NMR ANALYSIS OF INTRACELLULAR AMYLOID-BETA PEPTIDE

By

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*We also certify that written approval has been obtained

for any proprietary material contained therein.
This thesis is dedicated to my family and Sarah.
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<tr>
<td>Aβ</td>
<td>amyloid β</td>
</tr>
<tr>
<td>ACA</td>
<td>Affordable Care Act</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>APOE</td>
<td>apolipoprotein E</td>
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<td>APP</td>
<td>amyloid precursor protein</td>
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<tr>
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<td>β-secretase1</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CD</td>
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<td>colony forming unit</td>
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<td>CHG</td>
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<td>Compliment Receptor 1</td>
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<td>CSF</td>
<td>cerebral spinal fluids</td>
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<td>DMEM</td>
<td>Dubelco’s modified eagle medium</td>
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<td>dNTP</td>
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<td>EM</td>
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<td>fetal bovine serum</td>
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<td>FCM</td>
<td>flow cytometry</td>
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<td>Gene Expression Omnibus</td>
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<td>HBSS</td>
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<td>HFIP</td>
<td>hexafluorisopropanol</td>
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<td>INEPT</td>
<td>insensitive nuclei enhanced by polarization transfer</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>sAPPα</td>
<td>soluble amyloid precursor protein-α</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>soluble amyloid precursor protein-β</td>
</tr>
<tr>
<td>siRNA</td>
<td>small inhibiting RNA</td>
</tr>
<tr>
<td>SEC-R</td>
<td>serpin-enzyme complex receptor</td>
</tr>
<tr>
<td>SLO</td>
<td>Streptolysin-0</td>
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<td>side-scattered light</td>
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<td>SSIs</td>
<td>surgical site infections</td>
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<tr>
<td>STD</td>
<td>saturation transfer difference</td>
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<td>STE</td>
<td>stimulated echo</td>
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<td>trifluoro acetic acid</td>
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<tr>
<td>ThT</td>
<td>thioflavin-T</td>
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<tr>
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</tr>
<tr>
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<td>triggered receptor expressed on myeloid cells</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>α7nAChR</td>
<td>α7 nicotinic acetylcholine receptor</td>
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NMR Analysis of Intracellular Amyloid-Beta Peptide

Abstract

By

COLIN AGATISA-BOYLE

The primary goal of the present research is to study the earliest events of aggregation and eventual amyloid formation the Aβ peptide inside of living human cells using primarily NMR. The secondary goal is to examine the binding interaction between the Aβ peptide and melatonin in order to achieve a greater understanding of how melatonin behaves as an Aβ aggregation inhibitor.

Prior to using in-cell NMR to characterize the Aβ inside of living cells, the conditions used to introduce the Aβ peptide into living cells using streptolysin-O (SLO) were further optimized on previous work of Dr. Fang Han. Previously, we were able to introduce the Aβ into cells and reseal the cellular membrane in about 45% of SLO treated cells, as assessed by flow cytometry (FCM). Further efforts to optimize the SLO technique, improved this previous result and introduced Aβ into 57.6% of viable resealed cells. In order to confirm that the Aβ peptide was entering the cytoplasm and not sticking to the outer plasma membrane, images were acquired using confocal microscopy. By counter staining the plasma membranes
with CellMask™ Deep Red and acquiring a z-stack series of images, that the Aβ was distributed throughout the interior of the cells.

After optimizing the SLO technique, uniformly $^{15}$N labeled Aβ 1 – 40 was introduced into living cells, and 2D NMR heteronuclear single quantum coherence (HSQC) spectrum was acquired. The results showed twelve identifiable amino acid signals that had similar chemical shifts to the control Aβ spectrum. This result indicated the Aβ peptide was not adopting a new and folded tertiary structure inside the living cells. The effect of macromolecular crowding on Aβ was examined using polyethylene glycol (PEG) in an effort to aid in exploring the basis for the intracellular loss of signals. These studies involved acquiring HSQC of Aβ in solutions with various concentrations of PEG. Analysis of these spectra showed that a crowded molecular environment could not account for the loss of signals observed for intracellular Aβ.

Following these macromolecular crowding studies, the binding interaction between Aβ and cellular organelles was examined. Nuclei and mitochondria were individually isolated using differential centrifugation, and allowed to incubate in solution with Aβ, and finally 2D HSQC NMR spectra were acquired. The resulting spectra showed that three Aβ signals disappeared when the peptide was in the presence of isolated nuclei, and twelve signals disappeared when the peptide was in the presence of isolated mitochondria. Immediately following the completion of the HSQC experiments, the samples were centrifuged and an HSQC of the supernatants
were acquired. In both cases, no signals were observed indicating that the previous results were consistent with the Aβ bound to the cellular organelles and not from free Aβ in solution. Further examination of the cellular organelle spectra, it was observed that these organelles had a significant impact on the Leu-17 to Ala-21 region of the Aβ peptide. Previous studies have shown that this region is crucial to the aggregation of Aβ. Following this result, we had a F19A mutant Aβ 1 – 40 peptide, ¹⁵N labeled from Leu-17 to Ala-21, produced in order to determine if a mutation in this crucial region will prevent Aβ from binding to cellular organelles. This study is currently ongoing, but our initial results suggest that this mutation may in fact prevent Aβ from binding to the organelles.

The second project examined the interaction between melatonin (a hormone produced in the brain) and the Aβ peptide. Previous studies established that melatonin inhibits the aggregation of Aβ. Saturation transfer difference (STD) NMR was performed and showed that the melatonin-6H proton was in close proximity to the His-4H protons of Aβ. Following this result, we hypothesized that melatonin is able to prevent Aβ aggregation by inhibiting the formation of Asp-His salt bridges. This hypothesis was tested by performing 1D ¹³C NMR on a mixture of melatonin and ¹³C labeled at the side chain carbonyl of Asp-23 Aβ peptide. The result showed that in the presence of melatonin, the Asp-23 side chain carbonyl peak shifted when compared to the control, indicating that interruption of Asp-His salt bridges may be the mechanism by which melatonin inhibits Aβ aggregation.
The third project involved studying an experimental drug IXR4204 and its ability to inhibit Aβ aggregation. At the start of this project, this drug was undergoing a clinical trial for treatment of prostate cancer. For the present study HSQC NMR and circular dichroism (CD) were utilized. Overall the results showed that the drug interacts with Aβ monomers, but unfortunately accelerated Aβ aggregation.

The fourth project examined the abilities of different spices found in traditional Indian cuisine to inhibit Aβ aggregation. A variety of different techniques including $^1$H NMR, CD, and atomic force microscopy (AFM) were utilized and demonstrated that curcumin, anethole, tartaric acid and acetic acid all demonstrated some ability to inhibit Aβ aggregation.
CHAPTER 1
INTRODUCTION
1.1 Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder that is the leading cause of dementia worldwide (1,2,3,4). The most recent estimates claim that AD accounts for 60-80% of all cases of dementia (1,3,4). In the past, AD was defined strictly as a form of dementia, however in 2011 the definition of AD was expanded. Today, AD includes a wide spectrum of people from those who have AD related dementia, to those who only have a mild cognitive impairment (MCI), and even people who are asymptomatic but have the genetic predisposition to later develop AD (1,3,4). Currently, the only definitive way of diagnosing a patient with AD is to examine their brain post-mortem for extracellular Aβ plaques and intraneuronal neurofibrillary tau tangles (1,2,5,6). While there currently are no effective treatments to slow the progression of AD, the hope is that in the near future new treatments will be discovered. In order to aid in the development of new AD treatments, early AD biomarkers must be uncovered, and a greater understanding of the earliest stages of the disease must be achieved. The goal of this research is to study the earliest events of AD by examining the Aβ peptide inside of living human cells using NMR.

1.1 AD risk factors

AD is an age-related disease that slowly progresses in severity over the course of a person’s lifetime (1,4,5,6). It is currently believed that changes in the brain may occur over 20 years before the earliest symptoms of AD are manifested
(1,4,5,6). Other risk factors for AD include mutations in either the Amyloid Precursor Protein (APP) or presenilin (PS) genes (5,7,8), carrying the ApoE4 allele (9,10,11,12,13), family history of AD (1,2,14), MCI (1,15,16,17), head trauma (1,18), Down’s syndrome (19,20), high blood pressure or cholesterol (1,21,22,23,24,25), amount of education (1,26), and even lack of social engagement (1,27,28). The numerous symptoms of AD vary from person to person. These symptoms include difficulty remembering new information, difficulty completing daily tasks, confusion over time and date, easily misplacing things, difficulty with problem solving or making plans, poor decision making and lack of judgment, withdrawal from work and social activities, difficulty with finding the right words and speaking, difficulty reading or understanding spatial relationships, and changes in mood or personality (1,2,3).

1.2 Rates of AD in the United States

AD is the sixth leading cause of death in the United States, and is the only disease in the top 10 leading causes of death that is completely untreatable (1,2,3,29). It is currently estimated that there are 5.5 million people in the US with dementia related to AD, of which 5.3 million are over the age of 65 (1,2,3). In fact, 10 percent of all people over the age of 65 in the US have dementia due to AD (1). The US has one of the highest AD rates, compared to significantly lower rates worldwide, such as in India, where less than 2 percent of people over the age of 65 have AD (30,31). As a person's age increases past 65, their risk of developing
Alzheimer’s dementia drastically increases from about 3 percent of people aged 65 to 74, to 32 percent of people over the age of 85 (1). In the near future, the number of people in the US with AD will increase significantly as the baby boomer generation continues to age (1,2,3).

![Projected Number of People Age 65 and Older (Total and by Age Group) in the U.S. Population with Alzheimer’s Dementia, 2010 to 2050](image)

**Figure 1.1** Projection of the number of people who will develop AD in the US over the next 30 years. (1)

Current projections estimate that in the year 2050 there will be 13.8 million people over the age of 65 with Alzheimer’s dementia in the US alone (Figure 1.1) (1). It is estimated that in 2017, approximately 700,000 people over the age of 65 will die as a result of AD (32,33,34). Furthermore, in comparison to the other leading causes of death in the US that have decreased, deaths from AD have increased by 89 percent between 2000 and 2014 (Figure 1.2) (35).
Figure 1.2 Changes in some of the leading causes of death between 2000 and 2014. AD is the only disease that has seen an increase in its death rate. (1)

1.3 Financial burden of AD

The vast number of people living with AD both in the US and worldwide causes a significant financial burden. The average life expectancy of a patient age 65 and older with AD is 4 to 8 years after they are diagnosed (1,2,3). During this time span, on average person with AD will spend the last 40 percent experiencing the worst stages of dementia (1,34). During this time, people with AD typically live in nursing homes or with hospice, both of which are costly (1,32,33,34). For the year 2017, it is estimated that the total cost of care for AD will be $259 billion (1). Of this
$259 billion, Medicare and Medicaid are expected to pay $175 billion (1), a troubling statistic given the uncertain future of the Affordable Care Act (ACA) and the current expansion of Medicaid. The average person with AD currently receives $8,182 annually from Medicaid, and pays $10,315 out of pocket in expenses (36).

Furthermore, about two-thirds of all people living with AD are women (1,37), many of whom are widowed and living in poverty, leading to average Medicaid costs that are 70 percent higher than those for men (38).

Figure 1.3 Predicted annual cost of care for patients with AD in the US. In 2050, the financial burden for AD cost of care is expected to exceed $1.1 trillion each year (in today's dollars). (39)
Over the next three decades, the financial burden of Alzheimer’s cost of care in the US will increase dramatically. In 2050, approximately 13.8 million Americans will have Alzheimer’s dementia, resulting in an annual cost of care of $1.1 trillion in today’s dollars (Figure 1.3) (39). Unfortunately, the average annual budget of Alzheimer’s research funding is not expected to experience a similar increase. Currently, Alzheimer’s research receives about one fourth the amount of funding that heart disease research receives, and about one tenth of the funding for cancer research (Figure 1.4). Clearly, unless dramatic advances are made in the treatment of AD, the burden that AD will place on the US economy will be staggering.
Figure 1.4 Annual funding of AD research in comparison to the research funding of several other diseases. (1)

1.4 Current treatments for AD

While AD is an ever increasing public health problem, the options available to treat the disease are very limited. In the late 1990’s there were a total of five FDA approved drugs to treat AD; donepezil, galantamine, memantine, rivastigmine, and tacrine. Despite the fact that these drugs were approved to treat AD, none of them
actually treated the causes of AD. Instead, these drugs are used to ease some of the
symptoms associated with AD. Specifically donepezil, galantamine, rivastigmine,
and tacrine all target the cholinergic system, which is a system of neurons that are
activated by acetylcholine and are involved in cognitive functions such as memory
(40,41,42). Memantine targets the glutamatergic system, which is also involved in
memory (43,44). All of these drugs come with severe side effects such as nausea,
vomiting, diarrhea, severe headaches, dizziness, insomnia, hallucinations, confusion
and many more (45,46). The side effects for tacrine in particular are so severe that
in 2013 the FDA withdrew approval of the drug for use in the US (47). This
currently leaves only four FDA approved drugs to treat AD in the US, all of which
have questionable benefits on improving the quality of life of patients with AD
(45,46).

1.5 Pathology of AD

AD is characterized by the deposition of extracellular plaques, and
intraneuronal neurofibrillary tangles, located in the temporal neocortex and
hippocampal regions of the brain, which are associated with memory and cognition
(48,49,50,51,52). Neuritic plaques are associated with dead neurons, and decreases
in the number of synapses located in the immediate vicinity of the plaques
(48,53,54). The major protein component of the extracellular plaques is the Aβ
peptide (5,48,49,51,53,55), while hyperphosphorylated tau makes up the
neurofibrillary tangles (48,49,51,55,56). The Aβ peptide in extracellular plaques is found predominantly in two different forms, a 40 residue and a 42 residue form (48,49,51,53,58). Of the two Aβ 1 – 42 is far more aggregation prone and cytotoxic (48,49,51,53,58).


Aβ 1 - 40

Aβ 1 - 42

Figure 1.5 Sequences of Aβ 1 – 40 and Aβ 1 – 42. Aβ 1 – 42 differs by only the additional amino acids isoleucine and alanine, and is the more aggregation prone peptide.

The Aβ peptide is produced as a random coil monomer that, for reasons not fully understand, aggregates to form β-sheet oligomers in the brains of people with AD. Currently, the biological function of Aβ is unknown since it can be found in the brains and the cerebral spinal fluids (CSF) of healthy subjects (59,60,61). In the brains of people who develop AD, Aβ transitions from its native random coil conformation to a β-sheet conformation as it forms soluble oligomers. These oligomers in turn aggregate to form protofibrils, followed by mature fibrils, and eventually extracellular plaques (62,63,64). Aβ oligomers also trigger tau hyper-phosphorylation leading to intraneuronal fibrillary tangles of tau (53,65,66,67).
these different stages of Aβ aggregation, it has been found that soluble Aβ oligomers are the most cytotoxic to cultured neuronal cell lines (68,69,70), and have been found to reduce synaptic density (53,71,72). Furthermore, Aβ oligomers, fibrils and plaques all have the ability to act as nucleation sites, or catalysts, for the formation of additional Aβ oligomers (73,74). Over time, the brain’s natural Aβ clearance mechanisms become overwhelmed by the accumulation of Aβ aggregates, which leads to neuronal cell death and Alzheimer’s dementia. This process is known as the amyloid cascade hypothesis (75).

### 1.6 Biogenesis of Aβ

The Aβ peptide begins as a part of a much larger protein called the amyloid precursor protein (APP) (76,77). APP is a type I membrane spanning glycoprotein that is expressed throughout the body (78). The exact function of APP is still unknown, although it is believed to play a role in neural plasticity and synapse formation (79,80). APP can initially be cleaved at two different locations that lead to two different pathways, an amyloidogenic pathway and a non-amyloidogenic pathway. In the non-amyloidogenic pathway, APP is cleaved in the middle of the sequence of Aβ by the secretase protein α-secretase. This leads to the production of sAPP-α, which has been found to be neuroprotective (81). In the amyloidogenic pathway, APP is first cleaved at the N-terminal site of Aβ by β-secretase (BACE), after which it is cleaved a second time by γ-secretase at the C-terminus which is catalyzed by the proteins PS 1 and 2, producing Aβ of various lengths (53,77). While
APP is originally located on the outer plasma membrane of the cell, it is brought inside the cell in endosomes where it is cleaved to form Aβ (77,78,79); thus Aβ originates from within neurons.
Figure 1.6 Different APP processing pathways. APP can either be cleaved in the middle of the sequence for $\alpha\beta$ by $\alpha$- by, producing the neuroprotective fragment sAPP-$\alpha$, or it can be cleaved by $\beta$-secretase followed by $\gamma$-secretase to produce $\alpha\beta$. 
After being cleaved from APP, Aβ can either be shuttled outside of the cell, or be packaged into lysosomes for degradation (82,83). In addition to initially being found within neurons, Aβ can enter neurons from the extracellular environment. Several different Aβ receptors on the outer plasma membrane of neurons have been identified. These receptors include PrPc, serpin-enzyme complex receptor (SEC-R), insulin receptor, α7nicotinic acetylcholine receptor (α7nAChR), receptor for advanced glycosylation end-products (RAGE), and N-methyl-D-aspartate receptor (NMDA) to name a few (84). With respect to NMDA in particular, binding of Aβ to this receptor causes the endocytosis of the complex (82). Extracellular Aβ 1-42 has also been found to trigger increased expression of Aβ 1-42 through a prion-like pathway (85,86).

1.7 The amyloid cascade hypothesis

1.7.1 Criticisms of the amyloid hypothesis

Over the years, there has been a great deal of debate over the exact cause of AD. Currently, the most widely accepted theory as to the cause of AD is the amyloid hypothesis, discussed previously, which is not without its controversies. Critics of this hypothesis have raised several questions that have been recently studied and produced evidence in favor of the amyloid hypothesis. One of the main criticisms is that Aβ deposits can be found in the brains of healthy subjects who showed no signs of Alzheimer's dementia. However, it has been found that the Aβ deposits in the
brains of healthy people without AD are more diffuse, lack the fibrillar structure of AD plaques, and also do not show the characteristic signs of neuronal cell death and loss of synapses (72). Another key criticism of the amyloid hypothesis is the fact that Aβ aggregates seem to be both a cause and an effect of AD. It has been found that mutations in the genes for APP and PS 1 and 2 lead to an increase in Aβ production, which results in early-onset AD (48,53). On the other hand, individuals who are carriers for the ApoE-4 allele have reduced Aβ clearance, which leads to accumulation of Aβ aggregates (13,48,53,87,88,89,90,91).

Researchers have also had difficulty reproducing all of the symptoms of AD in mouse models (48,53). The initial AD mouse models used transgenic mice that overexpressed human APP in order to increase Aβ plaque load in the brain. However, it was found that this model did not produce all of the phenotypes necessary to mimic the disease, which led to doubts in the role Aβ plays in AD. Since then a double transgenic, and even more recently, a triple transgenic mouse model has been developed in which human APP, human Tau, and human PS are all expressed, which has proven to be a more effective model (48).

1.7.2 Evidence supporting the amyloid hypothesis

While the amyloid hypothesis has been challenged, it has thus far proven to be the best explanation for the causes of AD. Research has shown that there are two main pathways to the disease; both of which rely on the accumulation of Aβ peptide.
In the cases of early onset familial AD (FAD), inherited mutations in genes such as APP and presenilin 1 and 2 lead to an overproduction of the more pathogenic Aβ 1 – 42 that overwhelms the clearance mechanisms in the brain. Another genetic disorder that supports the amyloid hypothesis is Trisomy 21, or Down syndrome. People with Down syndrome have three copies of chromosome 21, where the gene for APP resides (92,93). As a result, these individuals produce more APP and subsequently Aβ, resulting in the development of AD usually in their mid 40’s (53). Interestingly, about 3-4% of people with Trisomy 21 have translocation Down syndrome in which the chromosomal material is rearranged and the extra chromosome 21 is not separate but attached to another chromosome. Although these individuals develop Down syndrome phenotype, they do not inherit three copies of the APP gene (94). Conversely, in a study of five families that developed early-onset AD, a rare duplication of the locus of chromosome 21 where APP is located was found. This resulted in an increased production of APP and Aβ similar to that of people with Down syndrome, but without the Down syndrome phenotype. Taken together, these two rare cases definitively show the role of increased Aβ production in cases of early-onset AD.

Recently, a missense mutation of APP was discovered in Icelandic and other Scandinavian populations (95). This mutation, called A673T, replaces the second amino acid of Aβ, alanine, with threonine. The result of this mutation is a decreased likelihood for APP cleavage by BACE, which results in a lower production of Aβ. Individuals who have this mutation were found to be significantly less likely to
develop AD, as well as to experience age-related cognitive decline (95). Subsequent studies found that this mutation increases the amount of sAPPα in relation to sAPPβ (96), and that sAPPα itself is neuroprotective (81). The discovery of the A673T mutation provided further proof to support the amyloid hypothesis.

The other main pathway that leads to late onset AD (LOAD) is a failure to clear Aβ in the brain. Early on in the study of AD, a connection was made between Aβ plaques and associated microglia cells (97). Microglia cells are a key component of the immune system in the brain, and are responsible for scavenging and clearing both soluble Aβ as well as Aβ plaques (98). Recent studies have found that microglia dysfunction plays a key role in increased Aβ plaque burden in the brains of people with LOAD (99). Three genes related to microglial function have been identified as potential risk factors for LOAD. These genes are Complement Receptor 1 (CR1) which regulates microglial activation and phagocytosis of Aβ (100), CD33 which also controls Aβ phagocytosis (101), and TREM2 which is responsible for continuing microglial cell response to Aβ deposits (102). Each of these genes has been found to be upregulated in AD patients (101,102,103), and mutation of TREM2 in particular has been connected to increased risk of AD (104).

Clearance of Aβ in the brain by microglia cells is also impeded in people who carry the ApoE4 allele (13,87,88,89,90,91). Apolipoprotein E (ApoE) is produced in the brain by astrocytes and microglia cells, and is responsible for cholesterol metabolism. In the early 1990’s, it was found that people who expressed the ApoE4
allele had a significantly higher risk of developing LOAD (13). The ApoE4 allele is now recognized as the strongest genetic risk factor for LOAD (1,13,48,53,91). People who are carriers of the ApoE4 allele have significantly diminished Aβ clearance in comparison to people who carry the ApoE2 and ApoE3 alleles (10,88,89). It has also been found that ApoE4 alters the response of microglia cells to Aβ deposits (105), and triggers a pro-inflammatory response by the brain’s immune system in comparison to the other ApoE isoforms (9). The role ApoE4 and microglia cell dysfunction play in the development of Aβ plaque burden in the brains of people with AD has provided further evidence in support of the amyloid hypothesis.

Currently there is no accepted biomarker that has been identified as an early warning sign for AD, like high cholesterol is recognized as a biomarker for atherosclerosis. Recent research has looked toward Aβ aggregation as a biomarker for AD and has produced some promising results, which provide further support for the amyloid hypothesis. It has been found that in comparison to healthy controls, Aβ 1-42 levels in the CSF are initially elevated before drastically declining approximately 25 years before the onset of symptoms in people who go on to develop AD (14). These changes in the CSF also precede Aβ deposits in the brain, an increase of tau in CSF, and neuronal cell death by about 15 years (14). Amyloid deposits in the brain have also been imaged in patients with varying degrees of cognitive decline using positron emission tomography (PET) imaging and the Pittsburgh compound B (PiB). PiB is an analog of thioflavin T that is radioactively
labeled with $^{14}$C, which binds fibrillar $\alpha$ (15,106,107). While PiB is capable of imaging fibrillar $\alpha$, it does not bind $\alpha$ oligomers or other forms of soluble $\alpha$ (108). Research using PiB to develop an agent that can be used to clinically diagnose AD is ongoing (109).

