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ORIGINAL PAPER

Studies of Interaction Between Cyanine Dye T-284 and Fibrillar Alpha-Synuclein

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Abstract A key feature of Parkinson's disease is the formation and accumulation of amyloid fibrils of the natively unfolded protein α-synuclein (ASN) inside neurons. Recently we have proposed novel sensitive monomethinecyanine dye T-284 as fluorescent probe for quantitative detection of ASN amyloid fibrils. In this study the T-284 dye complex with ASN fibril was characterized by means of fluorescence anisotropy, atomic force microscopy and time-resolved fluorescence techniques to give further insights into the mode of dye interaction with amyloid fibrils. The fluorescence anisotropy of T-284 was shown to noticeably increase upon addition of aggregated proteins indicating on stable dye/amyloid fibril complex formation. AFM imaging of fibrillar wild-type ASN revealed differences in heights between ASN fibrils alone and in presence of the T-284 dye (6.37±1.0 nm and 8.0±1.1 nm respectively), that is believed to be caused by embedding of T-284 dye molecules in the "binding channel" running along the fibril. Fluorescence decay analysis of the T-284 in complexes with fibrillar ASN variants revealed the fluorescence lifetime values for T-284/fibril complexes to be an order of magnitude higher as compared to the free dye. Also, the fluorescence decay of free T-284 was bi-exponential, while dye bound to protein yields tri-exponential decay. We suppose that in complexes with fibrillar ASN variants T-284 dye might exist in different "populations" due to interaction with fibrils in different conformers and ways. The exact binding mode of T-284 with ASN fibrils needs further studies. Studied parameters of dye/amyloid fibril complexes are important for the characterization and screening of newly-developed amyloid-sensitive dyes.

Keywords Cyanine dyes · α-synuclein · Amyloid fibrils · Fluorescent detection · Atomic force microscopy

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Introduction

Parkinson's disease is characterized by the accumulation of amyloid fibrils of the intrinsically disordered protein α-synuclein (ASN) inside neurons [1–5]. The extent and kinetics of the formation of amyloid fibrils are often assessed by measuring the fluorescence or other photophysical properties of dyes that bind specifically to the amyloid fibrils. The most commonly used dyes are Thioflavin T (Thio T) and Congo Red, although in recent years several new dyes have been proposed. The compound (trans,trans)-1-bromo-2,5-bis-(4-hydroxy)styrylbenzene (K114), derived from the structure of Congo Red, was shown to recognize a wide range of amyloid inclusions and has distinctive properties which allowed the quantitative monitoring of the formation of amyloid fibrils assembled from the amyloid-beta peptide, alpha-synuclein, and tau [6].

Celej et. al. proposed N-arylaminonaphthalene sulfonate (NAS) derivatives as noncovalent probes of ASN fibrillation [7]. Also in a search for improved fluorescence tools for studying amyloid formation the extrinsic multiple-emission probe 4′-(N,N-diethylamino)-3-hydroxyflavone (FE) was shown to have distinct spectroscopic signatures for amyloid fibrils formed by the wild-type and mutant ASN, presumably indicative of differences in supramolecular structure [8]. Recently we proposed cyanine dyes as specific fluorescent probes for aggregated protein structures. We demonstrated that mono- and trimethinecyanine dyes exhibit a specific increase in fluorescence intensity in the presence of fibrillar β-lactoglobulin [9], α-synuclein [10] and insulin (manuscript in preparation).

Amyloid fibrils are cross-beta filaments, where the protein molecules making up the β-sheet are arranged perpendicular to the long axis of the fibril [11, 12]. Despite the fact that several models have been proposed for the interaction between fluorescent dyes and amyloid fibrils, the understanding of the exact mechanism of these interactions remains incomplete. One model proposed for the commonly-used amyloid-specific dye Thioflavin T [13], suggests that the dye binds to the fibrils with its long axis parallel to the fibril axis by insertion into a "binding channel" running along the fibril. Klunk et al. also suggested the same type of interaction with fibrils for Congo Red [14, 15]. However, it also has been proposed that Congo Red may intercalate between protein molecules which form β-sheets and thus sits perpendicular to the fibril long axis [16]. It has further been suggested that in aqueous solutions at concentrations of 5–20 μM and higher, both Thioflavin T and Congo Red form long rod-like "micelles" and interact with amyloid fibrils in this supramolecular form [17, 18]. Therefore, models proposed for interaction of amyloid with binding dyes still remain incomplete and controversial.

