

Mechanism of thioflavin T binding to amyloid fibrils

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Abstract

Thioflavin T is a benzothiazole dye that exhibits enhanced fluorescence upon binding to amyloid fibrils and is commonly used to diagnose amyloid fibrils, both *ex vivo* and *in vitro*. In aqueous solutions, thioflavin T was found to exist as micelles at concentrations commonly used to monitor fibrils by fluorescence assay (~ 10 – $20 \mu\text{M}$). Specific conductivity changes were measured at varying concentration of thioflavin T and the critical micellar concentration was calculated to be $4.0 \pm 0.5 \mu\text{M}$. Interestingly, changes in the fluorescence excitation and emission of thioflavin T were also dependent on the micelle formation. The thioflavin T micelles of 3 nm diameter were directly visualized using atomic force microscopy, and bound thioflavin T micelles were observed along the fibril length for representative fibrils. Increasing concentration of thioflavin T above the critical micellar concentration shows increased numbers of micelles bound along the length of the amyloid fibrils. Thioflavin T micelles were disrupted at low pH as observed by atomic force microscopy and fluorescence enhancement upon binding of thioflavin T to amyloid fibrils also reduced by several-fold upon decreasing the pH to below 3. This suggests that positive charge on the thioflavin T molecule has a role in its micelle formation that then bind the amyloid fibrils. Our data suggests that the micelles of thioflavin T bind amyloid fibrils leading to enhancement of fluorescence emission.

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1. Introduction

Vassar and Culling (1959) introduced thioflavin T (ThT),¹ a cationic benzothiazole dye that shows enhanced fluorescence upon binding to amyloid in tissue sections. The detection of amyloid in the kidney with ThT after differentiation in acidic solutions was dem-

onstrated to be highly specific (Vassar and Culling, 1959). The superiority of ThT for detection of amyloid was further confirmed by the pathologists Saeed and Fine (1967) who carefully compared Congo red, crystal violet, van Gieson, and ThT dyes for their binding to several positive and negative control tissues.

In tissue sections, ThT was shown to bind to other connective tissues such as cartilage matrix, elastic fibers, and mucopolysaccharides (Kelényi, 1967; Vassar and Culling, 1959). Binding of ThT to DNA and RNA also demonstrates enhanced fluorescence (Canete et al., 1987; Vassar and Culling, 1959). Kelényi (1967) modified the staining conditions to lower pH (between

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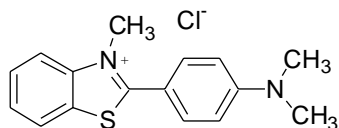
¹ ThT, thioflavin T; AFM, atomic force microscope; TBS, Tris buffered saline.

pH 0.8 and 2.8) compared to that described by Vassar and Culling (1959) to improve the specificity of staining for amyloid and reduced the staining to nucleic acids.

Due to the specificity of binding of thioflavin T to amyloid fibrils, it has found many applications such as diagnosis of amyloid in tissue sections using fluorescence microscopy (Hobbs and Morgan, 1963; Kelényi, 1967; Saeed and Fine, 1967; Vassar and Culling, 1959), monitoring extracted amyloid and in vitro amyloid fibril formation using fluorescence spectroscopy (LeVine, 1993; Naiki et al., 1989) and direct observation of amyloid fibril growth using total internal reflection fluorescence microscopy (Ban et al., 2003). More recently attempts are being made to detect amyloid- β in vivo using uncharged derivatives of thioflavin T that readily enter the brain (Klunk et al., 2001).

Kelényi (1967) studied a variety of thiazole dyes lacking the azo group, including primuline, thioflavin S and T by detailed analysis using paper chromatography, spectroscopy, and analytical ultracentrifugation, and described different components of each dye to explain the mechanism of specificity of these dyes. LeVine (1993, 1995) made considerable advances in understanding the fluorescence properties of ThT, and observed the appearance of a new excitation peak at 450 nm, upon binding to amyloid fibrils that is responsible for enhanced fluorescence emission at 482 nm.

The structure of thioflavin T, shown below, has a hydrophobic end with a dimethylamino group attached to a phenyl group, linked to a more polar benzothiazole group containing the polar N and S.



This combination of polar and hydrophobic regions creates the possibility for thioflavin T molecules to form micelles in aqueous solution, with hydrophobic interiors and the positively charged N pointing toward the solvent. There are suggestions that the thiazole nitrogen of the dye and hydroxyl groups of tissue structures form hydrogen bonds to give rise to specific binding of these dye molecules to amyloid and other tissue structures (Kelényi, 1967).

Despite its common use in the diagnosis of amyloid fibrils in ex vivo, in vitro, and animal model studies, not much is known about the mechanism of thioflavin T binding. The aim of the present work is to understand the mechanism of binding of thioflavin T to amyloid fibrils. The properties of ThT were studied in aqueous solutions as a function of the concentration. ThT

micelles bound to amyloid fibrils, was also observed using atomic force microscopy. This manuscript provides novel insight into the mechanism of thioflavin T binding to amyloid fibrils.

2. Experimental procedures

2.1. Thioflavin T solutions

ThT was purchased from Fluka and stock solutions were prepared by dissolving ~ 3 mg dry powder in 1 ml water. The solution was filtered through 0.22 μm syringe filters followed by measurement of the concentration by diluting the stock solution in ethanol and using an extinction coefficient of 26,620 $\text{M}^{-1} \text{cm}^{-1}$ at 416 nm (Wall et al., 1999). The stock solution was stored at 4 $^{\circ}\text{C}$ covered with foil and used for up to a month to make assay solutions by diluting either in water or desired buffer.

