МЕТОДИ БІОФІЗИЧНИХ ДОСЛІДЖЕНЬ

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NOVEL SQUARYLIUM DYES FOR DETECTION OF AMYLOID FIBRILS *IN VITRO*

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A series of novel symmetrical and asymmetrical squarylium dyes with the different substituents in the donor moieties have been tested for their ability to detect and characterize insulin and lysozyme amyloid fibrils prepared in acidic buffer at elevated temperature. The dye-protein binding parameters were estimated in terms of the one-site Langmuir adsorption model using the data of direct and reverse fluorimetric titrations. By comparing the dye quantum yields, binding affinities, and extents of the fluorescence enhancement in the protein-bound state, G6 and G7 were selected as the most prospective amyloid tracers. Furthermore, these probes provided evidence for the lower polarity of the lysozyme fibrillar grooves compared to insulin aggregates. The novel dyes G6 and G7 were recommended for amyloid fibril detection and characterization in the near-infrared region.
KEY WORDS: amyloid fibrils of insulin/lysozyme, binding model, quantum yield, squarylium dyes.

НОВІ СКВАРАЇНОВІ ЗОНДИ ДЛЯ ДЕТЕКТУВАННЯ АМІЛОЇДНИХ ФІБРИЛ *IN VITRO*

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Серія нових симетричних та асиметричних скваріанових зондів, що мали різні замісники у донорних групах, була протестована щодо здатності барвників детектувати та характеризувати амілоїдні фібрили інсуліну та лізоциму, отримані за умов низького pH та підвищеної температури. Параметри зв'язування зондів з білками оцінювали у рамках односайтової моделі Ленгмюра, використовуючи дані прямого та зворотнього флуориметричних титрувань. При порівнянні квантових виходів, спорідненості та ступеня зростання флуоресценції зондів, зв'язаних з білком, найбільш перспективними зондами виявились G6 та G7. Окрім цього, за допомогою даних зондів отримані докази на користь нижчої полярності жолобків фібрил лізоциму, порівняно з інсуліном. Нові зонди G6 та G7 були рекомендовані для детектування та дослідження структури амілоїдних фібрил у ближній інфрачервоній області.
КЛЮЧОВІ СЛОВА: амілоїдні фібрили інсуліну/лізоциму, модель зв'язування, квантовий вихід, скваріанові зонди.

НОВЫЕ СКВАРАИНОВЫЕ ЗОНДЫ ДЛЯ ДЕТЕКТИРОВАНИЯ АМИЛОИДНЫХ ФИБРИЛЛ *IN VITRO*

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Серия новых симметричных и асимметричных скварианновых зондов с разными заместителями в донорных группах была протестирована относительно способности красителей детектировать и характеризовать амилоидные фибриллы инсулина и лизоцима, полученные в условиях низкого pH и повышенной температуры. Параметры связывания зондов с белками оценивали в рамках односайтовой модели Ленгмюра, используя данные прямого и обратного флуориметрического титрования. При сравнении квантовых выходов, сродства и степени возрастания флуоресценции красителей, связанных с белком, наиболее перспективными зондами оказались G6 и G7. Кроме того, с помощью этих зондов были получены доказательства в пользу более низкой полярности желобков фибрилл лизоцима, по сравнению с инсулином. Новые зонды G6 и G7 были рекомендованы для детектирования и исследования структуры амилоидных фибрилл в ближней инфракрасной области.
КЛЮЧЕВЫЕ СЛОВА: амилоидные фибриллы инсулина/лизоцима, модель связывания, квантовый выход, скварианновые зонды.

Protein misfolding coupled with the formation of insoluble aggregates, amyloid fibrils, is is currently associated with a range of human diseases [1, 2]. One of the main approaches to detection and characterization of amyloid fibrils is based on monitoring the fluorescence changes of Thioflavin T (ThT) [3] and a range of novel markers [4]. Squarylium dyes, a subclass of cyanines, have been extensively used in sensor design [5, 6], for biological labeling [7–9], in photoconductive devices [10, 11] and photodynamic therapy (PDT) [12, 13]. In solution, this class of the organic fluorophores exhibits sharp and intense visible absorption within the "phototherapeutic window" (600–1000 nm), in which the depth of light penetration in tissues increases [14]. Furthermore, the probes possess significant triplet quantum yields [13] and high photostability owing to the presence of central squarate bridge [15]. The above properties render the squarylium photosensitizers for PDT capable of converting dissolved triplet oxygen to cytotoxic singlet oxygen, the process involved in the destruction of tumor cells [16]. Likewise, these dyes have been employed for the optical data storage due to their high ($>10^5$ $M^{-1}cm^{-1}$) extinction coefficients [17]. Importantly, squarylium compounds appeared to be effective reporters for examining protein-lipid interactions, e.g. in lysozyme-lipid systems [15, 18], studying structural transformations of protein molecules, for instance, native serum albumin [19] and detection of beta-lactoglobulin molten globule state [20].

