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The impact of post translational modification on the aggregation of the β -amyloid peptide in the presence or absence of lipids

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**The impact of post translational modification on the aggregation of the β -amyloid peptide
in the presence or absence of lipids**

Albert Walton Pilkington IV

Dissertation submitted to the
Eberly College of Arts and Science
at West Virginia University

in partial fulfillment of the requirements for the degree of

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in
Chemistry

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Morgantown, West Virginia
2019

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methionine oxidation, oligomers, polymorphic fibrils,

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ABSTRACT

The impact of post translational modification on the aggregation of the β -amyloid peptide in the presence or absence of lipids

Albert Walton Pilkington IV

Alzheimer's disease (AD) is a neurodegenerative condition afflicting 5.7 million Americans with no effective treatments. The pathology of AD is described by the formation of neurotoxic oligomers formed by the self-assembly of amyloid- β ($A\beta$). The affinity of oligomers for cellular membranes disrupts calcium homeostasis and is believed to be the underlying cause of neurotoxicity; however, due to the metastability of oligomers, it has been difficult to understand their role in membrane interactions and self-assembly. Here we utilize post translational modifications (PTM) to understand the role of specific amino acids on $A\beta_{40}$ -membrane interactions and self-assembly. We employ ThT, AFM, CD, and polydiacetylene assays (PDA) to determine how PTMs of Met35, Lys28, and Lys16 affect the aggregation rates, population distributions, morphologies, and secondary structure of $A\beta_{40}$ in the presence or absence of a model membrane system, total brain lipid extract (TBLE). Our results show that oxidative environments promote the selective oxidation of Met35 (Met35[O]). Decreases in the magnitude of $A\beta_{40}$ aggregation was observed along with an inhibition of oligomer structural transitions to fibrils. In the presence of total brain lipid extracts (TBLE), Met35[O] showed increased aggregation rates relative to $A\beta_{40}$ and reduced peptide-membrane interactions. Isolation of oxidative effects on TBLE and $A\beta_{40}$ showed that the oxidation of the membrane was responsible for reductions in peptide-membrane interactions. Subsequent studies chemically acetylated Lys16 and Lys28 to understand how the removal of lysine's cationic properties alters $A\beta$ self-assembly and membrane-binding. Here we report that lower levels of acetylation, predominantly affecting Lys16, reduced fibrilization and oligomerization of $A\beta_{40}$ while higher levels of acetylation affecting both Lys16 and Lys28 dramatically inhibited fibril formation and reduced oligomerization. In the presence of TBLE, aggregation of $A\beta_{40}$ was observed even under higher levels of acetylation. Under the influence of the membrane, fibril aggregates of $A\beta_{40}$ were observed with higher levels of acetylation producing annular aggregates suggesting the promotion of secondary aggregation pathways. Additionally, the magnitude of the peptide-membrane interactions of acetylated $A\beta$ and PDA/TBLE vesicles were reduced but not absent. Collectively, these results highlight the role specific amino acids play in the assembly of $A\beta$, and how the presence of membranes modulates peptide-membrane interactions and influence aggregation pathways

Dedication

I dedicate this Ph.D. dissertation to my family

Albert Walton Pilkington III

Michelle Lynn Plumlee-Herold

Aaron Michel Pilkington

Austin Lynn Pilkington

Jenna Marie Pilkington

Emmaline Elizabeth Pilkington

Joseph Lynn Pilkington

Milena Grace Pilkington

and to the memory of...

Albert Walton Pilkington II

Gwen Dean Pilkington

with a special dedication to the love of my life

Alissa Lauren Gulette

It is only with all your love, kindness, and patience that I can achieve my dreams...

Now Thus Now Thus

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List of symbols and abbreviations

AcK16	acetylated K16
AcK28	acetylated K28
AD	Alzheimer's disease
ADDL	amyloid-beta derived diffusible ligands
AFM	atomic force microscopy
AOT	sodium bis(2-ethylhexyl) sulfosuccinate
APP	amyloid precursor protein
A β	β -amyloid
A β 40Met35[O]	sulfoxide β -amyloid 1-40
A β 40Ox	oxidized β -amyloid 1-40
A β O	A β oligomers
BODIPY	boron-dipyrromethene
CD	circular dichroism
CHC	central hydrophobic core
CSF	cerebrospinal fluid
DMSO	dimethyl sulfoxide
EM	electron microscopy
EPR	electron paramagnetic resonance
FAD	familial Alzheimer's disease
H ₂ O ₂	hydrogen peroxide
HFIP	hexafluoroisopropanol

HMW	high molecular weight
HNE	4-hydroxy-2-nonenal
IM-MS	ion-mobility mass spectrometry
K16	lysine-16
K28	lysine-28
L:P	lipid-to-peptide
LC-MS	liquid chromatography mass spectrometry
LMW	low molecular weight
LPO	lipid hydroperoxide
LUV	large unilamellar vesicles
Met35	methionine-35
MS	mass spectrometry
NHSA	sulfo-N-hydroxysulfosuccinimide acetate
NMR	nuclear magnetic resonance
PDA	polydiacetylene assays
PICUP	photo-induced cross-linking of unmodified proteins
PTM	post-translational modification
PXRD	Powder X-ray diffraction
RFss	relative fluorescence at steady state
RM	reverse micelles
ROS	reactive oxygen species
$R_{\text{slope}}(\text{h}^{-1})$	relative slope of growth phase

sAD	sporadic Alzheimer's disease
SAXS	small angle X-ray scattering
SCXD	single crystal X-ray diffraction
SDS	sodium dodecyl sulfate
SEC	size-exclusion chromatography
SEM	standard error mean
TBLE	total brain lipid extract bilayers
TBLE _{Ox}	oxidized total brain lipid extract
TFA	trifluoroacetic acid
ThT	thioflavin-T
TIC	total ion chromatogram
t _{lag}	lag-phase time
TMAFM	tapping mode atomic force microscopy

1. Introduction: Mutations, modifications, and the membrane's role in the aggregation of A β associated with Alzheimer's disease

Alzheimer's disease (AD) is characterized by the accumulation and deposition of fibrillar aggregates of the amyloid- β (A β) peptide into neuritic plaques. These amyloid deposits were thought to play a central role in AD; however, the correlation between plaque load and disease is weak. Increasing evidence supports the notion that a variety of small, globular aggregates of A β , referred to broadly as A β oligomers (A β O), may in fact be the primary culprits associated with neurotoxicity. The aggregation of A β proceeds via a nucleation-dependent polymerization mechanism that is heavily influenced by changes in peptide sequence, environmental conditions, and preparatory steps. Familial point mutations of A β produce the early onset of Alzheimer's disease but only account for a small fraction of the number of AD cases. Recently, the analysis of sporadic AD patient's cerebrospinal fluid revealed the presence of post-translationally modified forms of A β and may represent changes to the physiochemical properties of A β that promote secondary aggregation pathways and toxic mechanisms. Experimental evidence shows AD patients exhibit altered membrane compositions. The presence of membrane surfaces also possesses the ability to modulate the aggregation of A β , stabilize toxic intermediates such as A β O, and are themselves targets of A β 's toxicity. Ultimately, understanding the interplay between A β and membranes, with respect to direct effects on A β sequence, modification, and environmental factors is important in elucidating the underlying causes of Alzheimer's disease.

