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INTERACTION OF NOVEL BENZANTHRONE DERIVATIVE WITH AMYLOID LYSOZYME

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A novel benzanthrone derivative AM18 was investigated with respect to its photophysical properties when bound to native, oligomeric and fibrillar hen egg white lysozyme. As shown by fluorimetric titration AM18 is more sensitive to pathogenic protein aggregates than Thioflavin T, however has no ability to differentiate between mature and immature lysozyme fibrils. The recovered affinity and fluorescence response of the novel probe to amyloid protein appeared to be similar to those of recently developed amyloid lysozyme-sensitive dyes like e. g. Nile Red and cyanine dye 7515. Despite the high increase of the probe emission in the presence of amyloid lysozyme compared to its fluorescence in buffer, the minimal amount that could be detected by 1 μM AM18 was 10 times lower for amyloid-native protein solutions due to high affinity of the dye for lysozyme monomers. In general, because of high quantum yields and "signal-to-noise" ratios in the presence of pathogenic protein aggregates AM18 appeared to be an effective tool for amyloid detection and characterization *in vitro*, being however unable to detect pathogenic protein aggregates *in vivo* like e.g. recently reported p-FTAA because of the sensitivity to lipids. Compared to previously reported AM3 a novel dye showed 2-fold lower "signal-to-noise" ratio in the presence of fibrillar lysozyme, and 2 fold lower blue shift of emission maximum. This tendency was explained in terms of decreased charge transfer from the donor to acceptor groupes of AM18 compared to AM3. Finally, as concluded from the comparison of AM18 and previously studied benzanthrone derivatives, the 5 nm – red edge excitation shift of AM18 is indicative of its possible binding to fibril "deep cavities", containing no water. High anisotropy values of amyloid-bound dye led us to conclusion that the enhanced fluorescence of the probe is associated with the decrease of the rotational motion of the amino-substitute about the benzanthrone unit. This is a sign of AM18 behaviour as a molecular rotor.

KEY WORDS: amyloid marker, affinity, dye, fluorescence, lysozyme, specificity.

ВЗАЄМОДІЯ ПОХІДНОЇ БЕНЗАНТРОНУ З АМІЛОЇДНИМ ЛІЗОЦИМОМ

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Досліджено фотофізичні властвості AM18, нової похідної бензантрону, при зв'язуванні з нативним, олігомерним та фібрилярним яєчним лізоцимом. За допомогою флуориметричного титрування показано, що AM18 більш чутливий до патогенних білкових агрегатів, ніж Тіофлавин Т, проте не може розрізняти зрілі та незрілі фібрили лізоциму. Водночас, отримані величини спорідненості та флуоресцентної відповіді нового зонду на присутність амілоїдного білу були одного порядку аналогічними параметрами нещодавно розроблених маркерів лізоциму, таких як, наприклад Нільський Червоний та ціаніновий барвник 7515. Незважаючи на чуттєве зростання флуоресценції зонду в присутності амілоїдного лізоциму відносно буферу, мінімальна кількість патогенних агрегатів, яку можно детектувати за допомогою 1 мкМ AM18, виявилася у 10 разів нижчою для розчину амілоїдного і нативного білку через високу спорідненість зонду до мономерів лізоциму. В цілому, внаслідок високих значень квантового виходу та відношень «сигнал – шум» у присутності патогенних білкових агрегатов, AM18 виявився ефективним інструментом для детектування та характеристики амілоїдів *in vitro*, проте нездатним виявляти

патогенні білкові агрегати *in vivo*, як наприклад p-FTAA, через чутливість до ліпідів. У порівнянні з дослідженим раніше зондом AM3 новий зонд показав у 2 раза менші значення відношення «сигнал – шум» та блакитного зсуву максимуму флуоресценції у присутності фібрилярного лізоциму. Це можна пояснити меншою ефективністю переносу заряду від донорної до акцепторної групи зонду AM18, порівняно з AM3. На основі порівняння барвника AM18 із раніше дослідженою серією бензантронових зондів зробили висновок про те, що 5 нм зсув максимуму флуоресценції зонду, що зумовлений ефектом «червоного зсуву», свідчить про можливе зв'язування AM18 у «глибоких канавках» фібрил, де немає молекул води. Високі значення анізотропії зв'язаного з амілоїдами зонда дозволяють зробити висновок про те, що зростання інтенсивності флуоресценції барвника пов'язано зі зменшенням обертального руху амінозамісника навколо бензантронового ядра, що дозволяє віднести AM18 до класу молекулярних роторів.
КЛЮЧОВІ СЛОВА: амілоїдний маркер, спорідненість, зонд, флуоресценція, лізоцим, специфічність.

