Protein aggregation studies: inhibiting and encouraging β-amyloid aggregation

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PROTEIN AGGREGATION STUDIES:
INHIBITING AND ENCOURAGING
β-AMYLOID AGGREGATION

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by

Jed Paul Aucoin
B.S., Nicholls State University, 1999
May 2004
Dedication

This dissertation is dedicated to:

My wife, Monique Aucoin
My parents, Fay and Harry Aucoin
My mother and father in-Law, Donna and Ronald Peltier
My brothers, Ward and Dewey
My sisters in-law, Lisa and Louise
My brothers in-law, Jeffrey and Jason
My nieces, Brooke, Taylor, and Zoe
My extended family
All of my friends
I am thankful for all of the love and support
Acknowledgments

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I would like to acknowledge Marcus Etienne and Yanwen Fu for synthesizing and preparing the peptide-based aggregation mediators that were used in this work. I also appreciate Martha Juban for performing the amino acid analysis of peptide solutions prepared in the laboratory. I would also like to thank Dr. Isiah Warner for allowing me to perform tensiometry work in his laboratory. I gratefully acknowledge the National Institutes of Health (NIH R01 AG17983-01A1) for funding this work.

I would like to extend my appreciation to my wife, Monique, for making me realize what is really important while achieving my goals. Thank you for loving me so much. I would also like to thank my mother, Fay, and father, Harry, for their unconditional love. I appreciate the moral and conservative upbringing you provided.

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I would like to thank my mother-in-law, Donna, and father-in-law, Ronald, for taking me in and taking care of us. I would also like to thank Jason and Jeffrey for being awesome brothers–in–law.
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I also would like to thank my fellow McCarley Group colleagues for helping me during my time here. I appreciate the humor and friendly environment of our group and I wish each and everyone success during their graduate studies.
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<td>α</td>
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<tr>
<td>AAA</td>
<td>amino acid analysis</td>
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<td>Aβ (1-40)</td>
<td>beta–amyloid 1-40</td>
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<tr>
<td>Aβ (10–35)</td>
<td>beta–amyloid 10-35</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>AMY-1</td>
<td>mediator with oligolysine tail on C terminus</td>
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<tr>
<td>AMY-2</td>
<td>mediator with oligolysine tail on N terminus</td>
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<tr>
<td>Beta</td>
<td>β</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
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M  molar
mg  milligram
ml  milliliters
mm  millimeter
mM  millimolar
μM  micromolar
mN  milliNewton
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl  sodium chloride
nM  nanomolar
NGF  nerve growth factor
PBS  phosphate buffer system
POPC  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
RAIR  reflection absorption infrared spectroscopy
RMS  root mean square
RPM  rotations per minute
RT  room temperature
SFM  scanning force microscopy
SEM  scanning electron microscopy
TEM  transmission electron microscopy
TTR  transthyretin
XPS  X–ray photoelectron spectroscopy
Abstract

This work demonstrates peptide-based aggregation mediation of fibril forming peptides, notably β-amyloid (1-40), Aβ₁₋₄₀. To achieve the goals of this work, Aβ₁₋₄₀ aggregation was governed by ionic strength, temperature, and pH. After controlling and reproducing Aβ₁₋₄₀ aggregate formation (fibrils), each peptide-based aggregation mediator was evaluated for its ability to self-aggregate and to possess surface-active properties. The incorporation of these mediators in Aβ₁₋₄₀ solutions changed the surface tension and produced aggregate complexes of mediator and Aβ₁₋₄₀. Evidence that Aβ₁₋₄₀ fibril formation may be mediated by disorder-to-order transitions has been observed by layer formation on top of a β-sheet monolayer of another fibril forming, but cyclic, peptide.

Governance of Aβ₁₋₄₀ aggregation was performed by controlling the environment of both the stock and sample conditions. Sample containment was important in controlling aggregation, as a certain brand of vessels induced Aβ₁₋₄₀ aggregation. The introduction of ionic strength and temperature also enabled studies at different aggregation stages of Aβ₁₋₄₀.

Each peptide-based aggregation mediator was characterized to determine any self-aggregation. It was found that AMY-1, developed at Louisiana State University, and an inhibitor developed by Dr. Regina Murphy at the University of Wisconsin sparsely aggregated. AMY-2, also developed at L.S.U., favored deposition on mica. The surface activity of each mediator was assessed by tensiometry. The Murphy inhibitor produced no change in surface activity over a wide concentration range, while AMY-1 and AMY-2 displayed surface activity and critical micelle concentrations just below 1 mM.

The introduction of some mediators caused changes in surface tension to solutions of Aβ₁₋₄₀; Murphy’s inhibitor increased the surface tension, whereas AMY-1 and AMY-2 decreased
the surface tension of Aβ\textsubscript{1-40} solutions. Complex formation between each mediator and Aβ\textsubscript{1-40} inhibited fibril development and instead created amorphous aggregates, as evidenced by scanning force and transmission electron microscopy.

The adsorption of a monolayer of Aβ\textsubscript{1-40} onto a β-sheet monolayer of cyclic peptides was observed. These cyclic peptides, initially thought to be fibril inhibitors, were found to be potent fibril formers themselves. These cyclic peptides attached only to exposed sites of highly oriented pyrolytic graphite. The ability for a surface to induce fibril formation may allow a more thorough understanding of Aβ\textsubscript{1-40} fibril formation.
Chapter 1

Goals, Research Synopsis, and Background

1.1 Research Goals

The primary goal of this research was to develop a methodology that would allow a reliable measurement of β–amyloid (Aβ) aggregation behavior in the presence and absence of peptide–based aggregation mediators in a near–physiological environment. It was proposed that these mediators can provide alternate non–toxic aggregation pathways. In order to achieve reproducible measurements of β-amyloid aggregation, the initial preparation in these studies required samples free of unwanted impurities that could lead to premature aggregation of Aβ. After precautionary methods were set in place, each peptide–based aggregation mediator was characterized according to the mediator’s surface activity and propensity to self-aggregate. The determination of surface-active properties employed the use of tensiometry for determinations of surface pressure at varying concentrations. Also, scanning force microscopy was used to observe any self-aggregating propensity of the mediators.

Upon completion of their characterization, each peptide–based aggregation mediator was analyzed for their specificity toward β–amyloid. Transmission electron microscopy and scanning force microscopy was used to define any disruption of Aβ fibril formation. The methodologies described herein provide a foundation for future efficacy studies of newly synthesized peptide–based aggregation mediators.

1.2 Research Synopsis

1.2.1 Preparation of Reproducible β–Amyloid Stock Solutions

The first research aim was to prepare a reliable and reproducible β–amyloid stock solution. The lyophilized peptide was obtained from Biosource (lot number Q9279). A stock solution free of impurities such as dust, bacteria, metal content, and premature Aβ aggregates
was needed for preparing samples with the same rate of aggregation. Possible contamination by bacteria was addressed by autoclaving all pipet tips and microcentrifuge tubes. Impurities such as metals were minimized by using only salts with purity above 99.99%. The removal of dust from buffered solutions and removal of premature Aβ aggregates was accomplished by filtering with non-rinsed inorganic Anotop 0.02 μm filters. Proper sample containment was also found to be important for eliminating premature Aβ aggregation, as it was found that the inner wall roughness of microcentrifuge tubes promoted aggregation of Aβ solutions. After removing any conditions that would promote uncontrolled or premature aggregation, the aggregation state of the Aβ solutions could then be studied over time and as a function of solution conditions.

1.2.2 Controlled Aβ Aggregation

Aβ aggregate formation could be induced by the presence of high ionic strength phosphate buffer at pH 7.4 and elevated temperature. The buffer used was near physiological conditions and consisted of 50 mM phosphate buffer and 150 mM NaCl at a pH of 7.4. Fibril formation of Aβ_{10-35} or Aβ_{1-40} under these aqueous solution conditions occurred slowly (3-5 days before fibrils observed). Due to such slow fibril formation, Aβ solutions were placed in an Eppendorf Thermostat plus incubator at 50 °C for up to 24 hours; SFM observed ~300 nm long protofibrils after just a 2 hour incubation at 50 °C.

1.2.3 Characterization of Peptide–Based Aggregation Mediators

These mediators were first characterized for self aggregation. Utilizing scanning force microscopy, it was found that some of the mediators self aggregated. Surface pressure measurements by tensiometry were also made of each inhibitor to determine any surface activity. Two inhibitors developed at Louisiana State University had a critical micelle concentration just below 1 mM in concentration. Therefore, all of the mediator/ Aβ studies were conducted at concentrations well below mediator micelle formation.
1.2.4 Determination of Mediator Efficacy on β–Amyloid Aggregation

Once the mediators were characterized individually, they were introduced into solutions containing β–amyloid. The introduction of each mediator into β–amyloid environments was to determine their specificity for interacting with β-amyloid and their effect on β–amyloid aggregate formation. The efficacy of each mediator was measured by its ability to form amorphous aggregate forms, dissolve fibrils, and inhibit fibril formation. Out of several aggregation mediators, one was found to be highly specific to β-amyloid. This mediator, AMY-2, aggregated rapidly with β–amyloid to produce a non–fibrillar amorphous aggregate. This research establishes groundwork for future mediator efficacy studies.

1.3 Background

1.3.1 Overview of β–Amyloid

Protein misfolding and fibril formation has been noted in several diseases. Misfolded intermediates of a protein, β-amyloid, have been found to form fibrils in the brain tissue of individuals with Alzheimer disease. The origin of this β-amyloid protein arises from an abundance of enzymatic cleavages of the neuron trans-membrane protein amyloid precursor protein (APP). The associations of these β-amyloid proteins during the construction of fibrils are known to harbor toxic effects upon neuronal cells.

Proposed options for alleviating the symptoms or progression of the disease involve controlling the body’s immune response to Aβ, lowering inflammation of brain tissue, and reconstructing already damaged regions of the AD brain. Many proposals have implicated Aβ as the major protagonist of Alzheimer’s disease progression; thus, multiple proposals have been made for the elimination or control of Aβ aggregate formation. The work presented in this document is devoted to the amelioration of toxicity through the mediation of β–amyloid aggregation.
1.3.2 Defining Proteins and Peptides

Proteins are central to all living systems due to their involvement in all aspects of cell life, immune protection, cell-cell communication, physical support (hair and skin), and muscle movement.\(^8,9\) Synthetically prepared proteins, shortened or cleaved proteins and proteins with unnatural amino acids can be referred to as peptides. Proteins and thus peptides are built from smaller individual amino acid units with each amino acid containing an \(\alpha\)-carbon bonded to an \(\alpha\)-amino group, an \(\alpha\)-carboxyl group, one hydrogen, and a side chain, R, of varying lengths and functionalities.\(^9\) The growth of a protein occurs by a condensation reaction between the free amine and carboxylic group of the different amino acids to form amide bonds.\(^9\)

1.3.3 The Driving Force Behind Protein Aggregation: Hydrophobic Clustering

Hydrophobic clustering or collapse is not caused by the mere chance encounters of hydrophobic domains, but is driven by the ordering of solvent (water) upon these domains.\(^9\) Water as a solid (ice) is more ordered than liquid water and thus has lower entropy. When ice is melted, liquid water molecules become less ordered and increase the entropy of the system. When a hydrophobic domain on a protein or peptide is placed in water, the water molecules are disturbed in its immediate vicinity. These water molecules compete with the hydrogen bonding nature of the hydrophobic interactions of the protein and orientate and thus order. In the presence of another hydrophobic domain, these ordered water molecules will become disturbed and are released with increased entropy. Thus, the dispersion of low entropy and ordered water molecules into less ordered and higher entropy water molecules provides a thermodynamically favorable event.\(^9\)
1.3.4 Protein Folding

Protein folding is driven by the need for a protein to sequester its hydrophobic side chains from water. This sequestering allows the polar groups of the protein’s backbone (NH and COOH) to hydrogen bond. The fold of a protein depends on the types of amino acids present within its protein sequence. The secondary structure of the α-helix is formed by the hydrogen bonding between the NH and CO groups of the same strand whereas the β-strand forms hydrogen bonding with other β-strands (Figure 1.1).

![Figure 1.1 Secondary Structures of Proteins. (A) α-helix. (B) β-sheet. The peptide backbones (blue line) can intramolecular hydrogen bond (red dotted line) as seen in the α-helix or intermolecular hydrogen bond (red dotted line) as seen in the β-sheet.](image)

These β-strands stabilize each other through intermolecular hydrogen bonding forming β-sheets. Tertiary structures build on these secondary structures to form other higher-order structures such as β-sheets, βαβ units, β-hairpins, and α-helix bundles. The fibrils of β-amyloid are composed of mainly β-sheets.

1.3.5 Protein Misfolding

If a protein were not to fold into some type of conformation, it would not function. Thus, folding is important to sustain life. However, conformational disordering results from the misfolding of the secondary structure of the protein, as in the cases of: Alzheimer’s (β–amyloid
protein deposits); amyotrophic lateral sclerosis or Lou Gehrig’s disease (mutations in superoxide dismutase protein); type 2 diabetes mellitus (Amylin 1-39); Creutzfeldt–Jakob disorder (prion protein); Huntington’s disease (Huntington protein); Parkinson’s (α-synuclein protein); and, familial amyloid polyneuropathy and hemodialysis–related amyloidosis (transthyretin protein).

Protein misfolding is usually a transition from the normal secondary structure of the protein into a β-sheet conformation. This transition may occur through genetic factors and sporadic expressions where conditions such as pH, ionic strength, nucleators, etc. play a role in the misfolding of the protein. Also, as in the case of prion disorders, infectious non-native β-sheet conformation proteins can induce protein misfolding of other proteins. The proposed lowering of activation energy necessary for protein/surface ordering and facilitation of an equilibrium shift toward the β-sheet conformation may help to explain the observed random coil to β-sheet transition of Aβ when interacting with lipids and phospholipids. Also, metabolites such as ozonized cholesterol have also been observed to initiate amyloid protein misfolding.

1.3.6 The Misfolding of a Protein May Lead to Fibril Formation

Misfolded proteins, especially in β-sheet Conformations can be further stabilized through aggregation or oligomerization (fibril formation). Protein misfolding has been the primary cause of fibril formation in several diseases. Fibril formation can be displayed by many proteins without much sequence similarity. Amyloid fibrils are likely to be composed of β–sheet subunits; however, evidence that even an α-helical protein can form fibrils under basic and high temperature conditions has been displayed by the protein muscle myoglobin.

1.3.7 Mechanism of Aβ Fibril Formation

Fibril formation is thought to consist of an aggregation pathway whose genesis contains a high entropic barrier and a thermodynamically unfavorable event. The aggregation and fibril
formation of Aβ consists of a conformational change from random coil (no structure) or α-helix into a β-strand.\textsuperscript{2} The pathogenesis pathway of the prion protein also contains this α-helix to β-strand transition.\textsuperscript{22} This transition has been noted to be slow in β-amyloid due to conformational minima at both the α and β conformers.\textsuperscript{16,23} Promotion of this conformation transition may be induced by the presence of non-amyloid artifacts.\textsuperscript{23}

The conformational change of β-amyloid is in part due to the hydrophobic collapse of the very hydrophobic C-terminal region of Aβ thereby limiting the rate of nucleation, and this hydrophobic interaction is further maximized by β-sheet conformation.\textsuperscript{2,21,24} The formation of these nuclei and fibril development are nucleation dependent and the kinetics of such fibril formation are determined by nucleation and fibril elongation rate.\textsuperscript{25}

The formation of nuclei as seen in Figure 1.2 is thermodynamically unfavorable, while the addition of monomers to existing nuclei is favorable.\textsuperscript{26} Monomers attach perpendicular to the proto-fibril axis with hydrogen bonding occurring parallel to this axis.\textsuperscript{27} These protofibrils contain roughly 1.2 to 2.1 monomers of Aβ\textsubscript{1-40} per each nm length of the protofibril.\textsuperscript{28} Lateral associations of proto-fibrils allow the construction of the mature fibril where the polar N terminal portion of β-amyloid (1-40) has been found to rest outside of the fibril core.\textsuperscript{24}

![Figure 1.2 Mechanisms of Protein Aggregation and Possible Route to Fibril Formation](image)

**Figure 1.2 Mechanisms of Protein Aggregation and Possible Route to Fibril Formation**
1.3.8 Clinical Observations Associated with Alzheimer’s Disease

The appearance of lesions or plaque formation in the brain has been identified in the post mortem brains of people identified as having Alzheimer’s disease. Intracellular deposits are commonly found in neurological diseases and are comprised of τ-protein tangles, but in Alzheimer’s disease, neural and non-neural cells contain oligomers of the peptide β-amyloid. The presence of intracellular β-amyloid is thought to predate the aggregation and fibril formation of this peptide outside of the cell. Extracellular deposits mainly found in the temporal lobe of the brain are mostly composed of β-amyloid fibrils, a misfolded and aggregated protein structure unique to Alzheimer’s disease. These amyloid fibrils have been found in the cerebral cortex among the leptomeningeal, meningeal, vascular, and cortical vessels. Furthermore, β-amyloid presence has been implicated as the main contributor to symptoms involving memory and cognition decline.

Early observations of fibrils were found through the Bielschowsky’s silver method of staining of post mortem brains in individuals with dementia by Alois Alzheimer. Further purification and characterization of amyloid fibrils employed congo red staining and polarization microscopy. One postmortem study of an individual afflicted with Alzheimer’s disease noted 20 grams of β-amyloid in a 500 gram brain or 10 nmol of β-amyloid per gram of wet tissue. Soluble Aβ has been found to be produced by various cell types, and has been found to circulate in the cerebrospinal fluid, urine, and blood of both AD and non–AD individuals. Concentrations of Aβ in AD patient serum are around 30 to 150 nM, 6 times higher than individuals without dementia.