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is a fatal neurodegenerative disorder that is the most prevalent form of dementia. Neuropathological and neurochemical hallmarks of AD include: synaptic loss and selective neuronal cell death; decreases in markers for certain neurotransmitters; and abnormalities in neurons and their processes as well as in the extracellular space. Two of the main features associated with AD are neurofibrillary tangles comprised of the protein tau and cerebrovascular, diffuse, and neuritic plaques composed predominantly of the amyloidogenic peptide amyloid- β (A β). These proteinaceous deposits of tau and A β consist of stable amyloid fibrils, which are β -sheet rich fibrous protein aggregates. Similar deposition of amyloid is associated with numerous other diseases.¹

In the early 90's, the amyloid cascade hypothesis was introduced, which postulated that A β aggregation and deposition directly lead to neuronal death, resulting in AD.² Yet, the correlation between plaques and cognitive dysfunction in AD has been questionable for years,³⁻⁷ and with A β -directed therapeutic strategies failing in numerous clinical trials, the role of A β in AD progression is being re-evaluated.⁸⁻¹¹ This has led to an enhanced research focus on diffuse, soluble aggregates of A β . Various small, globular aggregates of A β , referred to broadly as A β oligomers (A β O), were detected in AD patients a few decades ago,¹²⁻¹⁴ and these A β O were originally classified as being intermediates toward the formation of amyloid fibrils. Over the years, increasing evidence points toward A β O playing a central role in AD, as A β O correlate more strongly with AD progression in patients and animal models.¹⁵⁻¹⁹ For example, A β O formation and buildup occurs early compared with plaque buildup (much earlier than plaque

deposition) in the AD brain^{20–22} and CSF,²³ which has led to extensive efforts to develop assays to detect A β O_s for potential application as biomarkers.^{24–31}

An extensive body of evidence has linked high levels of A β O_s in the brain to a variety of pathogenic consequences associated with AD (summarized in Table 1). As such, there has been significant effort made to characterize A β O formation, structure, and biochemical/biophysical characteristics (such as interactions with other proteins and lipids) in the hope that underlying modes of A β O-related toxicity could be revealed.^{32–37}

Table 1.1: Toxic mechanisms associated with A β Os.

Toxic effects of A β Os	Model System	References
Reduction in neural plasticity	mice, rat	37–40
Stimulation of tau phosphorylation	cortical neurons (rat), hippocampal neurons, neuroblastoma, primary neurons, Tg-Mice	41–45
Choline acetyltransferase Inhibition	cholinergic cell lines	46,47
Oxidative stress	cortical neurons, hippocampal neurons, <i>in vitro</i> , neuroblastoma,	48–51
Endoplasmic reticulum stress	cortical astrocytes, cortical neurons (rat), fibroblasts, Tg-mice	45,52,53
Receptor disturbance	cortical neurons, hippocampal neurons	54–57
Insulin resistance	cortical neurons, hippocampal neurons, rat	42,57–59
Synapse deterioration	hippocampal neurons, pyramidal neurons (rat), Tg-mice,	21,35,56,60
Axonal transport	cortical neurons, hippocampal, <i>in vitro</i> , Tg-mice	61–64
Astrocytes/microglia effects	cortical astrocytes, Tg-mice	43,53,65,66
Cell cycle disruption	cortical neurons, Tg-mice,	67,68
Selective neuron death	mice	39,69
Inhibition of long-term potentiation	mice	37,40
Calcium dysregulation	cortical neurons (rat), hippocampal neurons, primary neurons	44,45,51,53
Modulation of metal toxicity	neuroblastoma	48
Cytoskeleton disruption	primary neurons	49
Modulation of Receptor/Channel Activity	hippocampal neurons	51

1.2 The A β Peptide

A β is an approximately 4 kDA peptide (typically 40–42 amino acids long) that is derived from the transmembrane portion of the amyloid precursor protein (APP; Figure 1). The production of A β is achieved by the sequential cleavage of APP by two membrane-bound endoprotease activities, β - and γ -secretase. The two predominant A β peptides produced are 40 and 42 amino acids in length, and these peptides are referred to as A β_{40} and A β_{42} respectively. A β is amphipathic in nature (having a predominately hydrophilic N-terminus and a predominately hydrophobic C-terminus), which is thought to drive its aggregation. As the C-terminal end of A β coincides with the transmembrane portion of APP, A β_{42} has a larger hydrophobic domain, making it more fibrillogenic compared to A β_{40} and deposits to a much greater extent in the brain.^{70–72} Only about 10% of APP is processed via this A β producing pathway. Most APP is cleaved by the α -secretase, generating a series of much more benign peptide fragments.

The hydrophilic N-terminal region of A β can adopt both an α -helical or β -sheet structure dependent on solution conditions, for example pH.^{73,74} The hydrophobic C-terminal end of A β has a propensity to adopt β -sheet structure upon aggregation independent of solvent conditions.^{73,74} Beyond its amphipathic nature, several other domains have been identified in A β . The different polymorphic fibril structures of A β are comprised of bundled β -sheets with backbones orthogonal to the fiber axis creating a cross- β structure,⁷⁵ and two β -strand forming domains (residues 11–21 and 29–39 respectively) that are separated by a turn/bend region (around residues 23–26) identified through various experimental and computational studies.^{76–79} The central

region of A β (residues 16–21), contained within one of the β -strands, has enhanced amyloidogenic properties and represents a hydrophobic core.⁸⁰

Figure 1.1 APP processing and subsequent A β amino acid sequence with specific domains of interest specified. Proteolytic cleavage of APP is initiated at residue 671 by β -secretase followed by either non-amyloidogenic processing, cleavage at residue 687 by α -secretase, or amyloidogenic processing, cleavage at residue 711, 713, 714, or 726 by γ -secretase. The amino acid sequence below highlights the region of APP from which A β is produced with the numbering referring the residues in A β . The orange highlighted region represents the intact A β_{42} sequence. Hydropathy indexes of individual residues are color coded according to hydrophobic (blue), slightly hydrophobic (light green), and hydrophilic (red). Regions of interest and cites of secretase activity within the APP and A β are indicated.

1.3 A β aggregation—a complex mechanism

The aggregation of A β (and other amyloid-forming proteins) is typically characterized in terms of fibril formation (Figure 1.2). A β fibril formation occurs via a complex aggregation pathway. Fundamentally, A β Os can be subdivided into species that are intermediates in fibrils formation (referred to as being on pathway) or species that do not directly lead to fibrils (referred to as being off pathway). This is a contributing factor to the immense heterogeneity observed in

A β O populations as will be discussed in more detail later. In general, amyloid formation proceeds via a nucleation dependent polymerization mechanism (Figure 1.3).^{81–83} With this mechanism, aggregation initially occurs via a slow nucleation phase (often called the lag phase) that involves the formation of a thermodynamically unfavorable critical nucleus that is associated with a transition from a native to non-native protein conformation. For A β , the critical nucleus is likely a multimeric species.^{84–86} Once the critical nucleus has formed, an elongation or growth phase (characterized by a relatively rapid extension of fibril aggregates) occurs. While numerical models can extract important parameters (lag phase times, elongation rates, critical nucleus size) from experimental data,⁸⁷ the actual aggregation pathway toward fibrils can be complicated. For example, other on pathway intermediates, like protofibrils, are also observed in A β aggregation. Protofibrils are amyloid-like, elongated aggregates with filament-like morphologies and are late-stage intermediate precursors on the aggregation pathway to fibrils. A key aspect that facilitates on and off pathway aggregation routes and complicates investigations of A β O is that they possess structural plasticity and are metastable and transient in nature.

