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Aggregation of thioflavin T and its new derivative in the presence of anionic polyelectrolyte

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ABSTRACT

Spectral properties of aqueous solutions of thioflavin T (ThT) and its new derivative, trans-2-[4-(dimethylamino) styryl]-3-ethyl-1,3-benzothiazolium perchlorate (DMASEBT) were studied in the presence of anionic polyelectrolyte sodium polystyrene sulfonate (PSS). It was shown that PSS promote the dye dimerization process. Quantum–chemical analysis of DMASEBT in monomer and dimer forms allowed to suggest that the dimers have a sandwich-like structure, i.e. H-aggregates can be formed. DMASEBT dimer formation in the presence of PSS leads to a 40 nm hypsochromic shift of the dye absorption spectrum and to quenching of its fluorescence. The PSS interaction with the monomeric dye leads to a 26 nm bathochromic shift of the absorption spectrum and to one order of magnitude increase in its fluorescence.

Keywords: *Aggregation, Amyloid Fibrils, Dimer, Polyelectrolyte, Thioflavin T, Thioflavin T Derivatives.*

1. INTRODUCTION

The fluorescent probe thioflavin T (ThT) (Figure 1A, Left Inset) is widely used for amyloid fibrils (AF) detection and testing [1–6]. ThT exhibits properties of fluorescent molecular rotor and its emission intensity depends on microenvironment viscosity and rigidity [7]. The binding of ThT to AF increases its fluorescence quantum yield by 2–3 orders of magnitude [8], which was explained by incorporation of ThT molecules into channels of the rigid β-sheet structure of the fibrils [1]. Recently new and improved probes were designed for AF detection. Fluorescent probes with two emission bands are of particular interest for AF staining since they allow to develop a sensitive ratiometric method [9]. Furthermore, in order to test amyloidosis in living cells and tissue, dyes that are sensitive to AF, such as ThT, but that absorb and fluoresce at longer wavelengths are required. The use of such a dye can decrease effects of both light scattering by AF in solutions (*in vitro*) and the absorption and fluorescence of biological tissues (*in vivo*). One of these dyes is the recently synthesized ThT analog, trans-2-[4-(dimethylamino) styryl]-3-ethyl-1,3-benzothiazolium perchlorate (DMASEBT) [10]. Both ThT and DMASEBT molecules exist in cationic form at pH 3–9.

The most difference between DMASEBT and ThT molecules are related to length of the "bridge" that connects the benzene and benzothiazole rings (Figure 1B, Inset). The absorption and fluorescence spectra of DMASEBT aqueous solutions are bathochromic shifted by approximately 100 nm compared to spectra of ThT aqueous solutions [10]. DMASEBT has a large Stokes shift in polar solvents. An increase in solution viscosity leads to significant increase in fluorescence quantum yield of DMASEBT. Such fluorescent properties are characteristic for molecules belonging to the class of molecular rotors. Fluorescence intensity enhancement in viscous solutions is related to decrease of the rate of twisted intramolecular charge transfer (TICT) process, associated with non-radiative deactivation of the excited state, due to restriction of the aromatic rings rotation relative each other [10].

Incorporation of the probe molecule into β-sheet structures of AF results in restriction of the twisting movement of its fragments relative to each other, and this restriction significantly decreases the rate constant of transition to the non-fluorescent TICT-state resulting in enhancement of fluorescence intensity [1, 7, 11]. However, the mechanisms by which ThT and its analogs are incorporated into amyloid fibrils and the stoichiometry of the dye–fibril complex are still actively debated. There are several points of view on the incorporation mechanism of ThT into amyloid fibrils. In particular, some researchers [12–15] argue that ThT incorporates into fibrils in the dimer, excimer, or even micellar forms. Therefore, the solution of this problem is very important.

