the other hand, at higher water concentration (>40 wt %), a bathochromic shift of the two most intense Hyp absorption bands is observed, and the spectrum at 80 wt % water content is similar to the one obtained after suspending Hyp in pure water.[15] At this concentration of water in the DMSO/water mixture, the extinction coefficient of Hyp at 600 nm is about four times lower (~10 000 M^{-1} cm^{-1}) in comparison with the corresponding value in DMSO. The spectral differences represent Hyp molecules' self-association caused by the presence of water. The nonexistence of isosbestic points in the absorption spectra of Hyp in different DMSO/water mixtures suggests that the transition of monomers to aggregates of Hyp is not a two-state process and that different

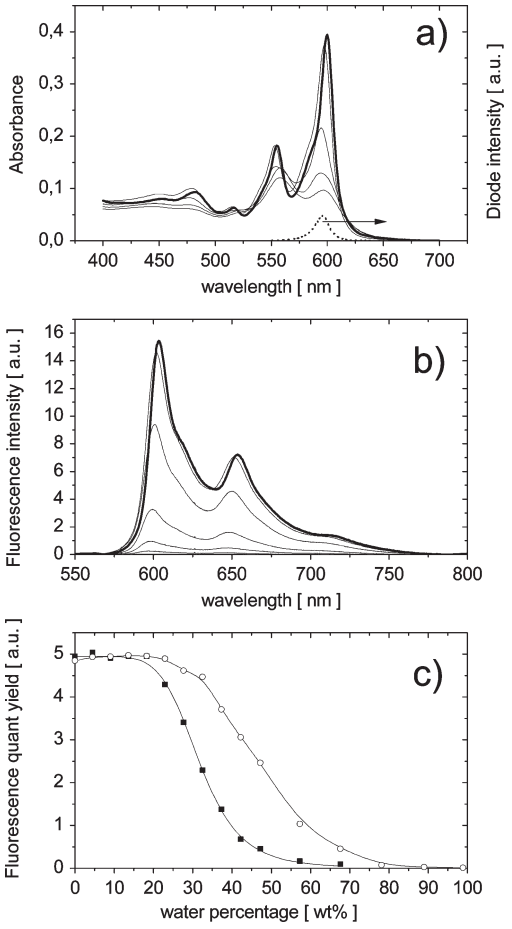

Figure 5. Absorption and fluorescence spectra of Hyp in different DMSO/water mixtures. (a) Absorbance of Hyp (molar concentration of 1×10^{-4} M) in DMSO/water mixtures containing 0 (thick line), 18, 37, 57, and 78 wt % water. The emission spectrum of the LED used for the diffusion coefficient measurements is also shown. (b) Fluorescence of Hyp (at molar concentration of 1×10^{-4} M) obtained with excitation wavelength of 488 nm in DMSO/water mixtures containing 0 (thick line), 18, 28, 37, 47, and 57 wt % water. (c) Fluorescence quantum yield of Hyp at molar concentration of 1×10^{-4} M (solid squares, 488 nm excitation) and 5×10^{-6} M (open circles, 550 nm excitation) as a function of water percentage in DMSO/water mixtures.

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types of Hyp aggregates are probably formed with the increase of water content in the DMSO/water mixture.

The fluorescence maxima of Hyp gradually shift toward shorter wavelengths as the water percentage increases (Figure 5b). It is usually assumed that only the monomer form of Hyp contributes to the fluorescence signal. Thus, the observed blue shift only expresses a higher probability for the formation of hydrogen bonds between Hyp and water molecules in DMSO/water mixture in comparison with pure DMSO. It was suggested that the hydrogen bonding does not involve the phenolic hydroxy groups but involves the carbonyl oxygens of Hyp.[13] This observation nicely corresponds with the blue shift of the absorption maxima of Hyp with the increase of water content in DMSO/water mixtures.

Figure 5c presents the dependence of the Hyp fluorescence quantum yield (the integrated fluorescence intensity normalized to the absorption at the excitation wavelength) on the water concentration in DMSO/water mixture. The results are shown for two different molar concentrations of Hyp: 1×10^{-4} and 5×10^{-6} M. The fluorescence intensity remains constant until water content reaches 15 and 25 wt % in DMSO/water mixture for 1×10^{-4} and 5×10^{-6} M concentrations of Hyp, respectively. This means that up to these values of water concentrations, almost all Hyp molecules remain in monomeric state. Further increase of the water concentration leads to a drop in Hyp fluorescence. The decrease is a consequence of the Hyp aggregates formation. The observed data suggest that 50% of Hyp molecules are still monomers at the water content of 30 and 45 wt % for 1×10^{-4} and 5×10^{-6} M concentrations of Hyp, respectively. The Hyp fluorescence is almost completely quenched at ~60 and ~80 wt % of water in DMSO. At lower Hyp concentration the rate of associative Hyp collisions is lower, which results in a higher abundance of Hyp monomers, and is reflected in the higher percentage of water needed for total fluorescence quenching.

