The applicability of Auramine O to the detection and characterization of lysozyme and serum albumin amyloid fibrils has been assessed using the fluorimetric titration and molecular docking. The parameters of the dye binding to native and fibrillar proteins were estimated in terms of the Langmuir adsorption model. It was found that Auramine O displays the high affinity for amyloid fibrils, being of the same order of magnitude as that of the classical amyloid markers. The dye also showed greater fluorescence response to lysozyme fibrils and lower sensitivity to the native protein, than Thioflavin T. Furthermore, unlike Thioflavin T, Auramine O was able to detect the morphological differences between lysozyme and albumin fibrils due to the shifts in the position of the emission maxima of the fibril-incorporated fluorophore. The molecular docking studies revealed that Auramine O and Thioflavin T form the most stable complexes with the G54_L56/S60_W62 groove of lysozyme fibrils, running parallel to the fibril axis. The results obtained suggest the contribution of both hydrophobic and electrostatic interactions to the stabilization of the dye complexes with amyloid fibrils.

**KEYWORDS:** Auramine O, Thioflavin T, amyloid marker, association constant, fluorescence quantum yield, molecular rotor, lysozyme, serum albumin, amyloid fibrils

**AURAMINE O AS POTENTIAL AMYLOID MARKER: FLUORESCENCE AND MOLECULAR DOCKING STUDIES**

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A wide variety of human disorders, viz. Alzheimer’s, Parkinson’s diseases, systemic amyloidosis, type II diabetes, etc. are characterized by the deposition of the specific protein aggregates, amyloid fibrils, in the extracellular matrix followed by functional disturbances in various tissues and organs [1]. Amyloid fibrils are the β-sheet-rich protein assemblies of ca. 5–10 nm in width and up to several micrometers in length [2]. Despite significant progress in understanding the molecular basis of amyloid fibril formation, a number of essential issues remain unclear. In particular, a great morphological heterogeneity of amyloids may hamper the medical diagnostics and development of anti-amyloid drugs, suggesting the necessity of the improved fibril detection strategies [2]. Furthermore, considering the amyloids as a new class of nanomaterials, the fabrication of the nanostructured amyloid films with the desired physical properties requires elucidating the effects of the environmental conditions on amyloid morphology. One of the most effective approaches to addressing these problems is based on the use of amyloid-specific fluorescent dyes. Of these, a fluorescent dye, Thioflavin T (ThT), has been widely employed for the identification and quantification of amyloid aggregates, and for the monitoring the fibrillization kinetics in real-time [13]. ThT association with the β-sheets composed of minimum 5 β-strands (that is typical for amyloid structures) results in a drastic increase in its fluorescence and significant red shift of the absorption maximum [14–16]. Despite the remarkable amyloid-specific spectral response of ThT, this dye may show false positive results in the presence of native proteins and lipids, sensitivity to the pH of the bulk, weak binding to the positively charged fibrils, etc. [17,18]. The above drawbacks of ThT strongly suggest the necessity of designing the novel amyloid markers. To exemplify, another dye, Auramine O (AuO) has been successfully employed to detect insulin aggregates [19]. Furthermore, unlike ThT, AuO “recognized” insulin fibrils due to the appearance of additional red-shifted emission band. However, the potential of Auramine O as amyloid marker is not yet fully assessed.

In view of this, the aim of the present study was to gain further insights into amyloid specificity of AuO through evaluating its applicability for the detection and characterization of lysozyme and albumin amyloid fibrils. Specifically, our goals were: i) to evaluate the quantitative parameters of the dye-protein binding using the fluorimetric titration; ii) to compare AuO and ThT responses to the presence of amyloid fibrils and native proteins; iii) to uncover the advantages of AuO over ThT; iv) to ascertain possible location of AuO within fibril structure using the molecular docking. Lysozyme is a small cationic protein, possessing antibacterial activity [4]. Lysozyme misfolding and aggregation is associated with the hereditary amyloidoses [5]. Hen egg white lysozyme (HEWL) is a structural analogue of the human variant, that is widely employed as a model protein in the amyloid studies [6,7]. The characterization of the interactions between lysozyme fibrils and small ligands, including the fluorescent dyes, may be useful for the design of the drugs against amyloidosis. Moreover, the HEWL amyloid films prepared in vitro showed remarkable mechanical properties, suitable for the development of the amyloid-based multifunctional materials [8].