Figure 1.2. Production and aggregation of $A\beta$. $A\beta$ is a cleavage product of APP, a transmembrane protein. Monomeric $A\beta$ transitions between ordered and disordered states. Once dimerization occurs, subsequent aggregation occurs either on pathway or off pathway with respect to fibril formation. The aggregates associated with these different pathways increase in molecular weight from the left to the right of the schematic. Fibrillization can proceed via several potential pathways that can populate various intermediate aggregate states, including oligomers and protofibrils. Off-pathway oligomers of various size may also form. Annular aggregates of $A\beta$ can also form and are thought to potentially be associated with forming pore-like structures.

Figure 1.3. Aggregation of Aβ via nucleation-dependent polymerization. The thioflavin-T (ThT) curve is representative of the aggregation of Aβ toward fibril aggregates. This sigmoidal curve is defined by three distinct regions. First, the initial lag-phase (green) is defined by the formation of LMW AβOs and HMW AβOs as Aβ forms the critical nucleus necessary for fibrillization. Aggregate species generated during the lag-phase are ThT-negative. Second, upon formation of the critical nucleus fibril formation is initiated and the elongation phase of the curve begins. This elongation/growth phase (pink) is dominated by a mixture of HMWAβOs which are ThT-negative, and to a greater extent small ThT-positive protofibril structures. Third, as the protofibrils increase in size and associate to form mature fibrils and plaques the ThT signal ultimately reaches a plateau (blue).

Further complicating the issue is the observation that Aβ can aggregate into a variety of morphologically distinct fibril structures, referred to as polymorphs.^{88–92} This phenomenon is predicated on subtle changes of environmental conditions associated with aggregation, and as a result, preparatory protocols employed in experiments determines the resulting Aβ fibril morphology.⁸⁹ While polymorphic aggregates are readily observed within *in vitro* studies using synthetic Aβ, polymorphic structures have been observed in amyloids derived from tissue, and it

is thought that variations in A β aggregate morphologies may play an important role in AD.^{93,94} For example, polymorphic aggregates and fibrils may result in distinct biological activities and levels of toxicity that could underlie variations in AD,⁷⁸ and distinct fibril structures can be directly associated with individual AD patients and clinical phenotype.^{95,96}

A complicating factor in evaluating and comparing studies aimed at elucidating A β formation, structure, and physiological impact is divergent experimental conditions, such as A β preparation protocols, heavily influences experimental outcomes. In terms of oligomers, the emergence of distinct fibril structure strongly suggests that there would also be distinct oligomeric precursors associated with their formation. Indeed, distinct A β O species can be observed within *in vitro* aggregation assays under conditions that result in fibril polymorphs (Figure 1.4).⁹⁷ For studies conducted with synthetic peptide, there are a variety of protocols used to solubilize A β (Table 1.2). Typically, these protocols consist of a disaggregation step and a reconstitution step. The disaggregation steps usually involve the use of hydrogen bond disrupting solvents, i.e., hexafluoroisopropanol (HFIP) or trifluoroacetic acid (TFA), that break down pre-existing aggregates within lyophilized stocks of A β . These solvents are often removed under vacuum, leaving a peptide film. The reconstitution step involves dissolving these peptide films into a solvent that facilitates dilution into an appropriate buffer. Dimethyl sulfoxide (DMSO) is often used, creating a concentrated stock that is diluted into the desired buffer. Disaggregation and reconstitution can also be facilitated by changes in pH. Sometimes reconstitution is performed directly into the final buffer. Importantly, variations in preparatory protocols indeed result in different populations of oligomers (Table 1.2 and Figure 1.4), and these variations can complicate direct comparisons between different reports within the literature. Further

complicating the issue, there are often distinctions observed between studies performed with synthetic A β and naturally derived A β .⁹⁸ In many studies aimed at elucidating activity of A β O_s, specific preparations are used to obtain a particular population of oligomer species. These are then directly applied to different model systems, ranging from cell culture to animal models. However, there is often a lack of effort to verify that once added to the model system (which can often be a pronounced change in chemical environment) that these A β O species do not dissociate or aggregate into a different A β O or A β aggregates. To truly relate specific A β O_s to a neurotoxic activity, effort should be invoked to attempt additional controls of this type.

Table 1.2. Representative disaggregation, reconstitution, and miscellaneous protocols for the preparation of A β and observed A β Os.

Classification	Disaggregation	Reconstitution	Miscellaneous	Result	References
ADDLs	None	F12 Media 4 °C	Centrifuged 14,000 $\times$ g for 10 mins	5–6 nm by AFM (height)	39
ADDLs	HFIP	DMSO at 5 mM A β	Sonicate 5 mins, dilute with DMEM/F12 Media	A11+	99
Globulomers	HFIP	DMSO at 5 mM A β	Sonicate 10 mins, dilute PBS + 0.05% SDS	16–56 kDa by SDS-PAGE	100
Globulomers	HFIP	DMSO at 5 mM A β	Dilute PBS + 0.05% SDS, Dialyze	38–48 kDa by SDS-PAGE	101
A β *56	HFIP	DMSO at 5 mM A β	Sonicate 20 min, PBS + 0.2% SDS incubate 6 h, dilute and incubated 18 h; centrifuge 3000 $\times$ g, dialysis	56 kDa by Native-PAGE	102
A β Os	HFIP	DMSO	F12 Media incubated at 4 °C for 24 h	1–4 nm by AFM	103,104
A β Os	HFIP/NH ₄ OH	10 mM Tris-HCl	Addition of Zn ²⁺	10–12 nm by AFM	99
A β Os	TFA/HFIP (2Xs)	2 mM NaOH	PBS Centrifuge 386,000 $\times$ g	2.5 nm by AFM	89,97
A β Os	TFA salt/2 mM NaOH, pH ~10.5, 1 min sonication 10% NH ₄ OH (w/v)	PBS	-	3–12 nm by AFM	105
A β Os	sonicated 5 mins, lyophilized	60 mM NaOH	-	1–10 nm by DLS	106
A β Os LMW	-	DMSO	Sonicate 1 min, centrifuge 16,000 $\times$ g, SEC, PBS, PICUP crosslinking	4–26 kDa by SDS-PAGE	107

Figure 1.4. Heterogeneity of AβOs. A series of atomic force microscopy images and size analysis, i.e. height histograms, of AβOs formed from synthetic Aβ that had been prepared by some of the protocols described in Table 2. The AβOs were prepared in the following way: (A) protocol provided in the AggreSure β-amyloid kits available from AnaSpec which consists of a reconstitution step directly into Tris buffer with bath sonication; (B) 10% NH₄OH disaggregation buffer, followed by additional treatment with HFIP, and reconstitution in 2 mM NaOH (pH > 11) with subsequent dilution into Tris buffer; (C) 10% NH₄OH disaggregation buffer, and reconstitution in 60 mM NaOH (pH > 11) with subsequent dilution into HEPES buffer; (D) HFIP disaggregation, reconstitution in DMSO, followed by dilution into PBS; (E) TFA disaggregation with sonication, an additional HFIP disaggregation step, reconstitution with NaOH, and dilution into PBS; (F) No disaggregation step and direct reconstitution in to phosphate buffer.

