# Analysis of α-Synuclein Fibril Formation *in vitro*

## Using Fluorescence to Monitor Protein Aggregation in Microplates

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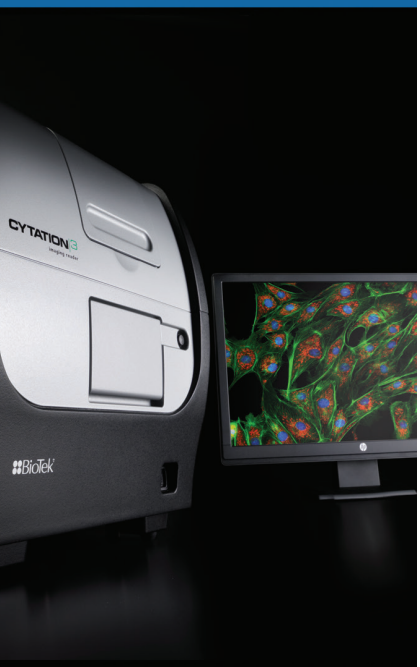


**Key Words:**

Parkinson Disease

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**Parkinson's disease is a debilitating neurological disorder characterized by selective and progressive degeneration of neurons in the substantia nigra pars compacta and by abnormal aggregation of α-synuclein. It is believed that cellular secretion of the protein fibrils or fibril precursors and subsequent endocytosis by other cells plays a role in Parkinson disease progression. Thus, the investigation of fibril formation in response to reaction conditions and their inhibition of formation by potential drug compounds are of particular importance. Here we describe the use of Thioflavin T dye to monitor the formation of α-synuclein fibril formation.**

### Introduction

Parkinson's disease is a progressive age-related neurodegenerative disorder characterized by resting tremors, bradykinesis and rigidity [1]. These symptoms have been attributed to the loss of dopaminergic neurons in the *substantia nigra pars compacta* region of the midbrain. On autopsy some of the surviving neurons in this region contain cytoplasmic inclusion bodies known as Lewy bodies, made up primarily of α-synuclein.

Alpha-synuclein is a highly abundant 140 amino acid protein that was originally identified by its association with synaptic vesicles of presynaptic nerve terminals [2]. Unlike most proteins of this size, α-synuclein in its monomeric form seems to have no folded structure and will spontaneously form fibrils in a nucleation dependant fashion [3]. Despite being cytosolic in nature, the protein has been found to be secreted via unconventional exocytosis [4]. This release has been shown to increase with cellular stress and the more aggregated forms are predominant in the secretion [5]. These data have lead to the suggestion that α-synuclein is a critical component in the spread of Lewy bodies with Parkinson disease progression [6].

There are a number of different methodologies available to investigate protein aggregation *in vitro* and *in vivo*. Separative methods such as electrophoresis, size exclusion chromatography, analytical centrifugation, and mass spectrometry identify polymer formation by the isolation of different protein species based on the physiologic characteristics, such as mass, charge and hydrophobicity [7]. These methods all have some advantages particularly in identifying fibril precursors and intermediates, depending on the available sample size or scientific question, but have limited throughput options. Spectroscopic methods such as turbidity, immunoassay (ELISA), and reporter dyes can be used with standardized microplates enabling significantly higher throughput.

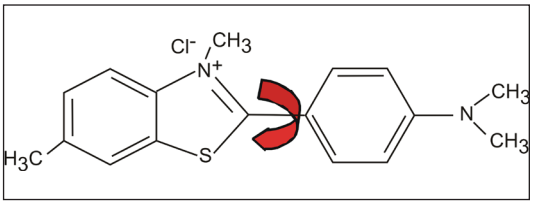



*Figure 1.* **Thioflavin T molecular structure.** The bond between benzothiazole and aminobenzene rings allows for torsional movement relative to one another, resulting in a nonfluorescent twisted internal charge transfer of energy when excited.

Turbidity has the ability to detect aggregates with a hydrodynamic radius greater than the wavelength of the incident light, typically 350 nm. The chief advantage of this technique is the fact that the protein does not need to be labeled and can be used for subsequent experimentation. The relative insensitivity and lack of discrimination between aggregate types (e.g.amyloid versus nonamyloid), limit its use to more of a production tests in the exogenous generation and purification of fibril reagents.

Immunoassay techniques, primarily ELISA can be used to detect the formation of amyloids and specific mutations and conformational variants with the use of appropriate antibodies. While quantitative, ELISA is an end point measurement and does not provide any kinetic information of fibril formation.

