CHARACTERIZATION OF BETA AMYLOID FORMATION AND

ITS EFFECT ON ALZHEIMER'S DISEASE

Thesis Supervisor:

_____

Steven T. Whitten, Ph.D.
Department of Chemistry and Biochemistry

Approved:

_____

Heather C. Galloway, Ph.D.
Director of the University Honors Program

CHARACTERIZATION OF BETA AMYLOID FORMATION AND ITS EFFECT ON ALZHEIMER'S DISEASE

HONORS THESIS

Presented to the Honors Committee of
Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for Graduation in the University Honors Program

by

Stephen Thomas Gates

San Marcos, Texas
May 2011

FAIR USE AND AUTHOR'S PERMISSION STATEMENT

Fair Use

Duplication Permission

ACKNOWLDGEMENTS

I am grateful for all of the opportunities I have been given over the last four years during my time at Texas State University-San Marcos. Dr. Heather Galloway has been of tremendous support and guidance to me since my freshman year, and it seems only fitting that she should help me conclude my college career through the approval and publication of this thesis. I would also like to thank Dr. Steve Whitten, who has been an invaluable resource to me during my academic career, first as a professor, and later as my research and thesis advisor.

I have been blessed with a tremendous support group, including numerous faculty and staff from across campus who have been instrumental in my success thus far. These individuals include Ms. Shannon Fitzpatrick, Dr. Margarita Arellano, Dr. Erik Malmberg, and Dr. Jennifer Irvin, among many others. Along with the full support of my family, friends, and girlfriend, I have made this university my home for the last four years and am forever grateful for the opportunities it has given me.

This manuscript was submitted on May 4th, 2011.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure **Page**

ABSTRACT

CHARACTERIZATION OF BETA AMYLOID FORMATION AND

ITS EFFECTS ON ALZHEIMER'S DISEASE

by

Stephen Thomas Gates

Texas State University-San Marcos

May 2011

SUPERVISING PROFESSOR: STEVEN T. WHITTEN, Ph.D.

Alzheimer's disease (AD) is a neurodegenerative disease and terminal form of dementia that affects more than 27 million people worldwide. Among a large group of protein folding diseases, AD acts through the deposition and aggregation of β-amyloid (Aβ) within the brain's blood vessels and outside senile plaques. The kinetics of Aβ42 was studied using thioflavin T fluorescence measurements to determine the formation of amyloid over time. Various purification techniques were compared in order to define the techniques necessary to effectively isolate monomeric Aβ42 before characterizing the formation of Aβ *in vitro.* The extreme conditions of ultracentrifugation were found to best isolate monomeric Aβ42. After further characterization of amyloidosis, future experimentation will utilize the screening of an oligopeptide library to identify sequences that bind to and possibly inhibit, promote, or dissolve formation of Aβ42 amyloid, which could lead to enhanced treatment of AD or the development of new drugs.

1. INTRODUCTION

1.1 *Alzheimer's Disease*

Alzheimer's disease (AD), a terminal form of dementia, currently affects more than 27 million people worldwide (*1*). Symptoms associated with the illness include cognitive dysfunction and neuronal death in the brain, both of which are indicative of a significant progressive neurodegenerative disease (*1*). Pathologically, amyloid plaques and neurofibrillary tangles have been deemed responsible for its onset (*2*). While AD patients can only truly be diagnosed post mortem, early diagnoses prove considerably accurate when detailed histories of progressive cognitive impairment and evidence of declining social or occupational functions are made available. In addition, certain medical tests, like that of blood, urine, or spinal fluid, and several scans, like computerized tomography (CT) and magnetic resonance imaging (MRI) are utilized by physicians to better diagnose dementia patients as having AD (*3*). Even so, success in the treatment of AD has historically been limited to treating only specific symptoms, as seen in the moderate success that cholinesterase inhibitors have had in delaying the degeneration of cognition (*1*).

The three existing cholinesterase inhibitors used in clinical trials, donepezil, galantamine, and rivastigmine, have had fairly significant success for patients with mild to moderate AD (*4*). There are a large number of patients, however, who lack any response at all, and even those who do often do so only for short time periods. The drugs

work by inhibiting the enzyme acetylcholinesterase, which acts to degrade acetylcholine neurotransmitters (*4*). When these neurotransmitters are not degraded, extracellular acetylcholine levels in the brain increase, leading to better communication between neurons and increased cognitive functionality. Unfortunately, experimental trials performed on rats or done *in vitro* have yielded more successful results than most of the ongoing clinical trials.

AD is only one of the many known protein folding diseases. *In vivo*, proteins are known to begin their natural folding process immediately after being translated on the ribosome (*5*). A large number of auxiliary proteins aid in this process, allowing the protein to fold efficiently and correctly, according to its genetic design, despite the highly crowded environment in which it is translated. Failure of a particular protein to fold correctly results in a disease belonging to the group of protein folding diseases, or amyloidoses (*5*). Such diseases involve the deposition of aggregated proteins, known as amyloids, in a variety of tissues (*5*). The term amyloid refers non-specifically to any protein aggregates that share a few common physiological features, which include having a mostly beta-sheet secondary structure and fibrillar morphology, as well as being insoluble to common solvents and resistant to proteases (*6*).

Three of the more commonly studied protein folding diseases are Huntington's disease, spongiform encephalopathy diseases, and Alzheimer's disease. In the first, a polyglutamine sequence (> 35 glutamines) in the amino-terminal region of the huntingtin protein (350 kDa) is released following proteolytic cleavage, initiating aggregation of the small polypeptides formed (*6*). Contrastingly, a neuronal cell-surface glycoprotein, called the prion protein (PrP, 34 kDa), initiates aggregation without requiring covalent

modification or proteolysis and is responsible for causing spongiform encephalopathy diseases, including “mad cow” disease (*6*). Recent research has shown promising results for the eventual treatment of prion diseases due to findings of certain oligopeptides that have the ability to disaggregate prion protein clusters *in vitro* (*7*). Such research showed that the addition of synthetic oligopeptides to a prion protein solution effectively disaggregated otherwise insoluble hamster PrP amyloid. In the experiment, 4 μg of synthetic peptide was added to 400 μL samples of 40 ng PrP in a 1 X phosphate buffered saline (PBS) solution. Following different periods of incubation, the three synthetic peptides, KFAKF, cyclo-CGKFAKFGC, and cyclo-CGGKFAKFGGC, yielded varied results in their respective abilities to disaggregate the hamster PrP amyloid. Both of the cyclo-peptides were able to significantly dissolve PrP clusters over time, as indicated by the western blot data (*7*).

