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Tri- and Pentamethine Cyanine Dyes for Fluorescent Detection of α-Synuclein Oligomeric Aggregates

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Abstract The pathogenesis of Parkinson's disease that is the second most common neurodegenerative disease is associated with formation of different aggregates of α-synuclein (ASN), namely oligomers and amyloid fibrils. Current research is aimed on the design of fluorescent dyes for the detection of oligomeric aggregates, which are considered to be toxic and morbific spices. Fluorescent properties of series of benzothiazole trimethine and pentamethine cyanines were characterized in free state and in presence of monomeric, oligomeric and fibrilar ASN. The dyes with wide aromatic systems and bulky phenyl and alkyl substituents that are potentially able to interact with hydrophobic regions of oligomeric aggregates were selected for the studies. For majority of studied dyes noticeable changes in fluorescence characteristics were shown in the presence of fibrillar or oligomeric ASN, while the dyes slightly responded on the presence of monomeric protein. For pentamethine cyanine SL-631 and trimethine cyanine SH-299 certain specificity to oligomeric aggregates over fibrils was observed. Using these dyes at 10^{-6} M concentration permits the detection of oligomeric ASN in the concentrations range of at least 0.2–2 microM. Pentamethine cyanine SL-631 is proposed as dye for fluorescent detection of oligomeric aggregates of ASN, while trimethine cyanine SH-299 is shown to be a sensitive probe both on oligomeric and fibrillar ASN. It is proposed that wide aromatic system of SL-631 pentamethine dye molecule could better fix on the less dense and structured oligomeric formation, while less bulky and more "crescent-shape" molecule of trimethine dye SH-299 could easier enter into the groove of beta-pleated structure.

Keywords Fluorescent probes · Amyloid fibrils · Oligomeric aggregates · Cyanine dyes · Alpha-synuclein

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Introduction

Parkinson's disease is the second most common neurodegenerative disease, a characteristic of which is the formation of amyloid fibrils of the protein α-synuclein (ASN) [1–4]. During the amyloidogenic pathway ASN molecules first form small oligomeric aggregates, which are considered as presumably toxic species [5, 6] and potentially more relevant to disease than the mature fibrils [7, 8]. A number of studies have suggested that oligomeric aggregates of fibrils may disrupt or permeabilize cellular membranes [9] leading to disruption of calcium regulation and cell-death. [10].

Generally, ASN oligomers are reported as spherical protein aggregates with sizes between 2–20 nm [11–13], although annular and tubular morphologies of aggregates have also been mentioned [13–15]. Oligomers are mainly considered to possess beta-sheet structure [9, 16], although presence of α-helical content has been shown as well [11]. Despite the beta-sheet content, the oligomers are structurally different than mature fibrils, and are considered a heterogeneous population of which the majority is off-pathway from the fibrillization process [17].

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As fluorescent reporters for detection of oligomeric aggregates the anilinonaphthalene sulfonate dyes (ANS, Bis-ANS) have been used [18, 19], and are known to bind with exposed hydrophobic surfaces. The disadvantage of these dyes is a lack of selectivity to oligomeric aggregates, since they are also known to bind with mature fibrils. The dyes Thioflavin T and Congo Red which are most commonly used for the detection of amyloid deposits [19, 20] are able to provide measurable signals only for mature amyloid fibrils. Thus there is an imperative to design dyes that are specific for these early aggregates.

Earlier we proposed mono- and trimethine cyanine dyes as fluorescent probes for quantitative detection of amyloid formations. These dyes exhibit a fluorescence signal in the presence of fibrillar proteins, with enhanced sensitivity to aggregated proteins and signal intensities comparable to or better than that of Thioflavin T. We have also shown the applicability of these dyes for monitoring of the fibrillization process [21–23].