Due to the possibility of employing squarylium dyes for a wide range of applications, there is a significant interest in the synthesis of compounds with the increased resistance to the nucleophilic attack (e.g., in alcohol solvents) [21], high solubility in aqueous solvents [22], decreased ability to the formation of J-aggregates [11], and the increased affinity for the proteins and lipids [20, 23]. The present study was aimed at testing the sensitivity of novel squarylium dyes to insulin and lysozyme amyloid fibrils. Specifically, our goal was two-fold: i) to assess the dye-protein binding parameters and the spectral properties of the protein-bound fluorophores; ii) to evaluate whether the examined dyes are suitable for uncovering the morphological differences between the fibrillar aggregates.

MATERIALS AND METHODS

Insulin from bovine pancreas and hen egg white lysozyme were purchased from Sigma (St. Louis, MO, USA). Thioflavin T was from Sigma (Steinheim, Germany). Squarylium dyes (Fig. 1A), referred to here as G1, G2, G4, G5, G6 and G7 were recently synthesized by Dr. Aleksey Vasilev at the Faculty of Chemistry of St. Kliment Ohridski University of Sofia. Stock solutions of the fluorophores were prepared by dissolving the dyes in dimethyl sulfoxide. The dye concentrations were determined spectrophotometrically using the extinction coefficients at absorption maxima 115450, 167300, 121900, 7500, 179000, 170000 and 23800 $M^{-1}cm^{-1}$ for G1, G2, G4, G5, G6, G7 and ThT, respectively. G1, G2, G4 and G6, G7 were excited at

610 and 590 nm, while G5 and ThT were excited at 490 and 420 nm, respectively. The binding to the amyloid fibrils resulted in the increase of the fluorescence at ~650 nm for most squarylium dyes (except G5), and at 545 (483) nm for G5 (ThT). Insulin/lysozyme amyloid fibrils were prepared by incubation of the protein (10/20 mg/ml) at 65 ˚C and pH 1.6 for 14/20 days without agitation. The amyloid nature of the fibrillar aggregates was confirmed by transmission electron microscopy (Fig. 1, B and C).

Association constants (K_a) and stoichiometry (n) of the dye-protein binding were estimated from the fluorimetric titration of the amyloid fibrils by the dyes and Thioflavin T (Fig. 2, Table 1) using the one-site Langmuir adsorption model. Additional parameter – the coefficient relating the measured fluorescence changes to the amount of bound probe (α), which is proportional to the difference of the dye quantum yields in a buffer solution and protein-associated state, was estimated from the dye titration by fibrillar proteins (Fig. 2A) [24]. Next, in order to circumvent the problem of a cross correlation between the parameters K_a and n, the amyloid fibrils were titrated by the dyes (Fig. 2A). The Gibbs free energy change (ΔG) was determined as: $\Delta G = -R \cdot T \cdot \ln(K_a)$, where $R = 8.31$ J/mol·K, and $T = 293$ K. Furthermore, the Scatchard plots for the dye binding to amyloid fibrils were calculated as: $B = (I(I_n) - I_0)/\alpha$, where $I(I_n)$ and I_0 are the dye fluorescence intensities in buffer solution and in the presence of amyloid fibrils (native protein), respectively; and $F = Z - B$, where Z, B, F are the total dye concentration, concentration of the protein-bound and free dye, respectively (Fig. 2B, inset figure). Finally, fluorescence response of the probes in the presence of fibrillar aggregates, or relative intensity increase for each dye (0.1 μM) bound to fibrillar protein (50 μM), was defined as $I(I_n)/I_0$ (Table 2), where I_0 and $I(I_n)$ were calculated using the dye-protein binding parameters. Quantum yields of protein-bound (Q, Q_n correspond to fibrillar and native protein states, respectively) G5 and Thioflavin T were estimated using the standard dye Rhodamine 101 (Q=0.91 in acidified ethanol) and the refractive indexes reported for fibrils and EtOH [24]. The Cy5 (Q=0.28) probe was used as a standard for calculation of the quantum yield of the other squarylium dyes.