FIG. 1. **Western blot depicting PrP cluster disaggregation following synthetic oligopeptide addition.** Three synthetic peptides, KFAKF (A), cyclo-CGKFAKFGC (B), and cyclo-CGGKFAKFGGC (C), were tested for their ability to disaggregate hamster PrP clusters, respectively (*7*). Using a western blot technique, PrP clusters were detected in each sample following different incubation conditions. A control of initial time point 0 h (F) was measured, as well as experimental samples after incubation for 62 h at 37 °C (I) and 62 h at 37 °C with periodic sonication (S).

Finally, in AD, the amyloid precursor protein (APP, 70 kDa) undergoes proteolytic cleavage, producing small, 4 kDa beta-amyloid peptides that aggregate and lead to the disease (*6*). Each of these protein folding diseases results from misfolded proteins, which are then either subject to proteolytic cleavage or spontaneous aggregation. These diseases differ, however, in the size and sequence of their respective polypeptides. In fact, huntingtin, PrP, and APP share no primary sequence homology and are not derived from similar sources (*6*). In this sense, although each disease's peptides aggregate in a manner similar to the others, the means by which they do so are unique.

In AD patients' brains, the uniqueness is the deposition and aggregation of β-amyloid (Aβ) found within the brain's blood vessels and outside senile plaques (*1*). Following the action of two proteases *in vivo*, β and γ secretase, Aβ is derived from APP and secreted by neurons and platelets, prompting the aggregation of the peptides (*8*). Aggregation occurs over time, as beta amyloid peptides join to become oligomers, then fibrils, and finally large plaques (*3)*. The build-up of intraneuronal neurofibrillary tangles leaves the brain cells completely ineffective and results in the complete degeneration of the affected neurons, eventually causing shrinking of the brain (*9*). *In vitro*, β-amyloids will also spontaneously aggregate when exposed to one another (*8*). Although numerous variations of the Aβ peptide are found to aggregate *in vivo*, the Aβ42 peptide is the predominant form of β-amyloid present in senile plaque formation during the early pathological stages (*10*). In addition, the majority of β-amyloid deposited in the brain contains 42 amino acids, also indicative of the Aβ42 peptide form (*8*).

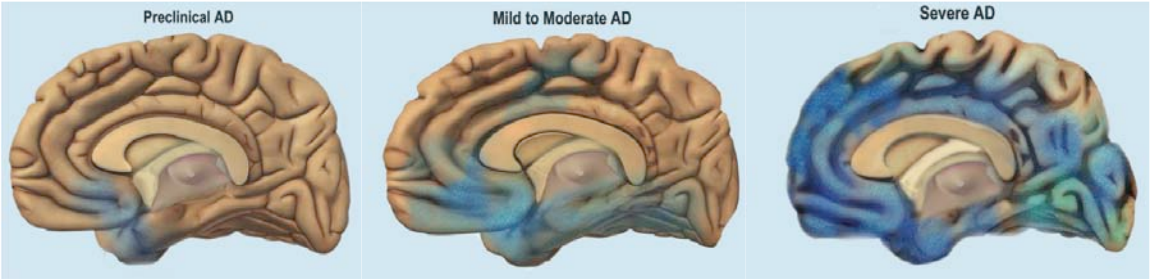

FIG. 2. **The effect of Alzheimer's disease on the brain**. The formation of large amyloid plaques result in neuronal death in the brain, as made evident in the above figure. The colored region on each image corresponds to dead brain cells, and is the cause for the reduced brain size that characterizes severe AD patients.

1.2 Experimental Techniques

Initial formation of amyloid, as well as changes in the amount of amyloid formed within a solution, can be detected through fluorescence spectroscopy using a fluorescent molecule that is able to bind to the aggregated proteins (*11*). Fluorescence spectroscopy is particularly useful because it is able to detect even miniscule amounts of fluorescent molecules present (*12*). The technique utilizes a source of ultraviolet light, set at a specific wavelength, to excite the molecules in solution. A detector then receives information in the form of visible, fluorescent light that is collected perpendicular to the sample, so as to prevent source light from contaminating the measurement (*12*). The information is then transmitted to a computer, where graphical data is generated.

In this study, the amount of protein aggregation directly corresponds with the fluorescence given off as the bound molecule absorbs light at a given wavelength before subsequently emitting light (fluorescence) of a longer wavelength, shorter frequency, and less energy (*11*). One such fluorescent molecule, thioflavin T (ThT), is a commonly used tool for determining amyloid fibril formation. ThT interacts strongly with amyloid fibrils,

and does so with high specificity, as it is unable to interact with monomeric peptides, regardless of whether they are in a folded or unfolded state (*13*). Therefore, binding of ThT specifically to Aβ42 fibrils can be tightly regulated.

In addition, the use of "click-peptide" Aβ42 prevents the premature, spontaneous formation of protein aggregates via a slight modification of the Aβ42 peptide backbone that is only fixed by a controlled action like a pH change immediately preceding experimentation, rendering the peptide active once again (*14*). Before the Aβ42 peptide resembles its native state, the backbone must be "clicked" into place at the twenty-sixth residue via an O-to-N intramolecular acyl migration (*14*). This migration occurs once the correct pH 7.4 conditions are attained. In controlling this change, the peptide can theoretically be maintained in its monomeric form until experimentation proceeds.