We have recently demonstrated that the benzothiazole monomethinecyanine dye T-284 (3-etylo-2-[(5-(N,N-diethylamino)-3-ethylbenzothiazol)methinyl]benzothiazole iodide) is a sensitive and specific probe for fluorescent detection of amyloid fibrils [10, 19]. This dye was shown to selectively bind to fibrillar wild-type ASN with significant emission intensity enhancement. The estimated equilibrium constant of T-284 dye binding to the ASN fibrils (K_b ~1.8×10^6 M^{-1}) was comparable with that reported for Thioflavin T complexed with aggregated synthetic peptides. Also, based on molecular dimension calculations, we suggested that T-284 inserts into "binding channels" with its long axis parallel to the ASN fibril axis, consistent with the model proposed for Thioflavin T [10].

The present study was aimed at characterizing the T-284 dye/ASN fibril complex by means of fluorescence anisotropy, time-resolved fluorescence, and atomic force microscopy techniques to give further insights into the mode of dye interaction with amyloid fibrils. These parameters are important for the characterization and screening of newly-developed amyloid-sensitive dyes.

Materials and methods

Reagents

Dimethyl sulfoxide (DMSO) and 10 mM TRIS-HCl buffer (pH 7.8) were used as solvents. DMSO, TRIS, HCl, Thioflavin T, bovine pancreas insulin and Ludox™ silica were purchased from "Sigma-Aldrich" (USA). Recombinant human ASN was expressed and purified as described in Ref. [20]. The T-284 dye was synthesized according to method described in [21]. The purity of the T-284 was >95%, and its structure was confirmed by ^{1}H NMR spectral method.

Induction of protein aggregation

Monomeric α-synuclein (wild-type and the two mutants A30P and A53T) at an initial concentration of 100 μM was incubated at 37 °C in 10 mM TRIS, 150 mM NaCl, pH 7.4 under constant stirring at 300 rpm in plastic vials. The volume per vial was 500 μl. Aggregation was monitored by Thio T binding until saturation of the fluorescence signal (after about 180 h). Bovine pancreas insulin was dissolved at 170 μM in 100 mM HCl. Fibrils were formed by shaking of the protein solutions at 750 rpm in a Thermomixer (Eppendorf) at 65 °C. The aggregation process was monitored by Thio T binding until saturation of the fluorescence signal after ~150 min.

The samples were stored at 4 °C. All aggregations were performed in triplicate. The presence of fibrils was confirmed with atomic force microscopy (AFM).

Preparation of stock solutions of dyes and biological molecules

2×10^{-3} M dye stock solutions were prepared by dissolving the dye in DMSO. The concentrations of proteins in stock solutions were 100 μM (α-synuclein variants) and 170 μM (insulin) for both native and fibrillar proteins. The fibrils used in all experiments were from the same batch.

Preparation of working solutions

Working solutions of free dye at 1 μM were prepared by dilution of the dye stock solution in 10 mM TRIS-HCl buffer (pH 7.8). Working solutions of dye/protein complexes were prepared by mixing of an aliquot of the dye stock solution and an aliquot of native or fibrillar protein in

500 μl of buffer. Concentration of the proteins in working solution ranged from 1 μM to 2 μM for both native and aggregated ASN variants and insulin.

Fluorescence anisotropy measurements

Steady-state fluorescence anisotropy measurements of the free T-284 dye, as well as its complexes with fibrillar proteins were performed using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon, France). Dye and proteins concentrations were 1 μM and 2 μM, respectively, in 10 mM TRIS-HCl buffer, pH 7.8. The anisotropy was calculated using the following equation: $A = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where A is the anisotropy, I is the intensity, V and H in the subscript represent the vertical or horizontal position of the excitation and emission polarizers, respectively. The G-factor was calculated as I_{HV}/I_{HH}. The anisotropy for the T-284 dye was measured by setting the excitation wavelength at 441 nm, and the emission wavelengths at 563 nm. Anisotropy measurements were performed in quartz cells (0.5 cm) at room temperature.

