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Research Article

Photophysical Properties of Fluorescent Probe Thioflavin T in Crowded Milieu

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Thioflavin T (ThT) is a widely used fluorescent probe of amyloid fibrils, which accompanies many serious neurodegenerative and other diseases. Until recently, examinations of processes of amyloid fibril formation in vitro were conducted in solutions whose properties were significantly different from those found inside the densely packed cells. Such crowded cellular milieu is typically simulated in vitro using concentrated solutions of inert polymers, which do not usually interact with proteins. However, these crowding agents can have a direct effect on the ThT molecule, and this effect must be taken into account. We examined the influence of PEG-400, PEG-12000, and Dextran-70 on the photophysical properties of ThT. It was shown that these crowding agents caused the red shift of the absorption, fluorescence excitation, and fluorescence spectra of ThT. Under these conditions, the increases of the molar extinction coefficient, fluorescence quantum yield, and excitation lifetime of ThT are also observed. However, these changes are significantly less pronounced than those observed for ThT bound to fibrils. It is concluded that, despite some effects of crowding agents on intrinsic fluorescent properties of ThT, this dye can be used as a probe of structure and formation of amyloid fibrils in crowded milieu in vitro.

1. Introduction

Amyloid fibrils are extended, β-sheet-rich regular protein aggregates. The formation and accumulation of amyloid fibrils as intra- and extracellular deposits accompany many serious diseases, such as Alzheimer's, Parkinson's, Huntington disease, prion diseases, systemic and nonsystemic amyloidoses, and diabetes [1–7]. In recent studies, it was shown that the amyloid fibril formation is characteristic not only for proteins associated with various pathogenic states but also for many pathology-unrelated proteins [8–12]. Many studies focus on searching for different agents that would have a therapeutic impact on amyloid fibrils (prevent or retard their formation) or would enable an investigation of the molecular mechanism of the fibril formation process and to study structure of these aggregates.

Fluorescent probes, such as benzothiazole dye thioflavin T (ThT), are widely and efficiently used for diagnosing amyloid fibril formation and studying fibrillar structure (Figure 1(a)) [13–16]. The advantage of ThT is in its high specificity of interaction with amyloid fibrils. It is considered that ThT practically does not interact with proteins in their native, unfolded, and partially folded states, with amorphous aggregates and protein oligomers. Despite the fact that some recent studies revealed that there are some exceptions from this rule (ThT was shown to bind to acetylcholinesterase [17, 18], serum albumins [19], and even α-helical poly(L-glutamic acid) [20]), only interaction of this dye with amyloid

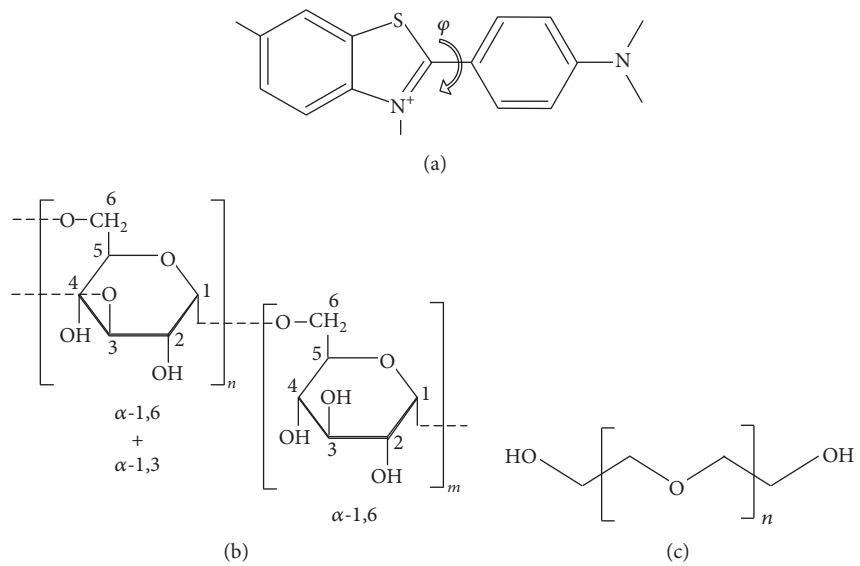

Figure 1: Structure of the fluorescent probe thioflavin T (ThT) and crowding agents. (a) ThT. The angle φ of internal rotation of benzothiazole and aminobenzene rings of ThT relative to each other is shown; (b) Dextran-70 (MW 70000 Da). It is shown that the straight chain of Dextran-70 consists of α-1,6 glycosidic linkages between glucose molecules, while branches begin from α-1,3 linkages; m: number of units (glucose molecules) with only α-1,6 glycosidic linkages and n: number of units with α-1,6 and α-1,3 linkages. (c) Polyethylene glycol (PEG). In the work we used PEG-400 and PEG-12000 with MW 400 Da (n = 9) and MW 12000 Da (n = 272), respectively.

fibrils is accompanied by the dramatic increase (by tens of thousands times) in the fluorescence quantum yield of ThT, in comparison with that determined for the unbound dye in aqueous solution (about 0.0001) [21]. It should be noted that the current understanding of the mechanisms of ThT interaction with fibrils was formed on the basis of in vitro studies in dilute buffer solutions. However, the total concentration of biomacromolecules (various proteins, nucleic acids, and carbohydrates) in the cellular environment in vivo, where proteins naturally function, is very high (50–400 mg/mL) [22–24]. The overcrowding of the cell environment results in a deficiency of free water [22, 25–29] and limited available space. The volume occupied by biomacromolecules becomes unavailable for other molecules (the excluded volume effect) [25, 27]. Such conditions of macromolecular crowding may have a significant impact on various biological processes including amyloid fibril formation [25, 30–32]. Macromolecular crowding conditions may have also impact directly on photophysical properties of fluorescence probes. However, the direct influence of crowding agent on the ThT molecule was not examined yet. To fill this gap, the aim of this work was to study the spectral properties of the fluorescent probe ThT in the conditions of macromolecular crowding.

To simulate the intracellular environment in vitro, various model crowding agents are effectively used [27, 31, 33]. Crowding agents are macromolecules with a wide range of molecular weights that do not specifically interact with examined proteins (inert). In our work, three different crowding agents were used: Dextran-70 (polysaccharide constructed from the D-glucose monomers connected by glycosidic linkages) that has the highest molecular mass 70000 Da and polyethylene glycol (PEG) that belongs to the class of polyether compounds with different molecular masses: ~12000 Da (PEG-12000) and 400 Da (PEG-400) (Figures 1(b) and 1(c)). Although solutions containing high concentrations of crowding agents are viscous, from the viewpoint of a dye molecule such solutions are characterized by high macroviscosity, which is different from the microviscosity phenomenon that can be defined as the friction experienced by a single probe molecule undergoing diffusion because of its interaction with its environment at the length scale comparable with the molecular size of the probe. To compare the effect of macro- and microviscosity, we also performed studies of the spectral properties of ThT in water-glycerol mixtures.