Falk et al. suggested that Hyp fluorescence at high water content could be also derived from aggregated Hyp species.[15] However, almost identical spectra of Hyp fluorescence for all water concentrations studied do not support this hypothesis.

The data obtained from the fluorescence experiments provide useful information about the dependence of the monomeric Hyp concentration on the water content in DMSO/water mixture. However, the data do not reveal the size of the Hyp aggregates formed under increasing water concentration. We attempted to obtain such information by determining the diffusion coefficient of Hyp in DMSO/water mixtures at various water contents.

Diffusion Coefficient Measurements and Calculations. Figure 6a shows the time dependence of the absorbance of Hyp dissolved in DMSO (c(Hyp) = 1×10^{-4} M) measured at three different positions (d = 1.15, 1.25, and 1.65 mm) in the diffusion-capillary tube. For a certain diffusion coefficient D and initial concentration of Hyp c_0, the concentration evolution of the solute depends only on the ratio of t/x^2 (see eq 4). It means that the shape of the $c(t)$ curves detected at different positions is identical with t/x^2 acting as a similarity parameter. The absorbances plotted against t/d^2 are depicted in Figure 6b. The measured data are multiplied by a correction factor of 1−1.2 to account for a different capillary diameter and/or beam settings at the three different positions. It is evident that all three curves overlap very well. This result indicates that there is no significant distortion of the experiment due to an uncontrolled mixing of the solvent in the upper and lower capillary at the beginning of the measurement.

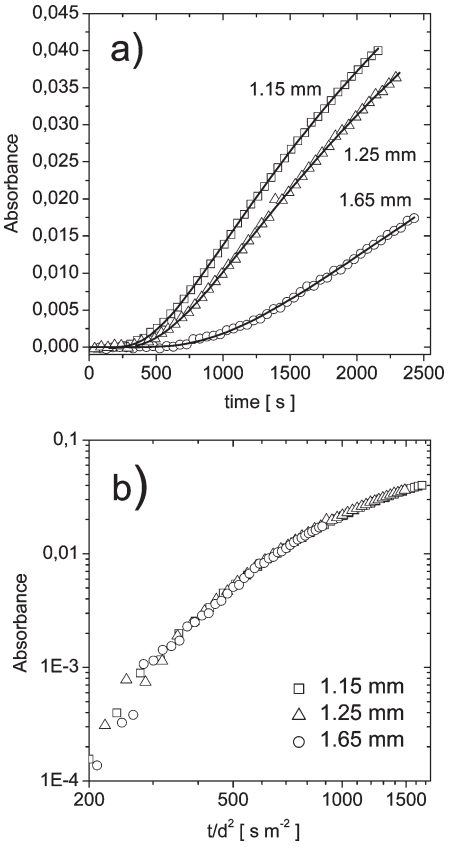

Figure 6. Time evolution of Hyp absorbance during the diffusion coefficient measurements in DMSO. Data measured at three different positions (d = 1.15, 1.25, and 1.65 mm) are plotted against (a) t and (b) t/d^2. Every fifth measured point is shown in the figure. The molar concentration of Hyp is 1×10^{-4} M.

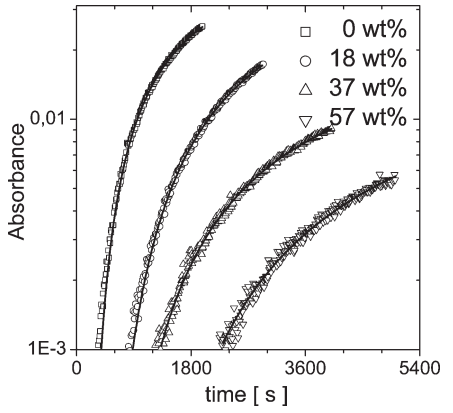

Figure 7. Time evolution of Hyp absorbance during the diffusion coefficient measurements in different DMSO/water mixtures. The four experimental curves belong to different concentrations (0, 18, 37, 57 wt %) of water in DMSO. Experimental data are fitted by eq 5.