Time-resolved fluorescence

Time-resolved measurements of T-284 dye fluorescence in free state and in complexes with fibrillar proteins were recorded using a FluoroMax-4 spectrophotometer (Horiba Jobin Yvon, France) equipped with time-correlated single photon counting capabilities. Excitation was provided by a pulsed NanoLED at 465 nm. The instrument response function was measured using a scattering reference solution of Ludox™ silica (Sigma-Aldrich, USA). Dye and proteins concentrations were 1 and 2 μM respectively in 10 mM TRIS-HCl buffer, pH 7.8; measurements were performed in quartz cells (0.5 cm) at room temperature. Other conditions for time-resolved measurements are given in the figure legend. For time-resolved spectra analysis and lifetime calculations the DAS6 v6.1 decay analysis software (Horiba) was used. The total lifetime values (τ) for the free dye and dye/amyloid fibril complexes were calculated using the following equation: $\tau = \tau_1 \times A_1/100 + \tau_2 \times A_2/100 + \tau_3 \times A_3/100$, where fluorescence lifetimes (τ_1, τ_2, τ_3) and their fractional amplitudes (A_1, A_2, A_3; in %) were obtained by fitting the fluorescence intensity decays of the dye in free state and in presence of fibrillar proteins to the minimum number of exponential terms that produced randomly distributed residuals using the DAS6 software.

Atomic force microscopy

For AFM measurements small aliquots (4 μl) of aggregate mixture (at time point ~180 h) were diluted in 10 mM TRIS-HCl buffer, 150 mM NaCl, pH 7.8, adsorbed onto mica, washed twice with 50 μl MilliQ water and gently dried under nitrogen gas. Tapping mode AFM height images were made on a custom built instrument, as has been described before [20]. Fibrils and dye/fibril complexes heights were measured using SPIP software (Image Metrology A/S, Lyngby, Denmark).

Results and discussion

Fluorescence anisotropy measurements of T-284 dye free in solution and complexed with amyloid fibrils

We have reported that the benzothiazole monomethinecyanine dye T-284 (Fig. 1) is an efficient fluorescent probe for detection of fibrillar wild type ASN (wt ASN). The characteristic fluorescence intensity increase of the T-284 dye in the presence of fibrillar ASN has been attributed to immobilization of the dye molecule in the "binding channel" of the fibril [10].

As the rate of reorientation of a particle is a function of its size, fluorescence anisotropy measurements could offer a sensitive and informative means of monitoring the interactions between the dye and the amyloid fibril. For a rigid system the maximum anisotropy value is ~0.4 whereas for a freely rotating small molecule the anisotropy values are significantly smaller. Accordingly, fluorescent dye/amyloid fibril complex formation will be accompanied by enhancement of the anisotropy value, as the rotation of the molecule is reduced as compared to free dye [22].

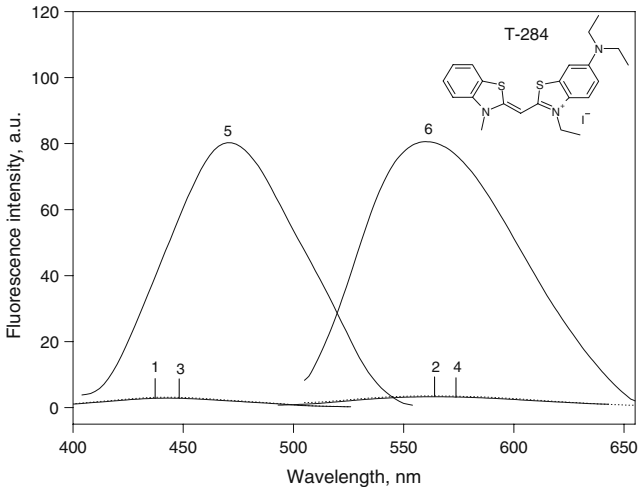


Fig. 1 Chemical structure of monomethine cyanine dye T-284 (1 μM) and its spectral properties in free state in TRIS-HCl buffer, pH=7.8 and in the presence of monomeric (2 μM) and fibrillar wt ASN (2 μM): fluorescence excitation (left) and emission (right) spectra of the T-284 in free state (1 and 2, respectively), in presence of monomeric wt ASN (3 and 4, respectively) and fibrillar wt ASN (5 and 6, respectively)

