Free and bound Thioflavin T molecules with ultrafast relaxation: implications for assessment of protein binding and aggregation

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Abstract: Fluorescent dye Thioflavin T (ThT) is a widely used probe for detection of amyloid fibrils, which are protein aggregates involved in the pathogenesis of neurodegenerative disorders. Upon the formation of a complex with amyloids, fluorescence quantum yield of ThT increases 1000-fold due to a dramatic reduction of the nonradiative decay rate. This is accompanied by a remarkable change of ThT fluorescence lifetime τ from ~1 ps to ~1000 ps, thus making it possible to assess ThT binding to different systems using τ as an indicator. However, when measuring ThT interaction with proteins, one can observe that binding affinity determined from ThT fluorescence intensity dependence on protein concentration may be orders of magnitude lower than that determined using τ. Here we show that this discrepancy at least partly originates from a limited temporal resolution when determining fluorescence lifetime of ThT in the ThT-protein system using the time-correlated single photon counting technique (TCSPC), which is usually characterized by a ~100 ps instrument response function. This results in the situation when a small fraction (~1%) of ThT molecules with a relatively slow decay (τ ~ 1000 ps) completely disguises the impact of ThT molecules with an ultrafast decay (τ ~ 1 ps) to the overall measured fluorescence decay curve. Moreover, using the femtosecond-resolved fluorescence up-conversion technique we demonstrate that not only free ThT molecules but also a subpopulation of protein-bound ThT molecules exhibits fluorescence decay on a 1 ps timescale. The obtained results are of critical importance for a reliable interpretation of protein binding and aggregation experiments when using a ThT assay with fluorescence lifetime determined by TCSPC as an indicator.

Keywords: time-correlated single photon counting, femtosecond-resolved fluorescence up-conversion, fluorescence lifetime, proteins, binding constant, thioflavin T

1 Introduction

The phenomenon of amyloids (amyloid fibrils, protein aggregates with a specific structure) formation is of high importance for biology and medicine due to its involvement into the pathogenesis of neurodegenerative disorders [1]. Numerous studies devoted to the investigation of mechanisms of amyloid formation and toxicity and development of inhibitors of fibril formation are based on *in vitro* experiments and make use of optical spectroscopy methods, including both label-free methods [2]

fluorescence spectroscopy with exogenous probes, to characterize fibrils. Thioflavin T (ThT) is the most used fluorescent probe to detect amyloid fibrils and monitor the kinetics of their formation [3]. Applicability of ThT is based on two facts: (1) ThT is considered to be a rather specific sensor for amyloids enriched in cross-β structures, i.e. it forms high-affinity complexes with them [4] and (2) fluorescence quantum yield of ThT is increased by three order of magnitude upon binding to amyloids [5]. The latter is due to rigid fixation of two parts of the bound ThT molecule, which diminishes an ultrafast excited state relaxation pathway connected with intramolecular charge transfer (ICT) observed for free ThT in aqueous solution [6]. Restriction of ThT rotation in viscous microenvironment results in a dramatic decrease of non-radiative decay rate and a concurrent increase of fluorescence intensity and fluorescence lifetime from ~1 ps to ~1000 ps [6; 7; 8].

Although ThT binding affinity is much lower for globular proteins compared to amyloids [9; 10; 11], biasing of ThT assay under excess of proteins with non-specific binding sites may occur [11; 12]. Moreover, amyloid fibrils are also known to possess both high and low-affinity sites for ThT [13; 14], and quantification of binding parameters towards different proteins and protein aggregates, as well as an assessment of ThT optical properties when bound to different low and high-affinity sites, remains of interest. The high sensitivity of optical properties to changes of viscosity [7] and polarity [15] of ThT microenvironment makes them convenient indicators for studying ThT interaction with proteins and other systems. That is, a saturating dependence of ThT fluorescence intensity, fluorescence lifetime or maximum of the ThT absorption spectrum on protein (or e.g. RNA) concentration in solution can be used to evaluate interaction parameters under certain assumptions about the binding stoichiometry [11; 16; 17; 18]. Considering changes of the non-radiative decay rate k_{nr} to be the main driver for fluorescence enhancement of ThT [7; 8], fluorescence intensity F of bound ThT could be expected to be linearly proportional to its fluorescence lifetime τ, as $F \sim k_r/(k_r + k_{nr}) = k_r \cdot \tau$, where k_r is the radiative decay rate. However, when measuring ThT binding to proteins, one may observe that saturation of τ dependence on protein concentration precedes that for F. In other words, increase of τ and its invariability on protein concentration could be indicative of the fact that all ThT is bound, however, further increase of F suggests that binding is not complete. A similar situation is observed upon measuring the kinetics of fibril formation when changes of τ occur earlier than that of F [19; 20].