Time evolution of the Hyp absorbance in the diffusion-capillary is shown in Figure 7 at different values of water content in DMSO. The diffusion of Hyp slows down as more water is added to the DMSO/water mixture. The effective diffusion coefficients D_{eff}—determined by fitting the experimental data (see Figure 7) by

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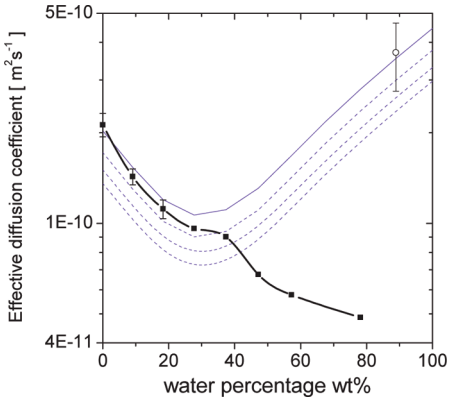

Figure 8. Experimental and calculated diffusion coefficients of Hyp^- aggregates. Solid squares: measured values of the effective diffusion coefficients of Hyp as a function of water percentage in DMSO. Open circle: the diffusion coefficient of Hyp in a pH 11 DMSO/buffer mixture. The molar concentration of Hyp is 1×10^{-4} M in all cases. Solid and dashed lines belong to calculated diffusion coefficients of stacked homochiral Hyp aggregates containing one and two, three, and four Hyp molecules, respectively.

Table 1. Calculated Ratio of the Friction Coefficient ξ and the Viscosity η for Stacked Homochiral Hyp^- Aggregates Consisting of n Molecules

n	ξ/η (10^{-10} m)
1	98.39
2	116.24
3	132.72
4	147.35

eq 5—are plotted in Figure 8 as a function of the water percentage in DMSO/water mixtures.

The theoretically calculated ratio of the friction coefficient ξ and the viscosity η (see eq 2) is tabulated for stacked homochiral Hyp^- aggregates consisting of n molecules in Table 1. These theoretical values can be combined—based on eq 2 and eq 1 — with the experimental viscosity data of Figure 3 to obtain a prediction for the diffusion coefficient of different Hyp^- aggregates. The obtained results are shown in Figure 8 as indicated with solid and dashed lines.

There is a good agreement of the measured and calculated diffusion coefficient values of Hyp monomers in pure DMSO (Figure 8). On the basis of this observation, we conclude that a possible solvation of Hyp by DMSO does not have a significant effect on its diffusion. The fluorescence spectroscopy data reveal that Hyp remains in its monomeric form for up to 15 wt % water content in DMSO (see Figure 5). However, the obtained diffusion coefficients in the presence of 10−20 wt % water in the DMSO/water mixture are slightly lower than the calculated values for the monomers. This is an indication that Hyp monomers are hydrated by water molecules, as would be expected taking the hydrophilic character of the hydroxyl groups of Hyp into account, and also as it was demonstrated by the blue shift of the absorption and fluorescence spectra of Hyp. At above 30 wt % water content, the Hyp diffusion coefficient still decreases gradually while the theoretically predicted values of the monomeric form increase toward higher water content (Figure 8). This decrease is a sign of Hyp aggregates formation. Our data indicate that the characteristic size of the aggregates increases as more water is added to DMSO. At 50 wt % water content in DMSO, the effective diffusion coefficient is about 30% smaller than the calculated value for the stacked Hyp tetramer. This is in a qualitative agreement with the NMR observations of Falk and Meyer.[15] It was shown that at 50 wt % water the aggregates consist of at least four Hyp molecules.[15] Our results provide direct evidence, in accordance with the previous suggestion,[12,13,25] that in aqueous environment Hyp forms aggregates with large molecular weight. We hypothesize that stacked H-aggregates are the main form of Hyp structure in water.

To demonstrate the existence of the monomeric Hyp at alkaline pH in aqueous environment, we have determined the diffusion coefficient of Hyp in DMSO/water (10/90) mixture at pH of 11 (Figure 8, open circle). This value is in a very good agreement with the calculated one. This is another proof that dianionic Hyp, present at high pH, is unable to form nonfluorescent aggregates at a concentration of 10^{-4} M.

CONCLUSIONS

The aggregation of Hyp molecules in DMSO/water mixtures was investigated by fluorescence spectroscopy and by diffusion coefficient measurements. Hyp remains in its monomeric form up to water content of ~15−25 wt % in DMSO, depending on the Hyp concentration. At higher water concentration, Hyp starts to aggregate which results in a decrease of the fluorescence intensity and a lower diffusion coefficient. A comparison of the experimentally determined diffusion coefficients with the theoretically calculated values shows that at high water content in DMSO/water mixture Hyp is able to form aggregates (presumably H-aggregates) with high molecular weight. A good agreement was found between the measured and calculated diffusion coefficients of Hyp monomers in pure DMSO and in alkaline pH. This work create a solid basis for the investigation of Hyp movement in structures of biological importance.

AUTHOR INFORMATION

Corresponding Author
*E-mail gregor.bano@upjs.sk.

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