Serum albumin is an all-α-anionic protein, acting as a carrier from blood stream to tissues and playing a key role in the regulation of osmotic pressure, thrombosis and coagulation [9]. Similar to HEWL, bovine serum albumin has been widely employed as a model protein in the studies of the protein fibrillization [10]. However, to the best of our knowledge, the structure of the serum albumin fibrils and the precise mechanisms of the protein aggregation are so far poorly understood. For example, the residues, which form the core cross-β-structure of the albumin fibrils have not yet been identified. Furthermore, serum albumin was reported to inhibit the Aβ-peptide and transthyretin fibril growth by the stabilization of the Aβ monomers [11,12]. Therefore, further investigation of albumin fibrilization and its effects on the amyloid fibril formation by the other proteins and peptides may prove of importance for the improvement of anti-amyloid strategies.

**EXPERIMENTAL SECTION**

**Materials**

Hen egg white lysozyme (Lz) and bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and used without further purification.

**Preparation of amyloid fibrils**

The reaction of amyloid fibril formation by the lysozyme and bovine serum albumin was carried out in 10 mM glycine buffer at pH 2 and 60 °C for 14 days. Protein concentration in the stock solutions was 10 mg/ml. Hereafter, the native protein forms are designated as LzN and BSAN, while the fibrillar forms – as LzF and BSAF.

**Fluorescence measurements**

Fluorescence spectra of ThT and AuO were measured with the spectrofluorimeter Shimadzu RF-6000 (Japan) at 25 °C using 10 nm excitation and emission slit widths. The excitation wavelength for both dyes was 440 nm.

**Binding model**

The quantitative characteristics of the dye-protein complexation were determined from the fluorimetric titration of ThT and AuO solutions by the native or fibrillar proteins. Specifically, the dependence of the dye fluorescence intensity increase (ΔI) on the protein concentration (P) was analyzed in terms of the Langmuir adsorption model [22].
where $Z_o$ is the total dye concentration, $K_a$ and $n$ are the association constant and stoichiometry of the dye-protein complexes, $\alpha$ is a coefficient proportional to the difference of ThT and AuO quantum yields in a buffer and protein-associated state. To make a more precise comparison of the dye sensitivity and specificity to the fibrils, fluorescence intensity increases of the fibril-/native protein-bound dyes $(I_{fibr/nat}/I_0)$ with respect to buffer, and those of the fibril-bound probes $(I_{fibr}/I_{nat})$ with respect to the native proteins, have been estimated. The value of the binding parameters, $K_a$, $n$, $\alpha$, derived from the fit of eq. 1 to the experimental data are presented in Table 1 for the fixed dye and protein concentrations 2 and 10 µM, respectively.

Molecular docking studies

The molecular docking was implemented to identify the possible binding sites of the native and fibrillar proteins for AuO and ThT. Lysozyme and albumin structures with PDB IDs 3A8Z and 4F5S, respectively, were chosen for the docking. The model amyloid fibril of lysozyme was constructed from the K-peptide (the residues 54-62 of wild-type protein) using the CreateFibril tool [23]. ThT and AuO structures were built in Avogadro [24], followed by the geometry optimization of the ground states of the dyes with the 6-31G(d,p) basis set using the GAMESS software [25]. The protein-dye complexes possessing a good shape complementarity and the lowest desolvation energy were obtained using the PatchDock web server [26]. Next, 10 best structures generated by the PatchDock, were refined with the FireDock, an algorithm, which optimizes the intermolecular binding by allowing the flexibility of the side chains and small rigid-body movements [27]. The results obtained from the FireDock were reported to be in a good agreement with the experimental data. The most stable dye-protein complexes were visualized by the Visual Molecular Dynamics software [28].

RESULTS AND DISCUSSION

As seen in Fig. 1, AuO and ThT are the small cationic dyes, possessing the electron-donating amino moieties on the aromatic groups. These dyes belong to the molecular rotor family due to the internal rotation of their fragments upon excitation, followed by the transition from the fluorescent locally excited (LE) state to the nonfluorescent twisted internal charge transfer (TICT) state [19,20]. In turn, steric restriction of the intramolecular twisting results in the dramatic increase in the fluorescence quantum yield of these fluorophores. The above mechanism was suggested to underlie the high sensitivity of ThT to amyloid fibrils due to the dye embedding into the fibril grooves, running parallel to the fibril axis, giving rise to significant increase in the potential energy of relative rotation of the benzothiazole and phenyl moieties [21].