The use of stains or fluorescent dyes can be used with high throughput assays, as well as diagnostic tools with fixed tissue slices. Congo red has been used for histological detection of amyloid fibrils for almost 100 years and is considered a defining criterion of amyloid.

When Congo red is bound to amyloid fibrils, it exhibits apple-green birefringence under polarized light [8-9]. Rotator dyes, such as Thioflavin T (ThT) are widely used for probing the presence of amyloid fibrils and especially for in situ monitoring of amyloid formation kinetics, since it generally does not affect the aggregation kinetics [10]. The formation of amyloid fibrils can be probed by monitoring the increase in fluorescence as a function of time. As is the case with turbidity, the ThT assay can be performed in a multiwell plate reader format.

ThT has a relatively low fluorescence in solution. Its chemical structure is such that of a molecular rotor-type fluorophore (Figure 1). In solution, the dye is a poor fluorophore as excited states relax through heat generation into the surrounding solution caused by the ability of the fluorophore to rotate around a central axis in its molecular structure. In the presence of protein aggregates of fibrils, the dye can slip into cavities produced by the quaternary structure of the protein (Figure 2). In this state, rotation is constrained, resulting in a significant increase in fluorescence quantum yield [11].

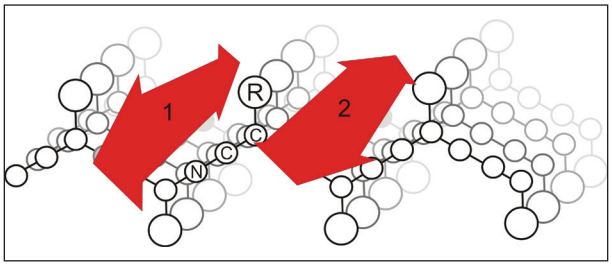



*Figure 2.* **Molecular rotator binding to ordered fibrils.** Ordered structures such as β-pleated sheets found in aggregation fibrils prevent rotation of Thioflavin T dye, resulting in enhanced fluorescence.

## Materials and Methods

Black-sided, clear bottom 96-well microplates (cat# 3603) were from Corning. TopSeal-A (cat # 6050195) adhesive plate sealers were from PerkinElmer. Thioflavin T (P/N T-3516) was from Sigma-Aldrich (St. Louis, MO). All chemicals used for stock solutions were reagent grade.

### Stock α-Synuclein Fibrils

α-Synuclein fibrils for fibril seeding were grown previously and stored at -80 °C until needed. Briefly α-synuclein fibrils were grown by incubating purified α-synuclein (100-200μM) in 50mM Tris-HCl, pH 7.5, 150mM KCl for several days at 37 °C, with orbital shaking (600 RPM) until samples appeared cloudy. Fibril formation was confirmed by Thioflavin T fluorescence. On the day of the experiment, α-synuclein fibrils were isolated by centrifugation for 30 minutes at 16,000 g. The supernatant was decanted and the fibril pellet was resuspended in 50mM Tris-HCl, pH 7.5, 150mM KCl. Final fibril concentration was determination by subtracting supernatant protein concentration, as determined by $A_{280}$ absorbance. Fibrils were then diluted to appropriate 10x stock concentrations.

### α-Synuclein Fibril Formation Assay

α-Synuclein fibril formation assays were prepared from a number of stock solutions. Unless otherwise stated, reaction conditions are as follows: 50 mM Tris-HCl pH 7.5; 150 mM KCl; 100 μM α-synuclein monomer; α-synuclein fibril seed (1 or 10 nM); and either 20 μM or 3 μM ThT in a final volume of 200 μL per well. During assay set up; water, Tris-HCl and KCl solutions are added to wells first, followed by α-synuclein monomer, then α-synuclein fibril seed. Assays are initiated by the addition of fluorescent dye. Plates are then sealed with clear adhesive plate sealers and plates loaded into the microplate reader.

### Reading Parameters

Fibril formation was assessed using either a Synergy™ 2, Synergy H1 or Cytation™ 3 Multi-Mode readers (BioTek Instruments, Winooski, VT). Measurements were made kinetically for 72 hours, with a read interval of 15 minutes. Reader temperature was set at 37 °C with continuous shaking between reads. ThT fluorescence intensity was measured using an excitation wavelength of 440nm and an emission of 480 nm. PMT gain was set at 35 for the Synergy 2 and 50 for the Synergy H1 and Cytation 3 readers. Fluorescence measurements are made from the bottom of the plate, with the top being sealed with an adhesive plate sealer to prevent evaporation.