1.3.9 Genetics

Several genetic markers have been found to be associated with AD. The genes which encode for amyloid precursor protein, presenlin-1, presenlin-2, and apolipoprotein E have been
found to be associated with Alzheimer’s disease. Some of these markers provide additional clues as to the age at which the onset of AD may occur. A genetic marker located on chromosome 21, when presented in a triplicate manner (Trisomy 21), causes both Down syndrome and early onset AD. The onset of AD above the age of 60 has been associated with a second genetic marker on chromosome 19. The onset of AD between the ages of 20 and 50 is most likely due to a presenlin 1 mutation on chromosome 14. Also, the development of AD in people above 50 years of age is sometimes caused by presenlin-2 mutation on chromosome 1.

1.3.10 The Origin of β-Amyloid in Alzheimer’s Disease

The peptide, β-amyloid, originates from a proteolytic cleavage of the amyloid precursor protein (APP), a human transmembrane protein reportedly needed for memory. The cleavage of this precursor protein occurs during normal cellular metabolism (Figure 1.3). In the healthy brain, a soluble fragment of the precursor protein is evolved during a two step cleavage initiated a cleavage of APP outside the cell by α-secretase followed by a second cleavage on APP at the cell membrane surface by γ-secretase. Furthermore, this cleavage occurs between the lysine (16) and leucine (17) amino acids located in the hydrophobic KLVFF region, removing the viability for Aβ aggregation and producing the peptide fragment p3 (Aβ17-40 or Aβ17-42).

In the pathogenic proteolytic processing of APP in the Alzheimer’s diseased brain, the initial cleavage of APP occurs by β-secretase and not α-secretase, leaving a longer cleaved APP at the cell membrane surface. This longer and cleaved APP region is removed from the cell surface by γ-secretase.

Cholesterol is thought to play a role in this pathogenic route. During uptake of cholesterol by the cell membrane, the membrane swells and allows a more predominant β-secretase cleavage to take place rather than an α-secretase cleavage of APP. 90% of the β-amyloid peptides produced during this later cleavage is (1-40) and 10% is β-amyloid (1-42).
1.3.11 A Description of Aβ Properties

The β-amyloid peptide is an amphipathic molecule containing both a hydrophilic N-terminus and a hydrophobic C-terminus. The propensity for β-amyloid to adopt a β-strand is in part due to the β-turn (figure 1.2 shown in green) stabilized by a salt bridge between residues aspartic acid (23) and lysine (28) and a hydrophobic region (Figure 1.4 shown in red) at residues (lysine(16), leucine(17), valine(18), phenylalanine(19), phenylalanine(20)). Replacement of these hydrophobic amino acids with hydrophilic amino acids has been found to inhibit β-amyloid fibril development. Although these sequences (KLVFF and VGSN) have been found to enable β-sheet folding and aggregate β-amyloid proteins, an Aβ protein with both of these same primary sequences can also adopt a conformation which is random coil and more vulnerable to protease degradation. The effect of environmental factors on protein conformation has been identified with β-amyloid and several other proteins; work in this area has led to the notion that any protein solution above some critical concentration will eventually aggregate as the correct fold of the protein begins to structurally change and aggregate into a more stable form.

![Figure 1.3 Cleavage of the Amyloid Precursor Protein (APP). (A) Normal processing of APP. (B) Abnormal processing of APP.](image-url)
Figure 1.4 Aβ₁₋₄₃. The Hydrophobic Region is in Red and the β-turn Region is in Green.

The β-amyloid structure can not be determined by solution NMR or X-ray crystallography due to its insoluble and non-crystalline nature. However, X-ray diffraction and Fourier-transform infrared spectroscopy (FTIR) both confirmed that short β-amyloid fragments favor an antiparallel β-sheet. Also, solid state NMR has been able to elucidate the structure of β-amyloid which was impossible with solution NMR. The solid state NMR method known as magic angle spinning further narrows resonances and can determine the structural constraints of β-amyloid peptides. Solid state nuclear magnetic resonance (NMR) identified full length Aβ as containing a cross-β motif which incorporates in-registry parallel β-sheets. The amyloid protofibril is composed of two interacting cross β-motifs forming a structure with 4 layers of in-registry parallel β-sheets (Figure 1.5).

Local acidic environments (endosomes and lysosomes) in the brain are thought to promote fibril formation. The propensity for Aβ to aggregate in such low pH conditions has also has been noted in vitro where pH environments below the isoelectric point of Aβ (1-43), PI 5.2, induce fibril forming behavior.

Another condition which induces Aβ aggregation is environmental metal content. Both Cu(II) and Zn(II) coordinate favorably to the histidines located on Aβ monomers and aid in Aβ intermolecular interactions.

H₂N-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAL-COOH
1.3.12 Protagonists of Aβ Aggregation

Local acidic environments (endosomes and lysosomes) in the brain are thought to promote fibril formation. The propensity for Aβ to aggregate in such low pH conditions has also been noted in vitro where pH environments below the isoelectric point of Aβ (1-43), pI 5.2, induce fibril forming behavior.

Another condition which induces Aβ aggregation is environmental metal content. Both Cu(II) and Zn(II) coordinate favorably to the histidines located on Aβ monomers and aid in Aβ intermolecular interactions.

1.3.13 Toxicity of Aβ

The mature amyloid fibril was previously thought to be the only toxic species of Aβ. The body’s immune response to remove Aβ deposits by microglial activation and production of arachnidonic acids and eicosanoids from these activated cells induce brain tissue
inflammation.\textsuperscript{37,46} This view is now challenged. Precursors are now thought to possess more toxicity due to their membrane pore forming capabilities.\textsuperscript{35,47,48}

Proteins such as prion and the islet amyloid polypeptide (amylin) have been identified as cell membrane pore formers.\textsuperscript{14,49,50} Evidence of this pore forming capability has been followed \textit{in vitro} with lipid bilayer studies; the sonication of Aβ with phospholipids destroys membrane fluidity.\textsuperscript{48} Electrostatic interactions between the cell membrane and Aβ are thought to drive pore formation.\textsuperscript{51} The electrostatic contribution of Aβ from the protein’s polar N-terminus mostly contains positive amino acids (arginine (5), lysine (16), and lysine (28)) that can bind to negatively charged and unnaturally exposed inner phospholipids.\textsuperscript{51}

Pore formation has been identified as the main contributor of calcium homeostasis disruption,\textsuperscript{50} synapse disruption, and uncontrollable nitric oxide release.\textsuperscript{52} Also, the vasoactive nature of Aβ has been noted to affect both the cerebral and peripheral vasculature, resulting in reduced blood flow, ischaemia and hypoperfusion.\textsuperscript{37}

1.4 Overview of Therapy for Alzheimer’s Disease

1.4.1 Detection of Alzheimer’s Disease Progression

The prognosis of AD and defining progression has been hindered in part to the unavailability of Aβ \textit{in vivo} assays. However, single photon computerized tomography (SPECT), has been proposed as a tool for quantifying Aβ aggregate in the brain.\textsuperscript{53,54} This tool would use radioiodinated ligands or γ-emitting technetium complexes as biomarkers for in vivo Aβ detection.\textsuperscript{53,54}

1.4.2 Removal of Immune Response and Symptomatic AD

The body’s immune response to the presence of Aβ is to activate microglial cells.\textsuperscript{6} These cells uptake Aβ and deposit themselves in extracellular compartments of the brain.\textsuperscript{32} Microglial presence has been recorded in plaque formations that are thought to promote fibril formation.\textsuperscript{32}
Although cell death may be induced by pore formation involving small Aβ aggregates, microglial activation indirectly causes even more harm to the brain by providing more nucleators for fibril development and cause brain inflammation. The use of anti-inflammatory drugs to limit brain inflammation has been effective, but heavy or long term application of such therapy may harm kidneys and other organs.6

1.4.3 Controlling Aβ Production

It is proposed that the production of Aβ can be slowed by decreasing the amount of APP expressed or eliminating its proteolytic cleavage. Removing APP expression entirely would unfavorably affect other needed proteins such as the Notch protein.55 However, a lithium secretase inhibitor has been found to reduce APP processing and production, but not interfere with the Notch protein.55 Another avenue is focused on blocking the cleavage of APP.56 The inhibition of cleavage of APP by β- and γ-secretases has been achieved with lipophilic dimethylaminoethyl tetralin and cyclohexylalanine–based statine. Another γ–secretase inhibitor, fenchylamine sulfonamide, was observed to eliminate β–amyloid production in cell culture.57

1.4.4 Aβ Vaccination

Auto-antibodies for β-amyloid proteins have been found in persons with AD. Antibodies or immunoglobulins are a part of the humoral immune response and are produced by plasma cells in a response to the appearance of a foreign motif (antigen or immunogen).9 However, the amount of antibodies may not be enough to have a huge impact. Auto–antibodies for the peptides Aβ 25-35 and Aβ 1-42 have been found in serum of persons with Alzheimer’s disease.58 The mucosal or nasal administration of transgenic mice containing platelet–derived growth factor promoter APP, PDAP with Aβ protein reduced the Aβ plaque of these mice later in life.59 Active vaccination of humans for Aβ was first employed by Elan pharmaceuticals.60 The injections of Aβ1-42 produced both antibody and T-cell responses by the patient; however, the
trial was ended due to hemorrhaging and brain inflammation. Therefore, active vaccination may be replaced by a more passive one. A passive approach to Aβ vaccination was also developed by Elan pharmaceuticals where Aβ antibodies were injected into mice. However, a possible side effect would be the development of an antibody for the Aβ antibody itself.

1.4.5 Initiation of Aβ Aggregation

Plaque formation in AD cannot be formed by Aβ alone and is stabilized by non-amyloid components. Such non–Aβ components are: polysaccharides, proteins (serum amyloid protein), and heavy metals such as Cu and Zn. Polysaccharides such as glycoaminoglycans are thought to bind plaques together by chaperoning and depositing with Aβ. Mice that were given a drug to disrupt this interaction, NC–75 or Alzhemed, had lower plaque burden. Proteins such as serum amyloid protein are known to coat and maintain the integrity of the senile plaque; removing serum amyloid protein would allow normal uptake of the now exposed senile plaque.

Metals have multiple relationships with Aβ by providing nucleation sites, and enhancing both Aβ aggregation and the stability of Aβ deposits. Thus, an agent that can remove unnecessary metals would potentially destabilize plaque formations. Since Cu (II) and Zn (II) have been found to bind favorably to the histidines located in β-amyloid, treatment with metal chelating agents may help to destroy the integrity of amyloid plaque formations.

1.4.6 Dissolving Pre-formed Amyloid Fibrils

Fibril dissolution has been proposed as a viable option for eliminating plaques. However, the subunits of these fibrils have been identified as being more toxic than the mature fibril and may increase the amount of these more toxic subunits. These subunits, oligomeric Aβ, have been found to be the main contributor of membrane pore formation and cell apoptosis. Studies with α–synuclein suggest that increasing the rate of the fibril forming pathway in order to prevent smaller oligomeric aggregates from forming pores in human cells may be an effective
strategy.\textsuperscript{64} Thus, successful fibril dissolution by constructs such as poly–L–lysine, polyethylene glycol, and peptides containing proline residues\textsuperscript{65} also need to keep smaller subunits from causing cell membrane damage.\textsuperscript{62}

1.4.7 Aβ Aggregate Mediation

Elimination of the fibril forming pathway of Aβ has been proposed using small molecules\textsuperscript{66}, peptide–based constructs, and surfactants. The mediator should have the ability to regulate β-amyloid aggregation and provide an alternate aggregation pathway which would result in a non–toxic form of aggregate (Figure 1.6).

1.4.8 Small Molecule Aβ Aggregate Mediation

Small molecules such as the dye congo red, the antibiotic rifampicin\textsuperscript{67} and anthracycline, 4’–iodo 4’–deoxydoxorubicin, have been found to prevent Aβ aggregation.\textsuperscript{61,68} Congo red has been observed to decrease the thermal folding temperature of the Aβ protein and inhibit fibril formation.\textsuperscript{69}

![Figure 1.6 Mediation of the Aβ Aggregation Pathway. Blue arrow indicates pathway to fibril formation. Orange arrow indicates the formation of amorphous/non-fibril aggregates of β-amyloid by aggregation mediators.](image)
1.4.9 Mediation of Aβ Aggregates by Surfactants

In the presence of sodium dodecyl sulfate SDS micelles, Aβ₄₀ has been found to remain monomeric and in an α-helix state while resting upon the periphery of the SDS micelle.⁷⁰,⁷¹ This conformation disables the pathway for amyloid fibril formation.⁷⁰,⁷¹ Other detergent molecules such as hexadecyl-N-methylpiperidinium (HMP) bromide have been shown to inhibit Aβ aggregation.³⁴ It was found that HMP bromide was specific to β-amyloid as it did not inhibit the formation of fibrils by other amyloidogenic peptides.³⁴

1.4.10 Peptide-Based Aβ Mediation Aggregation

Peptides which mimic β-strands have been prepared with the idea of forming a dimer with Aβ monomers by the self-formation of β-sheets and hydrogen bond action with the Aβ.⁷² Other peptides have been designed which incorporate amino acids with substituted methyl groups or alternating synthetic amino acids with large functionalities to also dimerize and prevent subsequent hydrogen bonding of Aβ.

A hydrophobic region of β-amyloid, residues 16-20, has been identified as a major contributor to amyloid fibril formation.²,⁸,⁷³-⁷⁵ This region, KLVFF, has been proposed as a useful recognition template for designing peptide-based aggregation mediators.⁸,⁴⁰,⁷³,⁷⁵,⁷⁶ Murphy et al. have constructed a peptide-based aggregation mediator containing the KLVFF region and a soluble oligolysine unit. This mediator allowed the regulation of Aβ aggregation and Aβ toxicity through specific binding and fibril inhibition.²,⁸,⁷⁵

Incorporating amino acids with substituents that disable hydrogen bonding between β-amyloid molecules has been found to inhibit fibril formation. A peptide-based aggregation mediator containing alternating amino acids with N-methyl groups throughout the hydrophobic KLVFFAE region, Aβ₁₆-₂₂, inhibited fibril formation.⁷⁷ Another mediator using N-methylated amino acids in the peptide Aβ₂₅-₃₅ has also been found to prevent fibril formation.⁷₈
The peptide based aggregation mediators designed and employed in the work presented here were obtained from Dr. Robert Hammer et al. at Louisiana State University. They contain alternating Cα-disubstituted alpha amino acids in the hydrophobic KLVFF β-amyloid region.\textsuperscript{79,80} These mediators were designed to hydrogen bond to the β-amyloid monomer on one side and disable any subsequent hydrogen bonding of β-amyloid by providing steric hindrance due to large functionalities of the synthetic amino acids.\textsuperscript{79,80} These peptide–based aggregation mediators were compared to the mediators developed by Dr. Regina Murphy, University of Wisconsin, who also used the KLVFF region as the main target of mediation.