To characterize the T-284 cyanine dye in complex with amyloid fibrils, fluorescence anisotropy measurements of the dye (at 1 μM) in the presence of 2 μM of fibrillar ASN (wild-type and disease-associated mutants A30P and A53T) and insulin were performed. Anisotropy was measured at the wavelengths of the respective excitation/fluorescence maxima of the dye or dye/protein complexes. For free T-284 dye excitation and emission maxima were at 441 nm and 563 nm respectively. The maximum of absorption spectrum of the dye in aqueous buffer was situated at 436 nm, having optical density of 0.17 o.u. (optical units) (data not presented). Upon addition of the fibrillar ASN variants and insulin the excitation maxima of T-284 dye were red-shifted to 460–470 nm, while emission maxima stayed almost unchanged (between 560–563 nm) (Table 1). Such spectra behavior could be explained by the change upon dye binding to fibrillar structure of the shapes and relative position of the dye ground and excited electronic state hypersurfaces as well as of possibilities of absorption and fluorescent transitions between them. It should be noticed, that almost no changes in the position, shape and intensity of the absorption spectra of the dye in complexes with fibrillar proteins were observed.

Free in solution, the T-284 dye demonstrated a low fluorescence anisotropy value of ~0.05±0.009 (Table 1) as expected. Upon addition of fibrillar proteins, however, the anisotropy increased remarkably (Table 1), and is strongly indicative of dye/amyloid fibril complex formation. For wild-type ASN, A30P ASN and insulin fibrils, T-284 showed relatively high steady-state anisotropy values (in the range from 0.21±0.009 to 0.3±0.002). Upon addition of fibrillar A53T ASN, the anisotropy of T-284 increased only to 0.16±0.002, suggesting altered binding for this mutant. The anisotropy values for dye/amyloid fibrils complexes obtained in this study are close to those previously described in literature for Thio T/HET-s fungal prion protein complexes (~0.27) [23]. Importantly, only a slight increase in the anisotropy values of the dye in the presence of all monomeric proteins species was observed. Previously, based on the literature [11] and by comparing data of fluorescence studies and molecular dimension calculations, we suggested that T-284 dye binds to the fibrillar ASN with its long axis parallel to the fibril axis via insertion into "binding channel" running along the fibril [10]. As "binding channel" could be formed only on the mature beta-pleated fibril, we could suggest that observed insignificant increase of T-284 fluorescence intensity in presence of monomeric proteins could be explained with the absence of dye/monomer protein association (Table 1).

T-284/ASN fibrils complex characterization using atomic force microscopy

Wild-type α-synuclein fibrils were grown in vitro as described in Materials and methods. Formation of the fibrils was verified by a Thioflavin T fluorescence assay and AFM imaging (after ~180 h of incubation) (Table 2).

The quantitative analysis of AFM images (made in tapping mode) of wt ASN fibrils reveals average height (i.e. the diameter) of fibrils to be 6.3±1.0 nm (Table 2). At the 180-h time point, where fibrils were observed, T-284 dye (2 μM) was added to the samples of wild-type ASN and incubated for two hours at room temperature before depositing the samples on mica. The amyloid fibril/dye complexes were directly visualized by AFM, and heights of 8.0±1.1 nm were measured (Table 2). Comparison of the average height measured for wild-type ASN alone and in the presence of dye revealed a statistically significant increase in fibrils heights upon addition of T-284.