ВЗАИМОДЕЙСТВИЕ ПРОИЗВОДНОГО БЕНЗАНТРОНА С АМИЛОИДНЫМ ЛИЗОЦИМОМ

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Исследованы фотофизические свойства AM18, нового производного бензантрона при связывании с нативным, олигомерным и фибриллярным яичным лизоцимом. С помощью флуориметрического титрования показано, что AM18 более чувствительный к патогенным белковым агрегатам, чем Тиофлавин Т, однако не может различать зрелые и незрелые фибриллы лизоцима. В то же время, полученные величины сродства и флуоресцентного ответа нового зонда на присутствие амилоидного белка оказались одного порядка со значениями недавно разработанных маркеров лизоцима как например, Нильский Красный и цианиновый краситель 7515. Несмотря на существенное возрастание флуоресценции зонда в присутствии амилоидного лизоцима относительно буфера, минимальное количество патогенных агрегатов, которое можно детектировать с помощью 1 мкМ зонда, оказалось в 10 раз ниже для раствора амилоида с нативным белком вследствие высокого сродства зонда к мономерам лизоцима. В целом, из-за высокого квантового выхода и отношения «сигнал – шум» в присутствии патогенных белковых агрегатов, AM18 оказался эффективным инструментом для детектирования и характеристики амилоидов *in vitro*, однако неспособным к обнаружению патогенных белковых агрегатов *in vivo*, как например p-FTAA, из-за чувствительности к липидам. По сравнению с исследованным ранее зондом AM3 новый зонд показал в 2 раза меньшие значения отношения «сигнал – шум» и голубого сдвига максимума в присутствии фибриллярного лизоцима. Это было объяснено меньшей эффективонстью величины переноса заряда от донорной к акцепторной группе зонда AM18 по сравнению с AM3. Наконец, из сравнения красителя AM18 и ранее изученной серии бензантроновых зондов сделан вывод о том, что 5 нм сдвиг максимума флуоресценции зонда, обусловленный эффектом «красного края», свидетельствует о возможном связывании AM18 в «глубоких канавках» фибрилл, где нет молекул воды. Высокие значения анизотропии связанного с амилоидами зонда позволяют сделать вывод о том, что увеличение интенсивности флуоресценции красителя связано с уменьшением вращательного движения аминозаместителя вокруг бензантронового ядра, что позволяет отнести AM18 к классу молекулярных роторов.
КЛЮЧЕВЫЕ СЛОВА: амилоидный маркер, сродство, зонд, флуоресценция, лизоцим, специфичность.

During the past decades fluorescent molecules that specifically target highly ordered fibrillar protein aggregates (amyloids) have found vast application in amyloid research area. The use of fluorescence spectroscopy for studying fibril structure and kinetics of amyloid formation may essentially help in developing new strategies for inhibition of protein aggregation. For instance, using the classical amyloid marker Thioflavin T, Nielsen and coworkers showed that the lag phase of the insulin fibrillization becomes longer with the decrease of the initial concentration of oligomeric seeds, added to the protein solution [1]. Mishra et al. employed the Nile Red fluorescent dye as the reporter molecule to demonstrate

that the lag phase of lysozyme fibrillization decreases with increasing the protein concentration [2]. Additionally, the typical sigmoidal dependencies of the fluorescence of amyloid specific probe on the time of protein incubation may be regarded as unambiguous proof of protein fibrillization with a certain nucleation time [3-5]. Despite considerable progress in this field, the development of novel fluorescent probes that display higher sensitivity to pathogenic protein aggregates compared to traditionally used fluorophores may provide new tools for uncovering the mechanisms of amyloid formation and toxicity. In addition to *in vitro* detection of pathogenic protein aggregates which is of great importance for medical diagnostics, extensive research efforts are focused on designing the amyloid imaging agents. Particularly, Makwana et al. used Coumarin 6 at nanomolar concentrations to give the characteristic of the bovine carbonic anhydrase fibrils [5] while Volkova and coworkers developed the cyanine dye T-284 showing the 80 times fluorescence intensity increase upon the addition of fibrillar α-synuclein [6]. However, despite the great number of the novel dyes, the ideal amyloid sensitive probe is to be found.