1.4 Familial Mutations and Post-Translational Modification of A β

A number of mutations within A β are associated with Familial forms of Alzheimer's disease (FAD), which are often characterized by early onset or other variations in disease progression. FAD mutations consist of single point mutations in the amino acid sequence of A β and produced a variety of AD symptoms including seizures, cardiovascular degradation, and cerebral amyloid angiopathy.¹⁰⁸ Primarily, FAD mutations of A β are located within the turn region and central hydrophobic core of A β (E22Q Dutch, E22K Italian, D23N Iowa, E22G Arctic,) which promote increased aggregation and stabilization of unique aggregate species (Figure 1.5). Additionally FAD mutations within the N-terminal region (English H6R, Tottori D7N) or C-terminal region (Piedmont L34V) also promote the accelerated fibril formation of A β .¹⁰⁸ For the FAD mutations mentioned above, the disease outcomes are principally associated with increased A β aggregation as APP processing remains unchanged. Additionally, some point mutations within A β slow A β aggregation (A21G Flemish and K16N) and instead promote increased secretion of A β resulting in early onset of AD.¹⁰⁸ Some FADs are associated with altered APP processing, such as increased production of A β or promoting A β 42 over A β 40. FADs, however, only make up 5% of the total cases of AD and do not explain the other 95% of sporadic Alzheimer's disease (sAD) cases.¹⁰⁹ Collectively, the ability of single point mutations to modify the A β aggregation process and resulting aggregate structures demonstrates that single amino acids within the peptide can heavily influence aggregation.

Figure 1.5. Genetic mutations and post translational modifications of the Aβ sequence. Familial mutations and post translation modifications of the Aβ amino acid sequence, highlighted in orange, within APP. Familial mutations are represented below the Aβ sequence with mutations that increase aggregation of Aβ colored red and mutations that slow aggregation highlighted in blue. Familial mutations that are responsible for increased production of Aβ are denoted with an asterisk. Located above the Aβ sequence are the post-translational modifications of Aβ with arrows denoting the location and type of modification. Hydrophathy indexes of individual residues are color coded according to hydrophobic (blue), slightly hydrophobic (light green), and hydrophilic (red). Familial and post translational modifications of Aβ shown here are not inclusive.

Aging is known to be the largest risk factor of Alzheimer's disease. The deregulation of cellular mechanisms governing post-translationally modified (PTMs) is also modified with increases in age.^{110–112} Analysis of sAD patient's senile plaques and cerebrospinal fluid (CSF) shows that Aβ is post-translationally modified.¹¹³ Whether PTMs of Aβ are directly responsible for the initiation of the amyloid cascade is still unclear; however, aggregation of tau has been directly linked its hyperphosphorylation. Post translational modifications of Aβ involve phosphorylation, oxidation, pyroglutamination, deamidation, and citrullination. A number of these modifications directly influence the Aβ aggregation process and resulting aggregate species.

Phosphorylation of A β *in vivo* occurs primarily at serine-8 and 26.^{114–116} Specifically, phosphorylation of A β at serine-8 is known to accelerate aggregation and promote oligomerization.¹¹⁴ Phosphorylated A β also exhibits increased toxicity in *Drosophila* models, and is found within the core of senile plaques suggesting its involvement in the early stages of aggregation.¹¹⁴ Pyroglutamination of A β (A β _pE3-40/42) involves the removal of two N-terminal amino acids, aspartic acid-1 and alanine-2, in the A β sequence. This truncation is followed by the dehydration and cyclization of the new N-terminus of A β by the enzyme glutaminy cyclase.¹¹⁷ This change in the N-terminal charge state of A β creates a peptide that is more hydrophobic and therefore A β _pE3-40/42 produces persistent oligomeric aggregates with increased rates of fibril formation.^{118,119} Furthermore, analysis of CSF in sAD patients revealed that the ratio of A β _pE3-40/42 to A β ₄₀/A β ₄₂ is increased suggesting pyroglutamination plays a role in late onset Alzheimer's disease.¹¹³ These observations further indicate that changes in the A β peptide at the single amino acid level can have a profound impact on the aggregation process, and hence AD.

A β can also undergo PTMs that are more frequently associated with the aging process.^{120,121} Oxidation of A β via reactive oxygen species (ROS) occurs primarily at methionine-35, in the absence of metals,^{122–125} and the modification of the hydrophobic methionine to a hydrophilic residue within the C-terminus region is suggested to reduce toxicity.^{126–128} Interestingly, further oxidation from the sulfoxide to sulfone derivative of methionine restores toxicity, providing evidence of the fine line between toxic and nontoxic forms of A β .¹²⁷ Additional evidence has implicated methionine in initiating radical chain reactions within cellular membranes leading to increased lipid peroxidation and the formation of ion-channel-like structures within the membrane.^{129–131} The increased lipid peroxidation appears to create a

feedback loop in which lipid peroxidation products like 4-hydroxy-2-nonenal (HNE) can PTM A β forming A β -HNE adduct which have an increased affinity for cellular membranes.¹³²

Deamidation and citrullination are PTMs that have also been suggested to be affected by the aging process.¹²⁰ Deamidation, involving the transition of an amide to a carboxylic acid, within A β results in the generation of a negative charge upon replacing asparagine-27 with aspartic acid via chemical modification. This deamidation significantly reduces the rate of fibril formation and promotes stable oligomeric intermediates of A $\beta_{40/42}$ with an antiparallel β -sheet structure which has been postulated to be a toxic conformation of A β O.¹³³ Citrullination of A β , involving the conversion of arginine-3 to an amide functional group, showed a marked reduction in fibrillization of A β_{40} but not for A β_{42} .¹³³ Citrullinated A β forms oligomeric and fibril structures, but was suggested to have alter β -sheet characteristic associated with fibril formation. Further analysis of AD brains reveals that citrullinated forms of A β are also localized within amyloid plaques.^{134,135} Collectively, point mutations of FADs and the PTM of A β found in sAD patients helps to illustrate the significant role that individual amino acids can play in aggregation, structure, and toxicity of A β .

1.5 The influence of membranes on A β aggregation

As previously discussed, A β aggregation is highly susceptible to sample preparation and environmental factors. While variations in sample preparation produce distinct polymorphs,⁸⁹ the presence of surfaces also modulates aggregate morphology. For example, A β aggregates into linear nanowires along the underlying lattice structure of graphite, and aggregation on mica yields A β O that coalesce into protofibrils.¹³⁶ In a physiological context, protein structure and dynamics are altered in the presence of a two-dimensional liquid environment provided by the

presence of lipid bilayers. The importance of studying the relationship between A β and membranes lies in the fact that the membrane compositions are altered in AD patients,^{137,138} plasma membrane damage occurs in AD,^{139–141} and the binding of A β to the plasma membrane is highly correlated with cell death.^{41,142}

The interaction of A β with lipid membranes has been well established;^{143,144} however, the nature of the peptide-membrane interactions is complex, as subtle changes in physiochemical properties of the peptide or membrane can greatly influence their interactions. The initial interaction of A β with membranes involves the insertion of A β into the hydrophobic region of the membrane or the adhesion of A β to the membrane surface. Numerous research efforts suggest that the mechanism by which A β binds to membranes is driven by electrostatic and hydrophobic interactions between the peptide and membrane (see review).¹⁴⁵ Moreover, the importance of lipid headgroup charges has been established with A β preferentially binding to anionic lipid headgroups^{146–148} more favorably over purely zwitterionic lipids.^{146–148} The charge of the membranes and the peptide to lipid ratio also have profound effects on the secondary structure of A β .^{145,149} Previous findings show that under low peptide to lipid ratios A β is primarily α -helical in nature with high peptide to lipid ratios favoring a β -sheet structure of A β .¹⁵⁰ Specific chemical modifications of a membrane's extent of unsaturation, conformational changes, variation in lipid headgroups, and acyl chains will also have an effect on the hydrophobic and electrostatic matching taking place at the protein-lipid interface of membranes.^{151,152} This is noteworthy as the plasma membrane of neurons are highly heterogeneous in nature, and changes in the composition or charge of lipids can result in differences of fibril elongation rates, nucleation times, and aggregate morphology.^{153,154}