The use of polyelectrolytes as model systems is interesting because aggregation of dye molecules (like acridine orange, methylene blue, rhodamine 6G, and others) was observed upon their interaction with polyelectrolytes [16–18]. Thus, study of aggregation processes of ThT and its derivatives in model systems, as well as an investigation of their spectral properties in aggregated forms in solution and when incorporated in ordered structures, are of current interest. For this reason, investigation of DMASEBT interaction with polyelectrolytes seems interesting. It should be noted that polyelectrolytes were reported to be initiators of amyloid fibril growth [19, 20] and it is necessary to take into account their interaction with ThT and its derivatives.

The paper reports investigation of spectral characteristics of ThT and its new derivative, DMASEBT, in the presence of sodium polystyrene sulfonate (PSS) (Figure 1A, Right Inset), which can significantly promote the dye dimerization process. Quantum–chemical calculations of DMASEBT dimer allowed to suggest that the dimers are non-fluorescent H-aggregates. It is concluded that the bathochromic shift of the absorption spectrum of ThT and DMASEBT in the presence of amyloid fibrils cannot be explained by the aggregation of dye molecules and the observed enhanced fluorescence upon dye binding to AF is caused by dye monomers incorporated into the fibrils.

2. EXPERIMENTAL SECTION

2.1. Materials.

DMASEBT was synthesized at Department of Laser Physics and Spectroscopy of Belarusian State University. Samples of thioflavin T (AnaSpec, USA) and PSS (Sigma-Aldrich, USA) with a molecular mass of 70 kDa were used without additional purification. Amyloid fibrils were prepared by incubating bovine insulin (Sigma, USA) at 37 °C in 20 % acetic acid containing 0.1 M NaCl at constant stirring for 24 hours. The insulin concentration in the solution was 2 mg/ml.

2.2. Methods.

Absorption spectra were recorded using Specord 200 spectrophotometer (Carl Zeiss, Germany), and steady-state fluorescence spectra were measured using CM 2203 spectrofluorimeter (Solar, Belarus). All spectral measurements were performed in aqueous solutions at pH 6.

Quantum-chemical calculations of DMASEBT dimer structures and energies were performed using FireFly 8.0.1 software [21]. The dimer geometry in the ground state was optimized by density functional theory method [22] corrected for dispersion interactions (the Grimm correction) [23] and using the hybrid functional B3LYP [24, 25] and the split valence basis set 6-31G. Calculations for all configurations of dimers were conducted in vacuum in absence of electric field.

3. RESULTS SECTION

3.1. ThT spectral properties in the presence of PSS.

Absorption spectrum of ThT in aqueous solution (pH 6) at micromolar concentration has maximum at 412 nm (Figure 1A, curve 1) [2]. Addition of anionic polyelectrolyte PSS into 3.5 μM ThT aqueous solution leads to a shift of the absorption spectrum. The direction of this shift depends on PSS concentration. At low (nanomolar) PSS concentration (approximately 4.5 nM) optical density decreases by ~10 % and absorption spectrum has insignificant 4 nm hypsochromic shift (Figure 1A, curve 2). When PSS concentration is large (> 4.5 nM) a bathochromic shift (15 nm at 0.4 μM PSS) is observed (Figure 1A, curve 3) and the optical density increases by 1.2 times.

It should be emphasized that the described spectral changes are mainly caused not by polyelectrolyte concentration (C_{PSS}) but by the ratio of amount of negatively charged sulfo groups SO_3^- in polyelectrolyte to the number of dye molecules: N_S:N_D. It can be calculated that for ThT concentration of $C_0 = 3.5$ μM and PSS concentration of C_{PSS} = 12 nM there are approximately 2.3 ThT molecules per each sulfo group of polyelectrolyte, i.e. the ratio N_S:$N_D = 0.44$ (Figure 1A, curve 2). If C_0 = 3.5 μM and $C_{PSS} = 0.4$ μM then N_S:$N_D \approx 38.8$ (Figure 1A, curve 3).

