Thioflavin T Binding to Amyloid Fibrils
Lauren Riggs
North Carolina State University
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Abstract

Treatment of Alzheimer’s disease (AD) is hampered by the fact that the disease progression cannot be tracked \textit{in vivo} at this time. Understanding the properties of Aβ amyloid fibrils associated with AD is imperative to finding a way to track the progression of the disease. Recently, fluorescent markers, derived from thioflavin T have been developed as markers of AD. This project examines the intrinsic fluorescence of thioflavin T as well as the fluorescence of thioflavin T bound to Aβ(1-40) amyloid fibrils with two distinct morphologies. The two fibril polymorphs produced different fluorescence emission intensities. The goal is to better understand the structural and binding properties of these amyloid fibrils in an attempt to provide earlier diagnosis of amyloid disorders, specifically AD.

Introduction

Amyloid is a type of insoluble fibrous protein aggregate. Amyloid fibrils play a role in some normal processes in the body, such as melanin formation. Amyloid, however, can accumulate in such a manner as to inhibit or degrade normal cellular function. Abnormal amyloid accumulations are frequently present in some neurodegenerative diseases, such as Alzheimer’s disease (AD). The study of amyloid peptide aggregation and accumulation as amyloid plaques has become a priority in research that spans a multitude of academic disciplines. The connection between
amyloid fibril accumulation and AD is a primary driving force behind much of this scientific interest. Congo red and thioflavin T (ThT), the dye used in our research, are the two dyes used most extensively for identification and localization of amyloid fibrils. The fluorescent dye, ThT, is known to show increased fluorescence upon binding to amyloid fibrils. Although much is known about amyloid fibrils, ThT, and the fact that ThT binds to amyloid fibrils, very little is known about the actual mechanism of the binding of ThT to amyloid fibrils. Research conducted in this laboratory will attempt to address some of these fundamental questions and further enhance our understanding of the mechanism of ThT binding to amyloid fibrils.

ThT, a cationic benzothiazole dye (see Figure 1 for chemical structure), has been used to identify amyloid fibrils since it was first shown (by Vassar and Culling in 1959 [1]) to demonstrate increased fluorescence upon binding to amyloid. Upon binding with amyloid, ThT experiences changes in both fluorescence emission and excitation spectra [2]. Subsequent studies of ThT binding to amyloid have shown that ThT is one of the most effective dyes used for this purpose. Recent research has offered several possible explanations for binding mechanisms. For example, ThT micelle formation was suggested by Khurana et al. [3]. Based upon increases in specific conductance as well as increases in fluorescence emission and excitation, Khurana and colleagues concluded that ThT molecules were forming micelles at concentrations above 4 µM ThT in aqueous solvents. Atomic force microscopy was used to visually reaffirm the presence of ThT micelles both alone and bound to amyloid fibrils [3].

Preliminary studies by Petkova et al. [4] involved two fibril morphologies of amyloid β peptide, residues 1 to 40, referred to henceforth as Aβ(1-40). A difference in
fluorescence emission was observed between quiescent and agitated fibrils, the two fibril types studied. Transmission electron microscopy (TEM) images of quiescent and agitated parent fibrils are seen in Figure 2. The two morphologies Petkova et al. studied [4] are similar to amyloid morphologies found in the human body. It is important to learn more about this difference in fluorescence for more accurate diagnosis of amyloid disorder. Determining the relationship between amount of fibril present and fluorescence would also help to establish a quantitative measure of amyloid deposition. Currently, the process of dye binding to amyloid is ill-understood and has previously only been examined in in vitro studies or in ex vivo studies. One goal of amyloid research is to be able to use fluorescent dye, specifically derivatives of ThT, in in vivo studies (Positron Emission Tomography, PET, imaging) in an attempt to track progression of amyloid-associated diseases at early stages. Because there is no cure or treatment for AD, delaying the progression of the disease if caught early on is currently the only hope for AD patients. Thus, early diagnosis is imperative at this point. With better understanding of the mechanism of binding, the usefulness of the dyes in early diagnosis of amyloid diseases such as AD will be increased.