Once bound, A β can then further migrate and aggregate along the membrane surface. Monomeric A β exhibits significant lateral mobility, but A β becomes increasingly immobile following oligomerization.¹⁵⁵ This lateral mobility of A β has also been related to the properties of the surface with highly hydrophilic surfaces inhibiting adhesion of A β and highly hydrophobic surfaces significantly slowing diffusion rates and inhibiting fibril formation.¹⁵⁶ It is hypothesized that the regulation of A β diffusion to a two-dimensional surface is the primary cause for increased rates of fibril formation in the presence of membrane surfaces. In this regard, surface density of A β on a lipid membrane also plays a role, making the peptide to lipid ratio an important parameter to control. Under high peptide to lipid ratios concentrations of A β on a single vesicle are increased effectively increasing the local concentration of A β at the surface and prompting accelerated oligomerization of A β and fibrillization. Low peptide to lipid ratios exert the opposite effect; wherein, the concentration of A β at the lipid surface is lower and the time it takes for A β to diffuse and aggregate significantly increased.¹⁴⁹

While membrane properties can influence the aggregation of A β ,^{153,157,158} features of the A β peptide also play a role in its interaction with the plasma membrane. For example, the aggregation of A β 40 and A β 42 on total brain lipid extract bilayers (TBLE) is different.^{159,160} Several point mutations with FADs promoted unique aggregate species on TBLE bilayers as well.¹⁶¹ This demonstrates that single amino acids within A β can play an important role in regulating the interaction of A β with the plasma membrane. Specifically, lysine in A β may be responsible for facilitating the electrostatic interactions between the peptide and anionic lipid headgroups. Lysine in other protein systems is known to play a role in membrane protein anchoring,¹⁶² cell-penetrating peptides,¹⁶³ and antimicrobial peptides.¹⁶⁴ These results suggest

that the peptide sequence and modification of A β are of equal importance in evaluating the interactions of A β and the membrane.

1.6 Conclusion

A β aggregation and deposition has long been associated with AD. The aggregation process and resulting aggregate species are highly dependent on the chemical properties of specific amino acids within A β . In addition, the interaction of A β with plasma membranes is also influenced by specific amino acids. Understanding the of each amino acid in these phenomena can provide novel therapeutic targets and strategies. Here, we explore how targeting specific amino acids with A β by chemically induced post-translational modifications alters the aggregation process, aggregate morphology, and interactions with model lipid membranes. In Chapter 2, we examine how increasing the concentration of H₂O₂, at physiologically relevant levels, post translationally modifies A β to its sulfoxide derivative Met35[O]. We go on to elucidate that oxidation of A β alters aggregation, secondary structural transitions, morphologies of aggregates, and oligomerization of A β in the absence of lipids. In addition, ThT assays are performed in the presence of TBLE and polydiacetylene assays (PDA; a lipid binding assay) are utilized to clarify the role of an oxidative environment on the A β -membrane interactions. Finally, we isolated the oxidative effects on A β and TBLE to determine the individual roles of oxidation on the peptide and membrane and determine the oxidation state of the membrane modulates the A β -membrane interaction. In Chapter 3, we chemically acetylate A β via the addition of sulfo-N-hydroxysulfosuccinimide acetate (NHSA) with the aim of understanding how the removal of lysines cationic charge through acetylation modifies the aggregation of A β in the presence and absence of TBLE. We observe the inhibition of fibril formation and oligomerization in the

absence of lipids with no observations of polymorphic structures. However, ThT assays performed in the presence of TBLE restore fibril formation in addition to producing polymorphic structures, annular aggregates, at higher acetylation concentrations. Finally, we conclude Chapter 3 by performing PDA assays to illustrate that acetylation of A β reduces membrane binding.

1.7 References

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2. Hydrogen peroxide modifies A β -membrane interactions with implications for A β ₄₀ aggregation

Alzheimer's disease (AD) is pathologically characterized by the formation of extracellular senile plaques, predominately comprised of aggregated β -amyloid (A β), deposited in the brain. A β aggregation can result in a myriad of distinct aggregate species, from soluble oligomers to insoluble fibrils. A β strongly interacts with membranes, which can be linked to a variety of potential toxic mechanism associated with AD. Oxidative damage accompanies the formation of A β aggregates, with a 10-50% proportion of A β aggregates being oxidized in vivo. Hydrogen peroxide (H₂O₂) is a reactive oxygen species implicated in several neurodegenerative diseases. Recent evidence has demonstrated that H₂O₂ concentration fluctuates rapidly in the brain, resulting in large concentration spikes, especially in the synaptic cleft. Here, the impact of environmental H₂O₂ on A β aggregation in the presence and absence of lipid membranes is investigated. A β ₄₀ was exposed to H₂O₂, resulting in the selective oxidation of methionine-35 (Met35) to produce A β ₄₀Met35[O]. While oxidation mildly reduced the rate of A β aggregation and produced a distinct fibril morphology at large H₂O₂ concentrations, H₂O₂ had a much more pronounced impact on A β aggregation in the presence of total brain lipid extract vesicles (TBLE). The impact of H₂O₂ on A β aggregation in the presence of lipids was associated with a reduced affinity of A β for the vesicle surface. However, this reduced vesicle affinity was predominately associated with lipid peroxidation rather than A β oxidation.

2.1 Introduction: The role of oxidation in Alzheimer's disease

A key pathological feature in Alzheimer's disease (AD) is the formation of senile plaques composed of the amyloid- β peptide (A β). A β is formed by the successive cleavage of the

transmembrane amyloid precursor protein (APP), resulting in varied lengths of A β , the most common being 40-42 residues long. Aggregation of A β results in the formation of insoluble plaques, fibrils, protofibrils, and soluble oligomeric intermediates; the latter of which is considered to be the dominant neurotoxic species.¹⁻³ Ninety-five percent of AD cases are sporadic, with aging being highly associated with developing AD.⁴

A contributing factor in aging is the continuous production of reactive oxygen species (ROS)⁵, which can lead to cellular and molecular damage^{6,7} and several enzymes function to counteract ROS.⁸ Oxidative modifications of polyunsaturated fatty acids play a role in age-dependent reduction of membrane fluidity by increasing the rigidity of lipid bilayer membranes due to steric hindrance restricting the movement of lipid acyl chains.^{9,10} A β oligomers preferentially accumulate near the plasma membrane of oxidatively damaged cells¹¹ and enhanced oxidative damage is observed in AD brains.¹² The A β -induced oxidative stress hypothesis states that the principle toxic mechanism underlying AD results from A β inducing oxidative stress within the brain.^{13,14} Numerous studies have provided evidence that implicates methionine-35 (Met35) in ROS generation, which can be associated with neurotoxicity.¹⁵