In this work, we aimed at the explanation of the asynchronous change of ThT fluorescence intensity and lifetime upon its interaction with proteins. By comparing the results of time-correlated single photon counting (TCSPC) techniques and femtosecond-resolved up-conversion spectroscopy we detected the existence of globular-protein bound ThT with ultrafast relaxation, as was observed previously for amyloids [21]. Further numerical modeling and time-resolved fluorescence measurements allowed for a detailed description of processes which underlay the investigated discrepancy.

2 Materials and Methods

2.1 Sample preparation

Thioflavin T (ThT) alpha-lactalbumin (α-LA, type I) were obtained from Sigma-Aldrich. All measurements were performed in a 20 mM Tris-HCl buffer at pH 7.4. Ionic strength was adjusted to 0.1 M by NaCl. A stock protein solution was prepared and then diluted to perform fluorescence titrations at a fixed ThT concentration of 2 μM.

2.2 Steady-state fluorescence measurements and time-resolved fluorescence measurements using TCSPC

Steady-state fluorescence measurements were performed on a FluoroMax-4 spectrofluorometer (Horiba Jobin-Yvon, Japan-France). ThT fluorescence spectra were obtained at 410 nm excitation (slit width 1 nm), which fits the maximum of its absorption spectrum [11] in the 425–660 nm range (slit width 2 nm).
Time-resolved fluorescence measurements of ThT were performed using the TCSPC technique on the custom-built fluorimeter [20]. Fluorescence was excited with a pulsed 405 nm laser diode (IOS, Saint Petersburg, Russia) delivering 11 pJ, 40 ps FWHM pulses, driven at a repetition rate of 10 MHz. The registration system included photomultiplier (PMC-100, Becker&Hickl, Germany) and a single photon counter module (SPC-130EM, Becker&Hickl). The FWHM of the instrument response function (IRF) for this setup was ~200 ps [11; 22].
The fluorescence decays were analyzed on the SPC Image software (Becker&Hickl) by fitting to the multi-exponential decay function, which is widely used procedure in fluorescence lifetime analysis [23; 24]:

$$F(\mathrm{t}) = \sum_{\mathrm{i}} a_{\mathrm{i}} \mathrm{e}^{-\frac{\mathrm{t}}{\tau_i}}, \qquad (1)$$

where a_{i} and τ_{i} are the pre-exponential factors (amplitudes and lifetimes) for the i-th component of the fluorescence decay. The mean fluorescence lifetime was determined as:

$$\tau_{\mathrm{mean}} = \sum_{\mathrm{i}} \tau_i a_{\mathrm{i}} \,/\, \sum_{\mathrm{i}} a_{\mathrm{i}} \,. \qquad (2)$$

Dissociation constant K_{d} was obtained by fitting the dependence of y, which corresponds to either fluorescence intensity at the maximum or mean fluorescence lifetime, on protein concentration C_{p}:

$$y(C_{\mathrm{P}}) = y_{max} \cdot \left(\frac{C_{\mathrm{ThT}} + C_{\mathrm{P}} + K_{\mathrm{d}}}{2} - \sqrt{\left(\frac{C_{\mathrm{ThT}} + C_{\mathrm{P}} + K_{\mathrm{d}}}{2} \right)^2 - C_{\mathrm{P}} \cdot C_{\mathrm{ThT}}} \right), \qquad (3)$$

where C_{P} and C_{ThT} are the total concentrations of protein and ligand (ThT) in solution [11]. The expression in parentheses represents the concentration of bound ThT.

