J Fluoresc (2011) 21:775–784
DOI 10.1007/s10895-010-0770-6

ORIGINAL PAPER

Hydroxy and Methoxy Substituted Thiacarbocyanines for Fluorescent Detection of Amyloid Formations

Kateryna D. Volkova · Vladyslava B. Kovalska · Mykhaylo Yu. Losytskyy · Kateryna O. Fal · Nadiya O. Derevyanko · Yuriy L. Slominskii · Olexiy I. Tolmachov · Sergiy M. Yarmoluk

Received: 7 June 2010 / Accepted: 16 November 2010 / Published online: 3 December 2010

Abstract In present paper series of trimethine cyanines modified in 5,5′- or 6,6′- position with hydroxy- or methoxy- substituents is studied for their ability to interact selectively with fibrillar formations. Processes of dye aggregation that accompany this interaction were also investigated. Meso-methyl trimethynecyanines with 5,5′-methoxy (7519) and hydroxy (7515) substituents strongly (up to 40 times) increase fluorescence intensity in the presence of fibrillar insulin, and also give noticeable fluorescent response on the presence of various aggregated proteins (lysozyme, β-lactoglobulin, α-synuclein A53T). 7519 and 7515 dyes can be used for fluorometric detection of fibrillar insulin at concentrations of approximately 1.5–120 microg/ml. For meso-ethyl substituted dye 7514 the ability to form H- and J-aggregates upon interaction with insulin fibrils was suggested. The model of the H- and J-aggregate packing in the protein fibrillar structure has been proposed.

Keywords Cyanine dyes · Amyloid proteins · Fluorescent detection · J-aggregates

K. D. Volkova · V. B. Kovalska (✉) · M. Y. Losytskyy · K. O. Fal · S. M. Yarmoluk
Institute of Molecular Biology and Genetics,
National Academy of Sciences of Ukraine,
150 Zabolotnogo St.,
03143 Kyiv, Ukraine
e-mail: v.b.kovalska@imbg.org.ua

N. O. Derevyanko · Y. L. Slominskii · O. I. Tolmachov
Institute of Organic Chemistry,
National Academy of Sciences of Ukraine,
Murmans'ka Str. 5,
02094 Kyiv, Ukraine

Introduction

The abnormal self-assembly of proteinaceous material into insoluble well-ordered fibrillar aggregates deposits is common to a large group of amyloid-associated disorders, such as Alzheimer's disease, Parkinson's disease, prion diseases, and type II diabetes [1–5]. Amyloidogenic proteins undergo an alternative folding pathway under stressful conditions leading to formation of fibrils having cross beta-sheet structure [6, 7].

The protein involved in the amyloid fibrils is specific for each disease. Despite of the fact that various amyloid-forming proteins do not reveal any simple sequence homology, all amyloid formations possess similar ultra-structural and physiochemical properties. Such formations could be detected using specific dye Thioflavin T (Thio T), which strongly increased fluorescence intensity upon dye/fibrill interaction [8–10].

Today cyanine dyes are widely used in various biomedical applications as highly efficient fluorescent probes for detection of nucleic acids and proteins [11, 12] and labels for biomolecules [13]. For the laboratory use unsymmetric monomethine dye T-284 and trimethine dye SH-516 (Fig. 1) were proposed that gave strong fluorescence response upon dye/fibril complex formation and were effective in the same detection range as Thio T [14]. As the advantage of these dyes comparing with Thio T, the good results reproducibility shown by cyanines in amyloid fibrils detection assay should be noted. Later the ability of meso-substituted trimethine cyanines to give fluorescent response on the presence of fibrillar proteins was demonstrated; also it was shown that presence of amino substituent in the dye heterocycle increases the affinity of the dye to amyloid formations [15].

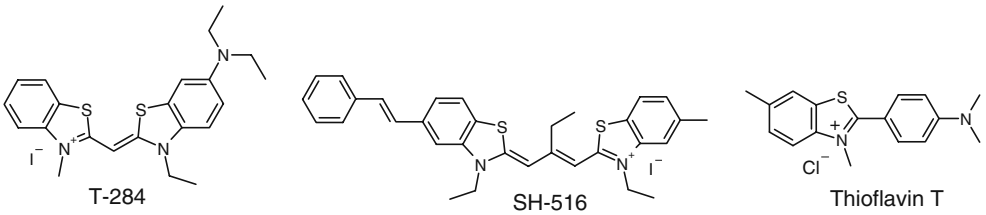

Fig. 1 Structure of cyanine dyes SH-516 and T-284, and Thioflavin T

Recently meso-methyl substituted trimethine cyanine dye 7519 (Fig. 2) containing methoxy substituents in 5,5′-positions of benzothiazole heterocycles was found to increase the fluorescence intensity significantly in presence of fibrillar insulin; the concentration range of fluorescent detection of fibrillar insulin with 7519 was estimated to be 1.5–120 μg/ml. This dye was also shown to be applicable for fluorescent detection of fibrillogenesis inhibition activity of potential inhibitors [16]. Hence, it is important to study the series of dyes related to 7519 in presence of fibrillar insulin in order to shed light on the influence of the dye structure on its binding to amyloid fibrils.

In present paper series of trimethine cyanines modified in 5,5′- or 6,6′- position with hydroxy- or methoxy-substituents is studied for their ability to interact selectively with fibrillar formations. Processes of dye aggregation that accompany this interaction are also investigated. For this purpose spectral-luminescent properties of the dyes in free form and in presence of monomeric and fibrillar insulin, which is chosen as model protein, are characterized. For the most efficient dyes, ability to bind with fibrillar protein of various amino acid compositions is estimated.

Experimental Section

Reagents

Anhydrous dimethylsulfoxide (DMSO) distilled under reduced pressure and 0.05 M Tris-HCl buffer (pH 7.9) were used as solvents. Methoxy- substituted trimethynecyanines 7514 and 7545 are synthesized similarly to their iodides [17]. For the synthesis of dyes 7513, 7519 and 7544 the same method was employed using ethylorthoformate or ethylorthoacetate instead of ethylorthopropionate. Hydroxy- substituted thiacarbocyanines 7520, 7523 and 7515 are obtained