Such unusual change of absorption spectra can be explained if we take into account two factors, 1) binding of the probe to polyelectrolyte and 2) interaction of the bound probe molecules with each other resulting in their aggregation. Indeed, in aqueous solution at neutral pH values ThT molecules exist in form of the cation. The most significant interaction responsible for dye molecules binding to polyelectrolyte is electrostatic attraction between ThT cations and negatively charged sulfo groups. At low N_S:N_D ratios (less than 1.0) there are several ThT molecules per each sulfo group. We believe that in the presence of nanomolar concentration of PSS repulsion between two ThT cations can be diminished and they can approach each other to a distance sufficient to form dimers. The quantum-chemical calculations of ThT dimer with different plausible structures have shown [26] that the sandwich-like structure of the dimer is the most stable one.

This leads to the conclusion that even at low (micromolar) dye concentrations ThT aggregates can be formed in the vicinity of sulfo groups. The aggregation of ThT molecules occurs at high concentration (10^{-3}–10^{-2} M) and this leads to hypsochromic shift of the absorption spectrum by ~6 nm [26]. The shape of the absorption spectrum of 10^{-2} M ThT in aqueous solution between two quartz glasses changes significant and the spectrum maximum shifts towards shorter wavelengths by 10 nm (Figure 1A, curve 4). But, in the presence of nanomolar PSS concentration, hypsochromic shift of ThT absorption spectrum is less, ~3 nm. We can say that in the presence of PSS the ThT aggregation appears spectrally weak.

In our opinion the bathochromic shift of ThT absorption spectrum observed at high N_S:N_D ratios is a result of the dye monomer binding to the polyelectrolyte. The similar spectral shift takes place when ThT molecules incorporate into AF [1, 2], DNA [27] and nanocavities [28]. This spectral changes were explained earlier as a result of a stabilization of the ground state energy upon interaction with a polar environment [1, 2, 29].

3.2. DMASEBT spectral properties in the presence of PSS.

It was found that the effect of PSS on DMASEBT spectral properties is much stronger than on ThT spectral properties. Absorption spectrum of DMASEBT in aqueous solution at concentration of $C_0 = 10$ μM has maximum at 514 nm (Figure 1B, curve 1), and DMASEBT fluorescence spectrum has maximum at 597 nm [10]. Addition of PSS into DMASEBT solution

significantly changes the absorption spectrum and this effect differs depending on N_S:N_D ratio. Thus, in the presence of 12 nM PSS (N_S:N_D = 0.4), absorption spectrum of 10 μM DMASEBT solution has maximum at 475 nm (Figure 1B, curve 2). Increasing PSS concentration leads to bathochromic shifts of DMASEBT absorption spectrum, and at 2.4 μM PSS (N_S:N_D ≈ 70) DMASEBT absorption spectrum has maximum at 540 nm (Figure 1B, curve 3).