1.4.11 Viable Peptide-Based Aggregation Mediators

The viability of a peptide-based aggregation mediator is determined by its ability to cross the blood brain barrier and withstand \textit{in vivo} conditions which could lead to mediator degradation.\textsuperscript{63} Low molecular weights are needed for crossing the blood brain barrier and the transport must be done without any degradation of the aggregation mediator.\textsuperscript{63} D–enantiomeric peptides have been found to be more stable \textit{in vivo} than L–amino acids due to their ability to withstand proteolysis.\textsuperscript{63,65,73}

1.4.12 Overview of Studies for Detecting β–Amyloid Aggregation and Cell Toxicity

Cell toxicity studies provide information on the ability of introduced drugs to control or eliminate the inherent toxicity of Aβ fibril formation.\textsuperscript{78} Such studies use the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction within the mitochondria as a measure of cell viability.\textsuperscript{78} Cell apoptosis is observed if the normal reduction of this dye decreases.\textsuperscript{78}

1.4.13 Binding Assays

Irreproducibility and high sample consumption has hampered past use of binding assays for measurements of mediator efficacy.\textsuperscript{81} The use of surface plasmon resonance (SPR) has
overcome these disadvantages.\textsuperscript{81} In SPR, an immobilized array of β–amyloid of a chosen length such as Aβ\textsubscript{10-35} is attached to a carboxymethyl dextran matrix.\textsuperscript{81} Once an array of Aβ monomers are attached to the matrix, introductions of mediators can be made to determine their binding affinity to Aβ.\textsuperscript{8,81}

1.4.14 Size and Growth Determinations of β–Amyloid Aggregates in Solution

Dynamic light scattering of β-amyloid containing solutions have been used to determine the kinetics of fibril growth in solution.\textsuperscript{25,82-87} Monomeric diffusion coefficients have been reported in the range of 6 x 10\textsuperscript{-7} and 17 x 10\textsuperscript{-7} cm\textsuperscript{2}s\textsuperscript{-1} depending on the solvation of the monomer.\textsuperscript{82} Molecular weight determinations of Aβ and Aβ/mediator aggregates can be obtained with analytical ultracentrifugation and size exclusion chromatography.\textsuperscript{25,88}

1.4.15 Determination of Amyloid Aggregate Size Adsorbed on Surfaces: Microscopy Studies of β–Amyloid

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been used to determine the structure of Aβ while physically adsorbed to a prepared substrate.\textsuperscript{6,34,65} Both TEM and SEM techniques employ non-amyloid materials (staining, coating) in order to image the amyloid aggregates on the surface.\textsuperscript{6,8,38,89-95}

Another microscopy technique, scanning force microscopy (SFM), has been exposed as a useful tool in determining β–amyloid aggregate morphology. Microscopy techniques are now focused on imaging amyloid in their native environment such as with \textit{in situ} tapping mode SFM.\textsuperscript{18}

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Chapter 2

Materials and Methods

2.1 Experimental

2.1.1 Chemicals and Products

The β-amyloid peptides, $\text{A}\beta_{10-35}$ (vendor number 03-153, 2902 g/mol), $\text{A}\beta_{25-35}$ (vendor number Q8160F, 1060 g/mol), and $\text{A}\beta_{1-40}$ (vendor number 03-136, 4331 g/mol, trifluoroacetic acid preparation, and lot numbers Q9279, Q10501) were obtained from Biosource International. Amylin fragment (20-29), (vendor number A-6097, 1009.1 g/mol) was purchased from Sigma-Aldrich. The KOH, phosphate buffer, and stock solutions of β–amyloid were filtered using an Anotop filter (sterile, 0.02 µm pore size/10 mm diameter) from Whatman. Deionized water was prepared by passing distilled water through a reverse-osmosis filter and then a series of organic and ion-exchange filters (Nanopure, Barnstead) to yield water of 18 MΩ cm resistivity. The 0.02 µm pore size Anotop filter was attached to a 3 mL latex–free Becton Dickinson & Co. syringe fitted with a cut pipet tip to deliver the solution. The low ionic strength phosphate buffer was prepared by diluting 15 M 99.999% phosphoric acid from Aldrich with 18 MΩ cm water, raising the pH of this diluted phosphoric acid solution to 7.4 with 1 M KOH, and then adding 18 MΩ cm water to achieve the desired concentration of phosphate buffer. The high ionic strength buffers were made with Puratronic metals basis salts (NaCl, NaF, and NaNO₃) from Alfa Aesar with purity greater than 99.99%. The 1 M KOH was prepared by dissolving semiconductor grade KOH pellets from Aldrich in 18 MΩ cm water. Samples were stored under nitrogen using non-colored Dot Scientific Inc. flat-top/graduated, polypropylene, 1.5 mL microcentrifuge tubes (vendor number 509-ftg) or in some instances, where noted here, in Sarstedt o-ring capped, 1.5 mL microcentrifuge tubes (vendor number
The pipette tips used were 1–200 µL, RNAse/DNAse-free, non-colored (Corning Inc.) pipette tips.

2.2 Methodologies

2.2.1 Proper Weighing of Amyloid Peptides

When adding the peptide to microcentrifuge tubes for dispensing purposes, it was noticed that the peptide is very much like charged dust; it tended to “jump” to things that came into contact with it. This attribute, coupled to β-amyloid and amylin peptides being fluffy, makes it difficult to handle. We adopted a method for transfer of the solid peptide that avoids some of these issues. We have found that gentle tapping of the container, usually a glass vial containing the lyophilized peptide, adjoined to an adjacent microcentrifuge tube allows for clear and easy transfer of the peptide. This procedure also prevents possible contamination associated with traditional weighing methods.

2.2.2 Preparation of β–Amyloid Stock Solutions

After weighing the peptide, it must be dissolved prior to introduction into the phosphate buffer. Attempts at dissolving the peptide in either phosphate buffer or phosphate buffer with added ionic strength (PBS), was found to cause premature or enhanced aggregate formation, supported by the observance of turbid solutions immediately upon dissolution of the β-amyloid solid. In an effort to prepare aqueous solutions of varying concentrations of β-amyloid under a variety of solution conditions, it became necessary to make fairly concentrated aqueous stock solutions of β-amyloid free of organic solvents (such as dimethyl sulfoxide–DMSO). A previous report has briefly commented on preparing such solvent–free stock solutions of Aβ1–

To eliminate aggregate formation from occurring during preparation of β-Amyloid, we dissolved the peptide in 0.02-micron-filtered Anotop filter obtained from Fischer VWR (vendor
number 28138-011) 10 mM semiconductor grade KOH “high pH” followed by vortexing with a Daigger Vortex Genie 2 until no particulates were observed. Approximately 20 µl of the filtered stock is used for amino acid analysis (AAA); 10 nanomoles or more peptide are needed for AAA detection.

2.2.3 Preparation of Low-Aggregation State Amyloid Solutions

Low-aggregation state amyloid solutions were prepared by introducing the β-amyloid stock solution to filtered phosphate buffer and phosphate buffered saline solutions. The 50 mM phosphate buffer and phosphate buffer with added “salt” solutions were filtered through 0.02 µm Anotop filters (Whatman) before combining with the filtered Aβ stock solution in KOH to produce the desired concentration of Aβ peptide. All samples were placed under nitrogen immediately after preparation to eliminate exposure to atmospheric carbon dioxide to ensure no development of carbonic acid and thus drops in pH within the solution. The samples were placed in 1.5 mL microcentrifuge tubes, which were then capped and wrapped with Parafilm. The Parafilm–sealed sample containers were then placed in a Nalgene 1000–mL, wide–mouth container and the Nalgene container subsequently sealed with Parafilm. The Nalgene container was periodically purged with nitrogen so as to prevent changes in peptide sample pH.

2.2.4 Determination of Amyloid Stock Solution Concentrations

Amino acid analysis (AAA) was used here to note possible differences in the concentration of Aβ in aqueous solutions before and after filtration through the 0.02 µm filters. In the amino acid analysis method used here, the peptide-containing sample was hydrolyzed, and the amino acid content of the individual residues was compared to known amounts of a standard, such as the nor-leucine amino acid. The amount of amino acids in the hydrolysate was determined by chromatographic separation (Dionex 6550 with acetonitrile-water gradient
containing 0.1% trifluoracetic acid, four replicates performed with the average and standard deviation reported here) coupled to an electrochemical detector (Dionex ED50). In order to ascertain the possible effects of filtering on Aβ solutions, a sample of Aβ₁₀₋₃₅ in ~10 mM KOH (pH 11.7) solution was prepared for amino acid analysis. Half of this sample was filtered with a 0.02 µm Anotop filter, and the other not. Both the unfiltered and filtered samples were hydrolyzed and subsequently analyzed for amino acid content. The peptide concentration for the unfiltered Aβ₁₀₋₃₅ was found to be 1.76 ± 0.01 mg/mL, whereas that of the filtered Aβ₁₀₋₃₅ was 1.74 ± 0.01 mg/mL, leading to an approximate loss of 20 µg/mL or 1.1 % of peptide, which is negligible.

AAA analysis was also used for determining whether any attribute of the longer and faster aggregating amyloid protein protein Aβ₁₋₄₀ would result in loss during the filtering process. Aβ₁₋₄₀ was dissolved in 10 mM KOH (pH 11) and filtered using an Anotop 0.02 µm filter. The unfiltered portion was 1.62 ± 0.03 mg/mL whereas the filtered portion was 1.55 ± 0.03 mg/mL noting a higher but small 70 µg/mL or 4% loss of peptide through filtering.

2.2.5 Preparation of Protein Aggregate-Inducing Solution Conditions

The addition of “salt” to the phosphate buffer was achieved using metals–basis or 99.99+% pure salts to reduce the possibility of complexation of the peptide with labeled impurity metal ions such as zinc, copper, or aluminum. Such metal ions have been shown to induce aggregation of β–amyloid peptide.²

2.2.6 Preparation of Amylin Stock Solutions

The normal stock solution preparation for β–amyloid has been used for preparing stock solutions of another fibril forming peptide, amylin (20-29). The full length of this peptide, amylin (1-37), (Sigma vendor number D-2162) was found to rapidly aggregate when basic
potassium hydroxide solution was added to lyophilized solid amylin\textsubscript{1-37} material. The two cysteine amino acids at residues 2 and 7 in amylin\textsubscript{1-37}, pKa of 8.5, enable the formation of a disulfide bridge. However, the amylin\textsubscript{20-29} human fragment does not contain these two amino acid residues and was found to readily dissolve in the basic KOH. The peptide can easily be filtered with a 0.02 µm Anotop filter and this peptide was found to form fibrils over time when added to a salt containing buffer.

### 2.2.7 Preparation of Peptide-Based Aggregation Mediator Solutions

The peptide-based aggregation mediators were dissolved in 18 MΩ water and filtered with a 0.02 micron Anotop filter into an autoclaved microcentrifuge tube. The concentration of the mediators was calculated to be 1mM, (verified by AAA). These mediator solutions were placed in a refrigerator at 8 °C.

### 2.2.8 Determining the pH of Minute Volumes of Peptide-Containing Solutions

The pH of each final sample was determined using a miniature (18 gauge) pH combination electrode model number MI–413 (Microelectrodes, Inc.) connected to a Denver Instrument Ultra Basic–5 pH meter. This miniprobe allows determinations of pH in microliter volume solutions. This is important, as most β-amyloid/aggregation mediator studies involve samples of much less than 1 ml of total volume. For pH measurements, a 5 µL aliquot was taken out of the sample and placed as a droplet onto a strip of buckled Parafilm; measuring the pH of the solution directly on a flat surface increases the possibility of mechanical breakage of the thin glass probe. The pH was measured before any analysis and included measurements before and after incubation periods.

### 2.2.9 Storage of β-Amyloid Samples for Analysis

All samples were placed under nitrogen to keep the solution pH from decreasing as a result of carbonic acid formation caused by exposure to atmospheric carbon dioxide. The
samples were placed in 0.5–ml microcentrifuge tubes, which were then capped and wrapped with Parafilm for storage. The microcentrifuge tubes were placed in Nalgen containers (vendor number MX21666/1000ml) which had two drilled holes with fitted septa. An (18-gauge) needle was placed in one of the two septa for exhaust; the other septum had a needle connected to a continuous nitrogen purge.

2.2.10 Preparation of β–Amyloid Samples for Analysis by Scanning Force Microscopy (SFM)

Samples were adsorbed onto two types of surfaces, atomically flat and hydrophilic mica (ASTM V–2 Grade 3 Ruby muscovite, Lawrence & Co.) or hydrophobic highly oriented pyrolytic graphite (HOPG) obtained from Arthur Moore at Advanced Ceramics Corporation. Cleavage and exposure of interior mica planes (which are atomically flat over large areas and ideal for SFM imaging) were achieved by placing a razor blade in the middle of the sheet edge layers of a 1 cm² piece of mica separating the layers by gripping one side with a pair of tweezers. After cleavage, the mica was blown dry with nitrogen to remove any particulates due to the cleaving process. A sample aliquot of 5 µL was then placed on the freshly exposed mica and allowed to remain for 5 minutes, unless otherwise noted. A fresh HOPG surface was prepared by placing a section of one-sided Scotch tape on one side of the graphite. The tape was then pressed and removed to reveal a smooth, shiny, and clean surface. Sample application on HOPG was the same as mica.

After a 5 minute adsorption step in most cases, the sample/substrate was rinsed with 400 µL of deionized water (fibrils or aggregates will remain adsorbed as noted later) followed by tilting the substrate and placing its edge on a Kimwipe to wick away the water. After the water rinse, the sample was gently blown dry with a stream of nitrogen. The mica and HOPG samples were then placed sample-exposed face up on a 15 mm metal specimen disc from Ted
Pella Inc. (vendor number 16218) with double–backed tape (Gluespot by Digital Instruments, catalog reference STKYDOT). When ready for imaging, each specimen disc was placed on top of the piezoelectric scanner of the SFM instrument. Samples not immediately imaged were stored in semiconductor wafer containers from Entegris (vendor number H22-101-0615 tray and number H22-10-0615) under an ordinary lab ambient environment.

2.2.11 Preparation of Samples for Transmission Electron Microscopy

All substrates, storage boxes, lens tissue, and chemicals used for transmission electron microscopy were purchased from Electron Microscopy Sciences. For electron microscopy, the sample solutions were adsorbed onto a thin carbon coated collodion layer placed on a copper grid.\(^3\) The collodion coating was used to keep the sample on the grid; deposition of carbon onto this grid was done to ensure thermal stability of the film. In order to make the collodion film, a pool of water was placed in a 150 mm x 15 mm polystyrene petri dish cover from Fisher (vendor number 08-757-14). The water was added until almost spilling over the sides of the petri dish. A sheet of Ross optical lens tissue (vendor number 71700) was placed on top of the pool of water and gently pulled across the surface to remove any particulates on the surface. A thin film of collodion was prepared by dropping roughly 20 µL of non-filtered 2% Collodion in amyl acetate (vendor number 12620-00) until the solution began to solidify and thicken on the surface. After the collodion film was formed, several 400 mesh Cu grids (vendor number G400–Cu) were placed onto the film and allowed to stand. Removal of the coated grids was accomplished by placing the paper used to separate Parafilm layers over the Collodion membrane until the entire paper was wet; this was to ensure proper adhesion of the grid to the Parafilm paper. The paper was then removed causing the grids and adsorbed Collodion film to be removed from the water/Collodion interface. After adsorbing the film onto the grid, the grids were placed in Petri dishes for 24 hours under ambient conditions to dry. A bench top Turbo
Denton vacuum evaporator was used to coat these grids with carbon in order to keep the Collodion from being vaporized by the thermal energy generated during the bombardment of incident electrons in the TEM.

Samples for TEM analyses were then prepared by placing the carbon/Collodion coated grid faced down on a 5–µL sample droplet for 1 minute. After this step, the excess droplet was wicked away by placing the edge of the grid onto a sheet of optical lens tissue. The grid is then placed on a droplet of 0.02 µm Anotop (Whatman) filtered 2% uranyl acetate (vendor number 22400) for a few seconds. The grid was then wicked away of excess liquid and placed in a grid storage box, (vendor number 71137), which holds 100 (2-3 mm) specimen support grids.

2.2.12 Preparation of Solutions for Tensiometry

Both the Du-Nuoy ring and Wilhelmy plate methods were used in these studies. For Wilhelmy sample preparations, the aliquot was placed into a 6 mm x 50 mm Kimble test tube, wrapped with a Kimwipe, and centrifuged in a larger plastic tube. The tubes containing the sample were centrifuged for 10 minutes at 2000 x G. After centrifugation, a gel loading pipet tip from United Scientific Products, Inc. (vendor number TGL-1000) was used to remove the supernatant of each sample. This supernatant was placed on a 25 mm watch glass from Aldrich (vendor number Z 50,919-1). Only 120 µL of sample was needed to make a measurement with a 1 cm x 2 cm Wilhelmy paper plate obtained from NIMA technology. The watch glass was immobilized on the back side of a scintillation cap to eliminate the watchglass from moving by capillary forces exerted from the Wilhelmy plate.

The plate was hooked onto the bottom of a hooked 50 mm long and 65.9 mg in weight platinum iridium wire (California Fine Wire Company vendor number 12881). The eye on top of this wire is fastened on the NIMA Technology type PS4 surface pressure sensor. The Wilhelmy plate method was used along with a Lab View program. After the solution was
placed on the 25 mm watch glass, the free plate was zeroed and brought to the surface of the droplet. Upon saturation, the plate was then lifted and zeroed. The plate was then lowered to the droplet; the surface tension was measured when the plate was just below the meniscus of the solution. Consecutive measurements were made by removing the plate and bringing the plate back to the surface of the droplet; each solution was measured 5 times.

2.2.13 Preparation of Samples for Dynamic Light Scattering

Sample tips, microcentrifuge tubes, and cells were rinsed with 0.2 micron Anotop-filtered 18 MΩ water to remove dust particulates. Removal of dust was important to remove unwanted scattering events that will eliminate coherence and destroy determinations of the diffusion coefficient and therefore size of the sample of interest.

When a sample was ready for scattering measurement, the sample cell was inserted into a bath of index matching solvent such as toluene; the cell was gently wiped with cotton fabric or lens cleaner to clear the windows and prevent scratching. The toluene must be 0.2 micron filtered to remove any dust particles that can scatter laser light.

2.2.14 Preparation of Samples for Reflection-Absorption Infrared Spectroscopy (RAIRS)

Reflection-absorption infrared spectroscopy (RAIRS) was conducted on a Thermo Nicolet Nexus 670 FTIR at an angle of 86°. A large and smooth piece of HOPG, prepared by removing the uppermost layer of the HOPG with tape, was used for surface analysis of peptide adsorption. Approximately 500 µL of 500 µM of each peptide was spread across a freshly cleaved HOPG surface and adsorbed for 5 minutes. A KBr pellet was made by mixing KBr with 1 mg of each peptide.
2.3 Instrumentation

2.3.1 Microscopy in General

Optical microscopes have enabled the determination of structures in the micron range. However, the visible wavelength of light (380-780 nanometers) used for such studies limits the detection of objects in the sub-micrometer range. Thus, imaging capability and resolution have increased with the employment of electron microscopy. The magnifications of microcopies increase when registering the scattering of the much shorter de Broglie wavelength of electrons (picometers) from a surface as in TEM or SEM. These techniques, however, require the use of delicate sample preparation and a vacuumed environment.

The development of the scanning tunneling microscope (STM) which uses platinum/iridium wire to provide a junction between the tip of this wire and a conductive surface can be used for determining structures adsorbed to surfaces at nanometer resolution. However, this technique was limited to work on conductive surfaces. Thus, this technique led to the development of the scanning force microscope (SFM) which collects information on the van der Waals interactions between a cantilever and a surface.