RESULTS AND DISCUSSION

The examined dyes possess low quantum yields in buffer, probably due to the high non-radiative decay (that may arise from the loss of the planarity because of steric restrictions) [25], although the main charge transfer (CT) process is primarily confined to the central cyclobutene ring [26] and the presence of the central four-member ring decreases the fluorophore internal rotation compared to that of cyanines [17, 27]. Indeed, all the novel probes had hydrocarbon and/or aromatic substituents in the donor

moieties perpendicular to the central cyclobutene ring and thus reduce the conjugation within the donor-acceptor-donor (D-A-D) system of the squaraines, as was proved by quantum-chemical calculations and will be described below. Furthermore, the dyes showed very low affinity for the native lysozyme and insulin (data not presented), in agreement with the results of the previous studies [27, 28]. Additionally, SO_3^- groups of G5 and G7 could suppress dye-protein interactions [6].

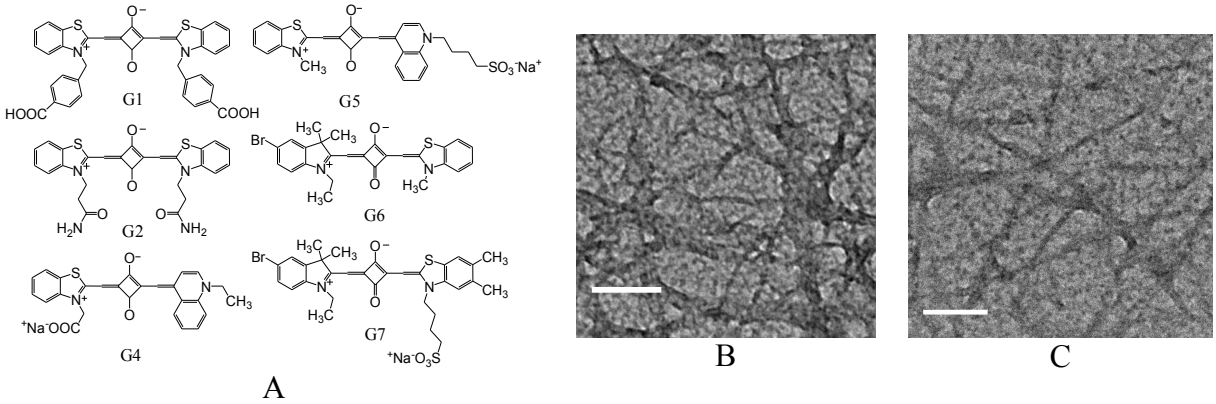

Fig. 1. Structural formulas of squarylium dyes (A). TEM images of insulin (B) and lysozyme (C) fibrils prepared by acidic denaturation at 65 ˚C. Scale bars are 100 nm.

Interestingly, the blue shifts of the dye absorption maxima could occur in the presence of electron withdrawing atoms (e.g., bromine), so that the absorption maxima of G6, G7 were observed at 590 nm, while for the other dyes these maxima were at 610 nm [26]. Emission maxima of the novel fluorophores (except G5) shifted up to 22 nm to the long-wavelength region upon binding to amyloid fibrils, the shifts being about 3–14 nm greater for lysozyme compared to insulin (Table 2, $\Delta\lambda$) [19,20]. Emission maximum of G5 showed *ca.* 40 nm shift to the shorter wavelength in the presence of lysozyme aggregates [28]. Perhaps, red shifts of the dye emission is due to the hydrophobic interactions, as was shown for the near-infrared cyanine IR806 [29], and the increase in the D-A-D CT character (or planarity) upon the dye incorporation into fibril structure [26], while the specific D-A-D character (e.g., the protonation of the SO_3^- group [30]) could lead to appearance of the additional absorption and emission maxima of G5 at ~490 and 545 nm, respectively [26]. In turn, the observed fluorescence intensity of the second population of G5 molecules at ~650 nm (when excited at 610 nm) remains invariant upon the dye-protein association. Thus, lysozyme binding sites for the squarylium dyes were found to be more hydrophobic as was also observed in our previous studies for ThT derivatives, although negatively charged G5 and G7 could bind to the positively charged lysozyme aggregates by the electrostatic interactions.