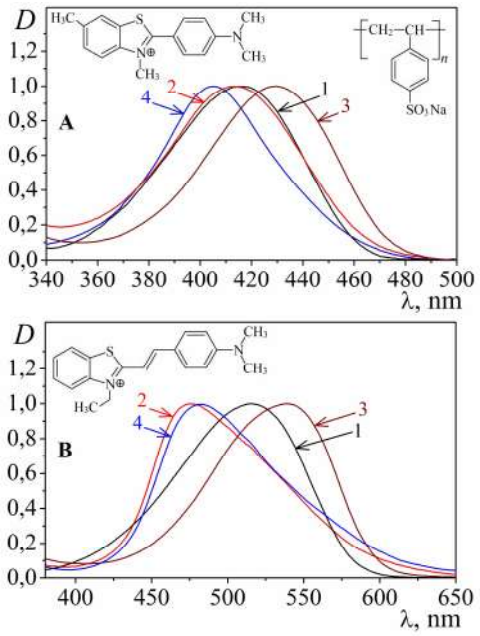

Figure 1. Normalized absorption spectra of ThT and DMASEBT in aqueous solution (pH 6, 22 °C) in the absence and presence of PSS. **Panel A.** Curves 1–3 were recorded for 3.5 μM ThT in the presence of 0 M, 4.5 nM and 0.4 μM PSS (ratio of N_S:N_D equals to 0, 0.44 and 38.8, respectively). For comparison, the normalized absorption spectrum of 10^{-2} M ThT aqueous solution between quartz glasses at 22 °C is shown (curve 4). **Left Inset:** chemical structure of ThT molecule. **Right Inset:** chemical structure of PSS. **Panel B.** Curves 1–3 were recorded for 10 μM DMASEBT in the presence of 0 M, 12 nM and 2.4 μM PSS (ratio of N_S:N_D equals to 0, 0.4 and 70, respectively). For comparison, the normalized absorption spectrum of 0.82 mM DMASEBT aqueous solution between quartz glasses at 22 °C is shown (curve 4). **Left Inset:** chemical structure of DMASEBT molecule.

It should be noted, that solutions with high DMASEBT concentrations, when the probability of aggregates formation is large, are characterized by a hypsochromic shift of the absorption spectrum. Concentrated DMASEBT solution (C_0 = 0.82 mM) between two quartz glasses at 22 °C has an absorbance maximum at 482 nm (Figure 1B, curve 4).

For more detailed study of PSS effect on spectral properties of the dye, we studied spectral characteristics of DMASEBT at low and high values of N_S:N_D. When the concentration of DMASEBT was 10 μM, the increase of PSS concentration from 0.0 to 12 nM led to a hypsochromic shift of DMASEBT absorption spectra from 500 to 475 nm and a change of its shape (Figure 2A). The fluorescence intensity of DMASEBT (λ_{exc} = 470 nm) decreased by one order of magnitude as PSS concentration increased from 1 to 12 nM. The shape and position of DMASEBT fluorescence spectra in these conditions remain unchanged (Figure 2A).

Further increase in PSS concentration from 0.1 to 2.4 μM causes significant increase in absorbance and fluorescence intensity of DMASEBT, a significant (from 475 to 540 nm) bathochromic shift of the absorption spectra and a comparatively small (3 nm) bathochromic shift of the fluorescence spectra (Figure 2B). We suggested that the increase in DMASEBT fluorescence was caused by restriction of the torsion rotation of the aromatic rings when the dye interacted with polyelectrolyte and consequently a decrease in the rate constant of the transition to TICT-state. We also proposed that at low N_S:N_D ratios these spectral changes were caused by DMASEBT aggregates formation induced by several dye molecules clustering in the vicinity of a negatively charged sulfo group of the polyelectrolyte.

We suggest that at low values of N_S:N_D the interaction of sulfo groups with dye monomers can be neglected. In this case the interaction of DMASEBT dimer with sulfo group can be determined as follows:

$$n\,\mathrm{DMASEBT}^{+} + \mathrm{SO}_3^{-} \leftrightarrow \left(\mathrm{DMASEBT}_n^{n+}\,\mathrm{SO}_3^{-}\right). \quad (1)$$

The constant of aggregate formation is determined as follows:

$$K_{aggr} = \frac{C_{aggr}}{C_f^n C_{sulf}}, \quad (2)$$

where C_{aggr} is concentration of dye aggregates, C_{sulf} is concentration of sulfo groups, and C_f is concentration of the free dye. Taking into account that concentration of the added dye is $C_0 = C_f + nC_{aggr}$, we have:

$$K_{aggr} = \frac{C_0 - C_f}{nC_f^n C_{sulf}}, \quad (3)$$

The logarithm of Eq. (3) gives:

$$\lg\left(C_0 - C_f\right) = n \lg C_f + \lg\left(nK_{aggr} C_{sulf}\right). \quad (4)$$