2.3 Fluorescence up-conversion spectroscopy

Time-resolved fluorescence measurements in the sub-picosecond time domain were carried out using a commercial femtosecond optically gated fluorescence kinetic measurement system FOG100 (CDP Ltd., Russia). General principles of the method are reviewed in [25]. The samples were excited by 100 fs

pulses at 405 nm (second harmonic of Ti:Sapphire oscillator Mia-Tai, Spectra Physics, USA). The excitation pulse repetition rate was 80 MHz. The fluorescence collected from the sample was focused on a 0.5-mm β-barium borate (BBO) crystal alongside with the other part of the beam (fundamental, 80 fs, 810 nm) acting as a gate pulse for frequency up-conversion. The gate pulse was delayed by a retroreflector on a stepper-motor controlled delay stage. The upconverted light was focused onto the entrance slit of a 160 mm double monochromator and detected by a photomultiplier tube. ThT fluorescence emission was collected at the 500±2 nm wavelength to reduce the impact of the Raman scattering of water to the measured signal. The IRF was measured as the temporal profile of the water Raman scattering signal, which is characterized by an ultrafast decay [26], at 470 nm. The reproducibility of the measurement was checked by 3 times measuring each decay trace. The samples were placed in 1 mm rotating cuvette (total volume of 1 ml) to avoid the photo-degradation of the sample.

2.4 Molecular docking simulations

The molecular docking simulation were performed similarly to that for the ThT-albumin system in our previous work [11]. The 3D structure of ThT was obtained from the PubChem database (CID: 16953) and α-lactalbumin crystal structure was extracted from the Protein Data Bank (PDB: 1F6S). The Autodock Vina software [27] was used for molecular docking simulations of ThT binding to α-lactalbumin as well as to evaluate ligand binding energies over the conformational search space. The simulation was performed using a blind docking approach, i.e. the search space included the whole protein globule, and the exhaustiveness parameter was set to 9. Prior to the simulation, hydrogen atoms were added to the protein model, and then Gasteiger partial charges were calculated and non-polar hydrogens were merged. Other parameters were set to default values. The binding modes with highest affinities for ThT were assessed and visualized using the PyMol software.

3 Results and Discussion

Figure 1A present the structure of a model protein used in this work, α-lactalbumin (α-LA). This protein is known to form fibrils at certain conditions [28], moreover, it was shown that α-LA in the native state binds ThT with affinity constant $K_d \approx 1$ mM [10]. Figure 1B presents the structure of the ThT probe, rotation around the bond shown with an arrow upon excitation of the free dye in solution results in ultrafast relaxation of the excited state with ~1 ps^{-1} rate and low quantum yield of ThT ($4 \cdot 10^{-4}$) [8]. Using the molecular docking approach, we assessed the possible location of ThT binding sites on α-LA and corresponding binding energies. It was observed that three clusters of possible ThT binding sites exist, which are characterized by close values of the free binding energy ranging from -5.5 kcal/mol to -4.8 kcal/mol, which correspond to K_d ranging from 100 μM to 300 μM.

