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Interaction of Thioflavin T with Amyloid Fibrils: Fluorescence Quantum Yield of Bound Dye

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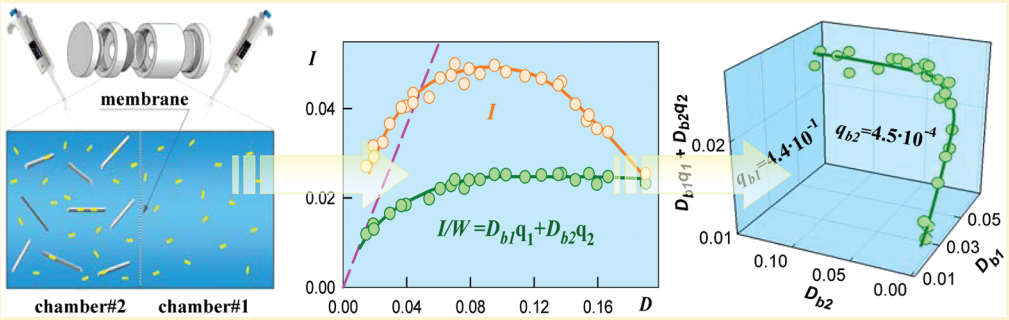

ABSTRACT: Benzothiazole dye thioflavin T (ThT) is a sensitive probe for amyloid fibril detection. The ThT probing is based on its unique ability to form highly fluorescent complexes with amyloid and amyloid-like fibrils. In this work we propose an approach of ThT fluorescence quantum yield determination based on two key points: (1) fluorescence intensity (I) presentation as a multiple of two factors one of which the correcting factor (W) depends only on total optical density of solution, while the other is a multiple of optical density and fluorescence quantum yield of ThT bound to amyloid fibrils or their sum in the case of several binding modes ($I = W \sum D_{bi}q_i$) and (2) sample and reference solutions preparation by equilibrium microdialysis. The last allows to determine the values of optical densities of free (D_f) and bound ($D_b = \sum D_{bi}$) dye. Thereafter, fluorescence quantum yield (q_{bi}) of ThT bound to sites of i binding mode can be determined by multiple linear regression. The fluorescence quantum yield of ThT molecules bound to the sites of two binding modes of lysozyme amyloid fibrils with high and low binding constants (7.5×10^6 and 5.6×10^4 M^{-1}) was found equal to 0.44 and 5×10^{-4}, respectively. The higher value of fluorescence quantum yield is larger than that for ThT in rigid isotropic solution (0.28), whereas the lower value is comparable to that of ThT in aqueous solution (1×10^{-4}). At the same time absorption spectra of ThT bound to these modes coincide (450 nm) and are red-shifted in comparison with that of free ThT in aqueous solution (412 nm).

INTRODUCTION

The fluorescence of extrinsic dyes is a tried and tested tool in biology and medicine for diagnostics and scientific research. In particular, thioflavin T (ThT) is a well-known tool for amyloid fibril detection, the deposition of which accompanies several serious diseases such as Alzheimer's and Parkinson's diseases, type II diabetes, and prion diseases.[1] ThT probing is based on the unique property of this dye to form highly fluorescent complexes with amyloid and amyloid-like fibrils.[2−4] Other than with acetylcholinesterase[5] and serum albumins,[6] ThT does not interact with globular proteins in a native state, nor does it interact with proteins in a molten globule and unfolded states or amorphous aggregates of proteins. The main reason of the dramatic increase of ThT fluorescence is explained by the restriction of the rotation motion of its benzothiazole and aminobenzoyl rings relative to each other in the excited state when ThT binds with amyloid fibrils.[7−9] The Krebs model of ThT binding to amyloid fibrils[10] suggests that the dye inserts itself into the grooves formed by the side chains of amino acids, making β-sheets. It is now evident that fibrils formed by different proteins, or even one protein under different conditions, are not identical.[11−14] The interaction of ThT with sites of different binding modes may also be different.[15−17] We suggested that the fluorescence quantum yield of ThT bound to amyloid fibrils depends not only on its internal mobility of benzothiazole and aminobenzoyl rings in the excited state but on its conformation in the ground state also.[18] That is why accurate determination of the fluorescence quantum yield of ThT bound to the sites of different binding modes of amyloid fibrils can be used for their structural investigations. This work shows how fluorescence quantum yield of ThT bound to the sites of different binding modes of amyloid fibrils can be determined using sample and reference solutions prepared by equilibrium microdialysis. The proposed approach is illustrated by the example of the determination of fluorescence quantum yield of ThT bound to lysozyme amyloid fibrils.