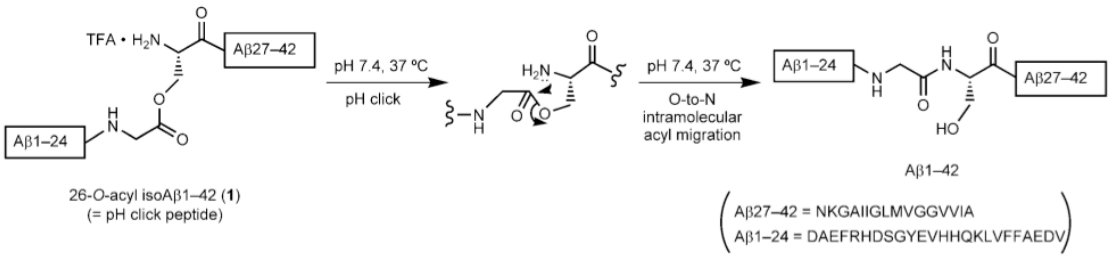

FIG. 3. **Chemistry of the Aβ42 "click-peptide."** "Click-peptide" Aβ42, when purchased, must undergo an O-to-N intramolecular acyl migration before acquiring its native conformation. This migration occurs in the peptide backbone at the twenty-sixth amino acid residue after proper pH conditions of 7.4 are reached (*14*).

Circular dichroism (CD) allows researchers to perform structural protein studies under normally operating protein conditions in solution, in addition to providing a means to depict changes in protein structure and measure the rate of that change (*15*). A type of

spectropolarimeter, a CD instrument measures the difference in absorbance between different polarized components of the protein, which is reported in units of ellipticity (θ) (*15*). CD studies are of particular importance due to the ability to recognize the monomeric or amyloid state of Aβ42 peptides. In utilizing this method before and after purification attempts, the state of the beta amyloid peptides can be determined.

Spectrophotometry allows for the determination of protein concentration. In addition to establishing exact numeric concentration values, it also confirms the presence of protein in solution. Such measurements are necessary during experimentation in order to determine or verify the concentration of protein, upon which kinetics studies are dependent. Concentration is determined using the Beer-Lambert Law:

$$A = \varepsilon\, c\, l$$

where A is the measured absorbance, ε is the extinction coefficient (L mol^{-1} cm^{-1}), c is the protein concentration (mol L^{-1}), and l is the path length that light travels through the sample (cm). For protein concentration determination, absorbance is measured by a spectrophotometer at 280 nm, which is the wavelength at which tyrosine, tryptophan, and phenylalanine amino acids absorb light (*16*). Figure 4 depicts these aromatic amino acids and their locations in Aβ42 peptide. The extinction coefficient used is that of tyrosine, 1490 L mol^{-1} cm^{-1}, because it absorbs more strongly than phenylalanine (*16, 17*). Along with knowledge of the path length, typically 1 cm through a quartz crystal cuvette, all information necessitated by the Beer-Lambert Law is provided or acquired to determine protein concentration.

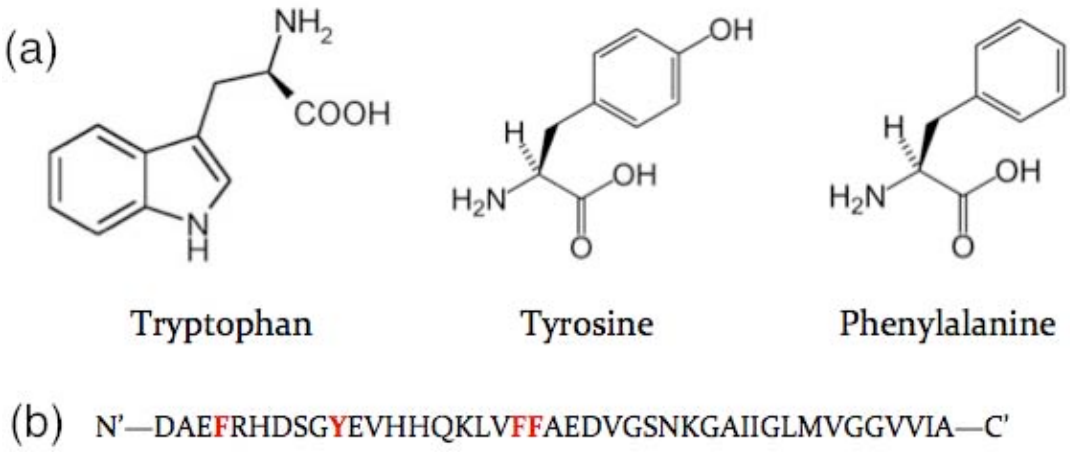

FIG. 4. **Aromatic amino acids and their presence in Aβ42**. The three aromatic amino acids, tryptophan (W), tyrosine (Y), and phenylalanine (F), are useful in determining protein concentration via spectrophotometric absorbency measurements and the Beer-Lambert Law (a). The sequence containing 42 amino acids depicts the primary structure of the Aβ42 peptide, with residues in red representing the aforementioned aromatic amino acids (b).

1.3 Goals

The goal of the current study was to define the techniques necessary to effectively isolate monomeric Aβ42 before characterizing the formation of beta amyloid *in vitro.* In using fluorescence spectroscopy to measure the increasing formation of peptide aggregates, kinetics studies were used to characterize the rate of amyloidosis. Such characterization regarding Aβ42 peptide aggregation could provide invaluable information that may be used to further studies on the effects of various protein interactions with beta amyloid. Research has shown that the addition of specific

oligopeptides to prion protein amyloid can act to dissolve the clusters *in vitro*. PrP diseases and AD share a commonality as protein folding diseases. Should certain oligopeptides be discovered that also disaggregate Aβ42 amyloid fibrils, such findings could ultimately help in the treatment of AD. For this reason, finding peptides that disaggregate Alzheimer's amyloid has been identified as a long-term goal for this project.