![Fig. 1. Chemical structures of the fluorescent dyes: a – Auramine O; b – Thioflavin T](image-url)

As shown in Fig. 2, the binding of AuO to the amyloid fibrils and native proteins manifests itself in the increase of the dye fluorescence intensity with the protein concentration. Likewise, emission maxima of the BSA-incorporated fluorophore showed ca. 10–12 nm shifts to the shorter wavelengths ($\lambda_{fibr/nat} - \lambda_0$, Table 1), suggesting the decreased polarity of the dye environment [29]. On the contrary, BSA-bound ThT did not show noticeable spectral shifts. Furthermore, emission maxima of the both dyes remained almost invariable in the presence of native and fibrillar lysozyme. According to these results, AuO seems to be more sensitive to the solvent polarity than ThT. The above spectral properties of AuO resemble those of Nile Red, another fluorescent amyloid marker, which showed the dependence of the emission maximum on the local polarity of amyloid binding sites [30].

To obtain the quantitative characteristics of the dye-protein binding the experimental dependences $\Delta I(P)$ were approximated by eq. (1) (Fig. 3). As seen in Table 1, AuO showed similar affinity for native and fibrillar proteins, and higher stoichiometry for BSAF, BSAN, than that of ThT. In turn, ThT possessed 10-fold higher $K_a$ value in the presence of lysozyme fibrils, as compared to that of lysozyme monomers. The greater $K_a$ values characteristic of the
AuO- and ThT-BSA complexes may arise from the enhanced hydrophobic dye-protein interactions. Likewise, at pH 7.4 BSA has a negative electric charge, while AuO and ThT are positively charged, assuming the increased electrostatic stabilization of the dye-protein complexes, as well [31]. The $K_a$ value characterizing the AuO-BSA binding also suggests the formation of fibrils of a certain morphology, differing from that observed by other authors. For instance, in the Mudliar’s work BSA (100 µM) was incubated at 65 ºC for 2 hours resulting in the fibril formation and the $K_a$ value lower by 2 orders of magnitude compared to that obtained in the present study [32].

Interestingly, unlike lysozyme, BSAN and BSAF have similar number of the dye binding sites, supposedly with different morphology. The large molecule of serum albumin possesses about 24 residues of the different subdomains with the very high aggregation propensity, estimated with AGGRESCAN [33], Zyggregator [34] and TANGO [35]. These residues presumably form fibril core, enabling the dye association with various fibril grooves that may increase the number of the dye binding sites. Indeed, Holm et al. reported drastic increase in the $\beta$-sheet content during BSA fibrillization, followed by ThT and Congo Red complexation with the different subtypes of the aggregates formed in parallel [36].

The comparison of the $\alpha$ values for the dye complexes with fibrillar and native proteins revealed higher fluorescence responses of the fluorophores to the presence of amyloid fibrils (Table 1). Furthermore, LzF-bound AuO showed greater signal-to noise ratio ($I_{\text{fibril}}/I_{\text{n}}$) compared to that of the BSAF-associated dye. In turn, ThT showed higher specificity for BSAF than that of LzF. Interestingly, the examined dyes possess up to 15-fold greater quantum yields in the presence of fibrillar and native BSA, as compared to LzN. In the case of BSAN this could be explained by the dye incorporation into the protein hydrophobic patches, viz. sites I and II [37]. In turn, stabilization of the locally excited state and the concomitant increase in the ThT quantum yield are predominantly due to the dye interactions with the long $\beta$-sheets, resulting in the lowered sensitivity of the fluorophore to hydrophobic pockets of BSAN [14]. Analysis of the quantitative characteristics of the dye-protein complexation revealed that AuO could be used as alternative to ThT for LzF detection.
Fig. 3. The isotherms of the dye binding to serum albumin and lysozyme  

a, b – AuO; c, d – ThT. AuO and ThT concentrations were 15 µM and 2 µM, respectively

Finally, the molecular docking was used to identify the possible protein binding sites for AuO and ThT. It appeared that AuO and ThT tend to associate with the similar sites on the native or fibrillar proteins (Figure 4). It can be assumed that both dyes associate with G54_L56/S60_W62 fibril groove of the anti-parallel β-sheet of the lysozyme.