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EXPERIMENTAL METHODS

ThT from Sigma (U.S.A.) and Fluka (Switzerland) was used after purification by crystallization from a mixture of acetonitrile with ethanol in a 3:1 ratio.[1] ThT "UltraPure grade" from AnaSpec (U.S.A.) was used without after-purification. ThT was dissolved in 2 mM Tris-HCl buffer (pH 7.7) with 150 mM NaCl. Fluorescent dye ATTO-425 from ATTO-TEC (Germany), lysozyme, and buffer components from Sigma (U.S.A.) were used without after-purification. Lysozyme amyloid fibrils were prepared as described previously.[19] Fluorescence measurements were performed with a homemade spectrofluorimeter[20] and a Cary Eclipse spectrofluorimeter (Varian, Australia). An PBS solution of fluorescent dye ATTO-425, whose fluorescent and absorption spectra were similar to that of ThT, was taken as a reference for determining the fluorescence quantum yield of ThT bound to fibrils. Fluorescence of ThT and ATTO-425 was excited at 435 nm. The spectral slits width was 10 nm in most of experiments. Change of spectral slits width did not influence the experimental results. The fluorescence quantum yield of ATTO-425 is 0.9 (ATTO-TEC Catalogue 2009/2010 p.14).

Equilibrium microdialysis was performed with a Harvard Apparatus/Amika (U.S.A.) device, which consists of two chambers (500 μL each) separated by a membrane (MWCO 10 000) impermeable to particles larger than 10 000 Da. Equilibrium microdialysis implies allocation of two interacting agents, a ligand and receptor, in two chambers (#2 and #1, respectively) divided by a membrane permeable to the ligand and impermeable to the receptor (Figure 1). In our case, the amyloid fibrils were formed in 0.05 M KH2PO4-NaOH buffer. pH 6.3 solution were placed in chamber #1. The concentration of fibrils in terms of amyloidogening protein concentration was 0.4 mg/mL. A 2-fold increase or decrease in fibril concentration did not influence the final results. The ThT solution in the same buffer, with an initial concentration C_0, was placed in chamber #2. After equilibration, the free ThT concentrations in chambers #1 and #2 were equal (C_f), while the total ThT concentration in chamber #1 was greater than that in chamber #2 by the concentration of the bound dye (C_b), i.e., $C_b = C_0 - 2C_f$.

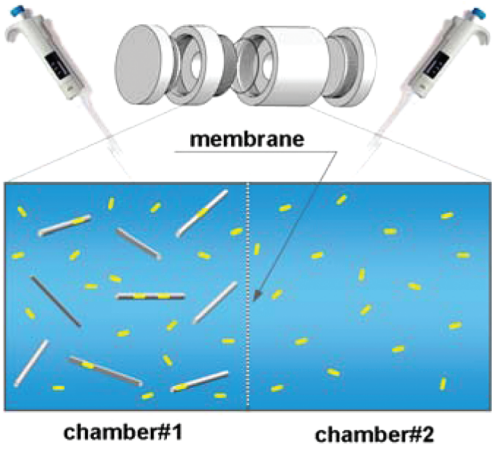

Figure 1. Principle of equilibrium microdialysis experiment. Details are in the text.

For performing equilibrium microdialysis, the devices were set on a rocking-bar in a thermostatted box for 48 h. All experiments were performed at 23 °C. In the test experiments, we put a solution of ThT of concentration C_0 in chamber #2 and the solvent in chamber #1. After 20 h of dialysis, the absorption spectra of samples from chambers #1 and #2 coincide ($D(\lambda)_{\#1} = D(\lambda)_{\#2}$). This means that 48 h is enough time to allow the dye to equilibrate between chambers #1 and #2. In addition, it was shown that $D(\lambda)_{\#1} = D(\lambda)_{\#2} = D(\lambda)_0/2$, which means that the dye does not bind to membrane or chambers walls. Optical densities of bound dye were determined as described previously.[21] In short, the absorption spectrum of the solution in chamber #1 represents the superposition of the absorption spectra of free ThT in a concentration C_f, ThT bound to fibrils in a concentration C_b ($D_b(\lambda)$) and the apparent absorption determined by light scattered by the fibrils ($D_{scat}(\lambda)$). The dependence of apparent optical density, determined by fibril light scattering, on λ was determined by equation: $D_{scat} = a\lambda^{-m}$. Coefficients a and m were determined from the linear part of the dependence $D(\lambda)_{\#1}$, where there is no active dye absorption, plotted in logarithmical coordinates $\log(D_{scat}) = f(\log(\lambda))$. The absorption spectra of ThT incorporated into amyloid fibrils was determined from the equation $D_b(\lambda) = D(\lambda)_{\#1} - D(\lambda)_{scat} - D(\lambda)_{\#2}$ (Figure 2). Absorption spectra were recorded by spectrophotometer U-3900H (Hitachi, Japan).