2. MATERIALS AND METHODS

2.1 Chemicals, Reagents, and Equipment

Human β-amyloid (1-42) (Aβ42, 4514 g/mol) was purchased from GenScript (Piscataway, NJ). Thioflavin T (ThT, 318.85 g/mol) was purchased from Acros Organics (Geel, Belgium). Glycine and monobasic, monohydrate sodium phosphate (NaH_2PO_4, 137.99 g/mol) were puchased from VWR (West Chester, PA), 0.1 % trifluoroacetic acid (TFA) was puchased from Mallinckrodt Baker, Inc (Phillipsburg, NJ), and 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, 168.04 g/mol) was purchased from Sigma-Aldrich (St. Louis, MO). Centrifugation was accomplished using either a Beckman TL-100 with a TLA-100.3 rotor and Beckman Centrifuge Tubes or a Beckman Coulter Microfuge 16 (Brea, CA). Safe-Lock 1.5 mL Eppendorf tubes were used to prepare solutions, which were incubated using an Eppendorf Thermomixer (Hamburg, Germany). Fisherbrand 25 mm syringe filters of molecular cutoff 0.22 μm were purchased from Fisher Scientific (Hampton, NH). Spectrophotometric readings were measured using a Beckman Coulter DU 730 Life Science UV/Vis Spectrophotometer (Brea, CA) and fluorescence was measured using a Varian Cary Eclipse Fluorescence Spectrophotometer with Cary Eclipse Scan Software (Palo Alto, CA). A Jasco J-710 Spectropolarimeter (Tokyo, Japan) was used to analyze amyloid conversion.

2.2 Isolation of Monomeric Aβ42

Click-peptide Aβ42 was dissolved in 0.1 % TFA to yield a 140 μM Aβ42 solution. Pre-formed aggregates were then removed via centrifugation or filtration using one of three different routes. Each of the procedures was compared and evaluated based on the ability to isolate pure monomeric Aβ42. In the first method, the sample was centrifuged at 16,000 x g for 30 min. Filtration was accomplished by passing the sample through a 0.22 μm cutoff syringe filter in the second method. The final method involved centrifuging the sample at 541,000 x g at 4 °C for 3 h. Regardless of the technique used, the top 510 uL of solution was removed and saved, and the bottom solution containing any pre-formed aggregates was discarded. All Aβ42 in 0.1 % TFA solutions were stored under conditions as described by Baducci et al. (*18*).

2.3 Confirmation of Monomeric Aβ42 and Determination of Concentration

Circular dichroism (CD) spectroscopy was used to determine the monomeric state of Aβ42 in 0.1 % TFA and phosphate buffer at times 0 h and 24 h. The CD spectra were measured at 37 °C using a 1.0 mm path length quartz cell, and eight scans were averaged to acquire the data. Following isolation of the top 510 uL of 140 μM Aβ42 solution, a spectrophotometric reading was used to determine the actual concentration of the sample. Absorbance of 60 uL of Aβ42 solution in a 1 mL total solution was measured at 280 nm using the tyrosine extinction coefficient of 1490 $M^{-1}cm^{-1}$.

2.4 Thioflavin T (ThT) Assay

An equal volume of phosphate buffer (0.2 M, pH 7.4) was immediately added to the 450 uL of 140 μM Aβ42 solution. Another equal volume of phosphate buffer (0.1 M, pH 7.4) was then added to the solution, yielding a 35 μM Aβ42 solution. Eighteen 100 uL solutions, triplicates of six time trials ranging from 0 h to 96 h, were immediately incubated at 37 °C while being shaken at a constant 600 rpm. Upon removal from incubation, 69 uL from each sample was immediately added to 2 mL of ThT solution (5 μM ThT, 50 mM glycine, pH 8.5) in a quartz crystal cuvette and the fluorescence emission was measured from 450 nm to 600 nm at an excitation wavelength of 442 nm at room temperature. In addition, ThT controls were measured for fluorescence before and after each time trial to ensure that no degradation of the light-sensitive solution occurred during the assay. These controls were measured as described by Levine (*19*). In addition, a blank fluorescence emission was measured using the same standards as those used for the experimental fluorescence emission measurements. New ThT solutions were prepared for each time trial, using the controls to determine the precision of measurements for each solution relative to the others.

3. RESULTS

In determining the increasing aggregation of Aβ42 over a period of time, ThT assays were employed. ThT solution (5 μM ThT, 50 mM glycine, pH 8.5) was newly prepared and measured for fluorescence immediately preceding and just after any given time point in each experiment to ensure consistency in its preparation. Figure 5 depicts the initial fluorescence readings that freshly prepared ThT solutions yield. The two control curves (a, b) act as standards to which any subsequent ThT solutions are compared. All fluorescence readings are expressed and compared using data acquired directly from the laboratory spectrometer, with arbitrary units and numerical values that are consistent and comparable.