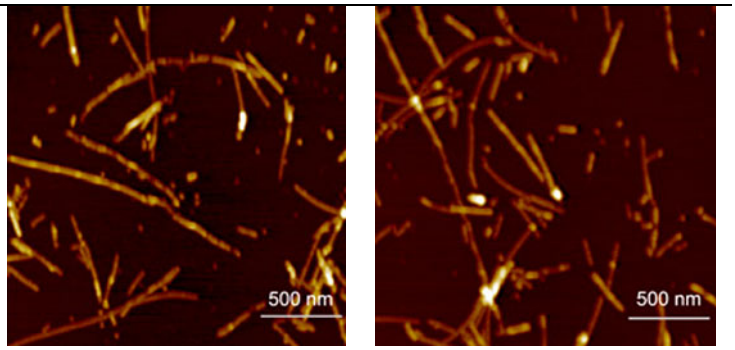
As already mentioned above, amyloid aggregates are considered to be cross-β filaments where the protein molecules making up the β-sheet are arranged perpendicular to the long axis of the fibril. Here, the side chains on each side of the β-sheet form neat rows ("binding channels"), running in the direction of the β-sheet. The width of the "binding channels" is the distance between every second residue, which in a β-sheet is 6.5–6.95Å. Based upon the dye/amyloid fibril binding model proposed by Krebs for Thioflavin T [11] and on the molecular dimensions of T-284 dye, we have previously suggested that this dye most likely inserts itself

Table 1 Spectral-luminescent characteristics and fluorescent steady-state anisotropy properties of T-284 dye free in solution and in the presence of fibrillar and monomeric proteins

Free T-284 dye			In presence of proteins:	wt ASN			A30P ASN			A53T ASN			Insulin		
λ_{ex}, nm	λ_{em}, nm	Anis		λ_{ex}, nm	λ_{em}, nm	Anis	λ_{ex}, nm	λ_{em}, nm	Anis	λ_{ex}, nm	λ_{em}, nm	Anis	λ_{ex}, nm	λ_{em}, nm	Anis
441	563	0.05 ±0.009	+ fibrillar protein	470	560	0.27 ±0.007	459	563	0.21 ±0.009	460	562	0.16 ±0.002	452	565	0.3 ±0.002
			+ monomeric protein	458	551	0.07 ±0.006	458	552	0.08 ±0.006	459	553	0.09 ±0.003	455	560	0.1 ±0.004

λ_{ex} (λ_{em})—maximum wavelength of fluorescence excitation (emission) spectrum; Anis—anisotropy value

Table 2 Morphological characteristics and AFM images of the wild-type α-synuclein fibrils and T-284 dye/wt ASN fibril complexes

	wt ASN	wt ASN + T-284 dye
AFM images		
N fibrils	34	23
Fibril height, nm	6.3±1.0	8.0±1.1

N fibrils is the number of fibrils analyzed. Heights values are averaged over all *N* fibrils, the standard deviation is used as measurement error. The images of fibrils and dye/fibril complexes were taken using tapping-mode AFM in air. Comparison of the average heights measured for wt ASN alone and in the presence of dye revealed the statistically significant increase in fibrils heights upon T-284 addition, which could point on dye/fibril interaction

into such binding channels formed on ASN fibrils, with its shortest axis perpendicular to the fibril axis [10]. The observed differences in heights between α-synuclein fibrils alone and in the presence of the dye are consistent with the possibility that T-284 molecules could insert in the "binding channel" running along the fibril.

Time-resolved fluorescence studies of T-284 free in solution and in presence of fibrillar ASN

The fluorescence decays of T-284 in free state and in fibrillar wild-type, A30P, and A53T ASN were fit with a multiexponential model. The fluorescence decay of T-284 in free form in buffer appeared to be bi-exponential having lifetime components of τ_1 ~2.43 ns (fractional amplitude 9%) and τ_2 ~0.04 ns (91%). The average lifetime value for the free dye in aqueous solution was τ ~0.25 ns, and is in accordance with a number of studies that show the fluorescence lifetimes of cyanine dyes in solution to be very short (~ps) [24].

Symmetric monomethine cyanines are well-known to exist basically in *trans* form due to relatively equally redistributed charge along the dye molecule that results in the equal order of the two methine bonds and thus to a rigid dye structure [25, 26]. However, in unsymmetrical monomethinecyanines the charge is located on the nitrogen atom of the more basic heterocycle, that results in a pronounced difference in methine bond orders. This makes the dye molecule more flexible and allows the rotation of heterocycle fragments around the single C-C bond of the methine

Table 3 Decay parameters of multi-exponential fit of T-284 free in buffer and in complexes with fibrillar wt ASN, A30Pand A53T