Fluorescence microscopy will be used for observing changes in fluorescence of ThT and ThT bound to β amyloid. Solid-state NMR experiments will be used in the future to attempt to locate the actual binding sites using specific residue labeled fibril samples. While the overall goal of this research is to discover the mechanism by which ThT binds to amyloid fibrils, some more specific and shorter term goals include identifying or verifying the binding ratio and locating the actual binding sites, both on the dye and on the amyloid fibrils.
**Pre-Investigation**

Since I became involved with this project in its very early stages, I was able to observe and participate in the planning of the project. I spent several weeks conducting background literature research, determining what was known with regards to the questions Dr. Petkova’s research hopes to address. Once a plan of action was identified, I began conducting research in the laboratory facilities of Dr. Stephen Hagen in the Physics Department. I also briefly used the laboratory facilities of Dr. Joanna Long at the McKnight Brain Institute.

I was involved in some of the groundwork for what will become one of Dr. Petkova’s research projects at the University of Florida. The first step was to attempt to reproduce results from previous studies and decide where to proceed from there.

**Investigation**

The ThT used in this experiment was obtained from ICN Biomedicals Inc. Amyloid fibril samples were parent and daughter amyloid β residues 1 to 40, Aβ(1-40), samples from a previous experiment [4]. Parent fibrils were grown by incubating fresh Aβ1-40 solutions and daughter fibrils were grown by seeding fresh solutions with sonicated fragments [4]. Fluorescence measurements for this project were taken using a Jasco FP 750 Spectrofluorometer, using a xenon arc lamp to generate light. A 4 ml quartz cuvette was filled with approximately 3.3 – 3.5 ml of solution for each fluorescence measurement. In accordance with Meredith’s assay [5], fluorescence emission spectra were taken from 465 to 550 nm with the excitation wavelength set at...
446 nm and fluorescence excitation spectra were taken from 350 to 475 nm with emission observed at 490 nm. An emission spectrum displays the fluorescence over a range of wavelengths when the sample is excited by light of a constant wavelength. An excitation spectrum displays the fluorescence at a constant wavelength when the wavelength of the light used to excite the sample varies. The temperature at which the data were taken ranged from approximately 20 to 24 °C (slightly different temperatures for different sets of measurements).

**Buffer Preparation**

Glycine hydrochloride (from Aldrich Chemical Company, Inc) was dissolved in deionized water to create a 0.5 L stock of 50 mM glycine buffer. To increase the pH from 1.94 to the desired pH of 8.5, approximately 6 ml of 3.0 N sodium hydroxide solution with 9 sodium hydroxide pellets was added to the buffer. Glycine buffer was also prepared following Meredith’s assay [5].

A second supply of buffer was created to study the effects, if any existed, of aged chemicals. Using glycine obtained from Fisher Scientific, a 0.2 L stock of 50 mM glycine buffer was mixed. This time the pH was raised from 5.16 to 8.49 by adding approximately 1.5 ml of a sodium hydroxide solution made by dissolving 2 pellets of solid sodium hydroxide in 10 ml of deionized water.

**ThT Fluorescence**

Concentration dependence of the intensity of ThT fluorescence was examined in an attempt to recognize micelle formation and to determine appropriate ThT
concentrations to use for fibril fluorescence measurements. Samples of 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 7, 10, 20, 50, 75, 100, 125, 150, 175, 200, 225, and 250 µM ThT concentrations were prepared. Amounts of ThT ranging from 2 to 4 mg were dissolved in a milliliter of ethanol. ThT stock was then diluted to the specified concentrations in glycine buffer of pH 8.5. Fluorescence emission and fluorescence excitation measurements were taken for each concentration of ThT. Several other ThT fluorescence characterization tests were run including determination of the effects of aging of ThT and the effects of the amount of ethanol present in ThT. These additional tests were done in order to improve the experimental protocol.

_ThT + Aβ Fluorescence_

Quiescent (incubated under dialysis conditions) and agitated (incubated under shaken conditions) amyloid β fibril samples were made of parent and daughter fibrils from a previous project [4]. The original stocks used for both quiescent and agitated fibrils were at 210 µM concentrations. Quiescent daughter fibrils were diluted to 0.00105, 0.0105, 0.07, 0.525, 2.1, and 5.25 µM concentrations. Agitated daughter fibrils were diluted to 0.0105, 0.525, 2.1, and 5.25 µM concentrations. Quiescent parent fibrils were diluted to 0.0105 µM concentration. ThT was added at a concentration of 0.1 µM to each of the concentrations of amyloid fibril. The combined ThT and amyloid fibril samples incubated for at least 30 minutes and then fluorescence emission and excitation measurements were taken. A second round of fibril fluorescence measurements was taken on some of the amyloid fibrils after placing the samples in a sonicating bath.
(Laboratory Supplies Co., Inc, Hicksville, NY, S4 model G112SP1T, 600V 80KC 0.5 amps) in order to break up any fibril clumps that might have been present.