2.2. Conductivity measurements

Conductivity was measured for ThT solutions ranging from 0.1 to 250 μM in water and dimethyl sulfoxide using either a YSI model 32 conductivity meter or a Orion conductivity meter. The conductivity meter used the constant current square wave principle, and the probe used was a platinum coated electrode YSI (3417) with a 1.0 cm cell constant. The probe was dipped in the ThT solutions and conductivity was recorded in $\mu\text{S}/\text{cm}$. The values of specific conductivity were plotted against ThT concentrations on a log scale.

2.3. Calculating critical micellar concentrations

CMC values were calculated by plotting linear regression of the changes in specific conductance against ThT concentrations, in both the low and the high concentration ranges, followed by calculating a concentration of ThT where both the linear regression values are the same (Mukerjee and Mysels, 1971).

2.4. Fluorescence assays

ThT fluorescence emission was measured with excitation at 450 nm and recording the spectrum between 465 and 565 nm with 5 nm slits using a FluoroMax 2 spectrofluorometer (Instruments S. A. Jobin Yuan-Spex). The excitation spectra were collected by setting the emission wavelength to 482 nm and collecting the spectrum between 300 and 470 nm with 5 nm slit widths, and 1 s integration time and 1 nm interval. Emission spectra between 465 and 565 nm were collected upon excitation at 450 nm. Excitation and emission spectra in the presence of amyloid fibrils were measured with varying concentrations

of ThT and 5 ng/ml amyloid (calculated based on starting protein concentration) before collecting the spectra.

2.5. Fluorescence anisotropy measurements

Anisotropy measurements were made using the Spex Fluorolog 2 autopolarizer accessory, which allowed the measurement of fluorescence emission with either normal or crossed positions of the horizontal and the vertical polarizers. The anisotropy values were calculated using the following equation:

$$A = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}),$$

where A is anisotropy and $G = I_{HV}/I_{HH}$, and V and H in the subscript represent the vertical or the horizontal position of the excitation and the emission polarizers. The position of the excitation polarizer is represented in the first place in the subscript followed by the position of the emission polarizer. For a rigid system the maximum anisotropy value is 0.4, for freely rotating small molecules the anisotropy values are considerably smaller. An increase in the size of the fluorescent molecule would be accompanied by an increase in the anisotropy as the rotation of the molecules is reduced. In the absence of any association, the anisotropy or polarization should be independent of the concentration. ThT anisotropy was measured by setting the excitation wavelength to 450 nm, emission wavelength to 482 nm, each slit was set to 10 nm and the intensity at all four positions was measured using the autopolarizer set to kinetic mode. The anisotropy value was measured averaged over 100 s, with measurements every 14 s.

2.6. In vitro fibril formation

Amyloid fibrils were made from 1 mg/ml insulin, and 0.5 mg/ml of the immunoglobulin light chain variable domain SMA, at pH 2.0 with stirring for 3–8 h, at 37 °C both in the absence and presence of 20 μ M ThT. Human calcitonin was incubated at 5 mg/ml concentration in water for a few days to form amyloid fibrils.

Fibrils were made from 8 μ M EDVAVYYCHQYYSS peptide solution in water (from a syringe filtered stock solution) by incubating the solution without stirring at 4 °C for several days. Forty-eight days ED fibrils were incubated with either 50 μ M ThT or 100 μ M Congo red solution and ultracentrifuged at 60,000 rpm to remove the excess dye. The pellet was resuspended in water before placing the sample on freshly cleaved mica.

2.7. Atomic force microscopy

Amyloid fibril suspensions of 0.05–0.5 mg/ml were incubated in the absence and presence of 20 μ M ThT or varying concentrations of ThT and deposited on freshly cleaved mica and dried immediately with nitro-

gen gas. The mica surface was then washed with deionized water to remove salts. The samples were imaged with either an Autoprobe CP AFM with a 25 μ m scanner (Park Scientific, Sunnyvale, CA, USA) at Department of Physics, University of California, Santa Cruz, USA, or a Bioscope with a Nanoscope IV controller and a 100 μ m scanner (Digital Instruments, Santa Barbara, CA, USA) at Centre for Cellular and Molecular Biology, Hyderabad, India, using non-contact mode. The images were taken in air, ambient conditions, at a scan frequency of less than 1 Hz, using either silicon nitride/silicon non-contact tips.

2.8. Diffusion coefficient determination using 2D DOSY NMR

NMR spectra were recorded in D₂O (Aldrich, 99.9% D), DMSO-*d*₆ (Aldrich, 99.9% D), and DMF-*d*₇ (Aldrich, 99.9% D) at room temperature using a Bruker advance DRX 300 MHz Fourier transform (FT) NMR spectrometer equipped with a 5 mm multinuclear inverse probehead with Z-shielded gradient. Chemical shifts are given on the δ scale and were referenced to TMS (0.0 ppm) for proton spectra. ¹H high-resolution 2D DOSY NMR experiment was performed at 25 °C using the BPP (LED) pulse sequence from the Bruker software library. Fifteen spectra were acquired, with gradient pulses of 1.5 ms ranging in strength from 1 to 30 G cm⁻¹ with a diffusion delay of 0.1 s in 4K data points. Processing was done using Bruker standard software for DOSY.

3. Results and discussion

3.1. Thioflavin T forms micelles in aqueous solvents

3.1.1. Electrical conductivity measurements

Electric conductivity is an established method used to determine critical micellar concentrations of polar surfactants in aqueous solutions (Mukerjee and Mysels, 1971). Concentration-dependent changes in the specific conductance of ThT in water was measured; the data was essentially constant at sub-micromolar concentrations, followed by a linear increase above 4 μ M ThT (Fig. 1A). To perform the electrical conductivity experiments, we chose de-ionised water conditions so that the salts in buffers do not interfere in the conductivity measurements.