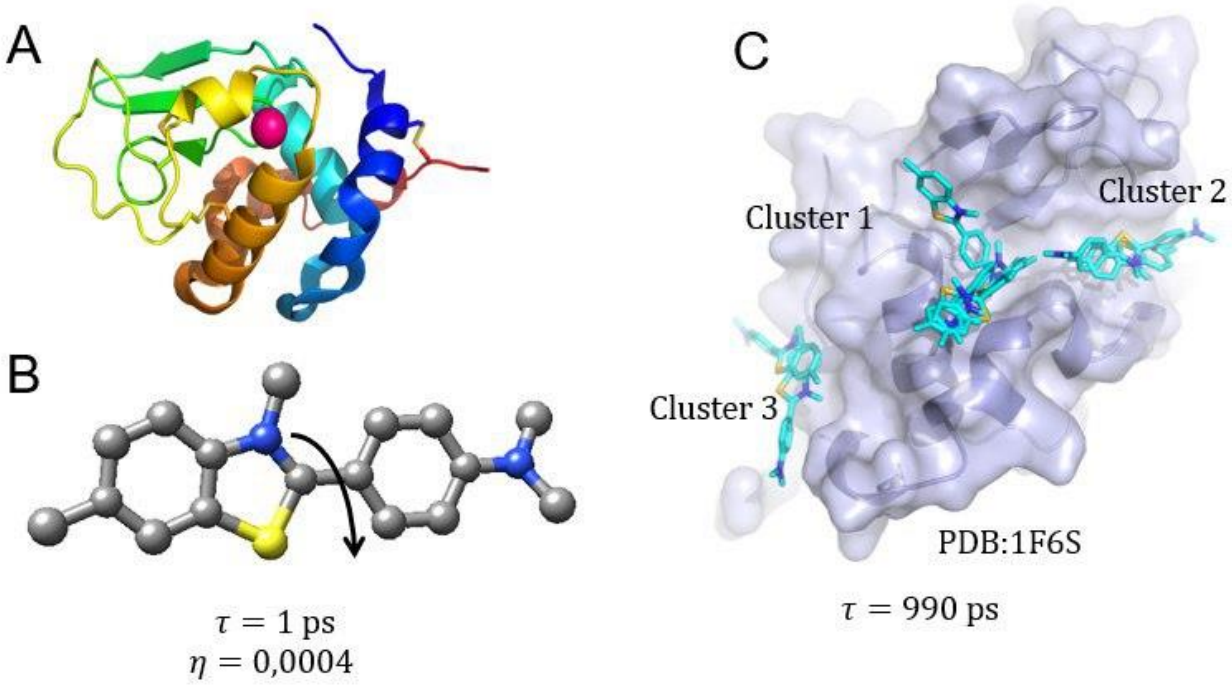

Figure 1. A) The structure of α-LA (PDB: 1F6S). The polypeptide chain is colored by gradient from blue (N) to red (C terminus). B) The structure of the ThT molecule (CID: 16953). C) The location of the molecular docking-predicted ThT binding sites on α-LA, which formed three preferential clusters characterized by similar binding energies, is shown.

Figure 2A demonstrates fluorescence spectra of ThT at different concentrations C_p of a model protein (α-lactalbumin, α-LA). ThT fluorescence intensity F increases upon addition of the protein that is indicative of interaction in the system (Fig. 2A). A similar trend was observed for ThT fluorescence lifetime, as can be seen from fluorescence decay curves presented in Fig. 2B. As for fluorescence lifetime of free ThT in aqueous solution is ~1 ps [6; 21], fluorescence decay curve in the absence of protein represents IRF of the TCSPC instrument. Adequate approximation of fluorescence decay in the presence of protein was reached by fitting to biexponential decay law (Eq. (1)), and a gradual increase in a mean lifetime up to 990±40 ps was observed. The dependence of F and ThT mean lifetime τ_m on protein concentration is shown in Fig. 2C: while τ_m reaches a plateau already at 50 μM of α-LA, a saturation of the F dependence on C_p occurs at much higher concentrations, ~1 mM.

The data presented in Fig. 2 raises two questions. First, while a ~1000-fold increase of τ_m (from ~1 ps (according to the reported data [6; 21]) up to 990 ps) was observed for bound ThT, fluorescence enhancement factor (EF) was as low as 34. This is not in agreement with the concept that both ThT fluorescence intensity and lifetime scale similarly due to a decrease of non-radiative decay rate upon binding, as observed in the case of amyloids, where EF is ~1000 [5]. Second, a clear asynchrony in the time course of F and τ_m is observed with the C_p increase, also suggesting that different processes (e.g. binding of ThT to different sites with different affinities) could be responsible for this effect.