After fresh supplies of ThT, glycine, and sodium hydroxide arrived, additional measurements were taken so as to eliminate the possible effects of aging in the chemicals used for the majority of the testing. Using the new chemicals, fluorescence emission and excitation measurements were taken with 0.0105 µM quiescent and agitated daughter fibrils with 0.1 µM ThT.

In attempt to confirm previous measurements, another protocol from Petkova et al. [4] was followed for binding of ThT to amyloid fibrils. For this procedure, quiescent and agitated daughter amyloid fibrils were diluted to 0.945 µM and combined with ~0.008 µM ThT. Fluorescence emission and excitation measurements were taken once without being sonicated, and a different set of measurements was taken on a second sample after sonication.

Results

ThT Fluorescence

When effects of ThT aging were analyzed, an increase in fluorescence was measured two days later when compared to the immediate fluorescence measurement, as seen on a 4 µM ThT sample in Figure 3. For this reason, fresh solutions of ThT were prepared to be used each day that fluorescence measurements were taken.

Comparison of fluorescence emission spectra of a constant concentration of ThT containing different amounts of ethanol showed essentially no difference, as seen in
Figure 4. This result led us to conclude that the amount of ethanol present did not have an effect on the ThT fluorescence measurements.

Full emission spectra of all ThT concentrations are shown in Figure 5. ThT fluorescence emission measurements showed a relatively steady increase in intensity up through 50 µM ThT. Above the 50 µM ThT concentration, the intensity decreased again relatively steadily through the remaining high ThT concentrations.

Excitation spectra also showed differences with changes in ThT concentrations (results not shown).

**ThT + Aβ Fluorescence**

Full emission spectra for the fluorescence of fibrils bound to 0.1 µM ThT are displayed in Figure 6. As a general trend, as fibril concentration increased, the intensity of the fluorescence emissions also increased. An unexpected result was the initial drop in intensity upon adding very low (0.00105 µM) amyloid fibril concentrations to the ThT. At the higher concentrations it is also evident that the agitated amyloid fibril samples produced more intense fluorescence emission spectra. This difference was much less apparent at low amyloid fibril concentrations.

The majority of amyloid research has been done measuring emission at 490 nm. Likewise, we analyzed the fluorescence emission at 490 nm. Figure 7 shows the fluorescence emission intensity at 490 nm of both fibril types in 0.1 µM ThT at varying fibril concentrations. Several measurements were taken (including both stirred and unstirred samples) at each concentration and are shown in Figure 7. Clearly fluorescence
emission intensity increases as fibril concentration increases, and fluorescence emission intensity of agitated fibrils tends to be higher than the intensity of quiescent fibrils.

A difference was also present in the emission spectra of parent and daughter fibrils. The quiescent parent fibrils showed significantly lower intensity than the quiescent daughter fibrils. Figure 8 displays the emission spectra of the quiescent parent and quiescent daughter fibrils.

Again, changes were observed in the excitation spectra of ThT bound to β amyloid (results not shown).

**Discussion**

The ThT fluorescence results showing a decrease in fluorescence above 50 µM ThT concentrations do not agree with the results of work of others, such as Khurana *et al.* [3], who claim that a sharp increase in fluorescence above 4 µM ThT concentrations is due to the formation of micelles at 4 µM, the critical micellar concentration. It is unclear why the results of our study differed from those performed by Khurana *et al.* Further testing would be required in order to confirm or dispute their results. In an article discussing accuracy and reproducibility of ThT assays, one of the main points noted is that fluorescence enhancement was greatest when ThT was present in a 15-fold molar excess [6]. Another suggested procedure was to prepare new working solutions of the dye daily and to cover buffer stock (newly prepared on a weekly basis) in foil and store at 4 °C [6]. In our assay, we used ThT at an excess of about 10-fold molar concentrations (0.0105 µM fibril and 0.1 µM ThT). The low ThT concentration was used in order to keep our ThT well below the critical micelle concentration. The plan was to also take the
same fluorescence measurements using a concentration of ThT well above the critical micelle concentration. The suggested procedure for preparing ThT solution was followed by preparing new working solutions daily; however, the buffer was not prepared weekly and was only sometimes stored at the 4 ºC temperature. The buffer was prepared initially in a large enough volume to last throughout the experimentation phase and was left capped but uncovered by foil at room temperature most of the time. While this should have no effect on the results (in fact, pH was measured to have remained at 8.5), in order to rule out any possible effects of the aging of the buffer, more measurements should be taken in the future with buffers of different ages and the pH of the buffer re-measured to make sure it is remaining fairly constant.