The increase in conductivity with increasing concentrations of thioflavin T is most likely indicative of a change in charge distribution that occurs by micelle formation, due to clustering of the positive charges of the ThT. Considering the amphiphilic nature of ThT, we believe that these micelles have a hydrophobic interior made of the dimethylaminophenyl group, with the

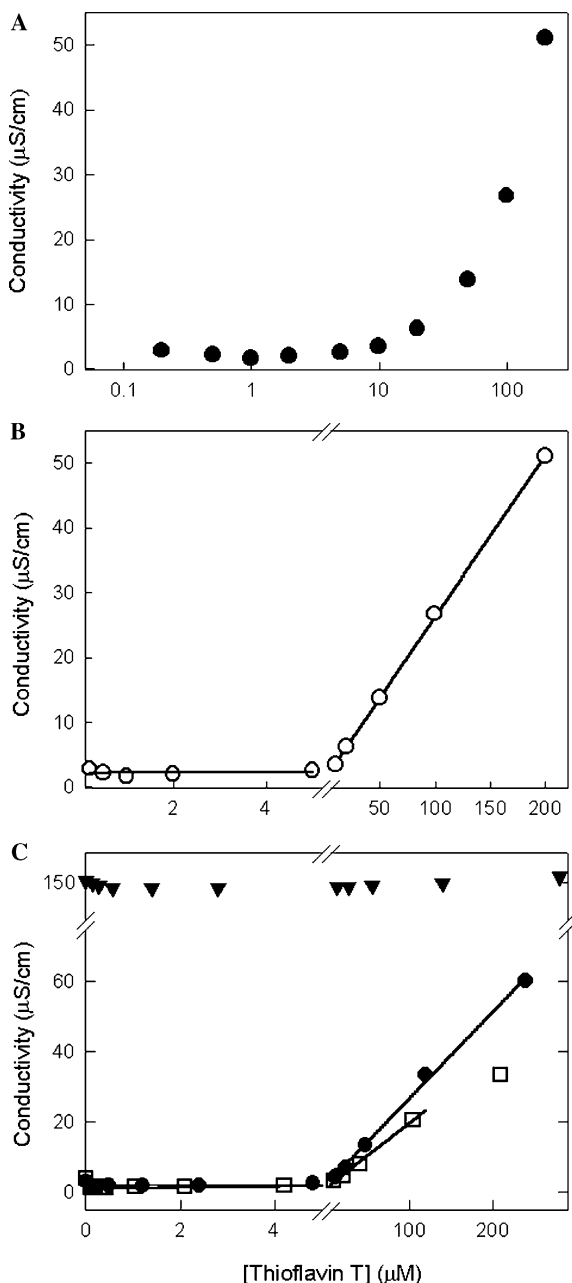


Fig. 1. Micelle formation in thioflavin T. Conductivity of ThT solutions concentration range between 0.1 and 200 μM in water were measured using a probe conductivity meter by alternating current and converted to specific conductance by multiplying with the cell constant of the probe and plotted against concentration (A). The specific conductance data were used to calculate critical micellar concentration by plotting linear regression in low and high concentration ranges of thioflavin T (B). Critical micellar concentration value of $4 \pm 0.5 \mu\text{M}$ was obtained by calculating the concentration of thioflavin T where the linear regressions at the low concentrations and high concentrations coincide. (C) Specific conductance with increasing thioflavin T concentration was measured in water (\bullet), methanol (\square), and in DMSO (\blacktriangledown). No change in specific conductance was observed in DMSO. The changes in specific conductance in water and methanol are indicative of the formation of micelles of thioflavin.

positively charged nitrogen of the benzothiazole pointing outwards. The specific conductivity data were used to calculate the critical micellar concentration of ThT by fitting linear regression to the conductivity data at lower concentration below 1 μM and above 10 μM and then calculating a concentration at which the two regression curves meet (Fig. 1B). The calculations gave a value of $4.0 \pm 0.5 \mu\text{M}$ as the critical micellar concentration of thioflavin T in water. No change in conductivity was observed with increasing concentration of thioflavin T in non-aqueous conducting solvent such as dimethyl sulfoxide with dielectric constant of 46 (Fig. 1C). In methanol and water having dielectric constants of 21 and 18, respectively, increase in conductivity was observed beyond critical micellar concentration of thioflavin T (Fig. 1C).

3.1.2. Concentration dependence of fluorescence of thioflavin T

The effect of ThT concentration on its intrinsic fluorescence properties was investigated. The intensity of the excitation at 450 nm (emission set at 482 nm) and emission at 482 nm (excitation at 450 nm) of aqueous ThT solutions from 0.01 to 100 μM are shown in Fig. 2A. Interestingly in the submicromolar concentration range, there is no significant change in the intensity of the excitation or emission spectra. This might be due to quenching effects of smaller order associative states of ThT molecules. Beyond 5 μM concentration however an increase in the intensity of both excitation and emission spectra was observed. This increase in intensity is closely related to the concentrations at which micelle formation occurs (Fig. 1B). These data suggest that the increase in fluorescence intensity of ThT is due to the micelle formation. The change in both excitation and emission intensity of ThT were also used to calculate the CMC (Fig. 2B) in the same way as with the specific conductance data (Fig. 1B). The CMC value calculated using changes in the excitation and emission intensities of thioflavin T with concentration was $3.75 \pm 0.5 \mu\text{M}$, which is comparable to the value of 4 μM calculated from the specific conductance data. Based on this analysis changes in the fluorescence excitation and emission properties of ThT also reflects its micelle formation.