In this work, we explored the ability of the benzanthrone derivative AM18 to act as noncovalent marker for aggregated forms of hen egg white lysozyme (HEWL), whose mutant human analogue is associated with hereditary systemic amyloidosis. Remarkable spectral characterisitics of AM18, such as low fluorescence in buffer and high affinity for lipids allowed us to recommend this dye as an effective amyloid imaging agent. An expanded knowledge on amyloid formation of HEWL has been obtained with the help of Thioflavin T (ThT), Nile Red, Michler's Hydrol Blue (MHB), 1-anilinonaphtalene-8-sulfonate (ANS), cyanine probe 7515 (Fig. 1). We used the above probes (especially ThT) as reference to evaluate the sensitivity of the novel fluorophore to amyloid HEWL.

MATERIALS AND METHODS

Chicken egg white lysozyme (HEWL) was purchased from Sigma (St. Louis, MO, USA). The fluorescent probe AM18 (Fig. 1) was synthesized at the Faculty of Natural Sciences and Mathematics of Daugavpils University, Latvia. Protein solution (3 mg/ml) was prepared as described previously [7]. The 8-day-old oligomers were withdrawn from the cuvette to study AM18 specificity to these aggregates. The formation of HEWL fibrils was observed over a time course of 30 days. The amyloid nature of fibrillar aggregates was confirmed in ThT assay. Native lysozyme solutions (3 mg/ml) in sodium phosphate buffer (pH 7.4) were also used for the fluorimetric titration. Steady-state fluorescence spectra were recorded with LS-55 spectrofluorimeter equipped with a magnetically stirred, thermostated cuvette holder (Perkin–Elmer Ltd., Beaconsfield, UK). Fluorescence measurements were performed at room temperature using 10 mm path-length quartz cuvettes. Emission spectra of AM18 were recorded in buffer and in the presence of native, fibrillar and prefibrillar lysozyme setting excitation wavelength at 470 nm. The emission maxima of the dye fluorescence in the presence of HEWL were around 565 nm. Fluorescence anisotropy measurements were conducted as described previously, setting the excitation and emission wavelengths at 470 and 565 nm [7]. All spectra were corrected for dilution effect. Quantitative characteristics of the fluorophores binding to lysozyme were calculated in terms of Langmuir adsorption model by analyzing the protein-induced changes in the probe fluorescence intensity (ΔI) at the wavelengths, corresponding to the dye emission maximum (~565 and ~478 nm for AM18 and ThT, respectively). Approximation of the experimental dependencies $\Delta I(C_P)$ (C_P and Z_0 are protein and dye concentrations, respectively) by the equation (1), allowed us to determine the dye-protein binding parameters (K_a – association constant, n – binding stoichiometry, a – molar fluorescence).

$$\Delta I = 0.5a\left[Z_0 + nC_P + 1/K_a - \sqrt{(Z_0 + nC_P + 1/K_a)^2 - 4nC_P Z_0} \right] \quad (1)$$

The quantum yield calculations were performed using the equation [8]:

$$Q = \frac{Q_S(1-10^{-A_S})S_P}{(1-10^{-A_P})S_S} \quad (2)$$

where Q_s is the quantum yield of a standard dye (Rhodamine 101) (equals 1 at the excitation of 450-465 nm), S_P and S_S are the integrated areas of the fluorescence spectra of dye and standard, respectively, A_P and A_S are absorbances at the dye and standard excitation wavelengths, respectively. Absorption measurements were performed with SF-46 spectrophotometer in 2 mm quartz cuvettes.

RESULTS AND DISCUSSION

AM18 is a small hydrophobic molecule, which shares similar structure with ABM, previously developed by Kirilova and coworkers for studying conformational changes of blood plasma albumin [9,10]. Each of these two dyes consists of benzanthrone unit as electron spacer and carbonyl group as electron acceptor, differing only by the electron donor moieties. Such conjugated system determines the dye sensitivity to the environmental polarity, i.e. their low fluorescence in aqueous solutions and enhanced emission in nonpolar media.