Decreases in fluorescence emissions of the fibril samples after sonication were also unexpected. These results do suggest a possible explanation, that is, sonication may have been too intense, so it not only broke up the fibrils, but it also dissolved some portion of the fibrils. The sonicating bath used was unfamiliar to the researchers, and therefore appropriate duration for sonication could have been misjudged. In order to determine whether the fibrils were simply sonicated for too long, additional experiments will need to be conducted. In these experiments, different times for sonication, as well as different fibril concentrations, should be tested.

Differences in emission spectra of quiescent and agitated fibrils were expected, based on the preliminary results of Petkova et al. [4]. In this study, significant differences in fluorescence were observed in the quiescent and agitated samples, which also displayed different morphologies and molecular structure. In agreement with the findings of Petkova et al. [4], we found that agitated fibrils showed higher emission
intensity than quiescent fibrils. The fluorescence differences noted in the quiescent parent and daughter fibrils can possibly be explained by a few physical differences in the two generations of fibrils. Parent fibrils usually consist of larger clumps; therefore, the ThT binding would be less efficient because the dye may not penetrate the clumped fibrils as well as isolated fibrils. Once proper sonication techniques have been established, parent and daughter fibril fluorescence should be reanalyzed after being sonicated to see if the results change or remain the same.

In addition to providing a more thorough investigation and analysis of the differences in fluorescence of quiescent and agitated fibrils, many aspects of the experimental protocol were investigated, so that future experimentation can be as exact and time-efficient as possible.

Several issues that need to be addressed in the longer term have been identified. Preliminary work by Lockhart et al. has identified three binding sites on amyloid fibrils with three different binding ratios [7]. Using information such as the known positive charge of ThT, structural models of Aβ fibrils, and the known charges of the amino acid residues that make up Aβ(1-40) amyloid fibrils, we propose a possible binding site involving the benzyl group of ThT interacting with residue F20 and the charge of ThT interacting with residue E22 on the agitated fibrils. Using labeled fibril samples and solid-state NMR techniques, the possible binding of the dye at these sites should be able to be observed or not observed, as changes or lack of changes in the fibril chemical structure which are caused by changes in the electronic environment of the fibril at these locations. Other possible binding sites will also be considered and analyzed through similar techniques.
Summary

The experimental protocol for analyzing fluorescence of ThT bound to Aβ(1-40) fibrils was analyzed and improved. ThT fluorescence dependence on both fibril concentration and fibril type was examined. Fluorescence was shown to increase as fibril concentration increases and agitated fibrils showed greater fluorescence than quiescent fibrils. This information, and the future plans of this experiment, will be important in designing a better PET-imaging marker for early identification of fibril formation associated with AD.

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References


Fig. 1. Chemical structure of the ThT molecule.
Fig. 2. TEM images of quiescent and agitated β-Amyloid fibrils. Parent fibrils [4].
Fig. 3. **Effects of aging on 4 µM ThT fluorescence emission spectra.** Fluorescence emission with freshly prepared ThT solution and the same ThT solution two days later. These spectra represent single measurements.
Fig. 4. **Effects of the amount of ethanol on ThT fluorescence emission spectra.**

These spectra represent single measurements.
Fig. 5. Thioflavin T fluorescence emission spectra. Fluorescence emission increases in intensity from 0.05 to 50 µM ThT. Emission then decreases in intensity from 50 to 250 µM ThT. These spectra represent averages of 1-4 measurements.
Fig. 6. ThT + Aβ fibril fluorescence emission spectra. Fluorescence emission spectra for quiescent (abbreviated qui, solid lines) and agitated (abbreviated agi, dashed lines) fibrils. All fibrils are bound to 0.1 µM ThT. These spectra represent averages of approximately 8 measurements per concentration.
Fig. 7. **Quiescent and agitated Aβ fibril fluorescence emission intensity at 490 nm.**

Each data point in this graph represents a single measurement.
Fig. 8. **Quiescent parent and quiescent daughter fibril fluorescence emission spectra.** 5.25 µM quiescent fibrils bound to 0.1 µM ThT. These spectra represent averages of approximately 8 measurements per fibril type.