2. Materials and Methods

2.1. Materials. The samples of "UltraPure Grade" ThT from AnaSpec (USA) were used without further purification. Samples of Dextran-70, PEG-400, and PEG-12000 from Sigma Aldrich (USA) and glycerol from Merck (Germany) were used. The stock solutions of ThT and crowding agents were prepared in purified Milli-Q water.

2.2. Preparation Solutions of Crowding Agents. Series of solutions of crowding agents were prepared over a wide range of concentrations from 0 to 400 mg/mL. The solutions of glycerol were prepared in the concentration range from 0 to 99%. Concentrations of crowding agents and glycerol were controlled refractometrically using a refractometer IRF-45452M (Russia). The dependence of the refractive index on the glycerol concentration was taken from the work [38]. For crowding agents the dependence of the refractive index on the

concentration of crowding agents obtained in our laboratory was used (data not shown; corresponding manuscript is in preparation).

2.3. Spectroscopic Studies. The absorption spectra of crowding agents and ThT were recorded using a U-3900H spectrophotometer (Hitachi, Japan). Fluorescence measurements were conducted using a Cary Eclipse spectrofluorimeter (Agilent Technologies, Australia). Fluorescence spectra were excited at the 412 nm (ThT absorption spectrum maximum in aqueous solution) and fluorescence excitation spectra were recorded at the 490 nm (ThT fluorescence spectrum maximum in aqueous solution). Recorded fluorescence intensity and fluorescence excitation spectra were corrected on the primary inner filter effect [39].

2.4. Fluorescence Intensity Correction on the Primary Inner Filter Effect. It is known that the concentrational dependence of the fluorescence intensity is nonlinear due to the so-called primary inner filter effect. The reasons for this effect include both the attenuation of the excitation light flux along its path through an absorbing solution (Beer–Lambert law) and the difference between the area that is illuminated by the excitation light and the working area from which the fluorescence light is gathered (as it is in the most widely used spectrofluorimeters with vertical slits).

In the case when the area illuminated by the excitation light coincides with the working area from which the fluorescence light is gathered (the situation for the spectrofluorimeter that was used in the present work), the recorded total fluorescence intensity $F(\lambda_{ex})$ is proportional to the fraction of the excitation light that is absorbed by the solution $(1-10^{-A_\Sigma})$. If one substance is responsible for both the absorption and fluorescence of a solution, then

$$\begin{aligned} F(\lambda_{ex}) &= k' I_0(\lambda_{ex})\,\Delta\lambda_{ex}\left(1-10^{-A_\Sigma}\right)q \\ &= k\frac{\left(1-10^{-A_{FL}}\right)}{A_{FL}}A_{FL}q. \end{aligned} \tag{1}$$

Here, $I_0(\lambda_{ex})\Delta\lambda_{ex}$ is the intensity of the excitation light at λ_{ex}, k' is a proportionality factor, $\Delta\lambda_{ex}$ is spectral width of the slits of the monochromator in the excitation pathway, and $k = k' I_0(\lambda_{ex})\Delta\lambda_{ex}$ is a normalization factor that is determined using a standard (a fluorescent substance with known fluorescence quantum yield) in the same experimental conditions (i.e., slits widths, photomultiplier voltage, and other pertinent factors) that were used in the experiment with the sample. The coefficient k is chosen such that the total fluorescence intensity of the standard and the sample gets physical meaning to the product of the absorbance and the fluorescence quantum yield:

$$F_0(\lambda_{ex}) = \frac{F(\lambda_{ex})}{W} = A_{FL}q. \tag{2}$$

Here, W is a correction factor that is determined from the total absorbance of the solution at λ_{ex}:

$$W = \frac{1-10^{-A_{FL}}}{A_{FL}}. \tag{3}$$

It is generally accepted that, for diluted solutions characterized by low absorbance, the fluorescence intensity is proportional to the concentration of the fluorescent substance and the primary inner filter effect is negligible [39]. However, we earlier showed that this assumption is not valid and fluorescence intensity values always require correction. In reality, the total fluorescence intensity is proportional to the absorbance (A) at only one point, when $A = 0$ (at $A = 0.1$, the deviation from linearity is 12%, and, at $A = 0.3$, the deviation is 38%). In the present work, (2) and (3) were used to determine the fluorescence quantum yield of ThT in the presence of crowding agents and in water-glycerol solutions.

2.5. Determination of the Fluorescence Quantum Yield. The fluorescence quantum yield of ThT was determined by the comparative method using the ratio

$$q = \frac{F_{0,ThT}}{F_{0,ATTO}}\frac{A_{FL,ATTO}}{A_{FL,ThT}}q_{ATTO}, \tag{4}$$

where q is the fluorescence quantum yield, q_{ATTO} is the fluorescence quantum yield of a standard dye ATTO-425, $F_{0,ATTO}$ and $F_{0,ThT}$ are the fluorescence intensities of ATTO-425 and ThT corrected on the primary inner filter effect, $A_{FL,ATTO}$ is the measured absorption of ATTO-425, and $A_{FL,ThT}$ is the measured absorption of ThT.

Equation (4) can be obtained by dividing the term by term relationship (2) for ThT and ATTO-425.

2.6. Time-Resolved Fluorescence Measurements. The fluorescence decay curves in the subnanosecond and nanosecond ranges were recorded by a spectrometer Pico Quant FluoTime 300 (Germany) with the Laser Diode Head LDH-C-440 (λ_{ex} = 440 nm). For registration of the ultrafast fluorescence decay curves in picosecond ranges and for the determination of the time dependence of the fluorescence intensity and the value of the fluorescence anisotropy, the upconversion device (FOG100, CDP, Russia) was used. For excitation, a cavity-dumped titanium:sapphire femtosecond laser (Mira, Coherent), which provided short pulses (120 fs) at a repetition rate of 85 MHz, was used. The samples were excited with the second harmonic light with excitation wavelength 420 nm, and the 490 nm fluorescence from the samples was upconverted by mixing it with the fundamental light pulse (gate pulse ~ 840 nm). The upconverted signal was measured using a photon counter after passing through a proper bandpass filter and a double monochromator. All of these measurements were performed in a rotating cell (1 mm path length) to ensure better heat dissipation and to avoid dye photodegradation. The measured emission decay was fit to a multiexponential function using the standard convolute-and-compare nonlinear least-squares procedure [40]. In this method, the convolution of the model exponential function