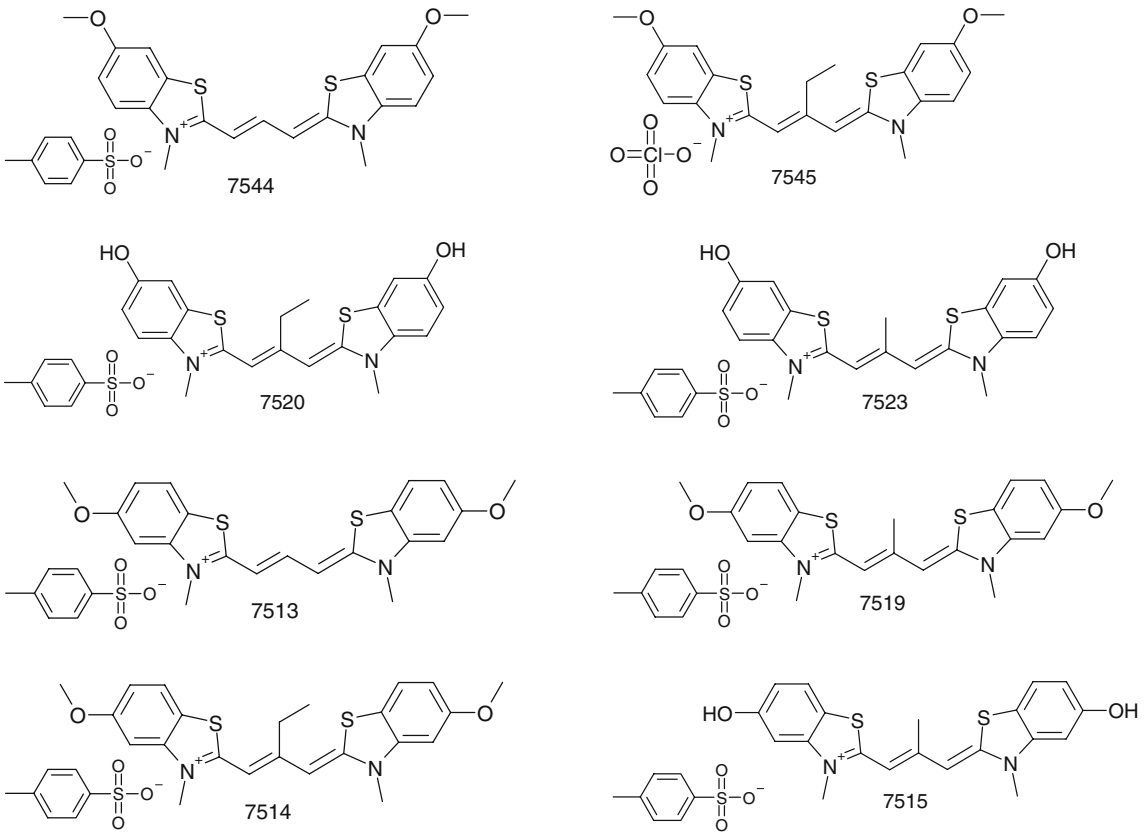

Fig. 2 Structures of studied trimethinecyanine dyes

starting from methyl p-toluensulfonate 6- (or 5)-hydroxybenzotiazole and triethyl esters of orthoacetic or orthopropionic acids like their N,N′-diethyl analogues unsubstituted in chromophore [18]. The structure and purity of the dyes are confirmed by the data of NMR ^{1}H spectra. ^{1}H NMR data of the dyes and their melting points are presented below.

Chicken egg white lysozyme, bovine pancreas insulin and β-lactoglobulin were purchased from Sigma (USA). The amyloid fibrils of lysozyme, bovine pancreas insulin and β-lactoglobulin were obtained as described [19, 20]. Recombinant human mutant α-synuclein (ASN) A53T (both fibrillar and monomer) was generously provided by Prof. V. Subramaniam (University of Twente, The Netherlands).

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 both native and fibrillar proteins in stock solutions were 10^{-4} M for ASN A53T; 1.7×10^{-4} M for insulin; 7.9×10^{-4} M for BLG and 10^{-3} M for lysozyme. The fibrils used in all experiments were from the same batch.

Preparation of Working Solutions

Working solutions of free dyes at 5×10^{-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 of an aliquot of the dye stock solution (2.5 μl) and an aliquot of native or fibrillar proteins in buffer. Concentrations of the proteins in working solutions were 10^{-6} M (ASN A53T, lysozyme and insulin) and 7.9×10^{-6} M (BLG) for both native and aggregated proteins. For the 7514 aggregation study, fibrillar insulin concentrations in working solutions were in the range of 0.85×10^{-6} M–5.1×10^{-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 Cary Eclipse fluorescence spectrophotometer (Varian, Australia). Fluorescence spectra were measured with excitation and emission slit widths set to 5 nm, and at a constant PMT voltage. 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 (except the study of 7514 aggregation study, for which the excitation wavelengths are provided in the text).

^{1}H NMR Spectra and Melting Points of Synthesized Dyes

6-Hydroxy-2-[(1E,3Z)-3-(6-Hydroxy-3-Methyl-1,3-Benzothiazol-2(3H)-ylidene)-2-Methylprop-1-enyl]-3-Methyl-1,3-Benzothiazol-3-ium p-toluensulfonate (7523)

DMSO-d_6: 2.281(s, 3H, CH_3), 2.521 (s, 3H, CH_3), 3.829 (s, 6H, N-CH_3), 6.307 (s, 2H, α-H chain), 7.014 (dd $_{J1=9.0\ Hz,\ J2=1.5\ Hz}$, 2H, Ar-H), 7.102 (d $_{J=8.1\ Hz}$, 2H, Ar-H), 7.396 (s, 1H, Ar-H), 7.481 (d $_{J=7.8\ Hz}$, 2H, Ar-H), 7.617 (d $_{J=9.0\ Hz}$, 2H, Ar-H), 9.993 (broadened s, 2H, OH). Melting Point 279–280 °C.

6-Methoxy-2-[(1E,3Z)-3-(6-Methoxy-3-Methyl-1,3-Benzothiazol-2(3H)-ylidene)prop-1-enyl]-3-Methyl-1,3-Benzothiazol-3-ium p-toluensulfonate (7544)

DMSO-d_6: 2.282 (s, 3H, CH_3), 3.721 (s, 6H, OCH_3), 3.753 (s, 6H, NCH_3), 6.346 (d $_{J=12.6\ Hz}$, 2H, α-H chain), 7.049 (d $_{J=9.3\ Hz}$, 2H, Ar-H), 7.110 (d $_{J=7.8\ Hz}$, 2H, Ar-H), 7.468 ($t_{J=12.8Hz}$, 1H, β-H chain), 7.468–7.588 (m, 6H, Ar-H). Melting Point 262–263 °C.