3.1.3. Fluorescence anisotropy measurements

Fluorescence anisotropy (Checovich et al., 1995) was measured to determine changes in the rotational relaxation time of ThT (reflecting changes in fluorescence particle size) at various concentrations between 10 nM and 100 μM in water (Fig. 3). ThT in aqueous solutions above 10 μM had a large anisotropy value, indicative of aggregates. Anisotropy data was not used to calculate the critical micellar concentration, because a change in anisotropy does not necessarily correspond to micelle formation in this system, since the formation

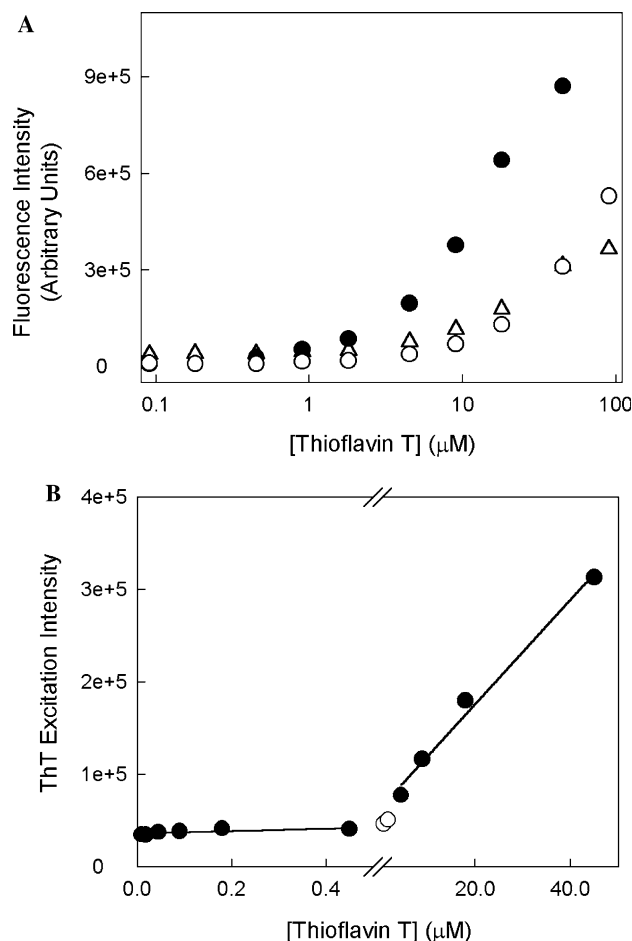


Fig. 2. Concentration-dependent changes in fluorescence properties of thioflavin T. (A) Concentration dependence of the fluorescence properties of ThT solutions was measured between 0.1 and 100 μM concentration of thioflavin T dye. The emission intensity was measured at 482 nm upon excitation at 450 nm both in the presence (●) and absence of (○) 5 ng/ml final concentration of immunoglobulin light chain V_L (SMA) amyloid fibrils. The excitation peak intensity (Δ) at its maxima 415 nm for the dye alone is plotted against ThT concentration. Linear regressions were plotted in the low and high concentration range for both emission and excitation data (B) and were used to calculate the CMC value of $3.75 \pm 0.5 \mu\text{M}$.

of smaller oligomers can also account for anisotropy changes. Based on the electrical conductivity data, we believe that the high anisotropy values obtained for concentrations of ThT above 10 μM represent micelle formation.

Another difference between the anisotropy and conductivity changes observed with increasing ThT concentrations is that saturation is observed in the anisotropy values above 10 μM thioflavin T, indicating that above this concentration the majority of ThT molecules exists as micelles and no change in micelle size is observed at higher concentrations. The specific conductance change, on the other hand, does not show saturation, due to the fact that with increasing concentrations, increasing numbers of ThT micelles are present and hence the conductivity value continues to increase.

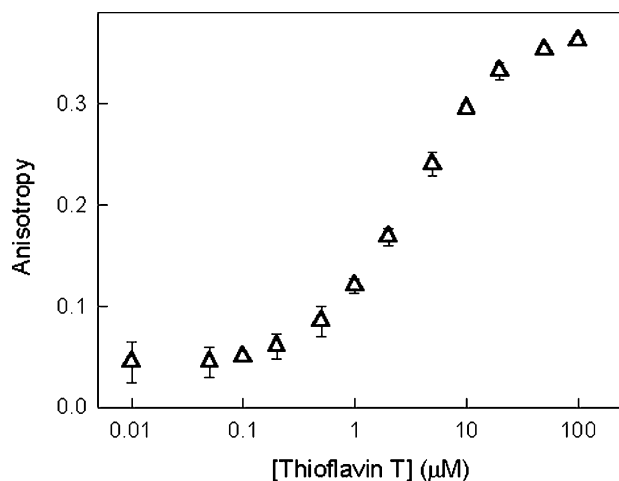


Fig. 3. Fluorescence anisotropy revealing association of thioflavin T molecules. Fluorescence anisotropy was measured for ThT solutions ranging from 0.01 to 100 μM , and is plotted as a log plot of thioflavin T concentration. The increase in anisotropy was observed starting at 0.5 μM concentration and it reached a maximum level at 20 μM .

3.1.4. Diffusion coefficient measurement by NMR

Non-covalent molecular association in solution reduces the translation motions of molecules, which in turn reflects their diffusion coefficient (Johnson, 1999). The micellar properties of thioflavin T was investigated in deuterated water (D_2O), $\text{DMSO-}d_6$ and $\text{DMF-}d_7$ by using DOSY ^1H NMR (Johnson, 1999) using an equimolar mixture of thioflavin T and sucrose where sucrose was acting as an external standard. Since the molecular weight of sucrose is 342 and thioflavin T is 318, the expectation is that the diffusion coefficient of sucrose will be lower. The diffusion coefficient in D_2O of sucrose was determined to be 4.65×10^{-8} and that of thioflavin T was 4.04×10^{-8} indicating molecular association in the latter and is further reinforcing the results obtained by conductivity and anisotropy. In DMSO and DMF, however, the diffusion coefficient of sucrose is lower than thioflavin T (diffusion coefficient of sucrose is 2.089×10^{-8} and thioflavin T is 2.434×10^{-8} in DMSO; diffusion coefficient of sucrose is 6.73×10^{-8} and thioflavin T is 8.27×10^{-8} in DMF) suggesting that both in DMSO and DMF thioflavin T is in monomeric state.