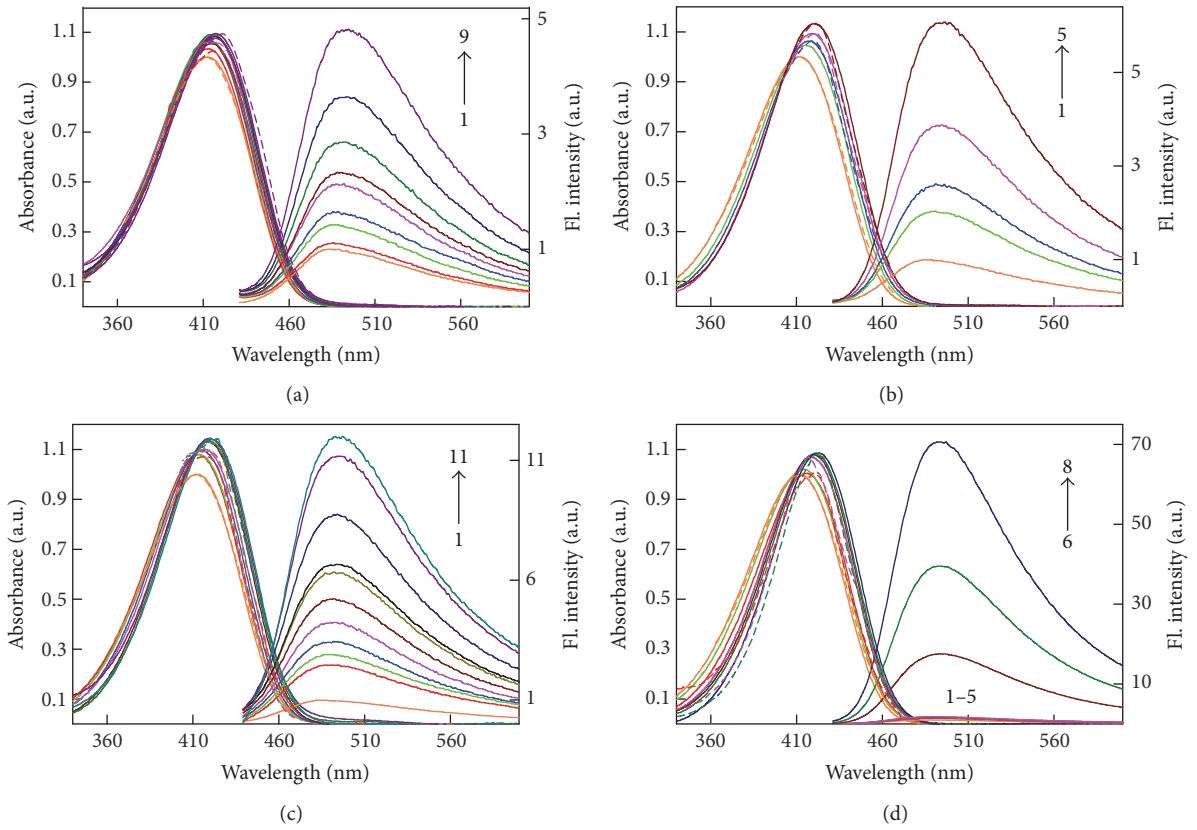

FIGURE 2: Absorption, fluorescence, and fluorescence excitation spectra of ThT in aqueous solutions in the presence of crowding agents and glycerol. (a) Absorption and fluorescence spectra (solid lines) of ThT in aqueous solutions in the presence of Dextran-70 in concentrations 0, 10, 50, 100, 150, 200, 250, 300, and 400 mg/mL (curves 1–9) and fluorescence excitation spectra (dashed lines) of ThT in aqueous solutions in the presence of Dextran-70 in concentrations 0, 10, 100, and 400 mg/mL (colors are similar to that of the absorption and fluorescence spectra) are presented. (b) Absorption and fluorescence spectra (solid lines) of ThT in aqueous solutions in the presence of PEG-400 in concentrations 0, 100, 200, 300, and 400 mg/mL (curves 1–5) and fluorescence excitation spectra (dashed lines) of ThT in aqueous solutions in the presence of PEG-400 in concentrations 0, 200, 300, and 400 mg/mL (colors are similar to that of the absorption and fluorescence spectra) are presented. (c) Absorption and fluorescence spectra (solid lines) of ThT in aqueous solutions in the presence of PEG-12000 in concentrations 0, 10, 20, 50, 100, 150, 200, 250, 300, 350, and 400 mg/mL (curves 1–11) and fluorescence excitation spectra (dashed lines) of ThT in aqueous solutions in the presence of PEG-12000 in concentrations 0, 10, 100, and 300 mg/mL (colors are similar to that of the absorption and fluorescence spectra) are presented. (d) Absorption and fluorescence spectra (solid lines) of ThT in aqueous-glycerol solutions with glycerol content of 0, 12, 25, 46, 61, 75, 89, and 99% (curves 1–8) and fluorescence excitation spectra (dashed lines) of ThT in aqueous-glycerol solutions with glycerol content of 0, 12, 61, and 89% (colors are similar to that of the absorption and fluorescence spectra) are presented; ThT concentration was ~$3 \cdot 10^{-5}$ M. Fluorescence spectra were excited at 412 nm and recorded at 490 nm.

with the instrument response function (IRF) was compared to the experimental data until a satisfactory fit was obtained. The IRF was measured using cross correlation of the excitation and fundamental gate pulse. A special program was used to analyze the decay curves [41]. The fitting routine was based on the nonlinear least-squares method. Minimization was performed according to Marquardt [42].

Fluorescence anisotropy was determined as follows: $r = (I_V^V - GI_H^V)/(I_V^V + 2GI_H^V)$, where I_V^V and I_H^V are vertical and horizontal components of fluorescence intensity excited by vertical polarized light and $G = I_V^H/I_H^H$ is coefficient which determines the different sensitivity of the registering system for vertical and horizontal components of fluorescence intensity.

3. Results and Discussion

3.1. Influence of Macromolecular Crowding Conditions on the Absorption and Fluorescence Spectra of ThT. Experimental results indicate that the presence of crowding agents or glycerol in the solution provides a red shift of the ThT absorption spectrum as compared with the absorption spectrum of the dye in aqueous solution (a maximum at λ = 412 nm). In Dextran-70 solutions, the smallest shift of the dye absorption spectra (about 5 nm at a concentration of the agent 400 mg/mL) (Figure 2(a)) compared with the absorption spectra of ThT in PEG-400 (Figure 2(b)) and PEG-12000 (Figure 2(c)) (about 9 nm at the concentrations of the crowding agents 400 mg/mL) solutions was observed. In solutions of ThT containing glycerol in a concentration

Table 1: Spectral properties of ThT in the presence of crowding agents*.