6-Methoxy-2-[(1E, 3Z)-3-(6-Methoxy-3-Methyl-1, 3-Benzothiazol-2(3H)-ylidene)-2-Ethyl-Prop-1-enyl]-3-Methyl-1, 3-Benzothiazol-3-ium Perchlorate (7545)

1.339(t, J=7.2 Hz, 3H, CH_3 (Et)), 2.882 (q J=6.9 Hz, 2H, CH_2 (Et)), 3.851 (s, 6H, OCH_3), 3.893 (s, 6H, NCH_3), 6.296 (s, 2H, α-H chain), 7.207 (d $_{J=9.0\ Hz}$, 2H, Ar-H), 7.675 (s, 2H, Ar-H), 7.746 (d $_{J=9.0\ Hz}$, 2H, Ar-H). Melting Point 246–247 °C.

6-Hydroxy-2-{(1E)-2-[(Z)-(6-Hydroxy-3-Methyl-1,3-Benzothiazol-2(3H)-ylidene)methyl]but-1-enyl}-3-Methyl-1,3-Benzothiazol-3-ium p-toluensulfonate (7520)

DMSO-d_6: 1.305 ($t_{J=6.9Hz}$, 3H, CH_3 (Et)), 2.277 (s, 3H, CH_3), 2.850 (q $_{J=7.2\ Hz}$, 2H, CH_2 (Et)), 3.840 (s, 6H, N-CH_3), 6.243–6.302 (d, 2H, α-H chain), 7.016 (d $_{J=8.7\ Hz}$, 2H, Ar-H), 7.103 (d $_{J=7.8\ Hz}$, 2H, Ar-H), 7.408 (s, 2H, Ar-H), 7.474 (d $_{J=7.8\ Hz}$, 2H, Ar-H), 7.630 (d $_{J=9.3\ Hz}$, 2H, Ar-H), 10.125 (broadened s, 2H, OH). Melting Point 267–268 °C.

5-Methoxy-2-[(1E,3Z)-3-(5-Methoxy-3-Methyl-1,3-Benzothiazol-2(3H)-ylidene)prop-1-enyl]-3-Methyl-1,3-Benzothiazol-3-ium p-toluensulfonate (7513)

DMSO-d_6: 2.282 (s, 3H, CH_3), 3.752 (s, 6H, OCH_3), 3.835 (s, 6H, NCH_3), 6.444 (d $_{J=12.6\ Hz}$, 2H, α-H chain), 6.967 (d $_{J=8.4\ Hz}$, 2H, Ar-H), 7.106 (d $_{J=8.1\ Hz}$, 2H, Ar-H), 7.209 (s, 2H, Ar-H), 7.477 (d $_{J=7.8\ Hz}$, 2H, Ar-H), 7.617

($t_{J=13.5\ Hz}$, 1H, β-H chain), 7.804 ($d_{J=8.7\ Hz}$, 2H, Ar-H). Melting Point 259–260 °C.

5-Methoxy-2-[(1E,3Z)-3-(5-Methoxy-3-Methyl-1,3-Benzothiazol-2(3H)-ylidene)-2-Methylprop-1-enyl]-3-Methyl-1,3-Benzothiazol-3-ium p-toluensulfonate (7519)

DMSO-d_6: 2.280 (s, 3H, CH_3), 2.540 (s, 3H, CH_3), 3.881 (s, 12H (6H N-CH_3+6H, OCH_3), 6.412 (s, 2H, α-H chain), 7.028 ($d_{J=8.7\ Hz}$, 2H, Ar-H), 7.103 ($d_{J=7.8\ Hz}$, 2H, Ar-H), 7.353 (s, 2H, Ar-H), 7.471 ($d_{J=8.4\ Hz}$, 2H, Ar-H), 7.908 ($d_{J=8.7\ Hz}$, 2H, Ar-H). Melting Point 246–247 °C.

5-Methoxy-2-{(1E)-2-[(Z)-(5-Methoxy-3-Methyl-1,3-Benzothiazol-2(3H)-ylidene)methyl]but-1-enyl}-3-Methyl-1,3-Benzothiazol-3-ium p-toluensulfonate (7514)

DMSO-d_6: 1.315 (s, 3H, CH_3), 2.277 ($t_{J=7.2\ Hz}$, 3H, CH_3 (Et)), 2.277 (s, 3H, CH_3), 2.875 ($q_{J=6.9\ Hz}$, 2H, CH_2 (Et)), 3.881 (s, 6H, OCH_3), 3.890 (s, 6H, N-CH_3), 6.336 (s, 2H, α-H chain), 7.027 ($dd_{J1=8.7,\ J2=2.4}$, 2H, Ar-H), 7.102 ($d_{J=8.1\ Hz}$, 2H, Ar-H), 7.366 ($d_{J=1.8\ Hz}$, 2H, Ar-H), 7.467 ($d_{J=8.4\ Hz}$, 2H, Ar-H), 7.911 ($d_{J=9.0\ Hz}$, 2H, Ar-H). Melting Point 150–151 °C.

5-Hydroxy-2-[(1E,3Z)-3-(5-Hydroxy-3-Methyl-1,3-Benzothiazol-2(3H)-ylidene)-2-Methylprop-1-enyl]-3-Methyl-1,3-Benzothiazol-3-ium p-toluensulfonate (7515)

DMSO-d_6: 2.278 (s, 3H, CH_3), 3.787 (s, 6H N-CH_3), 6.348 (s, 2H, α-H chain), 6.867 ($d_{J=9.0\ Hz}$, 2H, Ar-H), 7.103–7.121 (m (s+d), 4H, Ar-H), 7.480 ($d_{J=8.1\ Hz}$, 2H, Ar-H), 7.777 ($d_{J=9.0\ Hz}$, 2H, Ar-H), 10.230 (s, 2H, OH). Melting Point 276–277 °C.

Results and Discussion

Fluorescence Properties of the Dyes in Free Form and in Presence of Monomer Insulin

Spectral characteristics of the free trimethinecyanines in buffer and in presence of monomeric insulin are presented in Table 1. The dye Thio T that is commonly used for fluorescent detection of amyloid fibrils was taken as a reference dye.

For studied trimethine cyanine dyes maxima of excitation spectra were detected within the range from 558 to 573 nm, and fluorescence spectra maxima were situated between 574 and 588 nm. Values of Stokes shifts, observed for the dyes were small (between 9 and 19 nm) that is typical for trimethinecyanines. The low intrinsic emission values (I_0) were detected for the dyes containing substituents in the polymethine chain (2–20.4 a.u.). The intrinsic fluorescence intensities observed for the meso-unsubstituted trimethinecyanines 7544 and 7513 (91 and 60.3 a.u. respectively) were significantly higher than for their meso-substituted analogues. For Thio T the excitation and emission maxima were located at 410 and 472 nm, respectively; its intrinsic intensity was low (2.5.a.u).