Microscopes can be divided into two general categories: scanning and simultaneous imaging. Transmission electron microscopy can be conducted by both avenues, and the simultaneous imaging offered by TEM was used in the work presented here. Other microscopy techniques that use the scanning method are the scanning electron microscope (SEM) and varying modes of the scanning force microscopes, which will be further discussed.

2.3.2 Transmission Electron Microscopy

Research employed a JEOL 100 CX transmission electron microscope at an electron acceleration voltage of 80 kV. All film, developer, fixer, and other solutions were obtained from Electron Microscopy Sciences (EMS). The TEM film used was a 4489 multi-pack Kodak
electron microscope film (vendor number 1662238) with dimensions of 3.25" x 4" and an emulsion number of 31300201. D–19 developer by Kodak (vendor number 74200) was used to develop the film. This development was followed by Kodak Rapid Fixer, (vendor number 74312). After this step, the film was submerged in Kodak Photo–Flow solution (vendor number 74257) and allowed to dry. The negatives were then scanned into a computer using a DUOSCAN T/200 AGFA scanner operated by Fotolook 3.2 V3.60.60 software program on a PC. Adobe Photoshop 7.0 was used to further enhance the brightness and contrast of the images. Magnification bars were placed onto the pictures(1200 d.p.i.) and exported together to ensure correct representation of scale.

2.3.3 Theory of Transmission Electron Microscopy

In transmission electron microscopy (TEM), an electron gun containing a tungsten filament is used to generate electrons. The electrons are generated by thermionic emission when a negative high voltage, direct current is applied to the filament. A Wehnelt cylinder or shield completely surrounds this filament except for a small opening (2 to 3 nm in diameter) and operates at a higher negative voltage than the filament. The opening in the shield is to direct the electrons toward the anode just below the shield as seen in Figure 2.1. Once the electrons pass the anode, they are focused by the condenser lens onto the sample grid. The sample grid is usually a copper mesh grid with a support film. Before TEM analysis, specimens of interest are adsorbed to this film and typically stained using a heavy metal salt (uranyl acetate or phosphotungstic acid) which have high atomic numbers capable of scattering electrons.
Figure 2.2 depicts the transmission of electrons through the condenser lens to the sample grid. Any electrons not blocked by the sample upon the grid are then focused again by the objective lens. The objective lens and aperture define the aperture angle and depth of field. Once the image is formed, the electrons are passed through the intermediate (diffraction) lens to aid magnification. A final passage through projector lens further magnifies the electrons and increases the depth of focus.

After the electrons have passed the projector lens they are deposited onto a fluorescent screen containing a cellulose nitrate or gum arabic matrix containing fine ZnS/CdS particles. The small λ electron particles bombard this screen and cause an emission of longer λ which can be detected by the human eye. The resolution of this image (35-50 µm resolution on the screen compared to the 200 µm by the human eye) depends on the grain size of the embedded particles and the beam spread during the penetration of the screen layer. Beneath this screen is a shutter.
which can open and close to expose the photographic film. Advancement of the film opens the shutter allowing electrons to penetrate the film’s AgBr gelatin matrix. These electrons convert the AgBr to metallic Ag. Further production of metallic Ag occurs during the development of the film to provide the contrast needed for the image.

Figure 2.2 Image Formation During TEM Experiment

2.3.4 Scanning Electron Microscopy (SEM)

Another microscopic technique which uses electrons for image formation is the scanning electron microscope (SEM). Unlike TEM, the SEM uses its electromagnetic lenses to demagnify and focus electrons onto a conductive surface. The conductive surface can be a specimen of interest which has been carbon coated or a specimen which is conductive.
natively.\textsuperscript{6} The focused area of electrons is scanned in a raster pattern, as seen in Figure 2.3, across the surface causing the emission of low-energy secondary electrons from inelastic scattering events.\textsuperscript{6,7}

\textbf{Figure 2.3 Raster Pattern Offered by Scanning Electron Microscopes}

The collection of these electrons by a secondary electron detector are then converted into pixels on a monitor.\textsuperscript{6} The brightness of each pixel defines the amount of secondary electrons detected.\textsuperscript{6} Due to its depth of field, SEM allows for imaging of specimens of varying thickness.\textsuperscript{6}

\textbf{2.3.5 Scanning Tunneling Microscopy}

The formation of an image using the “raster pattern” technique across a surface has also been applied to scanning tunneling microscopy (STM). However, instead of scanning the surface with a focused amount of electrons, the surface is probed by a metallic wire (Pt/Ir) at a constant distance or constant current flow between tip and sample.
The distance between the tip and the surface is obtained by controlling the current between the tip and sample junctions through an applied voltage, Figure 2.4. The current is therefore a function of the bias voltage, $V$, the constant characteristic of conductors, $C$, and the spacing between the apex of the tip and the highest atom on the surface, $d$ as identified in Equation 2.1. This movement of the tip across the surface is obtained by piezoelectric motion which is detailed further under the scanning probe microscopy section.

**Equation 2.1**

$$I_t = V e^{-Cd}$$

### 2.3.6 Scanning Force Microscopy

A Nanoscope III Multimode scanning force microscope was used in Tapping Mode. Noncontact high frequency silicon cantilevers (NSC–15, MikroMasch) were used to obtain the SFM images. The non–contact NSC15 silicon cantilevers produced by MikroMasch used in these studies contained a reflective aluminum backside coating. These tips had a radius of curvature of 10 nm, a full tip cone angle less than 20°, and a tip height of 15-20 µm. The typical resonant frequency of these NSC15 cantilevers was 325 kHz with a force constant of 40 N/m. The scan parameters utilized were: 1.0-1.5 Hz scan rate and 0.6 integral and 0.2 proportional gains.
A scan size of up to 125 µm x 125 µm can be obtained with the vendor’s (Digital Instruments) software and a J–type piezoelectric scanner. After capturing the SFM images, a flatten procedure was performed, and the dimensions of the aggregates were determined using the manufacturer’s software. Topographical images are shown in grayscale, with brightest points in the image being the tallest features. The heights of aggregates are determined using a bump analysis algorithm.

2.3.7 Theory of Scanning Force Microscopy

A ceramic piezo-electric tube is used to produce the raster pattern used in probing the surface in scanning probe microscopy. Applying AC voltages to the conductive X and Y portions of the tube, as seen in Figure 2.5, distorts the shape of the piezo-electric tube and thus creates the motion, of the Z portion of the tube, which produces the raster pattern.

![Figure 2.5 Ceramic Scanner Piezo-electric Tube](image)

Scanning force microscopy, SFM, also known as atomic force or scanning probe microscopy, offers the ability to visualize β-amyloid aggregates on a surface. Such a method can be used to follow the aggregation behavior in solution by merely adsorbing the aggregates formed in solution onto an appropriate support (substrate). Information regarding the length and height (diameter) of aggregates can be obtained from SFM images. Because adsorbed
peptides and other biological materials are “soft”, a non-destructive SFM method is required, thus non-contact or Tapping Mode SFM is used. Tapping Mode SFM eliminates the shear forces created by contact SFM.⁸

Figure 2.6 Schematic of Tapping Mode Scanning Force Microscope

In Tapping Mode SFM, as seen in Figure 2.6, a cantilever made of crystalline silicon oscillates at a resonant frequency and set amplitude. A red 632.8 nm laser beam is reflected off the backside of the cantilever to a mirror, which in turn directs the laser beam onto a split photodiode detector. As the sample moves under the cantilever, the cantilever “taps” the surface on its downward position. As the cantilever approaches the surface, the cantilever encounters several forces (Figure 2.7). The forces used for detection in SFM are the van der Waals forces at the angstrom level above the sample.⁸
Any changes in the attractive or repulsive forces generated between the oscillating tip and the sample surface will change the frequency of the cantilever. The position of the laser upon the split photodiode detector changes due to this change. This change in position as a function of sample location is converted into an image such as height or amplitude. Tip convolution is of concern as the objects may appear larger than their apparent sizes due to the probe’s inability to completely resolve the surface feature.\footnote{9}

**2.3.8 Tensiometry**

Surface pressure measurements were conducted on solutions using the du Nuoy ring method on a Sigma 703 tensiometer. A platinum ring of 5.992 cm mean circumference (CSC Scientific Company) was employed for the evaluation of no less than 500 µL of starting volume of each cyclic peptide. The platinum ring was cleaned with a washing of absolute-200 proof ethanol from Aaper followed by 18 MΩ deionized water. An aliquot of the peptide solution was placed on a watch glass. The Pt ring was submerged in this aliquot and then removed repeatedly for 5 recordings of the surface pressure. Additional aliquots of 18 MΩ
water followed by agitation of the diluted solution upon the watchglass by pipettor allowed a range of concentrations to be studied.

2.3.9 Theory of Tensiometry

The surface tension of a solution is determined by how extensive the cohesive forces between the molecules are. In bulk solution, water can hydrogen bond in 4 positions, whereas at the surface, water molecules tend to place their hydrogen bonds closer to the hydrophobic air decreasing the amount of hydrogen bonding at the surface and thus causing excess energy for the molecule. This excess energy can be obtained by tensiometry where the excess free energy per unit area can be expressed as the force per length.

There are two types of tensiometry methods available: Wilhelmy plate and du Nuoy Ring. Most of the work done here was with the du-Nuoy ring setup where a platinum ring is used to determine the force of a solution just before film breakage shown in Figure 2.8.

![Du-Nuoy Ring Setup](Figure 2.8 Du-Nuoy Ring Setup)

The surface tension, $ST$, measured by the Du-Nuoy ring method is determined by the outer diameter of the ring, $d$, and the thickness of the ring, $t$, in meters as seen in Equation 2.2.

**Equation 2.2** $ST_{\text{ring}} = \frac{\text{Force}}{(\pi d + \pi(d-2t))}$

Thus, the surface tension is based on the force per perimeter of the platinum ring.\(^{10}\)
2.3.10 Dynamic Light Scattering

Light scattering measurements were conducted on a home-built dynamic light scattering (DLS) instrument, using either a Lexel Ar⁺ laser operated at 488 or 514.5 wavelength (adequate for low light scattering levels), or a Pacific Instruments photometer/ pulse-amplifier-discriminator, and a computer with an ALV-5000 multiple-τ digital auto correlator software version 5. The laser was directed into a vat containing the sample cell and a refractive index matching solvent, toluene $n = 1.5$. The scattered light was detected by a photomultiplier tube at some angle between about 15º or 150º. The fluctuations of the intensities of the scattered light were correlated by a computer containing the ALV 5000 autocorrelator card and associated software.

![Schematic of DLS Apparatus](image)

2.3.11 Theory of Dynamic Light Scattering

Light scattering can be divided into two techniques: static which measures the time-averaged intensities of light scattered from a solution of particles, and dynamic light scattering which measures the fluctuations of intensities of the scattered light. Static light scattering,
determinations of weight average molecular weight and radius of gyration, \( R_g \), can be made. In dynamic light scattering, determinations of the hydrodynamic (Stokes) radius, \( R_h \), of a scattering particle can be made.\(^\text{12}\)

The scattering of light from a particle (molecule) is achieved after the polarization of this particle by incident light.\(^\text{11}\) In most instruments, this incident light, often from an Ar\(^+\) laser, is polarized perpendicular to the plane defined by the incident beam and the detector.\(^\text{11}\) When the polarized light impinges on a particle, both a parallel and perpendicular polarization to the incident light plane occurs.\(^\text{11}\) However, most dynamic light scattering studies only measure the scattering the polarized scattering.\(^\text{11}\)

The fluctuations of scattered light appear to be random and uncorrelated after long periods of time, but inspection of these fluctuations at very small time intervals reveals that they are correlated. The persistence of the fluctuations can be described by an autocorrelation function. Normalizing this autocorrelation function enables the expression of the electric-field autocorrelation function:

\text{Equation 2.3} \quad g^{(1)}(\tau) = \exp(-\Gamma \tau)

where the decay rate \( \Gamma \) may be thought of as the inverse of the duration of an average fluctuation event. The diffusion coefficient or (speed) of the scatterer. The translational diffusion coefficient, \( D \), of the scatterer in medium is obtained from Equation 2.4:

\text{Equation 2.4} \quad D = \frac{\Gamma}{q^2}

where the scattering vector, \( q \), is the measure of the spatial frequency of the light scattered at an angle \( \theta \), as expressed by Equation 2.5.\(^\text{12}\)

\text{Equation 2.5} \quad q = \frac{4\pi n_o \sin(\theta/2)}{\lambda_o}
In Equation 2.5, the term \( n_0 \) is the refractive index of the solution and \( \lambda_0 \) is the wavelength of the scattered light in vacuo. From this equation the hydrodynamic radius, \( R_h \), of the scattering particle can be obtained by Equation 2.6.

**Equation 2.6**

\[
R_h = \frac{k_b T}{6 \pi \eta D}
\]

which is known as the Stokes-Einstein equation. The Boltzmann constant, \( k_b \), and solvent viscosity, \( \eta \) at a specified temperature, \( T \), are required to obtain \( R_h \).

Estimation of this hydrodynamic radius can be obtained using the Equation 2.7, where \( R_h \) is the hydrodynamic radius, \( M_w \) is the molecular weight of the peptide, \( \upsilon \) is the partial specific volume assumed to be 1.1 and \( N_A \) is Avogadro’s number.\(^{13}\)

**Equation 2.7**

\[
R_h = \left( \frac{3 M_w \upsilon}{4 \pi N_A} \right)^{1/3}
\]

**2.3.12 Reflection-Absorption Infrared Spectroscopy (RAIRS)**

Reflection absorption infrared spectroscopy (RAIRS) was conducted on a Thermo Nicolet Nexus 670 Fourier transform infrared spectrometer using a liquid-nitrogen cooled, wide band MCT detector. External reflection measurements were taken at 86º incidence using the Versatile Reflection Attachment with Retro-Mirror Accessory (Harrick, Ossining, NY). Corrections for \( \text{H}_2\text{O} \) and \( \text{CO}_2 \) were made prior to pristine HOPG background subtraction. A manual baseline was corrected for each spectrum. A 25 point smooth was performed by the vendor software (OMNIC 5.0).

**2.3.13 Theory of Reflection-Absorption Infrared Spectroscopy**

In RAIRS, incident infrared light impinges or and is reflected from a specular surface at equal angles (Figure 2.10).\(^{14}\) A specular surface displays a mirror like finish which has surface irregularities smaller than the wavelength of the incident IR light.\(^{14}\) If the irregularities in the material are larger than the wavelength of light, then scattering can occur.\(^{14}\) As the incident
light grazes the specular surface and also combines with the reflected light, a standing wave electric field is formed which can interact with the adsorbed species on the surface of the specular materials.\textsuperscript{14,15}

Only parallel ($p$) polarized light can be absorbed by immobilized entities on the specular surface, and therefore an incident beam of parallel-polarized light at a near grazing angle is used.\textsuperscript{14,15} The reason for the use of parallel polarized light is due to vector addition; perpendicular ($s$) polarized light undergoes destructive interference of its electronic vectors upon reflection from a surface, and it is therefore not suitable for interacting with adsorbed species.\textsuperscript{15} It has been found that at an angle of incidence of $88^\circ$ the reflectivity of light is 5000 times more at an angle of $0^\circ$ or normal.\textsuperscript{15} Thus, RAIRs is used to examine thin films containing very small quantities of materials.\textsuperscript{14}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{reflection-absorption-infrared-spectroscopy.png}
\caption{Reflection-Absorption Infrared Spectroscopy}
\end{figure}

\section*{2.4 References}


Chapter 3
Organic-Solvent-Free Preparations of β-Amyloid and Amyloid-like Peptides: Scanning Force Microscopy Studies

3.1 Introduction

Previous work by others has led to a basic understanding of the conditions needed to induce aggregation of various β-amyloid proteins in vitro.¹² To better represent in vivo conditions, employed here is a completely aqueous pathway for the preparation of β-amyloid solutions for in vitro studies. Our route does not involve the use of organic co-solvents; such co-solvents were previously used to successfully dissolve and prepare a variety of Aβ peptides for solution–phase analysis.¹² An alternative route is to solubilize the Aβ material in aqueous hydroxide and then lyophilize in the resulting solution so as to yield the “sodium salt” of the Aβ peptide, which can be used to prepare aqueous stock solutions.³

The focus is on the preparation of stable aqueous solutions of Aβ and Amyloid-like peptides and the determination of the conditions that lead to reproducible aggregation of Aβ and Amyloid-like samples in an aqueous environment closely resembling physiological conditions. The work described here is of importance to research in the thesis topic of Aβ aggregation mediation by peptide mimics in aqueous environments.⁴⁻⁹ For reliable and reproducible observations regarding the effects of aggregation mediators to be achieved, stable solutions of Aβ or other Amyloid-like peptides containing no co-solvents are necessary. Finally, the method used for storage of aqueous Aβ solutions is shown to be of great importance, due to the observation that the nature of the interior of the polymer storage container can induce aggregation of otherwise stable aqueous Aβ solutions.
In an effort to prepare aqueous solutions of varying concentrations of Aβ and Amyloid-like peptides under a variety of solution conditions, it became necessary to make concentrated aqueous stock solutions of Aβ_{10-35}, Aβ_{1-40}, and amylin_{20-29} free of organic solvents (such as dimethyl sulfoxide–DMSO). Although a previous report\textsuperscript{3} has briefly mentioned preparing such organic–solvent–free stock solutions of Aβ_{1-40} and another\textsuperscript{4} the formation of aqueous solutions from lyophilized “sodium salts” of Aβ_{1-40} and Aβ_{1-42}, we found it necessary to fully investigate the nature of Aβ_{10-35}, Aβ_{1-40}, and amylin_{20-29} in high pH aqueous solutions due to possible differences in the aggregation behavior of the various fragments in aqueous media free of organic co-solvents.