This work reports on the search for fluorescent dyes selective for oligomeric aggregates formations. Fluorescence spectroscopy was used to study cyanine dyes in the presence of monomeric ASN, oligomeric aggregates and amyloid fibrils. Tri- and pentamethine dyes (Fig. 1) with wide aromatic systems and bulky phenyl and alkyl substituents that are potentially able to interact with hydrophobic regions of oligomeric aggregates were screened for specific fluorescence signals. For the most efficient dyes found by the screening, we carried out a detailed characterization of their binding to aggregated ASN (oligomeric and fibrillar formations).

Materials and Methods

Reagents

Anhydrous dimethylsulfoxide (DMSO) distilled under reduced pressure and 0.05 M Tris–HCl buffer (pH 7.9) were used as solvents. Thiacarbocyanines of symmetrical structure 7519, T-307 and 7533 were obtained basing on quaternary salts of 2-methyl-5-methoxy- and 2-methyl-5-(6)-benzoylaminobenzothiazole by standard methods as described earlier [24]. The dyes SH-516, SH-349, SH-299 and SH-428 were synthesized from derivatives of 2-methyl-5-phenyl-, 2-methyl-5-(6)-styrylbenzothiazole, 2-methylthieno[2,3-e]benzothiazole according to the method of obtaining carbocyanines of unsymmetrical structure [24]. Dicarbocyanines SL-535, SL-631, SH-1082 and 2904y were obtained from corresponding quaternary salts of