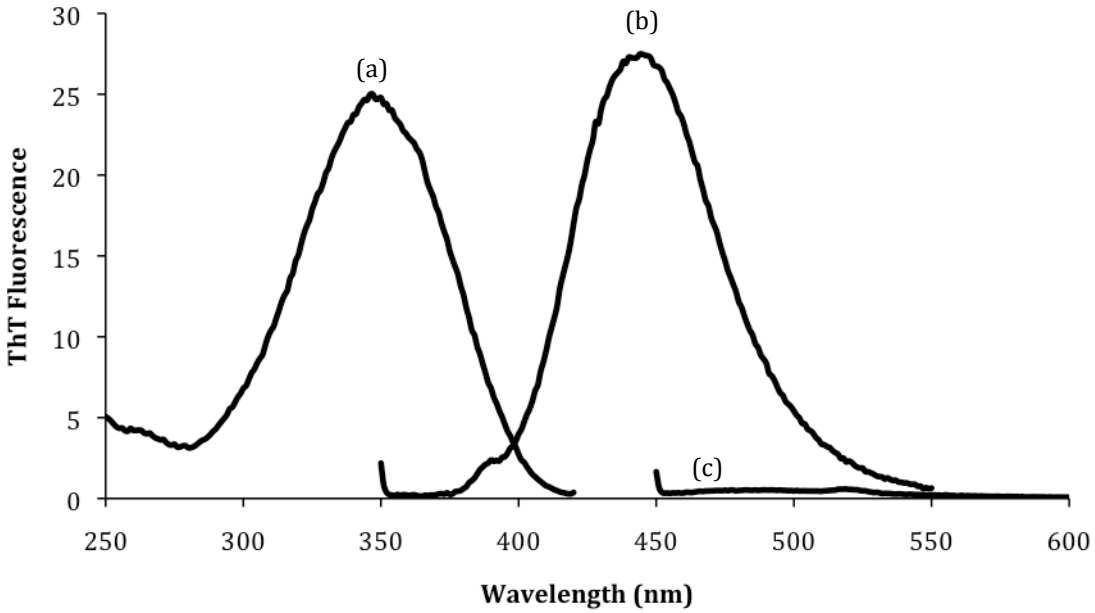

FIG. 5. **Thioflavin T fluorescence**. ThT solution (5 μM ThT, 50 mM glycine, pH 8.5) fluorescence controls were measured under a set of standard conditions to be used for subsequent ThT assays. Each newly prepared ThT solution was measured for fluorescence under these conditions immediately preceding and just after experimental readings were taken. Fluorescence emission from 350 nm to 550 nm at an excitation wavelength of 342 nm (b) and excitation from 250 nm to 420 nm at an excitation wavelength of 430 nm (a) were measured. A blank fluorescence emission was also measured from 450 nm to 600 nm at an excitation wavelength of 442 nm. (c).

In order to promote the intrinsic aggregate formation of Aβ42, a series of steps was employed. Aβ42 was dissolved in 0.1 % TFA and centrifuged at 16,000 x *g* for 30 min. After 268 μM Aβ42 was prepared from the supernatant and combined with the appropriate phosphate buffers, samples underwent incubation at 37 °C for allotted amounts of time (see Materials and Methods). Samples were removed from incubation after 0 h and 48 h, respectively, and immediately measured for fluorescence emission via a ThT assay. Experimental fluorescence readings were compared to that containing only ThT, used as a blank. Figure 6 depicts the curves that were generated from each of the

fluorescence scans. Curve (a) represents a baseline containing only the ThT blank, and therefore no amyloid formation. Curves (b) and (c) represent fluorescence as a result of amyloid formation after 0 h and 48 h, respectively. A threefold increase in fluorescence was observed from the measurements at 0 h to those at 48 h, with fluorescence maximums of 2.7 and 8.7, respectively, at 480 nm.

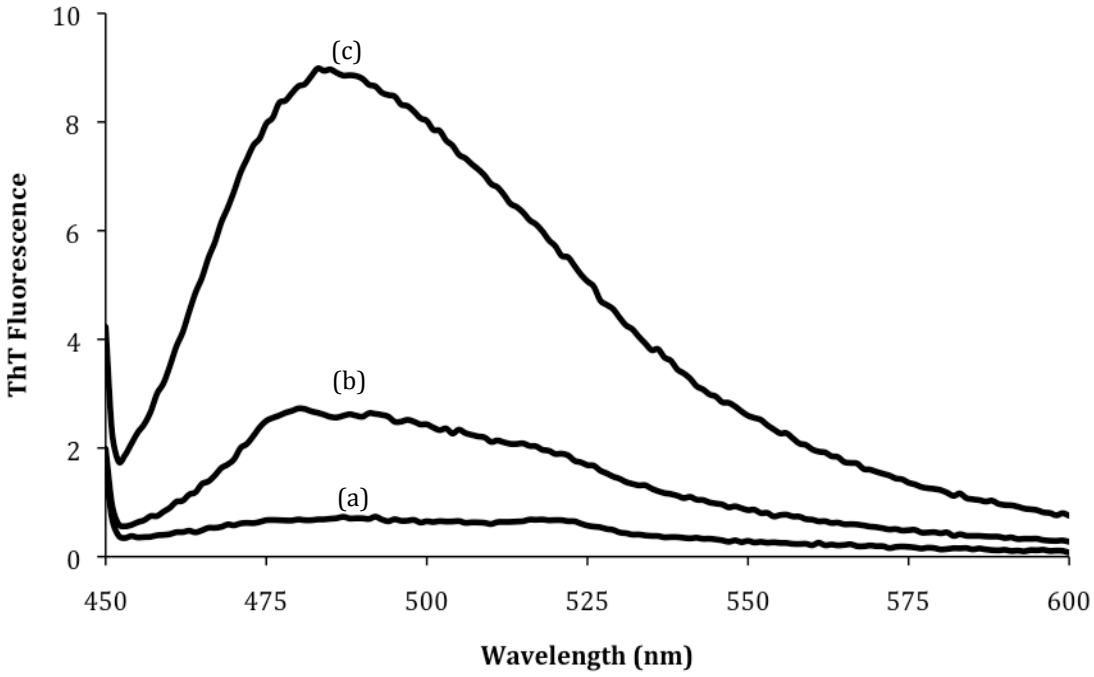

FIG. 6. **ThT assay of Aβ42 over 48 h.** Aβ42 (77 μM) samples were incubated over a 48 h time frame before being measured for fluorescence emission. A blank fluorescence emission containing only ThT (5 μM ThT, 50 mM glycine, pH 8.5) was measured from 450 nm to 600 nm at an excitation wavelength of 442 nm (a). Under identical spectroscopic conditions, 5 μM Aβ42 in ThT samples for time 0 h (b) and 48 h (c) were also measured for fluorescence emission.

The previous procedures were repeated, again to promote the intrinsic aggregate formation of Aβ42, except that 168 μM Aβ42 was prepared, combined with the appropriate phosphate buffers, and incubated for allotted amounts of time at 37 °C. Samples were removed from incubation after 0 h, 8 h, 24 h, 48 h, and 72 h, respectively, and immediately measured for fluorescence emission via a ThT assay. Experimental fluorescence readings were compared to that containing only ThT, used as a blank. Figure 7 depicts the curves that were generated from each of the fluorescence scans. Curve (a) represents a baseline containing only the ThT blank, and therefore no amyloid formation. Samples ranging from time 0 h to 72 h are depicted by curves (b), (f), (e), (c), and (d), listed chronologically. Unlike the results seen in Figure 6, the maximums on the curves in Figure 7 do not increase as a direct function of incubation time (i.e. the maximum fluorescence at 480 nm of the 8 h sample, 10.7, is higher than that of the 48 h sample, 7.9).

Verification that the Aβ42 protein began experimentation in its monomeric, non-amyloid form was conducted through circular dichroism (CD) spectroscopy. Portions of the aforementioned 0 h and 24 h samples were used in the CD spectroscopic measurement to generate the data seen in Figure 8. At a wavelength of 220 nm, the 0 h sample generated a larger negative ellipticity value than did the 24 h sample.