Object	Binding mode	$\lambda_{abs,max}$, nm	$\varepsilon \cdot 10^{-4}$, $M^{-1}cm^{-1}$	$\lambda_{fl,max}$, nm	q	q/q_0
ThT in aqueous solution [21]	—	412	3.2	490	0.0001	1
ThT in aqueous solution in the presence of Dextran-70 (400 mg/mL)	—	417	3.2	494	0.0005	5
ThT in aqueous solution in the presence of PEG-400 (400 mg/mL)	—	421	3.6	495	0.0008	8
ThT in aqueous solution in the presence of PEG-12000 (400 mg/mL)	—	420	3.6	497	0.0007	7
ThT in aqueous-glycerol solution with glycerol content 99% [21]	—	424	3.6	497	0.066	$6.6 \cdot 10^3$
ThT incorporated into insulin amyloid fibrils [34, 35]	1	449	7.9	488	0.72	$7.2 \cdot 10^4$
	2	448	2.3		0.27	$2.7 \cdot 10^4$
ThT incorporated into lysozyme amyloid fibrils [36, 37]	1	451	5.3	484	0.44	$4.4 \cdot 10^4$
	2	449	6.2		0.0001	1
ThT incorporated into amyloid fibrils formed from Abeta-peptide (1–42) [35]	1	442	8.3	488	0.19	$1.9 \cdot 10^4$
	2	437	1.8		0.02	$2 \cdot 10^3$

*For comparison the characteristics of ThT bound to amyloid fibrils are given.
ε: molar extinction coefficient, q: fluorescence quantum yield, q/q_0: fluorescence quantum yield normalized by the value in water (q_0), and $\lambda_{abs,max}$ and $\lambda_{fl,max}$: wavelengths of maximum of the absorption and fluorescence spectra, respectively. For ThT-amyloid fibrils interaction data shown for different binding modes (types of binding with significantly different binding parameters).

ranging from 0 to 99%, the red shift of absorption spectrum from 412 nm to 424 nm was also observed (Figure 2(d)). In the recent works it was shown that the absorption spectra of ThT bound to amyloid fibrils are more red shifted than in the presence of crowding agents or glycerol (Table 1). For example, ThT incorporated into amyloid fibrils formed from insulin, lysozyme, and $A\beta_{1-42}$ peptide has the maximum of absorption spectrum at 450, 449, and 440 nm, respectively [34–36]. Importantly, these red-shifted absorption spectra of ThT bound to fibrils can be recorded only when the samples are prepared by equilibrium microdialysis [43, 44]. The difference in the absorption spectra position can be explained by the fact that the charge of the ThT molecule is irregularly distributed between benzothiazole and aminobenzene rings in the ground and excited states. The excited state generated when the ThT molecule absorbs a light quantum is nonequilibrium. This nonequilibrium can be explained by the fact that the minimum energy in the excited state corresponds to the conformation of ThT with the angle between the planes of benzothiazole and aminobenzene rings $\varphi = 90\ (180)^\circ$. Also it can be explained by the fact that the solvation shell of the polar solvent formed at the excited state becomes nonequilibrium due to a substantial redistribution of the charge between benzothiazole and aminobenzene rings on the transition to the excited state. Interaction with the polar solvent makes the configuration of ThT molecules with the angle approaching $\varphi = 0\ (180)^\circ$ in an excited state more advantageous, while the angle φ approaching $\varphi = 90\ (270)^\circ$ increases the disequilibrium of the excited ThT molecules with the solvent molecules. Thus, the ground state is stabilized by the orientation interaction of the solvent molecules with the positively charged fragments of ThT, whereas the excited state is nonequilibrium. Therefore, the most blue-shifted absorption spectrum of ThT was observed in aqueous solution (since water is the most polar solvent) [45, 46]. So, we can assume that the red spectral shift of ThT absorption spectrum in the aqueous solution in the presence of crowding agents, glycerol, and amyloid fibrils is due to the changes in the polarity of its microenvironment. Position of the fluorescence excitation spectra of ThT corrected on the primary inner filter effect in the presence of crowding agents, as expected, practically coinciding with the position of the absorption spectra of this dye.

It is shown that, in the presence of crowding agents, a slight red shift of the ThT fluorescence spectra relative to the position of the spectrum of the dye in aqueous solution (a maximum at $\lambda_{em} = 490$ nm with excitation at $\lambda_{ex} = 412$ nm) takes place. The minimal red shift is observed in the presence of Dextran-70 (shift about 4 nm at a concentration of 400 mg/mL) (Figure 2(a)). The greatest influence on the position of the fluorescence spectra of the dye occurs in the presence of PEG-400 (Figure 2(b)) and PEG-12000 (Figure 2(c)) (shifts about 5 and 7 nm, respectively, at a concentration of the agents 400 mg/mL). The presence of glycerol in concentration range from 0 to 99% leads to a red shift of the ThT fluorescence spectrum of about 7 nm (Figure 2(d)). In the case of the dye interaction with amyloid fibrils, its fluorescence spectra are slightly blue shifted [34, 35, 37]. The observed shifts of the fluorescence spectra are less pronounced than shifts of the absorption spectra. This may be due to the fact that the fluorescence of ThT in the solutions is caused by the transition from a nonequilibrium excited state to the nonequilibrium ground state, and the position of the peak of fluorescence spectrum is not so sensitive to the solvent polarity as the position of the maximum of the absorption spectrum.

3.2. Molar Extinction Coefficient of ThT in the Presence of Crowding Agents. Our results showed there was not only a red shift of the ThT absorption spectrum under the influence of the crowding agents but also the change in the value of

molar extinction coefficient of the dye $\varepsilon(\lambda)$ evaluated using the following equation:

$$\varepsilon(\lambda) = \frac{A(\lambda)}{C \cdot l}, \quad (5)$$

where $A(\lambda)$ is the absorption measured at the wavelength λ, C is the concentration of the dye, and l is the optical pathway.

In the presence of PEG, there is an increase in the molar extinction coefficient as compared to the value determined for the dye in aqueous solution (about 31600 $M^{-1}\,cm^{-1}$): the value for PEG-400 (400 mg/mL) is about 35800 $M^{-1}\,cm^{-1}$ and that for PEG-12000 (400 mg/mL) is about 36100 $M^{-1}\,cm^{-1}$. In the presence of Dextran-70 (400 mg/mL), the changes in the molar extinction coefficient of the dye are almost nonexistent (about 32000 $M^{-1}\,cm^{-1}$). The value of the molar extinction coefficient of ThT in 99% glycerol is about 36000 $M^{-1}\,cm^{-1}$. In the recent works, a significant change in the value of the molar extinction coefficient of ThT bound to amyloid fibrils at both larger and smaller side was shown with the use of the samples prepared by equilibrium microdialysis (Table 1) [34–36]. This fact can be explained by different conformation of the dye molecules in water and water-glycerol solutions and in the presence of crowding agents or amyloid fibrils caused by changes in the microenvironment of ThT molecules.