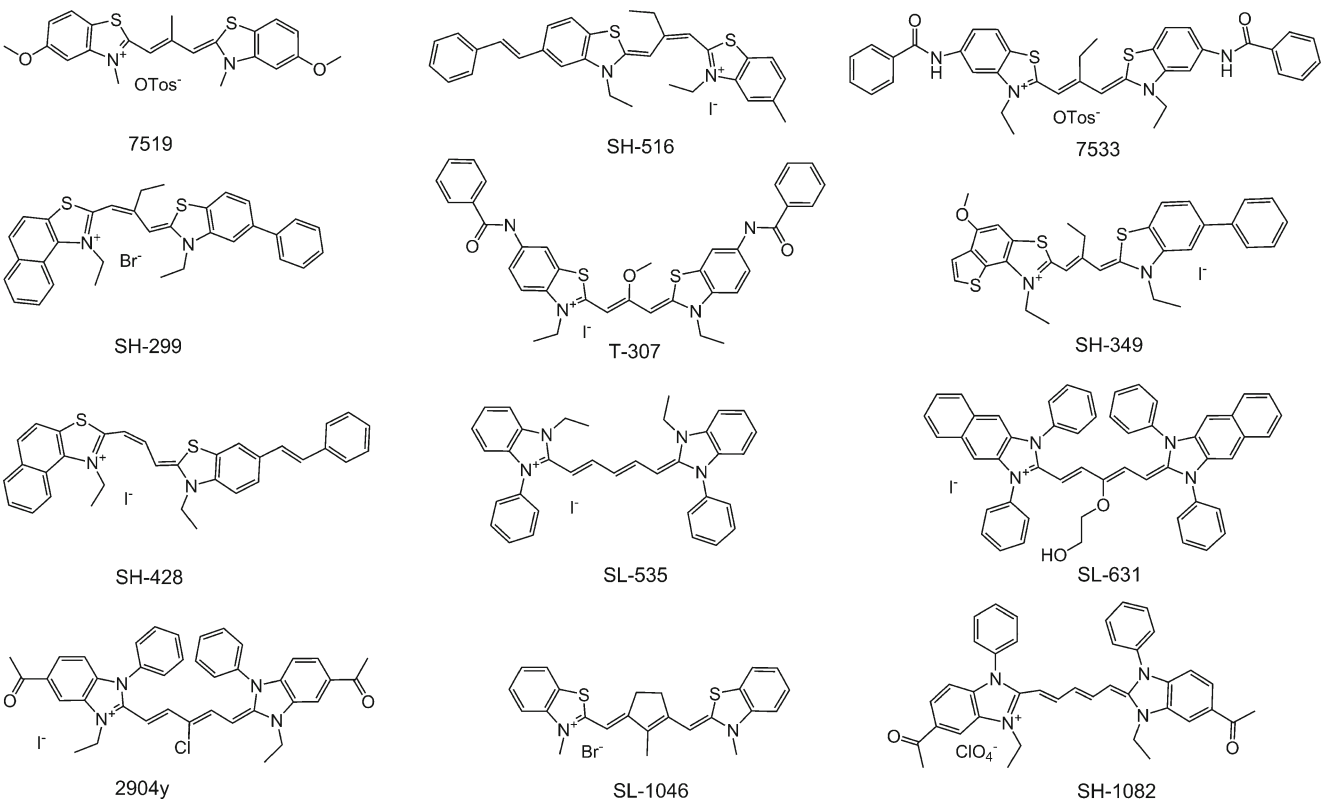

Fig. 1 Structures of studied tri- and pentamethine dyes

imidazole as described earlier [25, 26]. Synthesis of thiacarbocyanine SL-1046 was described in [27].

Recombinant human wild-type α-synuclein (ASN) was expressed and purified in 10 mM Tris–HCl, 50 mM NaCl, pH 7.4 as described earlier [28]. ASN oligomers were prepared as described previously [29]. In brief, monomeric ASN solution was dried in a vacuum evaporator and redissolved in MilliQ water at a 1 mM protein concentration. After incubating the ASN solution in an Eppendorf Thermo mixer with shaking at 1,250 rpm for 18 h at room temperature, the solution was filtered through a 0.2 μm Spin-X centrifugal filter (Corning) to remove possible large aggregates. The oligomeric species were purified by size-exclusion chromatography on a Superdex 200 (GE Healthcare) gel filtration column using 10 mM Tris–HCl, 50 mM NaCl, pH 7.4 as eluent. The ASN oligomer containing fractions were pooled and concentrated using a 10 kDa MWCO Vivaspin (Sartorius). The protein concentration was determined from the absorption at 275 nm.

ASN fibrils were prepared by incubating 100 μM ASN monomer solution in 10 mM Tris–HCl, 50 mM NaCl, pH7.4 in a Thermo mixer at 37 °C with shaking at 1,250 rpm. Fibril formation was continued until maximum fluorescence was reached in a Thioflavin T assay [28].

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 for monomer, oligomeric and fibrillar ASN in stock solutions were respectively 0.49×10^{-4} M, 0.24×10^{-4} M, and 10^{-4} M (monomer equivalent concentrations). The monomers, oligomers and fibrils used in all experiments were from the same batch.

Preparation of Working Solutions

Working solutions of free dyes at the concentration 10^{-6} M were prepared by dilution of the dye stock solution in 50 mM Tris–HCl buffer (pH 7.9). Working solutions of dye-protein complexes were prepared by mixing an aliquot of the dye stock solution and an aliquot of monomeric, oligomeric or fibrillar ASN protein in buffer. Concentrations of the proteins in working solutions were 0.5×10^{-6} M for monomer, oligomer and fibrillar proteins (monomer equivalent concentrations) and the dye concentration in the dye-protein working solution was 10^{-6} M. For the fluorescent titration experiment, concentrations of oligomer and fibrillar ASN were respectively 0.05–2×10^{-6} M and 0.1–5×10^{-6} M. All working solutions were prepared immediately before the experiment.

Spectroscopic Measurements

Absorption spectra were obtained with the help of the Specord M-40 spectrophotometer (Carl Zeiss, Germany). Fluorescence excitation and emission spectra were collected on a Cary Eclipse fluorescence spectrophotometer (Varian, Australia). Fluorescence spectra were measured with excitation and emission slit widths set to 5 nm. Spectroscopic measurements were performed in standard quartz cells (1×1 cm). All measurements were carried out at room temperature. Fluorescence emission for all dyes was excited at the corresponding excitation maxima (if not stated otherwise). Fluorescence quantum yield of SL-631 and SH-299 (10^{-6} M) in presence of fibrillar ASN (5×10^{-6} M) was measured using ethanol solutions of thiadicarbocyanine [30] and Rhodamine B [31] respectively as reference. For estimation of dyes binding constants to oligomeric ASN, the curve of dye fluorescent titration with oligomeric ASN was approximated with the equation [21]:

$$I = I_0 \times C_p \times K/(1 + C_p \times K)$$

where I is the dye fluorescence intensity in presence of the oligomer concentration C_p; K is the binding constant, and I_0 is the dye fluorescence intensity upon saturation (i.e. binding of all dye molecules to ASN oligomer). Since saturation was not reached in the experiment, I_0 was determined from the fit, as was the binding constant K.