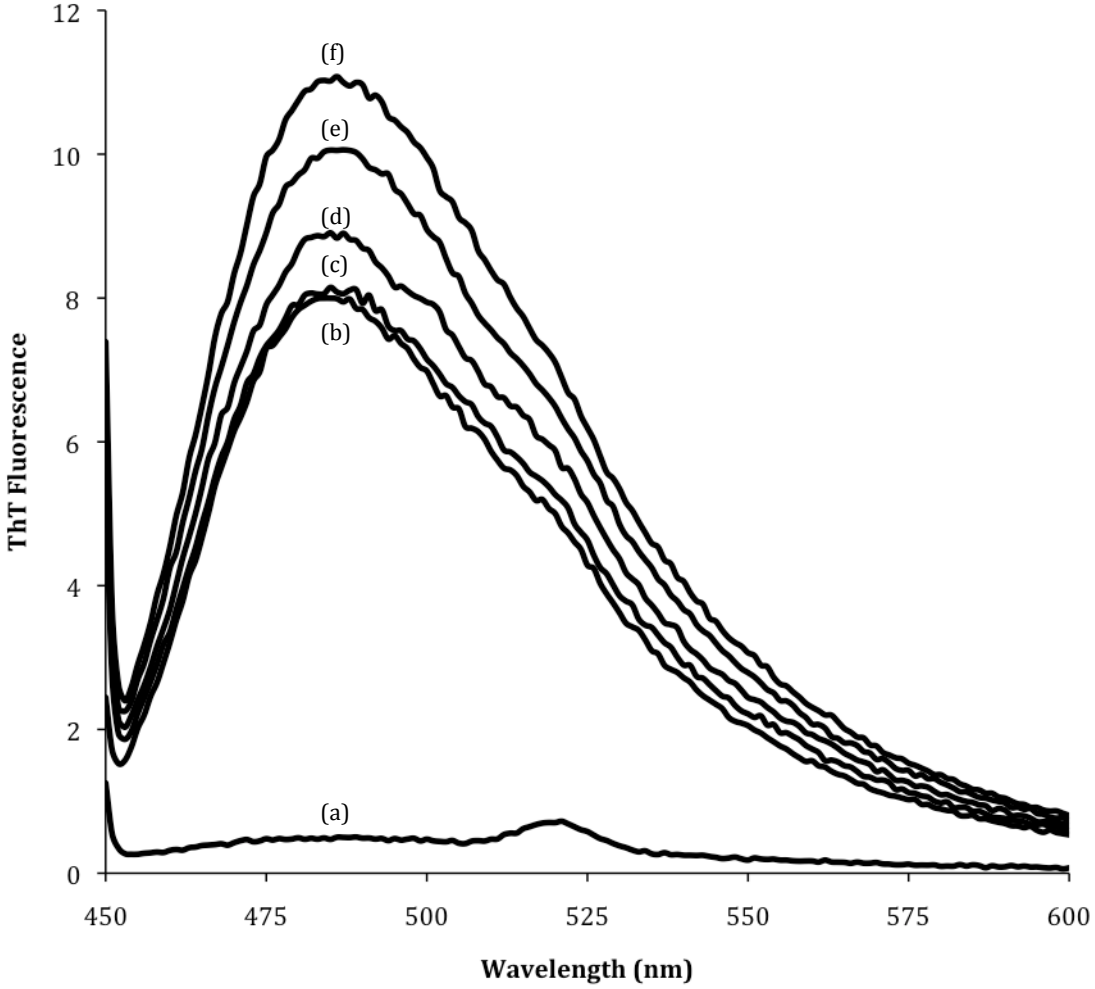

FIG. 7. **ThT assay of Aβ42 over 72 h.** Aβ42 (42 μM) samples were incubated over a 72 h time frame and measured for fluorescence emission at various times. A blank fluorescence emission containing only ThT (5 μM ThT, 50 mM glycine, pH 8.5) was measured from 450 nm to 600 nm at an excitation wavelength of 442 nm (a). Under identical spectroscopic conditions, 1.4 μM Aβ42 in ThT samples for times 0 h (b), 8 h (f), 24 h (e), 48 h (c), and 72 h (d) were also measured for fluorescence emission.

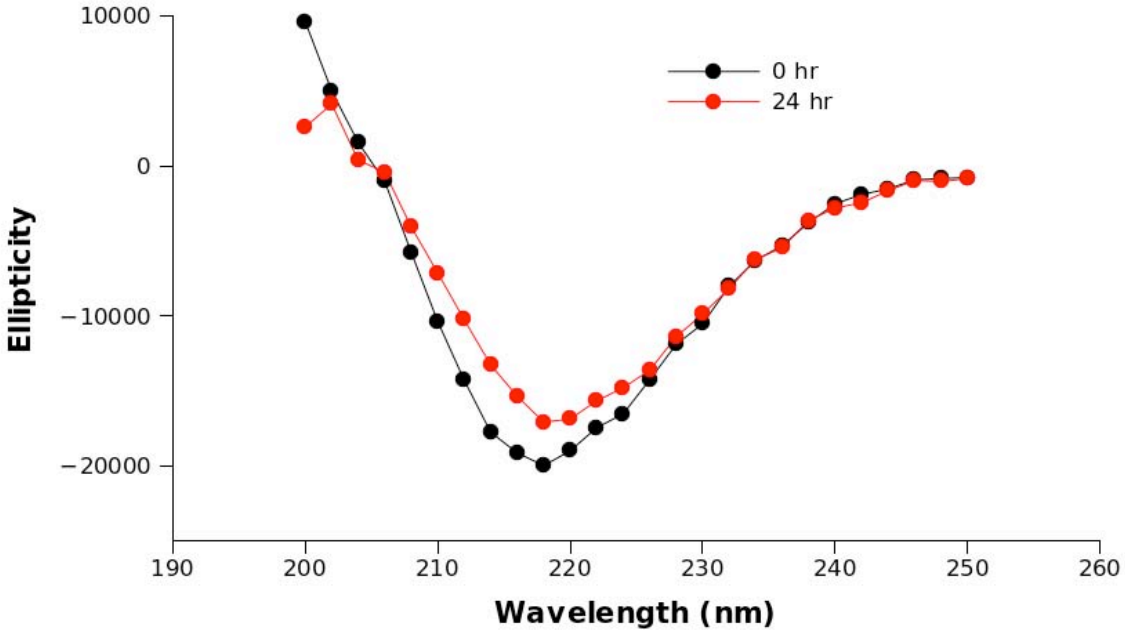

FIG. 8. **Circular dichroism (CD) spectroscopic determination of Aβ42's state.** CD spectroscopic measurements were obtained after 0 h and 24 h, respectively, to determine the formation or loss of amyloid following incubation. The lower curve depicts the ellipticity of the 0 h sample, which had a larger negative value than that of the 24 h sample at 220 nm.