Oxidized variants of A β have been detected in mass spectrometry studies of transgenic mice,¹⁶ Raman spectroscopy of intact senile plaques from AD patients,¹⁷ and post-mortem analysis of AD patient's cerebral spinal fluid with 10-50% of A β being oxidized.^{16,18} A β oxidation occurs at several residues, e.g. histidine and tyrosine, but these require transition metals.¹⁹ However, Met35 is the only residue susceptible to oxidation directly by hydrogen peroxide (H₂O₂).²⁰⁻²³ The oxidation of Met35 occurs via a hydroxyl radical addition to the thioether of methionine in aqueous media to its sulfoxide derivative.²⁴ The impact of oxidation on A β aggregation varies greatly in the literature (depending on experimental conditions) as

oxidation of A β has been observed to increase,^{25,26} decrease,^{21,23,27–29} or have no impact on aggregation.^{30–32} In some instances, A β oxidation inhibited the formation of intermediate aggregates associated with fibril formation. For example, oxidation of Met35 abolished the formation of critical nuclei associated with fibril formation,³³ and oxidized A β ₄₀ was limited to the formation of dimers.²² Yet other studies have reported the formation of higher ordered fibrils from Met35[O] substituted forms of A β .^{21,32,34,35}

The oxidation state of A β influences its toxic properties. Modification of Met35 to its sulfoxide derivative, A β ₄₂-Met35[O], abolished cellular toxicity in 9 to 11-day-old rat embryonic hippocampal neuronal cultures.³⁶ Further studies supported the notion that A β -Met35[O] reduced toxicity.^{32,37,38} Interestingly, Met35Val substitution enhances toxicity, illustrating that Met35 is not required for toxicity of A β ; however, the increased flexibility gained by the C-terminus upon this substitution allowed for a higher affinity of A β to lipid bilayers.³⁹

The interaction of A β and the plasma membrane has been associated with a variety of toxic mechanisms.^{40–42} Membrane surfaces can (i) modify peptide structure,⁴³ (ii) alter rates of amyloid nucleation and growth,⁴⁴ (iii) promote polymorphic aggregate structures,^{45,46} and (iv) stabilize intermediate aggregate species.^{47–49} ROS can also cause lipid peroxidation.⁵⁰ As oxidation of Met35 alters the chemical properties of A β , the ability of A β to bind and insert into lipid membranes would be altered with implications for the aggregation process occurring on the membrane surface. A potential phenomenon associated with A β /lipid interactions under oxidative conditions is the localization of A β to synapses⁵¹ and synaptotoxicity of A β oligomers, underlying neuronal loss in AD.^{51,52} Oxidation of Met35 reduces synaptotoxicity and dendrite retraction.³² Fast-scan cyclic voltammograms have measured, in real time, H₂O₂ dynamics in

vivo, suggesting that H₂O₂ fluctuations within the brain and synaptic regions occur ~4-11 times per minute,⁵³ creating an opportunity for acute oxidative damage of synaptic A β in the presence of the plasma membrane.

Here, we investigate how oxidative environments, produced by H₂O₂, influence A β ₄₀ aggregation in the presence and absence of model lipid membranes of total brain lipid extract (TBLE). TBLE is an excellent model system as it is comprised of a physiologically relevant ratio of lipids, i.e. acidic and neutral phospholipids, gangliosides, cholesterol, sphingolipids, and isoprenoids. We demonstrate that H₂O₂ reduces the total fibril load of A β ₄₀ in the absence of lipid until the H₂O₂ concentration reaches 6.0 mM. At this concentration, a polymorphic fibril structure is observed. With the introduction of TBLE vesicles, A β ₄₀ aggregation is delayed; however, the introduction of H₂O₂ begins to restore A β aggregation rates. This correlates with a reduced interaction between A β ₄₀ and the lipid bilayer caused by the presence of H₂O₂. The reduced affinity of A β ₄₀ for TBLE membranes is more strongly associated with the oxidation of lipids rather than the peptide.

2.2 Materials and Methods

2.2.1 A β ₄₀ Preparation — Synthetic β -Amyloid 1-40 (from AnaSpec Inc., San Jose, CA and Alfa Aesar, Tewksbury, MA) was equilibrated to room temperature for 30 min. Disaggregation of A β ₄₀ was achieved by adding 10% NH₄OH (w/v) to A β ₄₀ to obtain a concentration of 0.5 mg/mL. After 10 min of incubation and 5 min of bath sonication at room temperature, A β was aliquoted and snap frozen. Aliquots were lyophilized overnight and stored at -80°C. On the day of experiments, A β was equilibrated to room temperature and dissolved in a 60 mM NaOH solution, pH $\geq$ 11.23. The solution was gently pipette mixed and incubated for 10 mins at room temperature. Concentrations of the A β ₄₀ stock solutions, 1.5 mg/mL, was

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confirmed using a NanoDrop2000 (Thermo Scientific) at an absorbance of 280 nm with an extinction coefficient of $1440 \text{ M}^{-1} \text{ cm}^{-1}$. A litmus test verified the pH of the reconstituted A β to be ≥ 11.0 . Immediately, A β_{40} was used in experiments or other preparatory methods.

2.2.2 Preparation of Oxidized A β_{40} — Oxidized derivatives of A β_{40} were prepared by solubilizing lyophilized aliquots with a 60 mM NaOH solution and diluted to a final concentration of 20 μM in a 20 mM HEPES 150 mM NaCl buffer (pH = 7.23) containing 3% (w/v) H₂O₂, 980 mM. Samples were incubated for 3 h before being dialyzed against 18 M Ω water overnight in a Slide-A-Lyzer MINI Dialysis Device (Thermo Scientific, Waltham, MA) with a 3.5 kDa molecular weight cut off. Samples were alkalified for extraction by the addition of a 10% NH₄OH (w/v) solution. Extracts were lyophilized, re-disaggregated, and lyophilized again before storage at -80°C.

2.2.3 Liquid Chromatography Mass Spectrometry (LC-MS) — Lyophilized aliquots of A β_{40} were removed from -80°C and allowed to reach RT over the course of 30 min before being solubilized in 60 mM NaOH (pH ≥ 11.23) and incubated for 10 min at RT without agitation. Finally, the stock A β_{40} •NaOH solution was bath sonicated for 5 min. A β_{40} (20 μM) was incubated at 37°C in a HEPES buffering solution (20mM HEPES, 150mM NaCl pH = 7.23) with varying amounts of H₂O₂ (0, 0.1, 1.0, 3.0, 6.0 mM). Solutions were aliquoted for each oxidation condition and incubated without agitation. At specific time points (0, 15, 30, 45, 60, 120, 180, 240, and 300 min) aliquots of each oxidation condition were snap frozen and lyophilized overnight to remove H₂O₂. The following day, samples were diluted in 1% formic acid/water (v/v) right before being introduced to the LC-MS.

Chromatography was performed on a C18 column (ZORBAX RRHD Eclipse XDB 80Å C18, 2.1 x 100 mm, 1.8 μm ; Agilent, Santa Clara, CA) utilizing a mobile phase of 0.1%

formic/water (v/v) (%A) and 0.1% formic acid/acetonitrile (v/v) (%B) with a gradient of 5%B to 60%B over 4.5 min with a flowrate of 300 μ L/min. A Q Exactive (Thermo Scientific) mass spectrometer was operated in positive ion mode and scanned over a mass-to-charge (m/z) range of 500-2000 at a 70,000 resolution.

2.2.4 Mass Spectrometry Analysis — Percent oxidation was calculated by determining the area (total ion chromatogram, TIC) of the oxidized peak, retention time of ~5.5-5.7 min, and unoxidized peak, retention time of ~5.9-6.0 min, and dividing by the summation of the total peak area. Peak areas encompassed all charge states from m/z 500-2000. Unoxidized and oxidized A β ₄₀ ions were clearly resolved in the TIC as observed in the different mass spectra. However, the oxidized peak did contain minor contributions from the doubly oxidized sulfone peptide, but its contribution was consistently less than 1% and was considered negligible.