3.1.5. Visualizing thioflavin T micelles

Using atomic force microscopy, ThT micelles in water were directly visualized as spherical particles and the height of 3 ± 1 nm was measured (Figs. 4A and B). Assuming the micelles are spherical, this height corresponds to their diameter. An increase in the total number of micelles was observed by atomic force microscopy upon increasing the ThT concentration from 9 (Fig. 4A) to 90 μM (Fig. 4B), but no increase in the size of micelles was observed. With increasing concentration of ThT, more and more micelles are formed (Figs. 4A and B), causing a linear increase in excitation and emission

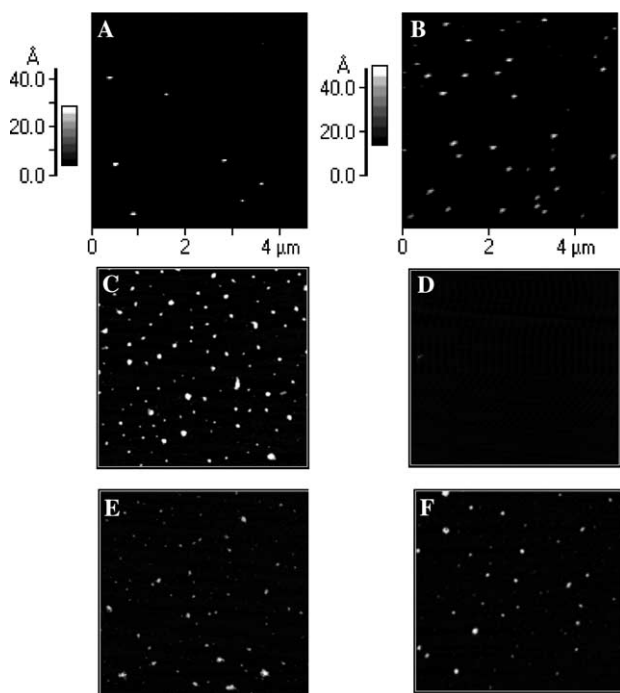


Fig. 4. Thioflavin T micelles visualized by atomic force microscopy. ThT aqueous solutions of 9 μM (A) and 90 μM (B) were placed on a freshly cleaved mica surface and air-dried before imaging using non-contact mode. A 10-fold increase in concentration of ThT shows increased number of micelles. The heights of the micelles remained constant with a mean height of 3 ± 1 nm at both the concentrations. 68 μM ThT in tris buffered saline pH 7.5 was placed on a freshly cleaved mica surface and washed with water (C), 1 N hydrochloric acid (D), 2 M sodium chloride (E), and 1 N sodium hydroxide (F), and air-dried before imaging using non-contact mode. The heights of the micelles ranges from 3 to 6 nm in TBS.

fluorescence intensities. If there was non-specific aggregation of thioflavin T with concentration then it is possible that the aggregate size would have increased with concentration. Analytical ultracentrifugation studies performed by Kelényi (1967) on thioflavin T and thioflavin S showed the presence of high molecular weight species. We believe that the macromolecular species observed by Kelényi (1967) correspond to the micelles we observe in our studies. We also observe thioflavin T micelles in Tris buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.5) as shown in Fig. 4C, the conditions in which we normally perform the fluorescence enhancement assay with amyloid fibrils. In TBS, we observed some variation in the thioflavin T micelle size and range was from 3 to 6 nm diameter as measured by the height measurement using atomic force microscopic images. This could be indicative of coalescing of equal size micelles in water to form slightly larger ones, due to the presence of low salt concentration. In fact longer storage of ThT solutions in TBS was observed to precipitate, possibly due to coalescing of ThT micelles and forming very large particles. When the thioflavin T micelles deposited on mica were washed with acid (1 N

HCl), high salt (2 M NaCl), and basic solution (1 N NaOH), no micelles were observed with acid wash, but both after high salt wash and basic wash micelles were observed as shown in Figs 4D–F, respectively. These observations suggest that ThT micelles formation involves charge interactions as changing the pH changes the ionic conditions and hence dissolves the micelles.

3.2. Micelles of ThT bind amyloid fibrils

3.2.1. Thioflavin T micelles observed along the length of amyloid fibrils by atomic force microscopy

Immunoglobulin light chain variable domain (V_L) fibrils were grown in vitro in the absence of ThT. At the 20-h time point, where protofilaments, protofibrils, and fibrils were observed (Ionescu-Zanetti et al., 1999), ThT was added to the samples and incubated before depositing the samples on mica (Fig. 5A and B). Interestingly no ThT micelles were observed on protofilaments (height 2.5 ± 0.5 nm) white arrows (Fig. 5B). Higher-order protofibrils and fibrils, however, had high points that could be accounted for by bound ThT micelles along the surface of the fibrils (Fig. 5A and B). The heights of the high points vary between 6.5 and 12 nm, corresponding to 3 nm ThT micelles bound to 4.0 nm protofibrils, or 6–8 nm fibrils. These data suggest the possibility that ThT micelles bind in the grooves of the twisted protofilaments in protofibrils or fibrils.