3.2 Behavior of Aβ_{10-35} in High pH, Low Ionic Strength Media and in Physiological pH, Low Ionic Strength Buffer

Shown in Figure 3.1 is a representative Tapping Mode SFM image obtained from a ~1 mM Aβ_{10-35} pH 11 solution incubated at room temperature under N\textsubscript{2} (to prevent pH drop due to CO\textsubscript{2} absorption reaction) for 3 weeks. The only features visible are ~2.5–nanometer–tall globular structures of Aβ_{10-35}; control experiments with Aβ–free buffers yielded featureless SFM images (bare mica). Although tip–sample convolution is a concern in imaging such small species, we report the length/width range of the globular deposits in Figure 3.1 to be 40 to 80 nm.\textsuperscript{11} There was no evidence for the presence of higher–order Aβ_{10-35} aggregates, as noted by the lack of any fibrillar species. These “non–aggregating” Aβ_{10-35} solutions can be maintained for 3 weeks, as long as pH is controlled; there is a need for a constant N\textsubscript{2} atmosphere because the solution is unbuffered (roughly 10 mM KOH) and therefore greatly affected by CO\textsubscript{2}. Dynamic light scattering measurements of 300 μM Aβ_{10-35} in 10 mM KOH (pH 11) at 90° displayed a hydrodynamic radius of 3.2 ± 0.4 nm during day one of sample preparation.
Figure 3.1 10 µm x 10 µm scanning force microscope image (on mica) obtained from a solution of 1 mM Aβ₁₀₋₃₅ in 10 mM KOH. Image was obtained after sample had been incubated for 3 weeks at room temperature. The z-range is 10 nm.

In order to alleviate the need for a N₂ atmosphere, we turned to making solutions of Aβ₁₀₋₃₅ in near physiological pH (~7.4) phosphate buffer. Upon addition of the necessary amounts of the 10 mM KOH solutions containing ~1 mM Aβ₁₀₋₃₅ to “salt–free” phosphate buffer, stock solutions of Aβ₁₀₋₃₅ that were pH stable over time could be prepared.

Shown in Figure 3.2 is a representative 10 µm x 10 µm SFM image of 300 µM Aβ₁₀₋₃₅ in 15 mM phosphate buffer solution; the solution was incubated at room temperature for a period of 8 days prior to its contents being examined by SFM. Similar to the observations for the 10 mM
KOH solutions of Aβ_{10-35}, no fibrillar species were observed in the SFM images, only small 0.8–3 nm–tall globular features were present, but in lower number.

Figure 3.2 10 µm x 10 µm scanning force microscope image (on mica) of 300 µM Aβ_{10-35} that had been incubated at room temperature for 8 days in pH 7.64, 15 mM phosphate buffer (no salt added). The z-range is 10 nm.

The methods described here for preparing Aβ_{10-35} stock solutions under either the high pH or near physiological pH conditions lead to solutions that do not contain higher order aggregates. As seen in Figures 3.1 and 3.2, the small peptide agglomerates do not appear to have any observable long-range order, as indicated by the lack of any fibrillar-like aggregates. In addition, the size and number of the small peptide agglomerates did not change during the timed
incubation periods, pointing to a stable behavior for the stock Aβ_{10-35} solutions. Observation of the stable nature of the Aβ_{10-35} solutions under low ionic strength conditions in either high pH or near-physiological pH is important because reproducible studies of aggregation inhibition depend on the initial aggregate state of the Aβ material to be non-fibrillar (a seeding effect has been observed in other work\textsuperscript{12}) and it must be the same for each preparation of stock Aβ solution. In addition, the β-amyloid solution preparation protocols should be simple in nature.

3.2.1 Preparation of β–Amyloid (1-40) Stock Solutions

Using the same protocol as that for Aβ_{10-35}, stable solutions of Aβ_{1-40} were obtained by dissolving the solid peptide in 10 mM KOH solutions, as noted by Figure 3.3. The basic solution kept Aβ_{1-40} stable for 3 weeks with substantial aggregate growth only observed when the pH would drop. This pH drop is probably due to carbonic acid formation from repeated exposure to air. Also, the solution’s stability may have been compromised by dust contamination during the retrieval of aliquots for sample preparations. Attempts to directly prepare Aβ_{1-40} stock solutions from solid peptide containing phosphate buffer in the absence or presence of added salt (ionic strength) resulted in rapid aggregation. This aggregation was apparent in minutes by visual observation of Aβ_{1-40} precipitation in the microcentrifuge tube. Thus, no filtering could be accomplished to salvage any soluble Aβ_{1-40}, as this aggregated material clogged the 0.02 µm Anotop filter, indicated by high syringe back pressure. The remaining aggregated material in the syringe was taken out and placed on mica for SFM analysis as seen in Figure 3.4. Scanning force microscopy analysis of this aggregated solution yielded micrographs displaying noted areas completely covered with aggregate formations, ranging in height from 5 to 13 nm.
Figure 3.3 15 µm x 15 µm scanning force microscope image (on mica) of filtered 500 µM Aβ_{1-40} adsorbed on mica after 5 weeks. Lyophilized peptide was dissolved in 10 mM KOH at pH 10, filtered with 0.02 µm Anotop filter and stored at 8 ºC. Aggregate height ranged from 2 to 12 nm. The z–range is 10 nm.
Figure 3.4 10 µm x 10 µm scanning force microscope images (on mica) of unfiltered 500 µM Aβ1-40 adsorbed on mica immediately after preparation. (A) Lyophilized peptide was dissolved directly in 50 mM phosphate buffer at pH 7.4; abundant aggregate height ranged from 5 to 24 nm. (B) Lyophilized peptide was dissolved in 50 mM phosphate buffer and 150 mM NaCl at pH 7.4; abundant aggregate height ranged from 5 to 13 nm. The z–range is 10 nm.
3.2.2 Aqueous Preparations of Amylin Peptide

The specificity of the peptide-based mediators with respect to Aβ aggregation required the use of amyloid-like peptides that form fibrils. Amylin20-29 was used for these specificity studies because it has been found to form amyloid-like fibrils, and it does not have the disulfide bridge contained in full-length amylin1-37. Amylin1-37 (formula weight 3903 g/mol) has sulfides originating from two cysteines on residues 2 and 7 in the sequence; this disulfide bridge is thought to have caused the rapid aggregation observed in these studies when this peptide was dissolved in 10 mM KOH. It has been observed that after 9 days at 8 ºC in pH 11.5 10 mM KOH, amylin20-29 readily aggregates. These aggregates were non-fibrillar in nature, but were large asymmetrical agglomerations greater than 150 nm in height; such large aggregations were difficult to resolve with SFM. Thus, the stability of stock solutions of amylin20-29 was lower than Aβ stock solutions, but it was still suitable for the specificity studies.

3.2.3 Effect of Sample Storage Containers on Aggregation Behavior of Aβ10-35

A key issue in the evaluation of mediators for the aggregation of Aβ peptides was the ability to reproducibly prepare stable solutions of the Aβ peptides in aqueous media free of influencing factors. In addition, controlled initiation of Aβ aggregation was necessary for such mediator studies. Once these two conditions were satisfied, successful studies were then completed regarding the possible effects of mediators on the aggregation of Aβ materials.

From the study of sample containment in two different types of poly(propylene) vials presented here, it is clear that surface-induced aggregation of Aβ10-35 is a key issue that must be addressed when using Aβ materials. Previous work by others indicates that the hydrophobicity and chemical nature of a surface can influence the aggregation behavior of Aβ materials. The SFM and XPS data presented here clearly demonstrate that chemically identical but
microscopically rough surfaces can significantly alter the kinetics, and most likely the pathway, of Aβ_{10–35} aggregation in aqueous media.

The X–ray photoelectron spectroscopy (XPS) data were obtained with a Kratos AXIS 165 X-ray spectrometer. The samples were prepared for analysis by cutting a small sliver of the wall of both the Dot Scientific and Sarstedt microtubes with a clean razor blade. The inner walls of the microtubes were positioned upward for exposure and both were clamped onto the same sample plate for analysis. An aluminum anode was used to produce the monochromatic K alpha X–rays. The presence of no other elements besides carbon on the surface of the poly(propylene) vials, Figure 3.5, as judged by XPS points to another surface characteristic influencing the aggregation of Aβ_{10–35}. We attribute the more rapid aggregation of Aβ_{10–35} in the Sarstedt tubes to their three-fold higher interior surface roughness (RMS roughness of 19.7 nm) versus the Dot Scientific microcentrifuge tubes (RMS roughness of 5.8 nm) as seen in Figure 3.6.

Results from a recent scanning force microscopy study of Aβ_{1–42} are suggestive of increased aggregation on nanoscopically rough surfaces.\textsuperscript{12} We conclude that the rougher poly(propylene) surfaces act as nucleation sites for the Aβ_{10–35}, which lead to production of structured Aβ_{10–35} seeds, which in turn cause aggregation of solution-phase Aβ_{10–35}.

The presence of seeds in solution has been shown to greatly accelerate the rate of Aβ aggregation.\textsuperscript{14} Similar effects to those observed here for Aβ_{10–35}, have been found with Aβ_{1–40}, and research conducted by students under the direction of Dr. Robin McCarley at Louisiana State University is currently exploring the role of surface chemistry and roughness in the aggregation pathway of various Aβ materials in aqueous solution.
Figure 3.5 X-ray photoelectron survey spectrum of interior of Dot Scientific and Sarstedt microcentrifuge tubes. No impurities (such as metals or surfactants) are evident as noted by the presence of only one peak corresponding to the carbon 1s signal from the poly (propylene) container.
Figure 3.6 10 μm x 10 μm scanning force microscope image of inner wall of microcentrifuge tube: (A) Dot Scientific, RMS roughness = 5.8 nm and (B) Sarstedt, RMS roughness = 19.7 nm. The z-range in both images is 200 nm.
Other influencing factors in the aggregation of Aβ peptides include the presence of non-Aβ seeds (heterogeneous nucleation) and metal ions. These potential aggregation facilitators can speed an otherwise slow nucleation and growth process. For example, we have noted that when solutions of Aβ_{10-35}, or the solutions used to dissolve the peptide, are not filtered through the 0.02 µm filters, aggregation occurs very rapidly and in an uncontrolled manner. SFM and light scattering experiments point to the presence of dust particles in these solutions as aggregation accelerants, further confirmed by a previous study.\textsuperscript{15} In addition, the use of high-purity materials for solution preparations so as to prevent metal ion or surfactant impurities is of great importance, and we have followed such a stringent protocol here.

3.2.4 Behavior of Aβ_{10-35} and Aβ_{1-40} in Physiological pH, High Ionic Strength Buffer

Amyloid β–peptides have previously been shown to aggregate more quickly in aqueous solutions containing salts, although the impact of the presence of organic co–solvents on this aggregation behavior is not known.\textsuperscript{10} We report here the addition of salts to aqueous solutions of Aβ_{10-35} and Aβ_{1-40} peptides containing no organic solvents.

In initial Aβ aggregation studies, Aβ_{10-35} were prepared in pH 7.5 phosphate buffer containing fixed concentrations of NaX salts: (X= Cl, F, and NO₃) and then examined their contents as a function of time using SFM. Shown in Figure 3.7 is a representative 10 µm x 10 µm Tapping Mode SFM image of 300 µM Aβ_{10-35} in 15 mM phosphate buffer containing 50 mM NaCl incubated at room temperature for 8 days and then exposed to mica. Examination of Figure 3.7 (and other aliquots obtained from similarly prepared solutions) led to observation of fibrillar Aβ_{10-35} structures varying in length from 40 nm to 1.2 µm and 7-12 nm in height. Such long Aβ_{10-35} fibrils were also observed when 50 mM NaF was present in the 300 µM Aβ_{10-35} 15 mM
phosphate buffer solutions. Roughly 6–nm–tall, 600 nm to 3 µm long fibrils were observed when NaF was present, see Figure 3.7 B.

Figure 3.7 10 µm x 10 µm scanning force microscope images (on mica) of 300 µM Aβ10–35 incubated for 8 days at room temperature in 15 mM phosphate buffer containing 50 mM (A) NaCl at pH 7.77, (B) NaF at pH 7.75, and (C) NaNO3 at pH 7.67. The z-range is 20 nm in all images.
In contrast to the cases for NaCl and NaF, no observable fibrils were found upon SFM inspection of mica surfaces exposed to 300 μM Aβ10-35 solutions in 15 mM phosphate buffer containing 50 mM NaNO3. Only 6–18 nm–tall spherical aggregates were found in SFM images obtained from such Aβ10-35 solutions containing NaNO3, as seen in Figure 3.7C. In general, high ionic strength environments led to formation of fibrillar aggregates or large agglomerates of Aβ10-35 in aqueous buffer solutions containing no organic co-solvents. This was evidenced by the presence of micrometer-long fibrils in the SFM images of Aβ10-35 solutions spotted on mica after incubation under buffered conditions when either 50 mM NaCl or NaF are present. Although no fibrils were produced during the 8-day incubation period when NaNO3 was used to increase the ionic strength, there were much larger number of peptide agglomerates that are much larger (2–20 times) than when no salt was present—compare Figures 3.2 and 3.7 C. At this time, it is unclear why this difference exists when nitrate is used versus chloride or fluoride, but it is possible that it is caused by interactions between the nitrate anion and the salt bridge of the full length Aβ. The cross β motif of the full length Aβ contains a salt-bridge between the 23rd residue, the negatively charged aspartate amino acid, and the 28th residue, the positively charged lysine amino acid. It is possible, that the nitrate anion may interact with this salt-bridge and thus keep the bridge from completion. This interference could keep the cross β motif (Figure 1.5) from forming and thus inhibit fibril development.

Aβ fibrillar structures have also been observed for Aβ1-40 prepared within phosphate buffered saline solutions (Figure 3.8). Thus, the sample preparation protocol allows the preparation of fibril forming aqueous solutions of both Aβ peptides.
Figure 3.8 10 μm x 10 μm scanning force microscope image (on mica) of 50 μM Aβ₁-₄₀ incubated for 8 days at room temperature in 50 mM phosphate buffer containing 150 mM NaCl at pH 7.4. Fibrils were 1-3 μm in length and 6-7 nm in height.

3.3 Conclusion

It has been demonstrated that the reproducible preparation of organic-solvent-free aqueous solutions of Aβ₁₀−₃₅ and Aβ₁−₄₀ do not result in Aβ higher-order aggregation for extended periods of time. Key to the successful preparation of these stable, concentrated Aβ₁₀−₃₅ and Aβ₁−₄₀ solutions is the removal of possible nucleation seeds (either Aβ or dust), exclusion of impurities during solution preparation, and careful consideration of the nature of the solution storage containers. In addition, it has been shown that formation of Aβ₁₀−₃₅ and Aβ₁−₄₀ fibrils and large aggregates can be triggered by addition of a variety of salts to these otherwise non-aggregating Aβ solutions. The nitrate anion may prevent the initial formation of the β-sheet rich Aβ aggregate, which enables Aβ fibril development, by sustaining a random coil or alpha helical Aβ conformer by interaction with the positively charged amino acid lysine utilized in the salt-bridge of Aβ₁−₄₀.
3.4 References


Chapter 4

Characterization of Aβ Aggregation Mediators

4.1 Designing Peptide-Based Aggregation Mediators

The proposal of using aggregation mediating peptides to eliminate the toxic aggregate pathway of β-amyloid has been supported by the observation that diffuse plaques of β-amyloid do not affect surrounding neurons or activate glial cells.\(^1\) Therefore, peptide-based aggregation mediators can be designed with the intent to cause aggregation of β-amyloid into a non-toxic species. The proposed mediators in this work are based on an amino acid sequence known to cause β-amyloid fibril formation.\(^2,3\) The binding affinity of this segment, KLVFF, of amino acids 16-22 in the full length sequence of β-amyloid, has been shown to bind specifically to much larger Aβ\(_{10-35}\) materials and also was beneficial in reducing cellular toxicity caused by the presence of β-amyloid.\(^4\) Therefore, peptides containing this amino acid sequence have been proposed as viable mediators of β-amyloid aggregation.

The Aβ aggregation mediators developed at Louisiana State University contain C\(_{\alpha,\alpha}\) disubstituted amino acids within this KLVFF sequence as seen in Figure 4.1.\(^5,6\) The alternating substitution of the KLVFF region with C\(_{\alpha,\alpha}\) disubstituted amino acids was designed to create an extended conformation of the mediator.\(^5,6\) The extended conformation enables the mediator to display β-strand conformation properties. This conformation allows possible β-strand interactions with the β-strand forming β-amyloid peptide.
Figure 4.1 Peptide–Based Aggregation Mediators. (A) Peptide aggregation mediator developed by Dr. Regina Murphy at the University of Wisconsin. (B) Mediators developed by Dr. Robert Hammer; mediators were defined by their lysine number and positions: AMY-1 \( x = 1, y = 6 \); AMY-2 \( x = 6, y = 1 \); AMY-3 \( x = 1, y = 1 \). The chemdraw drawings were provided by Marcus Etienne.
4.1.1 Peptide-Based Aggregation Mediator Studied

The peptide-based aggregation mediator developed by Dr. Regina Murphy at the University of Wisconsin with a molecular weight of 1420.9 g/mol contained the KLVFF region of β-amyloid and an oligolysine tail at its C-terminus to allow higher solubility. Two other mediators, AMY-1 and AMY-2, were developed by Dr. Robert Hammer and synthesized by Dr. Yanwen Fu and Marcus Etienne with a molecular weight of 1708 g/mol. Both AMY-1 and AMY-2 mediators contained a portion of the hydrophobic KLVFFAED region located in β-amyloid, but had substitutions of dibutylglycine (DIBG) for leucine (L), dibenzylglycine (DBZG) for phenylalanine (F) and a dipropylglycine (DPG) for an alanine (A). The differences between AMY-1 and AMY-2 lay in the position of the oligolysine tail; AMY-1 has the 6 lysines on its C-terminus, while, AMY-2 has its 6 lysines on its N-terminus.