1μM T-284	τ_1, ns	τ_2, ns	τ_3, ns	A_1, %	A_2, %	A_3, %	τ, ns	χ^2
in free form	2.43	0.04	–	9	91	–	0.25	1.30
+ fibrillar wt ASN	1.86	0.24	4.05	39	19	43	2.50	1.16
+ fibrillar A30P	1.26	0.11	3.19	26	20	54	2.07	1.02
+ fibrillar A53T	1.33	0.23	3.10	27	18	55	2.02	1.22

Fluorescence lifetimes (τ_1, τ_2, τ_3) and their fractional amplitudes (A_1, A_2, A_3) were obtained by fitting the decays of the dye in free state and in presence of fibrillar proteins to the minimum number of exponential terms that produced randomly distributed residuals; χ^2 is the reduced chi-square; τ is the total lifetime values, which was calculated using the following equation: $\tau = \tau_1 A_1/100 + \tau_2 \times A_2/100 + A_3/100$

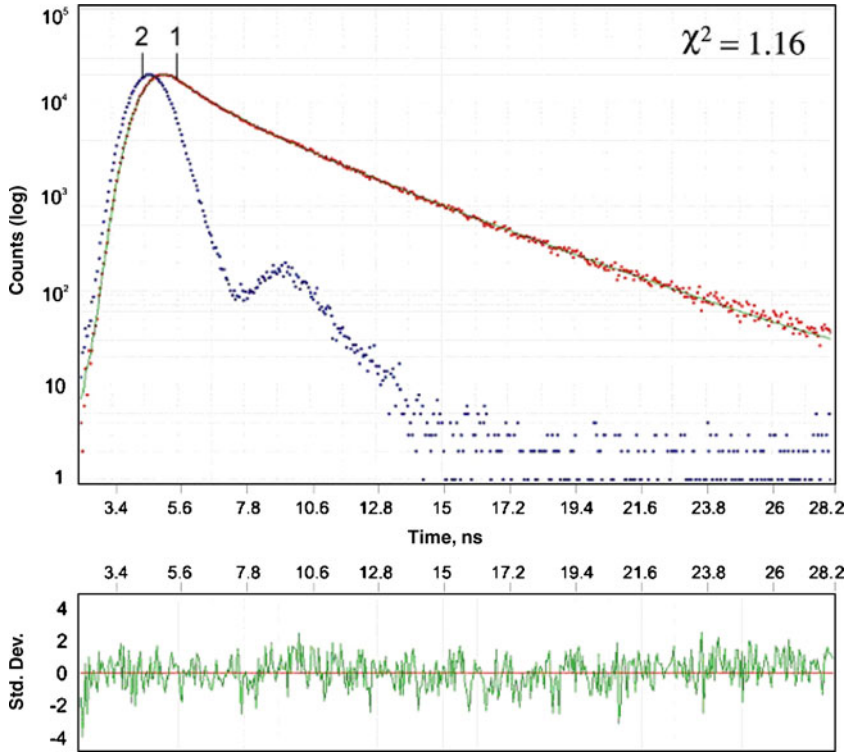


Fig. 2 Fluorescence decay of T-284 (1 μM) in presence of fibrillar wt ASN (2 μM) in TRIS-HCl buffer, pH=7.8 (1) vs. a scattering reference Ludox™ silica (2). Experimental curves was obtained using a 465 nm NanoLED, the experiment conditions were: coaxial delay = 80 ns, time to amplitude converter (TAC) range = 50 ns, peak preset = 20000 counts, repetition frequency rate = 1 MHz, synchronization delay = 0 ns. The data for dye/fibril complex presented is best fitted with a tri-exponential decay yielding a reduced chi-square χ^2=1.16

chain. Despite the lack of literature data concerning the possibility of unsymmetrical monomethines to adopt *cis* form, we suppose that in the case of the monomethinecyanine dye T-284, the presence of two distinct lifetime components for the free dye could be explained by the existence of its *trans* and *cis* conformers.

Because of its dominant contribution (91%), the shorter lifetime τ_2 ~0.04 ns could be assigned to the thermodynamically favorable *trans* state of T-284. Consequently, the minor lifetime component (9%) τ_1 ~2.43 ns is attributed to the *cis* conformation of the dye. Such two-exponential fluorescence decay was also observed for a number of trimethinecyanine dyes and has also been attributed to the presence of *cis* and *trans* conformers of the dyes, although the difference between the lifetime components was not as significant [27] as that observed here.