AFM was used to visualize amyloid fibrils of insulin grown in vitro in the presence and absence of 20 μM ThT. In the absence of ThT, the fibrils were smooth with a diameter, estimated by measuring the average heights of fibrils, of 3.5 ± 0.5 nm (Khurana et al., 2003). In the presence of ThT however, the fibrils showed a beaded appearance (Fig. 5C). The heights at the “bumps,” shown by red arrows corresponded to two ThT micelles, and green arrows show one ThT micelle bound to the fibril. According to the average height measured for insulin fibrils alone and thioflavin T micelles alone, the predicted heights for one or two micelles bound to an insulin fibril would be 6.5 ± 1 nm and 9.5 ± 1 nm, respectively. Not all fibrils showed evidence for binding of ThT micelles, some fibrils had no “bumps,” and parts of fibrils in between two “bumps” had heights comparable to fibrils grown in the absence of ThT (white arrows).

Increased numbers of thioflavin T micelles were observed along the length of human calcitonin amyloid fibrils (0.5 mg/ml final concentration) deposited on mica with increasing concentrations of thioflavin T as demonstrated in Figs. 6A and B with 7 and 2 μM thioflavin T, respectively. The micelles bound along the fibrils were not washed with 0.5 M NaCl (Fig. 6B). From the same stock of human calcitonin amyloid fibrils (5 ng/ml final concentration), we also observed enhanced fluorescence emission with varying concentration of thioflavin T

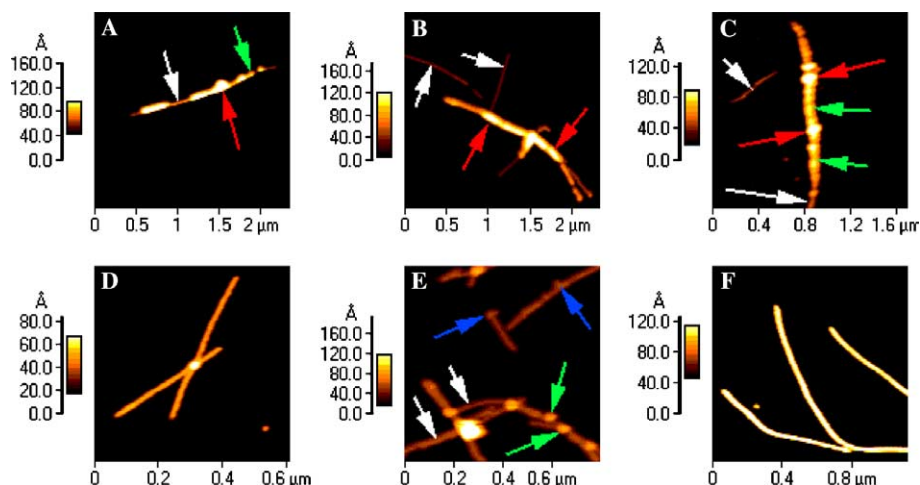


Fig. 5. Micelles of thioflavin T bind along the length of various amyloid fibrils. (A and B) ThT binding to immunoglobulin V_L fibrils. V_L fibrils at 0.5 mg/ml concentration were grown at pH 2.0 at 37 °C in 20 mM HCl in the absence of thioflavin T. At 20 h time point, the fibrils were incubated with 20 μ M ThT at a concentration of 0.05 mg/ml and the sample was dried on a freshly cleaved mica before imaging using atomic force microscopy. (A) Type II SMA fibrils with one (green arrow) or two (red arrows) ThT micelles bound on its surface. The heights of ThT micelles on the surface of fibrils range from 9 to 12 nm where white arrows marks the area where there is no ThT bound. (B) The smooth filaments with 2.5 ± 0.5 nm diameter shown by white arrows do not show any ThT micelles. (C) Insulin fibrils with heights of $\sim 3.5 \pm 0.5$ nm were observed shown by white arrows. Insulin fibrils studded with ThT micelles along its length were observed to be ranging from 5 to 10 nm in height. These numbers could account for one (green arrows) or two (red arrows) ThT micelles contributing to the increase in the height of fibrils at the marked spots. ED a 13 amino acid peptide derived from SMA sequence formed 4 m diameter fibrils, as shown in (D). ED fibrils bound to ThT are shown in (E) and bound to Congo red are shown in (F). ThT bound sample has regions marked by white arrows where no ThT micelle is bound and height is 4.0 nm, and green arrows point to areas where heights are 7.5–7.8 nm in height corresponding to one ThT micelle bound on ED fibril. Blue arrows point to regions where a ThT micelle of 3–4 nm seems to interact along the side of ED fibril without contributing a increase in height. (F) Congo red bound ED fibrils not showing characteristic features, but instead caused an increase in the diameter of ED fibrils from 4 to 10 nm.

(Fig. 6C). Increasing the concentrations of salt to 2 M washed off the amyloid fibrils from the surface and direct visualization of higher concentration of salt on thioflavin T micelles bound along the fibril length was not possible due to technical problems. This experiment was then performed using the enhanced thioflavin T fluorescence emission, which demonstrated a small decrease (30%) in the fluorescence emission with increasing the salt concentration from 0.5 to 2 M NaCl. This effect could be due to quenching effect of salt. This experiment indicates that salt does not remove the thioflavin T micelles bound to the amyloid fibrils. This experiment was also attempted using fluorescence light microscope where amyloid fibrils from human calcitonin were deposited on a glass slide and stained with thioflavin T and again some decrease in thioflavin T fluorescence was observed with salt washing from 0.5 M NaCl to 2 M NaCl, but thioflavin T fluorescence was observed even after washing with 2 M salt (data not shown).