1.8 **Objective and significance of research**

The objective of the research described in this thesis is to examine $\alpha$ inside living human cells to obtain a better understanding of the earliest stages of AD. We used NMR to examine $\alpha$ inside living human cells, a feat that had not been accomplished prior to this work. $\alpha$ was introduced into HeLa cells using the pore forming toxin streptolysin-O. The presence of the peptide within living cells was confirmed using flow cytometry and confocal microscopy. In-cell NMR spectra of uniformly $^{15}$N labeled $\alpha$ peptide show that intracellular $\alpha$ is likely binding to an unknown target. NMR was also used to demonstrate that $\alpha$ binds to nuclei and mitochondria that were isolated using differential centrifugation. These results may lead to a greater understanding of the earliest stages of AD, when the $\alpha$ peptide first begins to aggregate.

In addition to studying the $\alpha$ peptide *in vivo* using NMR, this thesis describes three other projects that examined the interaction between $\alpha$ and potential aggregation inhibitors. The first of these projects examines melatonin, a natural hormone found in the brain, and its binding interaction with $\alpha$ using STD
and $^{13}$C NMR. An experimental drug, IXR4204 was also examined for its potential ability to inhibit $\beta$ aggregation using HSQC NMR and circular dichroism (CD). Finally, several spices found in traditional Indian foods were studied for potential inhibition of $\beta$ aggregation using a combination of NMR, CD, and atomic force microscopy (AFM). These spices were chosen to address possible protective associations of their consumption and the lower incidence of AD among the population of India. The goal of these studies is to potentially aid in the discovery of novel AD therapeutics.
Chapter 2

INTRODUCTION OF Aβ PEPTIDE INSIDE LIVING CELLS
2.1 Introduction

The primary goal of this project was to observe the Aβ peptide in its native intracellular environment. This objective presented the challenge as to how best to introduce Aβ into living cells. For our purposes, we needed a technique that could reliably and reproducibly introduce Aβ into a human cell line that was cost effective, high throughput and could be accomplished in a short time span. Given Aβ's propensity to aggregate as time progresses, it was necessary to minimize the amount of time between when Aβ is first introduced in solution and the start of the NMR analysis. In addition, the chosen technique needs to use a minimal amount of Aβ, given the high cost of uniformly $^{15}$N labeled Aβ. There are several options for introducing proteins or peptides into living cells that each present their own challenges, as well as advantages and disadvantages, a few of which will be discussed.

2.1.1 Potential methods for introduction of the Aβ peptide into living cells

2.1.1.1 Transfection and transduction

Perhaps one of the most widely utilized techniques for introducing proteins or peptides into living cells is via transfection or transduction. These techniques allow for the introduction of genetic material, such as a sequence of DNA that codes for a specific gene, into living cells (110,111). Transfection refers to the
introduction of genetic material into eukaryotic cells via non-viral methods, while transduction refers to the viral method of introducing genetic material into eukaryotic cells. Once the desired gene is introduced into the targeted eukaryotic cell line, it is incorporated into the cell’s genome and expressed. There are several different methods of transfection that range from chemical-based transfection, to non-chemical means such as electroporation, and other physical methods such as micro-injection or the use of a gene gun (110,112). Transduction on the other hand utilizes the ability of different viruses such as the adenovirus to insert genetic material into human cell lines (110,113).

Comparing and contrasting these various different methods is beyond the scope of this project, but all are viable methods of potentially introducing Aβ into eukaryotic cells. However, while these methods are viable and well understood, they all share the same downfall that makes them unfeasible for our purposes. This major disadvantage is that while transfection can introduce the APP gene into human cells, and exploit the cells biochemical machinery to produce Aβ intracellularly, the produced Aβ will not be $^{15}$N labeled and thus be invisible to HSQC NMR. In order to make the cells produce uniformly $^{15}$N labeled Aβ, the cells would have to be grown in $^{15}$N enriched growth media, which can greatly increase the cost of an experiment. Growing cells in $^{15}$N enriched growth media also has the added disadvantage that all of the proteins produced by the cells will be $^{15}$N labeled, which could make isolating the signals for Aβ via NMR difficult. Finally, causing a human cell line to produce Aβ has the added problem of controlling the expression of the
APP gene. Overexpression of the APP gene may cause the high concentration of Aβ to kill the cells that are producing it. Conversely, if the expression of the APP gene is low, then it may be impossible to observe Aβ via NMR. Due to these reasons, high cost of 15N enriched growth media, difficulty in controlling Aβ production and thus concentration, transfection and transduction were ruled out for introducing Aβ into living cells.

2.1.1.2 Microinjection

Another widely used technique for introducing proteins into living cells is microinjection. This technique has several desirable advantages that could have been useful for our purposes. One of the major advantages of this technique is the small amount of peptide that would be required; volumes as low as 2 – 5 μL of a given protein stock solution are all that is required for microinjection (110,112,114). Using a small amount of Aβ is desirable given the high cost of this peptide when uniformly 15N labeled. Another advantage of microinjection is that it is a method of directly inserting a protein into a cell, and the location within the cell itself can be controlled (110). Proteins can be inserted into the cytoplasm or the nucleus of a cell using this technique. Finally, microinjection has the added advantage of being able to immediately conduct experiments on the cells after microinjection, without the need for lengthy incubation times (110,112,114).
While the advantages provided by microinjection of Aβ into living cells are numerous, there are several key disadvantages that ruled out this technique for our purposes. A major disadvantage of microinjection is the high cost of the specialized equipment necessary for the technique. Required equipment includes an inverted microscope and a micromanipulator, both of which are prohibitively expensive. However, the primary reason why microinjection was unfeasible for our needs is the extremely low throughput of this technique. At best, microinjection can insert protein into about 300 cells in a 15 min timespan, and this rate is dependent upon the skill of the practitioner (110). Simply put, in order to be able to observe $^{15}$N labeled Aβ inside living cells using NMR, we would need an amount of cells that is a factor of about $10^6$ greater than microinjection can produce.

### 2.1.1.3 Steptolysin O

After considering several of these different techniques, we settled on using the pore-forming toxin streptolysin O (SLO) to introduce Aβ into living cells. SLO is a hemolytic exotoxin that is produced by bacteria that belong to the genus streptococcus (115). Bacteria of this genus are responsible for a wide variety of diseases ranging from strep throat to scarlet fever (116). This toxin is capable of producing pores up to 35 nm in diameter in the plasma membrane of cells (117,118), which can allow proteins as large as 150 kDa, far larger than Aβ which is only 4 kDa, to diffuse into the cells (117,118). After the desired protein diffuses into the target cell line, the pores are sealed using a solution of calcium chloride. In total,
this entire process can be accomplished in about three hours, which allows for a quick turnover from introduction of a protein to NMR analysis.

Using SLO as a means of introducing Aβ into cells provided additional advantages. The reagents necessary for this technique including the SLO toxin are inexpensive. In addition, this technique can be performed on cells that are in suspension, thus providing an opportunity to optimize the volume of cells in relation to the amount of uniformly $^{15}$N labeled Aβ. Finally, this technique has been proven to be effective in introducing uniformly $^{15}$N labeled proteins into eukaryotic cells for the purpose of in-cell NMR (118). The only disadvantage is the use of a toxin that can lead to cell death if the experimental conditions are not optimized.

2.1.4 Flow cytometry

Prior to attempting any NMR experiments, we needed a means to confirm that we successfully introduced Aβ into living cells using the SLO technique. In order to quantify the presence of Aβ in cells that survived the introduction of the peptide, we used flow cytometry (FCM). FCM is a widely used technique in cell biology and medicine that employs a combination of light scattering and fluorescence spectroscopy to count, sort, and/or analyze cells using different biomarkers. This is accomplished by tagging a specific biomarker or protein of interest, in our case the Aβ peptide, with a fluorescent dye such as Dylight 488. The cells are then counterstained with another fluorescent dye, propidium iodide (PI),
that exclusively stains non-viable, or dead cells. As cells in suspension pass through the FCM, the machine compares the cells absorbance and emission of different fluorescent wavelengths to the cell’s forward scattered light (FSC) and side scattered light (SSC) (119). FSC can provide information about the size of the cell being measured, while SSC can provide information about the granularity of the cell in question (119). The granularity of a cell is an important factor that is used to determine if a cell is alive or dead; non-viable cells are more granular than viable cells (119). By comparing all of these factors, the FCM is then able to generate a plot that quantifies the number of viable cells that contain the desired protein or biomarker.

2.1.5 Confocal microscopy

In addition to using FCM to optimize our SLO technique and confirm the presence of Aβ inside of living cells, we used confocal microscopy to physically observe intracellular Aβ. Confocal microscopy is an incredibly useful tool that can be used for the acquisition of high-resolution fluorescent images of cells. One of the primary advantages of confocal microcopy over conventional fluorescence microscopy is the ability to change the depth of focus of the microscope. While traditional microscopes can only focus in the X and Y dimensions, a confocal microscope has the ability to also focus on specific planes in the Z dimension. This is accomplished by passing the light coming from the sample through a pinhole aperture, which allows the microscope to only focus on light from a specific focal
Confocal microscopy is also able to acquire images of cells that have been stained with several different fluorescent dyes and overlay the images, giving an in-depth look into the internal workings of a cell. However, this technique comes with a few disadvantages. Focusing on a single point in a given sample reduces the intensity of the light coming from the sample because all of the scattered light coming from the light source that aids in illuminating the sample is excluded. Additionally, given that confocal microscopy focuses only on a single point, in order to acquire a complete image, the sample must be scanned point by point, which can take some time. However, the end result is an extremely high-resolution image that can show miniscule structures inside of cells.

2.2 Materials and Methods

2.2.1 Dylight-488 labeled BSA and Aβ preparation

Prior to introducing Aβ into living cells, bovine serum albumin (BSA) was introduced into the cells in order to confirm the proper execution of the SLO technique. BSA was chosen because it is a cheap, inert protein that should not cause cell death when introduced into living cells. First, a 50 μM stock solution was prepared by dissolving BSA in phosphate buffer (0.1 M). The BSA stock solution (500 μL) was then added to a vial of DyLight 488 (50 μg) (Thermo Scientific), vortexed, and left to incubate at room temperature for 1 hr. Excess dye was then removed using a fluorescent dye removal column (Thermo Scientific).
DyLight 488 labeled Aβ 1–40 (rPeptide) was also prepared for use in confocal microscopy experiments. The fluorescently labeled Aβ stock (213 μM) was prepared by adding NaOH (1 mL, 10 mM) to a vial containing the Aβ powder (1 mg). This solution was sonicated for 10 mins to break up any aggregate seeds, and kept at -20 °C until use.

2.2.2 Flow cytometry

Growth medium was prepared by combining one bottle (500 mL) of DMEM high glucose with L-glutamine (ATCC), with fetal bovine serum (50 mL) (FBS) (ATCC), and penicillin-streptomycin 100x solution (5 mL). This gave a growth medium with 10% FBS and 100 IU/mL penicillin-streptomycin.

HeLa cells were grown in three 150 cm² canted neck flasks with vented caps (Corning) using 25 mL of growth medium in each flask. The cells were grown for a week; the medium was changed every two days until confluence was reached. Then the medium was aspirated, and PBS 1X (12 mL) (without calcium or magnesium, ATCC) was added to each flask and swirled. Next the PBS was aspirated and to each flask, trypsin (2.5 mL) (0.25% trypsin/0.53 mM EDTA in HBSS without calcium & magnesium, ATCC) was added, and the flasks were incubated at 37 °C for approximately 2 min. After incubation, the sides of the flasks were gently tapped to ensure all of the cells were no longer adhered. In order to neutralize the trypsin,
growth medium (12.5 mL) was added to each flask. The media from all three flasks was then pooled in one 50 mL conical vial, and centrifuged for 5 min at 1,000 rpm to pellet the cells. The supernatant was aspirated, and the cells were resuspended in cold PBS (15 mL). The cells were then centrifuged again for 5 min at 1,000 rpm, and the previous step was repeated.

The cell pellet was resuspended in 100 ng/mL of SLO (Sigma-Aldrich) in HBSS (VWR, without calcium, magnesium, or phenol red) and incubated at 4 °C for 30 min. After incubation, the cells were centrifuged for 5 min at 1,000 rpm, and resuspended in cold HBSS (8 mL). The solution was then split into two tubes and washed once more with cold HBSS. Once the cells had been washed, one aliquot was resuspended in 2.5 mg/mL bovine serum albumin (2 mL) (BSA) in HBSS, and the other aliquot was resuspended in Dylight 488 labeled 2.5 mg/mL BSA in HBSS (2 mL). The cells were then incubated for 30 min in a rocker at 37 °C, after which both aliquots were washed twice with cold HBSS.

In order to seal the pores that SLO had opened in the cell membranes, both aliquots of cells were then incubated in 2 mM CaCl₂ in HBSS for 30 min at 4 °C in a rocker, after which they were washed once in cold HBSS. After washing, the cells were pelleted by centrifuging for 5 min at 1,000 rpm, and each aliquot was resuspended in cold HBSS buffer (2 mL) and split into two, 1 mL portions. This produced four total aliquots of cells, two that contained unlabeled BSA, and two that contained Dylight 488 labeled BSA. All four aliquots were then pelleted by
centrifuging for 5 min at 1,000 rpm one last time. Next, one aliquot of Dylight 488 labeled cells and one aliquot of unlabeled cells were resuspended in HBSS (300 μL). The remaining two aliquots of cells, one labeled and one unlabeled, were resuspended in 50 μg/mL propidium iodide (300 μL) (Life Technologies Corporation) in HBSS that had been previously passed through a 0.45 μm filter to remove any PI aggregates. This produced a total of four different samples shown in Figure 2.1.

![Flow chart for flow cytometry sample preparation.](image)

**Figure 2.1** Flow chart for flow cytometry sample preparation.

Samples were then immediately analyzed by using a BD LSRII flow cytometer. All data was processed using FlowJo.

### 2.2.3 Confocal microscopy

HeLa cells were grown in 35 mm non-coated glass bottom dishes (MatTek) to a confluence of about 60-70%. A SLO concentration of 100 ng/mL was utilized as
described previously. However, Dylight-488 labeled Aβ 1–40 (rPeptide) was introduced into HeLa cells instead of BSA. The labeled Aβ (50 μM) was then added to the HeLa cells, and the pH was carefully adjusted from approximately 8.5 to 7.3 using dilute trifluoroacetic acid (TFA) (10 mM). After adding the Aβ peptide, the rest of the SLO technique was completed as previously described.

Prior to imaging, the cells were also counter stained using CellMask™ Deep Red. CellMask™ Deep Red is a red fluorescent dye that stains the plasma membrane. The buffer covering the cells was aspirated and CellMask™ Deep Red 1X (300 μL) in PBS was added to the cells. The cells were then incubated for 5 min at 37 °C, after which they were washed again three times with PBS. Following the staining, approximately 1.5 mL of PBS was added to the dish to cover the cells and the dish was placed on ice.

Confocal imaging was performed at the Lerner Research Institute, Cleveland Clinic, using a Leica™ SP8 upright multiphoton confocal microscope. All images were acquired using a 40x objective that simultaneously acquired images for both Dylight-488 labeled Aβ and CellMask™ Deep Red labeled plasma membranes. Z-stack scans of the labeled cells were acquired in order to image the distribution of Dylight-488 labeled Aβ throughout the entire cell volume. Image processing was done using Leica™ LSX software.
2.3 Results

2.3.1 Flow Cytometry Results

Our flow cytometry results show that SLO at a concentration of 100 ng/mL was able to successfully introduce fluorescently-labeled protein into living HeLa cells. Of the four different samples produced when performing FCM (Figure 2.1), the most important is the sample labeled with Dylight 488 and PI. The results for this particular sample can be seen below. As can be seen on the left in Figure 2.2, the sample injected into the FCM had a total of 19,487 hits, of which 68.5% or 12,031 were intact cells. The remaining hits are likely mostly cellular debris from ruptured cells. The middle box shown in Figure 2.2 is populated only by the hits that were gated from the box on the left. This particular plot shows a breakdown of how many of the intact cells are negative for propidium iodide. Of the 12,031 cells that populate this plot, 57.6% or 6928 of them are negative for propidium iodide, which indicates that these cells are still alive. Finally, the last plot on the right of Figure 2.2 shows a breakdown of the cells that were gated as PI negative from the middle plot. This last result shows that 99.3% of the intact live cells are positive for Dylight 488.

In total, 6879 out of 12031 or 57.2% of the intact cells in the sample were PI negative and Dylight 488 positive. This indicates that fluorescently labeled BSA was introduced into these cells, the pores created by SLO in the cell membranes were successfully sealed using CaCl$_2$, and these cells were still alive at the end of the procedure.
**Figure 2.2** Flow cytometry results showing the ungated sample (left), intact cells (middle), and viable cells that are Dylight 488 positive (right) for PI labeled HeLa cells containing Dylight 488 labeled BSA. Out of a total of 19,487 hits, 68.5% of these hits (12,031) were intact cells, and 57.6% of the intact cells (6,928) were negative for PI. Finally, 99.3% (6,879) of these live cells were positive for Dylight 488.

### 2.3.2 Confocal microscopy results

The confocal microscopy images we acquired clearly show that Dylight-488 labeled Aβ is located throughout the cytoplasm of the adherent HeLa cells. In addition to acquiring individual images of cells containing Aβ, Z-stack scans were acquired in order to observe the distribution of Aβ throughout the entire cell volume. Maximum projection images were created in LSX, which projected each of the Z-stack images into one picture that shows the distribution of Aβ throughout the HeLa cells (Figure 2.3). This image shows that the labeled Aβ, which fluoresces green, is contained within the interior of the HeLa cells and is not adhering to the outer plasma membrane, which is labeled with the red fluorescent dye.
**Figure 2.3** Maximum projection image of Dylight-488 labeled Aβ in HeLa cells counterstained with CellMask™ Deep Red. Fluorescently labeled Aβ (green fluorescence) can be seen throughout the cytoplasm of the HeLa cells.
2.4 Discussion

Before we could begin the process of examining Aβ inside living cells using NMR, it was necessary to confirm that we could successfully introduce Aβ into cells while keeping the cells alive. BSA was used in place of Aβ in the initial FCM study in order to ensure that the SLO technique was properly executed. Additionally, Dylight-488 labeled Aβ is significantly more expensive than buying the dye on its own, and labeling BSA. BSA was chosen initially because if Aβ was used and the majority of the cells did not survive the procedure, it would be impossible to determine if the SLO technique had been carried out incorrectly, or that the Aβ peptide itself was responsible for cell death.

Previously, Dr. Fang Han from Dr. Zagorski’s laboratory had optimized the SLO procedure described by Dr. Ogino et al. for use in HeLa cells (118). Her results found that the optimal SLO concentration for HeLa cells is 100 ng/mL (Figure 2.4), a result repeated prior to building upon her work. The previous results of Dr. Han showed that at a SLO concentration of 100 ng/mL, about 45% of the cells were Dylight-488 positive and PI negative, indicating that this population of cells was still alive and contained fluorescently labeled protein. Furthermore, this same SLO technique was used by Ogino et al. as a means of introducing fluorescently labeled thymosin β4 in HEK 293F cells; an efficiency of around 40% of cells were Dylight-488 positive and PI negative (118).
Figure 2.4  Plot of data correlating SLO concentration to percentage of cells that were Dylight-488 positive and PI negative (dark bars) and percentage of cells that were Dylight-488 positive and PI positive (light bars). A SLO concentration of 100 ng/mL was found to be the optimal concentration. (Figure from thesis of Dr. Fang Han)

Given the results from prior studies, my result of 57.6% of cells that were Dylight-488 positive and PI negative is very favorable. It should be noted that in contrast with the study performed by Ogino et al. who used HEK 293F cells (118), HeLa cells, which are a more robust cell line, were used in this study. In addition, in comparison to the study carried out by Dr. Fang Han, a few alterations were made in
the methodology. The present study used HBSS, which contains glucose in solution as the buffer of choice, as opposed to Dubelco’s Phosphate Buffered Saline (PBS) which does not contain glucose, as was used in Dr. Han’s study. Previously published studies that used the SLO technique used HBSS (118, 128). Perhaps more importantly though, the length of time of each incubation step performed in my study was limited to 30 min, while in Dr. Han’s study some incubation steps lasted longer. In the case of the SLO incubation step in Dr. Han’s study, the cells were incubated with the toxin for a total of 1 hr and 45 min. It is possible that this extended incubation period could have reduced cell viability.

Confocal microscopy was used to visually confirm the presence of Dylight-488 labeled Aβ inside living cells. The cells were counterstained using CellMask™ Deep Red, which stains the outer plasma membrane, in order to confirm that the fluorescent Aβ was located in the cell cytoplasm and not adhering to the plasma membrane. Our confocal results definitively show that the SLO technique is able to uniformly introduce the Aβ peptide into living human cells. Fluorescently labeled Aβ is clearly visible throughout the cytoplasm, and not stuck to the outer plasma membrane. Combined, these flow cytometry and confocal results demonstrate that the SLO technique we optimized for use in HeLa cells is effective at introducing Aβ into cells that remain viable.
Chapter 3

IN-CELL NMR STUDIES OF THE Aβ PEPTIDE
3.1 Introduction

3.1.1 Heteronuclear single quantum coherence (HSQC)

In order to view intracellular Aβ, we used heteronuclear single quantum coherence (HSQC) NMR experiments. This type of NMR experiment directly detects protons that are bound to a NMR sensitive heteronucleus, which is typically either $^{13}$C or $^{15}$N (122,123). While both $^{14}$N and $^{15}$N are NMR active, proteins are labeled with $^{15}$N because the low natural abundance of $^{15}$N (0.37%) ensure that signals detected via NMR are originating from the labeled protein. When studying peptides and proteins, typically the first NMR experiment performed is a HSQC on uniformly $^{15}$N labeled proteins. For each amino acid in a protein there is a peptide bond that contains a proton directly bound to a nitrogen atom, with the exception of proline (123). The side chain amines of those amino acids, such as arginine, which contain this group can also be detected using $^{15}$N HSQC. Observing the amide protons of a protein’s backbone can provide valuable structural information about the protein. When a protein is folded, the peaks corresponding to these amide protons tend to be well resolved and dispersed throughout the spectrum. In contrast, proteins that do not have a definite structure, such as monomeric Aβ, tend to have the majority of the amide proton peaks close together (within a 2 ppm range) and not very well resolved (123,124,125). Small changes in the chemical environment can also lead to noticeable shifts in the HSQC spectrum of the protein. This fact can prove valuable
in the study of random coil proteins such as Aβ, because should the protein fold and gain a definite structure, HSQC NMR could easily track this change.

As stated previously, HSQC NMR directly detects protons that are bound to a heteronucleus such as $^{15}$N, which is indirectly detected by the NMR. This is achieved by first magnetizing the proton in a H - $^{15}$N bond, then transferring the magnetization to the insensitive $^{15}$N nucleus through what is called an insensitive nucleus enhanced by polarization transfer or INEPT step. Following the transfer of magnetization, a small amount of time passes, called $t_1$, after which a retro INEPT step occurs, which transfers the magnetization back to the proton (Figure 3.1). After this step, the protons are allowed to relax, and the FID is recorded. Contained within the FID, is information about the chemical shift of the protons (T2 dimension) as well as the $^{15}$N atoms (T1 dimension) (122,123)

![Figure 3.1 Example of a $^{15}$N HSQC pulse sequence. Magnetization is transferred from protons to $^{15}$N via an INEPT step. After a time delay, or $t_1$, the magnetization is passed back to the protons via a retro INEPT step, and the FID is recorded.](http://www.cryst.bbk.ac.uk/PPS2/projects/schirra/html/2dnmr.htm)
3.1.2 In-cell NMR

Since the invention of NMR in 1945, the magnetic field strengths of the machines have steadily increased, drastically improving the sensitivity and resolution of NMR experiments. NMR has been used to study proteins and nucleotides since the 1960s, with the first protein solution structure solved in 1984 by Wüthrich et al. (126,127). Today, NMR is a powerful tool used in conjunction with X-ray crystallography and molecular modeling programs, to solve the solution structures of many different proteins, DNA, and RNA. However, physiological or in vivo conditions, such as the environment of living cells, is vastly different from solution, or in vitro conditions, in which proteins are typically studied using NMR. The cytosol of a cell is a crowded environment that contains a variety of macromolecules such as siRNAs, proteins, dNTPs etc. all of which can have transient interactions with a peptide such as Aβ and potentially alter its structure. Thus, to get a more accurate structural representation of a protein using NMR, it must be observed in its native intracellular environment.