Onset Time Determination

The fluorescent signal over time produces a sigmoidal shaped curve that is best described with a 4-parameter logistics fit of the data. The onset time is represented by the interception of the rapidly changing portion of the curve and the nearly horizontal lower portion of the curve (Figure 3). This can be calculated mathematically as described previously [12].

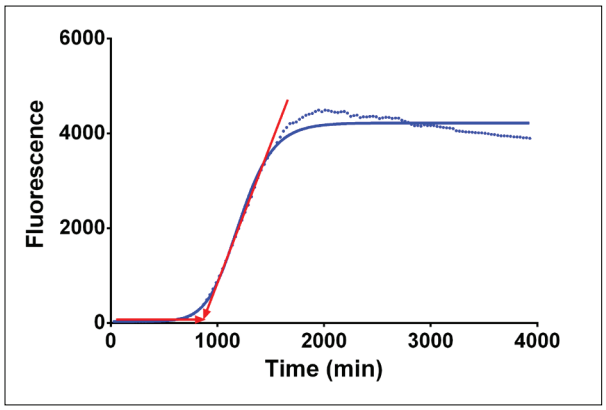



*Figure 3.* Schematic of Onset Time Determination from Sigmoidal shaped plot.

## Results

The fluorescence of known fibril concentrations was measured by incubating ThT with known concentrations of previously formed protein fibrils. ThT demonstrates a linear relationship between fluorescence and fibril concentration (Figure 4). Because the fluorescence of a fixed amount of dye increases in relation to fibril concentration the fluorescence signal can be used to assess fibril formation.

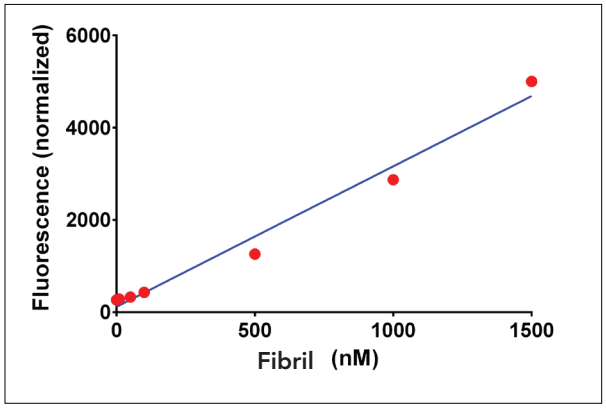
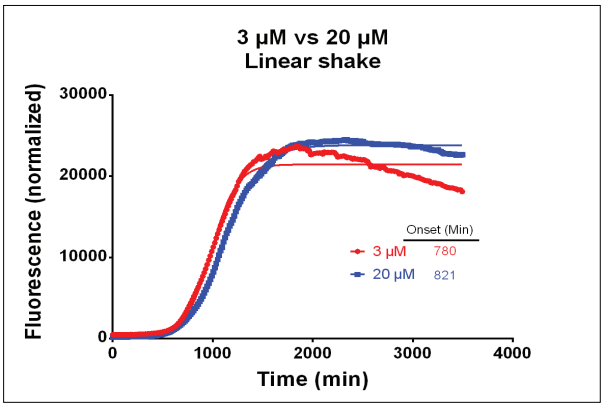



*Figure 4.* **ThioflavinT fluorescence in the presence of α-Synuclein fibrils.** Thioflavin T at a concentration of 3 µM was incubated with fibrils at 37 °C in standard assay buffer (pH 7.5) for 15 minutes prior to fluorescence determination.

The Synergy readers offer both linear as well as orbital shaking patterns. ThT is reported in the literature to be used at a concentration of 20 µM. This high concentration of reporter dye is in part due to the use of less sensitive instrumentation in the past. Newer, more sensitive instrumentation could conceivably be used with lower dye concentrations. The effect of the rotator dye on fibril formation was demonstrated in Figure 5. When lower concentrations of ThT were used in conjunction with a linear shake very little difference in the onset time was observed between a 3- and a 20-µM reporter dye concentration (Figure 5A). Kinetic fluorescent curves with normalized data show very similar curves. Note that the raw fluorescent signal in the 20 µM ThT reactions were substantially greater than the 3 µM reactions (data not shown). With an orbital shake pattern the 20 µM ThT reactions had a significantly shorter onset time than that observed with 3 µM ThT (Figure 5B). These data agree with prior work with amyloid that showed ThT forms micelles at concentrations above 3-4 µM and that theses micelles assist in fibril formation [13].