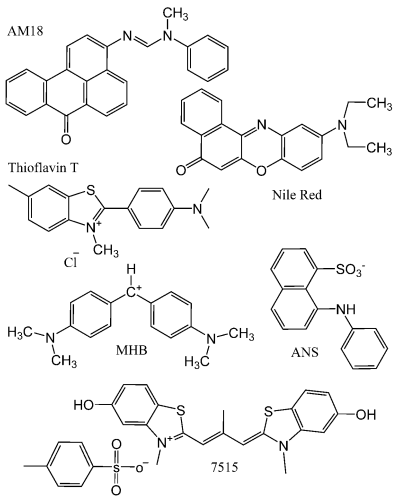

Fig.1. AM18 and the extrinsic fluorescent dyes: Thioflavin T, Nile Red, Michler's Hydrol Blue (MHB), 1-anilinonaphtalene-8-sulfonate (ANS) and cyanine probe 7515 used in recent studies for amyloid lysozyme detection and characterization

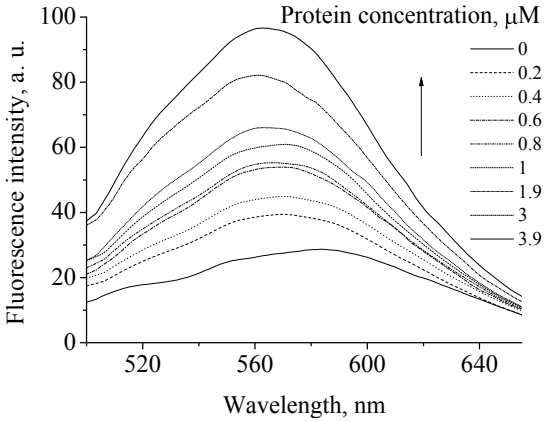

Fig. 2. AM18 fluorescence emission spectra in presence of native HEWL. The dye concentration was 4.5 μM.

As seen in Fig. 2, addition of the monomeric HEWL to AM18 solution in buffer leads to the fluorescence increase indicating the dye binding to native protein. The same tendency is observed for AM18 associated with oligomeric (Fig. 3) and fibrillar HEWL (Fig. 4), but with higher fluorescence increases. In general, amyloid-sensitive fluorophores, e.g. cyanine dye 7515 or ANS (Fig. 1) interact poorly with protein monomers, showing no or very weak fluorescence changes [3,11]. As illustrated in Fig. 5, the emission intensity of fibril-bound dye at the wavelength of 565 nm, corresponding to fluorescence maximum, turned out to be ~1.3 and ~12 times higher than that of oligomer-bound and free probe, respectively. Altogether,

higher fluorescence response was observed for amyloid-bound AM18 compared to the probe bound to monomeric lysozyme.

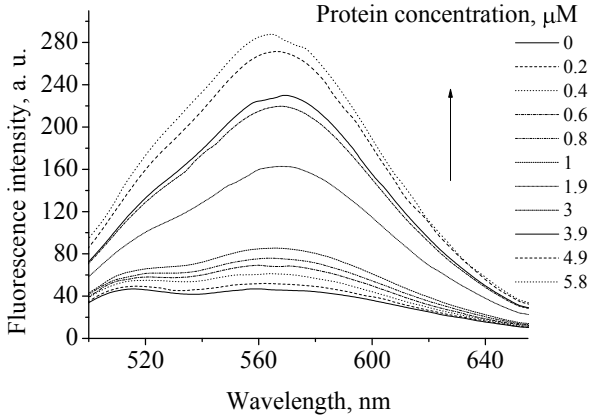

Fig. 3. AM18 emission spectra in presence of oligomeric HEWL. The dye concentration was 4.5 μM.

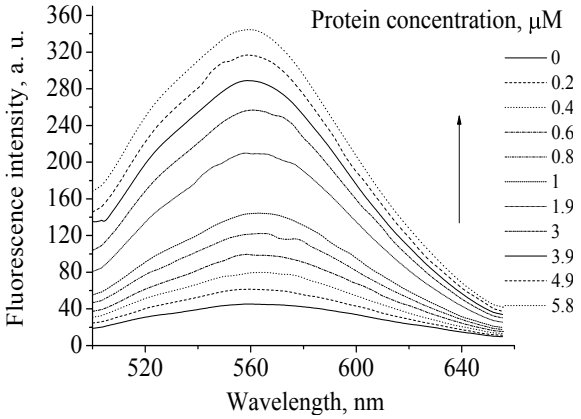

Fig. 4. AM18 emission spectra in presence of fibrillar HEWL. The dye concentration was 4.5 μM.