In-cell NMR is a fairly new NMR technique, first described in 2005 (128), which has become a valuable tool in understanding proteins in their native environment. The initial in-cell NMR experiments observed proteins in E. coli, but more recent studies have expanded the technique to eukaryotic cells (118,129). Using this technique, intrinsically disordered proteins such as FlgM, a peptide only twice the size of Aβ that originates from Salmonella typhimurium, have been
observed to fold and gain structure when inside a cell (130). In-cell NMR has also been used to observe α-synuclein, the protein responsible for causing Parkinson’s disease. Like Aβ, α-synuclein is an intrinsically disordered protein that forms amyloid aggregates in neurons (131). Controversy over the structure of this protein arose when a study in 2011 suggested that in reality α-synuclein formed helical tetramers in cells, and that it was not an intrinsically disordered protein (132). The authors of the study reasoned that the established view that α-synuclein is disordered is an artifact of harsh protein isolation methods and therefore does not reflect its native structure (132). However, a subsequent study in which in-cell NMR was able to view intracellular α-synuclein proved that the native form of the protein is in fact disordered (133). These studies show how effective a tool in-cell NMR can be on gleaning structural information regarding intracellular proteins and peptides.

3.1.3 Molecular crowding

The interiors of living cells are very crowded environments. Contained within the cytoplasm are countless proteins, molecules of RNA and DNA of varying sizes, as well as a complex cytoskeleton. As an example, a single *E. coli* bacterium can contain over 4000 different proteins, in a volume smaller than a eukaryotic cell (134). One of the effects of molecular crowding is an effective increase in the concentration of a given protein due to a smaller volume of cytosol in relation to total cell volume, available as a solvent (135,136). Larger molecules experience greater effects from molecular crowding (135,136); for example α-synuclein would
be affected by molecular crowding more than Aβ is, given that α-synuclein is about three times larger. The crowded cytosol can have a significant influence over the folding of proteins (135,136). Protein misfolding and aggregation may also be the result of molecular crowding (136,137).

In addition to the effect molecular crowding can have on protein folding, it can also present significant challenges in relation to the observation of intracellular proteins using NMR. The crowded environment of the cell cytosol causes molecules in solution to tumble at a slower rate (135,136,137). The rate at which a molecule “tumbles” in solution, is known as its rotational correlation time. Shorter rotational correlation times, i.e. a faster tumble rate, result in longer T2 relaxation times which lead to sharper NMR peaks. Conversely, if a molecule has a long rotational correlation time due to molecular crowding, the T2 relaxation time of the molecule will be much shorter resulting in broad, poorly resolved NMR peaks (138). Despite the challenge molecular crowding presents, it is still possible to obtain well resolved in-cell NMR spectra (118,124,125,129).

3.2 Materials and Methods

3.2.1 Aβ 1-40 peptide preparation

The Aβ stock solution was prepared by first adding NaOH (1 mL, 10 mM) to a vial containing 1 mg of uniformly $^{15}$N labeled Aβ 1-40 (rPeptide, Cat # A-1101-2),
giving a 213 μM solution. This vial was capped and sonicated for 10 minutes to ensure all Aβ had been dissolved, and any potential aggregates were broken up. A working solution was prepared by diluting the Aβ stock solution (118 μL) in phosphate buffer (332 μL, 20 mM) with D₂O (50 μL) added so that the NMR would be able to lock. The pH of the working solution was adjusted to 7.3 using dilute TFA (10 mM), and the solution was transferred to a 5 mm Shigemi NMR tube.

The NMR experiment was performed on a Bruker 900 MHz spectrometer equipped with a high-sensitivity cyroprobe. The sample temperature was maintained at 8 °C. ¹H-¹⁵N HSQC data was acquired with 1024 points in the direct dimension and 48 points in the indirect dimension. The HSQC experiment was run for 22 hours, and the H₂O signal was suppressed by coherence selection using pulse field gradients. Spectra were processed by zero-filling the indirectly detected dimension into 256 points before Fourier transformation. Square sine-bell window function with π/2 shift was applied for processing the FID in both dimensions. To increase the signal to noise ratio, a 10 Hz exponential multiplication window function was applied in the F2 dimension.

3.2.2 Incubated Aβ 1-40

Subsequent in-cell NMR experiments require incubation periods at 37 °C, which could potentially cause Aβ to aggregate. Thus it was necessary to examine the effect of this incubation period on Aβ. First, the Aβ stock solution (118 μL, 213
μM) was diluted in phosphate buffer (332 μL, 20 mM) with D₂O (50 μL) in a microcentrifuge tube. The pH of this solution was adjusted to 7.3 using TFA (10 mM). The solution was then placed in a 37 °C incubator for 1.5 hr with rocking, which equals the total amount of time cells were incubated with Aβ in a subsequent in-cell NMR experiment. After incubation, the solution was transferred to a 5 mm Shigemi NMR tube, and a ¹H-¹⁵N HSQC was performed as described in the previous section.

### 3.2.3 SLO titration experiment

HeLa cells were grown in three 150 cm² flasks as described previously. These cells were trypsinized and washed twice with cold PBS. After the cells had been washed twice, they were resuspended in cold HBSS (4 mL) to which SLO (400 μL, 4 μg/mL) was added, giving a 100 ng/mL final SLO concentration. The cells were then placed on a rocker in a 4 °C cold room for 30 min. Two washes using cold HBSS (10 mL) were then performed. The cells were then resuspended in cold phosphate buffer (766 μL, 20 mM) with uniformly labeled ¹⁵N Aβ 1-40 stock (234 μL, 213 μM) giving a 50 μM Aβ concentration. The pH was adjusted to 7.3 using TFA (10 mM). This tube was incubated on a rocker at 37 °C for 30 min. After this incubation, an additional amount of the uniformly labeled ¹⁵N Aβ 1-40 stock (125 μL, 213 μM) was added, which raised the Aβ concentration to 75 μM, and the tube was once again placed on a rocker at 37 °C for 30 min. Once this incubation period finished, a final aliquot of uniformly labeled ¹⁵N Aβ 1-40 stock (150 μL, 213 μM) was
added to give a final Aβ concentration of 100 μM, and the tube was incubated one final time at 37 °C for 30 min with rocking.

Upon completion of the incubation, the cells were washed twice with cold phosphate buffer (20 mM). To seal the pores that SLO had opened in the cell membranes, the cells were then incubated in phosphate buffer (20 mM) containing CaCl₂ (2 mM) for 1 hr at 4 °C on a rocker, after which they were washed twice in cold phosphate buffer. Once the pores in the cell membranes were sealed, the cells were resuspended in cold phosphate buffer (315 μL, 20 mM), with added D₂O (35 μL). The cells were then placed in a 5 mm Shigemi NMR tube, and a ¹H-¹⁵N HSQC was performed as described previously.

3.2.4 Molecular crowding experiment

A 50% w/v PEG stock solution was prepared by dissolving polyethylene glycol (PEG) 6000 (Sigma-Aldrich) in Milli-Q water. Three different Aβ 1-40 solutions with final PEG concentrations of 10%, 20%, and 30% respectively, were prepared. For each solution, the final concentration of Aβ was 50 μM of uniformly ¹⁵N labeled Aβ 1-40 peptide in deuterated phosphate buffer (20 mM). The pH of each solution was adjusted to 7.3 using TFA (10 mM), and each solution was placed in 5 mm Shigemi NMR tubes. Given the length of each NMR experiment (24 hr), the three samples were prepared and analyzed on different days. The experiments were performed on a Bruker 900 MHz spectrometer equipped with a high-
sensitivity cyroprobe. The sample temperature was maintained at 8 °C. $^1$H-$^{15}$N HSQC data was acquired with 1024 points in the direct dimension and 48 points in the indirect dimension. The HSQC experiment was run for 22 hours, and the H$_2$O signal was suppressed by coherence selection using pulse field gradients. Spectra were processed by zero-filling the indirectly detected dimension into 256 points before Fourier transformation. Square sine-bell window function with $\pi/2$ shift was applied for processing the FID in both dimensions. To increase the signal to noise ratio, a 10 Hz exponential multiplication window function was applied.

3.3. Results

3.3.1 In-cell NMR

The control spectra for Aβ can be seen in Figure 3.2, however given that the methods for the SLO introduction of Aβ peptide into living cells involves a lengthy incubation at 37 °C which could promote Aβ aggregation, this does not provide an adequate control. To address this, in an additional experiment Aβ peptide was exposed to the same 1.5 hr incubation at 37 °C as was used in the SLO experiment. The resulting spectrum, which can be seen in Figure 3.3, show slightly more noise than can be observed in Figure 3.2, when Aβ was not incubated. However, the spectrum is still very clean with well resolved peaks throughout its entirety.
Figure 3.2 HSQC of 50 μM Aβ 1-40. Each blue peak is representative on a proton directly bound to a $^{15}$N atom.

A comparison between incubated and non-incubated Aβ can be seen in Figure 3.4. This overlay shows that every amino acid visible in the original Aβ control spectrum is still present when Aβ was incubated. The only differences between the two are the signals for Ser-8 and Lys-16, which have both shifted upfield in the proton dimension. These differences are minor, and all future
comparison spectra will be using the incubated Aβ control, to ensure that the peptide is being subjected to identical conditions.

**Figure 3.3** HSQC of 50 μM Aβ 1-40 that was aged for 1.5 hr at 37 °C.
Figure 3.4 Aβ 1-40 controls HSQC overlay. Aβ that was not incubated can be seen in blue, and Aβ that was incubated for 1.5 hr at 37 °C can be seen in black. The notable differences can be seen with Ser-8 and Lys-16, which have both shifted upfield slightly in the incubated spectrum.
3.3.2 SLO experiment

Intracellular Aβ peptide was successfully observed inside living cells using $^{15}$N HSQC NMR, as can be seen in Figure 3.5.

**Figure 3.5** HSQC spectrum of intracellular Aβ 1-40 observed inside living HeLa cells.
Figure 3.6 Intracellular Aβ 1-40 (blue) overlaid on Aβ 1-40 control (grey). Identifiable intracellular peaks have been labeled.

As can be seen in Figure 3.6, intracellular Aβ shows significant loss of signals, with only 9 out of the 40 amino acids identifiable, as well as signal broadening, which can be seen primarily in the region of Asp-23, Lys-28, and Met-35, in addition to Leu-17. Despite lowering the contour levels, the missing signals were not detected. In addition, to these nine backbone signals, the side-chain peaks for Arg-5 are also barely detectable. Of the nine signals observed in Figure 3.7, six are located
in the hydrophobic second half of the peptide, and five of the nine are hydrophobic amino acids.

The weak peaks that have been identified as Phe-20 and Val-36, have shifted slightly downfield in the proton dimension. At lower contour levels the peaks for Phe-20 and Val-36 become more prominent (Figure 3.7), however more noise begins to appear in regions of the spectra where amino acid signals in the control spectra are not located.
Figure 3.7 Expanded contour plot showing where the main body of the Aβ peptide is located, and at a lower contour level. The signals for Val-36 and Phe-20 are more prominent at this contour level. Weak signals for Arg-5, and Glu-11 are also present.

The signals for Arg-5 and Glu-11, which were not seen in Figure 3.6, are also weakly visible at a lower contour level in Figure 3.7.
3.3.3 Molecular crowding

The cytosol of living cells is a much more crowded environment than a pure aqueous solution. Previous studies have shown that small molecules diffuse about four times slower in the cytosol in comparison to water given how crowded the interior of a cell is (135). In these experiments, PEG 6000 was used as a molecular crowding agent to simulate a crowded cell cytosol to explore if a more crowded solution had an effect on the Aβ peptide. When in solution with 10% and 20% w/v PEG, Aβ was affected by the increased viscosity of the solution.

Figure 3.8 50 μM Aβ control (black) vs. 50 μM Aβ in 10% w/v PEG (red).
At a PEG concentration of 10% w/v, the signals for Aβ are broadened in comparison to the control Aβ spectrum (Figure 3.8). While the Aβ signals have clearly broadened, no significant shifts in any of the peaks are seen. Two signals in the control spectrum, Asp-7, and Phe-19, are no longer visible in the 10% PEG spectrum. Met-35 is also not clearly identifiable in the 10% PEG spectrum, but it is possible that its signal broadened enough to merge with other nearby Aβ signals.

Figure 3.8 50 µM Aβ control (black) vs. 50 µM Aβ in 20% w/v PEG (red)

In comparison to the 10% PEG solution, the presence of 20% w/v PEG had a greater effect on Aβ (Figure 3.9). Nearly all of the signals for Aβ in the above spectrum have shifted to some degree. As was observed in the 10% PEG spectrum,
two signals Asp-7, and Phe-19, are no longer visible in the 20% PEG spectrum.

Additionally, this spectrum is noisier in comparison to the 10% PEG spectrum as can be seen in the streaking at 111 ppm in the $^{15}$N dimension, owing to the higher PEG concentration.

**Figure 3.10** 50 μM Aβ in 30% w/v PEG. No identifiable Aβ signals.

The final concentration of PEG tested proved to be too high, as no signals for Aβ can be observed in Figure 3.10.
3.4 Discussion

Our results show the first observation of intracellular Aβ peptide inside living human cells using NMR. Previous in-cell NMR results have only shown much more stable proteins such as alpha-synuclein and TTHA1718 (125,133). Observing intracellular Aβ provided significant challenges, given that the peptide can aggregate at higher concentrations and temperatures, as well as physiological pH. However, given these difficulties, we were able to optimize the conditions to observe intracellular Aβ using NMR.

3.4.1 Incubated Aβ

Initially an HSQC spectrum of un-aged Aβ 1–40 was acquired to be used as a control in subsequent comparisons of in-cell NMR experiments. However, after troubleshooting and optimizing the SLO technique to introduce Aβ into living cells, it became apparent that the effects of aging Aβ at 37 °C must be observed, given that this step was necessary to introduce Aβ into HeLa cells. Elevated temperatures accelerate Aβ aggregation, thus it was necessary to take this additional step of aging a new Aβ control to be able to rule out the 37 °C incubation period as solely responsible for any changes observed for intracellular Aβ. After aging uniformly $^{15}$N labeled Aβ for 1.5 hr at 37 °C and obtaining a HSQC spectrum, when compared to a control Aβ spectrum, it was observed that this incubation period had little effect on
Aβ (Figure 3.5). The spectrum obtained from this experiment (Figure 3.4) was subsequently used as the reference spectrum for all future experiments.

3.4.2 In-cell Aβ

Once it was confirmed that incubation of Aβ for 1.5 hr, a step necessary for the SLO technique, does not have an effect on an HSQC of Aβ, uniformly $^{15}$N labeled Aβ was introduced into living HeLa cells. Previously our group had shown that HeLa cells containing Aβ introduced via the SLO technique remain viable for a period of 24 hr when maintained at 8 °C, which is the length of time and temperature that a sample would experience when HSQCs are performed. In-cell NMR experiments were run several times to confirm that initial results were repeatable, as well as to optimize conditions to maximize the observed signal to noise. Initial in-cell experiments introduced Aβ into SLO treated HeLa cells at a concentration of 50 μM. This concentration is the standard concentration that the Zagorski group works with Aβ given the Aβ peptide propensity to aggregate at higher concentrations.

After several attempts at a concentration of 50 μM that produced reproducible results, it was decided to devise a way to introduce more Aβ into the HeLa cells. The final method that was settled upon was to titrate Aβ into a sample of SLO treated HeLa cells. Over the course of the incubation period the concentration of Aβ that the HeLa cells were exposed to rose from 50 μM to 100 μM. The objective of titrating Aβ into solution over the course of the incubation was to drive more Aβ
to enter the HeLa cells, while preventing the concentration of extracellular Aβ in solution from becoming high enough to cause the peptide to aggregate and kill the cells.

This titration experiment proved to be successful, significant loss of signal was observed for intracellular Aβ, with only nine of the forty amino acids identifiable (Figure 3.7). When the contour layer of the spectrum was lowered as shown in Figure 3.8, two additional signals became identifiable, raising the total to eleven amino acids identified. In addition to the loss of the majority of the signals for Aβ, significant broadening was observed in the region of the spectrum where Asp-23, Lys-28 and Met-35 are located, and the signals for Phe-20 and Val-36 shifted downfield in the proton dimension. It is important to note that six of the identifiable amino acids are located in the largely hydrophobic tail of the Aβ peptide. While the majority of the amino acid signals were no longer visible, this result provides a valuable insight into what is occurring within the cell with Aβ. If the Aβ was free and monomeric in the cytoplasm, the resulting NMR spectrum would look similar to that of the control. Conversely, if Aβ were to fold and adopt a structure when in its native environment, one would expect the group of amino acids signals found in the middle of the control spectrum to spread out. However, neither of these possibilities occurred, which indicates that one of two alternative outcomes for Aβ is occurring. As previously discussed, the cytoplasm of living cells is a crowded environment, in which non-specific binding interactions slow the tumbling rate of cytosolic proteins, resulting in NMR signal broadening. The other possibility is that
Aβ is interacting and potentially binding to a specific intracellular target. Additional experimentation is required to determine if non-specific or specific binding interactions are the cause for the observed loss of signals.

3.4.3 Molecular crowding

The objective of the molecular crowding experiments was to determine if the crowded cytosol of a living cell could be the cause of the previously described in-cell NMR results. Three different concentrations of PEG 6000 were prepared to simulate successively more crowded molecular environments. As Figure 3.9 demonstrated, when Aβ was in solution with 10% w/v PEG, the more crowded molecular environment had a minimal effect on Aβ HSQC signals. Slight shifts in the Aβ signals are observable, but given the minimal amount of signal shifting it is clear that the Aβ peptide had not adopted a different secondary structure. The Aβ signals had also broadened as expected due to the slower tumbling rate of the Aβ peptide in the more viscous 10% PEG solution. In comparison to the control all but three of the Aβ peptide’s signals are easily identifiable, which is in contrast to the drastic amount of signal loss seen for intracellular Aβ.

The molecular crowding results for Aβ with 20% PEG 6000 were similar to those when Aβ was in solution with 10% w/v PEG. Shifts in the signals for Aβ were once again observed (Figure 3.10), but to a larger degree this time. However, while the signal shifts were more dramatic, it does not appear that Aβ has lost its random
coil secondary structure. Again, some loss of signal was observed with two signals no longer present in comparison to the control, but it does not compare to the loss of the majority of signals seen for intracellular Aβ. Some difficulty arose when attempting to acquire HSQC spectra for Aβ with 20% PEG, owing to the higher concentration of PEG in this sample. These difficulties manifest themselves in the streaks of noise seen at 111 ppm and to a lesser extent 128 ppm in the $^{15}$N dimension. Extensive measures were taken to maximize the signal to noise ratio, but it proved impossible to completely eliminate the noise from PEG.

Finally, an experiment was attempted with 30% w/v PEG 6000. Unfortunately, this concentration proved to be too high to be able to acquire readable spectra. Multiple attempts at this experiment were made, with efforts aimed at trying to suppress the high amounts of noise coming from PEG, but they were unsuccessful.

These results taken together prove that results observed for intracellular Aβ cannot be explained to be due to the crowded molecular environment found in the cytosol of a living cell. Instead, these molecular crowding results suggest that the observed intracellular Aβ results are likely due to the Aβ peptide interacting with some intracellular target.
Chapter 4

CELL ORGANELLE ISOLATION AND
INTERACTION WITH Aβ PEPTIDE
4.1 Introduction

Recently, an increasing amount of AD research has focused on the intraneuronal accumulation of the Aβ peptide, and how this affects disease pathology. This area of research began when monoclonal antibodies were designed to target Aβ peptides immuno-labeled individual neurons in cortical brain slices of AD patients, in addition to extracellular plaques (66,85,139). At the time these findings were published, Aβ was thought to only accumulate in extracellular plaques and vessels inside the brain. Initially this finding was controversial because the monoclonal antibody that immuno-labeled intraneuronal Aβ was specifically targeted to the 17 – 24 amino acid region of the Aβ peptide, can also be found in APP (139). Critics of the hypothesis pointed out that this method could not distinguish between possible intraneuronal Aβ and APP, which is far more abundant in the brain. In order to address this issue, new antibodies were developed that only recognized the C-terminus of Aβ 1 – 40 and 1 – 42 (85, 140). These antibodies had the ability to distinguish Aβ peptides from APP, because they relied on the presence of the C-terminus, in addition to a sequence of amino acids in Aβ.

Once acceptance of the intracellular Aβ hypothesis became accepted, investigators saw the implications that intracellular Aβ can have on the progression of AD. As was discussed previously, the cytosol is a crowded environment that effectively raises the concentration of a given macromolecule, and Aβ becomes increasingly aggregation prone as its concentration increases. Additionally, the
intracellular concentration of metal ions, which promote Aβ aggregation, is higher than that found in plasma. Finally, the pH of different organelles such as lysosomes can be lower than the typical physiological pH of 7.3, yet another important factor that can lead to Aβ aggregation (141).

4.1.1 Aβ located in multivesicular bodies

Following the earlier studies that found evidence of intracellular Aβ, subsequent efforts have focused on trying to locate specific regions inside cells where Aβ resides. In 2002, immunoelectron microscopy (immune-EM) was used to directly observe Aβ 1 – 42 contained in endosomal multivesicular bodies (MVBs) (142). Not only was Aβ observed in neurons originating in transgenic mice and rats, but it was also observed in MVBs from human AD patients (142). Furthermore, this study was unable to detect intracellular Aβ in the brains of APP knockout mice, establishing that the results were not false positives. Subsequent immuno-EM and fluorescent microscopy studies have found MVBs that contained Aβ adjacent to mitochondria, and even within the mitochondrial cristae (143). The mitochondria play a role in processing many proteins, although not APP (144,145,146). Instead, it is theorized that Aβ is trafficked to mitochondria from MVBs, where the peptide can then wreak havoc on the cell’s metabolism (147,148,149).
4.1.2 Mitochondrial dysfunction in relation to Aβ

The mitochondria in neurons of patients with AD show signs of dysfunction when compared to those of age-matched control patients without AD (147,149). In AD patients, a decrease in the membrane potential of neuronal mitochondria is observed, along with a reduction in oxygen consumption and an increase in reactive oxygen species (ROS) that leads to oxidative stress (150). Mitochondrial dysfunction has also been connected to the phosphorylation of tau, the other protein that is connected to AD, and is the major component in neurofibrillar tangles (151,152). While there is a clear connection between mitochondria dysfunction and AD, it is not yet known whether Aβ accumulation leads to mitochondrial dysfunction or if, in fact, the inverse is true.

4.1.3 Aβ located near cell nuclei

While the majority of research on intracellular Aβ has identified MVBs and mitochondria as locations for Aβ accumulation, the Aβ peptide has also been observed close to, and even within the nucleus. Studies performed by the Glabe laboratory using a fluorescently labeled antibody called M78 that stains fibrillar proteins forming parallel β-sheets, such as Aβ, have shown nuclear and perinuclear staining of cortical brain slices of both transgenic mice, and more importantly, human subjects with AD pathology (153,154). Interestingly, it was discovered that M78 primarily stained the nuclear regions of neurons from patients with mild
cognitive impairment (MCI), and that as the AD pathology progressed, the amount of M78 staining decreased (153). This result suggests that the association of Aβ with the nucleus of neurons could play a role in the early stages of the disease. Neurons that were positive for M78 had abnormal pathology as well; their nuclear envelopes were swollen, and the DNA contained within had been fragmented (153). Additionally, extracellular plaques co-stained for M78 and DAPI, a nuclear stain, suggesting that plaques in AD brains are the result of the death of individual neurons. Upon rupturing, neurons laden with Aβ would release their contents forming the core of a plaque, and releasing neurotoxic Aβ oligomers into the extracellular space, which could then go on to damage surrounding neurons (155).