2.2.5 Sample preparation for ¹H NMR and analysis of measurements — ¹H NMR experiments were run on an INOVA^{Unity} 600 MHz spectrometer (proton frequency, 599.67 MHz) equipped with a triple-resonance z-axis pulsed field gradient 5 mm probe. A β ₄₀ samples were oxidized, as mentioned above, for 3 h or 24 h at ambient temperature without agitation. Lyophilized aliquots of A β ₄₀ or oxidized A β ₄₀ were solubilized in 60 mM NaOD in D₂O and diluted to 10 μ M with a 100 mM Na₃PO₄ deuterated buffer. Then a 700 μ L solution was placed in 5 mm NMR tube and the ¹H NMR spectra with a PRESAT sequence were acquired for 30 min to 1 h. The FIDs of the ¹H NMR spectra were processed using the commercially available NMR software package ACD/Spectrus Processor 2017.2 (Advanced Chemistry Development, Inc; <http://>, File Version S70S41, Build 98721, 19 Dec 2017). ¹H NMR chemical shifts were referenced to a residual proton peak (HDO) of water at 4.63 ppm, and the FID's of ¹H NMR were processed with a line broadening function of 2 Hz.

2.2.6 Thioflavin-T Fluorescence Assay (ThT) — Lyophilized aliquots of A β ₄₀ were removed from -80°C storage and allowed to reach RT over the course of 30 min. A β ₄₀ was solubilized with 60 mM NaOH (pH $\geq$ 11.23), incubated, and sonicated for 5 min before being diluted into a HEPES buffer (20 mM HEPES, 150 mM NaCl pH = 7.23) at 20 μ M A β ₄₀, as previously mentioned. The formation of higher ordered β -sheet aggregates was monitored via a thioflavin-T (ThT; Sigma Aldrich, St. Louis, MO) assay. In a 96-well plate A β ₄₀ was aggregated in a HEPES buffering (20 mM HEPES 150 mM NaCl pH = 7.23) solution containing ThT at a 1.5 molar ratio to A β ₄₀, with varying final concentrations of H₂O₂ (0, 0.1, 1.0, 3.0, 6.0 mM) relative to A β 's final concentration. Additionally, to facilitate homogenous mixing across the 96-well plate and enhance the reproducibility of aggregation kinetics 3 mm borosilicate beads were added to each well. Well plates were sealed and reactions were performed at 37°C. Fluorescence intensities were measured at excitation and emission wavelengths of 440 nm and 484 nm, respectively, utilizing a SpectraMax M5 or M2 Multi-Mode plate reader (Molecular Devices, Sunnyvale, CA) to measure in 5 min interval readings with 10 s shaking between intervals. Assays performed in the presence of TBLE were carried out using the same procedure (see Lipid Vesicle Preparation). The ThT signal in the presence of TBLE exhibited only a slight increase in fluorescence in the background signal which was constant across all variables and subtracted out during baseline correction.

Data analysis was performed employing GraphPad Prism 6 to average across triplicates, baseline correct, and perform error analysis reported as a standard error mean (SEM). Kinetic parameters of aggregation such as nucleation lag-phase time (t_{lag}) were obtained empirically by fitting the ThT traces for each experiment to a Boltzmann sigmodal function, Equation 1.^{54,55}

$$Y = (y_i + m_i x) + \frac{y_f + m_f x}{1 + e^{-\left(\frac{x - x_o}{\tau}\right)}} \quad (1)$$

Utilizing GraphPad Prism 6 the initial lag-phase and plateau regions of the curves are defined by linear fits where the initial fluorescence and slope are defined by, y_i and m_i , and the final fluorescence and slope as, y_f and m_f , respectively. The time to reach the half maximum of the curve is defined by x_o and the elongation time constant of the growth phase is defined as τ . As a nonideal sigmodal function, no constraints were applied. A least squares fitting operation was then performed for a maximum iteration limit of 1000. The lag-phase time was calculated from the kinetic parameters, $t_{lag} = x_o - 2\tau$. The slope of the growth phase is associated with the growth of fibrils. The slope was calculated by normalizing the ThT traces to the mean maximum response of control group (no H₂O₂ added) within each independent experiment for comparison across experiments. To obtain the relative slope of the growth phase, $R_{slope}(h^{-1})$ the linear fits of the growth phase were performed by defining the boundaries of the fit to only include the linear region between the lag-phase and the plateau region as visually determined for each ThT curve. The maximum signal of fluorescence at steady state was calculate using a linear fit over the plateau region of the sigmodal curve. This was accomplished by adjusting the starting point of the data to be fit and moving it further back to optimize the r^2 value. Initial starting point points were visually determined to be near the inflection point where the sigmoidal curve enters the plateau region. The fits were performed from these starting points to the end of the data set. Once the boundaries were defined, the average fluorescence signal within this plateau region was determined. For comparison between trials, these were converted to the relative fluorescence at steady state (RF_{ss}) by dividing each value by the control experiment (no H₂O₂ for that experiment). All kinetic parameters were then averaged from triplicates of independent experiments to determine the mean $\pm$ SEM. Significance was reported as greater than 95%

confidence calculated using a standard Student's T-test to determine variations in parameters across varied H₂O₂ concentrations.

2.2.7 Atomic force microscopy — A β ₄₀ was prepared in the same manner as the ThT assays. Samples were incubated using a Spectra Max M5 plater reader's shaker and incubator, 37°C, to maintain the same experimental conditions as used in the ThT assays. At 1 h intervals. 2 μ L samples were taken and deposited on freshly cleaved mica. Samples were allowed to incubate for 1 min before being washed with 200 μ L of 18 M Ω water and dried immediately with compressed air. Imaging of samples was performed on a Nanoscope V Multimode Atomic Force Microscope (AFM) (Veeco, Santa Barbara, CA) with a closed-loop vertical engage J-scanner. All images were obtained in tapping mode under *ex-situ* conditions utilizing a diving-board-shaped silicon oxide cantilever with a spring constant of 40 N/m and the resonance frequency of ~300 kHz.

AFM image processing and analysis was performed in Matlab (MathWorks, Natick, MA) by utilizing the imaging processing toolbox.⁵⁶ A 3rd order polynomial flattening algorithm was used to correct for background curvature. Binary maps were created by using an established height threshold ($z = 0.99$ nm), and discrete aggregates were selected by implementing recognition algorithms to the binary maps. Physical features of individual aggregates (e.g., height, volume, diameter, shape factor, length, aspect ratio, length, and area covered) were measured. A minimum of five 5×5 μ m AFM images for each time point was analyzed, resulting in a minimum of 125 μ m² of surface area analyzed.

The number of oligomeric aggregates per unit area was determined through a filtering algorithm. Thresholds for filtering oligomeric aggregates were defined as heights between 1.0 and 10 nm with an aspect ratio between 1.0-2.5. Fibril aggregates per unit area were counted

individually by hand and divided by total area of the image.^{56,57} The percent of the mica surface covered by fibrils was determined by filtering out non-fibrillar aggregates (based on aspect ratio) from the above mentioned binary map. The remaining number of pixels in the binary map associated with fibril aggregates were summed and divided by the total number of pixels in the AFM image. Populations of oligomers and fibrils, along with the percent area covered by fibrils, were plotted using GraphPad Prism 6 to average across replicates and perform error analysis reported as a standard error mean (SEM).