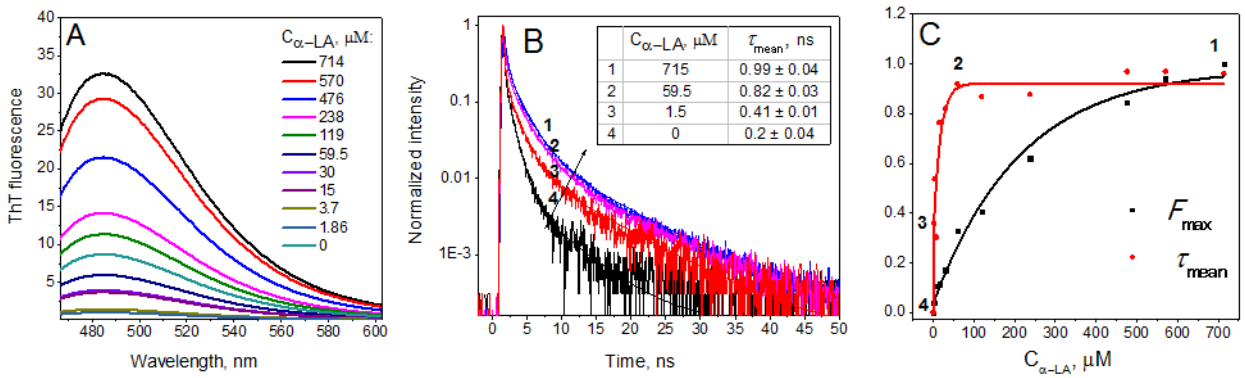

Figure 2. A) Fluorescence spectra of ThT and different protein (α-LA) concentrations obtained at 410 nm excitation. B) Fluorescence decay curves for ThT at different protein concentrations obtained at 405 nm excitation. Solid lines correspond to fitting to the biexponential decay law. C) The dependence of maximum ThT fluorescence intensity (red circles) and mean fluorescence lifetime (black squares) on protein concentration. Solid lines correspond to fitting by the 1:1 binding model with K_d = 12±2 μM for the red curve and K_d = 520±100 μM for the black curve (Eq. 3). The curves are normalized to the (0,1) interval.

To analyse this fact, we performed numerical modelling of fluorescence decay curves in a two-component system with the following parameters: $\tau_1 << \tau_{IRF}$ and $\tau_2 >> \tau_{IRF,}$ where τ_{IRF} is the FWHM of the IRF. Such a relation holds true for the ThT-protein system, where τ_1 = 1 ps corresponds to free ThT, τ_2 = 990 ps corresponds to bound ThT, and τ_{IRF} ~ 200 ps. Fluorescence decay in this system can be described by the following equation:

$$\frac{dn_i}{dt} = I(t) \cdot \sigma \cdot (1 - n_i) - n_i/\tau_i, \quad (4)$$

where n_i, $\sigma_{i,}$ and τ_i correspond to the concentration of the excited state, absorption cross-section and fluorescence lifetime of the i-th component, respectively, and I(t) is the temporal profile of the laser pulse. For simplicity, we further consider that IRF of the system is determined solely by the laser pulse duration, i.e. $\tau_{IRF} = \tau_{pulse,}$ as taking into account detector response time leads to the same results. The observed fluorescence decay of this two-component system can be presented as:

$$F(t) = C_1 \cdot n_1(t) \cdot k_{r1} + C_2 \cdot n_2(t) \cdot k_{r2}, \quad (5)$$

where C_i and k_{ri} are concentrations and radiative decay rates of the i-th component. Figure 3A presents the model fluorescence decay curves for the described two-component system at different C_2/C_1 ratios and equal absorption cross-section and radiative decay rate values. Laser pulse was taken as a Gaussian curve with 200 ps FWHM.