A.




B.

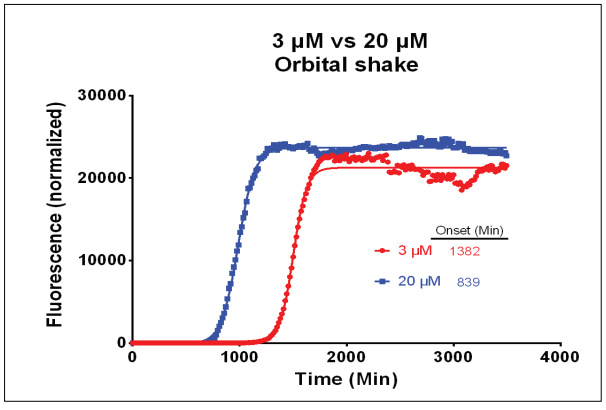



*Figure 5.* **Comparison of linear and orbital shaking.** Two different ThT concentrations were compared using either a linear shake or an orbital shaking pattern.

The effect of salt on α-synuclein fibril formation was determined. The presence of 150 mM KCl or NaCl resulted in very similar kinetic curves when 20 µM ThT was used as a tracer. While the presence of salt produced greater fluorescent signal than samples lacking salt, the onset time for salt-free samples was 30% less (Figure 6). Three-dimensional protein structures such as β-pleated sheets that make up the fibrils depend on hydrogen-bonding to form. While the presence of ions is not absolutely necessary, their presence has been shown to assist in their formation through salt bridges in prion protein [14]. The residual ions from the Tris-HCl buffer in the reaction mix could potentially serve this function in reactions lacking KCl or NaCl.

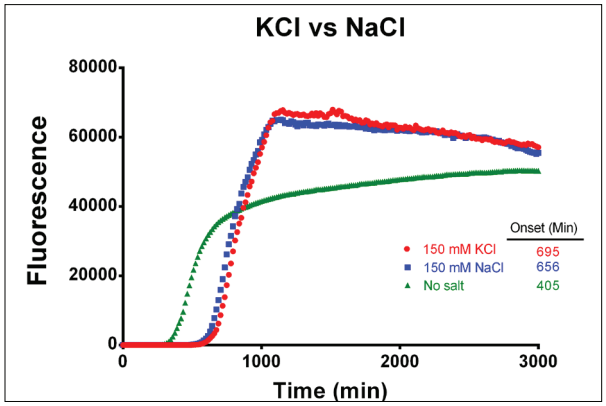



*Figure 6.* **Effect of Salt on Fibril formation.** 20 µM ThT dye was used to detect Synuclein fibril formation in the presence or absence of 150 mM KCl or NaCl at a pH of 4.9.

To improve repeatability, reactions are usually seeded with low amounts of pre-formed α-synuclein fibril. The influence of different concentrations of seed fibril was demonstrated in Figure 7, where the effect of 1 nM and 10 nM fibril seed concentrations were compared. While the calculated onset time for both concentrations was relatively close, the rate and extent of fibril formation, as measured by fluorescence was greater with the higher fibril seed concentration.

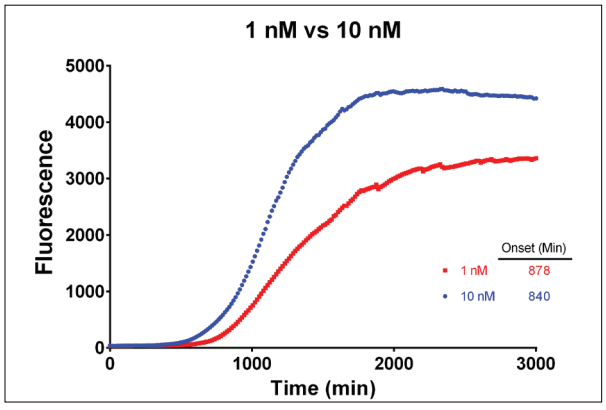



*Figure 7.* **Effect of Synuclein fibril seed concentration on fibril formation.** Values represent the mean fluorescence of 5 separate samples at each time point.