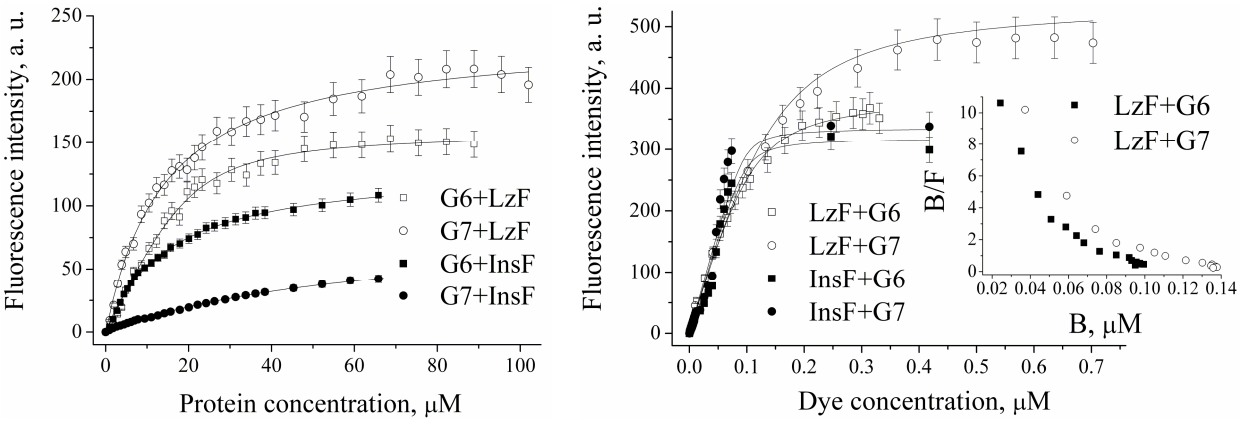

Fig. 2. The isotherms of G6 and G7 binding to fibrillar lysozyme and insulin. G6 and G7 concentrations in the presence of lysozyme (insulin) fibrils were 0.043 (0.017) and 0.063 (0.008) μM, respectively (A). Lysozyme and insulin concentrations were 4.8 and 4.1 μM, respectively (B). The inset figure shows the Scatchard plots for the dye binding to amyloid fibrils (B).

As seen in Table 1, the dye-protein binding parameters obtained via titration of the dyes by the fibrils are cross-correlated, because there exist the pairs with high affinity and low stoichiometry or low affinity and high stoichiometry, while the stoichiometry values should be approximately similar for the dyes of one class. The titration of the protein by the dye resulted in the close values of K_a and n. In the presence of amyloid fibrils the highest values of n, K_a and ΔG were found for the dyes G6 and G7 (Table 2). Presumably, hydrophobic interactions play a crucial role in the dye-protein binding, while the electrostatic repulsion could lead to the decreased dye-insulin binding parameters (the insulin grooves should be quite thick compared to lysozyme ones (Figure 1, B and C), thus, there is no steric hindrance for the dye-insulin binding). Furthermore, carboxyl groups of the protein could form a strong halogen bond with bromine of G6 and G7 [17]. The Scatchard plots for G6 and G7 in the presence of lysozyme fibrils (Fig. 2B, inset figure) are not linear, and the fit with the two-site absorption model yielded the characteristics of the sites with high and low affinity (data not presented).

In turn, long hydrocarbon tails in the donor moieties of G1 and G2 induce the decrease of their association with the fibrils due to the steric hindrance (if the planarity of the dye decreases because of the presence of the bulky substituents, it will complicate their incorporation into the fibril grooves [25]), although these tails increase the affinity for native protein [20]. Indeed, the dihedral angles between the long hydrocarbon substituents in the thiazole (naphtalene) part of the donor moieties and the central cyclobutene ring were about 90 degrees, as was obtained from the ground state geometry optimization with 6-31(d,p) basis using density functional theory (data not presented). Thus, due to the high widths of G1 and G2, they hardly

penetrate into the amyloid fibril grooves. Notably, besides hydrophobic interaction, which seem to be the main determinant of the dye-fibril binding, we suppose that the formation of H-bonds between the carbonyl group of cyclobutene ring and sulfur atom in benzothiazole cycle of the dye with amino group and carboxyl group of the peptide backbone could occur, as well [20]. Furthermore, zwitterionic and negatively charged dyes can interact with the proteins electrostatically, as follows from their higher affinities for lysozyme fibrils compared to Thioflavin T (Table 1, K_a) [31].