4.1.4 Isolation of Organelles via Differential Centrifugation

The accumulation of Aβ in and around cell organelles may play a vital role in the progression of AD. Our in-cell NMR results demonstrated that intracellular Aβ peptide is potentially interacting with some target within the cell. We chose to examine nuclei and mitochondria as potential targets for Aβ. In order to study the interaction between Aβ and nuclei and mitochondria individually, we utilized differential centrifugation to isolate the organelles. Differential centrifugation is a technique that is used to isolate specific cell organelles based upon their densities. This is accomplished by first suspending cells in a hypotonic buffer which causes the cells to swell followed by lysing them using a homogenizer. The cell lysate is then placed in a centrifuge, and spun at a relatively low speed to remove cellular debris.
The supernatant is then also centrifuged, this time at a higher g-force in order to pellet nuclei. If the objective is to isolate smaller organelles such as mitochondria, these steps are then repeated using progressively higher g-forces, until the desired organelle is isolated. Once the desired organelle has been pelleted, the supernatant is extracted and discarded, leaving behind the isolated and purified organelle.

**Figure 4.1** Diagram depicting the steps of differential centrifugation. After each step in the process, progressively higher g forces are necessary to pellet organelles of lesser densities. Upon pelleting the desired organelle, the supernatant is extracted and discarded, allowing the organelle to be processed. (Thomasione)
4.2 Materials and Methods

4.2.1 Nuclei extraction and incubation with the Aβ peptide

HeLa cells were grown, trypsinized, and washed as previously described in Chapter 3. After the cells had been washed twice with cold PBS, the cells were resuspended in three times the volume of the packed cells (approximately 3 mL) in hypotonic buffer (10 mM HEPES, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT) and incubated on ice for 15 min. The cells were split into two equal volumes (in order to prevent overflowing in the homogenizer) and homogenized using a chilled glass-dounce homogenizer using 60 strokes. The cell lysates were pooled and centrifuged at 230 g (International Clinical Centrifuge Model No. 49845H, 213 rotor) for 10 min at 4 °C. Supernatant was discarded, and the nuclear pellet was resuspended in sucrose solution 1 (3 mL) (0.25 M sucrose, 10 mM MgCl$_2$) and laid over sucrose solution 2 (3 mL) (0.88 sucrose, 0.5 mM MgCl$_2$). Two distinct layers were observed at this point, and the sucrose gradient was centrifuged at 3000 g (International Clinical Centrifuge Model No. 49845H, 213 rotor) for 10 min at 4 °C. The resulting pellet was cell nuclei, and the supernatant was discarded.

Nuclei were resuspended in phosphate buffer (268 μL, 20 mM), to which uniformly $^{15}$N labeled 1-40 Aβ peptide (82 μL, 213 μM) was added, giving a final peptide concentration of 50 μM. The pH was adjusted to 7.3 using dilute TFA, and the nuclei were incubated at 37 °C for 20 min. The nuclei were then centrifuged at
3000 g for 10 min at 4 °C, and the supernatant was discarded. The nuclei were then resuspended in cold PBS (1 mL), then centrifuged again at 3000 g for 10 min at 4 °C. This washing step was repeated one more time. After washing, the nuclei were resuspended in phosphate buffer (450 μL, 20 mM) with D₂O (50 μL). The pH was adjusted again to 7.3 using TFA (10 mM), and the solution was transferred to a 5 mm Shigemi NMR tube. The experiments were performed on a Bruker 900 MHz spectrometer equipped with a high-sensitivity cyroprobe. The sample temperature was maintained at 8 °C. ¹H-¹⁵N HSQC data were acquired with 1024 points in the direct dimension and 48 points in the indirect dimension. Data for the HSQC experiment were acquired in 22 hours, and the H₂O signal was suppressed by coherence selection using pulse field gradients. Spectra were processed by zero-filling the indirectly detected dimension into 256 points before Fourier transformation. Square sine-bell window function with π/2 shift was applied for processing the FID in the F1 dimension. To increase the signal to noise ratio, a 10 Hz exponential multiplication window function was applied in the F2 dimension.

In order to confirm that any observed signals were the result of Aβ peptide bound to nuclei, and not free Aβ in solution, immediately after the HSQC was completed, the solution was placed in a 1.5 mL centrifuge tube, and centrifuged at 3000 g for 10 min at 4 °C. The supernatant was placed in a clean 5 mm Shigemi NMR tube and the above HSQC experiment was repeated.
4.2.2 Mitochondria extraction and incubation with Aβ peptide

HeLa cells were grown in three 150 cm² canted neck flasks with vented caps as previously described and pooled in PBS. Mitochondria extraction was performed using a mitochondria isolation kit for cultured cells (Thermo Scientific). The pooled cells from the previous step were resuspended in mitochondria isolation reagent A (800 μL), briefly vortexed and then put on ice for 2 min. The cells were then placed in a glass dounce homogenizer, and homogenized using 60 strokes while keeping the homogenizer on ice. To the homogenizer, mitochondria isolation reagent C (800 μL) was added, and the solution was transferred to a micro centrifuge tube. The homogenizer was washed with reagent A (200 μL), and pooled with the cell lysate. The cell lysate was then centrifuged at 700 g for 10 min at 4 °C in order to remove cellular debris. The supernatant was transferred to a new micro centrifuge tube and centrifuged at 5,000 g for 15 min at 4 °C. After centrifugation, the supernatant was discarded, the pellet was resuspended in reagent C (500 μL), and centrifuged one last time at 12,000 g for 7 min at 4 °C; the resulting pellet is the mitochondria. Supernatant was discarded, and the pellet was resuspended in cold phosphate buffer (383 μL, 20 mM) with uniformly $^{15}$N labeled 1-40 Aβ peptide (117 μL, 213 μM) to give a final Aβ concentration of 50 μM. The pH was adjusted to 7.3 using dilute TFA, and the solution was placed in a 37 °C rocking incubator for 1 hr. After the incubation was complete, the solution was centrifuged at 12,000 g for 7 min at 4 °C. The pellet was washed twice using cold phosphate buffer (20 mM), using the previous centrifugation step each time. Given the small size of the final
mitochondria pellet, the mitochondria were resuspended in only 250 μL of phosphate buffer (20 mM) with 10% D₂O instead of the usual 500 μL. This smaller volume necessitated the use of a smaller 3 mm NMR tube (Norell Select Series 3 mm), instead of the usual Shigemi 5 mm NMR tubes. The 3 mm NMR tubes allow a smaller volume of sample to cover the entirety of the NMR probe coils. An adapter was used to make the 3 mm tubes compatible with the spinner in the 900 MHz NMR (Norell, Optimizer insert for spinner turbines). The experiments were performed as previously described on a Bruker 900 MHz spectrometer equipped with a high-sensitivity cyroprobe.

In order to confirm that any observed signals were the result of Aβ peptide bound to nuclei, and not free Aβ in solution, immediately after the above HSQC was completed the solution was placed in a 1.5 mL centrifuge tube, and centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was placed in a clean 3 mm NMR tube and the above HSQC experiment was repeated.

4.2.3 Mutant peptide production and stock preparation

Following our nuclei and mitochondria results, we decided to design an experiment which would observe the effect a point mutation in the Leu-17 to Ala-21 sequence of Aβ had on the peptide’s ability to bind to cellular organelles. For this experiment, we contracted Dr. Smarajit Bandyopadhyay of the Molecular Biotechnology Core at the Lerner Research Institute to produce a mutant version of
Aβ 1 – 40. The mutant that we had prepared substituted Ala for Phe-19. In an effort to minimize the cost of the mutant F19A peptide, only the sequence from Leu-17 to Ala-21 was $^{15}$N labeled (Figure 4.2).

DAEFRHDSGYEVHHQKLVAFAEDVGSNKGAIIGLMVGGVV

**Figure 4.2** Sequence of mutant F19A Aβ 1 – 40. Alanine was substituted for phenylalanine-19. The amino acids highlighted in red were $^{15}$N labeled.

A stock solution (213 μM) for the mutant F19A Aβ peptide was prepared by dissolving the Aβ peptide (1 mg) in NaOH (1 mL, 10 mM). This stock solution was sonicated for 10 min in order to break up and aggregate seeds that may be present. Next, a control sample was prepared by combing the mutant F19A Aβ peptide stock solution (117 μL, 213 μM) with phosphate buffer (333 μL, 20 mM) and D$_2$O (50 μL), giving a final Aβ concentration of 50 μM. The pH was adjusted to 7.3 using TFA (10 mM), and the sample was then placed in a 5 mM Shigemi Tube. An HSQC was acquired on a Bruker 900 MHz spectrometer equipped with a high-sensitivity cyroprobe as previously described.

**4.2.4 Nuclei extraction and incubation with mutant peptide**

Mitochondria were isolated from HeLa as previously described. The isolated mitochondria were then resuspended in phosphate buffer (283 μL, 20 mM), to which uniformly $^{15}$N labeled mutant F19A Aβ peptide (82 μL, 213 μM) was added, giving a
final peptide concentration of 50 μM. The pH was adjusted to 7.3 using dilute TFA, and the nuclei were incubated at 37 °C for 20 min. The nuclei were then centrifuged at 3000 g for 10 min at 4 °C, and the supernatant was discarded. The nuclei were then resuspended in cold PBS (1 mL), then centrifuged again at 3000 g for 10 min at 4 °C. This washing step was repeated on more time. After washing, the nuclei were resuspended in deuterated phosphate buffer (250 μL, 20 mM). The pH was adjusted again to 7.3 using deuterated TFA (10 mM), and the solution was transferred to a 3 mm Norell NMR tube. An adapter was used to make the 3 mm tubes compatible with the spinner in the 900 MHz NMR. An HSQC was acquired on a Bruker 900 MHz spectrometer equipped with a high-sensitivity cyroprobe as previously described.

4.2.5 Mitochondria extraction and incubation with mutant peptide

Mitochondria were isolated from HeLa as previously described. The isolated mitochondria were then resuspended in phosphate buffer (283 μL, 20 mM), to which uniformly 15N labeled mutant F19A Aβ peptide (82 μL, 213 μM) was added, giving a final peptide concentration of 50 μM. The pH was adjusted to 7.3 using dilute TFA, and the mitochondria were incubated at 37 °C for 20 min. The mitochondria were then centrifuged at 12,000 g for 7 minutes at 4 °C, and the supernatant was discarded. The mitochondria were then resuspended in cold PBS (1 mL), then centrifuged again at 12,000 g for 7 minutes at 4 °C. This washing step was repeated on more time. After washing, the mitochondria were resuspended in deuterated phosphate buffer (250 μL, 20 mM). The pH was adjusted again to 7.3 using
deuterated TFA (10 mM), and the solution was transferred to a 3 mm Norell NMR tube. An adapter was used to make the 3 mm tubes compatible with the spinner in the 900 MHz NMR. An HSQC was acquired on a Bruker 900 MHz spectrometer equipped with a high-sensitivity cyroprobe as previously described.

4.3 Results

4.3.1 Nuclei Results

The HSQC spectra for isolated nuclei incubated with Aβ 1-40 is shown in Figure 4.3. The contour levels were lowered to the point where noise started to appear, however, it is obvious that some signals have broadened and are no longer visible.
To observe the full effect the isolated nuclei had on the Aβ signals, the HSQC spectrum from the isolated nuclei was overlayed on the control Aβ 1-40 spectra. As Figure 4.4 shows, several Aβ signals bound to nuclei have either shifted, broadened, or disappeared when compared to the control. Three signals, Ser-8, Leu-17, and Glu-22 are no longer visible in the nuclei spectrum. Signals that have shifted include
Arg-5, Lys-16, and Val-18. Finally, Glu-3, Arg-5, Tyr-10, Val-18, Ala-21, Gly-25, and Asn-27 all had their signal intensities significantly diminished, and Phe-4, Glu-11, Asp-23, Lys-28, and Met-35 experienced significant signal broadening. Additionally, the Arg-5 side chain showed extra peaks not present in the control spectrum.
Figure 4.4 HSQC of Aβ 1-40 w/ nuclei (red signals) overlaid on control spectrum of Aβ 1 – 40 (black signals). Signals that are either missing or shifted in the Aβ 1-40 w/ nuclei spectrum are labeled. The contour levels were brought down in both spectra to the point where noise started to appear in an effort to observe any additional signals in the Aβ 1-40 w/ nuclei spectrum.
After the experiment with isolated nuclei and Aβ 1-40 was performed, the sample was centrifuged and an additional HSQC was run on the resulting supernatant. The resulting spectrum was processed in the same manner as the previous experiments, and the contour was lowered to the point where noise was prevalent in order to potentially observe any Aβ signals. As Figure 4.5 shows, no Aβ signals can be observed; the spectrum consists entirely of noise showing that Aβ was not present free in solution.

Figure 4.5 HSQC of supernatant isolated after centrifugation of the Aβ 1-40 w/ mitochondria sample. No signals originating from Aβ 1 – 40 can be identified.
To compare the results obtained from the isolated nuclei experiment to the previous in-cell results, the nuclei spectrum was superimposed with the in-cell spectrum (Figure 4.6). These two spectra have signals for Glu-3, Phe-4, Phe-20, Asp-23, Val-24, Lys-28, Leu-34, and Val-36 as well as one of the Arg-5 side chain signals in common. The in-cell data has one signal present, Leu-17, that is not observed in the nuclei data. While there are some similarities between these two results, the majority of signals seen in the nuclei data are not present in the in-cell data.

**Figure 4.6** HSQC of Aβ 1-40 w/ nuclei (red signals) overlaid on spectra of in-cell Aβ 1-40 (blue signals). More signals are present in the Aβ 1-40 w/ nuclei spectrum in relation to the in-cell spectrum, however, the two spectra share several identifiable signals in common.
Figure 4.7 Overlay spectrum of Aβ 1-40 w/ nuclei (red signals), in-cell Aβ 1-40 (blue signals), and control Aβ 1-40 (black signals).

4.3.2 Mitochondria results

Aβ peptide was observed to bind to mitochondria as demonstrated in Figure 4.8. The contour level was lowered to the point where noise started to appear; however, it is obvious that some signals have either broadened or are no longer visible. In order to observe the full effect the isolated mitochondria had on the Aβ signals, the HSQC spectrum from the isolated mitochondria was overlayed on the control Aβ 1-40 spectra. As Figure 4.9 shows, several Aβ signals bound to
mitochondria have shifted, broadened, or disappeared when compared to the control.

Figure 4.8 HSQC of Aβ 1-40 w/ mitochondria. The contour level was lowered to the point where noise started to appear in an effort to observe all of the signals present in the spectrum.

Twelve amino acids peaks are no longer present in the spectrum of Aβ 1-40 with isolated mitochondria (Figure 4.9). These signals correspond to Phe-4, Ser-8, Gly-9, Tyr-10, Leu-17, Phe-19, Ala-21, Glu-22, Val-24, Ser-26, and Ile-32. Eight
signals have shifted, corresponding to Ala-2, Arg-5, Glu-11, Lys-16, Phe-20, Asp-23, Leu-34, and Val-40. Finally, the amino acids Ala-2, Glu-3, Arg-5, Glu-11, Val-12, Phe-20, Gly-25, Ile-31, Leu-34, Gly-37, Gly-38, and Val-40 all had significantly diminished signal intensities.

Figure 4.9 HSQC Aβ 1-40 w/ mitochondria (purple signals) overlaid on the control spectrum of Aβ 1-40 (black signals). All of the missing peaks in spectrum containing mitochondria have been labeled.
After the experiment with isolated mitochondria and Aβ 1-40 was performed, the sample was centrifuged and an additional HSQC was ran on the supernatant. The resulting spectrum was processed in the same manner as the previous experiments, and the contour was lowered to the point where noise was prevalent in order to potentially observe any Aβ signals. As Figure 4.10 shows, no Aβ signals can be observed; the spectrum consists entirely of noise indicating that Aβ was not present free in solution.
Figure 4.10 HSQC of supernatant isolated after centrifugation of the Aβ 1-40 w/ mitochondria sample. No identifiable signals originating from Aβ 1-40 are observable

To compare the results obtained from the isolated mitochondria experiment to the previous in-cell results, the mitochondria spectrum was superimposed on the in-cell spectrum (Figure 4.11). These two spectra have signals for Glu-3, Phe-20, Asp-23, Val-24, Lys-28, Leu-34, Met-35 and Val-36 as well as the Arg-5 side chain signals in common. The in-cell data has one signal present, Leu-17, that is not
observed in the mitochondria data. While there are some similarities between these two results, the majority of signals seen in the mitochondria data are not present in the in-cell data.

**Figure 4.11** HSQC A\(\beta\) 1-40 w/ mitochondria (purple signals) overlaid on spectrum of in-cell A\(\beta\) 1-40 (blue signals). More signals are present in the A\(\beta\) 1-40 w/ mitochondria spectrum in relation to the in-cell spectrum; however, the two spectra share several identifiable signals in common.
Figure 4.12 HSQC overlay spectrum of Aβ 1-40 w/ mitochondria (purple signals), in-cell Aβ 1-40 (blue signals), and control Aβ 1-40 (black signals).

The mitochondrial results were compared to the results obtained from isolated nuclei in Figure 4.13. While very similar, these two spectra have a few slight differences in their identifiable signals. There are only two signals, Val-12 and Gly-25, that are present in only the mitochondria spectrum. With regard to the nuclei spectrum, there are a total of six signals present, Phe-4, Gly-9, Tyr-10, Ala-21, Ser-26, and Ile-32, that are only present in this spectrum. Small signals are present in the mitochondria spectrum in the areas of Phe-4 and Ile-32, but the intensities of these signals are so small that they cannot be definitively identified as signals and
not noise. Another important contrast to note between these two spectra is that while the mitochondria spectrum has fewer signals, it has greater resolution in the main body of the peptide (between 8.4 and 8.6 ppm).

Figure 4.13 HSQC of Aβ 1-40 w/ mitochondria (black signals) overlaid on spectrum of Aβ 1-40 w/ nuclei (red signals). While the two spectra share several common signals, there are a few differences. Overall, there are more signals present in the Aβ 1-40 w/ nuclei spectrum, but the Aβ 1-40 w/ mitochondria spectrum has better resolution.
4.3.3 Mutant peptide results

Prior to observing any potential interactions between the mutant F19A Aβ peptide and cellular organelles, a control spectrum of the mutant peptide was acquired. A comparison spectrum of the mutant peptide vs. a wild type (WT) Aβ can be seen in Figure 4.14. The tentative identities of the mutant peptide peaks are labeled.

Figure 4.14 Comparison HSQC spectrum of mutant F19A Aβ 1-40 peptide (red signals) vs. WT Aβ 1-40 peptide (black signals). The tentative identities of the mutant peptide peaks are labeled.
After acquiring control spectra of the mutant F19A A\(\beta\) 1-40 peptide, the mutant was incubated with nuclei and mitochondria individually to assess interaction with the organelles. The spectra for isolated nuclei with the mutant peptide (Figure 4.15) and isolated mitochondria with the mutant peptide (data not shown) showed similar results. In both cases, no identifiable signals for the mutant peptide could be observed after extensive processing.

![Figure 4.15](image)

**Figure 4.15** HSQC spectra of mutant F19A A\(\beta\) 1-40 peptide with isolated nuclei. No identifiable signals are present.
4.4 Discussion

4.4.1 Nuclei discussion

Our results show that Aβ 1-40 interacts with nuclei isolated from HeLa cells using differential centrifugation. In total, eighteen of the amino acids in Aβ have been affected in some manner, which indicates that an interaction occurred when the peptide was incubated with isolated nuclei. Of these eighteen amino acids, ten are hydrophilic and eight are hydrophobic (Chart 4.1). As this chart shows, a much larger percentage of the hydrophilic amino acids have been affected in relation to the hydrophobic amino acids. Of the eight hydrophobic amino acids that were affected, only Gly-25 and Met-35 are found in the hydrophobic tail of the peptide.

![Chart 4.1: Comparison of affected hydrophilic and hydrophobic amino acids, to the total number of amino acids of each type in Aβ 1-40.](chart4.1.png)
There are a total of sixteen hydrophilic amino acids in Aβ 1-40; however, they are not all visible in a HSQC spectrum of the peptide. The hydrophilic amino acids that are not visible to NMR are Asp-1, His-6, Asp-7, His-13 and His-14. That leaves eleven hydrophilic amino acids in Aβ 1-40 that can be observed in the control spectrum (Table 4.1). Of these eleven hydrophilic amino acids, only Ser-26 was unaffected when Aβ 1-40 was incubated with isolated nuclei.

<table>
<thead>
<tr>
<th>Hydrophilic Amino Acids</th>
<th>Hydrophobic Amino Acids</th>
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</table>

Table 4.1 List of affected amino acids when Aβ 1 - 40 is in the presence of isolated nuclei.

In contrast, only eight of the twenty-four hydrophobic amino acids in Aβ 1-40 had their signals affected by an interaction with nuclei. There were seventeen amino acids that were not affected by Aβ's interaction with nuclei, of which sixteen were hydrophobic amino acids. Fourteen of the sixteen unaffected hydrophobic amino acids are located in the second half, or hydrophobic tail, of the peptide.
To ensure that the signals observed in the HSQC were only coming from \( \text{A}\beta \) bound to nuclei, after the experiment had concluded, the supernatant was isolated and examined using the same HSQC parameters. The resulting spectrum (Figure 4.5) showed no identifiable peaks and only noise was visible. This result demonstrates that signals observed in Figure 4.4 are exclusively coming from \( \text{A}\beta \) directly attached to the isolated nuclei in the sample, and not peptide that may be free in solution.

The results demonstrating the interaction of \( \text{A}\beta \) with isolated nuclei show similarities to those found from intracellular \( \text{A}\beta \) (Figures 4.6 and 4.7). Eight of the nine identified peaks for intracellular \( \text{A}\beta \) were also present in nuclei-bound \( \text{A}\beta \). Only Leu-17 was present in intracellular \( \text{A}\beta \) but not nuclei-bound \( \text{A}\beta \). While there are significantly more peaks present in nuclei-bound \( \text{A}\beta \), this could be the result of the isolated nuclei being in suspension in phosphate buffer as opposed to cell cytosol which, as previously discussed, is crowded and increases the rotational correlation time of the peptide. An increase of rotational correlation time has the effect of decreasing T2 relaxation time, which can cause signals to broaden to the point of no longer being present in the NMR spectrum.

These results suggest that the hydrophilic amino acids in \( \text{A}\beta \) could be interacting with the hydrophilic phospholipid heads of the nucleus membrane. Given that there was no difference seen in the peaks corresponding to the majority of hydrophobic amino acids in \( \text{A}\beta \) with nuclei when compared to the control (Figure
4.4) and that these unaffected amino acids are mostly found in the second half of the peptide, this would leave the hydrophobic tail of the peptide free in the cell cytosol.

### 4.4.2 Mitochondria Discussion

As with previously discussed nuclei results, the present results show that Aβ 1-40 interacts with mitochondria isolated from HeLa cells using differential centrifugation. The signals for twelve amino acids have disappeared, eight amino acid signals have shifted, and an additional five amino acids (not counted in the previous two groups) have had their signal intensities significantly diminished. Thus, twenty-six amino acids have been affected by interactions with mitochondria.

<table>
<thead>
<tr>
<th>Hydrophilic Amino Acids</th>
<th>Hydrophobic Amino Acids</th>
</tr>
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</table>

**Table 4.2** List of affected amino acids when Aβ 1 – 40 is in the presence of isolated mitochondria.
Out of these twenty-six amino acids, eight are hydrophilic and eighteen are hydrophobic, thus there is a significant bias towards hydrophobic amino acids. As was mentioned previously with regard to data obtained from isolated nuclei, out of a total of sixteen hydrophilic amino acids in Aβ 1-40, only eleven are visible in the HSQC spectrum of the peptide. The hydrophilic amino acids that are not visible to NMR are Asp-1, His-6, Asp-7, His-13 and His-14. Of these eleven hydrophilic amino acids, only Glu-15, Asp-27 and Lys-28 were unaffected when Aβ 1-40 was incubated with isolated mitochondria.