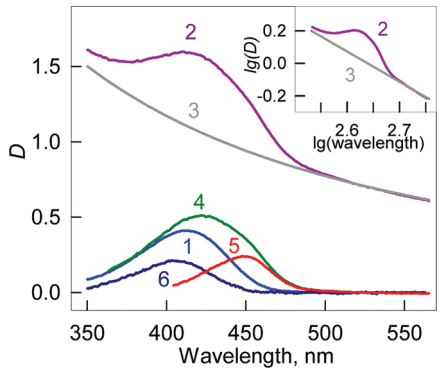

Figure 2. Absorption spectra of thioflavin T (ThT) incorporated in lysozyme amyloid fibrils. Curves 1 and 2 represent absorption spectra of solution in chamber #2 (free ThT in concentration C_f) and in chamber #1 (superposition of absorption spectra of free ThT in concentration C_f, ThT bound to fibrils in concentration C_b and apparent absorption caused by fibril light scattering) after reaching equilibrium. Curve 3 is the optical density determined by fibril light scattering: $D_{scat} = a\lambda^{-m}$. Coefficients a and m were determined from the linear part of curve 2 (where there is no active dye absorption) plotted in logarithmical coordinates $\log(D_{scat}) = f(\log(\lambda))$, inset, curve 3. Curve 4 is the total absorption of free and bound dye after light-scattering subtraction ($D(\lambda)_{\#1} - D_{scat}$). Curve 5 is the absorption spectra of ThT incorporated in amyloid fibrils $D_b(\lambda) = D(\lambda)_{\#1} - D(\lambda)_{scat} - D(\lambda)_{\#2}$ (the difference between 4 and 1 spectra). Curve 6 is the absorption spectra of free dye in a concentration equal to that of bound dye ($D(\lambda)_0 - 2D(\lambda)_2$).

RESULTS AND DISCUSSION

Fluorescence intensity of ThT in aqueous solution is very low but dramatically increases when it incorporates into amyloid fibrils. However, there are no data on the fluorescence quantum yield of the dye incorporated into amyloid fibrils. The problem of the determination of fluorescence quantum yield of ThT bound to amyloid fibrils is really difficult because a solution of ThT in the presence of amyloid fibrils is an equilibrium system

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Table 1. Characteristics of ThT Bound to Amyloid Fibrils and Free Dye in Aqueous Solution[a]

	λ_{max}, nm	mode no., i	$\varepsilon_{i,max} \times 10^{-4}$, M^{-1} cm^{-1}	$\varepsilon_{i,435} \times 10^{-4}$, M^{-1} cm^{-1}	$K_{bi} \times 10^{-5}$, M^{-1}	n_i	q_i
ThT bound to lysozyme amyloid fibrils	449	1	5.1	3.7	75	0.11	0.44
	449	2	6.7	5.8	0.56	0.24	0.0005
free ThT in aqueous solution	412		3.2	2.0			0.0001

[a]All values for ThT bound to lysozyme amyloid fibrils except the values of q_i, are taken from the work by Sulatskaya et al.;[21] all values for free ThT in aqueous solution are taken from the work by Sulatskaya et al.[18]

of free dye and dye bound to fibrils. Until recently, the evaluation of the concentrations of free or bound ThT was an unsolved problem.

In the previous work we showed how the equilibrium concentrations of free ThT and ThT bound to fibrils can be determined for solutions of ThT in the presence of amyloid fibrils with the use of equilibrium microdialysis.[21] Surprising, equilibrium microdialysis inherently designed for determination of affinity and stoichiometry of ligand−receptor interaction was never used for investigations of ThT binding with fibrils before. At the same time only equilibrium microdialysis allowed to obtain the true reference solution (free ThT) for the sample solution containing ThT and amyloid fibrils. The recorded optical density of reference solution (D_f) allowed to obtain the concentration of free dye (C_f) and consequently the concentration of dye bound to fibrils (C_b). A number of equilibrium microdialysis procedures performed for different concentrations of input ThT (C_0) allows to obtain the dependence of C_b on C_f and to determine ThT−amyloid fibril binding parameters by multiple nonlinear regression: the number of binding modes i, the number of binding sites n_i and binding constants K_{bi} of each mode. On the basis of the obtained date, concentrations of the dye bound to the sites of each mode can be determined. Further, the record of absorption spectrum of the sample solution (ThT in the presence of amyloid fibrils) relative to reference solution (free ThT) each prepared in one equilibrium microdialysis experiment allowed to obtain absorption spectrum of the ThT bound to fibrils $D_b(\lambda) = l\sum C_{bi}\varepsilon_{bi}(\lambda)$. The last procedure performed for a set of solutions obtained by equilibrium microdialysis (the same solutions for which the values of n_i and K_{bi} were determined) allowed to calculate molar extinction coefficients of ThT bound to the sites of the different binding modes ($\varepsilon_{bi}(\lambda)$) by multiple linear regression. The values of n_i, K_{bi}, and $\varepsilon_{bi}(\lambda)$ determined for ThT bound to lysozyme amyloid fibrils earlier[21] are given in Table 1. Finally, on the basis of C_{bi} and ε_{bi} the optical densities of ThT bound to the sites of the different binding modes D_{bi} can be calculated.