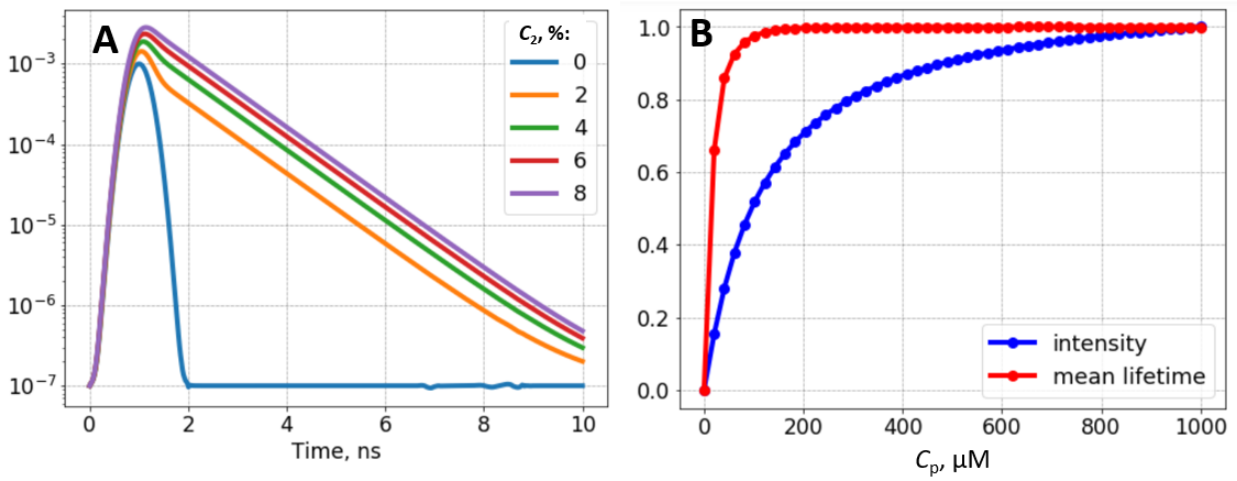

Figure 3. A) Fluorescence decay curves for the two-component system calculated using Equations (4-5) for different C_2/C_1 (shown in % in the figure field). The following parameters were used: τ_1 = 1 ps, τ_2 = 990 ps, τ_{pulse} = 200 ps. B) The dependence of integral fluorescence intensity and fluorescence lifetime of ThT in the presence of different protein concentrations calculated using Eq. (3-5). K_d = 500 μM. The curves are normalized to the (0,1) interval.

While the amplitude of the second (slow) component increases throughout the laser pulse due to the effective accumulation of excited molecules with $\tau > \tau_{pulse}$, the amplitude of the first (fast) component is low due to its immediate relaxation after photon absorption. Hence, already at C_2/C_1~5% the fast component is barely seen, and fitting of the corresponding curve to biexponential decay results in lifetime

values similar to that of the one (slow) component approximation. That is, the amplitudes of components of fluorescence decay a_i in Eq. (1) at finite τ_{pulse} are determined not only by their concentrations but also by their fluorescence lifetimes: at equal concentrations the ratio $a_2/a_1 \approx \tau_{pulse}/\tau_1$.

Next, we performed the following procedure. For the affinity constant K_d = 500 μM and C_{ThT} = 2 μM concentrations of free (C_1) and bound (C_2) ThT were calculated for different C_p using Eq. (3). For these C_1 and C_2 values, fluorescence decay curves were simulated using Eqs. (4-5) and then fitted to the biexponential decay law (Eq. (1)). The obtained mean lifetime values, as well as fluorescence intensities calculated as integrals under fluorescence decay curves, were plotted as a function of C_p (Fig. 3B). This approach allowed for simulation and qualitative analysis of the data presented in Fig. 2C.

Figure 3B demonstrates the same trend as was observed in the experiment (Fig. 2C): fluorescence lifetime of ThT reaches a plateau at C_p much lower than that required for fluorescence intensity saturation, hence, being fitted to Equation (3), these plots would provide for significantly different K_d values. From this perspective, asynchronous changes of F and τ for ThT with protein concentration can be readily explained: while F saturates after complete binding of ThT, a much lower fraction of bound ThT is required to completely mask the fast component in fluorescence decay and saturate the $\tau(C_p)$ dependence.

However, one fact still needs to be explained. That is, even at protein concentrations when ThT fluorescence intensity reaches a plateau, and all ThT can be considered to be bound by protein, the EF is as low as 4, i.e. significantly lower than the increase of ThT fluorescence lifetime. To clarify this issue, we address the results presented in [21], where it was shown that a weakly fluorescent subpopulation of bound ThT molecules exists in the ThT-insulin fibrils system. This fraction of molecules was not observed in TCSPC experiments due to characteristic ultrafast relaxation (~2 ps) and could be only enlightened using femtosecond-resolved techniques [21]. This is in agreement with the prediction of the two-component model described above: the component with $\tau_1 << \tau_{IRF}$ cannot be seen even at low contribution from a component with $\tau >> \tau_{IRF}$. As a result, the existence of a significant fraction of weakly fluorescent bound ThT molecules leads to low values of EF at a high apparent fluorescence lifetime value.