3.3. Fluorescence Quantum Yield of ThT in the Crowded Milieu. Fluorescence quantum yields of ThT in crowded conditions were calculated by using a reference dye ATTO-425 with known fluorescence quantum yield (q = 0.9) and spectral characteristics similar to the spectral characteristics of ThT. The smallest increase in the fluorescence quantum yield of ThT (about 5 times) compared with the quantum yield of the dye in aqueous solution (about 0.0001 [21]) occurs in the presence of Dextran-70 (400 mg/mL), while the addition of PEG-400 (400 mg/mL) and PEG-12000 (400 mg/mL) leads to an about 7-8 times increase in the fluorescence quantum yield of the dye (Table 1). The presence of glycerol at concentrations from 0 to 99% leads to a more than 650-fold increase in the fluorescence quantum yield. The increase in the fluorescence quantum yield of ThT in solutions of crowding agents may be due to the molecular rotor nature of ThT, with the benzothiazole and aminobenzene rings of this dye being able to be rotated relative to each other in the excited state. According to the chemical quantum calculation data, the nonradiative transition to the ground state occurs under transition of the molecule to a state with an angle between the fragments close to 90° [45]. This fact leads to a low fluorescence quantum yield of the free ThT in aqueous solution. We believe that the increase in the fluorescence quantum yield of ThT in solutions of crowding agents is caused by the restriction of the dye fragments rotation relative to each other in the excited state due to the rigidity of the microenvironment. Furthermore, the variation of the quantum yield value may be due to the differences in the conformations of the dye molecule in the ground state in aqueous solution and in the presence of crowding agents. It can be assumed that the higher values of the fluorescence quantum yields and molar extinction coefficients of ThT correspond to a more planar conformation of the dye molecule. For comparison, the values of the fluorescence quantum yield and molar extinction coefficient of ThT incorporated into amyloid fibrils formed from lysozyme, insulin, and Aβ-peptide are shown in Table 1 [34, 35, 37]. It can be noticed that the increase in the fluorescence quantum yield of ThT in the presence of crowding agents is significantly smaller (by orders of magnitude) than that observed for the dye interacting with the amyloid fibrils.

3.4. Fluorescence Lifetime of ThT in the Crowded Medium. As discussed above, rotation of the ThT fragments relative to each other in the excited state leads to the conformation with the disturbed π-electron conjugated system ($\varphi \approx 90^{\circ}$) and, consequently, to the radiationless deactivation of the excited state of ThT molecules. In aqueous solutions, the rate of this process is much higher compared to the rotation of ThT molecule as a whole, and the fluorescence lifetime is determined by the rate of internal rotation of benzothiazole and aminobenzene rings relative to each other. In our recent works, we experimentally determined the fluorescence lifetime of the excited state of free ThT in aqueous solutions with the use of the values of radiative lifetime and fluorescence quantum yield of ThT (1 ps) [21] and the dye fluorescence decay curves (0.98 ps) [47].

In the present work, using the femtosecond time-resolved fluorescence, we showed that, in the presence of crowding agents in concentration of 400 mg/mL, the florescence lifetime of ThT increases about an order of magnitude. Figure 3 serves as an example and shows the dye decay curves of fluorescence for ThT in the presence of PEG-400 in the concentration of 400 mg/mL (Figure 3(a)) and glycerol in the concentration of 46% (Figure 3(b)). Observed increase of the dye fluorescence lifetime with the increase of crowding agents (Figure 3(c)) and glycerol (Figure 3(d)) concentrations can be caused by the restriction of the rotational motions of ThT fragments relative to each other in the excited state and by the decrease of the rate of the radiationless deactivation of the dye molecules. For the same reason there is an increase in the dye fluorescence lifetime when it binds to amyloid fibrils. However, in this case, this value increases to nanoseconds.

3.5. Fluorescence Anisotropy of ThT in the Presence of Crowding Agents. Direction of the transition dipole moment of ThT coincides with the axis of the internal rotation of the benzothiazole and aminobenzene rings relative to each other. Therefore, the relative rotation of fragments cannot change the direction of the transition dipole moment of ThT. As relative rotation of the dye fragments (which leads to radiationless deactivation of the dye molecules) is two orders of magnitude faster than the rotation of the ThT molecule as a whole, the molecule does not have time to change its spatial orientation during the lifetime of the excited state. That is why the fluorescence anisotropy in aqueous medium is very high, close to the limiting value 0.4 (Figure 4(a)) [47]. Although, in viscous isotropic solutions (99% glycerol), the characteristic time of both processes (rotations) increases, the fluorescence