Results and Discussion

Spectral Characteristics of Penthamethine Dyes and Their Complexes with Monomeric and Aggregated ASN

Characteristics of fluorescence spectra of free pentamethine dyes and in the presence of monomeric ASN are presented in Table 1.

For unbound pentamethine cyanines with exception of dye SL-535 excitation maxima were situated at 604–650 nm, and emission maxima at 615–665 nm. Small Stokes shift values from 11 to 20 nm and rather low fluorescence intensities (4.8–30 a.u.) were observed for these dyes. The pentamethine cyanine SL-535 in buffer solution demonstrated very low emission level, and had no clear emission and excitation maxima.

Addition of monomeric ASN results in the slight shift (~1–4 nm) of emission and excitation maxima of pentamethine dyes SL-631, 2904y, SH-1082 and SL-1046, in slight changes of Stokes shifts values (~7–24 nm) and in some loss of dye emission intensity (observed intensities 4.3–26.1 a.u.). For the dye SL-535 noticeable bathochromic shift of maxima and insignificant emission increase were

Table 1 Spectral-luminescent properties of pentamethine dyes in free state and in presence of monomeric, oligomeric, and fibrillar ASN

dye	Free dye			Monomer ASN			Fibrillar ASN			Oligomeric ASN		
	λex, nm	λem, nm	I_f	λex, nm	λem, nm	I_M	λex, nm	λem, nm	I_F	λex, nm	λem, nm	I_O
SL-535	~645	~700	~0.1	~700	~730	~0.3	725	730	14	729	737	3.9
SL-631	650	665	4.8	650	662	4.5	672	683	9	664	678	70
2904y	606	628	30.5	607	631	26.1	624	645	32	619	639	48
SH-1082	616	636	5.6	619	640	4.3	646	660	45	636	654	70
SL-1046	604	615	13	607	614	8	628	632	28	624	630	28

λex – excitation wavelength, λem- emission wavelength, I_f, I_M, I_F, I_O – fluorescence intensity of correspondingly free dye, in presence of monomeric, fibrillar and oligomeric ASN

recorded. The fluorescence intensity levels still remain very low and no clear maxima were observed.

Spectral characteristics of pentamethine dyes in presence of fibrillar and oligomeric ASN are presented in Table 1.

The pentamethine dyes studied demonstrated spectral response upon addition of fibrillar protein. Excitation and emission maxima of dyes in presence of fibrillar ASN demonstrated noticeable bathochromic shifts of ~17–30 nm relative to the maxima of the free dyes. Dye 2904y slightly changes its emission in the presence of fibrillar ASN. For other pentamethines, fluorescence emission increased between a factor of 2 for the dye SL-631 and 140 (due to the negligible fluorescence of free dye) observed for dye SL-535. Emission intensity values of the dyes in complexes with aggregated protein were between 9 and 45 a.u.

In the presence of oligomeric ASN in most cases spectral maxima of pentamethine dyes were situated between corresponding maxima of free dyes and dyes in complexes with fibrillar ASN. Excitation maxima are situated between 619 and 729 nm, and emission maxima are between 630 and 737 nm. In presence of oligomeric ASN for all studied pentamethines emission increases in ~1.6–39 times. Fluorescence intensities of the dyes in complexes with oligomers were between 3.9 a.u. for SL-535 and 70 a.u. for SL-631 and SH-1082. It should be noted that for all studied pentamethine dyes, emission enhancement was higher in the presence of oligomeric ASN than fibrillar ASN with exception of SL-535 and SL-1046 (for the last dye enhancement values are equal).