The same solutions prepared by equilibrium microdialysis, for which ThT binding parameters and optical density were determined, can be used for determination of the fluorescence quantum yields of ThT bound to the sites of the different binding modes of amyloid fibrils. This is possible because fluorescence intensity can be presented as a multiple of two factors: correcting factor W which depends on the total optical density of a solution and the sum of the products of fluorescence quantum yields and optical densities of the ThT bound to the sites of the different binding modes ($I = W(\sum D_{bi}q_{bi})$). In the next sections we explain why such presentation of fluorescence intensity is valid and show how correcting factor W can be determined. Thereafter, by example of interaction of ThT with lysozyme amyloid fibrils we show how to calculate the values of q_{bi} on the basis of the record of fluorescence intensity and determined D_{bi} values for a set of solutions prepared by equilibrium microdialysis experiments with different C_0.

Dependence of the Fluorescence Intensity on the Optical Density of the Fluorescent Substance and the Total Optical Density of the Solution. The fluorescence intensity of any single-component solution of fluorescent dye can be presented as follows (see, e.g., refs 22 and 23):

$$I = kI_0(1 - T)q_{FL} = kI_0(1 - 10^{-D})q_{FL} \quad (1)$$

Here I_0 is the intensity of the excitation light, k is the proportionality coefficient, $(1 - T)$ is the fraction of excitation light absorbed by the solution, $T = 10^{-D}$ is transmission, where D is the optical density of solution, and q_{FL} is the fluorescence quantum yield of the dye. Thus fluorescence intensity of the dye solution is proportional to the part of the excitation light absorbed by solution ($1-10^{-D}$), but not to the concentration of the fluorescent dye. It is evident that the dependence of I on D is a curve with saturation ($I = kI_0q_{FL}$ at $D \to \infty$).

Fluorescence intensity is measured in arbitrary units. It can be normalized ($kI_0 = 1$) so that at $D \to \infty$ fluorescence intensity will be numerically equal to fluorescence quantum yield of the dye $I = q_{FL}$. Figure 3a shows the dependence of the fluorescent intensity of the dye ATTO-425 solution, which was used in this work as a standard substance with known quantum yield, on the optical density of solution. When, as for ATTO-425 solutions, the total optical density of solution D coincides with the optical density of fluorescent substance D_{FL}, the eq 1 can be written as follows

$$I = kI_0\frac{1 - 10^{-D}}{D}D_{FL}q_{FL} \quad (2)$$

Note should be taken that even experienced researchers who do not specialize in fluorescence techniques do not take into account the fact that the plateau of the dependence of fluorescence intensity on the optical density of the fluorescent substance is its general property and that it does not necessarily indicate the saturation of binding centers. That is why, nearly all existing data on the parameters of ThT binding to amyloid fibrils have been based on the assumptions that the recorded fluorescence intensity is proportional to the concentration of bound dye ($I = kC_b$) and that the dependence of fluorescence intensity on C_b reaches a plateau when all binding sites (nC_p, where C_p is the concentration protein solution used for amyloid fibril formation, n is the number of dye binding sites on the amyloid fibrils per protein molecule) are occupied ($I_{max} = knC_p$).[24]

For a solution consisting of two components, one of which absorbs (with optical density D_{ABS}) but does not fluoresce, while the other absorbs (with optical density D_{FL}) and

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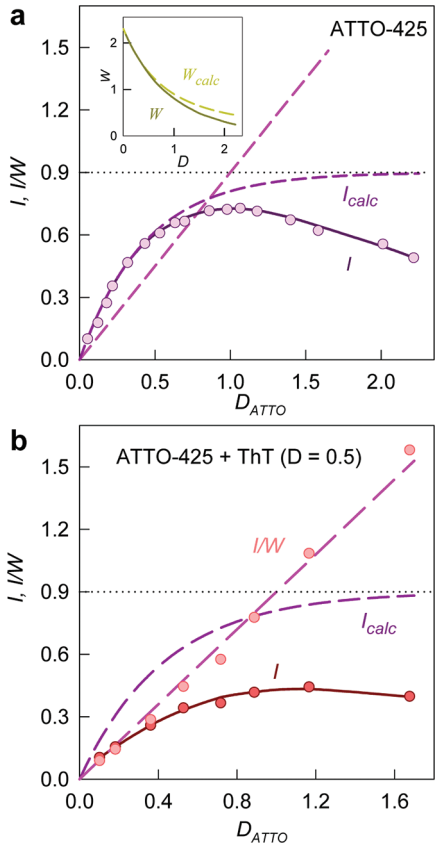