4.1.2 Assessment of Possible Self-Aggregating Behavior of Inhibitors

Scanning force microscopy was used to note any apparent self-aggregation of the peptide-based mediators, as outlined in Figure 4.2. It was found that AMY-2 readily self-aggregates, as indicated by observation of apparent monolayers on mica 1.3 ± 0.1 nm in height. AMY-1 had aggregates in the range of 1 to 17 nm in height, whereas the mediator created by Dr. Regina Murphy resulted in aggregates that are 1-8 nm in height.

4.1.3 Assessment of Surface-Active Properties of Peptide-Based Aggregation Mediators

The surface active properties of each peptide-based aggregation mediator were examined to determine whether each mediator had any surfactant-like properties, Figure 4.3. Due to an observed drop in surface tension around 1mM, both AMY-1 and AMY-2 mediators probably have a critical micelle concentration (CMC) near this concentration.
Figure 4.2 10 µm x 10 µm SFM images of 50 µM mediators in 50 mM phosphate/150 mM NaCl at pH 7.4. (A) AMY-1, (B) AMY-2, (C) Murphy. Samples were incubated at room temperature after an initial incubation at 37 °C for 1.5 hours. White bar is 1 µm in length. Z-range is 20 nm.
The small volumes used (200 µL) to reduce sample consumption, and the 25 mm diameter watch glass to enable immersion of the du-Nuoy ring demonstrated a surface tension of water to be 83.1 ± 1. This value was much larger than the 72.5 ± 0.5 surface tension of water found at a volume of 400 µL of water in a 50 mm watch glass. Therefore, Figure 4.3 had high trend values for surface activity due to the nature of sample reduction. Still, the effort proves that surface activity occurs with increasing AMY-1 and AMY-2 concentration and concentration has no effect for the inhibitor developed by Dr. Regina Murphy. Other experiments for determining surface activity were mainly with peptide based aggregation solutions of concentrations at 50 µM and with volumes at 1000 µL allowing measurements with the large 50 mm watch glass.

The mediator developed by Dr. Regina Murphy did not display any apparent change in surface activity over the concentration range studied; this inhibitor was found by Dr. Regina Murphy’s group to increase the surface tension of solutions.\(^7\) The CMC of each mediator increased our understanding of the role of each mediator when in an environment of β-amyloid peptide. The 1 mM CMC value of AMY-1 and AMY-2 is 20 fold larger than the concentrations of these inhibitors used in the aggregation inhibition/mediation studies in Chapter 6. Therefore, interaction of AMY-1 and AMY-2 at the intended concentrations with β-amyloid should involve specific interactions instead of the presence of secondary structure stabilizing micelles, such as has been found for Aβ\(_{1-40}\) in a sodium dodecyl sulfate (SDS) micellar environment.\(^8,9\)

**4.1.4 Conclusion**

The surface activity of each peptide-based aggregation mediator was investigated to determine whether β-amyloid interacts specifically with each mediator in a more or less
Figure 4.3 Plot of Surface Pressure Measurements of Peptide-Based Aggregation Mediators. Measurements for AMY-1 were obtained in pH 11, 10 mM KOH; AMY-2 in pH 4.7, deionized 18 MΩ water; and Murphy in pH 4.5, deionized 18 MΩ water.

monomeric state and not interacting with a possible micellar form of the mediators. It was found that at the concentrations of inhibitors used in the Aβ aggregation mediation studies (Chapter 6) there were no micellar species present. Also, SFM analysis of each mediator alone allowed any observation of inhibitor self-aggregating ability. It was found that AMY-2 favored monolayer adsorption on mica, whereas AMY-1 and the mediator developed by Dr. Regina Murphy displayed only a small ability to form small agglomerates on mica.

4.2 References


Chapter 5
Properties of β-Amyloid at the Air/Water Interface in the Presence or Absence of Aggregation Mediators

5.1 Background on Surface Activity of Amyloid-Containing Solutions and Stabilization of Aβ Aggregates by Surfactants

Aβ protein fragments resemble amphipathic surfactants owing to their composition of both a polar N-termini and a hydrophobic C-termini; fragments of β-amyloid from Aβ1-33 to Aβ1-43 have been noted to be surface active with an average critical micelle concentration around 25 µm.1 Aβ1-40 has been found to fold into a β sheet conformation at the air-water interface.2 Studies of β-amyloid under acidic conditions yielded an observation of spherocylindrical micelle structures with a length of 11 nm and a width of 4.8 nm (containing 30-50 Aβ monomers).3 The addition of the surfactant sodium dodecyl sulphate (SDS), above its critical micelle concentration, to solutions of β-amyloid has led to stabilization of the amyloid protein as an α-helical structure.4 Below the critical micelle concentration of SDS, Aβ forms fibrils as a result of rapid conversion of initially α-helical conformations to β-sheet structures.5

5.2 Surface Activity of β-Amyloid in the Presence and Absence of Mediators

The surface activities of β-amyloid solutions containing peptide based aggregation mediators were assessed. The surface tension of H₂O is 72 mN/m. The surface tension of Aβ1-40 at 50 µM is substantially lower than each one of the mediators studied individually (Figure 5.1). However, the introduction of AMY-1 or AMY-2 additionally lowered the surface tension of β-amyloid, whereas the Murphy inhibitor increased the surface tension of the solution.6 This increase in the surface tension of Aβ1-40 solutions by the Murphy inhibitor has been reported by Dr. Regina Murphy and is thought to aid in increasing the rate of Aβ1-40 aggregation.6 The surface tension of the peptide, Aβ25-35, which does not contain the KLVFF sequence, did not display any surface activity alone. Again, AMY-1 and AMY-2 lowered the surface
tension of this peptide, but the surface tension was not affected by the presence of the Murphy inhibitor (Figure 5.2 A). The lowering of Aβ25-35 was probably due to the stronger surfactant activity of AMY-1, because the surface tension was close to AMY-1 alone. AMY-2 lowered the surface tension of Aβ25-35 below that of AMY-2 alone. AMY-2 therefore lowers the surface tension of both Aβ1-40 and Aβ25-35 solutions below the value of AMY-2 solutions at 50 µM.

A similar effect was observed with amylin20-29. Again, addition of AMY-1 and AMY-2 to amylin solutions lowered the surface tension slightly, with Murphy having no effect (Figure 5.2 B). AMY-1 or Murphy did not drop amylin20-29 lower than their own individual surface tensions, but addition of AMY-2 to amylin20-29 solutions lowered the surface tension a considerable amount.

5.3 Conclusion

A binary mixture of surface-active agents can complex and form a compound with even higher surface activity than the agents alone (Figure 5.3). This was observed in the case of AMY-2 with Aβ1-40, Aβ25-35, and amylin20-29. AMY-2 formed a complex which each fibril forming peptide having greater surface activity than AMY-2 or each fibril forming peptide would have alone. However, AMY-1 was more selective and only complexes with Aβ1-40 to form a more surface-active complex. The mediator developed by Dr. Regina Murphy increased the surface tension of Aβ1-40 only, had no effect on Aβ25-35, and moderately lowered the surface tension of amylin20-29. Therefore, AMY-1 displayed some selectivity in complex formation with Aβ1-40 due to the observation that AMY-1 surface tension dropped when only in the presence of Aβ1-40.
Figure 5.1 Surface pressure measurements of mediators alone (A) and in the presence of Aβ1-40 (B) at given molar ratios.
Figure 5.2  Surface pressure measurements of $\text{A}B_{25-35}$ (A) and amylin$_{20-29}$ (B) in the presence or absence of mediators.
Figure 5.3 Representation of how individual fibril-forming peptides (A) and mediators (B) are less surface-active than the amyloid-mediator complex alone (C).

5.4 References


Chapter 6

Mediation of Aβ Aggregation

6.1 Initial Aggregation Mediation Studies

Research was initially conducted on understanding the aggregation of Aβ10-35 due to its slower aggregation rate than the larger β-amyloid peptides Aβ1-40 and Aβ1-42. This peptide still contained the necessary amino acids, namely those in the hydrophobic and the β-turn regions of the full-length complement. Aβ10-35 was used for my early investigations of the first available peptide-based aggregation mediator, AMY-1 developed at Louisiana State University. AMY-1 was found to inhibit fibril formation of Aβ10-35 and form spherical agglomerates of various sizes.

6.1.1 Studies of AMY-1 Inhibitor in the Presence of Aβ10-35

It was determined that at a ratio of 9:1 of AMY-1 to Aβ10-35, significant differences in aggregation were observed when compared to a control of only Aβ10-35 (Figure 6.1). This control was a 50 µM Aβ10-35 sample in 15 mM phosphate buffer and 150 mM NaCl at pH 7.3. This sample was incubated at 37 °C for 14 hours and was maintained at a pH of 7.3, until an aliquot was spotted onto mica and subsequently imaged by Tapping mode SFM. Images obtained from an aliquot of the control displayed an extensive network of 560 µm to 700 µm long fibrils having heights 2-28 nm. This fibril formation was not present in the sample containing the AMY-1 mediator. The sample with the 9:1 AMY-1 mediator to Aβ10-35 ratio experienced the same conditions as the control. An SFM image obtained from the 9:1 sample at the same time point as the control exhibited only very small spherical aggregates that were on average 61.8 ± 2 nm wide and 2.1 ± 0.8 nm tall. Interestingly, the differences between the sample without the AMY-1
mediator and with mediator are quite striking, as noted by the lack of fibrils or large agglomerates in the AMY-1 containing sample.

Figure 6.1 10 µm x 10 µm SFM images on mica showing: (A) 50 µM Aβ10-35 with fibrils 560 µm and 700 µm in length with a height range of 2-28 nm, (B) 9:1, 450 µM AMY-1 to 50 µM Aβ10-35, average aggregate is 61.8 ± 2 nm in width and 2.1 ± 0.8 nm in height. The z-range is 10 nm. Solution conditions were 15 mM phosphate buffer and 150 mM NaCl at pH 7.3. This sample was incubated at 37 °C for 14 hours.
The images shown here are representative of 5 replicate measurements (images) obtained and all images yielded similar features with and without the AMY-1 inhibitor. AMY-1 was found to inhibit amyloid fibril formation at a 1:1 ratio of mediator to $A\beta_{10-35}$ in an environment of 18 mM phosphate buffer at an ionic strength of 116 mM NaCl at pH 7.4. This sample was observed over a period of 9 days with the last time point displayed in Figure 6.2. The control, $300 \mu M \ A\beta_{10-35}$, exhibited large 1.5-$\mu$m wide and 143-nm tall aggregates. These large structures were not seen in the samples containing AMY-1. Apparent changes in the mechanism of aggregate formation were observed with the introduction of AMY-1. The 1:1 AMY-1:$A\beta_{10-35}$ sample exhibited an average particle width of $193 \pm 52$ nm and height of $15 \pm 3$ nm. Under identical conditions, a 3:1 ratio of AMY-1 to $A\beta_{10-35}$ led to the observation of particles that are a bit larger, with $305 \pm 26$ nm wide and $49 \pm 6$ nm tall species observed.

6.2 Aggregation Mediation Studies with $A\beta_{1-40}$

In order to investigate the general efficacy of the AMY mediator series, a more extensive comparison study was undertaken with $A\beta_{1-40}$. The $A\beta_{1-40}$ peptide is more relevant to in vivo work due to its more rapid aggregation under physiological buffer conditions. Mediator efficacy studies involved observing any possible aggregates formed by $A\beta_{1-40}$ in the presence and the absence of each mediator (AMY-1, AMY-2, and Murphy inhibitor). After incubation at 37 ºC for 1.5 hours, $A\beta_{1-40}$ alone displayed an abundance of spherical aggregates (> an estimated thousand in field of view), and were around 3 nm in height with more linear-like aggregates which were 140-470 nm in length comprised of the spherical aggregates. These early “pearl like” structures have been identified elsewhere as the precursor (protofibril) of the much larger $\beta$-amyloid fibril (Figure 6.3A).
Figure 6.2 10 µm x 10 µm SFM images on mica showing: (A) 300 µM Aβ_{10-35}, (B) 1:1 300 µM AMY-1/300 µM Aβ_{10-35} at pH 7.7. Solution conditions were 18 mM phosphate buffer at an ionic strength of 116 mM NaCl at pH 7.4. The image shown here was taken after 9 days at room temperature. Z-range was 300 nm.

Initial visual observation of the AMY-2 interaction with Aβ_{1-40} provided a unique appearance of rapid aggregation in solution. When AMY-2 was added to Aβ_{1-40} solutions at a 1:1 ratio (50 µM each), the solution rapidly became opaque and took on a colloidal appearance.
(30 minutes). Such rapid aggregation was not observed when either Aβ1-40 or AMY-2 was alone under the same conditions and identical times.

A scanning force microscopy image further details the extent of AMY-2 interaction with Aβ1-40 after a 1.5 hour incubation at 37 ºC. SFM images from a 1:1 AMY-2:Aβ1-40 ratio displayed large amorphous aggregates which are assumed to be of the β-amyloid and AMY-2 interaction product. These non-fibrillar aggregates were measured to be 70-117 nm in height and were clustered together. Both AMY-1 and Murphy mediation of Aβ1-40 aggregation displayed aggregated material without the “pearl-like” spherical aggregates (protofibrils) observed in the Aβ1-40 alone solutions.

A second study designed to explore the later stages of Aβ1-40 aggregation to form higher-order aggregates (fibrils) was performed. Images of 50 µM Aβ1-40 alone, pointed out the presence of elongated fibrils greater than 10 µm in length and roughly 8 nm in height Figure 6.4A. This sample, after an incubation at 50 ºC for 1.5 hours, exhibited no fibrils, but rather a small number of amorphous aggregates of varying height (3-9 nm). Along with these small amorphous aggregates, a monolayer of adsorbed material (0.5 nm in height) was observed and probably contributed by the product. It is important to note that the sample of the AMY-2:Aβ1-40 was taken from the middle of the microcentrifuge tube; it was apparent that the majority of the material had settled to the bottom of the storage container. Samples from the 1:1 AMY-1 and Aβ1-40 solution had aggregates 8-17 nm in height, as well as a layer of adsorbed material (0.6 nm in height), as seen in Figure 6.4B. However, as noted in Figure 6.4D, a fibrillar structure (696 nm in length) and aggregates 3-7 nm in height from the sample containing 1:1 of Dr. Regina Murphy’s peptide and Aβ1-40.

Transmission electron microscopy work on Aβ1-40 after one week at room temperature in 50 mM phosphate buffer and 150 mM NaCl at pH 7.4 resulted in significant fibril formation.
(Figure 6.5A). At a ratio of 1:1 AMY-1 to Aβ₁₋₄₀, also in 50 mM phosphate buffer and 150 mM NaCl at pH 7.4, only amorphous agglomerates were observed (Figure 6.5B) similar to the aggregates seen in Figure 6.2B. The aggregation mediation of Aβ₁₋₄₀ by AMY-2 under identical conditions also encompasses the formation of non-fibrillar aggregates. The inhibitor developed by Dr. Regina Murphy resulted in slightly larger aggregates, but were also amorphous in nature (Figure 6.5 C, D).

6.3 Overall Observation of Aggregation Mediator Efficacy

Measurements of the surface activity by tensiometry of each mediator alone have revealed that each of these mediators displayed no micelle formation at the concentrations used in our studies (50 µM). Dr. Regina Murphy at the University of Wisconsin has reported that the inhibitor, KLVFF(K)₆, increases the surface tension of Aβ₁₋₄₀ containing solutions; this observation was also found in our work detailed in Chapter 5, Figure 5.1B.¹ Dr. Regina Murphy’s group believed that the increased rate of Aβ aggregation was caused by this increase in surface tension, but also found that increases in surface tension alone did not increase Aβ₁₋₄₀ aggregation.¹ This increased rate of aggregation was also found at Louisiana State University by dynamic light scattering as a 276 nm aggregate of 1:1 Aβ₁₋₄₀ :Murphy grew into a larger 712 nm aggregate after 1.5 hours at 40º C.