The dye isotherms (Fig. 6) derived from the probe titration with HEWL were approximated by the Eq. (1), thus yielding the dye-protein binding parameters (Table 1). The same was done for ThT binding curves (Table 2). Analysis of the dye affinities (K_a) revealed an order lower association constants of ThT compared to AM18, in contrast to the similarity of the affinities of AM18 and fibril-specific Nile Red [12]. It should be pointed out that despite different pH of the Nile Red (pH = 1.6) and AM18 (pH = 7.4) assays with amyloid HEWL, used for their K_a determination, the comparison of the K_a values is possible because of a slight pH-dependence of Nile Red fluorescence [12].

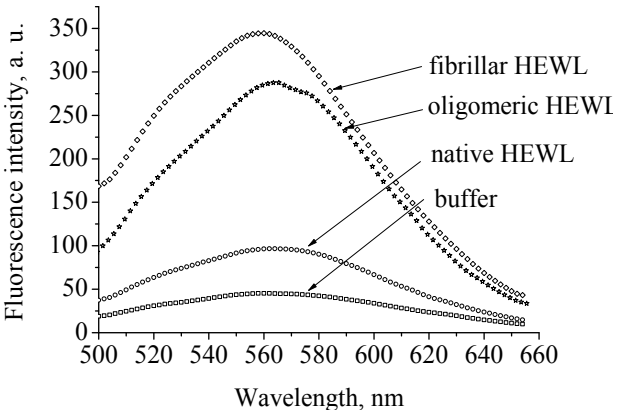

Fig. 5. AM18 emission spectra in buffer and in presence of native, oligomeric and fibrillar HEWL. The dye concentration was 4.5 μM. Protein concentration was 5.8 μM for oligomeric and fibrillar HEWL, and 3.9 μM – for native HEWL.

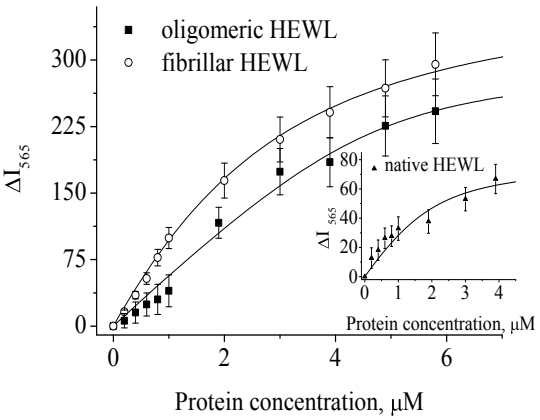

Fig. 6. Binding curves of AM18 to native, oligomeric and fibrillar HEWL. The dye concentration was 4.5 μM.

By comparing the values of molar fluorescence (a), proportional to the dye quantum yield, we concluded that AM18 displayed higher quantum yield in the presence of fibrillar HEWL, than ThT. Additionally, fibril-bound AM18 showed ~ 3 times lower binding stoichiometry (n) than classical amyloid marker. This indicates that despite close molecular dimensions of neutral dye AM18 (1.6×0.7 nm) and positive by charged ThT (1.5×0.6 nm) the dye charge plays an important role in the determining of the protein binding affinity. Regarding to the

different values of AM18 binding parameters in the presence of native, oligomeric and fibrillar HEWL, the dye affinity for monomeric protein appeared to be ~ 3 times higher than that for amyloid HEWL. Hence, despite the probable existence of specific dye-monomeric protein interaction (because of high K_a), accompanied by a slight fluorescence enhancement, AM18 displayed higher sensitivity to oligomeric and fibrillar HEWL due to ~3 – 5 higher molar fluorescence values (Table 1).

Table 1. Parameters of the AM18 complexation with 3 forms of HEWL

HEWL type	Ka, μM^{-1}	a, μM^{-1}	n, mol/mol	Q	I/I0	I/(I0+Inat)	Emission shift, nm	Anisotropy
native	0.6±0.1	18±5	2.5±1	0.069	3.2	-	0	*
oligomeric	0.22±0.05	130±35	0.9±0.3	0.187	5.4	1.4	0	0.262±0.003
fibrillar	0.2±0.03	96±22	2.4±0. 4	0.207	5.5	1.9	-2 nm	0.286±0.003

The recovered binding parameters seem to be very useful for comparison of the amyloid specificities of fluorescent dyes through calculating the fluorescence intensity and amount of amyloid-bound probe (B, changing from 0 to 1) at different dye and protein concentrations (Eq. (1)).