3.2.2. Enhancement in thioflavin T fluorescence emission upon binding to amyloid fibrils

Several fold increase in the emission fluorescence of thioflavin T is observed at 482 nm upon binding to amyloid fibrils. Constant amount of amyloid fibrils was added to varying thioflavin T concentrations and emission fluorescence was measured (Fig. 2A). For this

experiment it is important that we add limiting concentration of amyloid fibrils for example 0.005 mg/ml of fibril solution. If higher concentration of amyloid fibrils is added then enhancement of thioflavin T emission is observed even below the critical micellar concentration of thioflavin T. It is possible that a higher concentration of amyloid provides some sort of hydrophobic surface to thioflavin T and micelles are formed due to change of environment. Some enhancement in thioflavin T emission was observed even at concentrations below 4 μ M thioflavin T, but above 4 μ M ThT (critical micellar concentration), a very significant increase in the thioflavin T fluorescence emission was observed ranging from 5- to 50-fold increase upon addition of amyloid fibrils. We also observe a reduction in the fluorescence enhancement of thioflavin T upon binding to amyloid fibrils when the pH of the solution showing enhancement was reduced (data not shown). These observations led us to propose that it is the thioflavin T micelles that bind the amyloid fibrils and cause enhancement in thioflavin T fluorescence emission. It is possible that the monomeric thioflavin T binds amyloid fibrils and causes enhanced fluorescence emission, but the direct observation of the thioflavin T micelles bound along the length of the amyloid fibrils, shown above favors the hypothesis that it is the binding of the thioflavin T micelles that causes enhancement of fluorescence. In addition, when the amyloid fibrils were incubated with increasing

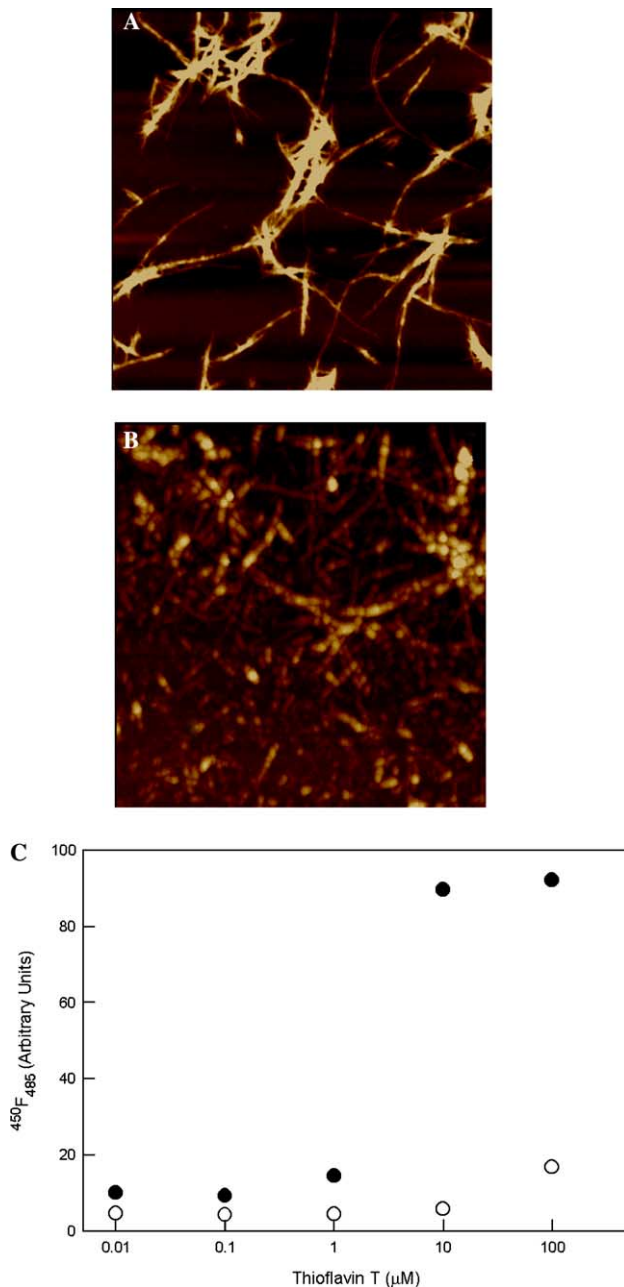


Fig. 6. Increasing concentration of thioflavin T shows increased number of thioflavin T micelles bound to amyloid fibrils. (A) 7 μM and (B) 21 μM thioflavin T incubated with the human calcitonin amyloid fibrils on the mica surface prior to imaging the sample in non-contact mode using AFM. (A) Only a few high spots indicative of thioflavin T are observed along the fibril length as the increase in height corresponds to the size of thioflavin T micelles, whereas in (B) all the fibrils are covered with thioflavin T micelles that are clearly observed as spotted fibrils all along the length of human calcitonin amyloid fibrils. (C) A plot of increase in fluorescence emission of thioflavin T alone with increasing concentration of thioflavin T alone (open symbols) and with 0.005 mg/ml human calcitonin amyloid fibrils (closed symbols).

concentration of thioflavin T above the critical micellar concentration more and more micelles were observed bound to the amyloid fibrils by atomic force microscopy (Figs. 6A and B).

3.3. Comparing the mechanism of thioflavin T and Congo red binding to amyloid fibrils

A peptide, EDVAVYYCHQYYYS (ED) corresponding to a fragment of the Ig light chain SMA, formed fibrils spontaneously in water upon incubation for several days. Formation of ED fibrils was monitored using ThT binding and atomic force microscopy. ED fibrils were 2 nm in diameter up to 30 days and after 2 months laterally aggregated to 4 nm diameter (Fig. 5D). Interestingly no periodicity was observed in either the 2 or the 4 nm ED fibrils in contrast to that observed in the SMA fibrils (Ionescu-Zanetti et al., 1999). ED fibrils (4 nm diameter) were visualized using atomic force microscopy after binding of ThT and Congo red dyes. Interestingly, as observed for immunoglobulin V_L domain fibrils (Figs. 5A and B) and insulin (Fig. 5C), thioflavin T micelles were observed bound to ED fibrils (Fig. 5E). In addition to observing regions where height increases in ED fibrils due to one ThT micelle binding green arrows, we also observed sites which could be a ThT micelle bound along the side of ED fibrils (blue arrows) without causing a change in height. In contrast, Congo red, which is also used to stain amyloid fibrils, did not show characteristic micelles bound to ED fibrils, instead an increase in the ED fibril heights to 10 ± 0.5 nm was observed (Fig. 5E). This increase in the diameter of ED fibrils could be due to the elongated hydrophobic Congo red molecules binding along the length of the fibrils in an end-on manner, or through Congo red-induced lateral aggregation of ED fibrils. Similarly, we also observed doubling of the heights of SMA V_L fibrils in the presence of Congo red, using atomic force microscopy (data not shown). Congo red is not specific for amyloid fibrils, it induces oligomer formation in several native proteins (Khurana et al., 2001), thus the lateral aggregation of ED and SMA fibrils in the presence of Congo red may be comparable to the Congo red-induced oligomer formation in native proteins.