With regard to the hydrophobic amino acids, seventeen of the twenty-four total hydrophobic amino acids in Aβ 1-40 had been affected in some manner. Of these seventeen, eight are no longer visible, four have shifted in the mitochondria spectrum, and only ten amino acids were not affected by the interaction between Aβ and isolated mitochondria, of which seven are located after position 27, or the hydrophobic tail.

Perhaps the most important piece of information gleaned from these studies is the interaction of nuclei and mitochondria with the Leu-17 to Ala-21 portion of Aβ. Previous studies have shown that this sequence of five amino acids is crucial to the aggregation of Aβ (156). If there is a single mutation among these amino acids, it has been observed that it hinders Aβ’s ability to aggregate and eventually form amyloid deposits. Three of these five crucial amino acids (Leu-17, Val-18, Phe-19) were affected when Aβ was bound to nuclei, and four of these five amino acids (Leu-
17, Val-18, Phe-19, Ala-21) are not visible in the mitochondria spectrum, Figure 4.9. The last of these five, Phe-20, has a diminished intensity, and has also shifted. These results suggest that this crucial sequence for Aβ aggregation, may also be equally important in the interaction with cell organelles.

### 4.4.3 Mutant peptide discussion

Following our findings that the Leu-17 to Ala-21 region of the Aβ 1-40 peptide is greatly affected by interaction between the peptide and cellular organelles, we decided to examine the effects of a point mutation in this region on the ability of Aβ to bind to nuclei and mitochondria. Our hypothesis was that a mutation in this key region for Aβ aggregation would prevent the mutant peptide from binding to cellular organelles, as we previously observed with WT Aβ. As noted previously, we contracted the Molecular Biotechnology Core at LRI to produce a mutant F19A Aβ 1-40 peptide. Given that the cost to produce a uniformly $^{15}$N mutant peptide was prohibitively high, we decided to $^{15}$N label only the region from Val-18 to Ala-21. After acquiring the mutant, a control HSQC spectrum was taken and compared to the WT Aβ 1-40 control (Figure 4.14), and the tentative identities of the peaks were labeled. The peaks for Leu-17, Val-18, Phe-20 and Ala-21 of the mutant peptide are all in close proximity to the amino acids in the WT control. Given that the F19A mutation occurs in the middle of the five $^{15}$N labeled peptide sequence, it is understandable that chemical shifts in the mutant peptide are observed in relation to the WT control.
When the F19A mutant Aβ peptide was added to isolated cellular organelles in the same manner as previously described, the resulting spectra showed no identifiable signals. At first glance, this result seems to support our hypothesis that a mutation in the key Leu-17 to Ala-21 region would prevent Aβ from binding to cellular organelles. However, the possibility that the mutant Aβ is tightly bound to the cellular organelles, causing the NMR signals to be unobservable, cannot be ruled out. In order to definitively determine if the mutant F19A Aβ peptide is able to bind to cellular organelles, an additional mutant Aβ peptide is currently being produced. This peptide will retain the same F19A mutation and five $^{15}$N labeled sequence as the previous mutant, but, in addition, each glycine in the peptide will be $^{15}$N labeled (Figure 4.16).

**Figure 4.16** Sequence of the second mutant F19A Aβ peptide. The amino acids highlighted in red are $^{15}$N labeled.

In our previous results that used uniformly $^{15}$N labeled WT Aβ 1-40, five of the six glycines were visible when the peptide was bound to nuclei and mitochondria. Once we are able to repeat this experiment using the new mutant peptide, we should be able to say definitively if the mutation prevents Aβ from binding to cellular organelles.
Figure 4.17 Depiction of proposed Aβ interaction with phospholipid membranes of cellular organelles. The hydrophilic amino acids interact with the hydrophilic phospholipid heads, leaving the hydrophobic amino acids of Aβ exposed in the cytosol free to bind to monomeric Aβ.

In summary, our results show that nearly all of the hydrophilic amino acids of Aβ were affected by the peptide’s interaction with both nuclei and mitochondria. The hydrophobic amino acids of Aβ were also affected by the interaction with these organelles, but not to the extent seen with the hydrophilic amino acids. In both cases, the majority of the hydrophobic amino acids that were not affected by the interaction between Aβ and the organelles were located in the hydrophobic tail of the peptide. Thus, our results suggest that while the hydrophilic amino acids of Aβ
may be responsible for holding the peptide in place on the surface of the phospholipid membranes of the two organelles, while the hydrophobic tail of the peptide is exposed in the cytosol, free to interact with monomeric Aβ in solution (Figure 4.17). This particular hypothesis may be significant in identifying the possible first nucleation step in the aggregation of Aβ. Currently, the mechanism in which Aβ first begins to aggregate in the brains of patients who develop Alzheimer’s disease is unknown. Much is known about subsequent steps in the following cascade of aggregation. Aβ oligomers catalyze the formation of more oligomers, which can then go on to form fibrils and plaques both of which are also able to act as nucleation sites for the formation of even more Aβ oligomers. Understanding the first step in the chain reaction of aggregation could someday lead to a target for future drug studies. Because our results suggest Aβ has different binding partners, identifying phospholipids and membrane proteins that mitochondria and nuclei share and ruling them out as potential binding partners is of great importance.
Chapter 5

MELATONIN INTERACTION WITH THE Aβ PEPTIDE
5.1 Introduction

Melatonin is a hormone that is naturally produced in the pineal gland of vertebrates (158). The primary purpose of melatonin is to regulate circadian rhythm, or the sleep-wake cycle. It has also been found that melatonin has antioxidative effects, and can protect against free-radicals in the brain (159). Because of melatonin’s natural role in circadian rhythm, it has been used to treat sleep disorders with limited success (160,161). More recently, melatonin has shown promise as an AD therapeutic due to its ability to inhibit Aβ aggregation (162,163). The goal of this study was to determine specifically how melatonin is able to inhibit Aβ aggregation.

5.1.1 Physiological role of melatonin

During the day melatonin levels in the blood stream are low, but when the sun sets the pineal gland begins to produce melatonin which leads to drowsiness, and eventually sleep (164). Once the sun rises, the pineal gland stops producing melatonin, eventually leading to wakefulness. Studies have found that artificial light affects production of melatonin, and can have a negative impact on sleep cycles (164,165,166). In the past, before the wide spread use of artificial light in homes, the sleep patterns of people were very different than they are in modern times (167). Before the invention of the lightbulb, people tended to sleep in two four-hour periods during the night, with a brief period of wakefulness between the two. As a
result of artificial light delaying the production of melatonin by the pineal gland, sleeping disorders have seen an increase in the modern world (165, 166, 168). Among the many different treatments for sleeping disorders, melatonin has shown limited success (160, 161), but it has been shown to be an effective treatment for jet lag (169). Recently, a review found that there is insufficient evidence to support melatonin being a more effective treatment for sleep disorders than a placebo (170).

### 5.1.2 Melatonin inhibits Aβ aggregation

While melatonin is perhaps not as effective at treating sleep disorders as originally thought, it has shown an ability to inhibit Aβ aggregation. Melatonin levels may play an important role in the development of AD given that its production is reduced in older people, and is reduced dramatically in patients with AD (171). Melatonin was found to be effective at preventing the death of cultured neuroblastoma cells that were exposed to Aβ (172). Initially, it was thought that the antioxidant properties of melatonin were associated with its neuroprotective effects, due to the role oxidative stress has in the development of AD. However, later studies that compared melatonin to other antioxidants found that these substitutes were not as effective Aβ aggregation inhibitors as melatonin. Circular dichroism (CD) was used to show that in comparison to melatonin, two alternative free radical scavengers (NAT and PBN) were ineffective at preventing Aβ from forming β-sheet secondary structures (162). In addition, EM was used to physically observe Aβ fibrils, which were not present when melatonin was added to Aβ. It was
found using $^1$H NMR spectroscopy that melatonin caused the Aβ histidine signals to shift, indicating that this is likely the source of the interaction between the two. Our investigation of melatonin’s ability to inhibit Aβ aggregation focused on melatonin’s interaction with the histidines of Aβ.

5.1.3 Role of His-Asp salt bridges in Aβ aggregation

The aggregation of the Aβ peptide is highly dependant upon pH (141). Aβ will only aggregate when the pH of the solution is between 6 and 7.4. This has to do primarily with the pKa of the histidine side-chain which is approximately 6. At physiological pH, the side chain of histidine still has some positive charge with approximately one protonated side chain for every ten non-protonated side chains. Positively charge histidine side chains are then able to hydrogen bond to negatively charged aspartic acid side chains, and form salt bridges (173). His-Asp salt bridges, as well as those formed between other cationic and anionic amino acids, are an important interaction in the formation of the structures of countless other proteins such as G. thermodenitrificans EstGtA2 which is a bacterial lipolytic enzyme (174). This particular salt bridge has been found to be vital in the initial stages of Aβ aggregation. Molecular modeling, followed by examination by EM of Aβ peptide fragments found that the sequence HQKLVFFAED (Aβ 14 – 23) is the shortest Aβ fragment that will form fibrils (175) at physiological pH. When histidine is not positively charged at higher pHs, the Aβ peptide will not aggregate (175). Thus, the his-asp salt bridge is an interaction that can potentially be targeted to prevent Aβ
aggregation.

5.1.4 Saturation transfer difference (STD) NMR

Previous studies have shown a clear interaction between melatonin and the histidine residues of Aβ (162). In order to acquire further information about this interaction, we employed the technique of saturation transfer difference (STD) NMR, a powerful tool that is used to study ligand-receptor binding. This is achieved by observing the nuclear Overhauser effect (NOE) between protons of the ligand and protons of the receptor. The NOE is a through space spin-spin coupling that occurs between protons in close proximity, up to 6 Å, of each other (176). Typically, NOEs are used to help solve the three dimensional structures of molecules. In the case of STD NMR, ligand-receptor binding information is acquired by selectively saturating the receptor and observing the transfer of saturation to the ligand via NOEs. In order to observe this effect, two different spectra must be acquired; one in which the receptor is saturated on-resonance where a signal of interest is located, and in the other the receptor is saturated off-resonance where no signals are located (177). One spectrum is then subtracted from the other using NMR processing software, resulting in a difference spectrum. The only signals present in the difference spectra are those responsible for the binding interaction between the ligand and the receptor.
5.2 Materials and Methods

5.2.1 Melatonin and Aβ sample preparations for STD NMR

It was important for this saturation transfer difference (STD) experiment to minimize the amount of H2O that was present in the samples that were subjected to NMR. In order to achieve this goal, fresh deuterated 20 mM phosphate buffer was prepared by dissolving NaH2PO4 (0.031g) and NaHPO4 (0.109g) in D2O (50 mL) (Cambridge Isotopes) from a new, unopened bottle. The pH was adjusted to 7.4 using small amounts of NaH2PO4 to lower the pH. The buffer was placed in a flame dried 200 mL jar with teflon tape covering the jar’s threading. After each time the jar was capped, it was further sealed by wrapping Para-film™ around the outside of the cap.

The stock solution for melatonin was prepared by dissolving melatonin (1.16 mg) (Sigma-Aldrich) in the above deuterated phosphate buffer (10 mL, 20 mM). This gave a melatonin stock solution of 500 μM.

Prior to weighing the Aβ peptide, a solution of 10 mM NaOD was prepared by combining fresh 40% wt. (50 μL, 10 M) NaOD (Sigma-Aldrich) with fresh D2O (50 mL). Next, Aβ 1-40 (1 mg) (Anaspec) was weighed and dissolved in this solution (1 mL, 10 mM) to produce a Aβ 1-40 stock concentration of 213 μM. This solution was sonicated for 15 mins prior to each use.
Next, two samples were prepared: one control sample containing only Aβ 1-40, and the other containing Aβ 1-40 plus melatonin. The control sample was prepared by taking Aβ 1-40 stock solution (117 μL, 213 μM) and bringing the total volume up to 500 μL with deuterated phosphate buffer (20 mM), which gives a final Aβ concentration of 50 μM. The pH was adjusted to 7.3 using deuterated trifluoracetic acid (10 mM)(Sigma-Aldrich). For the other sample, Aβ 1-40 stock solution (117 μL, 213 μM) was combined with the melatonin stock solution (100 μL, 500 μM), and the volume was adjusted to 500 μL with deuterated phosphate buffer (20 mM). This produced a final concentration for Aβ of 50 μM, and 100 μM for melatonin. The pH was adjusted to 7.3 using deuterated trifluoracetic acid (10 mM) as it was for the control sample. The two samples were placed in separate 5 mm NMR tubes (Shigemi, Precision Thin Wall Tubes), and kept on ice.

5.2.2 STD NMR experiment methods

First a 1D NMR spectrum was taken for the control Aβ 1-40 using a Bruker 900 MHz spectrometer equipped with a high-sensitivity cryoprobe. The sample temperature was lowered to 8 °C, and the number of scans was increased to 1024 in order to improve the signal to noise ratio. Next, a 1D NMR spectrum was taken for the sample containing both melatonin and Aβ 1-40 using the same conditions as the control spectrum. Both spectra were briefly analyzed using the machine’s Top Spin 3.0 software in order to observe shifts in the signals corresponding to Aβ’s histidine
2H and 4H peaks of the imidazole ring in the sample of Aβ with melatonin, in reference to the Aβ control. The shifts observed in these signals were expected (162), and were used to confirm that the two samples were properly prepared.

For the saturation transfer NMR experiments, three different spectra were obtained. In the first, the sample containing both melatonin and Aβ was selectively irradiated at 6.82 ppm, the location for the Aβ His-4H peak. For the second spectrum, the same sample was irradiated at 7.8 ppm, the location for the Aβ His-2H peak. A final reference spectrum was acquired, this time with the sample being irradiated at 5 ppm, which is a location in the spectrum where no signals are observed. It is necessary to irradiate the reference spectrum in such a manner to ensure that the process of irradiating the sample alone is not the cause of any observed changes in the spectra.

In order to observe the STD results, two distinct difference spectra were prepared using the NMR processing software MNova. For the first difference spectrum, the spectrum that was irradiated at 6.82 ppm was subtracted from the reference spectrum that was irradiated at 5 ppm. The second difference spectrum was prepared by subtracting the spectrum irradiated at 7.8 ppm from the reference spectrum.
5.2.3 Melatonin and Aβ $^{13}$C NMR experiment

For the $^{13}$C NMR experiments, an Aβ 1-42 peptide that was labelled with $^{13}$C only on the side chain of Ala-2 and the carbonyls of Asp-23 was used. The Aβ stock solution was prepared by dissolving the peptide (1 mg) in NaOD (1 mL, 10 mM), giving an Aβ concentration of 213 μM. This solution was sonicated for 15 mins prior to each use.

Three different samples were prepared for NMR experiments: an Aβ control, a melatonin control, and a sample containing melatonin and Aβ in a 2:1 ratio. For the Aβ control, the $^{13}$C labelled Aβ 1-40 stock solution (117 μL) was added to deuterated phosphate buffer (383 μL, 20 mM), and the pH was adjusted to 7.3 using deuterated trifluoroacetic acid (10 mM). The melatonin sample was prepared by adding melatonin stock solution (100 μL, 500 μM) to deuterated phosphate buffer (400 μL, 20 mM). Finally, the last sample was prepared by combining the $^{13}$C labelled Aβ 1-40 stock solution (117 μL) with melatonin stock solution (100 μL, 500 μM), and the total volume was brought to 500 μL using deuterated phosphate buffer (20 mM). Once again, the pH was adjusted to 7.3 using deuterated trifluoroacetic acid (10 mM). All samples were kept on ice until they were inserted into the NMR spectrometer. In addition, a small amount of 1,4-dioxane was added to each sample as a reference.
Each sample was run on a Bruker 500 MHz NMR equipped with a high-sensitivity cryoprobe. Given the low concentrations of the samples, the number of scans for each experiment was increased to 34K, resulting in an experiment that ran for approximately 30 hours. All spectra were analyzed using MNova.

5.3 Results

5.3.1 STD NMR results

Prior to performing any STD NMR experiments, control spectra were acquired for Aβ 1-40 alone, as well as for Aβ 1-40 with melatonin. The goal of this experiment was to determine if an upfield shift occurs for the Aβ His 2-H and His 4-H signals.
As was expected, the peaks corresponding to His-2H and His-4H shifted upfield 0.02 ppm and 0.01 ppm respectively in the downfield region for the mixture of Aβ 1-40 with 2 molar equivalents of melatonin in relation to the control Aβ spectrum (Figure 5.1). This result indicated that it was appropriate to proceed to STD NMR experiments.

**Figure 5.1** Aβ 1-40 vs. Aβ 1-40 w/ melatonin downfield. Red line: 50 µM Aβ 1-40. Blue line: 50 µM Aβ 1-40 with 100 µM melatonin.
Figure 5.2 Aβ 1-40 vs. Aβ 1-40 w/ melatonin upfield. Red line: 50 µM Aβ 1-40.

Blue line: 50 µM Aβ 1-40 with 100 µM melatonin.

The upfield region of the control spectrum was also examined for any potential effects of melatonin on Aβ. While there are differences in the intensities of a few peaks, most notably the peaks at 0.95 ppm and 1.1 ppm, there were no observed shifts of any of the Aβ peaks.
Figure 5.3  Aβ 1-40 vs. Aβ 1-40 w/ melatonin full spectrum. Red line: 50 µM  Aβ 1-40. Blue line: 50 µM  Aβ 1-40 with 100 µM melatonin.
**Figure 5.4** His-4H irradiated vs. reference spectra downfield. Red line: 50 µM Aβ 1-40 with 100 µM melatonin irradiated at 6.82 ppm. Blue line: reference spectrum of 50 µM Aβ 1-40 with 100 µM melatonin irradiated at 5 ppm.

Figure 5.4 illustrates a comparison of two saturation transfer difference (STD) spectra. The blue line correspondes to the reference spectrum of 50 µM Aβ 1-40 with 100 µM melatonin irradiated at 5 ppm where no peaks are observed. The red line corresponds to the spectrum of 50 µM Aβ 1-40 with 100 µM melatonin irradiated at 6.82 ppm where the peak for the Histidine 4H proton is located in Aβ 1-40. This spectrum shows that the intensity of the signal for melatonin’s 6H proton is decreased in comparison to the reference. This indicates proximity between the
His-4H proton of Aβ 1-40 and melatonin. The triplet at 6.9 ppm also corresponds to His protons, whereas the peaks at 7.0 ppm and 6.6 ppm correspond to Aβ 1-40 Phe and Tyr-3,5H protons, respectively. No similar effects were observed in upfield regions of the spectrum (Figure 5.5).

Figure 5.5 His-4H irradiated vs. reference spectra full. Red line: 50 µM Aβ 1-40 with 100 µM melatonin irradiated at 6.82 ppm. Blue line: reference spectrum of 50 µM Aβ 1-40 with 100 µM melatonin irradiated at 5 ppm.
Figure 5.6  Downfield spectral region of reference spectrum (A) and difference spectrum of 50 µM Aβ 1-40 with 100 µM melatonin irradiated at 6.82 ppm (B).

A comparison of the reference spectrum (spectrum A), and the difference spectrum (spectrum B) can be seen in Figure 5.6. The peaks corresponding to His-4H are observable in the difference spectrum as expected, because these protons were selectively irradiated in order to observe any transient binding with melatonin. The signal for the melatonin 6H proton can be observed in the difference spectrum, which indicates that there is an interaction between this proton and the His-4H proton of monomeric Aβ 1-40. There was no effect observed in the upfield region of the difference spectrum (data not shown).
Figure 5.7 Downfield spectral region of reference spectrum (blue) and STD spectrum of 50 µM Aβ 1-40 with 100 µM melatonin irradiated at 7.8 ppm (red).
Figure 5.8 Downfield spectral region of reference spectrum (A) and difference spectrum of 50 µM Aβ 1-40 with 100 µM melatonin irradiated at 7.8 ppm (B). No melatonin signals are present in the difference spectrum.

Another STD experiment was performed with irradiation at 7.8 ppm, the location of the Aβ 1-40 His-2H proton. As Figure 5.7 shows, no effect was observed for any melatonin signals in the downfield region, which is in contrast to the STD experiment when the sample was irradiated at 6.82 ppm (Figs. 5.5-5.6). The difference spectrum (Figure 5.8B) clearly shows no melatonin signals, indicating that no transfer of saturation from His-2H to melatonin occurred. Likewise, no effect was observed in the upfield region of the above spectrum (Figure 5.9). These
results indicate that Aβ 1-40 is not in close proximity to the His-2H proton of melatonin.

Figure 5.9 Upfield spectral region of reference spectrum (blue) and STD spectrum of 50 μM Aβ 1-40 with 100 μM melatonin irradiated at 7.8 ppm (red). No effect observed in irradiated spectrum.

5.3.2 Melatonin and Aβ $^{13}$C NMR results

A $^{13}$C 1D NMR control spectrum of Aβ 1-42 was acquired in order confirm the presence of both $^{13}$C labeled carbonyls in Asp-23. As Figure 5.10 shows, both carbonyl peaks were clearly identified.
When this control spectrum is compared to a spectrum of the 2:1 mixture of melatonin with \(^{13}\text{C}\) labelled A\(\beta\) 1-42 (Figure 5.11), both Asp-23 carbonyls are clearly affected by the presence of melatonin. The backbone carbonyl, located at 171.1 ppm, shifted downfield 0.7 ppm, and the side-chain carbonyl located at 164 ppm in the control is indistinguishable from the background noise.
Figure 5.11 Comparison of $^{13}$C labelled A$\beta$ 1-42 control (spectrum A) and 2:1 mixture of melatonin with $^{13}$C labelled A$\beta$ 1-42 (spectrum B).

5.4 Discussion

5.4.1 STD NMR discussion

The specific aim of these studies was to determine the exact location of the binding site between A$\beta$ 1-40 and melatonin. Previous studies have identified the histidines of A$\beta$ as a possible binding site for melatonin, and have shown that melatonin inhibits the aggregation of A$\beta$. In order to better understand the ligand to
receptor binding interaction between these two molecules, STD NMR was performed. STD NMR is a powerful tool that can identify NOEs between a ligand and its receptor, an indication of a close proximity (<4-5 Å) between the two.

When working with Aβ, it is crucial that the pHs of all the samples are identical. Small variations in pH between samples can lead to shifts in the histidine peaks of Aβ in a 1D proton NMR spectrum, that could be incorrectly attributed to interactions that are not actually occurring. This possibility was ruled out in our studies by initially acquiring a spectrum of the mixture of melatonin and Aβ, and comparing it to a control spectrum of just Aβ. The resulting comparisons (Figures 5.1 and 5.2) do show slight shifts in the locations of the Aβ histidine peaks; however no other differences in the peaks for Aβ were observed, indicating that the samples had been properly prepared.

There were two possible locations for the binding interaction between Aβ and melatonin. These specific sites are the His-2H and the His-4H proton of Aβ, whose 1D proton NMR peaks shift when Aβ is in the presence of melatonin (Figure 5.1). Each site was selectively irradiated, and the resulting spectra were subtracted from a reference spectrum which was irradiated at a location where no peaks are observed. The resulting spectrum is the difference spectrum, where the saturation is transferred from the receptor (Aβ in this case) to the ligand (melatonin) via the nuclear Overhauser effect. In the difference spectrum, any signals from the ligand that are present come from protons that are in close proximity to the receptor. Our
STD NMR results show that the melatonin 6H proton is observed in the difference spectrum when Aβ’s His-4H proton is irradiated (Figure 5.6). This result indicates there is a transient binding interaction between melatonin and the histidines of Aβ that causes these two protons to come in close proximity to each other. Another difference spectrum was prepared, in which the His-2H proton of Aβ was irradiated; however, in this case, no interaction with melatonin was observed (Figure 5.12). This result indicates that while melatonin may come in close contact with Aβ’s His-4H proton, it does not come as close to the His-2H proton.