A second explanation for the presence of two components in the fluorescence decay could be proposed. Since the dimethylamino group is an organic base and demonstrates electron donor properties, hydrogen bond formation between dimethylamino group and water molecules could be suggested. Formation of such complexes potentially hinder the internal rotation of the dye molecule and thus could lead to the appearance of a long lifetime component in the fluorescence decay, while the short lifetime component corresponds to dye molecules that are not complexed with water.

Time-resolved fluorescence analysis indicated that the average lifetime values for T-284 in the presence of fibrillar proteins are an order of magnitude higher (τ ~2.02–2.50 ns) than those for free dye (τ ~0.25 ns) (Table 3, Fig. 2). Moreover, fluorescence decays of T-284 dye in the presence of fibrillar ASN variants showed tri-exponential decay kinetics with lifetimes of τ_1 ~1.26–1.86 ns, τ_2 ~0.11–0.24 ns, and τ_3 ~3.10–4.05 ns (Table 3). It should be noted, that increasing of concentration of fibrillar wild type, A30P and A53T ASN did not significantly influenced on fluorescence lifetime of the dye in complex with fibrils (data not presented).

Earlier Lindgren et al. observed three lifetime components for Thio-T bound to transthyretin fibrils, ranging from 0.1 to 3.9 ns, that was explained by existence of different microenvironments for the bound dye [28]. The presence of distinct dye binding sites on ASN fibrils, which are differently exposed to water has been proposed by Celej et al. [7, 29]. Thus long lifetime components in spectra of T-284 in ASN presence could be attributed to complexes of

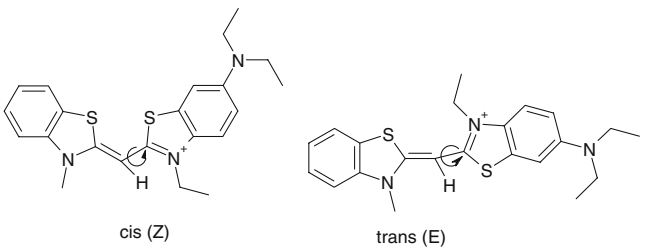


Fig. 3 Proposed cis- and trans- conformers of T-284 dye

dyes with different sites on the fibril or to formation of complexes of different structure.

Furthermore the dye molecule could bind to the ASN fibril either in *trans* or in *cis* conformation by different binding modes (Fig. 3). Upon increasing of dye concentration virtually no changes in lifetime components of T-284 were noticed (data not presented), suggesting an insignificant contribution of the free dye to the measured lifetimes.

We conclude that in the presence of the fibrillar proteins studied, T-284 dye might exist in different "populations" due to interaction with fibrils in different conformers and binding modes. The exact binding mode of specific T-284 conformers with ASN fibrils will require further studies.

Conclusions

1. Monomethinecyanine dye T-284 showed low (~0.05) fluorescence anisotropy value in the absence of fibrillar proteins, while upon addition of aggregated proteins this value increased up to 0.3, indicative of highly ordered dye/amyloid fibril complex formation.
2. The quantitative analysis of AFM images of fibrillar wild-type α-synuclein reveals differences in heights between ASN fibrils alone and in presence of the T-284 dye (6.37±1.0 nm and 8.0±1.1 nm respectively). We argue that the increase in the fibril diameter could be caused by embedding of T-284 dye molecules in the "binding channel" running along the fibril.
3. Fluorescence decay analysis of the T-284 dye in complexes with fibrillar ASN variants revealed the fluorescence lifetime values for T-284/fibril complexes to be an order of magnitude higher as compared to those of the free dye (τ ~2.02–2.50 ns and τ ~0.25 ns respectively). The fluorescence decay of free T-284 was bi-exponential, while dye bound to protein yields tri-exponential decay. We suggest that in the presence of the fibrillar ASN variants, T-284 dye might exist in different "populations" due to interaction of fibrils with different conformers of the tested dye or different dye-fibrils binding modes. The exact mechanism of T-284 interaction with ASN fibrils needs further studies.

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