Table 1.

Binding parameters of novel squarylium dyes associated with amyloid fibrils

Dye	Insulin					Lysozyme				
	Dye titration by the protein			Direct and reverse titrations		Dye titration by the protein			Direct and reverse titrations	
	K_a, μM^{-1}	n	α, μM^{-1}	K_a, μM^{-1}	n	K_a, μM^{-1}	n	α, μM^{-1}	K_a, μM^{-1}	n
G1	12	0.014	2693	11	0.006	22	0.005	457	2.5	0.015
G2	129	0.002	1330	0.7	0.030	156	0.002	1692	2.5	0.009
G4	389	0.001	1289	0.5	0.040	0.12	0.52	918	3.5	0.013
G5	0.003	44	1526	0.2	0.007	0.006	19	1020	0.4	0.093
G6	2.2	0.03	7708	23	0.010	101	0.003	3700	57	0.027
G7	0.6	0.03	10000	24	0.010	0.27	0.27	3707	33	0.028
ThT	0.2	0.16	7295	1.1	0.020	16	0.01	3700	0.8	0.049

Table 2.

Spectral properties of novel squarylium dyes associated with amyloid fibrils.

Dye	Insulin						Lysozyme					
	ΔG, kJ/ mol	Q	Q_n	I/I_0	I/I_{nat}	$\Delta\lambda$, nm	ΔG, kJ/ mol	Q	Q_n	I/I_0	I/I_{nat}	$\Delta\lambda$, nm
G1	-39	0.13	n/d	6	n/d	18	-36	0.15	0.23	6	3.3	22
G2	-33	0.08	n/d	13	n/d	19	-36	0.19	0.05	4	2.7	22
G4	-32	0.09	n/d	17	n/d	16	-37	0.10	0.19	7	5	22
G5	-30	0.76	n/d	7.5	n/d	3	-31	0.21	0.54	45	25	-40
G6	-41	0.55	n/d	38	n/d	12	-43	0.45	0.43	21	9	19
G7	-41	0.28	n/d	92	n/d	0	-42	0.38	0.67	37	16	14
ThT	-34	0.85	0.07	209	17	-6	-33	0.83	0.09	136	49	-5

Furthermore, G5, G6 and G7 showed the highest quantum yield (Q), fluorescence enhancement (I/I_0) and preference to the fibrils (I/I_{nat}) (compared to native protein) (Table 2). The increase of the dye quantum yield in the presence of fibrils is likely to reflect the decreased polarity and high rigidity of the fluorophore environment [22].

These results are in accordance with the previous studies of the sensitivity of squarylium dye to the molten globule state of amyloidogenic protein beta-lactoglobulin [20]. Surprisingly, the quantum yield of the dyes turned out to be lower in the presence of lysozyme fibrils compared to the native protein (Q_n), although their sensitivity to lysozyme monomers is negligible due to the very low affinity and stoichiometry, and the native protein detection limit is one order higher than that of the fibrils. Therefore, at low protein concentrations (up to 150 μM) the dye fluorescence increases only in the presence of fibrils. Interestingly, G5 was the most sensitive to the amyloid morphology due to the highest differences in the quantum yields and spectral shifts of the insulin- and lysozyme-bound fluorophores. High value of Q in the presence of insulin could be explained by the dye-protein hydrophobic interactions, while the blue emission shift ca. 40 nm in the presence of lysozyme is probably due to the specific dye-protein interactions (Tables 1 and 2). Furthermore, G6 and G7 were found to be more stable than the other dyes (probably due to the lower subjection to the nucleophilic attack) and possess higher affinity for amyloid fibrils and extinction coefficients compared to G5 and other squarylium dyes. Interestingly, G4 could have low protein sensitivity as compared to that of G5, G6 and G7, probably due to the electrostatic interactions of the carbonyl group with the fibrils [6] and/or lower D-A-D CT character that increases the energy barrier for the formation of the planar dye structure upon fibril incorporation [26].

In conclusion, novel squarylium probes G6 and G7 can be recommended for the amyloid fibril detection and characterization in the near-infrared region, as judged from their high sensitivity to lysozyme and insulin fibrils, photostability and large extinction coefficients [17]. Furthermore, the potential of their application in the amyloid detection and photodynamic therapy could be tested in vivo, as well, because the squaraines appeared to be nontoxic to biological systems [13]. This work was supported by CIMO Fellowship (KV).

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