![Figure 5.12 Predicted stabilizing interaction between histidine and melatonin.](image)

When model representations of melatonin and histidine are arranged in such a manner that melatonin’s 6H proton is in close proximity to Aβ’s His-4H proton, there is the possibility of a hydrogen bond between the protonated amine on
histidine's side chain, and the methoxy group of melatonin. An alternate resonance form of protonated histidine can also form a possible hydrogen bond with melatonin (Figure 5.13), while keeping the melatonin's 6H proton is in close proximity to Aβ's His-4H proton.

![Figure 5.13](image)

**Figure 5.13** Alternative representation of predicted stabilizing interaction between histidine and melatonin.

In both of these resonance forms of histidine where melatonin's 6H and Aβ's His-4H protons are next to each other, it can be seen that the His-2H proton is on the opposite side of the histidine imidazole ring. If the hydrogen bond shown between the protonated amines of the histidine side chain and the methoxy group of melatonin is responsible for the interaction between these two species, then this
could explain why our STD NMR results show that the His-2H proton has no NOE with melatonin.

At physiological pH (7.3) aspartic acid is negatively charged, and while above histidine's pKa of ~6.0, some histidine molecules have a positive charge. This allows a hydrogen bond to form between these two amino acids. The formation of salt-bridges between aspartic acid and histidine is a key interaction in the aggregation of Aβ. Given our STD NMR results, we speculate that melatonin may interfere with the formation of this salt-bridge, which could explain melatonin's ability to impede Aβ aggregation.

![Figure 5.14](image)

**Figure 5.14** Melatonin possibly inhibits the formation of Asp-His salt-bridges.
5.4.2 Melatonin and Aβ $^{13}$C NMR discussion

The possibility that melatonin could potentially inhibit the formation of Asp-His salt-bridges was explored using $^{13}$C NMR and Aβ that was $^{13}$C labelled at Asp-23 carbonyls. As Figure 5.11 shows, both Asp-23 carbonyls are affected by the presence of melatonin. This interaction between melatonin and aspartic acid caused the backbone carbonyl in Asp-23 to shift downfield. Perhaps more importantly, this result suggests that the presence of melatonin in the sample is causing the Asp-23 side chain carbonyl to relax faster, thus diminishing its signal. This is a clear indication that melatonin is interacting with this carbonyl in some manner. This result taken in conjunction with previous CD studies showing melatonin’s ability to prevent Aβ aggregation, as well as our STD NMR results, strongly suggest that melatonin indeed inhibits the formation of Asp-His salt bridges in Aβ.
CHAPTER 6

EXPERIMENTAL DRUG IXR4204
6.1 Introduction

Despite over two decades of research, a new drug for AD treatment has not yet appeared. In 1996, there were a total of five therapeutics for patients with AD; donepezil, galantamine, memantine, rivastigmine, and tacrine. As previously mentioned, none of these drugs actually treats the causes of AD or were effective at slowing the progress of the disease, instead they were targeted to ease the symptoms that AD patients experience. However, these drugs come at the cost of severe side effects that cause the families of many patients to stop their use. In some cases, the side effects are so severe that since the late 90’s the FDA has withdrawn approval for tacrine, leaving only four therapeutic options (47). Recent efforts at new AD therapeutics have included Aβ aggregation inhibitors, antibodies that target Aβ to enhance clearance of extracellular plaques, and even repurposed chemotherapeutics, a few of which are discussed below.

6.1.1 Retinoid X receptor (RXR) agonists

Recently, attempts at finding new AD therapeutic have expanded to include repurposing chemotherapeutics, in particular those that are retinoid X receptor (RXR) agonists. In the liver, RXRs form heterodimers with either peroxisome proliferator-activated receptor γ (PPARγ) or liver X receptors (LXRs), which leads to
the transcription of apolipoprotein E (ApoE) (8). ApoE plays a clear role in the pathogenesis of AD in patients who have the E4 allele, which raises their risk of developing AD from 20% to 90% (178). In 2012 the Landreth group at Case Western Reserve University found that the RXR agonist bexarotene is able to simultaneously activate the RXR-PPARγ and RXR-LXR pathways, leading to a significant rise in ApoE production in transgenic mice (179). This rise in ApoE production subsequently leads to a significant decrease in soluble Aβ peptides in the brain, lower plaque burden, and increases cognitive function in the mouse model. These results showed that bexarotene, also known as Targretin®, had the potential to be used to treat AD. An added advantage of bexarotene’s potential as an AD therapeutic is that it is already FDA approved for use in humans as a drug to treat certain types of skin cancer (180). However, bexarotene does not come without its downsides. In addition to boosting ApoE transcription, bexarotene also promotes transcription of fatty acids, leading to a rise of triglycerides and cholesterol in patients who take the drug (181).

While the initial findings for bexarotene were very exciting, subsequent experiments aimed at reproducing the results were not as successful. These follow up studies were able to confirm that bexarotene reduces soluble Aβ levels in the brain, as well as its effect on improving memory in their mouse models (182,183); however none of these studies were able to reproduce the finding of a lowered plaque burden in the brains of their mice (182,183,184,185). Furthermore, in two
Phase 1b human trials aimed at testing the ability of bexarotene to decrease soluble Aβ in cerebral spinal fluid (CSF), the trials failed to reach their targeted goal (186). Following the failure of these two initial human trials, a Phase 2a trial also showed that bexarotene failed to lower plaque burden in the brains of patients who had been diagnosed with AD (186).

6.1.2 Experimental drug IXR4204

![Structure of experimental drug IXR4204](image)

Despite the shortcomings of human trials testing bexarotene, research has continued in the area of RXR agonists as potential AD therapeutics. In 2012, the Zagorski group was approached by Dr. Pasinetti of Mt. Sinai School of Medicine, to test a different RXR agonist, IXR4204. At the time, IXR4204 was a prostate cancer drug that was undergoing Phase 2 trials. This drug was found to be highly selective for RXRs in relation to retinoic acid receptors (RARs), activation of which can lead to negative side effects (179). Previously, IXR4204 was shown to disaggregate Aβ fibrils taken from the brains of patients with AD (Figure 6.1). The objective of our
study was to examine this drug’s interaction with Aβ using NMR, as well as its ability to inhibit Aβ aggregation using circular dichroism (CD).

**Figure 6.1** EM images of Aβ fibrils taken from the brains of AD patients. IXR4204 was found to disaggregate these fibrils. (Wang et al. 2013)
6.1.3 Circular dichroism (CD)

In order to observe the effect that IXR4204 has on Aβ aggregation, we used CD. CD is a type of spectroscopy that utilizes plane-polarized light that is commonly used to examine the secondary structures of proteins. Depending on the conformation the protein, the CD will afford one of three characteristic curves (Figure 6.2). Using this technique, we could track the conformational changes in Aβ as it transitioned from a random coil conformation to β-sheet as it aggregated.
Figure 6.2 CD plot of the three characteristic curves of proteins with α-helix (triangles), β-sheet (squares), or random coil (circles) secondary structures. (Khan et al.)
6.2 Materials and Methods

6.2.1 Aβ 1-42 and IXR4204 stock solution preparation

Two different Aβ 1-42 stock solutions were prepared, unlabeled stock and uniformly $^{15}$N labeled. For both stock solutions, the solution ($709 \mu$M) was prepared by dissolving the respective Aβ peptide (0.8 mg, Anaspec for the unlabeled peptide, rPeptide for the uniformly $^{15}$N labeled peptide) in NH$_4$OH (200 μL, 1% v/v). To ensure that no aggregate seeds were present, the solution was then sonicated for 10 min. Care was taken to keep the Aβ 1-42 sample cold at all times.

The stock solution for the experimental drug IXR4204 was prepared by dissolving IXR4204 (1 mg) in absolute ethanol (1 mL), which gives a final IXR4204 solution of 2.6 mM.

6.2.2 HSQC NMR

At the start of this study a HSQC NMR spectrum was acquired in order to observe if the experimental drug IXR4204 interacts with Aβ 1-42. The sample was
prepared by combining the stock solution of IXR4204 (6.5 μL, 2.6 mM) with the uniformly $^{15}$N labeled Aβ 1-42 stock solution (49.5 μL, 709 μM) in phosphate buffer (215.5 μL, 20 mM) with absolute ethanol added (28.5 μL), and finally D$_2$O (50 μL) so the NMR could lock on to the sample. This gives final concentrations of 50 μM IXR4204 and 100 μM Aβ 1-42. The pH was adjusted from approximately 8.5 to 7.3 using dilute TFA, and the sample was placed in a 5 mm Shigemi NMR tube. While the sample was prepared and transported to the NMR spectrometer, it was kept on ice in order to prevent the Aβ 1-42 from aggregating. An HSQC was acquired on a Bruker 900 MHz spectrometer equipped with a high-sensitivity cyroprobe, as was previously described in Chapter 3.

6.2.3 CD experiment

Circular dichroism can be used to determine the secondary structure of proteins, and was used to monitor the transition from random coil to beta sheet for the Aβ peptide. IXR4204 is insoluble in water, but is soluble in ethanol. Therefore, in all circular dichroism experiments, the solvent used was 10% ethanol in phosphate buffer.

First, a control spectrum was obtained for Aβ 1-42 alone. The control was prepared by combining the Aβ 1-42 stock solution (50 μL, 709 μM) was added to
phosphate buffer (265 μL, 20 mM) with ethanol added (35 μL), giving a final Aβ 1-42 concentration of 100 μM. The pH was adjusted to 7.3 using dilute TFA. This working solution was added to a 1 mm flat CD cell (Hellma), and inserted into a Jasco J-810 CD. CD measurements were made for each sample at six different time points: 0 hr, 21 hr, 48 hr, 72 hr, 96 hr, and one week. Measurements were taken every 1 nm from 175 nm and 270 nm; each sample measured three times at each wavelength, and the CD data was averaged.

To test IXR4202’s ability to inhibit Aβ aggregation, the drug was mixed with Aβ at various concentrations and followed by CD. In the first of these experiments, the stock solution of IXR4204 (6.5 μL, 2.6 mM) was added to the Aβ 1-42 stock solution (49.5 μL, 709 μM) in phosphate buffer (265.5 μL, 20 mM) with absolute ethanol (28.5 μL). The pH was adjusted to 7.3 using dilute TFA. This gives final concentrations of 50 μM IXR4204 and 100 μM Aβ 1-42. This solution was then added to a 1 mm flat CD cell, and inserted into the CD, after which measurements were taken over the course of one week.

The previous experiment was repeated, but with IXR4204’s concentration at 100 μM. The working solution was prepared by combing the IXR 4204 stock solution (13 μL, 2600 μM) with the Aβ 1-42 stock solution (49.5 μL, 709 μM) in phosphate buffer (265.5 μL, 20 mM) with absolute ethanol added (28.5 μL). The pH was adjusted to 7.3 using dilute TFA. This gives final concentrations of 100 μM
IXR4204 and 100 μM Aβ 1-42. This solution was then added to a 1 mm flat CD cell, and inserted into CD. CD measurements were taken over the course of one week. All of the samples were allowed to age at room temperature for the duration of the experiment.

### 6.3 Results

#### 6.3.1 HSQC NMR results

The HSQC NMR results clearly show that IXR4204 interacts with Aβ 1-42, and four amino acid signals, Ser-8, Ser-26, Ile-31, and Gly-37, are no longer visible when it was in the presence of IXR4204. However, it is possible that the signal for one of these amino acids has shifted dramatically to become the peak labeled with an asterisk at the top of Figure 6.3. Given that the nearest identified peak in the control, Gly-38, has a corresponding identifiable peak in the drug spectrum, it is not possible to identify this unknown signal. In addition to the missing peaks, a large number of amino acids have had their signals shifted. These amino acids include Ala-2, Gly-9, Lys-16, Val-18, Phe-19, Glu-22, Val-24, Gly-29, Ala-30, Ile-32, Gly-33, Gly-38, Val-40, and Ile-41. Of the eighteen total amino acids of Aβ 1-42 that were affected by the presence of IXR4204, only four (Ser-8, Lys-16, Glu-22, Ser-26) are hydrophilic amino acids.
Figure 6.3 HSQC of Aβ 1-42 w/ IXR4204 (Red peaks) vs. Aβ 1-42 Control (Black peaks). The identity of the peak labeled with an asterisk is unknown.

6.3.2 CD results

Following the HSQC NMR results that showed an interaction between Aβ 1-42 and IXR4204, CD experiments were performed to investigate if IXR4204 could inhibit the Aβ 1-42 random coil to β-sheet conversion, along with subsequent aggregation. Two different ratios of Aβ 1-42 and IXR4204 were examined: 2:1 ratio
and 1:1. Over the course of one week, the samples were tested at several different time intervals to observe how the secondary structure of Aβ changed. The results for the control of Aβ 1-42 (Figure 6.4) show that the peptide had already begun to transition from an initial random coil conformation to a β-sheet conformation at 21 hr. By 48 hr, the Aβ control had completely adopted a β-sheet conformation.

The results for both samples when Aβ 1-42 was in the presence of IXR4204 were surprising. When Aβ was in a 2:1 ratio with IXR4204 (Figure 6.5), the peptide had almost completely transitioned to a β-sheet conformation at 21 hr. Furthermore, when Aβ was in solution with a higher concentration of IXR4204 (Figure 6.6), the peptide had completely adopted the β-sheet conformation at 18 hr. A comparison of the results from the control sample with the sample of Aβ 1-42 with IXR4204 at a 2:1 ratio can be seen in Figure 4. This plot clearly demonstrates how much quicker Aβ transitioned to the β-sheet conformation when in the presence of IXR4204.
**Figure 6.4** CD Results for control Aβ 1-42 (100 μM). Aβ 1-42 begins in a random coil configuration, then begins to transition to a β-sheet conformation by 21 hr. By 48 hr, the peptide has fully adopted a β-sheet secondary structure.
Figure 6.5 CD Results for Aβ 1-42 (100 μM) with IXR4204 (50 μM). While in the presence of the drug, Aβ had already adopted a β-sheet secondary structure by 21 hr.
Figure 6.6 CD Results for Aβ 1-42 (100 μM) with IXR4204 (100 μM). When the concentration of IXR4204 was increased, Aβ aggregated even faster, adopting a β-sheet conformation at 18 hr.
Figure 6.7 Comparison of CD Results for Aβ 1-42 control with Aβ 1-42 with IXR4204 (50 μM).

6.4 Discussion

Prior experiments established that the drug IXR4204, currently undergoing Phase II trials as a prostate cancer chemotherapeutic, had the potential to also be
used as a treatment for AD. EM imaging had demonstrated IXR4204’s ability to break up preformed Aβ fibrils (Figure 6.1).

To further investigate this drug’s potential ability to treat Alzheimer’s Disease, we examined IXR4204’s interaction with Aβ 1-42 using HSQC NMR and CD. Our HSQC NMR results showed a clear interaction between IXR4204 and Aβ 1-42. A total of eighteen amino acids in Aβ 1-42, four hydrophilic and fourteen hydrophobic, were effected by the presence of IXR4204. The clear bias towards an interaction with the hydrophobic amino acids of Aβ 1-42 can possibly be explained by the hydrophobic nature of IXR4204.

Given our NMR results showed an interaction between Aβ 1-42 and IXR4204, next the drug’s ability to inhibit the aggregation of Aβ 1-42 monomers was examined. If it could be proven that IXR4204 inhibits Aβ aggregation, then this drug would have a great potential to be used in Alzheimer’s disease treatment. Unfortunately, our CD results showed that instead of inhibiting Aβ aggregation, IXR4204 actually accelerated Aβ aggregation. In the control, Aβ 1-42 was observed to have begun its transition from a random coil configuration, to a β-sheet conformation at 21 hr, and had completely transitioned to β-sheet at 48 hr. To show potential as an AD therapeutic, IXR4204 would then have to have delayed the aggregation of Aβ 1-42 at least 48 hr. However, our results showed that by 21 hr when Aβ 1-42 was in a 2:1 ratio with IXR4204, it had already adopted the β-sheet
conformation, indicating that it had already begun to aggregate. Furthermore, when the concentration of IXR4204 was doubled, Aβ 1-42 aggregated even faster, having completely transitioned to β-sheet at only 18 hr. These results definitively prove that IXR4204 cannot be used directly as an aggregation inhibitor in the treatment of AD.
CHAPTER 7

EFFECTS OF SPICES ON Aβ AGGREGATION
7.1 Introduction

Each year the number of people who develop AD in the U.S. and around the globe continues to grow, but the number of treatment options available to these patients has remained stagnant. This has led many researchers to look towards natural products as a possible solution to this looming healthcare crisis. Current statistics shows that in the U.S., one out of every ten people age 65 and older have AD (1,2,3). In comparison, Mediterranean and Indian populations have significantly lower incidences of AD, and less than 2% of the Indian population over 65 have AD (31). While genetics certainly play a large role, connections have been made between different cultural diets and disease states (187,188,189). Many studies have made a connection with strict adherence to the Mediterranean diet and lower incidences of AD (187,190,191). Spices used in Indian cuisines have also been connected to a lower chance of developing AD (192). In this study, we attempted to combine bioinformatics with analytical tools to target specific compounds found in Indian spices as potential AD therapeutics.

7.1.1 Health benefits of the Mediterranean diet

The Mediterranean diet has become widely popular for its potential health benefits. Multiple studies have shown associations between the diet and lower risks of obesity, hypertension, and cancer, in addition to AD (187,188,189,190,191). One particular finding that arose from studying the Mediterranean diet in relation to AD,
that entered the public consciousness, is that drinking red wine could have neuro-protective effects. The initial findings that identified resveratrol as a potentially beneficial natural product came from a study that found that resveratrol extends the lifespan of *Saccharomyces cerevisiae*, a type of yeast (193). Follow-up studies however, have not found a clear association between resveratrol and extended human life spans (194). Resveratrol has a very low bioavailability, and less than 1% of resveratrol consumed becomes bioavailable (195). However, it has been found that resveratrol can be safely taken orally at very large dosages, up to 2 grams per day, and has an effect on Aβ levels in CSF when taken at such doses (194).

7.1.2 Health Benefits of Indian Cuisine

Traditional Indian cuisine has also recently been scrutinized for natural products that can protect against AD. Compared to the US population, the occurrence of AD and other forms of dementia in India is drastically lower, with an incidence of less than 2% among the Indian people over the age of 60 (189,196,197). One compound in particular, curcumin the primary micronutrient component of turmeric, has been found to have many health benefits (192). In addition to anti-inflammatory and anti-oxidant properties, curcumin has also been found to have neuro-protective effects in mouse models of AD (198), and anti-aggregation effects for Aβ (199,200,201). Given curcumin’s well-established ability to inhibit Aβ aggregation, other spices examined in this study were compared to curcumin, which was tested concurrently. Another micronutrient that is found in
nearly all Indian foods is capsaicin, the compound responsible for making chili peppers taste spicy. Capsaicin has also been linked to positive health benefits, such as increased metabolism (202), use as a topical analgesic (203), as well as cardiovascular disease protective effects due to capsaicin's capacity as a vasodilator (202, 203). In this study, we also examined capsaicin's ability as an Aβ aggregation inhibitor, both alone and in conjunction with curcumin.

7.1.3 Identification of spices using bioinformatics

Traditional Indian cuisine uses a wide variety of spices in addition to turmeric and chili peppers, some of which could potentially help prevent the aggregation of Aβ. In an effort to identify additional natural products that could aid in the treatment of AD, a comprehensive bioinformatics study was performed by Swathi Srinivasan. This study collected data from Gene Expression Omnibus (GEO), Online Mendelian Inheritance of Man (OMIM), ClinVar, Genome-wide Association Studies (GWAS) and Search Tool for Interacting Chemicals (STITCH) databases in conjunction with the Ingenuity Pathway Analysis software to build gene profiles of both AD associated genes, and genes that are targeted by a wide variety of different spices. Spices that targeted genes associated with AD were identified, four of which were selected and examined in our study. These spices were anise, tamarind, turmeric and chili peppers.
7.1.4 Atomic Force Microscopy (AFM)

Atomic Force Microscopy (AFM) was used to observe the effects that curcumin and capsaicin had on the size of Aβ aggregates. This imaging technique is able to measure a sample with a resolution of a fraction of a nanometer, and then create a topographic image from the data (204). Unlike traditional light microscopy, AFM uses an extremely fine probe to physically scan the surface of the sample. There are two types of imaging modes that the AFM can use: contact mode in which the tip is dragged along the surface of the sample, and tapping mode, which is the imaging mode that we employed. In tapping mode, the cantilever on which the probe tip is located is oscillated at its resonance frequency. The tip is then scanned in close proximity to the sample surface. When the tip closely approaches a surface, atomic forces, such as the Van der Waals force, cause the frequency of the cantilever to change, which is measured and used to map the surface of the sample.

7.2 Materials and Methods

7.2.1 Spice study CD

Circular dichroism (CD) was used to observe if the spices curcumin and capsaicin are able to inhibit Aβ aggregation. To start, stock solutions were prepared for Aβ 1-40, curcumin and capsaicin. For the Aβ stock, Aβ 1-40 (1 mg) (Anaspec)
was dissolved in NaOH (1 mL, 10 mM), and sonicated for 10 min to ensure Aβ was solubilized and monomeric. The Aβ stock was kept at 4 °C at all times. Stock solutions for curcumin (Sigma-Aldrich) and capsaicin (Sigma-Aldrich) were prepared by dissolving 1 mg of each spice respectively in Milli-Q water (10 mL).

Next, a total of four working solutions were prepared: a control Aβ solution, a 1:2 mixture of Aβ and curcumin, a 1:2 mixture of Aβ and capsaicin, and finally a 1:2:2 mixture of Aβ, curcumin, and capsaicin. For each working solution (350 μL) the stock solutions were diluted in phosphate buffer (20 mM) and the final concentration of Aβ was 50 μM, while the spices had final concentrations of 100 μM. The pH of each solution was adjusted to 7.3 using trifluoroacetic acid (10 mM), and placed in individual 1 mm CD cells (Hellma), capped, and sealed using Teflon™ tape. CD measurements were made for each sample at four different time points: 0 hr, 24 hr, 72 hr, and 96 hr. Measurements were taken every 1 nm from 175 nm and 270 nm; each sample was measured three times at each time point, and the CD data was averaged. Between measurements, the samples were allowed to age at room temperature.

Following the conclusion of the previous CD experiment, we decided to repeat the experiment using a different solvent. Curcumin and capsaicin have low solubilities in water, but these spices are soluble in 30% hexafluoroisopropanol (HFIP) aqueous solutions. For this experiment, the Aβ stock solution (200 μM) was prepared by dissolving Aβ 1-42 (0.45 mg) (Anaspec) in HFIP (525 μL) (Sigma-Aldrich). This solution was then sonicated for 10 min to ensure Aβ was solubilized.
and monomeric and kept at 4 °C. The curcumin stock solution (8.7 mM) was prepared by dissolving curcumin (3.2 mg) in HFIP (1 mL) and vortexed. For the capsaicin stock solution (3.9 mM), capsaicin (0.6 mg) was also dissolved in HFIP (0.5 mL) and vortexed. Next, a total of four working solutions were prepared: a control Aβ solution, a 1:2 mixture of Aβ and curcumin, and a 1:2 mixture of Aβ and capsaicin. For each working solution (350 μL) the final concentration of Aβ was 50 μM, while the spices had final concentrations of 100 μM. In addition, each solution was diluted in phosphate buffer (20 mM) with HFIP added so that the final HFIP concentration in each sample was 30%. The pH of each solution was then adjusted to 7.3 using NaOH (10 mM), and placed in individual 1 mm CD cells, capped, and sealed using Teflon™ tape. CD measurements were made as previously described for each sample at four different time points: 0 hr, 24 hr, 72 hr, and 7 days. In between measurements, the samples were allowed to age at room temperature.