Figure 3. Fluorophore's dependence of fluorescence intensity on optical density and on total optical density of the solution. (a) The dependences of fluorescence intensity on the optical density (D_{ATTO}) of the fluorescent dye ATTO-425 with a known quantum yield (q = 0.9), calculated as $I_{calc} = ((1 - 10^{-D})/D)Dq$ and experimentally recorded I. The dependences $W_{calc} = ((1 - 10^{-D})/D)$ and $W = I/Dq$ on total optical density are given in the inset. The straight line is the dependence of Dq on D, calculated as $Dq = I_{calc}/W_{calc} = I/W$. (b) The calculated ($I_{calc}$) and experimentally recorded (I) dependencies of fluorescence intensity of the two component solution containing fluorescent dye ATTO-425 and ThT on optical density of ATTO-425. D_{ThT} = 0.5. Dots are the limiting value of I at $D \to \infty$. The straight line is the dependence of Dq on D, calculated as $Dq = I_{calc}/W_{calc} = I/W$.

fluoresces (with quantum yield q_{FL}) fluorescence intensity will be as follows:

$$I = kI_0(1 - T_{FL+ABS})\frac{D_{FL}}{D_{FL} + D_{ABS}}q_{FL} = kI_0\frac{1 - 10^{-D}}{D}D_{FL}q_{FL} \quad (3)$$

Here $D_{FL}/(D_{FL} + D_{ABS})$ is the part of light absorbed by the fluorescent component and $D = D_{FL} + D_{ABS}$ is the total optical density of the solution. As in the case of the one component solution, fluorescence intensity can be normalized ($kI_0 = 1$) so that $I = D_{FL}/(D_{FL} + D_{ABS})q_{FL}$ at $D_{FL} + D_{ABS} \to \infty$, and consequently $I = q_{FL}$ at $D_{FL} \to \infty$ and $I = 0$ at $D_{ABS} \to \infty$. Figure 3b shows the dependence of fluorescence intensity of PBS solution of the fluorescent dye ATTO-425 on its optical density ($D_{FL} = D_{ATTO}$) in the presence of ThT which in these conditions absorbs but does not fluoresce ($D_{ABS} = D_{ThT}$).

Finally, if in solution along with a nonfluorescent component (D_{ABS}) there are several fluorescent components, eq 3 will be as follows:

$$I = kI_0\frac{1 - 10^{-D}}{D}\sum_i D_{FLi}q_{FLi} = kI_0\frac{1 - 10^{-D}}{D}D_{FL}\sum_i \frac{D_{FLi}}{D_{FL}}q_{FLi} \quad (4)$$

where $D = D_{FL} + D_{ABS}$ is the total optical density of solution and $D_{FL} = \sum_i D_{FLi}$.

Thus, it is evident that in eqs 2−4, two factors can be selected. One of the factors depends on the total optical density of the solution only and does not depend on the relative contributions of the optical densities of the fluorescent and nonfluorescent components:

$$W_{calc} = \frac{1 - 10^{-D}}{D} \quad (5)$$

The other factor is a product of the optical density and the quantum yield of the fluorescent component ($D_{FL}q_{FL}$) or the sum of the product of these values if there are several fluorescent components in solution.

The dependence of W_{calc} on the total optical density of solution D calculated based on eq 5 is presented in Figure 3a (insert). The fluorescence intensity corrected on the factor depending on total optical density of solution I/W_{calc} will be proportional to the optical density of fluorescent components if there is only one fluorescent component. For multicomponent system the dependence will be also linear if the contributions of fluorescent components do not change with the increase in total optical density (D_{FLi}/D_{FL}) = const. In the other cases, this dependence becomes nonlinear.