In following the procedure that called for a 0.22 μm cutoff syringe filtration, Aβ42 was dissolved in 0.1 % TFA and filtered. Subsequent determination of its concentration yielded a significantly small spectrophotometric reading that indicated an Aβ42 concentration of 0.7 μM in the filtrate. Due to the lack of beta amyloid protein in the solution, no further experimentation or ThT assay was performed on the sample.

The ultracentrifugation of Aβ42 dissolved in 0.1 % TFA at 541,000 x g and 4 °C for 3 h, however, did yield spectrophotometric readings indicative of Aβ42 presence. This procedure yielded visible precipitation, from which the supernatant was removed for concentration determination measurements, and found to be 140 μM.

4. DISCUSSION

4.1 Conclusions

The tendency of β-amyloid peptides to spontaneously aggregate *in vivo* lends way to a troubling and life changing illness, Alzheimer's disease. Its propensity to also aggregate spontaneously *in vitro*, however, allows for research to be conducted in order to study the disease. In analyzing the kinetics by which these β-amyloids, specifically Aβ42, aggregate with each other, we will soon be able to study any altering affects that the introduction of various oligopeptides may have on the amyloid fibrils.

Before attempting to determine the effect of oligopeptides on Aβ42 aggregation, the spontaneous formation of peptide amyloid was first evaluated. In doing so, thioflavin T was the fluorescent agent used to bind aggregates and relay amyloid formation through graphical data. As a result, a primary and essential goal concerned having readily available, freshly prepared, and consistent ThT solutions prior to and following experimentation. A series of fluorescence detections was used as the controls to determine whether the ThT was reliable or degraded. Subsequent measurements whose curves yielded smaller maximum values than the original controls or a deviation in shape were presumably tainted or improperly prepared. Throughout experimentation, ThT solution purity allowed for the determination of valid and consistent results.

Upon obtaining Aβ42 click-peptides, the protein was dissolved in 0.1 % TFA and existing amyloid fibrils were removed by a variety of methods. In the first, light

centrifugation was applied and the supernatant was saved. Spectroscopic absorbency measurements suggested the presence of protein in the supernatant, allowing for a ThT assay to be performed. Initial incubation of samples for 0 h and 48 h, respectively, yielded data consistent with what was expected, a direct correlation in incubation time and amyloid formation (Figure 6). The subsequent experiment, however, failed to validate those initial findings (Figure 7). Similar to the results in the first graph, initial addition of Aβ42 sample to ThT in the subsequent experiment prompted an increase in fluorescence relative to the ThT blank. Such a response is clearly indicative of amyloid fibril presence in solution. However, only data in which the 0 h sample's plot remained significantly near that of the ThT blank would indicate completely pure, monomeric beta amyloid. In addition, the other samples, whose incubation times ranged from 8 h to 72 h, did not yield fluorescence increases in direct correlation with incubation time, as would be expected (*20*). In fact, the results seem scattered with no trends except that the lowest peak signified Aβ42 after 0 h of incubation. Due to the inconsistency in data from one trial to the next, it is clear that amyloid fibrils, in significant capacity, were present prior to the addition of phosphate buffer. Despite the Aβ42 click-peptides being engineered to resist their natural conformation away from ideal pH, it is likely that aggregation was able to occur, thus promoting an inconsistent set of data as a function of incubation time. Light centrifugation, therefore, was ruled out as an alternative method for isolating purely monomeric Aβ42.

In attempting a crude form of filtration to isolate and discard unwanted amyloid fibrils, a 0.22 μm-cutoff syringe filter was used to yield a filtrate. Upon spectrophotometric analysis, however, nearly all protein had been removed from the

solution. For obvious reasons, use of this filtration system was deemed unable to effectively isolate monomeric Aβ42, likely because of some adsorption or attractive interaction between Aβ42 peptide and the filter's membrane. A variety of research has in fact shown that different types of nitrocellulose membranes and the like often have a tendency to adsorb proteins under filtration conditions (21).

Contrary to the previous two methods, ultracentrifugation under more extreme conditions was able to visibly produce a pellet, likely to be unwanted, pre-formed Aβ42 aggregation. Without any preformed amyloid fibrils, this technique was able to yield an Aβ42 solution ready for kinetic studies, 140 μM. As seen in a prior study, exposure of the Aβ42 solution to 541,000 x *g* under vacuum at 4 °C for a prolonged period also effectively rid the sample of any existing amyloid (*22*).

At all times, Aβ42 in 0.1 % TFA was stored as described by Baducci et al., at -80 °C (10). Under such conditions, the Aβ42 solution could last for weeks without degradation or unforeseen spontaneous amyloid formation. Only the addition of phosphate buffer allowed the click-peptides to return to their native state and begin to aggregate.

4.2 Experimental Findings

Additional experimental observations drew unforeseen conclusions, which will prove useful for future experimentation. Initial protocol called for a variety of small 1.5 mL and 0.5 mL tubes to be used during incubation at 37 °C and 600 rpm. For samples incubated longer than 24 h, however, only safe-lock 1.5 mL Eppendorf tubes ensured that

all solution remained in the tube. In many of the tubes, condensation collected on the undersides of the caps. Some 48 h samples were lost completely from evaporation in other tubes, so safe-lock tubes were employed for all remaining experiments.