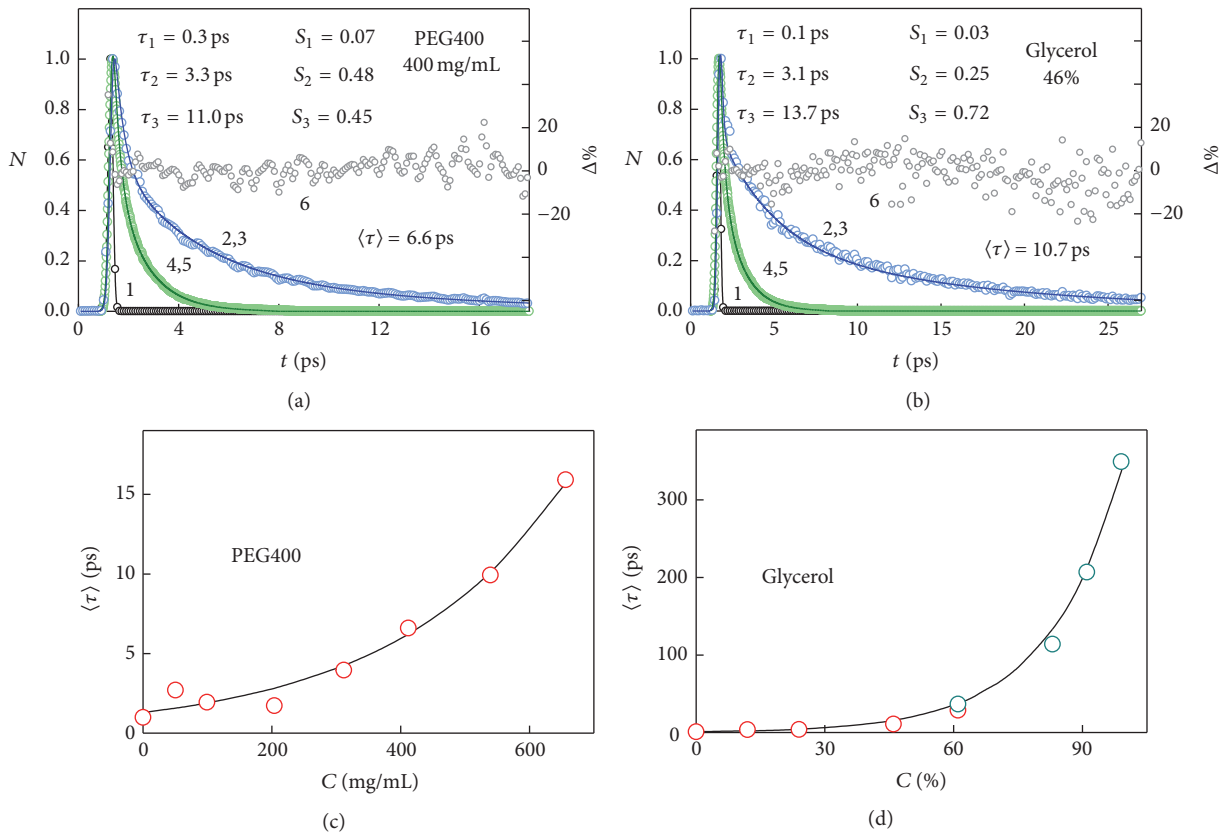

FIGURE 3: ThT fluorescence lifetime in aqueous solution, in aqueous-glycerol solutions, and in aqueous solutions in the presence of crowding agents. The excitation laser impulse profiles (black curves), experimental fluorescence decay curves (light blue circles), best-fit for a three-exponential decay models (blue curves), and the deviation between the experimental and calculated decay curves (grey circles) for ThT in aqueous solutions in the presence of PEG-400 and glycerol 46% are shown on (a) and (b), respectively. For comparison on (a) and (b) experimental fluorescence decay curves (light green circles) and best-fit calculated fluorescence decay curve (green curves) for ThT in aqueous solution are also represented. $N(t)$ is the number of registered impulses in the time interval $(t + \Delta t)$ normalized to unity in the maximum. The dependence of ThT fluorescence lifetime on the concentration of PEG-400 and glycerol is shown on (c) and (d), respectively. The results presented on (a), (b), and (c) were obtained with the use of spectrometer FOG100; the data presented on (d) for low concentrations of glycerol were obtained with the use of spectrometer FOG100 (red circles) and for high concentrations of glycerol were obtained with the use of spectrometer FluoTime 300 (blue circles).

anisotropy of ThT remains the same (Figure 4(d)) [47]. For comparison the results for ThT in the presence of lower concentration of glycerol (46%) are also shown (Figure 4(c)). In the current work we found that the fluorescence anisotropy of ThT in the presence of crowding agents is also high, being close to the limiting value (Figure 4(b)).

Interestingly, the fluorescence anisotropy of the ThT bound to amyloid fibrils, though being very high, can also be slightly lower than that of the ThT anisotropy in aqueous solution. At first glance it seems strange. However, the ThT bound to amyloid fibrils loses its peculiarities of molecule rotor, as the rotation of benzothiazole and aminobenzene rings is restricted in the bound state. First of all, this is manifested in the significant increase in the fluorescence lifetime and the fluorescence intensity on ThT binding to fibrils. Furthermore, in the excited state, the ThT molecule would tend to achieve the energy minimum at $\varphi \approx 90^{\circ}$, whereas the rigid environment could hinder this process. This can lead to the molecular deformation, which causes changes in the direction of the transition dipole moment that result in the decrease in the fluorescence anisotropy.

4. Conclusions

Our results show that the presence of crowding agents (Dextran-70, PEG-400, and PEG-12000) and glycerol in the solution leads to a red shift of absorption, fluorescence excitation, and fluorescence spectra of ThT relative to the spectrum of the dye in aqueous solution, as well as to the increase in its molar extinction coefficient and fluorescence quantum yield (5–8 times). We assume that variation of the photophysical characteristics of the dye in the presence of crowding agents and glycerol may be due to the differences in the microenvironment of the dye molecules, which causes alterations in the ThT conformation and rigidity of the molecule fragments torsional rotation relative to each other in the excited state. It is shown that ThT can be used as