Experimentally Determined Dependence of the Correcting Factor *W* on the Total Optical Density of the Solution. The experimental dependence of fluorescence intensity on the optical density of the fluorescent substance can differ from the calculated one. The fact that the recorded fluorescence intensity, after reaching some value of optical density, begins to decrease with an increase in the content of the fluorescent substance is a general property of such dependences (Figure 3a,b) and does not indicate the existence of self-quenching or dye aggregation, as it has been frequently suggested (see e.g., refs 25−27). In reality, the dependence of fluorescence intensity (I) on the optical density of the fluorescent substance (D_{FL}) differs from the calculated one because an increase in the total optical density of the solution results in an increased absorption of excitation light by the solution layers adjacent to the front wall of the spectrofluorimeter cell. The detection system of the spectrofluorimeter "sees" only the central part of the cell, which is reached by a respectively smaller amount of excitation light. Because of this effect, the recorded fluorescence intensity begins to diminish after the optical density reaches a certain value. The effects discussed above depend on the particular instrument used in the experiment and must be taken into account. This can be performed by replacing W_{calc} with an experimentally determined value W. The experimental dependence of W on the total optical density of the solution was determined based on the experimentally recorded dependence of the fluorescence intensity of ATTO-425 on its optical density ($W = (I/I_0k)/Dq$,

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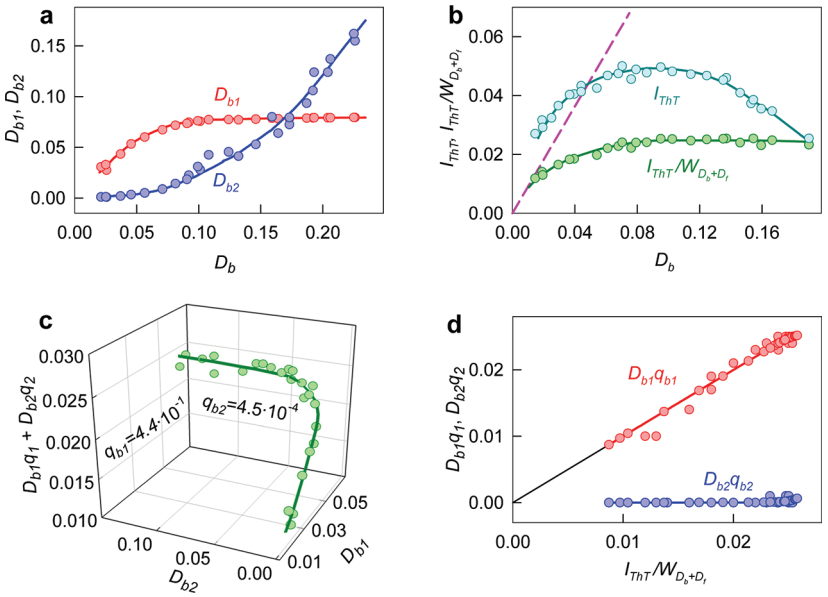

Figure 4. Determination of the fluorescence quantum yield of ThT bound to amyloid fibril. (a) Optical density of ThT bound to amyloid fibril as a superposition of optical densities of the dye bound to sites of mode 1 (D_{b1}) and mode 2 (D_{b2}). (b) The dependencies of the experimentally recorded fluorescence intensity (I_{ThT}) and corrected fluorescence intensity ($I_{ThT}/W_{D_b+D_f}$) of ThT bound to lysozyme amyloid fibril on its optical density (D_b). The dashed line is the dependence of Dq on D for the fluorescent dye ATTO-425 (etalon with $q = 0.9$). Fluorescence measurements were conducted using a homemade spectrofluorimeter[20] and a commercial Cary Eclipse spectrofluorimeter (Varian, Australia). (c) 3D dependence of $D_{b1}q_{b1}+D_{b2}q_{b2}$ on D_{b1} and D_{b2}. Experimental data, best-fit curve and the values of q_{b1} and q_{b2}, determined from multiple nonlinear regressions are presented. (d) Corrected fluorescence intensities of $I_{ThT}/W_{D_b+D_f}$ as a superposition of the corrected fluorescence intensities of the dye bound to mode 1 ($D_{b1}q_{b1}$) and mode 2 ($D_{b2}q_{b2}$).

Figure 3a, insert). The factor kI_0must be chosen so that at low optical density experimental and calculated curves $I = f(D)$ and $W = f(D)$ coincide. When $D \to 0$, $W = W_{calc} = 2.303$. The value of W as the value of W_{calc} is determined only by the total optical density of the solution and does not depend on the contribution of the optical density of the fluorescent substance. The possibility of using the obtained experimental dependence of W on optical density for determination of the fluorescence quantum yield of fluorescent component was tested on a two-component PBS solution containing ATTO-425 and ThT, which, in these conditions, absorbs but does not fluoresce (Figure 3 b). Thus, subject to the above the value of W_{calc} must be replaced with W in eqs 2−4.