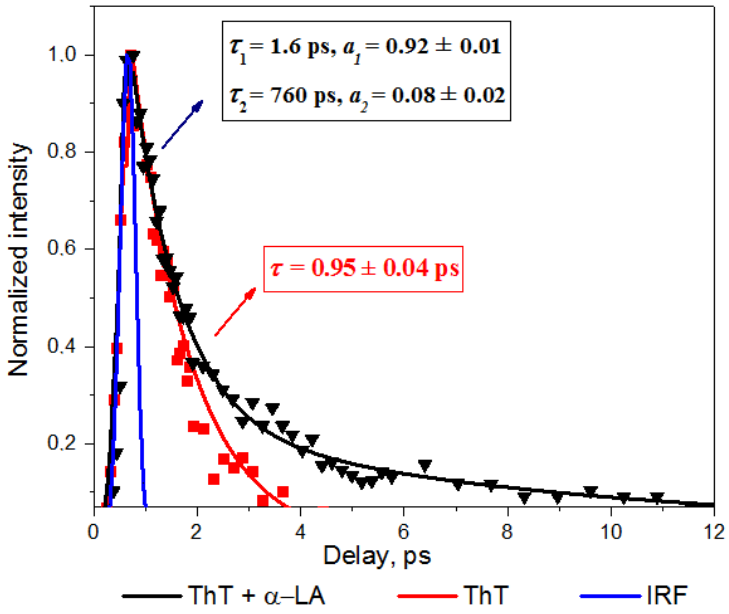

Figure 4. Fluorescence decay curves of ThT aqueous solution and ThT-αLA system (C_p = 714 μM) and their fits (solid lines) to monoexponential and biexponential decay law, respectively. The IRF corresponds to the temporal profile of the water Raman scattering signal.

To show that this hypothesis holds true in our case, we demonstrated the existence of weakly fluorescent bound ThT with ultrafast relaxation in the ThT-α-LA system using fluorescence up-conversion spectroscopy. Figure 4 demonstrates fluorescence decay for free ThT and ThT in the presence of 1 mM of α-LA, where, according to fluorescence titration experiments (Fig. 2C), all ThT is protein-bound. As can be seen, the decay time for free ThT was 1 ps, in agreement with the reported data [6; 21], while τ_{IRF} of the setup measured using the water Raman scattering signal was 200 fs. Importantly, a predominant fast component with a 1.6 ps lifetime (92 %) was observed for the ThT-protein system, while the amplitude of the slow component in fluorescence decay was significantly lower (only 8 %). As at the K_d value determined from the $F(C_p)$ dependence no more than 10% of ThT could be unbound at the protein concentration used in the fluorescence up-conversion experiments, the ~10-fold excess of the short component in Fig. 4 clearly show the existence of bound ThT molecules subpopulation with ultrafast decay. This fact explains the low EF for the ThT-α-LA system and provides additional evidence that the asynchronous change of fluorescence intensity and a lifetime with protein concentration is due to the masking of the fast component in fluorescence decay when using setups with ~200 ps IRF.

4 Conclusion

In this work, we studied the interaction of a model globular protein, α-LA, with ThT. Although ThT is considered to be an amyloid-specific probe, it exhibits also low-affinity binding to globular oligomeric proteins devoid of amyloid-like motifs in their structure. Investigation of ThT optical properties, namely, fluorescence intensity and fluorescence lifetime decay, at different protein concentration revealed the following trend, which is typical for the protein-ThT systems. It was observed that ThT fluorescence lifetimes reach a plateau at protein concentrations much lower than those for the fluorescence intensity saturation. Moreover, while an increase of ThT lifetime from ~1 ps up to ~990 ps was observed, its fluorescence intensity increased only 35-fold. To explain the observed discrepancy, numerical modelling of a two-component system with ultrafast (i.e. <<IRF) and slow components was performed. It was demonstrated that the presence of even a small fraction of slow component effectively masks the impact of the ultrafast component, hence, the observed saturation of ThT lifetime at low concentration of the added protein could be due to the appearance of a small amount of the ThT-protein complex. Next, sub-picosecond resolved fluorescence up-conversion techniques demonstrated the existence of bound ThT subpopulation with ultrafast relaxation, which could be responsible for the low fluorescence enhancement of ThT when bound to α-LA. We consider the obtained results to be of general significance when investigating fluorescence-based ThT interaction with systems exhibiting low-affinity binding, e.g. non-specific aggregates and globular proteins, as the observed effects (low enhancement of ThT fluorescence upon binding and earlier saturation of ThT fluorescence lifetime during protein aggregation) are also typical when monitoring fibril formation kinetics.

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