Upon addition of the monomeric insulin to the solution, for the majority of dyes the slight shift of fluorescence excitation maxima (up to 3 nm) was observed. For the cyanines 7519, 7514 and 7515 excitation maxima positions did not shift. Positions of emission maxima in the presence of native insulin for almost all of the dyes were slightly shifted to the short-wavelength region (maximum shift was 5 nm for 7519), while for cyanine 7545 it remained unchanged. Stokes shifts for the studied dyes in momomeric insulin presence were in the similar range as for the free dyes buffer solution, namely between 8 and 17 nm.

The addition of native insulin resulted into the slight increasing of emission intensity for the dyes with hydroxyl substituents in heterocycles (7515, 7520, 7523). For other dyes, which contain methoxy substituents the noticeable decrease of fluorescence signal was observed. Especially such decrease (up to 3 times comparing with buffer solution) was pronounced for the meso-unsubstituted dye 7513. It should be noted that addition of monomer insulin slightly influenced fluorescent characteristics of Thio T.

Spectral Properties of the Dyes in the Presence of Fibrillar Insulin

Fluorescence characteristics of the cyanines in the presence of fibrillar insulin are presented in Table 1.

The addition of fibrillar insulin to the dye solutions resulted in a bathochromic shift of excitation maxima positions (16–25 nm) compared to those for free dyes. Emission maxima positions for the studied dyes in the presence of fibrillar insulin were red-shifted by 9–20 nm relatively to those in buffer. The wavelengths of excitation and emission maxima of Thio T were respectively 449 nm and 481 nm. The addition of fibrillar insulin to the trimethine cyanine dyes solutions led to the fluorescence intensity increase in ~1.3–43 times. The exception was meso-ethyl substituted dye 7514, containing methoxy groups in 5,5′-positions, which decreased fluorescence intensity in fibrillar insulin presence (I_f/I_0=0.74).

This was caused by strong aggregation processes of the dye, which occurred in the presence of fibrillar protein. The maximum corresponding to the dye aggregates was also manifested in the emission spectrum (as described in Section "Aggregation of the Dye 7514 in Presence of Fibrillar Insulin"). It should be also noted, that further increase of insulin fibrils concentration (up to 3.4 μM)

Table 1 Selected characteristics of fluorescence spectra of the dyes in free form, in presence of native and fibrillar insulin

Dye name	Free dye			In presence of native insulin			In presence of fibrillar insulin		
	λ_{ex}, nm	λ_{em}, nm	I_0, a.u	λ_{ex}, nm	λ_{em}, nm	I_m, a.u	λ_{ex}, nm	λ_{em}, nm	I_f, a.u
Thio T	410	472	2.5	410	473	2.7	449	481	40
7513	573	588	60.3	570	586	23.7	597	605	78.52
7514	567	576	13.4	567	575	8.7	590	594	10
								632*	6
7515	567	578	2	567	576	2.2	585	595	86
7519	562	581	5	562	576	3.5	580	590	129
7520	564	579	4.3	566	576	6	584	599	13.4
7523	558	574	3	556	573	4.1	583	593	27.4
7544	566	582	91	565	580	70	590	599	120
7545	566	578	20.4	565	578	15	582	594	43

λ_{ex}—excitation maximum wavelength, λ_{em}—emission maximum wavelength, I_0—intrinsic fluorescence intensity of the dye, I_m—emission intensity of dye in the presence of monomer protein, I_f,—emission intensity of dye in the presence of aggregated protein, a.u.—arbitrary units, *—aggregation band.

resulted in about 10-fold emission enhancement for 7514 dye. Another meso-ethyl substituted dye (7545) containing 6,6′-methoxy groups also demonstrated sharp intensity enhancement (from ~2 up to ~15 times) upon increase of fibrillar insulin concentration (from 1 μM up to 2 μM) (data are not presented).

At the same time, for the meso-ethyl substituted dye containing hydroxyl groups (7520) moderate emission intensity increase (I_f/I_0 up to ~3.1) was observed. In this case further increase of insulin concentration only slightly enhanced fluorescence intensity of the dye.

The high emission level in presence of fibrillar insulin was noticed for meso-unsibstituted cyanines 7544 and 7513. However, because of high intrinsic fluorescence of these dyes, the observed value of emission increasing was insignificant (I_f/I_0 ratio ~1.3).

It should be admitted that the highest emission increase in the presence of fibrillar insulin was observed for meso-methyl substituted dyes 7523, 7519 and 7515 (I_f/I_0 was respectively ~9.1, ~25.8 and ~43 times) (Fig. 3). The 5,5′-substituted cyanines 7519 and 7515 in complex with insulin demonstrated the top emission enhancement value (up to ~43 times) together with noticeable fluorescence intensity, which considerably exceeded that for Thio T.

Based on these data we can conclude that presence and structure of meso-substituent is critical for the ability of dye to increase emission in the presence of fibrillar insulin. Dyes with meso-methyl subsistent were selected for further determination of their specificity to fibrillar proteins of various amino acids composition. Besides, for obtaining of amyloid-sensitive dyes the incorporation of 5,5′- substituents seems to be more efficient compared with 6,6′-substituents.

Sensitivity of Dyes to the Fibrillar Proteins of Various Amino Acid Compositions

To study the ability of dyes to give fluorescence response in the presence of fibrillar proteins of various amino acid composition, such proteins as BLG, lysozyme and mutant ASN A53T in both monomer and fibrillar forms were used. Experiments were carried out for the meso-methyl-substituted dyes (7519, 7515 and 7523) and obtained results are presented in the Table 2. It was shown that dyes gave significant fluorescent response in presence of used amyloid proteins. Excitation and emission maxima of dyes in complexes with studied fibrillar proteins were close to these observed in presence of insulin.

For the 7519 dye, that contains methoxy groups in 5,5′-position, the lowest emission increasing upon addition of fibrillar proteins (in about 12 times) was observed in the presence of lysozyme (I_f=57 a.u.). Emission intensity values of this dye in complexes with fibrillar BLG and ASN A53T were higher (80 and 95 a.u. correspondingly). At the same time this dye slightly changed its emission in the presence of monomeric form of all proteins studied.

The dye 7515 containing hydroxyl groups in 5,5′-positions gave the same fluorescent response (intensity increase in about 11 times) when complexed with BLG and ASN A53T (38 and 36 a.u. correspondingly), while its emission in the presence of lysozyme was higher - about 52 a.u.(about 16 times). This dye slightly (in ~2.9 times) increased emission intensity upon addition of monomeric BLG, but it remained unchangeable in presence of monomeric lysozyme and ASN A53T.