An increased rate of Aβ aggregation was also identified with the AMY-2 mediator developed at Louisiana State University. AMY-2 increases the rate of Aβ aggregation but decreases the surface tension. There must be another mechanism such as complex formations between Aβ and mediators which can change the surface tension of the Aβ containing solution and also enhance the rate of Aβ aggregation.
Figure 6.3 5 µm x 5 µm SFM images on mica demonstrate aggregation mediation of Aβ₄₀ at 50 µM with added mediators and an inhibitor. (A) Small precursor aggregates and protofibrils were observed for 50 µM Aβ₄₀. (B) 1:1 AMY-1:Aβ₄₀. (C) Large amorphous aggregates of 1:1 (Aβ₄₀ : AMY-2). (D) 1:1 Murphy and Aβ₄₀. Samples were incubated at 37 °C for 1.5 hours. Solution conditions were 50 mM phosphate and 150 mM NaCl. pH of samples was 7.4.
Figure 6.4 5 µm x 5 µm SFM images on mica obtained from solutions of Aβ₁-₄₀ in the presence and absence of peptide-based aggregation mediators. Samples were incubated at 50 °C for 1.5 hrs in 50 mM phosphate buffer and 150 mM NaCl. (A) 50 µM Aβ₁-₄₀ alone; (B) 1:1 with AMY-1; (C) 1:1 with AMY-2; (D) 1:1 with Murphy inhibitor. White bar is 1 micron in length. Z-range is 10 nm.
Figure 6.5 Transmission electron micrographs of 50 µM $\text{A}\beta_{1-40}$ in the presence or absence of peptide-based aggregation mediators. (A) $\text{A}\beta_{1-40}$ alone, (B) 1:1 with AMY-1, (C) 1:1 with AMY-2, (D) 1:1 with Murphy after 9 days at room temperature in 50 mM phosphate and 150 mM NaCl at pH 7.4.

Even though the mediators at the concentrations studied (50 µM) do not form micelles, there is reason to believe that indeed micelles are a part of these studies; $\text{A}\beta_{1-40}$ has a micelle concentration at 25 µM. Therefore, interactions between each mediator in the bulk of the solution may involve a capping of, intercalation of the core, or insertion into the periphery of the $\text{A}\beta$ micelle as seen in Figure 6.6.
Figure 6.6 Generalized Model of Aβ1-40 micelle (red indicates polar N-terminus) interacting with an Aβ aggregation mediator (blue indicates oligolysine unit). (A) Mediator intercalates the core of the Aβ micelle. (B) Insertion of mediator into Aβ micelle. (C) Capping of micelle with mediator.

The differences in the SFM and TEM data suggest the sample preparation of the two techniques provide detail about structures in different locations of solution. SFM sample preparation involves placing an aliquot after mixing of the sample onto mica, whereas in TEM, sample preparation requires the placement of a grid on top of a droplet of an aliquot of agitated Aβ1-40 solution. The placement of this drop may determine what types of aggregates were seen as any surface-active complexes may have been seen more easily by TEM than SFM. However, both SFM and TEM, found that AMY-1, AMY-2, and Murphy inhibitor formed aggregates with Aβ1-40 which differed to normally seen aggregate stages of Aβ fibril formation in the absence of such mediators.

6.4 Conclusion

Aggregation mediation and fibril inhibition can be directly observed by SFM and TEM analysis. The very large aggregate formed by the 1:1 interaction of AMY-2 and Aβ1-40 is thought to be caused by the position of the lysines on the N-terminus of the peptide. The observation of
large aggregate complexes between Aβ1-40 and an aggregation mediator with an oligolysine unit on its N-terminus of KLVFF has been previously reported by Dr. Regina Murphy.3,4

6.5 References


7.1 Introduction

Many pathological diseases are associated with the presence of bundles and plaques containing fibril formations. A majority of these fibril forming peptides contain a β-sheet motif. The cyclic peptides presented here form β-sheet conformations in both solution and while adsorbed on HOPG. The ability to form assemblies and fibrils were observed on HOPG, but not on C18-terminated Au. The adsorption and propagation of these cyclic peptides on thermally provided pits on HOPG provide evidence that the edge of graphite is important in anchoring these cyclic peptides. The seeding of β-amyloid 1-40 was evident on previously exposed cyclic peptide HOPG surfaces by the observation of layers and nucleations ontop of these β-amyloid layers.

A model for describing fibril formation in general remains elusive as each fibril-forming peptide contains different attributes. We describe in this work, two cyclic peptides which contain both a hydrophobic segment similar to that of β-amyloid protein and a synthetic β-turn which model the β-amyloid protein. In doing so, we have found these cyclic peptides to be potent fibril formers in solution, and they nucleate and form fibrillar structures at select locations on highly-oriented pyrolytic graphite (HOPG).

7.2 Fibril-Forming Cyclic Peptides

The versatility of peptide tubule or fibril construction and their capabilities are evidenced by the different designs from which they are built. The fabrication of tubules of cyclo-[(L-Phe-D-N-MeAla-)4] has been proposed for size-selective transportation through membranes. The transportation of charge in microelectronics may be accomplished with junctions across biotin-
coated Au substrates made from avidin-coated peptide tubules. The presence of a hydrophobic cavity and specific internal diameter enables the cysteine-based cyclo-bisamide tubule to aid in intercalation and delivery of target drugs.

7.3 An Interest in Fibril Formation

Our interest in fibril formation is the result of their role in neurodegenerative diseases, such as Alzheimer’s disease. The fibril formation associated with these proteins originate from a misfold in the protein under favorable conditions. Protein misfolding has been identified as the major player in a host of diseases in which the native conformation of the protein is converted into a more β–sheet conformer which also is resistant to proteolysis and treatment. Proteins which are rich in this ability to adopt β–sheet conformations have been noted in several proteins with dissimilar sequences. The aggregation of these proteins further stabilize such β conformations by screening hydrophobic sections from the bulk solution. β-sheet rich proteins, such as β–amyloid, transthyretin, huntington, prion, and amylin proteins, are known to aggregate into fibril structures. However, even α–helical proteins, under certain conditions can be made to convert into fibrils.

The β–sheet forming peptide, β–amyloid (1-40), a main cleavage fragment of the neuron trans-membrane, amyloid-precursor protein, has been noted to cause amyloid fibrils in the brains of individuals stricken with Alzheimer’s disease. The ability of this peptide to convert to fibrils is due in part to two essential components, a very hydrophobic amino acid sequence component adjacent to an amino acid sequence region which adopts a β-turn. This hydrophobic amino acid sequence, KLVFF, is being used by researchers as a part of a recognition element within designed constructs which would antagonize the toxic conformational pathway of β–amyloid. However, KLVFF itself does not form fibrils. The models developed herein contain the
KLVFF sequence known to promote aggregation, and a synthetic β-turn to resemble the VGSN turn found in β-amyloid protein.

\[
\begin{align*}
&\text{A} \\
&G-K-L-V-F-F-P_D \\
&G-Q-L-I-K-K-G \end{align*}
\]

\[
\begin{align*}
&\text{B} \\
&G-K-L-V-F-F-P_D \\
&G-Q-K-K-K-K-G \end{align*}
\]

Figure 7.1 Cyclic peptides developed by Dr. Mark McLaughlin. (A) MCP-1 and (B) MCP-2.
7.4 Materials and Methods

7.4.1 Peptide Synthesis and Purification

The cyclic peptides were synthesized in the Louisiana State University Protein Facility. A solid-phase, continuous-flow chemistry on an Applied Biosystems PIONEER instrument was used in the synthesis of these cyclic peptides. An initial linear peptide was constructed on a PAL-PEG-PS resin using F-moc protected amino acids and TBTU and HOBT activation. Use of allyl-protected glutamic acid allowed deprotection with tetrakistriphenylphosphine palladium, followed by cyclization with PyAOP activation, as the final step of a fully automated synthesis. Cleavage of the peptide from the resin was accomplished using a trifluoroacetic acid-based reagent (88% TFA, 5% water, 5% phenol, 2% triisopropylsilane) for 2 hours, followed by a precipitation in cold diethyl ether. The crude peptide was purified by reverse-phase HPLC on a Waters Delta Prep system, incorporating a Waters 486 detector operating at 220 nm wavelength and a Waters C4 Delta-Pak column (10 nm pore size, 15 μm particle size), 200 x 25 mm. The gradient was composed of water and acetonitrile, each containing 0.1% TFA. Initial conditions were 90% water/10% acetonitrile. Final conditions were 60 % water and 40 % acetonitrile, run over 60 minutes. The molecules of interest eluted at 22% acetonitrile. Fractions were freeze-dried prior to checking the purity of each on a Vydac 218TP54 column, using a Waters 600S pump and a Waters 996 photodiode array detector. Fractions with purities >95% determined by HPLC were pooled and dried again. The net peptide content of the finished product was determined by quantitative amino acid analysis on a Dionex AAA-Direct system. Molecular weights of these pure peptides were verified by MALDI mass spectrometry. The peptide was stored as lyophilized powder at –20 °C.
7.4.2 Preparation of Samples for Scanning Force Microscopy

Sample preparation was accomplished by placing an aliquot of 5 µL of 50 µM MCP-1 or MCP-2 in 18 MΩ deionized water (final pH 2.9) on various surfaces. Such surfaces used for SFM were hydrophilic mica (ASTM V–2 Grade 3 ruby muscovite, Lawrence & Co.) and hydrophobic highly-oriented pyrolytic graphite obtained from Dr. Arthur Moore at Advanced Ceramic Corporation. HOPG surfaces possessing mono-atomic deep pit domains were achieved by heating the HOPG at 650 °C for 30 minutes in a furnace in air. Another hydrophobic surface, C18-terminated Au, was prepared in our laboratory by adsorbing octadecane-1-thiol terminated thiol on Au-coated mica [(Au 111)].

Silicon (111) wafers of n-type (Ar-doped) Si (111) were also employed in our studies and were obtained from Virginia Semiconductor. The silicon was prepared by removing organic materials from the surface with RCA-1 cleaning (1:1:6) NH₄OH: H₂O₂: H₂O and removing the SiO₂ layer with etching techniques NH₄F: HF.14-17 This left a hydride-terminated Si surface, which has been shown to be hydrophobic and ordered.

Cleavage of mica was done by separating two thin sheets of (1 cm x 1 cm) mica using a razor blade and then pulling them apart using tweezers. The cleavage of graphite was done by removing a thin veneer of graphite from a bulk sample with the aid of one-sided Scotch tape. The freshly exposed surfaces were then placed face up on a flat surface, and an aliquot of 5 µL sample was placed ontop this surface for 5 minutes. The surface was then gently dried with a stream of nitrogen. After drying, the substrates were glued to a magnetic puck with the aid of a Gluespot disk from Digital Instruments (vendor catalog reference code STKYDOT). The samples were either imaged immediately by scanning force microscopy immediately or after storage inside an Entegris polypropylene wafer (vendor number H22-101-0615 and H22-10-06115 for the tray and lid) for later analysis.
7.4.3 Preparation of Samples for Circular Dichroism

An Aviv circular dichroism spectrometer model 62 DS with Igor Pro version 4.0 software was used to plot the secondary structure of these cyclic peptides. Both cyclic peptides were dissolved in an aqueous solution at pH 3.

7.4.4 Preparation of Samples for Dynamic Light Scattering

Solutions were analyzed by a blue line 488 nm laser at 100 mW output. Samples were placed in screw capped Pyrex 9826 tubes (vendor number 60827-453) from VWR Scientific. Measurements could be obtained with volumes as low as 250 µL in these tubes. The samples were kept at room temperature between DLS measurements except for incubations at 50 °C. Multi-run measurements were performed every 60 seconds at a scattering angle of 90°. A third cumulant fit algorithm on the ALV software was used to determine the diffusion coefficient and size of the particle of interest.

7.4.5 Preparation of Samples for Tensiometry

Surface pressure measurements were conducted on solutions using the du Nuoy ring method on a Sigma 703 tensiometer. A platinum ring from (CSC Scientific Company) was able to detect 500 µL of starting volume of each cyclic peptide. The peptide solution was placed on an ethanol-cleaned watch glass and the ring was submerged and then removed for 5 recordings of the surface pressure. The addition of 18 MΩ water and agitation of the solution on the watchglass by pipettor action allowed a range of concentrations to be studied.

7.5 Discovery of the Fibril-Forming Nature of These Cyclic Peptides

Assays used for determining the efficacy of peptide constructs to inhibit fibril formation revealed fibril morphology previously unknown to β-Amyloid. This unknown, long (sometimes greater than 20 microns), and very linear fibril morphology was found to coexist along with the
smaller β-amyloid fibrils. Therefore, these cyclic peptides did not prevent the fibril formation of β-amyloid, but thrived as potent fibril formers of their own.

Even though these cyclic peptides (MCP-1 and MCP-2) formed fibrils, their apparent affinity for template formation and aggregation on hydrophobic surfaces provides an analogy of the possible conformational changes of proteins which occur at the membrane surface in vivo.¹⁸ Also, such surface induced aggregation may enable the building of constructs for nanostructure design.¹⁸

Figure 7.2 SFM image of MCP-2 and Aβ₁–4₀ fibrils adsorbed on HOPG obtained by incubating 50 mM phosphate and 150 mM NaCl at pH 7.2 for 14 hrs. at 37 °C. Image height range is 3 nm and white bar is 1 µm in length.
7.6 Microscopic Evidence of Fibril Formation

Evidence of the fibril forming nature of these cyclic peptides was obtained from scanning force microscopy (SFM). Images obtained with this method exhibited adsorbed layers of MCP-1 and MCP-2 with fibril formation only on the hydrophobic surface (HOPG). The HOPG step edges evidently act as nucleation sites for formation of MCP-1 and MCP-2 layers. Sub-monolayers seem to run nearly orthogonal to the step edge on graphite surfaces but tend to veer away from an adjoining upper atomic plane (step edge) as the peptide structures approach the adjoining step edge, as seen in Figure 7.3. The deposits on mica of MCP-1 aggregates (Figure 7.3 A) measured 5.9 ± 1.0 nm in height whereas MCP-2 deposition (Figure 7.3 C) were 1.7 ± 0.6 nm high aggregates. These MCP-1 and MCP-2 aggregates on mica do not seem to possess any order. However, very different morphologies of the cyclic peptides were observed when HOPG was used as the substrate. HOPG surfaces exposed to MCP-1 and MCP-2 for 5 min. all exhibited fractal-like deposits which appear to have been initiated at the step edges of the HOPG atomic planes (step height is 3.4 Å for HOPG). In addition, the surfaces for both MCP-1 and MCP-2 possess > 10 µm-long fibrillar structures. These fibrillar structures grow from the presence of the existing monolayers. This observation of fibril formation initiated by an ordered monolayer on HOPG has also been noted with elastin-like peptides.\(^1\)

Control experiments with pH 3 solutions not containing MCP-1 or MCP-2 do not lead to the observation of any adsorbed fractal species nor any fibrillar material, indicating the cyclic peptides were responsible for the fractal and fibrillar deposits. MCP-1 has a monolayer much smaller in height than MCP-2. MCP-1 monolayer height is 0.47 ± 0.1 nm (Figure 7.3 B) whereas MCP-2 monolayer height is 1.7 ± 0.2 nm as seen in Figure 7.3 D. The fibrils formed from MCP-1 were 2.3, 3.4 and 4.1 nm in height (Figure 7.3 B), whereas the large fibril seen in MCP-2 was 8.1 nm in height. Possible lateral associations of fibrils formed by MCP-1 and the
large fibril formations of MCP-2 are displayed in Figure 7.5. Cross-sectional analyses of MCP-1 fibrils yielded values of $7.4 \pm 0.4$ nm for the height of the fibrils, whereas the MCP-2 fibrils were $\sim 9$ nm in height with assemblies 1.4 to 1.6 nm in height (Figure 7.4).

Time dependent studies of each cyclic peptide are represented in Figure 7.5. At an adsorption time of 5 seconds, MCP-1 displayed a monolayer of material, after 5 minutes, fractal crystallization took place, and after 30 minutes of exposure to HOPG, fibrils were seen on the surface. MCP-2 displayed different attributes at the same adsorption times. MCP-2 displayed small spherical agglomerations with small propagating structures at a 5 second adsorption and further propagation of these structures at 5 minutes. After a 30 minute adsorption, fibrils were observed.

7.7 Dependence of Fibril Formation on Hydrophobicity or Surface Orientation of Substrate [(C$_{18}$H$_{37}$S-Au(111)]

Hydrophobic C18-terminated Au surfaces were employed so as to allow the determination of whether fibril formation was influenced by surface hydrophobicity. The hydrophobic C18-thiol monolayer on Au(111) did not display any apparent assembly or fibril formation for either cyclic peptide (Figure 7.6). However, the exposure of HOPG to an aliquot from the same solution of these cyclic peptides led to observations of large fibrils (upwards of 20 microns in length) on (0001) HOPG.

To further probe the role of surface orientation with fibril formation, a surface with a different orientation, Si(111), was employed. No fibril formation from either cyclic peptide was observed on Si(111) before cleaning. Si(111) without cleaning has a very hydrophobic surface due to a layer of SiO$_2$ and carbonaceous deposits.$^{19}$ However, cleaned Si(111) displays a surface with each Si atom possessing a hydrogen bond perpendicular to the surface.$^{20}$
Figure 7.3 5 µm x 5 µm SFM image of fibril formation on highly-oriented pyrolytic graphite of peptides dissolved in 18 MΩ deionized water (pH 2.9) at 5 min. adsorption. (A) 50 µM MCP-1 on mica, (B) 50 µM MCP-1 on HOPG, (C) 50 µM MCP-2 on mica, (D) 50 µM MCP-2 on HOPG. White bar is 1 µm in length.
Figure 7.4 Cross-sectional analyses of both fibrils and fractal assemblies on HOPG. (A) MCP-1 fibrils of 7.1, 7.9, and 7.4 nm in height. (B) MCP-2 fibril is 9.1 nm in height and fractal assemblies were either 1.4 or 1.6 nm in height.
Figure 7.5 Time dependent evidence provided by SFM images of MCP-1 and MCP-2. Images are of MCP-1 (A) and MCP-2 (B) after a 5 second adsorption, MCP-1 (C) and MCP-2 (D) after a 5 minute adsorption, and MCP-1 (E) and MCP-2 (F) after 30 minutes of adsorption. Samples were at pH 3. Z-range was 10 nm.
Both MCP-1 and MCP-2 were found not to form fractal crystallizations on cleaned Si(111). However, the cleaned Si(111) displayed a roughness that may not be suitable for cyclic peptide adsorption and propagation (data not shown).