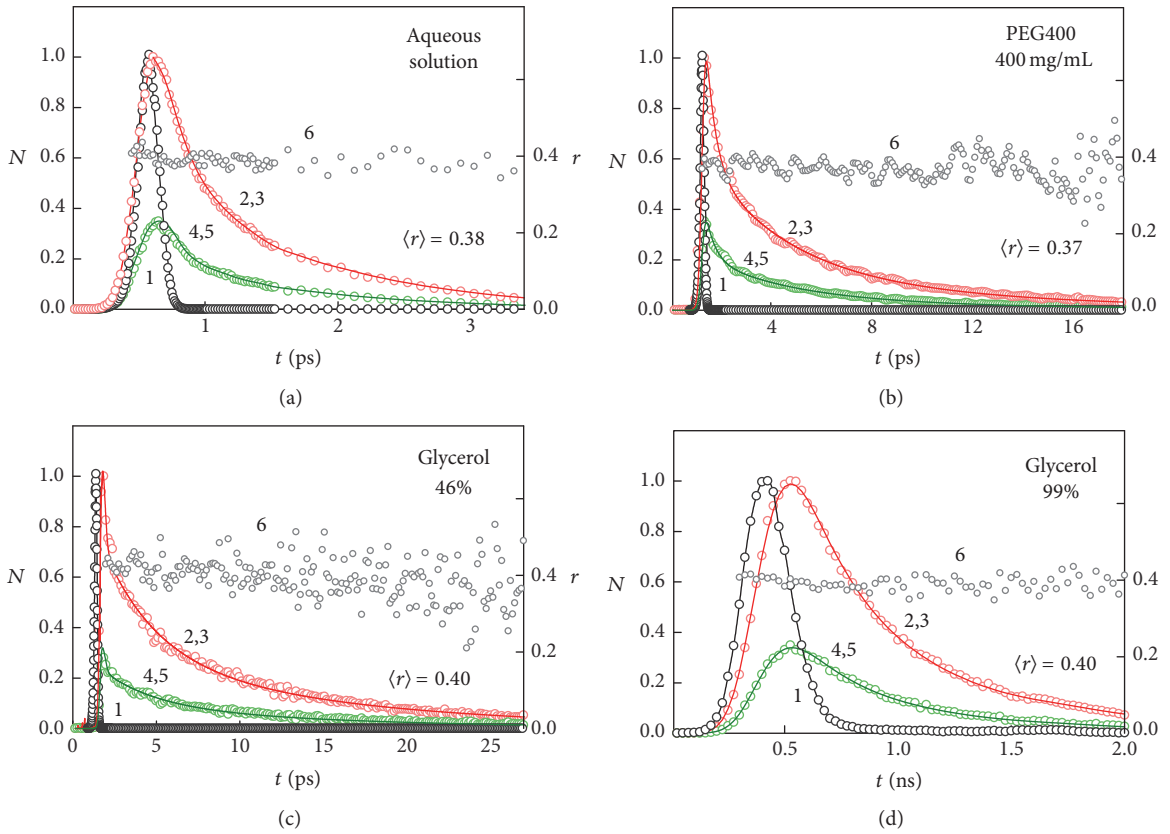

Figure 4: Fluorescence anisotropy of ThT in aqueous solution, in aqueous-glycerol solutions, and in aqueous solutions in the presence of crowding agents. The excitation laser impulse profile (1), the experimental and best-fit calculated decay curves of the vertical (2,3) and horizontal (4,5) components of the fluorescence excited by vertically polarized laser impulses at 420 nm and the change in fluorescence anisotropy over time (6) for ThT in aqueous solution (a), ThT in aqueous solution in the presence of PEG-400 in concentration 400 mg/mL (b), ThT in aqueous solution in the presence of 46% glycerol (c), and ThT in aqueous solution in the presence of 99% glycerol (d) are shown. $N(t)$ is the number of registered impulses in the time interval $(t+\Delta t)$ normalized to unity in the maximum. The data presented on (a), (b), and (c) were obtained with the use of spectrometer FOG100 and the data presented on (d) were obtained with the use of spectrometer FluoTime 300.

a marker for amyloid fibril formation not only in dilute aqueous solutions but also in modeled macromolecular crowding conditions in vitro, which simulate fibrillogenesis conditions in vivo, because the photophysical characteristics of the dye in the presence of crowding agents vary considerably less in comparison with changes induced for ThT bound to amyloid fibrils. Results of this work expand current understanding of the possibility of using fluorescent probes in conditions close to the intracellular environment.

Competing Interests

The authors declare that there are no competing interests regarding the publication of the paper.

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References

[1] J. D. Harper, C. M. Lieber, and P. T. Lansbury Jr., "Atomic force microscopic imaging of seeded fibril formation and fibril branching by the Alzheimer's disease amyloid-β protein," *Chemistry and Biology*, vol. 4, no. 12, pp. 951–959, 1997.

[2] J. W. Kelly, "Amyloid fibril formation and protein misassembly: a structural quest for insights into amyloid and prion diseases," *Structure*, vol. 5, no. 5, pp. 595–600, 1997.

[3] R. W. Carrell and B. Gooptu, "Conformational changes and disease—serpins, prions and Alzheimer's," *Current Opinion in Structural Biology*, vol. 8, no. 6, pp. 799–809, 1998.

[4] M. Hashimoto and E. Masliah, "Alpha-synuclein in Lewy body disease and Alzheimer's disease," *Brain Pathology*, vol. 9, no. 4, pp. 707–720, 1999.

[5] E. H. Koo, P. T. Lansbury, and J. W. Kelly, "Amyloid diseases: abnormal protein aggregation in neurodegeneration," *Proceedings of the National Academy of Sciences*, vol. 96, no. 18, pp. 9989–9990, 1999.

[6] V. N. Uversky, A. Talapatra, J. R. Gillespie, and A. L. Fink, "Protein deposits as the molecular basis of amyloidosis. Part I.

Systemic amyloidoses," *Medical Science Monitor*, vol. 5, no. 5, pp. 1001–1012, 1999.

[7] V. N. Uversky, A. Talapatra, J. R. Gillespie, and A. L. Fink, "Protein deposits as the molecular basis of amyloidosis. II. Localized amyloidosis and neurodegenerative disorders," *Medical Science Monitor*, vol. 5, no. 6, pp. 1238–1254, 1999.

[8] C. M. Dobson, "Protein misfolding, evolution and disease," *Trends in Biochemical Sciences*, vol. 24, no. 9, pp. 329–332, 1999.

[9] V. N. Uversky and A. L. Fink, "Conformational constraints for amyloid fibrillation: the importance of being unfolded," *Biochimica et Biophysica Acta*, vol. 1698, no. 2, pp. 131–153, 2004.

[10] F. Chiti and C. M. Dobson, "Protein misfolding, functional amyloid, and human disease," *Annual Review of Biochemistry*, vol. 75, pp. 333–366, 2006.

[11] M. Fändrich, M. A. Fletcher, and C. M. Dobson, "Amyloid fibrils from muscle myoglobin," *Nature*, vol. 410, no. 6825, pp. 165–166, 2001.

[12] T. A. Pertinhez, M. Bouchard, E. J. Tomlinson et al., "Amyloid fibril formation by a helical cytochrome," *FEBS Letters*, vol. 495, no. 3, pp. 184–186, 2001.

[13] H. Naiki, K. Higuchi, M. Hosokawa, and T. Takeda, "Fluorometric determination of amyloid fibrils in vitro using the fluorescent dye, thioflavine T," *Analytical Biochemistry*, vol. 177, no. 2, pp. 244–249, 1989.

[14] H. LeVine III, "Thioflavine T interaction with synthetic Alzheimer's disease β-amyloid peptides: detection of amyloid aggregation in solution," *Protein Science*, vol. 2, no. 3, pp. 404–410, 1993.

[15] H. LeVine III, "Quantification of beta-sheet amyloid fibril structures with thioflavin T," *Methods in Enzymology*, vol. 309, pp. 274–284, 1999.

[16] D. Allsop, L. Swanson, S. Moore et al., "Fluorescence anisotropy: a method for early detection of Alzheimer β-peptide (Aβ) aggregation," *Biochemical and Biophysical Research Communications*, vol. 285, no. 1, pp. 58–63, 2001.

[17] G. V. De Ferrari, W. D. Mallender, N. C. Inestrosa, and T. L. Rosenberry, "Thioflavin T is a fluorescent probe of the acetylcholinesterase peripheral site that reveals conformational interactions between the peripheral and acylation sites," *Journal of Biological Chemistry*, vol. 276, no. 26, pp. 23282–23287, 2001.

[18] M. Harel, L. K. Sonoda, I. Silman, J. L. Sussman, and T. L. Rosenberry, "Crystal structure of thioflavin T bound to the peripheral site of Torpedo californica acetylcholinesterase reveals how thioflavin T acts as a sensitive fluorescent reporter of ligand binding to the acylation site," *Journal of the American Chemical Society*, vol. 130, no. 25, pp. 7856–7861, 2008.

[19] P. Sen, S. Fatima, B. Ahmad, and R. H. Khan, "Interactions of thioflavin T with serum albumins: spectroscopic analyses," *Spectrochimica Acta—Part A: Molecular and Biomolecular Spectroscopy*, vol. 74, no. 1, pp. 94–99, 2009.

[20] V. Babenko and W. Dzwolak, "Thioflavin T forms a non-fluorescent complex with α-helical poly-l-glutamic acid," *Chemical Communications*, vol. 47, no. 38, pp. 10686–10688, 2011.

[21] A. I. Sulatskaya, A. A. Maskevich, I. M. Kuznetsova, V. N. Uversky, and K. K. Turoverov, "Fluorescence quantum yield of thioflavin T in rigid isotropic solution and incorporated into the amyloid fibrils," *PLoS ONE*, vol. 5, no. 10, Article ID e15385, 2010.