Unexpectedly high level of emission was observed for the 6,6′-hydroxy substituted dye 7523 in complex with fibrillar BLG (134 a.u., emission increasing is about 48

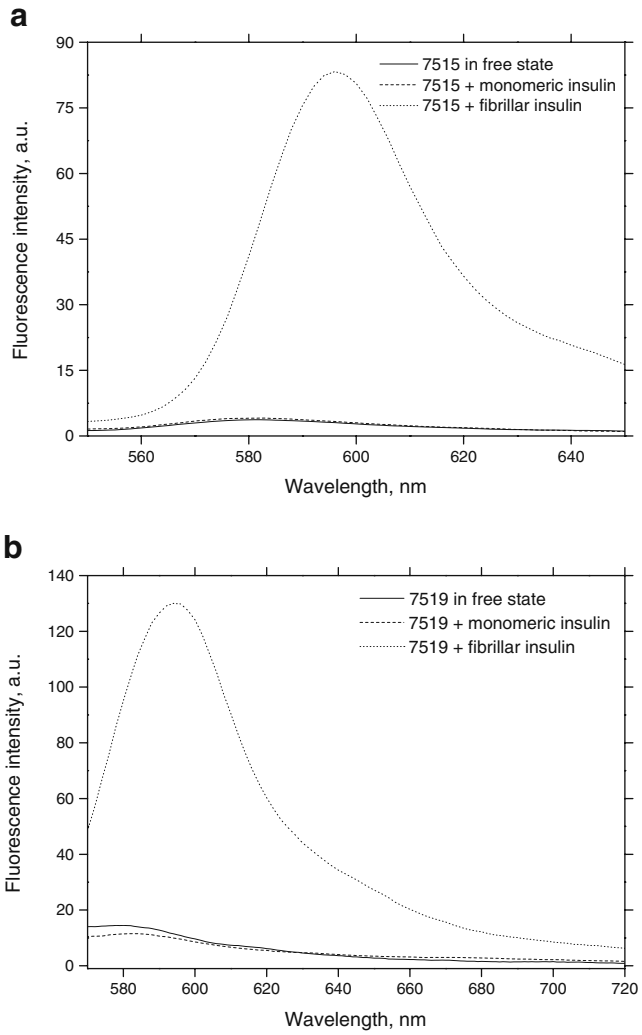

Fig. 3 Emission spectra of cyanine dyes 7515 (A) and 7519 (B) in TRIS-HCl buffer, pH=7.9 and in presence of monomeric and aggregated into fibrils insulin

times). In the presence of fibrillar lysozyme and ASN A53T the dye fluorescence was considerably lower (24.4 and 44 a.u. correspondingly). It should be mentioned that in presence of monomeric BLG noticeable (about in ~10 times) emission increase was observed, while addition of other monomeric proteins insignificantly changed fluorescence of the dye. Such essential emission enhancement both in the presence of fibrillar and monomeric BLG could be explained with the unspecific external binding of 7523 dye to this protein.

Determination of the Linear Range of Fibrillar Insulin Detection with the Dye 7519

For the trimethinecyanine 7519, estimation of the effective detection range of fibrillar insulin was described earlier [16]. Here the mentioned range was evaluated more carefully by titration of a 5 μM solution of the dye with increasing amounts of protein. Figure 4 shows the dependence of fluorescence intensity of the dye on the concentration of added fibrillar insulin. The linear range and detection limits for 7519 were calculated from the measured fluorescence intensities. The lower detection limit was considered to be the protein concentration, in presence of which fluorescence intensity of the dye increased two times as compared to free dye.

It was shown, that fluorescence intensity of the 7519 increased two times (up to 11 a.u.) in presence of fibrillar insulin in concentration of ~1.5 μg/ml. The maximum fluorescent intensity value for this dye (391 a.u.) was registered in the presence of ~120 μg/ml of fibrillar insulin. In such a way, use of 7519 dye allows quantitative detection of fibrillar insulin in the range of 1.5 μg/ml–120 μg/ml. The obtained detection limits are comparative to those for commercially available amyloid-sensitive dyes [21, 22].

Table 2 Fluorescent characteristics of dyes 7515, 7519 and 7523 in presence of various aminoacids composition proteins in monomer ([M]) and fibrillar ([F]) forms

Dye	Free dye			In presence of BLG				In presence of Lys				In presence of ASN A53T			
	λ_{ex}, nm	λ_{em}, nm	I, a.u	[F]			[M]	[F]			[M]	[F]			[M]
				λ_{ex}, nm	λ_{em}, nm	I_f, a.u	I_m, a.u	λ_{ex}, nm	λ_{em}, nm	I_f, a.u	I_m, a.u	λ_{ex}, nm	λ_{em}, nm	I_f, a.u	I_m, a.u
Thio T	408	470	2.2	447	476	22.3	5.3	440	480	8.2	3.1	451	480	34.7	2.7
7515	566	580	3.4	586	593	38	9.8	587	597	52	3.3	581	592	36	3.0
7519	560	578	4.6	583	591	79.7	4.9	584	593	57	4.6	581	591	95	3.8
7523	558	574	2.8	583	593	134	27	568	594	24.4	4.2	578	591	44	2.7

λ_{ex}—excitation maximum wavelength, λ_{em}—emission maximum wavelength, I_0—intrinsic fluorescence intensity of dye, I_m—emission intensity of dye in the presence of monomeric protein, I_f—emission intensity of dye in the presence of aggregated protein, a.u.—arbitrary units; BLG—β-lactoglobulin; Lys—lysozyme; ASN A53T—α-synuclein, mutant form A53T.

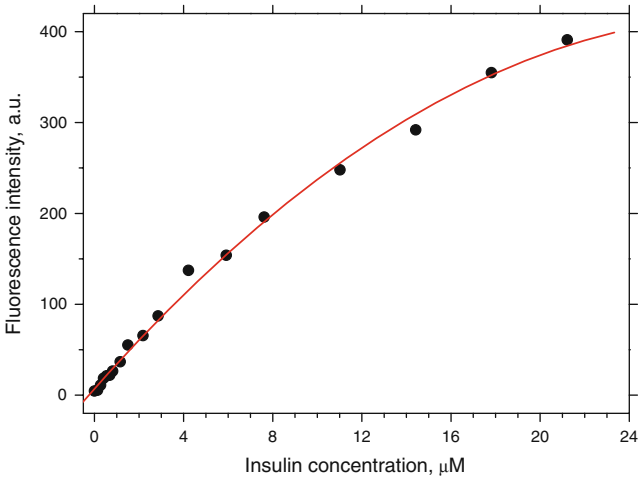

Fig. 4 Dependence of fluorescence intensity of cyanine 7519 (5×10^{-6} M) on the fibrillar insulin concentration (in TRIS-HCl buffer, pH=7.9)

Aggregation of the Dye 7514 in Presence of Fibrillar Insulin

As it was mentioned above, in the fluorescence spectra of the dye 7514 in presence of fibrillar insulin we registered the band shifted to the long-wavelength region relatively to that of monomer dye. It was supposed that this band could belong to the 7514 J-aggregates formed on the insulin fibrils. Thus the spectral properties of 7514 in presence of fibrillar insulin were studied in detail.