7.2.2 Spice study NMR

In order to observe the effects of curcumin and capsaicin on Aβ, 1D proton NMR spectroscopy was utilized. First a fresh stock solution of Aβ (220 μM) was prepared by dissolving Aβ 1-42 (0.5 mg) (Anaspec) in deuterated hexafluoroisopropanol (500 μL) (dHFIP). This solution was sonicated for 10 min to ensure the Aβ was solubilized and monomeric, then kept at 4 °C. Stock solutions of capsaicin and curcumin were also prepared at 1 mM in dHFIP. A working solution of Aβ 1-42 (50 μM) and capsaicin (100 μM) (Sigma-Aldrich) was prepared by
diluting the respective stock solutions in deuterated phosphate buffer (20 mM) and kept at 4°C. Similarly, a working solution of Aβ 1-42 (50 μM) and curcumin (100 μM) (Sigma-Aldrich) was prepared as the Aβ and capsaicin solution. The pH of these working solutions was brought to 7.3 using NaOD (10 mM).

In addition, three control solutions of Aβ 1-42 (50 μM), capsaicin (100 μM), and curcumin (100 μM) respectively were prepared by diluting the respective stock solutions in deuterated phosphate buffer (20 mM) and kept at 4 °C. As was done before, the pH of these control solutions was brought to 7.3 using NaOD (10 mM).

One-dimensional 1H NMR experiments were performed on all of the above samples using a Bruker Ascend 500 NMR with an ADVANCE III HD probe. Each sample was kept on ice prior to NMR analysis, and each experiment was run for 256 scans in order to provide greater signal to noise ratios. All data was processed using MNOVA.

7.2.3 AFM

The size of Aβ aggregates were measured using AFM. The first step in this experiment was to determine the optimal concentration of Aβ to be used in subsequent trials. If the Aβ concentration is too high, it could produce structures that are too large to accurately measure via AFM, and if the concentration is too low
then any structures that Aβ produces could potentially be too small to be observed over the background noise.

A fresh Aβ 1-42 (Anaspec) stock solution (200 μM) was prepared in NaOH (10 mM) as previously described. Next, three different Aβ dilutions (100 μM, 10 μM, 1 μM) were prepared via serial dilutions in phosphate buffer (20 mM). The pH of each solution was adjusted to 7.3 using TFA (10 mM). The three solutions were then aged at room temperature for a period of 9 days.

Each sample (10 μL) was then dropped directly onto the center of a freshly cleaved muscovite mica chip, and allowed to stand for 1 min at room temperature. Care was taken to avoid contact of the sample with the edges of the mica chip because a large amount of mica dust is present on the edges of mica chips. After standing for 1 min, the mica chips were tilted and a Kim-wipe™ was placed underneath. The mica chips were then carefully rinsed with Milli-Q water (2 x 100 μL) that was dropped directly on the center of the chip. This step removed unbound peptide, as well as the salts present from the phosphate buffer. Next, the mica chips were dried using a stream of nitrogen gas. Upon drying, the samples were then imaged using a Nanoscope IIIa controller (Digital Instruments) equipped with a multimode scanning probe microscope. All measurements were carried out in a blind fashion; the technician acquiring the images did not know the identity of each sample, and was tasked to acquire images that were representative of each sample. The measurements were acquired in the Tapping Mode™ using ambient conditions.
using an etched single-beam silicon cantilever (125 μm), with a nominal spring constant (20 – 100 N/m). The following settings were also used: RMS drive amplitude of 30 -300 mV, set point amplitude of 0.5 – 1.2 V, drive frequency of 270 – 300 kHz, scan rate of 1 – 3 Hz. The protocol was derived from the thesis of Dr. Rehka Srinivasan Ph.D.

Once the optimal Aβ concentration for AFM was determined, the effect of curcumin and capsaicin on the size of Aβ aggregates was studied. Three different samples were prepared: an Aβ 1-42 control (10 μM), a mixture of Aβ 1-42 (10 μM) with curcumin (20 μM), and a mixture of Aβ 1-42 (10 μM) with capsaicin (20 μM). The solution of each sample contained 30% HFIP, in order to ensure the spices stayed in solution with Aβ. In this case, the Aβ stock solution was prepared by dissolving Aβ 1-42 (1 mg) into HFIP (1 mL). It was crucial that the Aβ stock was prepared in HFIP and not NaOH in order to prevent dramatic fluctuations in pH, which could easily cause Aβ 1-42 to prematurely aggregate, given the need for each sample to be in 30% HFIP. As was done before, the pH of each solution was adjusted to 7.3 using NaOH (10 mM) because each sample was initially acidic. Finally, the three solutions were then aged at room temperature in 1 mm CD cells that were sealed using Teflon™ tape. While the samples aged, all three were examined by CD in order to monitor the progression of peptide aggregation. At the end of 9 days, all three samples were prepared for AFM analysis as described above using freshly cleaved mica, Milli-Q water rinses, and a nitrogen stream to dry the samples. Once dried, the samples were imaged using AFM as previously described.
7.2.4 Methods for the CD and NMR examination of additional spices

Upon completion of the initial CD and NMR studies on curcumin and capsaicin, we examined the potential of three additional compounds to prevent Aβ aggregation. The additional compounds were trans-anethole, tartaric acid, and acetic acid, which are found in the spices anise and tamarind. These compounds were identified as potential therapeutics from a bioinformatics study performed by Swathi Srinivasan.

For these additional studies, a fresh Aβ stock solution (318 μM) was prepared by dissolving Aβ 1-42 (1.1 mg) (Anaspec) in HFIP (0.75 mL). This solution was sonicated as previously described in order to ensure that the peptide was completely monomeric. Next, stock solutions for each of the previously listed compounds were prepared as well as a stock solution for curcumin. The stock solutions for trans-anethole (5 mM) (Sigma-Aldrich) and acetic acid (5 mM) (Sigma-Aldrich) were prepared by diluting these compounds in HFIP and Milli-Q water respectively. The stock solution for tartaric acid (7.9 mM) was prepared by dissolving tartaric acid (11.9 mg) (Sigma-Aldrich) in Milli-Q water (10 mL). Lastly, the stock solution for curcumin (1 mM) was prepared by dissolving curcumin (3.7 mg) in Milli-Q water (10 mL). The stock solutions for tartaric acid and curcumin were not prepared at the same concentrations as those for trans-anethole and acetic acid due to the difficulty of measuring exact amounts at such small scales.
Next, working solutions were prepared for each of the four compounds plus an Aβ control. These solutions were prepared with a 1:2 ratio between Aβ 1-42 (50 μM) and each individual compound (100 μM) in phosphate buffer (20 mM) with 30% HFIP. Curcumin and trans-anethole were the only compounds that have low water solubilites, but the decision was made to include 30% HFIP in each sample in order to remain consistent among all of the tested compounds. The pH of each sample was then adjusted to 7.3 using NaOH (10 mM). The samples were then placed in individual 1 mm CD cells, capped, and sealed using Teflon™ tape. CD measurements were made for each sample at four different time points: 0 hr, 24 hr, 48 hr, and 72 hr. In between measurements, the samples were allowed to age at room temperature.

In addition to CD analysis, each of the above four compounds, in conjunction with Aβ 1-42, were also examined via 1D ¹H NMR. New stock solutions for Aβ 1-42, trans-anethole, acetic acid, tartaric acid and curcumin were prepared as previously described with the modification of preparing each stock solution using deuterated solvents. Next, working solutions were prepared for each of the four compounds plus an Aβ control. These solutions were prepared with a 1:2 ratio of Aβ 1-42 (50 μM) to each individual compound (100 μM) in deuterated phosphate buffer (20 mM) with 30% dHFIP. The pH of each sample was then adjusted to 7.3 using NaOD (10 mM).
Finally, one-dimensional $^1\text{H}$ NMR experiments were performed on all above samples using a Bruker Ascend 800 NMR spectrometer with an ADVANCE III HD probe. Each sample was kept on ice prior to NMR analysis, and each experiment was run for 256 scans in order to provide greater signal to noise ratios. All data was processed using MNOVA.

### 7.3 Results

#### 7.3.1 Aqueous solution CD results

During the initial experiment in this study, we examined the ability of curcumin and capsaicin, two compounds found in the spices of traditional Indian food, to inhibit $\text{A}\beta$ aggregation using CD. Both of the compounds were tested at a 2:1 spice to $\text{A}\beta$ 1-40 ratio in aqueous solutions. Additionally, a mixture of both compounds was examined for their combined ability to inhibit $\text{A}\beta$ aggregation. The reasoning behind preparing this mixture was that these spices are typically found together in a variety of different foods. After examining the progress of $\text{A}\beta$ aggregation in all of these samples over the period of four days (96 hr), it was apparent that curcumin and capsaicin both aided in preventing $\text{A}\beta$ 1-40 from progressing from its initial random coil conformation to a $\beta$-sheet conformation, that indicates the first stages of aggregation. In each of the four CD spectra of Figure 7.1, the control sample of $\text{A}\beta$ 1-40 has the highest minimum found at 195 nm.
Similarly, the same trend is found in each of the spectra for the other samples of curcumin, capsaicin, and the mixture of the two. In each case, curcumin has the lowest minimum, followed by the mixture of spices, and finally capsaicin. These results suggest that these compounds aid in preventing Aβ aggregation.

7.3.2 CD results in 30% HFIP

While our initial CD results demonstrated the abilities of curcumin and capsaicin to inhibit Aβ aggregation, a few additional observations were made. While Aβ did not aggregate in any of the samples in which a spice was added, the control sample of Aβ 1-40 also did not fully transition to a β-sheet configuration, indicating that the samples were not examined for a sufficient amount of time. Additionally, while the spices did appear to have an effect on Aβ, both curcumin and capsaicin have low water solubilities. At the end of this experiment, precipitates of the spices were seen at the bottom of the CD cuvettes. Finally, the mixture of both curcumin and capsaicin did not demonstrate greater ability to inhibit Aβ aggregation; rather the curve for the mixture was between those of curcumin and capsaicin.

To address these issues, a few changes were made and the experiment was repeated. Instead of Aβ 1-40, the more pathogenic and aggregation prone Aβ 1-42 was utilized. The solvent used in the experiment was also changed from purely Milli-Q water, to 30% HFIP in Milli-Q water in order to ensure that curcumin and capsaicin remained in solution for the entire duration of the experiment.
Figure 7.1 CD spectra of 50 μM Aβ 1-40 (purple), 100 μM curcumin + 50 μM Aβ 1-40 (black), 100 μM capsaicin + 50 μM Aβ 1-40 (red), and mixture of 100 μM curcumin + 100 μM capsaicin + 50 μM Aβ 1-40. In each case, the curve for the Aβ 1-40 control has the highest minimum, indicating it is farthest along in its transition to β-sheet.
When the previous experiment was repeated using 30% HFIP, significant differences were observed among the three samples tested. Over the course of seven days, the Aβ 1-42 control transitioned to a β-sheet conformation. Unlike the previous study in which Aβ was initially in a random coil conformation when in a purely aqueous environment, in this experiment Aβ started out in an alpha-helix conformation. This difference in conformation due to solvent effects is well understood for Aβ (141) and not the result of some odd occurrence.

The Aβ control (Figure 7.2) demonstrates that during the experiment, the peptide gradually transitioned from random coil to β-sheet over the course of 72 hr, and by seven days the peptide had completely transitioned to a β-sheet conformation. As expected, the curcumin sample showed no signs of Aβ aggregation over the course of the experiment. One data point that should be noted in the Aβ control, as well as the curcumin samples, is the 48 hr time point. In both cases, at 48 hr, the curve does not match the trend observed at the other time points. In the Aβ control, the peptide slowly transitioned to β-sheet, while in this case it remained essentially at the same point as an α-helix for the curcumin sample. It is possible that changes in the environment around the CD machine are responsible for these readings at this time point.

In this experiment, the capsaicin sample (Figure 7.2) is notably different from the other samples. In this case, it appears that capsaicin immediately caused the Aβ 1-42 to form β-sheet complexes and aggregate, given the noisy curve that
roughly correlates to a typical β-sheet CD curve. Most likely, the curves are noisy because Aβ aggregates had grown large enough to precipitate, thus obscuring the light pathway through the CD cell, and increasing the amount of noise in this sample. This result is in direct contradiction to our previous capsaicin findings, which indicates that an increase of capsaicin solubility has a significant impact on its interaction with Aβ.
Figure 7.2 CD spectra of 50 μM Aβ 1-42 (top left), 100 μM curcumin + 50 μM Aβ 1-42 (top right), and 100 μM capsaicin + 50 μM Aβ 1-42 (bottom left). The solvent for each of the samples is 30% HFIP in Milli-Q water. Over the course of seven days, the Aβ 1-42 control transitions to β-sheet, while the curcumin sample stays as α-helix. The capsaicin sample is in β-sheet, and had already begun precipitating out of solution at the 0 hr time point.
A comparison of the samples at three different time points can be seen in Figure 7.3. This representation clearly shows how curcumin inhibits aggregation in comparison to the Aβ control.

**Figure 7.3** Comparison of CD spectra of 50 μM Aβ 1-42 (black), 100 μM curcumin + 50 μM Aβ 1-42 (blue), and 100 μM capsaicin + 50 μM Aβ 1-42 (red) at 0 hr, 72 hr, and 7 days. The solvent for each of the samples is 30% HFIP in Milli-Q water. The spectra clearly demonstrate curcumin's ability to inhibit Aβ aggregation.
7.3.3 Curcumin and Capsaicin NMR Results

The downfield spectral region for curcumin, Aβ 1-42, and a 1:2 mixture of curcumin with Aβ 1-42 are shown in Figure 7.4. In the Aβ 1-42 spectra there are three well-resolved peaks just above 8 ppm that correspond to the His-2H signal. The spectra corresponding to Aβ 1-42 + curcumin shows that only two of these three peaks are present, and the middle of the three has been shifted up-field slightly. In addition, the peak at 8.3 ppm in the curcumin spectra also shifts up-field in the Aβ 1-42 + curcumin spectra. This peak likely corresponds to the alcohol proton found in the enol form of curcumin (Figure 7.5). These results are indicative of an interaction between curcumin and the Aβ peptide.
Figure 7.4 Downfield 1H NMR spectra region (500 MHz) of 100 μM curcumin, 50 μM Aβ 1-42 + 100 μM curcumin, and 50 μM Aβ 1-42. Signals that experienced a change in chemical shift are connected with dotted lines. The His-2H signal in the Aβ 1-42 with an asterisk is no longer seen in the Aβ 1-42 + curcumin spectra.
Figure 7.5 The enol form of curcumin. The peak seen in the curcumin NMR spectrum just above 8.3 ppm is likely the middle alcohol proton in the above structure.

The downfield spectral region for capsaicin, Aβ 1-42, and a 1:2 mixture of capsaicin with Aβ 1-42 are shown in Figure 7.6. In the Aβ 1-42 spectra there are three well-resolved peaks just above 8 ppm that correspond to the His-2H signal. As previously observed when Aβ was combined with curcumin, the spectra corresponding to Aβ 1-42 + capsaicin shows that only two of these three peaks are present, and the middle of the three is slightly shifted up-field. In addition, the peak above 8.3 ppm in the capsaicin spectra also shifts up-field in the Aβ 1-42 + capsaicin spectra. This peak corresponds to the amide proton of capsaicin. These results are indicative of an interaction between capsaicin and the Aβ peptide.
Figure 7.6 Downfield 1H NMR spectra region (500 MHz) of 100 µM capsaicin, 50 µM Aβ 1-42 + 100 µM capsaicin, and 50 µM Aβ 1-42. Signals that experienced a change in chemical shift are connected with dotted lines. The His-2H signal in the Aβ 1-42 with an asterisk is no longer seen in the Aβ 1-42 + capsaicin spectra.

7.3.4 AFM results

After obtaining CD and NMR results that examined the interaction between curcumin and capsaicin with Aβ, we employed atomic force microscopy in an effort to directly observe the physical effects of these spices on Aβ aggregates. As the
samples for AFM analysis aged, the progress of Aβ aggregation was monitored using CD. After aging for nine days, it was observed that Aβ 1-42 control (Figure 7.7) had begun to aggregate and the decision was made to image the samples using AFM. As had been observed in the previously described CD experiment, the capsaicin sample showed that it progressed to the point of producing insoluble aggregates, while the curcumin sample showed no signs of aggregation.

Figure 7.7 CD comparison of Aβ 1 – 42 (black), curcumin (blue) and capsaicin (red) after each sample had been aged for 9 days.
After AFM imaging, the results reflected what was observed via CD (Figure 7.7). The AFM image of the Aβ 1–42 control (50 µM) (Figure 8) shows large aggregates that have a maximum height of 5 nm, that are likely Aβ pre-fibrils formed when Aβ oligomers begin to start assembling into larger structures.

![AFM image of 50 µM Aβ 1-42 aged 9 days.](image)

**Figure 7.8** AFM image of 50 µM Aβ 1-42 aged 9 days. The large structures (yellow) are Aβ aggregates.

The AFM results for the capsaicin (100 µM) + Aβ 1-42 (50 µM) sample also clearly reflected the CD results. It was observed via CD that this sample had not only aggregated, but had formed oligomers large enough that they began to precipitate
out of solution. Aβ fibrils are clearly visible (Figure 7.9) in the AFM image. Contrary to the aggregates observed in the Aβ 1-42 control, the aggregates in this sample are much more highly organized.

**Figure 7.9** AFM image of 100 μM capsaicin + 50 μM Aβ 1-42 aged 9 days. Aβ fibrils are clearly visible (yellow).

Finally, the curcumin (100 μM) + Aβ 1-42 (50 μM) sample also reflected the observations made via CD. The AFM results for the curcumin sample (Figure 7.10) did not display the large aggregates that were seen in the Aβ 1-42 control as well as
the capsaicin sample. Instead, smaller, approximately spherical-shaped structures are observed, which are likely early Aβ oligomers.

Figure 7.10 AFM image of 100 µM curcumin + 50 µM Aβ 1-42 aged 9 days. The yellow structures are likely Aβ oligomers.

7.3.5 CD examination of additional spices

Upon completion of the previous analysis of the ability of curcumin and capsaicin to inhibit Aβ aggregation, three additional compounds were studied and compared to curcumin. These compounds were acetic acid, anethole and tartaric
acid, which are components of the spices chili pepper, anise and tamarind. This particular experiment was terminated after 72 hr due to the fact that the Aβ 1–42 control had already started to precipitate at the 48 hr time point, and no changes were observed in any of the spices.
Figure 7.11 CD spectra of 50 μM Aβ 1-42 (top left), 100 μM acetic acid + 50 μM Aβ 1-42 (top right), 100 μM anethole + 50 μM Aβ 1-42 (bottom left), and 100 μM tartaric acid + 50 μM Aβ 1-42 (bottom right). The solvent for each of the samples is 30% HFIP in Milli-Q water. Between the 24 and 48 hr time points, the Aβ 1-42 control started to precipitate (Figure 11), and had almost completely fallen out of solution by 72 hr. In contrast, acetic acid, anethole, and tartaric acid all prevented the Aβ in solution with from aggregating.
Figure 7.12 Comparison of CD spectra of 50 μM Aβ1-42 (black), 100 μM acetic acid + 50 μM Aβ1-42 (red), 100 μM anethole + 50 μM Aβ1-42 (blue), 100 μM curcumin + 50 μM Aβ1-42 (purple), and 100 μM tartaric acid + 50 μM Aβ1-42 (green) at each of the time points.

A comparison of each of the compounds plus curcumin, versus the Aβ1-42 control can be seen above in Figure 7.12. This comparison shows that while the Aβ control rapidly aggregates within 48 hr, when Aβ is in solution with each of these spices it remains unchanged in an α-helix secondary conformation. It is also notable that none of the spices distinguish themselves in their effectiveness of preventing Aβ aggregation.
The final experiment of this project involved the acquisition of a series of proton NMR spectra for each of the spices that were tested. A comparison of the downfield region for each of the compounds can be seen below in Figure 7.13. In each of the shown cases, the respective spice had an effect on both of the histidine signals (His-2H, and His-4H) of Aβ 1-42. The chemicals shifts of these protons have shifted up-field to vary degrees when Aβ 1-42 was in the presence of a spice. Slight shifts in the signals for Phe and Tyr-3,5H can also be observed. Finally, the peaks for the various aromatic protons in Aβ 1-42 located 6.8 and 7 ppm are not as well resolved in the cases of acetic acid, anethole, and capsaicin. It is important to note that each of the samples were prepared identically and at the same time, and that all of the spectra were acquired within several minutes of each other.
Figure 7.13 Comparison of $^1$H NMR spectra for 50 μM Aβ 1-42 (A), 100 μM acetic acid + 50 μM Aβ 1-42 (B), 100 μM anethole + 50 μM Aβ 1-42 (C), 100 μM capsaicin acid + 50 μM Aβ 1-42 (D), 100 μM curcumin + 50 μM Aβ 1-42 (E), and 100 μM tartaric acid + 50 μM Aβ 1-42 (F). Signals that experienced a change in chemical shift are connected with dotted lines.

7.4 Discussion

Curcumin’s ability to act as an Aβ aggregation inhibitor has been previously studied and is well understood (199,200,201). The goal of this project was to examine compounds found in other spices that have shown a potential association
with preventing the onset of AD and to compare their effectiveness to that of curcumin. Initially, another goal of this project was to determine if a mixture of spices could potentially have an additive effect on preventing Aβ aggregation. This concept originated from the fact that food is prepared using a combination of spices in order to enhance flavor. Thus, if individual spices showed small effects on Aβ aggregation, then a combination of spices would show a cumulative effect. However, our results did not demonstrate such a trend. In our initial pilot study that was designed to compare capsaicin’s potential for Aβ aggregation to that of curcumin, a mixture of the two spices was also examined. As was expected, curcumin was very effective at preventing the aggregation of Aβ 1-40 over the course of 96 hr. Capsaicin also showed the ability to inhibit Aβ aggregation, albeit to a lesser extent than that of curcumin. Interestingly, the mixture of the two spices did not show a cumulative effect on inhibiting Aβ aggregation; instead the CD curves for the mixture seemed to be almost an average of those of curcumin and capsaicin at all time points. Thus, it appeared that the spices did not work synergistically. Rather, it seemed as if capsaicin’s lesser ability to inhibit Aβ aggregation actually negatively effected the impact that curcumin had on inhibiting aggregation.

At the end of this initial pilot study a few additional observations were made. Particularly, at the end of the experiment the two spices had noticeably precipitated to some extent. Consequently, their respective stock solutions were warmed in order to enhance their solubility. When these stock solutions returned to room temperature, no precipitation was observed. In particular curcumin, which is a
yellow-orange compound, produced a solution of the same color. As was previously noted, while precipitates were observed at the end of the 96 hr CD experiment, the samples containing curcumin still remained a faint yellow-orange color, indicating that there was still curcumin in solution. However, given that at least a portion of the spices fell out of solution, we decided to utilize 30% HFIP in Milli-Q water in the follow-up experiment in an effort to keep the spices in solution. Initially, a few other alternatives to HFIP were tested, one of which was DMSO. In the DMSO trial runs, a drop of DMSO was added to the working solutions in order to enhance the solubility of the spices. This proved to be extremely effective at keeping the spices in solution; however, the DMSO in solution completely obscured the CD results at the smaller wavelengths (data not shown). It was ultimately decided to use 30% HFIP because it would keep curcumin and capsaicin in solution, and the Aβ peptide has previously been studied by CD in solutions containing HFIP. In the presence of HFIP monomeric Aβ adopts a α-helix secondary conformation instead of its usual random coil conformation in purely aqueous solutions. This is the only difference the change in solvent has on Aβ however; the peptide still aggregates as it normally would, and aggregation can be tracked via CD by observing the transition to a β-sheet secondary conformation.

In addition to changing the solvent to 30% HFIP, we decided to change from the Aβ 1-40 peptide to the Aβ 1-42 peptide. This decision was made because in the previous experiment the Aβ 1-40 peptide had not yet adopted a β-sheet conformation at the end of 96 hr. The Aβ 1-40 peptide was used initially because it
is a much easier peptide to handle given it is less aggregation prone. This characteristic, while useful in sample preparation, proved detrimental in the pilot study because the Aβ did not aggregate. Using the Aβ 1 – 42 peptide has the added benefit of being the more pathogenic peptide to study; if a spice is able to inhibit Aβ 1 – 42 aggregation, it is potentially better suited as a therapeutic agent.