Table 2. Parameters of the ThT complexation with lysozyme

Protein type	Ka, μM^{-1}	a, μM^{-1}	n, mol/mol	Q	I/I0	I/(I0+Inat)	Emission shift, nm
native	0.03±0.01	7.1±1.8	10.3±3.4	0.009	1.3	-	0
fibrillar	0.04±0.01	22±5	7.6±2.1	0.03	2.8	0.87	0

Using the B values, that correspond to AM18 (ThT) and HEWL concentrations at the last titration point, we corrected the quantum yield values (Q), putting into Eq. (2) $A_P \cdot B$ instead of A_P, i.e. the absorbance of amyloid-bound dye in solution instead of that for total probe concentration. As seen from Tables 1 and 2, for fibril- and native-bound AM18 Q values turned out to be ~ 7 times higher than corresponding parameters for ThT with Q of fibril-bound dye being ~ 3 times higher than that of native-bound one. However, despite similar quantum yields of these two probes, bound to native protein, the Q value of fibril-bound AM18 was 10 times higher than that of free dye, but the Q increase of ThT equaled 4. Thus, AM18 showed higher absolute and relative Q values in the presence of fibrillar HEWL, than ThT. On the other hand, the Q values of fibril- and oligomeric-bound AM18 were similar, indicating that the novel dye is capable of detecting both types of pathogenic protein aggregates. Furthermore, the 3-fold Q increase of the amyloid-bound probe, compared to native-bound one, gave us the confidence to easy amyloid detection, not limited by the presence of monomeric protein. However AM18 cannot distinguish between oligomeric and fibrillar HEWL neither by Q values like e.g. ANS, showing much higher fluorescence intensity in the presence of bovine carbonic anhydrase II molten globule than that in presence of fibrillar HEWL; nor by emission maxima changes like e.g. MHB, whose excitation and emission maxima in the presence of fibrillar HEWL are shifted to shorter wavelengths

compared to those in the presence of insulin ones [12,13]. This is because of very small blue shift (~ 2 nm) observed for fibril-bound dye (Table 1), compared to oligomeric-bound one, that along with close *Q* values complicates the independent detection of two types of pathogenic HEWL aggregates.

To characterize the dye specificity to amyloid fibrils one should know the minimal protein concentration, which probe is able to detect. For this reason the fluorescence intensities of 1 μM fibril-bound AM18 and ThT were calculated (at 565 and 478 nm, respectively, corresponding to the dyes' fluorescence maxima), setting the concentration of HEWL at 0, 0.5, 1.5 and 15 μM. Next, the "signal-to-noise" ratios, i.e. the ratios of fibril-bound dye fluorescence (I) to that of free dye (I_0), were determined. It should be mentioned here that the fluorescence intensity of 1 μM of free AM18 at 565 nm could be easy calculated as a ratio of the fluorescence intensity of 4.5 μM dye (that was obtained experimentally) to 4.5, because of the proportionality between the dye concentration and its fluorescence. As shown in Fig. 7, if the lower detection limit (first detection limit) is considered to be the protein concentration, in the presence of which fluorescence intensity of the dye increased *two* times as compared to free dye, it appears to be ~ 0.5 and ~ 1.5 μM for AM18 and ThT, respectively, i.e. the lowest HEWL amount, that could be detected by AM18 is ~ 3 times higher than that detected by ThT.

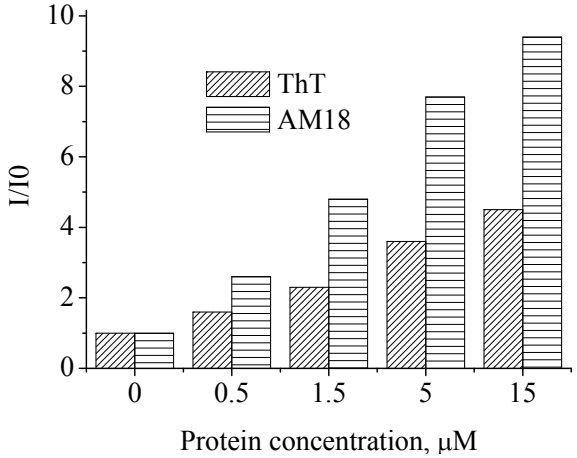

Fig. 7. The bar graph of Thioflavin T and AM18 signal-to-noise ratios at the emission wavelength 565 nm. I and I0 are the fluorescences of fibril-bound dye and free dye in buffer, respectively. The concentration of each dye was 1 μM.