Dyes SL-631 and SH-1082 demonstrate in the presence of oligomeric ASN sufficient emission increase (14.6 and 12.5 times respectively) and fluorescent intensity (70 a.u.), also these dyes are not sensitive to the presence of monomeric protein. Moreover dye SL-631 has shown noticeable specificity to oligomeric ASN over fibrillar protein. This can be explained by the wide aromatic system of this dye molecule that probably could better fix on less dense and structured oligomeric formations, than in regularly structured grooves of beta-sheets of mature fibrils.

Spectral Characteristics of Trimethine Dyes and Their Complexes with Monomeric and Aggregated ASN

Spectral characteristics of free trimethine dyes and in the presence of monomeric ASN are presented in Table 2. We have earlier determined that the trimethine cyanines SH-516 and 7519 are sensitive to fibrillar proteins [22, 23], and these dyes were also investigated as probes for detection of ASN oligomeric aggregates.

The excitation maxima of the trimethine dyes studied are in the range 525–590 nm. Four of these dyes T-307, 7533, SH-299 and SH-349, demonstrate extremely large values of Stokes shifts (~65–236 nm), with emission maxima in the range 630–776 nm. Such significant shifts of emission maxima are probably connected with aggregation processes that occur in free dye solution. Three of the dyes, 7519, SH-516 and SH-323, have rather small values of Stokes shift (10–20 nm) with emission maxima ~570–603 nm. These maxima are thought to correspond to emission of dyes in monomeric form. All trimethine cyanines demonstrated low emission intensity (0.6–1.75 a.u.) free in solution.

Trimethine dyes slightly change their spectral properties in the presence of monomeric ASN. For these dyes shift of emission and excitation maxima positions of up to 12 nm and changes in fluorescence intensity in 0.63–1.3 times were observed.

Spectral characteristics of trimethine dyes in presence of fibrillar and oligomeric ASN are presented in Table 2. The trimethine cyanines studied demonstrated a noticeable response upon addition of fibrillar ASN. For dyes SH-349, SH-299, 7533 and T-307 in complexes with fibrillar protein, significant shifts of excitation and emission maxima positions compared to those of free dyes were observed. These shifts are attributed to interaction of monomeric dye molecules with fibrillar protein. For dyes 7519, SH-516 and SH-323 red shifts of excitation and emission maxima of ~2–20 nm were observed. Excitation maxima of studied dyes ranged between 570 and 600 nm, and emission maxima between 583 and 610 nm, with Stokes shift values ~9–18 nm. Fluorescence intensities increased ~2.9–57 times and emission intensities of

Table 2 Spectral-luminescent properties of trimethine dyes in free state and in presence of monomeric, oligomeric, and fibrillar ASN

dye	Free dye			Monomer ASN			Fibrillar ASN			Oligomeric ASN		
	λex, nm	λem, nm	I_f	λex, nm	λem, nm	I_M	λex, nm	λem, nm	I_F	λex, nm	λem, nm	I_O
7519	561	581	1.75	560	575	2	581	590	100	578	588	17.2
SH-349	540	776	1.5	540	766	1.5	600	609	9.5	595	608	15.8
SH-516	560	570	0.6	560	570	0.8	570	583	12.1	577	584	14.5
7533	565	630	1.15	570	620	1.5	580	593	12.5	583	592	10
SH- 299	543	645	1.6	544	645	1.3	592	606	12	590	602	30
SH-428	590	603	1.4	590	600	0.9	592	610	4	590	616	4
T-307	525	712	1.2	534	700	0.75	586	598	9	583	591	3

λex – excitation wavelength, λem- emission wavelength, I_f, I_M, I_F, I_O – fluorescence intensity of correspondingly free dye, in presence of monomeric, fibrillar and oligomeric ASN

~4–100 a.u. were detected. The most pronounced fluorescent response and emission intensity value were observed for the trimethine dye 7519, which was earlier reported as a suitable probe for insuline fibril formation.