Fluorescence of ThT Solutions in the Presence of Amyloid Fibrils. Dependence of the Corrected Fluorescence Intensity *I*/*W* on the Optical Density of Bound Dye. The solution of ThT in the presence of amyloid fibrils is a two-component system in which some fraction of molecules (free ThT, unbound to fibrils) absorbs the excitation light (with optical density $D_f \equiv D_{ABS}$) but does not fluoresce, while the other fraction of ThT molecules (ThT bound to fibrils) absorbs the excitation light ($D_b \equiv D_{FL}$) and fluoresces with quantum yield q_b. The total optical density of this solution is $D_b + D_f$. By assuming that all binding sites of ThT with fibrils are identical and independent of each other, the fluorescence intensity is determined as follows:

$$I_{ThT}/W_{D_b+D_f} = D_b q_b \quad (6)$$

where $I_{ThT} = I/kI_0$, I is recorded fluorescence intensity, k is normalizing coefficient (see eq 1), which can be determined on the basis of fluorescence intensity recorded for the fluorescent dye with known fluorescence quantum yield (in our case for ATTO-425):

$$I_{ATTO}/W_{ATTO} = D_{ATTO}q_{ATTO} \quad (7)$$

where $I_{ATTO} = I/kI_0$. If there are several binding modes in fibrils, this equation will be as:

$$I_{ThT}/W_{D_b+D_f} = \sum_i D_{bi}q_{bi} = D_b \sum_i \frac{D_{bi}}{D_b} q_{bi} \quad (8)$$

where D_{bi} and q_{bi} are the optical densities and fluorescence quantum yields of dye molecules bound to sites of i mode. In eqs 6 and 8, $W_{D_b+D_f}$ is an experimentally recorded correcting multiplier. To determine the dependence of the corrected fluorescence intensity $I_{ThT}/W_{D_b+D_f}$ on the optical density of bound dye D_b, it is necessary, to know not only D_b but also the total optical density the of solution, $D_b + D_f$. The last value is needed for choosing correcting multiplier $W_{D_b+D_f}$. As was said above, the values of D_b and D_f can be obtained by recording optical density of the sample and reference solutions obtained by equilibrium microdialysis.

Determination of the Fluorescence Quantum Yield of ThT Bound to Fibrils. Fluorescence quantum yield of ThT bound to fibrils is determined on the basis of fluorescence intensity and optical density of solutions prepared by equilibrium microdialysis. If all binding sites in fibrils are identical and independent (one binding mode) the value of q_b is determined as an average of several values independently measured for samples with different dye concentrations after equilibrium microdialysis. As it was shown earlier[21] lysozyme amyloid fibrils have two binding modes. Figure 4b shows the dependences of I_{ThT} and $I_{ThT}/W_{D_b+D_f}$ of ThT bound to lysozyme fibrils on its optical density (D_b). As expected, in

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the case of two binding modes, the dependence of $I_{ThT}/W_{D_b+D_f}$ on D_b is not linear. These dependences allow the determination of the fluorescence quantum yield of ThT bound to sites of different binding modes.

To determine the values of q_{b1} and q_{b2}, it is necessary to know data sets of three related values D_{b1}, D_{b2} and $D_{b1}q_{b1} + D_{b2}q_{b2}$ that correspond to the several equilibrium microdialysis experiments (for different C_0 and, consequently, different D_b) . The values of D_{b1} and D_{b2} for each D_b can be determined on the basis of the values of the molar extinction coefficients and concentrations of ThT molecules bound to the sites of two modes in samples obtained after equilibrium microdialysis: $D_{b1} = \varepsilon_{b1}C_{b1}l$ and $D_{b2} = \varepsilon_{b2}C_{b2}l$. The description of the determination ε_{b1} and C_{b1} is given in the work by Sulatskaya et al.[21] The values of $(D_{b1}q_{b1} + D_{b2}q_{b2})$ can be determined on the basis of fluorescence experiment using eq 8. The dependence of $D_{b1}$and D_{b2} on D_b are given in Figure 4a. Data for plotting this dependence are taken from our previous work.[21] The dependence of $(D_{b1}q_{b1}+D_{b2}q_{b2})$ on D_b is given in Figure 4b. The fluorescence quantum yields of the dye bound to the sites of each binding mode can be determined on the basis of the large sets of values D_{b1}, D_{b2} and $D_{b1}q_{b1} + D_{b2}q_{b2}$ by multiple linear regression. The results are given in Figure 4c and in Table 1. The contribution of the fluorescence intensity of ThT bound to each mode in the total fluorescence intensity is presented in Figure 4d.

CONCLUSIONS

In this work we propose an approach of ThT fluorescence quantum yield determination based on two key points: (1) fluorescence intensity (I_{ThT}) presentation as a multiple of two factors one of which the correcting factor (W) depends only on total optical density of solution (the dependence of W on total optical density of solution can be determined beforehand), while the other is a multiple of optical density and fluorescence quantum yield of ThT bound to amyloid fibrils or their sum in the case of several binding modes ($I_{ThT}/W = \sum D_{bi}q_{bi}$) and (2) sample and reference solutions preparation by equilibrium microdialysis. The last allows to determine the values of optical densities of free (D_f) and bound ($D_b = \sum D_{bi}$) dye, as well as optical densities of the dye bound to the sites of each binding mode (D_{bi}).