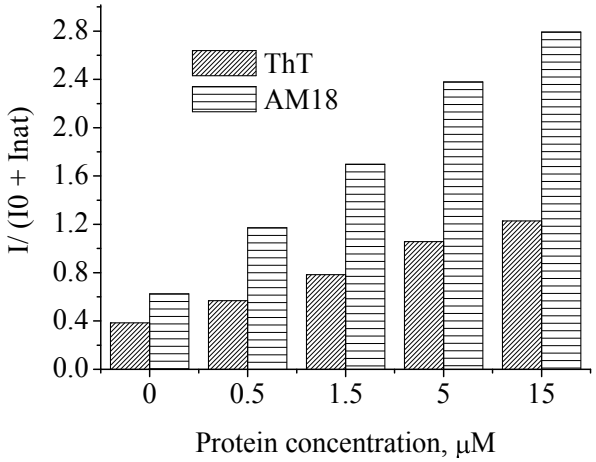

Fig. 8. The bar graph of Thioflavin T and AM18 signal-to-noise ratios at the emission wavelength 565 nm. I and Inat are the fluorescence intensities of fibril- and oligomer-bound dye, respectively. I0 corresponds to the fluorescence of the free dye in buffer. The concentration of each dye was 1 μM.

Moreover, the detection limit corresponding to the protein concentration, in presence of which fluorescence intensity of the dye increased *four* times (for higher data reliability) as compared to free dye (second detection limit), only the 1.5 and 15 μM HEWL amounts could be detected by AM18 and ThT, respectively. In the last case the novel amino-benzanthrone displays an order higher sensitivity to fibrillar HEWL. In addition to the above parameters other "signal-to-noise" ratios are represented in Fig. 8, where "signal" is the fluorescence intensity of fibril-bound AM18 (I) and ThT, and the "noise" was taken as the sum of the free dye signal (I_0) and the fluorescence of the native-bound probe. Obviously, significant decrease of the second "signal-to-noise" ratio is observed compared to the first one. This is because of AM18 and ThT specificity to monomeric HEWL, which affects the relative *Q* values. According to the above estimations, the minimal fibrillar HEWL concentration which

could be detected by AM18 and ThT is 5 and over 15 μM, respectively. Similar estimations of the detection limits were done by Volkova and Qin, who showed that fluorescence intensity of the cyanine dye 7519 (5 μM) increased two times in the presence of fibrillar insulin (1.5 μg/ml) and the fluorescence intensity of the ThT dimer diThT-PEG2 (5 μM) enhanced two times in the presence of the same amount of amyloid beta peptide [11,14]. We attribute the obtained results to higher sensitivity of AM18 to fibrillar HEWL compared with ThT. It should be pointed out that I/I_0 and $I/(I_0+I_{nat})$ estimaties (these values represented in the Tables 1 and 2 correspond to 1 and 2 μM dye and protein concentration, respectively) along with *Q* values seem to be the most effective parameters, characterizing the dye sensitivity to amyloid protein. Whereas the first value represents the dye fluorescence response to the addition of pathogenic protein aggregates, the second one shows whether/ how much amyloid detection is limited by the presence of the monomeric protein in the sample, and the third parameter indicates the absolute value of the dye fluorescence increase in the presence of amyloid protein. Additionally, *Q* value is important in the cases, when very low fluorescence of free dye along with low fluorescence in the presence of amyloid aggregates (which could lead to mistakes in comparing the relative *Q* values of the dyes) give high relative fluorescence responses. At the same time low *Q* is indicative of the weak probe sensitivity to amyloid protein. Simultaneously, the above binding parameters (K_a, a, n) serve as the tools for revealing more useful quantities as well as for characterizing the amyloid binding sites for the novel dye. As seen from the Tables 1 and 2, I/I_0 and $I/(I_0+I_{nat})$ of fibril-bound AM18 appeared to be ~ 2 times higher than corresponding values of ThT, and similar to those of oligomer-bound AM18, confirming again the inability of the aminobenzanthrone dye to distinguish between mature and immature fibrils.