Spectral maxima of dyes in the presence of oligomeric ASN were close to those in complexes with fibrillar ASN. Excitation maxima of the dyes range between 577 and 590 nm, and emission maxima between 584 and 616 nm, with observed Stokes shifts values between 7 and 26 nm. The emission intensities of trimethine dyes increase in the presence of oligomeric ASN, ranging from 2.5 (T-307) up to 24 (SH-516) times, with emission intensity values between 3 and 30 a.u.. For the dyes SH-349 and SH-516, the emission intensity of the dyes in the presence of oligomeric ASN was somewhat higher than that in the presence of fibrillar protein. Amyloid sensitive dye 7519 gave about 5 times less intensive emission in complex with oligomeric ASN compared with fibrillar protein. Dye SH-299 demonstrated noticeable specificity to oligomeric ASN compared to fibrillar and monomeric protein (observed emission enhancement respectively 18.7, 7.5 and 0.81 times).

Fluorescent Titration of the Dyes SL-631 (a) and SH-299 (b) with Oligomeric ASN

We further performed a fluorescence titration of the dyes SL-631 and SH-299 with oligomeric ASN (Fig. 2) to characterize the response of these dyes in more detail. The dye concentration was 10^{-6} M, while that of oligomeric ASN was between 0.05 and 2×10^{-6} M (monomer equivalent concentration).

At low concentrations, the addition of oligomeric ASN does not perceptibly change the fluorescence intensity of SH-299, and even decreases that of SL-631. This could be due to formation of non- or weak-fluorescent dye aggregates in the presence of oligomer, as it was reported for some of cyanines in presence of biological molecules at high dye-to-biomolecule concentrations ratios [32]. Further increase of the protein concentration leads to binding of the dye molecules to the oligomers mostly in monomeric form and to increase in dye fluorescence intensity. If we define the oligomer concentration leading to a doubling of dye fluorescence intensity to be the limit of oligomer detection with the dye, this limit could be estimated as 0.2 microM for both

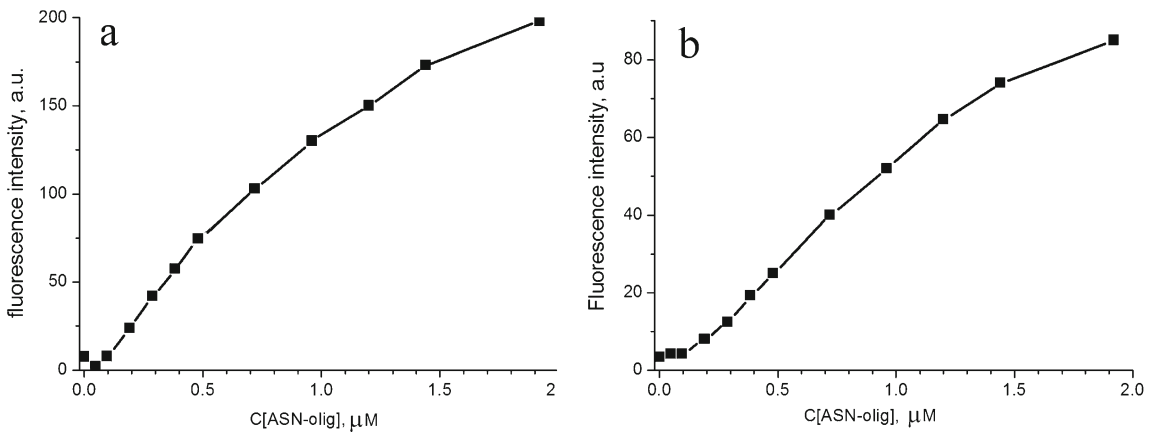

Fig. 2 Fluorescent titration of the dyes SL-631 (**a**) and SH-299 (**b**) with the oligomeric ASN. Dyes concentration was 10^{-6} M