This work is the terminal one in a series of two works where we showed how on the basis of solutions prepared by equilibrium microdialysis we can obtain information about fibrils−ThT binding parameters (the number of binding modes i, the number of binding sites of each mode n_i and binding constant of each binding mode K_{bi}), absorption spectrum (or spectra in the case of several binding modes) of ThT bound to fibrils and fluorescence quantum yield (or quantum yields in the case of several binding modes) of ThT bound to fibrils.

Initially, we input the solution of free ThT (dye concentration is C_0) in one chamber and the suspension of amyloid fibril in the other chamber (protein concentration which forms amyloid fibrils is C_p) divided by a membrane permeable to the ligand and impermeable to the receptor. Equilibrium microdialysis experiments are done for a large number of different C_0. The experiments done with these sample and reference solutions allow to obtain information about ThT−amyloid fibrils binding parameters and concentration (points 1.1−1.3), absorption spectra (points 2.1−2.2), and fluorescence quantum yields (points 3.1−3.2) of ThT bound to sites of different binding modes of fibrils:

1.1 first, optical density of reference solution (D_f) is recorded and the concentration of free dye (C_f) is determined. The concentration of bound dye (C_b) is determined on the basis of C_0 and C_f

1.2 such experiments done for a set of solutions obtained by equilibrium microdialysis give the dependence of $C_b = f(C_f)$ on the basis of which, binding parameters (i, n_i, and K_{bi}) are determined by multiple nonlinear regression

1.3 on the basis of the values n_i, K_{bi}, C_f and C_p the concentration of ThT bound to binding sites of different modes (C_{bi}) are determined

2.1 the absorption spectrum of bound ThT $D_b(\lambda)$ is recorded using the sample and reference solutions prepared by equilibrium microdialysis

2.2 the absorption spectra $D_b(\lambda)$ recorded for a set of solutions obtained by equilibrium microdialysis together with C_{bi} (determined according point 1.3) allow to determine molar extinction coefficient of ThT bound to binding sites of each mode ($\varepsilon_{bi}(\lambda)$) by multiple linear regression; on the basis of C_{bi} and ε_{bi} the values D_{bi} are calculated

3.1 fluorescence intensity (I_{ThT}) of the ThT solutions in the presence of amyloid fibrils (sample solution obtained by equilibrium microdialysis) is recorded. The value $I_{ThT}/W_{D_b+D_f}$ is calculated on the basis of total optical density of solution ($D_b + D_f$) (points 1.1 and 2.1) and the dependence of correcting coefficient W on total optical density of solution determined beforehand. Then value $I_{ThT}/(W_{D_b+D_f})$ is normalized in the term of the multiple of optical density and fluorescence quantum yield. Normalizing coefficient is determined using the solution of the dye with the known quantum yield. The corrected and normalized value $I_{ThT}/W_{D_b+D_f}$ equals $D_{b1}q_{b1} + D_{b2}q_{b2}$. For the same solutions D_{b1} and D_{b2} are determined (see point 2.2)

3.2 a set of values $(D_{b1}q_{b1} + D_{b2}q_{b2})$, D_{b1}, and D_{b2}, obtained for a large series of solutions obtained by equilibrium microdialysis allows us to determine q_{b1} and q_{b2} by multiple linear regression

The proposed approach of determination fluorescence quantum yield was test on lysozyme amyloid fibrils. As has been predicted,[18] the fluorescence quantum yield of ThT bound to amyloid fibrils depends not only on the restriction of high-frequency rotational oscillations of benzothiazole and aminobenzoyl rings of ThT molecule against each other in the excited state, but also on the molecular conformation of ThT in the ground state and could be both larger or smaller than that of the rigid isotropic solution ($q = 0.28$). It has been shown that in the case of lysozyme amyloid fibrils, the ThT molecules that are bound to fibrils with higher binding constants have higher fluorescence quantum yield ($q = 0.44$), while ThT molecules which bind to fibrils with low binding constants, have a very low fluorescence quantum yield ($q = 0.0005$). The existence of a binding mode at which ThT has the same order of magnitude of fluorescence quantum yield as it has in water[18] ($q = 0.0001$) was reliably shown. Interestingly, the absorption spectra of ThT bound to both modes coincide and is significantly red-shifted ($\lambda_{max} = 449$ nm) in comparison to that of ThT in aqueous solution ($\lambda_{max} = 412$ nm).

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Notes

The authors declare no competing financial interest.

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