[22] S. B. Zimmerman and S. O. Trach, "Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of Escherichia coli," *Journal of Molecular Biology*, vol. 222, no. 3, pp. 599–620, 1991.

[23] B. van den Berg, R. J. Ellis, and C. M. Dobson, "Effects of macromolecular crowding on protein folding and aggregation," *The EMBO Journal*, vol. 18, no. 24, pp. 6927–6933, 1999.

[24] G. Rivas, F. Ferrone, and J. Herzfeld, "Life in a crowded world," *EMBO reports*, vol. 5, no. 1, pp. 23–27, 2004.

[25] S. B. Zimmerman and A. P. Minton, "Macromolecular crowding: biochemical, biophysical, and physiological consequences," *Annual Review of Biophysics and Biomolecular Structure*, vol. 22, pp. 27–65, 1993.

[26] A. B. Fulton, "How crowded is the cytoplasm?" *Cell*, vol. 30, no. 2, pp. 345–347, 1982.

[27] A. P. Minton, "Influence of excluded volume upon macromolecular structure and associations in 'crowded' media," *Current Opinion in Biotechnology*, vol. 8, no. 1, pp. 65–69, 1997.

[28] R. J. Ellis, "Macromolecular crowding: obvious but underappreciated," *Trends in Biochemical Sciences*, vol. 26, no. 10, pp. 597–604, 2001.

[29] A. P. Minton, "Protein folding: thickening the broth," *Current Biology*, vol. 10, no. 3, pp. R97–R99, 2000.

[30] A. P. Minton, "Models for excluded volume interaction between an unfolded protein and rigid macromolecular cosolutes: macromolecular crowding and protein stability revisited," *Biophysical Journal*, vol. 88, no. 2, pp. 971–985, 2005.

[31] D. M. Hatters, A. P. Minton, and G. J. Howlett, "Macromolecular crowding accelerates amyloid formation by human apolipoprotein C-II," *The Journal of Biological Chemistry*, vol. 277, no. 10, pp. 7824–7830, 2002.

[32] I. Kuznetsova, K. Turoverov, and V. Uversky, "What macromolecular crowding can do to a protein," *International Journal of Molecular Sciences*, vol. 15, no. 12, pp. 23090–23140, 2014.

[33] O. V. Stepanenko, O. I. Povarova, A. I. Sulatskaya et al., "Protein unfolding in crowded milieu: what crowding can do to a protein undergoing unfolding?" *Journal of Biomolecular Structure & Dynamics*, vol. 34, no. 10, pp. 2155–2170, 2016.

[34] I. M. Kuznetsova, A. I. Sulatskaya, V. N. Uversky, and K. K. Turoverov, "Analyzing thioflavin T binding to amyloid fibrils by an equilibrium microdialysis-based technique," *PLOS ONE*, vol. 7, no. 2, Article ID e30724, 2012.

[35] I. M. Kuznetsova, A. I. Sulatskaya, V. N. Uversky, and K. K. Turoverov, "A new trend in the experimental methodology for the analysis of the thioflavin T binding to amyloid fibrils," *Molecular Neurobiology*, vol. 45, no. 3, pp. 488–498, 2012.

[36] A. I. Sulatskaya, I. M. Kuznetsova, and K. K. Turoverov, "Interaction of thioflavin T with amyloid fibrils: stoichiometry and affinity of dye binding, absorption spectra of bound dye," *Journal of Physical Chemistry B*, vol. 115, no. 39, pp. 11519–11524, 2011.

[37] A. I. Sulatskaya, I. M. Kuznetsova, and K. K. Turoverov, "Interaction of thioflavin T with amyloid fibrils: fluorescence quantum yield of bound dye," *Journal of Physical Chemistry B*, vol. 116, no. 8, pp. 2538–2544, 2012.

[38] Glycerine Producers' Association, *Physical Properties of Glycerine and Its Solutions*, Glycerine Producers' Association, New York, NY, USA, 1963.

[39] A. V. Fonin, A. I. Sulatskaya, I. M. Kuznetsova, and K. K. Turoverov, "Fluorescence of dyes in solutions with high absorbance. Inner filter effect correction," *PLoS ONE*, vol. 9, no. 7, Article ID e103878, 2014.

[40] D. V. P. O'Connor, *Time-Correlated Single Photon Counting*, Academic Press, New York, NY, USA, 1984.

[41] K. K. Turoverov, A. G. Biktashev, A. V. Dorofeiuk, and I. M. Kuznetsova, "A complex of apparatus and programs for the measurement of spectral, polarization and kinetic characteristics of fluorescence in solution," *Tsitologiia*, vol. 40, no. 8-9, pp. 806–817, 1998.

[42] D. W. Marquardt, "An Algorithm for Least-Squares Estimation of Nonlinear Parameters," *Journal of the Society for Industrial and Applied Mathematics*, vol. 11, no. 2, pp. 431–441, 1963.

[43] A. I. Sulatskaya, O. I. Povarova, I. M. Kuznetsova, V. N. Uversky, and K. K. Turoverov, "Binding stoichiometry and affinity of fluorescent dyes to proteins in different structural states," *Methods in Molecular Biology*, vol. 895, pp. 441–460, 2012.

[44] I. M. Kuznetsova, A. I. Sulatskaya, O. I. Povarova, and K. K. Turoverov, "Reevaluation of ans binding to human and bovine serum albumins: key role of equilibrium microdialysis in ligand—receptor binding characterization," *PLoS ONE*, vol. 7, no. 7, Article ID e40845, 2012.

[45] A. A. Maskevich, V. I. Stsiapura, V. A. Kuzmitsky et al., "Spectral properties of thioflavin T in solvents with different dielectric properties and in a fibril-incorporated form," *Journal of Proteome Research*, vol. 6, no. 4, pp. 1392–1401, 2007.

[46] K. K. Turoverov, I. M. Kuznetsova, A. A. Maskevich, V. I. Stepuro, V. A. Kuzmitsky, and V. N. Uversky, "ThT as an instrument for testing and investigation of amyloid and amyloid-like fibrils," in *Proceedings of the International Conference on Lasers, Applications, and Technologies 2007: Environmental Monitoring and Ecological Applications; Optical Sensors in Biological, Chemical, and Engineering Technologies; and Femtosecond Laser Pulse Filamentation*, Proceedings of SPIE, Minsk, Belarus, June 2007.

[47] I. M. Kuznetsova, A. I. Sulatskaya, A. A. Maskevich, V. N. Uversky, and K. K. Turoverov, "High fluorescence anisotropy of thioflavin T in aqueous solution resulting from its molecular rotor nature," *Analytical Chemistry*, vol. 88, no. 1, pp. 718–724, 2016.