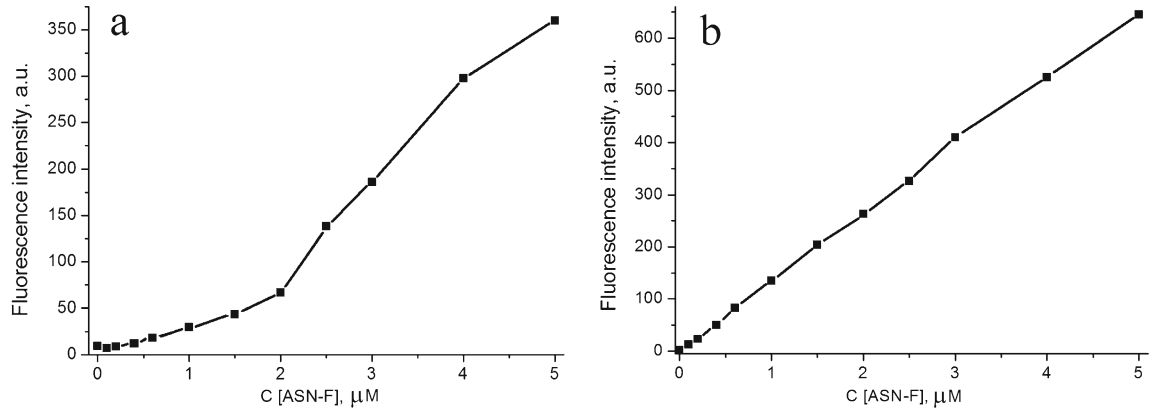

Fig. 3 Fluorescent titration of the dyes SL-631 (**a**) and SH-299 (**b**) with the fibrillar ASN. Dyes concentration was 10^{-6} M

dyes SL-631 and SH-299. The fluorescence intensity monotonously grows at least to 2 microM. Thus at a dye concentration of 10^{-6} M we are able to detect oligomeric ASN in the concentration range of ~0.2–2 microM.

The fluorescence titration data were used to make a rough estimate of the binding constant of the dye to oligomeric ASN. The ASN oligomers studied were shown to contain significant β-sheet structure [17]. If we consider the dye molecules to bind into the grooves of the β-structure, the concentration of the binding sites could be considered much higher than this of the bound dye molecules at least for the concentrations range about 1–2 microM of oligomers. Thus the dye to oligomer binding constant was roughly estimated using five points corresponding to the highest oligomer concentrations studied. The obtained value was equal to $2.4\pm0.8\times10^5$ M^{-1} for SH-299 and $3.8\pm0.5\times10^5$ M^{-1} for SL-631. At the same time these values were calculated using the protein concentration in ASN molecules, while, on one hand, a dye molecule occupies only small part of the oligomerized globule. On the other hand, significant part of the aminoacid sequence forming oligomer is buried inside the oligomer and thus is not accessible to dye binding. Thus the number of dye binding sites of the oligomeric ASN that is necessary to calculate the true binding constant is unknown. Besides, influence of the dye aggregation (both in the solution and on the oligomeric ASN molecule) cannot be completely excluded even at the highest oligomer concentrations used for calculations. Thus the obtained binding constant values could be considered as illustrative only.

Fluorescent Titration of the Dyes SL-631 (a) and SH-299 (b) with Fibrillar ASN

For the dyes SL-631 and SH-299 studied above in presence of oligomeric ASN, the fluorescent response on the addition of fibrillar ASN was also investigated. For this fluorescent titration of 10^{-6} M of the dyes with ASN mature fibrils (concentration between 0.1 and 5×10^{-6} M of ASN molecules) was performed (Fig. 3).