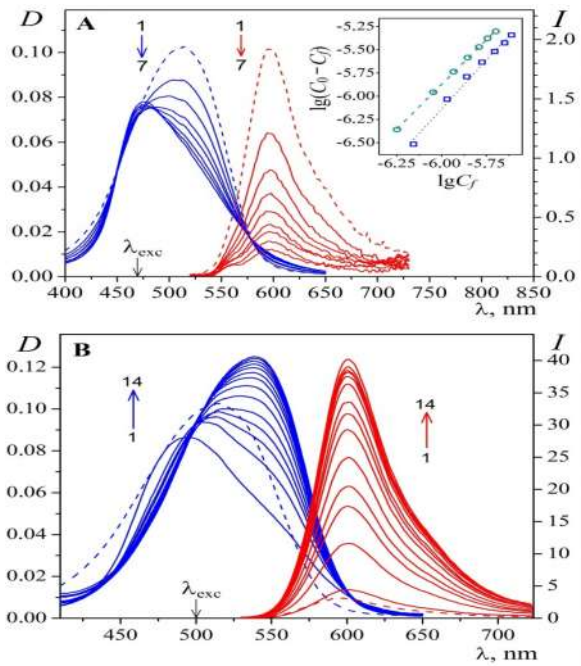

Figure 2. The influence of PSS on absorption and fluorescence spectra of DMASEBT. **Panel A.** Absorption (blue) and fluorescence (red, λ_{exc} = 470 nm) spectra of DMASEBT (C_0 = 10 μM) in aqueous solutions (dashed lines) and in the presence of PSS at concentrations of 1, 2, 3, 4, 6, 8 and 12 nM (1–7 solid lines), which correspond to low N_S:N_D ratios. **Inset:** the dependence of $\lg(C_0 - C_f)$ on $\lg C_f$ for aqueous DMASEBT solutions in the presence of 4.5 nM (squares) and 6.0 nM PSS (circles). **Panel B.** Absorption (blue) and fluorescence (red, λ_{exc} = 520 nm) spectra

of DMASEBT (C_0 = 10 μM) in aqueous solutions (dashed lines) and in the presence of PSS at concentrations of 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, and 2.4 μM (solid lines 1–14), which correspond to intermediate and high N_S:N_D ratios.

To obtain order of aggregation n, we measured absorption spectra of solutions at different dye concentrations and constant polyelectrolyte concentration. Two series of experiments were performed for 4.5 and 6.0 nM PSS. The obtained spectra were approximated as sum of two lognormal functions that describe the absorption spectra of aggregated and free molecules. Parameters of absorption spectrum for the free dye molecule were obtained by fitting the spectrum of the dye in the absence of PSS using lognormal function. Concentration C_f of free molecules was determined by absorbance at 500 nm using coefficient of molar extinction for free dye at 500 nm: ε_f = 9800 $M^{-1}cm^{-1}$.

According to Eq. 4, the slope of $\lg(C_0 - C_f)$ dependences on $\lg C_f$ (Figure 2A, Inset) is equal to order of aggregation n. For PSS concentrations 4.5 and 6.0 nM, the aggregation order values were calculated to be 2.10 ± 0.09 and 1.97 ± 0.02, respectively. Thus, the obtained results demonstrate that the aggregates of dye molecules in the presence of PSS are dimers. It can be stated that dimer formation leads to fluorescence quenching because at these PSS concentrations, the fluorescence intensity of DMASEBT decreases (Figure 2A).

3.3. Structure and photophysical properties of DMASEBT dimers. Quantum-chemical calculations.

To determine the structure of DMASEBT dimer, we performed quantum-chemical calculations of two dye molecules with different spatial orientations. We examined the possibility of formation of the following dimer structures: 1) sandwich-like structure (Figure 3B); 2) structure with molecules oriented perpendicular to one another; and 3) "head-to-tail" structure, in which the molecules were located in a line one after another. To determine stability of the different structures of DMASEBT dimers, we calculated the dependence of the ground state energy on distance between the molecules. The obtained dependencies (Figure 3B) show that the most stable dimers have the sandwich-like structure because the corresponding curve (Figure 3C, curve 1) has the deepest energy minimum. Earlier, similar dependencies were also obtained for ThT dimer with sandwich-like structure [26].