The results from this subsequent experiment using curcumin and capsaicin in 30% HFIP had an unexpected result (Figure 2). Both the Aβ 1 – 42 control and curcumin behaved as expected. Over the course of the 7 day experiment, the Aβ control gradually transitioned from its initial α-helix conformation to a β-sheet conformation as it aggregated. Similarly, the Aβ 1 – 42 in the curcumin sample consistently remained in a α-helix conformation, with only minor deviations in the CD measurements. The capsaicin sample, however, did not produce results similar to those seen when this spice was in water. Instead, the CD results show that even at the initial 0 hr time point, capsaicin caused the Aβ 1 – 42 in the solution to aggregate. The curve seen in the capsaicin plot roughly corresponds to that of a β-sheet, but more importantly, it is very noisy. This is likely an indication that insoluble Aβ oligomers that were in suspension obscured and scattered the light that was passing through the CD cuvette. Taken together, these results indicate that curcumin inhibited Aβ aggregation in relation to the control, while capsaicin actually accelerated the rate at which Aβ aggregated.
Following the previous CD results, 1D proton NMR spectra were acquired for freshly prepared Aβ, curcumin and capsaicin samples. This experiment was carried out in an effort to elucidate specific interactions these spices have with the Aβ peptide. The NMR results showed that both curcumin and capsaicin interacted with the His-2H protons of Aβ, given that the corresponding peaks experienced up-field shifts. In addition to the changes in chemical shifts of these peaks, the peak for one of the three His-2H protons was no longer visible when Aβ was in the presence of both curcumin and capsaicin. No other significant changes were observed for the Aβ signals. The changes observed in Aβ’s His-2H signals indicate that both curcumin and capsaicin are interacting with these corresponding protons in some manner.

The fact that both curcumin and capsaicin show similar NMR results, but drastically different CD results, begs the question: why did curcumin inhibit Aβ aggregation but capsaicin aided Aβ aggregation in our experiments. The answer may lie in the structures of these two compounds. Curcumin has hydrophilic groups on both ends of its structure, as well as an enol group between the two phenyl groups. On the other hand, while capsaicin shares a methoxyphenol group with curcumin, it also has a hydrophobic hydrocarbon tail. It may be possible that this hydrophobic tail may promote aggregation of Aβ, which is considered a hydrophobic peptide. In order elucidate how these spices are interacting with histidine and producing their observed CD results, further experimentation is required. STD NMR, discussed in previous chapters, could be used to further examine the ligand-receptor interaction occurring between these spices and Aβ.
To further examine the effects curcumin and capsaicin have on Aβ, fresh samples were prepared, aged, and imaged using AFM. When working with Aβ, and especially Aβ 1–42, it is crucial that the Aβ stock is freshly prepared each time because the peptide may behave differently if minute amounts of aggregate seeds are present. Thus it is never advisable to use Aβ from multiple sources in a single experiment or to compare newly acquired results to older Aβ controls. Prior to AFM analysis, the three different samples, Aβ 1-42 control, curcumin + Aβ, and capsaicin + Aβ, were aged for several days and monitored via CD. After nine days, it was decided to proceed to AFM analysis. This decision was based on the fact that the Aβ 1–42 control had started to aggregate (Figure 7), while the curcumin sample was still monomeric. CD analysis also showed that the capsaicin sample had caused Aβ to form insoluble aggregates, as was seen in the prior CD experiments. Upon imaging, the AFM results (Figures 8–10) reflected what was observed via CD. Large aggregated structures were visible in the Aβ 1–42 control, and the capsaicin sample clearly showed Aβ fibrils. The curcumin sample also displayed smaller structures which are likely Aβ oligomers. This result did not reflect the observation made with CD that showed the Aβ in the curcumin sample in a monomeric state. However, the presence of these Aβ oligomers could be explained by the method of preparing samples for AFM. AFM is not performed in solution; it requires the sample to be dried and adhered to the surface of the mica chip. One of the primary factors that leads to Aβ aggregation is concentration of the peptide. When a sample of Aβ in solution is dried, the concentration of Aβ increases as the volume of the solution decreases. Thus, it is possible that as the curcumin sample was dried for
AFM analysis, the Aβ in solution began to aggregate as its concentration spiked. Despite the presence of the oligomers in the AFM image of the curcumin sample, this result still reflected the observations made with CD just prior to the preparation of the AFM sample. The formation of Aβ oligomers preceded the formation of prefibrils and fibrils; thus the curcumin AFM result reflects prior observations made via CD.

Following the initial studies aimed at only curcumin and capsaicin, a bioinformatics study of natural products performed by Swathi Srinivasan identified several different spices as having potential therapeutic benefits for Alzheimer’s Disease. This study identified four spices, turmeric, chili pepper, anise and tamarind, as promising candidates for AD therapeutics. From these four spices, five compounds in particular were singled out: acetic acid, anethole, tartaric acid, as well as curcumin and capsaicin. Given curcumin and capsaicin had already been studied extensively, we decided to focus on the three new compounds. Since curcumin had shown promising results in our prior studies, it was also analyzed concurrently the new compounds in order to rule out the potential that slight differences in sample preparation were responsible for the new observations.

The first experiment performed on these additional compounds was CD. In this round of CD analysis, the Aβ 1–42 control aggregated by the 48 hr time point, and had almost completely precipitated out of solution by 72 hr. During the prior CD experiment, the Aβ 1–42 control did not aggregate until 7 days after it had been
prepared. This once again demonstrates the importance of always comparing results to an Aβ control that was prepared and analyzed concurrently. However, despite the fact that samples for acetic acid, anethole, curcumin, and tartaric acid were prepared with an Aβ stock that aggregated much faster than in prior experiments, over the course of 72 hr none of these samples showed signs of Aβ aggregation. A comparison of these compounds (Figure 12) shows that they were all equally effective at preventing Aβ aggregation in comparison to the control. It should also be noted that while these three new compounds are water soluble, they were all analyzed in a 30% HFIP solution. This allowed consistency with our prior analyses, as well as the ability to directly compare their results to that of the curcumin sample that was also in this round of experiments.

Finally, the last experiment performed in this study was another series of proton NMR experiments. Once again, fresh samples were prepared for all four of the compounds in addition to a control. Similarly to the previous NMR results, all of the new compounds had an effect on the His-2H protons of Aβ. In each case, the His-2H signals shifted up field. The signals for the His-4H protons also experienced up field shifts in all of the compounds to varying degrees. In addition to these histidine signals, the signals for Phe and Tyr-3,5H experienced slight changes in their chemical shifts. A possible explanation for these changes in chemical shifts during this experiment but not in prior NMR analyses could be due to the higher 800 MHz field strength used in these experiments. Higher NMR field strengths lead to greater resolution of individual peaks, as well as signal to noise. Again, in order to gain a
greater understanding as to how acetic acid, anethole, and tartaric acid specifically interact with Aβ, additional STD NMR experiments need to be performed.

While our results show some promise in demonstrating that the spices turmeric, chili pepper, anise and tamarind may be able to prevent Aβ aggregation in humans, further testing is required. The bioinformatics study that identified acetic acid, anethole and tartaric acid as having potential therapeutic effects did not specifically identify these compounds as Aβ aggregation inhibitors. Instead, these spices were identified as having an involvement in several different biochemical pathways that can lead to the onset of Alzheimer’s disease. It is entirely possible that these spices may aid in preventing AD, but their mechanism of action is different from that which our project studied. Additionally, this study examined these compounds directly on the Aβ peptide, and not any possible metabolites of these compounds. Perhaps one of these spices can act as an Aβ aggregation inhibitor, but the compound responsible for the inhibition of aggregation is actually a metabolite. Finally, our study looked at the individual direct effects of these compounds on Aβ, which does not reflect how these spices are used in cuisine. In reality these spices are not individually consumed on their own, since a combination of spices are almost always employed when preparing food. Furthermore, these spices are typically cooked under high heat, and in some instances, pressure. These variables that could significantly alter the chemistry of these spices were not examined in our experiments, but offer an interesting new area that can be explored in future studies.
Chapter 8

Conclusions and Future Directions
8.1 Conclusions and significance

The connection between the Aβ peptide and AD is widely accepted; however, the complete mechanism in which the Aβ peptide begins to aggregate in the brains of patients with AD is not completely understood. Previous studies of the aggregation of the Aβ peptide have only been performed in vitro, which does not provide a good representation of in vivo conditions. The primary objective of the research covered in this thesis was to use NMR to examine the Aβ peptide in living human cells (in vivo), a feat that had yet to be accomplished. Additionally, prior in vivo studies have established a connection between Aβ accumulation and cellular organelle dysfunction using fluorescent imaging. This thesis expanded upon these observations using NMR to investigate the binding interactions between the Aβ peptide and isolated organelles. The objective of these studies was to gain a greater understanding of the Aβ peptide in its native intracellular environment in an effort to elucidate further areas of inquiry that could lead to future therapeutic options.

The second objective of this thesis was to examine several different compounds that could act as Aβ aggregation inhibitors. Efforts were made to determine if melatonin, the experimental drug IXR4204, and several natural products could act as Aβ aggregation inhibitors. This area of research is significant because the identification of a compound that acts as an early Aβ aggregation inhibitor could provide clinical benefits and potentially lead to the development of the first drug that prevents Aβ aggregation in AD.
8.1.1 In-cell NMR observation of Aβ

Prior to attempting to observe intracellular Aβ using NMR, we first needed to confirm that the peptide was successfully introduced into viable cells. Previous work done by Dr. Fang Han of the Zagorski group had determined the optimal concentration of SLO to introduce the peptide into HeLa cells. The first objective of this thesis was to recreate these results, and potentially improve upon them. Through trial and error, flow cytometry results showed that it was possible to improve the previous SLO protocol, enabling introduction of labeled Aβ peptide into 57% of viable cells. This result was extremely significant given how difficult it is to detect Aβ within living cells using NMR; any increase in the amount of signal coming from the sample aids in its detection. Confocal microscopy was used to confirm that this improved SLO protocol introduced Aβ into the cell cytoplasm, as opposed to causing the Aβ to adhere to the plasma membrane.

Using the improved SLO protocol, successful detection of uniformly $^{15}$N labeled Aβ inside of living HeLa cells was seen by HSQC NMR. The Zagorski group is the first to successfully detect intracellular Aβ in this manner. The results acquired from this experiment indicated that the majority of the amino acids signals of Aβ had broadened to the point where they were no longer detectable, which indicates that Aβ is not adopting a folded structure *in vivo*, a finding that has not been observed in *in vitro* experiments. Many previous in-cell NMR studies have experienced similar losses in NMR signals due to extensive signal broadening.
The signal broadening of many in-cell NMR experiments has been attributed to non-specific and specific interactions between the protein being studied and intracellular targets. Non-specific interactions include interactions between the protein and various macromolecules, the cytoskeleton, and nucleic acids, which have the effect of slowing the tumbling rate of the protein, thereby decreasing the T2 relaxation rate that leads to broadened NMR signals.

The cell cytoplasm is a very crowded environment, therefore molecular crowding experiments using PEG were performed to examine the effects of non-specific binding interactions on Aβ. These experiments were unable to reproduce the in-cell NMR result. Taken in conjunction with the in-cell NMR results, this indicates that non-specific interactions do not account for the signal loss and broadening we observed from intracellular Aβ; instead it is likely that a specific binding interaction is occurring between Aβ and some unknown target.

Using PEG as a molecular crowding agent in 2D NMR experiments came with a significant drawback. Solutions composed of higher concentrations of PEG produced spectra that contained a great deal of noise originating from the PEG. At a PEG concentration of 30\% w/v, the intensity of the nearby PEG O-H signal drowned out any possible signals originating from the Aβ peptide. Efforts were made to suppress the PEG signal; however, these attempts proved to be unsuccessful. Given the difficulties that using PEG as a molecular crowding agent presented, alternative
crowding agents such as dextran or ubiquitin may have been better choices and warrant further investigation.

Rather than study the effects of non-specific binding interactions on the Aβ peptide using molecular crowding experiments, a better alternative may have been to use in-cell NMR to observe Aβ within *E. coli*. Observing the Aβ peptide inside a prokaryotic cell line would eliminate the possibility of Aβ interacting with its specific human intracellular targets. Such an experiment would allow for the study of non-specific binding interactions on intracellular Aβ exclusively. This technique has recently been utilized by the Barbieri laboratory to restore the in-cell NMR signals originating from PFN1 (208).

Given that non-specific binding interactions could not account for the loss of signal observed for in-cell Aβ, we examined if specific binding interactions occurs between Aβ and nuclei and mitochondria that were isolated using differential centrifugation. Previous in-cell NMR studies have found that specific interactions between the protein of interest and its physiological binding partners can lead to broadened NMR signals (205,212). We chose to examine nuclei and mitochondria for further examination based on the findings of several recently published studies that showed that Aβ accumulation is associated with dysfunction of these organelles (147,148,149153). Amyloid aggregates have been observed within the nuclei of neurons from post mortem AD patients, leading to a swelling of the nuclear envelope and fragmentation of the DNA contained inside (153). Mitochondrial
dysfunction is also closely associated with AD; Aβ has been observed in MVBs in close proximity to mitochondria, and within the mitochondria themselves in post mortem cortical brain slices of AD patients.

Our examination of the interaction between the Aβ peptide and cellular organelles showed that Aβ was indeed binding to the isolated organelles, given that no Aβ signal was detected when the supernatants of these samples were also examined by NMR. These experimental findings, taken in conjunction with the previous molecular crowding observations, suggest that specific binding interactions occurring between Aβ and targets on the surface of cellular organelles primarily account for the loss of signals that we observed for intracellular Aβ. In addition, the spectra acquired of these isolated organelles with Aβ showed some similarity to the previous in-cell results, indicating that previous observation of intracellular Aβ may be originating from Aβ bound to organelles inside the living cells. Further examination of the nuclei and mitochondria spectra demonstrated that the hydrophilic amino acids of Aβ were affected to a greater extent than the hydrophobic amino acids of Aβ. Perhaps more importantly, the sequence from Leu-17 to Ala-21, a region key for Aβ aggregation, was greatly affected by the binding interaction between Aβ and the cellular organelles.

Recently it has been shown that introduction of point mutations into a protein of interest and repeating in-cell NMR experiments on the mutated peptide in both eukaryotic and prokaryotic cells can lead to recovery of NMR signals.
Examination of the mutated peptide can lead to a greater understanding of the effects of non-specific and specific binding interactions on the protein (208). In light of the nuclei and mitochondria results, we hypothesized that a mutation in the Leu-17 to Ala-21 region of the Aβ peptide, a sequence crucial for aggregation, would prevent the peptide from binding to these organelles. Currently, the Zagorski group is working on examining the effects of such a point mutation on the ability of Aβ to bind to cellular organelles, with the creation of a F19A Aβ1 – 40 mutant. Unfortunately, the production of a uniformly ¹⁵N labeled F19A Aβ mutant is prohibitively expensive. Therefore, to allay the cost of producing the mutant peptide, we chose to produce an F19A Aβ mutant that was ¹⁵N labeled only from Leu-17 to Ala-21. We chose this region to study further as it is the sequence crucial to aggregation. The findings based on this mutant Aβ peptide and cellular organelles have proven to be inconclusive. No identifiable peaks were observed in the HSQC spectra taken of the mutant peptide and cellular organelles. This finding is an indication that the introduction of the point mutation inhibited the binding interaction between the mutant peptide and the organelles, as we hypothesized. However, these results cannot eliminate the possibility that the mutant peptide was tightly binding to the organelles, thus leading to the observed loss in NMR signals. Further experimentation is therefore needed using a more extensively ¹⁵N labeled F19A Aβ mutant. In the future, once the Zagorski group has acquired the new mutant Aβ, these experiments will be repeated to determine if this point mutation prevents specific binding interactions between the peptide and the cellular organelles. In addition, in order to conclusively test our hypothesis, it will be
necessary to examine the effect of mutations outside of the Leu-17 to Ala-21 region on the peptide’s ability to bind to cellular organelles.

As noted previously, point mutations can lead to a restoration of in-cell NMR signals (208). If a fully $^{15}$N labeled F19A Aβ mutant were to be acquired in the future, it would be informative to use in-cell NMR to observe if the mutation causes a restoration of in-cell NMR signals. Additionally, similar experiments could be conducted with different Aβ mutants, such as the E22K Italian mutation or the D23N Iowa mutation, that have been linked to FAD or early-onset AD. It would be interesting to observe if these more neurotoxic mutants would have a greater or lower number of in-cell NMR signals in comparison to a WT control. Given these mutant peptides greater neurotoxic properties, we would expect that their mutations would not inhibit their abilities to bind to cellular organelles. Unfortunately, while companies such as Anaspec produce various familial AD mutants, they do not offer uniformly $^{15}$N labeled versions of these peptides in their catalogs. It is likely that custom orders of uniformly $^{15}$N labeled versions of these mutants would also prove to be cost prohibitive with current funding sources.

While our results demonstrate that the Aβ peptide binds to isolated nuclei and mitochondria in in vitro, our findings do not prove that the same interaction occurs in vivo. The possibility that Aβ found in the cytosol does not produce the same interactions cannot yet be ruled out. In order to determine if these in vitro binding interactions were the result of an artifact in the experimental design, a
similar experiment would need to be performed examining the interaction between isolated organelles and an alternative protein, such as ubiquitin or alpha-synuclein. Alpha synuclein would be an interesting substitute due to the fact that, like Aβ, it is an intrinsically disordered protein that forms β-sheet structures as it aggregates. If no interaction was observed between the isolated organelles and a substitute protein, such findings would strengthen the case that Aβ specifically binds to nuclei and mitochondria, and that the observed binding interaction is not the result of Aβ indiscriminately sticking to anything it is in solution with.

In order to definitively determine the exact intracellular location of Aβ, a series of experiments would be necessary in which cells containing labeled Aβ peptide are lysed, the lysate is separated via differential centrifugation into its different components, and the fractions are analyzed for the presence of Aβ. Additionally, a series of stimulated-transfer echo (STE) diffusion NMR experiments could be performed on both intracellular Aβ and Aβ bound to isolated nuclei and mitochondria. STE NMR can be used to estimate the size of the compartment a protein is contained within, as well as determine if a protein is free in solution or bound to a surface. Several different groups have managed to find intracellular Aβ trapped in endosomes and other MVBs in the brains of human subjects, as well as in rodent models of AD (142,143). It is possible that once Aβ is introduced into living cells using SLO, the peptide finds its way into MVBs. Using STE NMR would help determine if the intracellular Aβ that I observed is either free in the cytosol, or contained within MVBs. This NMR technique could also be extremely useful in
determining if the Aβ that was detected is bound to nuclei and/or mitochondria, either on the surface of these organelles, or is within the organelles. Determination of the precise location of the Aβ could be valuable in identifying specific binding partners of Aβ and potential targets for therapeutic drugs.

Finally, I believe that experiments using fluorescent correlation spectroscopy (FCS) would be extremely useful to help determine binding partners of Aβ that are found in the membranes of nuclei and mitochondria. FCS cannot only determine if a protein is binding to a specific phospholipid or membrane protein, but also the binding affinity of the interaction. This technique could be used to find specific binding partners of Aβ, which could also help identify targets for future AD therapeutics.

8.1.2 Melatonin’s interaction with Aβ

The secondary goal of this thesis was to examine the potential of several different compounds to inhibit Aβ aggregation. The first of these experiments examined melatonin, a naturally produced hormone in the brain that had been previously considered protective effect against AD. Using STD NMR, it was ascertained that the 6H proton of melatonin is in close proximity to the 4H proton of histidine in Aβ, which could allow melatonin to interrupt the aspartic acid-histidine salt bridges that are necessary for Aβ to aggregate. The effect melatonin has on Asp-
23 in Aβ was also examined using $^{13}$C NMR, and it was found that melatonin had an effect on the signals for the Asp-23 carbonyls. This result was significant because it suggested that melatonin is capable of interrupting the histidine-aspartic acid salt bridges that aid in Aβ aggregation.

In the future, the binding interaction between melatonin and Aβ monomers and oligomers will be studied using isothermal titration calorimetry (ITC) and fluorescence spectroscopy. These techniques will be able to examine the kinetics of this binding interaction, and determine if melatonin preferentially binds to Aβ monomers over Aβ oligomers.

**8.1.3 IXR4204**

Early in my training in the Zagorski lab, our group was approached by Dr. Pasinetti of Mt. Sinai’s School of Medicine, New York, to test the ability of an experimental drug, IXR4204, to inhibit Aβ aggregation. First, the interaction between IXR4204 and Aβ was examined using HSQC NMR, and it was found that the drug caused several amino acid signals of the Aβ to shift, indicating that the drug was binding to Aβ. Next, the effect IXR4204 has on Aβ aggregation was examined using CD. Unfortunately, this experiment revealed that rather than inhibiting Aβ aggregation, IXR4204 accelerated Aβ aggregation. While this result suggested that this drug does not have the potential to be an aggregation inhibitor, it may still prove to be an AD therapeutic through its action as an RXR agonist.
8.1.4 Spice studies

Finally, several different spices that are commonly found in traditional Indian cuisine were examined for their potential to inhibit Aβ aggregation. Several different techniques, including CD, AFM and NMR, were employed in these studies. My results showed that curcumin, acetic acid, anethole, and tartaric acid all demonstrated the ability to inhibit Aβ aggregation in vitro using CD. NMR was used to learn more about the binding interaction of each spice with Aβ, and it was found that they all affected the histidines of Aβ. AFM was also used to physically observe the effect that curcumin and capsaicin had on the size of Aβ aggregates. My results may provide some explanation as to why the rate of AD in Indian populations is appreciably lower than that found here in the US.

While this study suggests a possible mechanism to explain how a diet of traditional Indian cuisine may protect against the development of AD, further experimentation must be carried out in order to determine if these spices are neuroprotective. The biggest shortcoming of this study was the solvent used to study the effect of these spices on Aβ aggregation. In order to dissolve curcumin and capsaicin, an aqueous solution containing 30% HFIP was required. This solvent was selected because of the well understood effect HFIP has on the secondary structure of Aβ as it aggregates. Rather than transitioning from random coil monomers to β-sheet oligomers, the Aβ peptide begins as α-helix monomers in HFIP and transitions to β-sheet oligomers as it aggregates. While this model has been
used to study Aβ aggregation in the past, it does not mimic in vivo conditions. Therefore, the effects of these spices on Aβ aggregation in vivo cannot be definitively determined using this experimental method.

In order to determine the specific manner in which these spices interact with the Aβ peptide, additional experimentation is required. 1D proton NMR experiments showed that these spices caused a chemical shift in the histidine residues of the Aβ peptide. In the future, STD NMR experiments can be performed to help characterize the interaction that occurs between the Aβ peptide and the spices. Additionally, ITC can be used to determine the $K_D$ of these binding interactions. It is likely that the spices tested are not equally capable of preventing Aβ aggregation, thus determination of their binding constants would allow for the identification of the most potent aggregation inhibitors.

This research can also be expanded upon in the future by observing the effects that several of these compounds in conjunction have on Aβ aggregation. In Indian cuisine, these spices are not consumed individually; many different spices are used in the composition of each dish. Additionally, when Indian food is prepared, these spices are cooked which could significantly alter their chemical structures. This study did not adequately simulate either of these realities; future experiments must take these factors into account.
Finally, in the future, EM, instead of AFM, could be used to examine the effects of these spices on Aβ aggregates. The downside of using AFM to study Aβ is the requirement that the peptide must be bound to the mica surface. In this study, this was accomplished by studying dried samples. This presented the problem that the concentration of Aβ peptide in solution to drastically increase as the sample dried, which could lead to the formation of Aβ aggregates that may have not been present in solution. In contrast to AFM, EM can be used to study molecules that are in solution, thereby eliminating the possibility that the aggregates observed in this study by AFM are artifacts that arose as part of the sample preparation.
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