Table 1. Quantitative characteristics of the dye-protein binding

<table>
<thead>
<tr>
<th>Dye</th>
<th>Protein</th>
<th>$\lambda_0^*$, nm</th>
<th>$\lambda_{fibr/nat} - \lambda_0^{**}$, nm</th>
<th>$K_a$, µM$^{-1}$</th>
<th>$n$</th>
<th>$\alpha$, µM$^{-1}$</th>
<th>$I_{fibr/nat} / I_0$</th>
<th>$I_{fibr} / I_{nat}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuO</td>
<td>BSAF</td>
<td>514</td>
<td>-12</td>
<td>1.20±0.25</td>
<td>2.60±0.51</td>
<td>800</td>
<td>26.7</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>BSAN</td>
<td></td>
<td>-10</td>
<td>2.00±0.41</td>
<td>2.90±0.32</td>
<td>500</td>
<td>17.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LzF</td>
<td></td>
<td>-4</td>
<td>1.40±0.30</td>
<td>1.50±0.30</td>
<td>102</td>
<td>3.9</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>LzN</td>
<td></td>
<td>0</td>
<td>0.40±0.08</td>
<td>1.20±0.24</td>
<td>21</td>
<td>1.4</td>
<td>-</td>
</tr>
<tr>
<td>ThT</td>
<td>BSAF</td>
<td>480</td>
<td>-2</td>
<td>0.73±0.15</td>
<td>0.35±0.07</td>
<td>4800</td>
<td>17.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>BSAN</td>
<td></td>
<td>0</td>
<td>1.70±0.37</td>
<td>1.90±0.03</td>
<td>174</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LzF</td>
<td></td>
<td>-3</td>
<td>0.20±0.04</td>
<td>0.11±0.02</td>
<td>4020</td>
<td>3.5</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>LzN</td>
<td></td>
<td>0</td>
<td>0.02±0.00</td>
<td>7.50±0.15</td>
<td>260</td>
<td>1.8</td>
<td>-</td>
</tr>
</tbody>
</table>

*Emission maximum of the free dye in buffer; **Difference between the emission maxima of the protein-bound and free dye fibril via hydrophobic interactions [38]. According to our quantum-chemical calculations, AuO length is ca. ~1.3 nm, being shorter than that of ThT (ca. ~1.5 nm). Therefore, AuO may associate with 4 β-strands of the β-sheet, while ThT is likely to associate with 5 β-strands [39].

Furthermore, AuO and ThT tend to associate with the IB domain of BSAN (the residues L115, P117, L122, E125, F133, K136, Y137, E140, Y160, R185) and the active center of LzN (the residues I58, N59, W62, W63, I98, D101, N103, A107, W108) [40,41]. Interestingly, Mudliar et al. reported AuO binding to the hydrophobic IIA domain of BSA, i.e. to the specific ligand-binding site [32,42]. These discrepancies could be due to the fact that Mudliar employed AutoDock software, while the simple PatchDock/FireDock tools used in the present study give less precise results. The
above data support our suggestion about the contribution of both hydrophobic and electrostatic dye-protein interactions in the stabilization of the dye-protein complexes [43].

Fig. 4. Schematic representation of the most stable dye-protein complexes obtained by the molecular docking

a – AuO associated with the lysozyme fibril represented by the β-sheet formed from the K-peptide. The dye is bound to the G54_L56/S60_W62 fibril groove of the anti-parallel β-sheet; b – ThT associated with the same lysozyme fibril groove as AuO; c – AuO bound to the native serum albumin. The residues L115, P117, L122, E125, F133, K136, Y137, E140, Y160, R185 represent the protein binding site for the dye; d - AuO bound to the native lysozyme. The residues I58, N59, W62, W63, I98, D101, N103, A107, W108 represent the protein binding site for the dye.

CONCLUSIONS

• The quantitative parameters for the Auramine O binding to fibrillar and native lysozyme and bovine serum albumin have been estimated, revealing the possibility of the dye application for the detection and characterization of amyloid fibrils;
• Auramine O showed the dependence of the position of the emission maximum on the polarity of the amyloid binding sites. This feature could be employed for the structural differentiation of lysozyme and serum albumin amyloid fibrils;
• Simple docking studies revealed that Auramine O associates with the specific fibril binding sites, viz. the grooves, running parallel to the fibril axis.
• Both fluorescence and docking studies indicate that hydrophobic and electrostatic dye-protein interactions play an essential role in the stabilization of the dye-protein complexes.

REFERENCES