Absorption spectra of the dye in free form and in presence of different concentrations of fibrillar insulin are presented in Fig. 5. In the free dye spectrum, the band with maximum near 553 nm corresponding to the free dye monomer could be observed. Besides, bands near 450, 492 and 512 nm which are possibly connected with the dye H-aggregates were manifested.

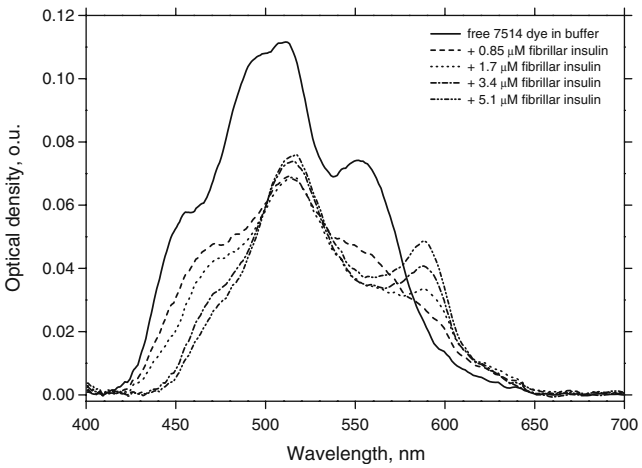

Fig. 5 Absorption spectra of 7514 (5×10^{-6} M) in free form in TRIS-HCl buffer, pH=7.9 and in presence of fibrillar insulin (0.85–5.1 μM)

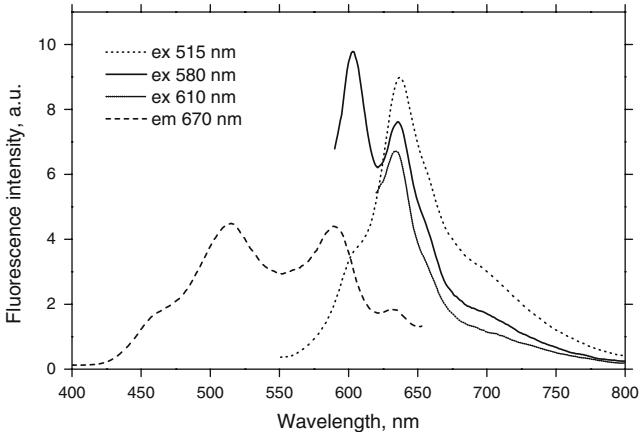

Fig. 6 Fluorescence emission (1–3) and excitation (4) spectra of 7514 (5×10^{-6} M) in presence of fibrillar insulin (1.7 μM) in TRIS-HCl buffer, pH=7.9. Fluorescence emission was excited at 515 nm, 580 nm and 610 nm, fluorescence excitation spectrum (dashed line) was registered for 670 nm emission

Upon fibrillar insulin addition, several changes occurred in the absorption spectrum. First, the free dye monomer maximum decreased, while the new maximum appears at 590 nm corresponding to the monomer dye/fibril complexes. Second, the H-aggregate bands intensities decrease is observed. While the short-wavelength H-bands decrease almost completely upon fibrils addition, the band at 515 nm is almost constant at the fibrils concentrations studied. Finally, the small shoulder near 630 nm appears upon addition of fibrillar insulin (Fig. 5). Its possible nature will be discussed below.

Fluorescence excitation and emission spectra of 7514 (5×10^{-6} M) in presence of 1.7 μM of fibrillar insulin are presented at Fig. 6. It could be seen that the spectra

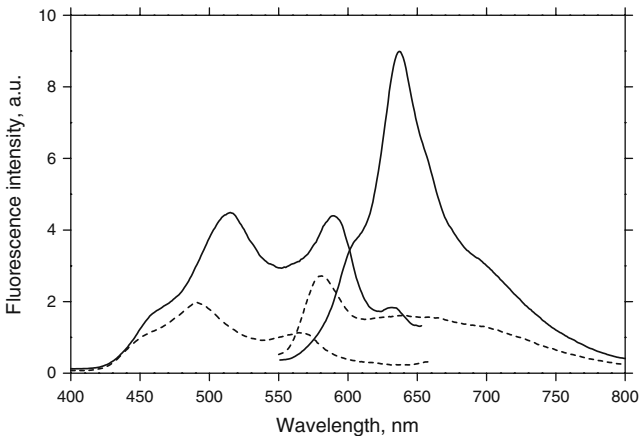

Fig. 7 Fluorescence excitation (*left*) and emission (*right*) spectra of 7514 (5×10^{-6} M) in free state in TRIS-HCl buffer, pH=7.9 (*dash*) and in presence of 1.7 μM of fibrillar insulin (*solid*). Fluorescence emission was excited at 515 nm, fluorescence excitation spectrum was registered for 670 nm emission

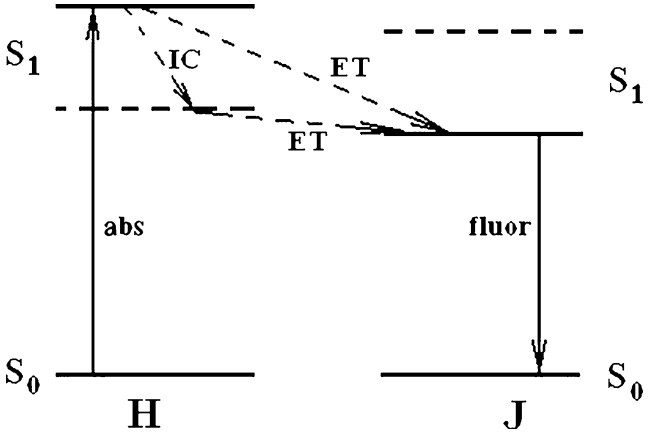

Fig. 8 Schematic representation of the excitation energy transfer from H-aggregates to J-aggregates. S_0 are the ground electronic energy levels, while S_1 are the excited levels that are split (absorption transitions are possible to the levels marked by solid lines and prohibited to these marked by dashed lines). Solid and dashed arrows are radiative (absorption (abs) and fluorescence (fl)) and non-radiative (energy transfer (ET) and internal conversion (IC)) transitions respectively. Dashed arrows show possible pathways of the excitation energy transfer from H- to J-aggregate