3.4. Specificity of thioflavin T binding

Thioflavin T is known to bind to many structures other than amyloid, including nucleic acids, keratin, and elastin fibrils. The importance of electrostatic interactions in ThT binding is evident in the binding to the negatively charged nucleic acids. Presumably, the positive charges on the surface of the ThT micelles interacts with the negative charges on the nucleic acids to give strong binding, leading to enhanced fluorescence (Caneite et al., 1987). This binding is considerably reduced at low pH (Kelényi, 1967) indicating a role of charged interactions in binding of thioflavin dye micelles to nucleic acids. To learn more about the specificity and preferential binding of ThT to amyloid fibrils, the

interaction of ThT with several in vitro and in vivo protein aggregates was examined.

3.4.1. Thioflavin T binds Inclusion bodies but not other amorphous aggregates

Inclusion bodies of interleukin-2 were tested for ThT binding. In contrast to native interleukin-2, its inclusion bodies induced a 50- to 100-fold enhancement of ThT fluorescence. Other protein aggregates, such as aggregated P22 tail-spike protein, heat-induced protein aggregates from HeLa cells, and amorphous aggregates of SMA V_L , did not result in enhanced ThT fluorescence.

3.4.2. Thioflavin T specificity toward protein and peptide fibrils

Amyloid fibrils derived from a variety of proteins, e.g., A- β peptide, insulin, transthyretin, α -synuclein, amylin, lysozyme, Ig light chains, have been demonstrated to significantly enhance ThT fluorescence upon binding. We tested two 13 amino acid peptides, KLEG (KLKLELELELG) (Lazo and Downing, 1997) and ED (EDVAVYYCHQYYYS) both of which formed fibrils in water (Gillespie J.R. and Fink A.L., unpublished observations) for ThT binding. Only ED peptide showed binding of ThT, both by enhanced fluorescence signal, and by micelles bound along the fibril length. No enhanced ThT fluorescence was observed in the presence of KLEG peptide, or its fibrils. This might be related to the positive charges on KLEG (due to three lysine residues) that could repel the positively charged micelles of ThT. ED peptide, on the other hand, does not have positively charged residues (other than histidine that acquires a positive charge only pH lower than physiological pH) and can bind the micelles of ThT leading to the enhancement in fluorescence emission of ThT as with other protein fibrils. These observations are consistent with the positive charges on the outside of the ThT micelles leading to selectivity due to specific electrostatic interactions to amyloid fibrils (as well as nucleic acids).

3.5. Enhanced fluorescence of thioflavin T micelles upon binding to amyloid fibrils

As suggested by Kelényi (1967) it is possible that binding of ThT micelles involves hydrogen bond formation between charged nitrogen in the thiazole group to amyloid fibrils. The benzothiazole moiety is a combination of a hydrophobic phenyl ring linked to a thiazole ring with positively charged nitrogen. Based on the structure, the conductivity results and binding specificity we believe that the benzothiazole group is on the surface of the micelles of ThT. Micelles of ThT have a low fluorescence emission upon excitation at 450 nm in water. Several fold enhancement in ThT fluorescence emission is observed only upon binding to amyloid fibrils

(LeVine, 1993). It is possible that the environment of the benzothiazole moiety is responsible for the enhanced fluorescence upon binding to amyloid fibrils. We suggest that the micelles of ThT formed in aqueous solvent might be hydrogen-bonding to amyloid fibrils leading to changes in excitation spectrum that in turn cause enhancement in fluorescence emission.

3.6. Does thioflavin T dye bind the crossed- β structure in amyloid fibrils?

There are suggestions that the dyes Congo red and thioflavin T bind the crossed- β structure in the amyloid fibrils. We have earlier demonstrated that Congo red can bind several proteins with varying secondary structure and does not have any specificity for β -structure (Khurana et al., 2001). Krebs et al. (2005) suggest that thioflavin T binds along the β -strands in the amyloid fibrils. Since thioflavin T binds both nucleic acids (Canete et al., 1987) and amyloid fibrils (Vassar and Culling, 1959), it is unlikely that the dye is specific for β -sheet structure. We suggest that the binding of thioflavin T to nucleic acids is purely based on charged interactions as the positive charge of the dye can interact with the negative charges on the nucleic acids and is completely abolished by lowering the pH (Kelényi, 1967). A highly charged peptide that forms amyloid fibrils shows no binding to thioflavin T both by AFM and enhanced fluorescence emission. We do demonstrate clearly that the micelles of thioflavin T bind the amyloid fibrils possibly involving both ionic and hydrophobic interactions.

4. Conclusions

Thioflavin T molecules forms micelles above 4 μ M concentration in aqueous solvents as observed by the increase in the specific conductance. Formation of micelles is also associated with increased fluorescence excitation and emission of ThT. Micelles of ThT alone and bound to the surface of amyloid fibrils were observed by atomic force microscopy. Binding of ThT micelles to amyloid fibrils causes changes in the excitation spectra and enhanced emission fluorescence.

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