Figure 7.6 5 µm x 5 µm SFM images of C18-thiol terminated Au. (A) C18-terminated thiol on Au alone; (B) MCP-1 on C18; (C) MCP-2 on C18.
7.8 Cyclic Peptide Character Defines Fibril Formation

These cyclic peptides adopt a β-sheet conformation in both solution and while adsorbed on HOPG evidenced by circular dichroism (CD) and reflection absorption infrared spectroscopy (RAIRS). The formation of both cyclic peptide fibrils on HOPG surfaces have been noted at both acidic (pH = 2.9) and neutral (pH = 7.4) conditions (Figure 7.7). The formation of these fibrils is thought to depend on the overall charge on the cyclic peptide.

HOPG is comprised of carbon atoms covalently bonded through $sp^2$ bonding within each layer. Although HOPG is inert along its basal plane, the step edges have faster electron kinetics and may undergo surface oxidation leading to a creation of oxidized carbon species on the edge plane. Thus, the edge plane of HOPG has been found to be more reactive and prone to adsorption than at the basal plane.22 Each cyclic peptide contains lysines which have a terminal pKa of (10.8).24 At both acidic and neutral pH, the surface carboxyl group is partially ionized leaving a negatively charged surface domain with which a positive NH$_3^+$ from lysine can interact.

In a high pH environment (pH of 12), the cyclic peptides do not have a positive charge and the adsorbed layers do not display the fractal crystallization or large fibril formations identified at a pH below the terminal lysine pKa. The lack of aggregate order may be attributed to the lack of positive terminal amine groups available to interact with the still negatively charged carboxyl group found on the HOPG.24

7.9 Microscopic Evidence of Cyclic Peptide Nucleation at Exposed HOPG Domains

Heated HOPG surfaces containing monoatomic-deep pits were observed to cause nucleation and radial growth of cyclic peptide away from the periphery of the HOPG pits as seen in Figure 7.4.
Figure 7.7 10 µm x 10 µm SFM images of cyclic peptide solutions adsorbed on HOPG. (A) MCP-1 at pH 7.4; (B) MCP-2 at pH 7.4; (C) MCP-1 at pH 12; (D) MCP-2 at pH 12. White bar is 1 µm. Z-range is 10 nm.
MCP-1 displayed a monolayer of 1.6 ± 0.4 nm in height around the outside of the pit of HOPG.

MCP-2 displayed a monolayer of 1.8 ± 0.2 nm in height around the outside of a 0.83 nm deep HOPG pit.

7.10 Behavior of Cyclic Peptide Solutions

The appearance of the cyclic peptide containing solutions when placed onto substrates used for scanning probe microscopy (graphite, mica, gold, and silicon) was very hydrophobic. The removal of aliquots from these solutions displayed little surface activity as the solutions did not foam when agitated. Negligible surface activity of both cyclic peptides was observed by tensiometry as detailed in Figure 7.6. These solutions tended to bead well on various surfaces and displayed this attribute on the inner walls of polypropylene microcentrifuge tubes and the
inner walls of pipet tips. Both MCP-1 and MCP-2 did not display any significant surface activity and both easily dissolved in aqueous solutions.

Figure 7.9 Surface pressure measurements of MCP-1, represented as triangles, and MCP-2, represented as squares. Aqueous cyclic peptide solutions were at a pH of 3. Concentrations were 50 µM, 500 µM, 1 mM, and 2 mM in deionized water.

7.11 Solution Studies of Possible Cyclic Peptide Growth in the Absence of HOPG

Dynamic light scattering was used to determine whether these fibrils evolve in solution or evolve only when the peptide is near a nucleating and hydrophobic surface. In solution, it was found that the MCP-1 cyclic peptide grew at a faster rate than did MCP-2 (Figure 7.7). MCP-1 grew large structures after 3 days whereas MCP-2 had a small amount of growth over 6 days. At 3 mM MCP-2, the particle size was $75 \pm 0.8$ nm on the first day and increased to $89 \pm 2$ nm at 3 days incubated at room temperature and at a pH of 3.

For MCP-1 under similar conditions, initial particle size was $82 \pm 2$ nm on the first day and $142 \pm 18$ nm on the third day of incubation. However, no correlation in size between light scattering and microscopy could be made on either HOPG or mica. Once the solution was placed upon graphite, the cyclic peptides grew into assemblies and fibrils longer than the sizes
recorded in solution by light scattering and furthermore aliquots of these solutions spotted on mica displayed smaller sizes than were observed in solution.

**Figure 7.10 DLS Detection of the Growth of Each Cyclic Peptide in Solution at a pH of 3.**

### 7.12 Circular Dichroism Studies of Cyclic Peptides

The secondary structure of each cyclic peptide was assessed in its dissolved state (aqueous solution) at pH 3. Figure 7.11 provides evidence which shows that both cyclic peptides adopt a β-sheet conformation. This β-sheet conformation has also been identified using RAIRS.
7.13 **Infrared Studies of Cyclic Peptide Adsorption on HOPG**

A shift in the amide I and II bands was observed for each cyclic peptide when adsorbed on HOPG. Within an isotropic medium such as KBr, MCP-1 had an amide I band position of 1671 cm\(^{-1}\), indicative of a $\beta$-sheet, and an amide band II of 1531 cm\(^{-1}\). Also, MCP-2 had an amide I band of 1676 cm\(^{-1}\), indicative of $\beta$-sheet, and an amide II band of 1537 cm\(^{-1}\).

When adsorbed to HOPG, MCP-1 displayed an amide I band at 1691 cm\(^{-1}\), also indicative of $\beta$-sheet, and an amide II band at 1556 cm\(^{-1}\). MCP-2 displayed an amide I band at 1691 cm\(^{-1}\) and an amide II band at 1559 cm\(^{-1}\).

7.14 **Adsorbed Cyclic Peptides Enable Aggregation of $\beta$-Amyloid (1-40)**

Monolayers of $\mathrm{A\beta}_{1-40}$ were found to be produced when adsorbed to an existing MCP covered surface. Surfaces chosen for depositing $\beta$-amyloid were from the 5 minute adsorptions of either MCP-1 or 2 (Figure 7.6 C, D). $\mathrm{A\beta}_{1-40}$ adsorbed on pristine HOPG displayed only small
aggregates of 1-11 nm in height (Figure 7.13A). Figure 7.13B demonstrates Aβ_{1-40} deposition on a previously deposited MCP-2 surface shown before (Figure 7.6D).

![RAIRS spectra of Amide I and II bands occur when cyclic peptides are adsorbed on HOPG. (A) 0.5 mg of MCP-1 and MCP-2 in an isotropic medium such as KBr. (B) 500 µM MCP-1 and MCP-2 at pH 3 adsorbed on HOPG.]

Here, Aβ_{1-40} covered the pre-existing layer of MCP-2 and had nucleations of 10-16 nm on top of the layer. Aβ_{1-40} deposition on pre-existing MCP-1 monolayer (Figure 7.6 C) displayed an adsorbed layer of β-amyloid and nucleations of 6-13 nm in height (Figure 7.13 C). The pre-existing monolayer of either cyclic peptide is thought to induce a disorder to order transition which would allow fibril formation of β-amyloid to take place. Such substrate-derived formation of fibrils has been reported with elastin-like peptides.¹
Figure 7.13  5 µm x 5 µm SFM images of Aβ1-40 adsorption on (A) HOPG; (B) HOPG with pre-existing MCP-2 layer; (C) HOPG with pre-existing MCP-1 layer. White bar is 1 µm. Z-range is 10 nm.

7.15 Model of Peptide Nucleation and Growth on HOPG

Models for the orientation and growth of each cyclic peptide have been proposed in Figure 7.14. In Figure 7.14 A, MCP-1 is proposed to lay parallel and propagate across the basal plane of HOPG after an initial adsorption to the step edge of HOPG. SFM software cross-sectional analysis of the MCP-1 monolayer demonstrated a monolayer height of 4 Å. MCP-2, in
Figure 7.14 B displayed a monolayer height by cross-sectional analysis of 17 Å due to the standing of the cyclic peptide monomer on the basal plane.

Figure 7.14 Models of Cyclic Peptide Adsorption on HOPG. (A) MCP-1 laying parallel to the surface and (B) MCP-2 facing perpendicular to the surface. Each blue ring represents one monomer of cyclic peptide.

7.16 Conclusion

Scanning force microscopy images of cyclic peptides adsorbed to hydrophobic graphite provided evidence of surface-induced self-assembly. The hydrophobic surface has been identified as a promoter of protein conformation.\textsuperscript{18,25,26} Observations that amyloid deposition orientates according to the symmetry of the graphite surface also was believed to play a factor here in cyclic peptide fibril formation.\textsuperscript{18,26} Cyclic peptide organization on the surface began with the adsorption of cyclic peptide aggregates followed by propagation of such aggregates in
orientated directions. These orientations allow the nucleation and growth of fibrils. Evidence of this phenomenon was identified with heated HOPG. The production of pits in the HOPG created sites where both cyclic peptides could nucleate and grow radially around the periphery of the pit. The presence of either carboxyl or phenolic groups on these exposed sites is thought to induce nucleation. This model serves a possible explanation to the work done on HOPG with β-amyloid proteins.

7.17 References


Chapter 8

Summary of Conclusions and Future Directions

8.1 Summary of Conclusions

8.1.1 β-Amyloid Aggregation Can Be Controlled

A stable initial stock solution environment is critical for eliminating non β-amyloid nucleation and aggregate formation. It is highly suggested that removal of non–amyloid nucleating sites such as dust, metal impurities, and rough inner walled vials should be performed. These potential nucleation facilitators can speed an otherwise slow–nucleation and growth process. The rapid presence of turbid stock solutions after dissolving the peptide in rough inner walled vials has led to the belief that inner wall roughness can play a major role in the amount of aggregate formation. Elimination of dust and pre-formed β-amyloid aggregates can be removed by filtering. While removing uncontrollable aggregation, inducing and controlling β-amyloid aggregate formation can be done with the aid of increasing the ionic strength of the buffer system used.

8.1.2 Characterization of Peptide Aggregation Mediators

The surface activity of each peptide-based aggregation mediator at various concentrations enabled a determination of the extent of each mediator’s surfactant-like properties. It was found that both AMY-1 and AMY-2 display surface activity around 1 mM, whereas, the mediator developed by Dr. Regina Murphy did not. Even though surface activity was found for AMY-1 and AMY-2, the concentrations at which they would form micelles are 20 fold larger than the concentrations found to disrupt Aβ1-40 fibril formation. Thus, the interactions between each mediator and Aβ1-40 are not one involving micellar interactions. It was also important to ensure that each mediator did not aggregate substantially with itself. SFM studies confirmed that AMY-1 and Murphy sparsely self-aggregated whereas AMY-2 tended to form monolayers on mica.
8.1.3 Surface Activity Aids in Determining Selectivity

The surface-active properties of the peptide-aggregation mediator and fibril forming peptides were also assessed by tensiometry to understand the interactions made between them before any aggregation occurred. It was found that the complex formed between AMY-1 and Aβ_{1-40} was more surface-active than AMY-1 or Aβ_{1-40} alone. AMY-2 was found to form a more surface-active complex with Aβ_{1-40}, Aβ_{25-35}, and Amylin_{20-29}. The mediator developed by Dr. Regina Murphy did not form a complex which had a higher surface active property than the mediator or fibril forming peptide alone. Therefore, AMY-1 formed a complex with only Aβ_{1-40} which was more surface-active than the mediator or Aβ_{1-40} alone.

8.1.4 Efficacy of Aggregation Mediators

AMY-1, AMY-2, and Dr. Regina Murphy’s mediator all displayed an ability to disrupt fibril formation. AMY-1 was able to mediate Aβ_{1-40} amyloid aggregation and produced spherical agglomerations. The mediator developed by Dr. Regina Murphy mediated the aggregation of Aβ_{1-40} in the form of amorphous aggregates and branched linear aggregates, but no fibril morphologies similar to Aβ_{1-40} fibrils were present. AMY-2 displayed rapid interaction and aggregation with Aβ_{1-40} in the form of a colloidal precipitate. It was found that AMY-2 was able to form large amorphous aggregates and inhibited fibril formation.

8.1.5 Cyclic Peptides Generate a Model for Protein Aggregation and Fibril Formation

Although initially proposed as inhibitors of fibril formation, both MCP-1 and MCP-2 displayed significant fibril formation of their own. Their β-sheet properties found by CD and RAIRS are properties associated with many fibril forming peptides.¹ Their ability to template only on edge planes of HOPG is thought to be driven by electrostatic interactions between the lysines located on the cyclic peptides and the oxidized carbon species located on the edge of the HOPG.² ⁴ Evidence of electrostatic interactions was supported by the observation of cyclic
peptide adsorption and radial propagation from thermally induced holes in HOPG and also the inability of cyclic peptide fractal crystallization or large fibril formation at pH 11 (terminal Lysine pka is 10.8). The monolayers of each cyclic peptide on HOPG have been shown to promote $A\beta_{1-40}$ monolayer formation and nucleation demonstrating the disorder to order transition needed for aggregation and eventual fibril formation.$^5$

### 8.2 Future Directions

The work presented here mainly dealt with the end point of the interactions between $A\beta_{1-40}$ and peptide-based aggregation mediators. A more comprehensive study is needed to determine exactly the modes of interaction between $A\beta_{1-40}$ and a mediator. Understanding the affinity for each mediator to bind to $A\beta_{1-40}$ could be accomplished using surface plasmon resonance (SPR) to determine the binding constants of each mediator (AMY-1, AMY-2, and others) to an immobilized array of $A\beta_{1-40}$. Binding isotherms can be used to describe these mediator/$A\beta_{1-40}$ complex formations at the air-water interface.$^7$

Another tool for determining modes of interaction would be to detect the interactions made between mediators and $A\beta_{1-40}$ tethered to Au nano particles by in situ RAIRS. This would give insight into whether the interaction with each mediator creates an $\alpha$-helix, parallel or antiparallel $\beta$-sheet conformation of $A\beta_{1-40}$. $\beta$-Sheet conformations on surfaces, such as found with MCP-1 and 2 on HOPG, may induce $A\beta_{1-40}$ fibril formation. Cyclic peptide monolayers on HOPG have been observed to induce $A\beta_{1-40}$ layer formation. In situ Tapping Mode SFM could be used to watch the growth of these fibrils with time.

Understanding the role of these cyclic peptides on adsorbing onto HOPG is also needed. The capping of exposed sites of HOPG with tertiary butyl groups would aid in determining whether the oxidized carbon species located on these sites are the main factor in templating the cyclic peptides described here.$^4$ In doing so, it is hoped that the elimination of negative charge
on these sites will allow for investigation of whether any electrostatic interactions occur between HOPG and the lysine groups on each cyclic peptide. Additionally, the question of the reversibility of the cyclic peptide assembly process could be probed by making the fibrillar species on HOPG followed by changing the pH or ionic strength (or maybe addition of surfactants). If the fibrils de-assemble, then such assemblies might be useful in molecule delivery applications.

From a materials science point of view, one could think of using the HOPG surfaces to make fibrils and subsequently isolate them to see if they possess any void space (are they tubes or true fibrils?). One could use scanning electron microscopy to change the orientation of these tubules or fibrils to see if they are indeed hollow. The outcomes from such endeavors might lead to the possibility of using the cyclic peptide assemblies as templates for making metallic, inorganic semi-conductor or polymeric nanostructures. For example, certain metal ions/metal ion complexes might coordinate to the amino acid residues of the assemblies and then their subsequent reduction would lead to production of metallic nanometer-diameter wires with lengths on the micron scale. Furthermore, Fullerene nanotubes, such as single-walled carbon nanotubes or multi-walled carbon nanotubes, terminated with carboxylic acids\(^8\), could be used to make protein-coated nanotubes, which might make the Fullerene tubes amenable to use in a variety of media or allow for fabrication of complex protein-carbon nanotube structures.

8.3 References


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Vita

Jed Paul Aucoin was born in Thibodaux, Louisiana, on March 3, 1977. For 22 years, he resided in Des Allemands, Louisiana. He obtained a bachelor’s degree in chemistry at Nicholls State University located in Thibodaux, Louisiana, in 1999. During his undergraduate years at Nicholls State University, he was involved in collecting information on the bioaccumulation of heavy metals in the aquatic life and soil of a local lake under the direction of Dr. James Beck and Dr. Keshav Mandhare. Also during this time, he was involved in the Louisiana Systematic Initiatives Program (LaSIP) under the directions of Dr. Glenn Lo and Dr. Judy Chauvin. Upon entering the graduate program at Louisiana State University, he joined the group of Dr. Robin McCarley and is currently completing the degree requirements for the degree of Doctor of Philosophy in chemistry. Upon completion of this doctoral program, Jed accepted a position as a chemist for the Lockheed Martin Manned Space Systems Corporation in New Orleans, Louisiana.