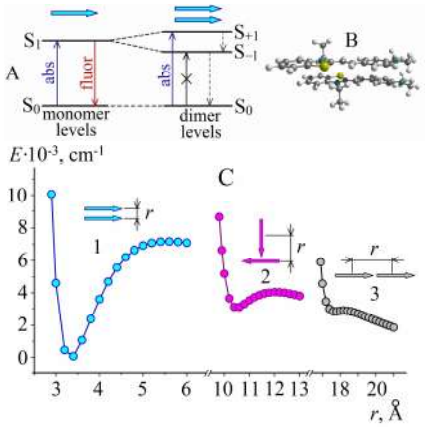

Figure 3. Panel A. Exciton energy diagram for H-aggregate (solid arrows are allowed transitions; dashed arrows are non-fluorescent transition). **Panel B.** Optimized sandwich-like structure of DMASEBT dimer. **Panel C.** Dependences of DMASEBT dimer ground state energy on distance between the molecules for different structure types (arrows indicate relative molecular locations within the dimer; the lowest dimer energy value was taken as zero value).

The hypsochromic shift of the absorption spectrum and fluorescence quenching due to the formation of dye dimers can be explained using an exciton model [30, 31]. According to the exciton theory, when H-aggregates form, the excited state S_1 with energy E_1 splits into two exciton states S_{-1} and S_{+1} with energies E_{-1} and E_{+1}, respectively, with $E_{-1} < E_1$, and $E_{+1} > E_1$ (Figure 3A) [32]. In this case the transition from the ground state S_0 to the state S_{-1} is forbidden; therefore, photoexcitation leads to a transition to the state S_{+1} and the transition energy is greater than in the monomer case, which leads to the hypsochromic shift of the absorption spectrum. It is important to note that deactivation of the excited state S_{+1} occurs through a non-radiative transition to the state S_{-1}, after which, a non-radiative transition to the ground state occurs. Hence, in the case of the H-aggregate, fluorescence quenching takes place. The hypsochromic shift of the absorption spectrum and the fluorescence quenching are specific features of H-aggregates. On the basis of this information, we supposed that at nanomolar concentrations of polyelectrolyte, DMASEBT molecules form non-fluorescent H-aggregates.

On the basis of the quantum-chemical calculations, it has been also established that oscillator strength of the transition from the S_{-1} state to the ground state S_0 is approximately 0.004. The low value of the oscillator strength indicates that this transition is forbidden, which is consistent with exciton theory. Earlier it was shown that for DMASEBT molecule with planar conformation, the oscillator strength of the $S_1 \rightarrow S_0$ transition is close to 1 [10]. Due to torsional relaxation, the conjugated fragments rotate relative one another, and the molecule transits to the non-fluorescent TICT-state, for which oscillator strength equals to zero. However, it is important to note that during dimer formation, no torsional rotation of the fragments occurs because the other molecule prevents this rotation. Nevertheless, we attribute the decrease in fluorescence intensity of DMASEBT dimer to the low values of the oscillator strength for the $S_{-1} \rightarrow S_0$ transition but not to the torsional relaxation into the TICT-state.

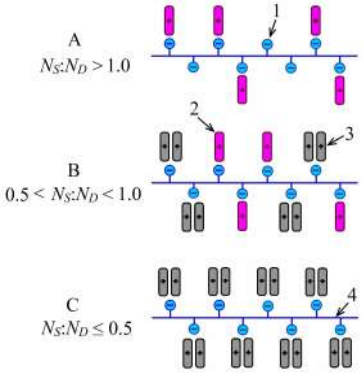

Figure 4. The model of DMASEBT interaction with PSS at different N_S:N_D ratios (1 – negatively charged sulfo group; 2 – fluorescent dye molecule; 3 – non-fluorescent dye dimer; 4 – polyelectrolyte chain).

We proposed the model of DMASEBT interaction with PSS at different N_S:N_D ratios (Figure 4). At high N_S:N_D ratios (more than 1.0) the number of sulfo groups is large compared to the number of dye molecules, so that the latter bind to polyelectrolyte in monomeric form (Figure 4, A). When dye

concentration is increased and the number of sulfo groups becomes less than the number of DMASEBT molecules, the probability of non-fluorescent dye dimers appearance increases (Figure 4, B). If the N_S:N_D ratio is equal or below 0.5, almost all dye molecules form dimers on binding to PSS (Figure 4, C).