Importantly, knowledge, gained from the study of AM18 and HEWL interactions may be useful for *in vitro* amyloid detection and characterization by the novel 3-amino substituted benzanthrone dye. However, in contrast to e.g. tiophene derivative p-FTAA, which was shown to cross the mouse blood-brain barrier and stain the amyloid beta protein deposits, AM18 cannot detect amyloid plaques *in vivo* because of its high sensitivity to lipids (similar to other amyloid markers like ThT) [15]. In spite of this drawback novel fluorophore proved proven to be more sensitive to amyloid HEWL than ThT and therefore it can serve as practical research tool for studying protein aggregation diseases.

As AM18 turned out to be a potential amyloid detecto, characterization of its protein binding sites seems to be essential for amyloid HEWL investigations with this benzanthrone derivative. Because of the different n and charge values it is unlikely that AM18 binds to the same fibril sites, as ThT, i.e. inserts into the same "binding channels" running along the fibril axis [16]. One way to characterize the polarity of the protein binding sites is to determine the dye emission maxima in solvents with different polarities and to compare the emission maximum position of the amyloid-bound probe with the calibration curve. For instance, this was done by Mishra and coworkers, who proved the fibrillar HEWL binding sites for Nile Red to have the dielectric constant ~ 10 [12]. In our study we used simplified approach, just comparing the emission maxima of the free and amyloid-bound AM18. However, despite high *Q* and "signal-to-noise" ratios, the dye displayed low sensitivity to the environmental polarity showing 2 nm blue shift in the presence of fibrillar HEWL and no response to the addition of oligomeric protein (Table 1). This is opposite to the behavior of another amino-derivative of benzanthrone – AM3, showed 8 nm blue shift or decrease of the micro-environmental polarity in the presence of fibrillar HEWL [7]. Lower AM18 sensitivity to the polarity changes which is also displayeded in 2 times lower I/I_0 value compared to AM3 could be explained by the decreased charge transfer from the donor to acceptor groupes. The

latter is probably related to lower electron donating power of the benzanthrone amino-substituent in 3C-position, corresponding to AM18 dye, compared to that of AM3 [17]. By comparing the red edge excitation shift values (REES) of AM18 and previously investigated series of amino-benzanthrone dyes we concluded that AM18 can be be localized on fibril "dry interface", or in deep cavities, containing no water, thus resulting in low REES ~ 5 nm [7].

Finally, high anisotropy values of amyloid-bound AM18, which were also used for the characterization of protein aggregation, allowed us the possibility to draw a conclusion that the enhanced fluorescence of the probe is associated with the decrease of the rotational motion of the amino-substitute about the benzanthrone unit [18]. This is indicative of AM18 behavior as a molecular rotor. Besides, the anisotropy of amyloid-bound dye (Table 1), appeared to be 9 % higher in the presence of fibrillar protein compared to oligomeric one, ppointing to higher rotational restrictions of fibril-bound dye [18]. Interestingly, the anisotropy of AM18 in 30-day-old HEWL solution in 80% of EtOH, which was incubated without agitation and was used as a control, reached 0.265, the value similar to that of the dye bound to 8-day-old oligomers (0.263). Moreover, the *Q* values of the probe in the presence of oligomer and control sample were very close (the difference was 20%). This fact reflects the existence of fibril growth under denaturing conditions without agitation, but this process is slowed down because of the decreased probability of collisions between protein molecules [7].

CONCLUSIONS

To conclude, novel fluorescent dye AM18 proved to be a good alternative to ThT for *in vitro* amyloid detection, displaying sensitivity to mature as well as immature fibrils and higher "signal-to-noise ratio" compared to classical marker ThT. Interestingly, despite different structure of AM18 and the reference amyloid markers, their affinities and fluorescence responses to amyloid HEWL appeared to be of the same order (except ThT), which is indicative of a small morphology difference between amyloid fibrils prepared at acidic (for the reference dyes) and neutral (for AM18) pH. At the same time, examination of the pH dependence of the fluorescence of amyloid-bound AM18 and its sensitivity to pathogenic aggregates of different proteins may help to answer the question about the versatility of the dye application. Finally, such attractive properties of amyloid markers as an ability to distinguish fibrils and oligomers as well as very low fluorescence in buffer, are expected to be found among the benzanthrones with other amino-substitutes than that of AM18 in our on-going experiments.

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