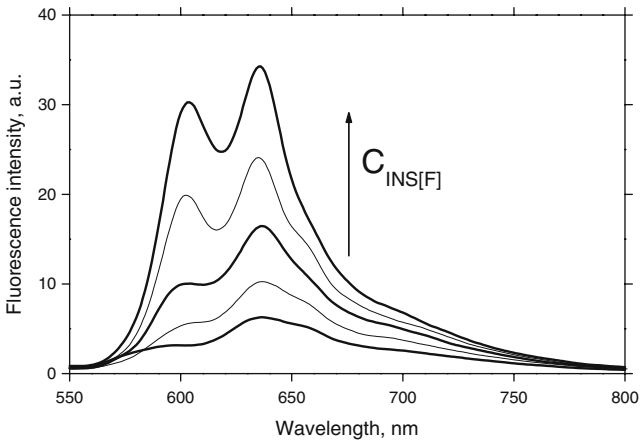

Fig. 9 Fluorescence emission spectra of 7514 (5×10^{-6} M) in presence of different fibrillar insulin concentration (0.85, 1.7, 3.4, 5.1 and 6.8 μM) excited at 515 nm (TRIS-HCl buffer, pH=7.9). Arrow points the insulin concentration increase

contained several bands. Among them, first of all, emission band at 603 nm should be named, which corresponded to the excitation band at 590 nm. These bands could be attributed to the emission of the monomer dye/fibrillar insulin complexes. Second, the excitation (633 nm) and emission (636 nm) maxima, which were not observed for the free dye solution, could be attributed to the dye J-aggregates formed on the insulin fibrils due to the narrow bands, long-wavelength position and small Stokes shift.

Third, the excitation maximum at 515 nm is also connected with the J-band at 636 nm. Besides, if we compare spectra excited at 515 nm and 610 nm (Fig. 6), we clearly see the long-wavelength emission near the 636-nm J-band in the former case. Thus the 515-nm excitation maximum corresponded at least partially to the non-intensive broad emission band near 700 nm. Such bands are characteristic for the fluorescence emission of the H-aggregates [23, 24]. It is seen from the Fig. 7 that the 515 nm excitation band is not manifested in the free dye spectrum. This leads us to the conclusion that the 7514 H-aggregates are formed on the insulin fibrils.

At the same time, as it was mentioned above the 515 nm maximum was manifested in the excitation spectrum of the 636 nm J-aggregate emission. This could be explained by the excitation energy transfer (FRET) from the dye H-aggregates formed on the fibrils to the J-aggregates (Fig. 8). The second possible explanation was that the 515 nm excitation band is the complicated one and includes, except the H-aggregate band, another allowed absorption transition of J-aggregates [25].

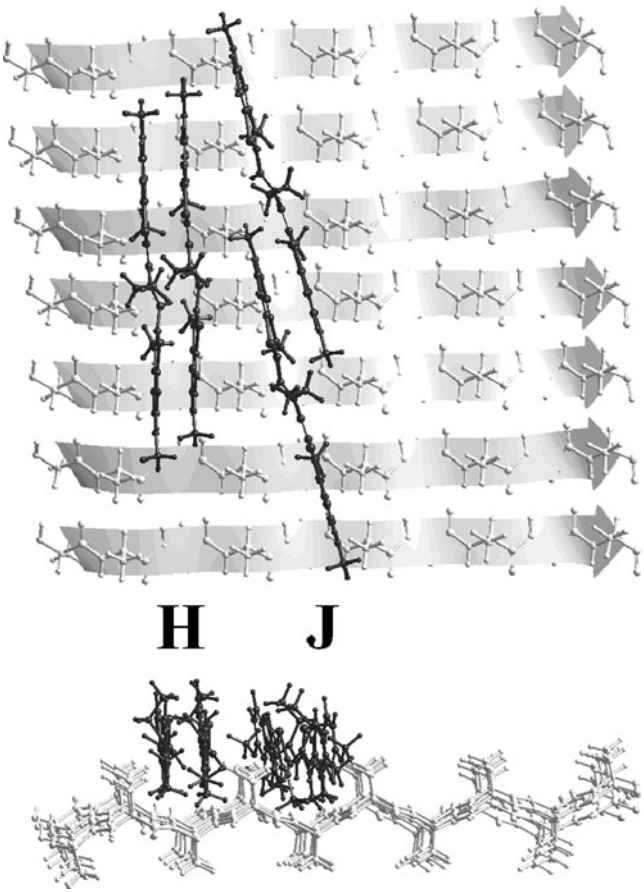

Fig. 10 Possible structures of H- and J-aggregates formed on the fibrillar insulin, built with the help of the HyperChem 8.0 program package, seen from various sides. Fibrillar structure was modeled as the parallel poly-alanine β-sheet. J-aggregate was built as a dimer for the demonstration of the aggregate structure, but it is supposed to consist of higher number of dye molecules

We have also compared the emission spectrum dependence on the increasing concentrations of fibrillar insulin (Fig. 9), that resulted in the enhancement of relative contribution of first the J-aggregate (at 636 nm) and than the bound monomer (at 603 nm) bands, while this of the H-aggregates decreased. Such behavior is consistent with our assumptions about the bands nature. We could suppose that the H-aggregate structure formed on the fibril was more compact than the J-aggregate one, since the H- and J-aggregates generally had the "card-pack" and "head-to-tail" structures respectively [26]. Thus the rather small protein-to-dye concentrations ratios were the most appropriate conditions for the dyes H-aggregate formation on the fibrils that was also supported by the absorption spectra (Fig. 5). Upon increasing the fibrillar insulin concentration, we suggested the dye molecules to form J-aggregates or to bind to the fibrils as monomers.

Based on the conclusion about the formation of both H-aggregate and J-aggregate structures of 7514 on the insulin fibrils, and an assumption of the energy transfer between them, we proposed the possible structure of these aggregates packing in the fibril (Fig. 10). It was supposed that the dyes binding to the fibril occurred in the groove of the beta-sheet [27]. Besides, since we have supposed that the excitation energy transfer takes place from the H- to the J-aggregates, the model was built so that the transition dipole moments of H- and J-aggregate structures were oriented in parallel one to another.

Conclusions

Series of benzothiazole trimethine cyanine dyes, containing methoxy or hydroxy substituents was studied by spectral-luminescent methods as possible fluorescent probes for detection of amyloid formations using insulin as model protein.