The effect of hydrogen ion concentration was also examined. While the calculated onset time for reactions at pH 7.5 was shorter, the degree of fluorescence change was very modest as compared to reactions at pH 4.9 (Figure 8). Interestingly, the increase in fluorescence for both reactions occurred over approximately the same amount of time, albeit at much different degrees of change. These data agree with findings from amyloid formation, which occurs more readily at lower pH [15].

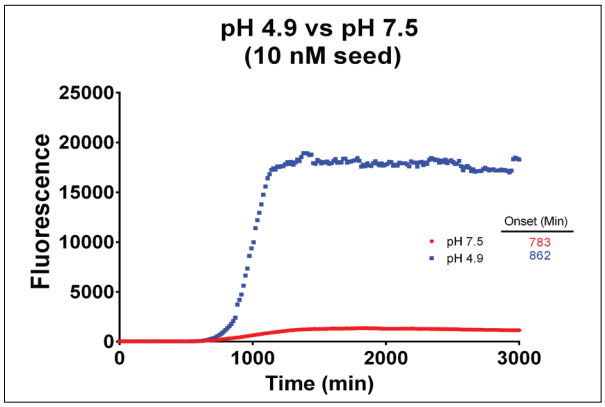



*Figure 8.* Effect of pH on α-Synuclein fibril formation.

## Discussion

These data demonstrate that α-synuclein fibril formation can be tracked using ThT dye in the Synergy™ readers from BioTek. ThT produces a linear response relative to the amount of α-synuclein fibril that is present. However, ThT itself may have properties that artificially assist in fibril formation. When used at concentrations above 4 µM it has been shown to form micelles, which have been suggested to aid in the formation of amyloid β-pleated sheets [13]. While a similar phenomenon may occur with α-synuclein fibrils, the rapidity of fibril formation with ThT at concentrations below the CMC suggests that the dye might have other properties that aid in fibril formation independent of micelles.

These assays require a kinetic fluorescence determination with temperature control and continuous shaking over long periods of time. The Synergy readers have been designed with 4-zone temperature control to maintain temperature and minimize variations across the entire plate. Robust shaking has been integrated into the instruments for years of use. The concentration dependence suggests that orbital shaking is more vigorous and provides more shear than linear. The presence of more ThT could serve as a stabilizing factor that promotes fibril formation through the stabilization of microfibrils, allowing them to form larger sheets rather than immediately dissipating.

Because fluorescence intensity is not an absolute measure of the amount of formed aggregated protein, care must be taken when using this technique to quantitate protein production. The presence of salt (KCl or NaCl) can promote fibril formation through salt bridge formation and protonation of molecules with pH levels below the pKa through hydrogen bonding. However, these same factors can markedly affect fluorescent quantum yield of the reporter dye independent of any protein fibril formation [20]. Depletion of dye can also present a problem. As the fluorescent dye binds increasing amount of aggregated protein the fluorescence increases until all of the fluorescent dye is bound. Once the free dye is consumed fluorescent signal no longer increases despite the continual formation of aggregate. Linearity between fluorescence intensity and protein concentration should be verified by a standard curve if the fluorescence intensity at the plateau phase is used quantitatively.

There are a number of protein aggregation related diseases that can be investigated in a similar fashion as described here for Parkinson disease. For example, Alzheimer's disease is associated with fibril extracellular deposits of amyloid in dense formations referred to as senile plaques, Amyloid monomers are soluble proteins that are primarily alpha helical in membranes, but undergo a change to form β-sheet rich structures when present at high concentration [16]. In addition the disease has been referred to as a tauopathy as a result of abnormal aggregation of the microtubule associated tau protein, which when hyperphosphorylated can accumulate as paired helical filaments [17]. Polyglutamine expansion genetic disorders such as Huntington's disease have mutations in specific genes resulting in malformed proteins. These proteins are resistant to protease activity and cannot be cleared by neuronal cells. As they accumulate they form aggregates with fibrils that have β-sheet structures [18]. Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, have as their common element misformed prion protein [19]. The normal protein, $prPrP^{C}$,is a 35-35kDa protein with one disulfide bond, a mainly alpha-helical structure and is digestible by protease. An abnormal conformation of the protein, known as $PrP^{sc}$, is resistant to protease, can serve as an infection catalyst to convert more protein from previously uninfected cells, and forms -sheet structures that can be detected by rotator fluorescent dyes [15].

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