Additionally, 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was believed to quench, or stabilize, any amyloid formation upon removing samples from incubation. The fluorescence measurements that followed the quenching of samples, however, yielded fluorescence equivalent to that of the baseline ThT blank. It was clear at that point that HFIP worked simply to dissolve any amyloid that formed, which was also a conclusion of a previous study (*23*). The solubility of Aβ42 in HFIP is especially significant for the β(34-42) region, which does not readily dissolve in other, more common solvents *in vitro* (*23*). For this reason, the use of HFIP in dissolving any aggregate formation *in vitro* is greatly worthwhile, although it is inapplicable *in vivo* because of the organic chemical's hazardous qualities.

4.3 Future Experimentation

The correlation of AD symptoms with the deposition and aggregation of β-amyloid in blood vessels in the brain has sparked a significant amount of research on the disease. While AD continues to be a major factor in the lives of those who are affected by the disease, research into the kinetics of amyloid fibrils may prove effective in limiting the effect of or abolishing the symptoms. After further experimentation using the extreme conditions of ultracentrifugation to isolate monomeric Aβ42, the kinetics of spontaneous agreggation *in vitro* will be better understood. Future experimentation will then utilize the

screening of an oligopeptide library to identify sequences that may bind to and alter the aggregation of the Aβ42 peptides, as seen in a previous PrP study. In measuring the relative fluorescence in a number of Aβ42+oligopeptide samples, peptides may be identified that inhibit, promote, or dissolve formation of Aβ42 amyloid. Screening more oligopeptides may lead to knowledge of peptides that can dissolve amyloid fibril complexes and lead to enhanced treatment of AD or the development of new drugs.

REFERENCES

1. Lee, J. S., and Park, C. B. (2010) *Biomaterials* **31**, 6789-6795

2. Ballard, C., Gauthier, S., Corbett, A., Brayne, C., Aarsland, D., and Jones, E. (2011) *Lancet* **10**, 61349

3. Alzheimer's Disease Fact Sheet. National Institutes of Health (November 2008), http://www.nia.nih.gov/Alzheimers/Publications/adfact.htm

4. Pepeu, G. and Giovannini, M. G. (2010) *Chem.-Biol. Interact.* **187**, 403-408

5. Dobson, C. M. (2001) *Phil. Trans. R. Soc. Lond. B* **356**, 133-145

6. Murphey, R. M. (2002) *Annu. Rev. Biomed. Eng.* **4**, 155-174

7. Personal correspondence, Claudio A. Soto, University of Texas Medical School in Houston

8. Seppala, T. T., Herukka, S. K., Hanninen, T., Tervo, S., Hallikainen, M., Soininen, H., and Pirttila, T. (2010) *J. Neurol. Neurosur. Ps.* **81**, 1123-1127

9. Shoghi-Jadid, K., Barrio, J. R., Kepe, V., Wu, H. M., Small, G. W., Phelps, M. E., and Huang, S. C. (2005) *Nucl. Med. Biol.* **32**, 337-351

10. Fukuda, T., Matsumoto, E., Onogi, S., and Miura, Y. (2010) *Bioconjugate Chem.* **21**, 1079-1086

11. Croney, J. C., Jameson, D. M., and Learmonth, R. P. (2001) *Biochem. Mol. Biol. Edu.* **29**, 60-65

12. Lakowicz, J. R. (Ed.). (2000) *Topics in Fluorescence Spectroscopy* (Volume 6). New York: New York Kluwer Academic/Plenum Publishers.

13. Sulatskaya, A. I., Maskevich, A. A., Kuznetsova, I. M., Uversky, V. N., and Turoverov, K. K. (2010) *PLoS One* **10**, e15385

14. Taniguchi, A., Sohma, Y., Kimura, M., Skwarczynski, M., Nagaon, T., Okada, T., Ikeda, K., Hayashi, Y., Kimura, T., Hirota, S., Matsuzaki, K., and Kiso, Y. (2006) *J. Pept. Sci.* **12**, 823-828

15. Kelly, S. M., Jess, T. J., and Price, N. C. (2005) *Biochim. Biophys. Acta* **1751**, 119-139

16. Aitken, A. and Learmonth, M. (1996) The Protein Protocols Handbook. J. M. Walker (Ed.), *Protein Determination by UV Absorption* (pp. 3-6). New Jersey: Humana Press Inc.

17. Pace, C. N., Vajdos, F., Grimsley, G., and Gray, T. (1995) *Protein Sci.* **4**, 2411-2423

18. Balducci, C., Beeg, M., Stravalaci, M., Bastone, A., Sclip, A., Biasini, E., Tapella, L., Colombo, L., Manzoni, C., Borsello, T., Chiesa, R., Gobbi, M., Salmona, M., and Forloni, G. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107**, 2295-2300

19. Levine, H. (1999) *Methods Enzymol.* **309**, 274-284

20. Bourhim, M., Kruzel, M., Srikrishnan, T., and Nicotera, T. (2007) *J. Neurosci. Meth.* **160**, 264-268

21. Birk, H. W., Kistner, A., Wizemann, V., and Schutterle, G. (1995) *Artif. Organs* **19**, 411-415

22. Taniguchi, A., Sohma, Y., Hirayama, Y., Mukai, H., Kimura, T., Hayashi, Y., Matsuzaki, K., and Kiso, Y. (2009) *ChemBioChem.* **10**, 710-715

23. Halverson, K., Fraser, P. E., Kirschner, D. A., and Ladsbury, P. T. (1990) *Biochemistry* **29**, 2639-2644

VITA

Stephen Thomas Gates was born in Shreveport, Louisiana on February 4, 1989 to Thomas and Sheryl Gates. After graduating Salutatorian from Samuel Clemens High School in Schertz, Texas in 2007, he entered Texas State University-San Marcos. He received his Bachelor of Science in Biochemistry from Texas State upon graduating Summa Cum Laude, with Honors, in May 2011. He matriculated into the University of Texas Health Science Center School of Medicine in San Antonio in August 2011.

Permanent Address: 1016 Linden Grove Dr.
Schertz, Texas 78154

This thesis was typed by Stephen T. Gates.