Dyes 7519 and 7515 in presence with fibrillar proteins demonstrated the highest emission increase (26 and 43 times approximately) and fluorescence intensity level that significantly overpasses that of Thio T. Besides they give strong fluorescent response on the presence of various aggregated proteins (lysozyme, BLG and ASN A53T). For dye 7519 detection range of fibrillar insulin was 1.5 μg/ml–120 μg/ml, which is comparable with those of commonly used dyes. Thus cyanines 7519 and 7515 could be proposed for detection and quantification of amyloid formations *in vitro*.

Meso-ethyl substituted dye 7514 forms fluorescent J-aggregates as well as low-intensive H-aggregates bound to the fibrillar structure of insulin. Excitation energy transfer from H-aggregates to J-aggregates was suggested. The model of the H- and J-aggregate packing in the protein fibrillar structure was proposed.

Acknowledgements This work was supported by a STCU-NASU grant №4936. We are grateful for Professor Vinod Subramaniam (University of Twente, the Netherlands) for providing monomeric and fibrillar alpha-synuclein mutant A53T.

References

1. Agorogiannis EI, Agorogiannis GI, Papadimitriou A, Hadjigeorgiou GM (2004) Protein misfolding in neurodegenerative diseases. Neuropathol Appl Neurobiol 30:215–224
2. Cohen FE (1999) Protein misfolding and prion diseases. J Mol Biol 293:313–320
3. Cohen FE, Kelly JW (2003) Therapeutic approaches to protein-misfolding diseases. Nature 426:905–909
4. Dobson CM (1999) Protein misfolding, evolution and disease. Trends Biochem Sci 24:329–332
5. Dobson CM (2003) Protein folding and misfolding. Nature 426:884–890
6. Harper JD, Lansbury PT (1997) Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. Annu Rev Biochem 66:385–407
7. McLaurin J, Yang DS, Yip CM, Fraser PE (2000) Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. J Struct Biol 130:259–270
8. Naiki H, Higuchi K, Hosokawa M, Takeda T (1989) Fluorometric determination of amyloid fibrils in vitro using the fluorescent dye, Thioflavin T1. Anal Biochem 177:244–249
9. LeVine H 3rd (1993) Thioflavine T interaction with synthetic Alzheimer's disease β-amyloid peptides: detection of amyloid aggregation in solution. Protein Sci 2:404–409
10. Foguel D, Suarez MC, Ferrao-Gonzales AD, Porto TC, Palmieri L, Einsiedler CM, Andrade LR, Lashuel HA, Lansbury PT, Kelly JW, Silva JL (2003) Dissociation of amyloid fibrils of α-synuclein and transthyretin by pressure reveals their reversible nature and the formation of water-excluded cavities. Proc Natl Acad Sci USA 100:9831–9836
11. Volkova KD, Kovalska VB, Yarmoluk SM (2007) Modern techniques for protein detection on polyacrylamide gels: problems arising from the use of dyes of undisclosed structures for scientific purposes. Biotech Histochem 82(4–5):201–208
12. Yarmoluk SM, Kovalska VB, Lukashov SS, Slominskii YL (1999) Interaction of cyanine dyes with nucleic acids. XII. β-substituted carbocyanines as possible fluorescent probes for nucleic acids detection. Bioorg Med Chem Lett 9(12):1677–1678
13. Haugland RP (2005) The handbook: A guide to fluorescent probes and labeling technologies, 10th edn. Eugene, OR
14. Volkova KD, Kovalska VB, Balanda AO, Vermeij RJ, Subramaniam V, Slominskii YuL, Yarmoluk SM (2007) Cyanine dye–protein interactions: looking for fluorescent probes for amyloid structures. J Biochem Biophys Meth 70:727–733
15. Volkova KD, Kovalska VB, Balanda AO, Slominskii YuL, Tolmachev OI, Subramaniam V, Yarmoluk SM (2008) Studies of mono- and trimethine cyanine dyes as specific fluorescent probes for fibrillar β-lactoglobulin detection. Ukr Bioorg Acta 6(2):15–21
16. Volkova KD, Kovalska VB, Inshin D, Slominskii YL, Tolmachev OI, Yarmoluk SM (2010) Novel fluorescent trimethine cyanine dye 7519 for amyloid fibril inhibition assay. Biotechnic & Histochemistry Early Online, 1–4
17. Levkoev II, Sveshnikov NN, Heifetz SA (1946) Research in area of cyanine dyes. Zh Obshch Khim 16(9):1489–1494

18. Kiprianov AI, Dashevskaya BI (1949) Cyanine dyes from isomeric 2-methylhydroxybenzotiazoles. Zh Obshch Khim 19 (6):1158–1166
19. Volkova KD, Kovalska VB, Segers-Nolten GM, Veldhuis G, Subramaniam V, Yarmoluk SM (2009) Explorations of the application of cyanine dyes for quantitative α-synuclein detection. Biotech Histochem 84(2):55–61
20. Arnaudov N, deVries R, Ippel H, vanMierlo CPM (2003) Multiple steps during the formation of β-lactoglobulin fibrils. Biomacromolecules 4:1614–1622
21. Crystal AS, Giasson BI, Crowe A, Kung MP, Zhuang ZP, Trojanowski JQ, Lee VMY (2003) A comparison of amyloid fibrillogenesis using the novel fluorescent compound K114. J Neurochem 86(6):1359–1368
22. Ahn JS, Lee JH, Kim JH, Paik SR (2007) Novel method for quantitative determination of amyloid fibrils of alpha-synuclein and amyloid beta/A4 protein by using resveratrol. Anal Biochem 367(2):259–265
23. Ogul'chansky TYu, Yashchuk VM, Losytskyy MYu, Kocheshev IO, Yarmoluk SM (2000) Interaction of cyanine dyes with nucleic acids. XVII. Towards an aggregation of cyanine dyes in solutions as a factor facilitating nucleic acid detection. Spectrochim Acta A 56(4):805–814
24. Ogul'chansky TYu, Losytskyy MYu, Kovalska VB, Yashchuk VM, Yarmoluk SM (2001) Interactions of cyanine dyes with nucleic acids. XXIV. Aggregation of monomethine cyanine dyes in presence of DNA and its manifestation in absorption and fluorescence spectra. Spectrochim Acta A Mol Biomol Spectrosc 57(7):1525–1532
25. Losytskyy MYu, Yashchuk VM, Lukashov SS, Yarmoluk SM (2002) Davydov splitting in spectra of cyanine dye J-aggregates, formed on the polynucleotide. J Fluoresc 12(1):109–112
26. Kasha M (1963) Energy transfer mechanisms and the molecular exciton model for molecular aggregates. Radiat Res 20:55–71
27. Krebs MRH, Bromley EHC, Donald AM (2005) The binding of thioflavin T to amyloid fibrils: localisation and implications. J Struct Biol 149:30–37