3.4. Spectral changes of DMASEBT aqueous solutions in the presence of insulin amyloid fibrils.

Earlier it was shown that ThT derivative, DMASEBT, is very sensitive fluorescent probe for AF detection [10]. Spectral characteristics of 10 μM aqueous DMASEBT solution in the presence of insulin amyloid fibrils demonstrated that absorption spectrum of the dye bound to fibrils experiences a significant bathochromic shift of approximately 50 nm (Figure 5), while the fluorescence spectrum is slightly shifted (approximately 3 nm) towards longer wavelengths, and fluorescence intensity (in the presence of 0.20 mg/ml insulin) is almost 2 orders of magnitude higher than that in aqueous solutions (Figure 5). On the other hand, at nanomolar concentrations of polyelectrolyte when the probability of dimers formation is high the absorption spectrum of DMASEBT is considerably hypsochromic shifted (~40 nm), the fluorescence spectrum is not shifted, and the fluorescence intensity significantly decreases (Figure 3, A).

Thus, it can be concluded that spectral changes of DMASEBT aqueous solution in the presence of fibrils can not be explained by the incorporation of the dye molecules into fibrils in the aggregated form. On the basis of obtained results we consider that DMASEBT molecules incorporate into fibrils in monomeric form. Similar spectral properties are exhibited by ThT in the presence of amyloid fibrils [1, 2]. Since the molecules of ThT and DMASEBT are structurally similar we believe that ThT molecules are also incorporated into fibrils in monomeric form. We suggest that the other models of ThT and its derivatives binding in AF (in the dimer, excimer of micellar form) [12–15] are incorrect.

Our results also demonstrate that the use of ionic polyelectrolytes makes it possible to study ionic dye aggregation processes at low (micromolar) concentrations when the optical density of solutions is low and inner filter effects are negligible.

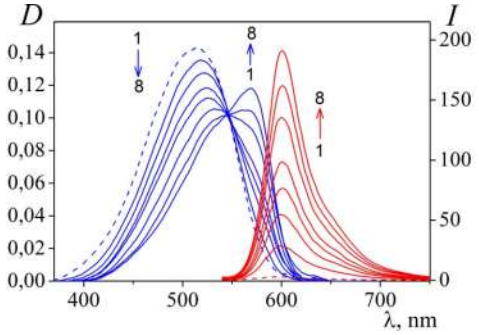

Figure 5. DMASEBT absorption (blue) and fluorescence (red, λ_{exc} = 550 nm) spectra in aqueous solution (pH 6) in the presence of amyloid fibrils; dashed lines 1 indicate the DMASEBT spectra without amyloid fibrils; solid lines 2–8 are DMASEBT spectra in the presence of amyloid fibrils at insulin concentration of 0.02, 0.04, 0.06, 0.08, 0.12, 0.15 and 0.20 mg/ml.

4. CONCLUSIONS

The anionic polyelectrolyte PSS has significant effect on absorption and fluorescence spectra of ThT and its new derivative, DMASEBT, in aqueous solutions. PSS promote the dimerization process of DMASEBT and of ThT. But the ThT dimers formation under the effect of PSS appears spectrally weak.

Formation of dimer molecules of DMASEBT, promoted in the presence of PSS, is accompanied by hypsochromic shift of the absorption spectrum by 40 nm, while DMASEBT binding to fibrils is accompanied by bathochromic shift of the absorption spectrum by 50 nm. The obtained data allow us to conclude that spectral changes of DMASEBT solutions, as well as that of ThT, in the presence of fibrils cannot be explained by the aggregation of dye molecules and it is a result of their incorporation into the fibrils in the monomeric form.

The use of ionic polyelectrolytes makes it possible to study ionic dye aggregation processes at micromolar concentrations when the optical density of the solutions is low.

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