The addition of the fibrillar ASN to the SL-631 solution first results in the decrease in dye fluorescence intensity as it was observed for the case of oligomeric ASN. Thus the formation of non-fluorescent aggregates could be supposed for SL-631 molecules in presence of fibrillar ASN as well as oligomeric ASN. At the same time, for SH-299 addition of fibrillar ASN leads to apparently linear growth of the dye fluorescence intensity at the wavelength of the emission maximum. Meanwhile, besides the emission maximum at about 607 nm (with corresponding excitation peak near 599 nm) that is due to SH-299 monomer molecules bound to ASN fibrils, a maximum at 648 nm (with corresponding excitation peak near 647 nm) appears at low ASN concentrations which we attribute to SH-299 fluorescent J-aggregates formed on ASN fibrils (Fig. 4). We note that

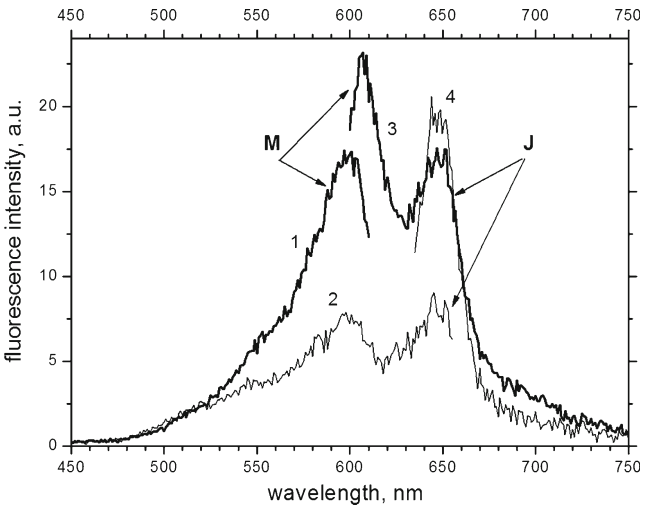

Fig. 4 Fluorescence excitation (1,2) and emission (3,4) spectra of the dye SH-299 (1 microM) in presence of the fibrillar ASN (0.2 microM). Emission wavelengths 620 nm (1) and 665 nm (2); excitation wavelengths 590 nm (3) and 625 nm (4). M and J – maxima corresponding to monomers and J-aggregates of the dye respectively

formation of fluorescent J-aggregates of cyanine dye on fibrillar insulin was described by us earlier [23]. Further increase of the fibrillar ASN concentration leads to enhancement in dye fluorescence intensity. As is seen from Fig. 3 the limit of fibrillar ASN detection using the 10^{-6} M dye concentration could be estimated as 0.6 microM for SL-631 and less than 0.1 microM for SH-299, and detection is possible up to at least 5 microM of fibrillar ASN.

In addition, we determined the fluorescence quantum yield of 1 microM concentration of the dyes SL-631 and SH-299 in the presence of 5 microM of fibrillar ASN, yielding values of 0.07 and 0.13 for SL-631 and SH-299 respectively. The true fluorescence quantum yield of the dye bound to ASN fibrils could be even higher since only part of the dye was bound to ASN fibril at the concentrations mentioned.

Conclusions

1. Series of benzothiazole trimethine and pentamethine cyanine dyes with wide aromatic systems and bulky phenyl and alkyl substituents were studied as potential fluorescent probes for selective detection of oligomeric aggregates of ASN. For the majority of dyes studied noticeable changes in fluorescence characteristics occur in the presence of fibrillar or oligomeric ASN, while the dyes show only a very small response in the presence of monomeric protein. For pentamethine cyanine dye SL-631 and trimethine SH-299 certain specificity to oligomeric aggregates over mature fibrils was observed.
2. It is proposed that the wide aromatic system of SL-631 pentamethine dye molecule could better fix on less dense and structured oligomeric formation, than in regularly structured grooves of beta-sheets of mature fibrils. At the same time less bulky and more "crescent shape" molecule of trimethine dye SH-299 could easier enter into the groove formed by beta-sheets.
3. We consider pentamethine cyanine SL-631 could be proposed as dye for fluorescent detection of oligomeric aggregates of ASN, while trimethine cyanine SH-299 is shown to be a sensitive probe both on oligomeric and fibrillar ASN spices. Using these dyes at 10^{-6} M permits the detection of oligomeric ASN in the concentrations range of at least 0.2–2 microM. The limit of fibrillar ASN detection with SH-299 is less than 0.1 microM, and detection is possible up to at least 5 microM of fibrillar ASN.

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