2.2.8 Circular Dichroism Spectroscopy — Samples containing 20 μM $\text{A}\beta_{40}$ and different concentrations of H_2O_2 (0, 1.0, 6.0 mM) in a 10 mM K_2HPO_4 50 mM Na_2SO_4 (pH = 7.23) buffer, were prepared in a 96-well plate and incubated on a Spectra Max M5 plate reader, as described previously here. Samples were taken in 1 h intervals and transferred to a 1-mm quartz cell. A Jasco-810 spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan) was used to record spectra from 190-260 nm at 0.5 nm resolution at ambient temperature. An average of five spectra were collected, averaged, and repeated three more times over the course of 1 h to ensure that any continued aggregation had not significantly changed spectra between the initial and final reading. Deconvolution of spectra was performed using BeStSel (<http://bestsel.elte.hu/>)^{58,59} and plotted using GraphPad Prism 6. Experiments were performed alongside their equivalent ThT assay experiment to ensure the accuracy of aggregation kinetics.

2.2.9 Lipid Vesicle Preparation — Lyophilized total brain lipid extracts (TBLE; Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform at 25 mg/mL, aliquoted into 5 mg amounts, dried *in vacuo* producing a transparent film, and stored at -20°C . Oxidation of TBLE samples was performed by hydrating the lipid film with a 3% (w/v) H_2O_2 solution and incubating at 60°C and 1400 rpm for 3 h on an Eppendorf Thermomixer. Unoxidized and oxidized TBLE

samples were hydrated with a HEPES buffer (20 mM HEPES 150 mM NaCl buffer pH = 7.23) and incubated for 30 min at 60°C and 1400 rpm in a Thermomixer. Lipid samples were subjected to 7 freeze-thaw cycles followed by 30 mins of bath sonication. Lipid stocks were heated and kept at 60°C during extrusion through 100 nm polycarbonate membrane filter. Extruded lipids were kept at 37°C before being added to a 96-well plate for a final concentration of 0.432 mg/mL. To determine the extent of lipid peroxidation for pre-oxidized lipid experiments, a Lipid Hydroperoxide (LPO) Assay Kit (Abnova) was used.

2.2.10 Preparation of TBLE/Polydiacetylene Vesicles (PDA) — Five-milligram aliquots of TBLE or TBLE_{Ox} were dissolved in a 1:1 chloroform: ethanol solution. Separately 8.4 mg of 10,12-tricosadiynoic acid (GFS Chemicals, Columbus, OH) was also dissolved in a 1:1 chloroform: ethanol solution. The two were combined in a glass vial at a 60:40 mass ratio of PDA to TBLE and solvent was removed *in vacuo* to produce a TBLE/PDA film. Eight milliliters of a HEPES buffer (20 mM HEPES 150 mM NaCl buffer pH = 7.23) was heated to 75°C and used to hydrate the TBLE/PDA film. The solution was probe sonicated for 10 mins at 180 W then stored at 4°C, protected from light, overnight. The following day the TBLE/PDA or TBLE_{Ox}/PDA vesicles are transferred to a 100-mL beaker and stirred under a black box while being irradiated at 254 nm for 10 mins through the utilization of a TLC lamp. Experiments were conducted in a Costar 96 black clear flat bottom well plate with the addition of 3 mm borosilicate glass beads. Utilizing a SpectraMax M2 or M5 Multi-Mode plate reader (Molecular Devices, Sunnyvale, CA), the rates of peptide-lipid interactions were observed fluorescently under ambient temperature and at excitation and emission wavelengths of 485 nm and 560 nm, respectively. Data analysis was performed using the software suite GraphPad Prism 6. Briefly,

mean triplicates were baseline corrected with the matching vehicle controls and adjusted relative to the minimum and maximum experimental variable.

2.3 Results

2.3.1 Oxidation of A β ₄₀ via H₂O₂ is selective for methionine-35. To determine the extent and selectivity of A β ₄₀ oxidation in the presence of H₂O₂, 20 μ M solutions of A β ₄₀ were exposed to H₂O₂ at concentrations ranging from 0.1 mM to 6.0 mM. The resulting solutions were incubated at room temperature without agitation. Aliquots were taken at various time points ranging from 0 min to 300 min and accurate protein masses were determined from charge state envelopes within high-resolution MS spectra (Figure. 2.1).

Figure 2.1. H₂O₂ oxidation of A β ₄₀ is selective for methionine-35. (A) Mass spectrum from 600-1200 m/z illustrating that the +4, +5, +6 m/z charge states exhibit a 16 Da mass shift when unoxidized A β ₄₀, Met (green), is exposed to H₂O₂ producing a singly-oxidized A β ₄₀ variant, MetOx (red). (B) Percent oxidation of A β ₄₀ as determined from the oxidized peak area (TIC) of A β ₄₀ relative to the total A β ₄₀ peak area. A β ₄₀ was incubated under varied H₂O₂ concentrations 0.0 mM (green), 0.1 mM (blue), 1.0 mM (purple), 3.0 mM (orange), 6.0 mM (red), and varied lengths of time indicated by symbols (see key). Lines represent the mean $\pm$ SEM percent oxidation over the time course of the experiments. (C) The ¹H NMR spectrum of A β ₄₀ dissolved in 60 mM NaOD and diluted to 10 μ M in 100 mM Na₃PO₄ deuterated buffer at pH 7.23. Total acquisition time was over 30-60 min. The ¹H NMR spectrum was referenced to the residual proton peak of HDO at 4.63 ppm. In the ¹H NMR spectrum of unoxidized A β ₄₀ (black) the singlet peak 1.99 ppm belongs to ϵ CH₃ of Met. Oxidation of A β ₄₀ was achieved by addition of 3% H₂O₂ (w/v) for 3 h and 24 h before its removal via lyophilization. The ¹H NMR spectra (blue and red) exhibited downfield shifts indicated by arrows at 2.55 ppm and 2.95 ppm which were assigned to ϵ CH₃ of Met-sulfoxide and Met-sulfone, respectively.

The mass spectra showed a single oxidation event consistent with the oxidation of Met35 to a sulfoxide derivative under all conditions from the analysis of the +4, +5, and +6 charge state ions. Oxidation of A β ₄₀ with H₂O₂ concentrations up to 6.0 mM predominately resulted in the sulfoxide rather than the sulfone. By comparing the spectral peaks for the unoxidized states (+4 ion at m/z 1083, +5 ion at m/z 866.8, +6 ion at m/z 722.5) to the respective oxidized peaks (+4 ion at m/z 1087, +5 ion at m/z 870.0, +6 ion at m/z 725.2), the extent of oxidation at different H₂O₂ concentrations was determined as a function of time. Without the addition of H₂O₂, a small amount of oxidized A β ₄₀ was present (6.3% on average over all time points with 18% of A β being oxidized by 5 h of incubation), which is inherent to the peptide preparation and disaggregation procedure (Figure. 2.1B). The addition of H₂O₂ at 5:1 molar ratio (0.1 mM H₂O₂) did not significantly increase the extent of Met35 oxidation (6.8% on average over all time points with 17% of A β being oxidized by 5 h of incubation). At 50:1 molar ratio of H₂O₂ (1.0 mM H₂O₂) a significant increase in Met35 oxidation is observed (14% on average over all time points with 36% of A β being oxidized by 5 h of incubation). Additional increases in H₂O₂ (150:1 and 300:1) yielded increases in Met35 oxidation (24% and 36% on average over all time points with 50% and 67% of A β being oxidized by 5 h of incubation, respectively). The observed selective oxidation of Met35 by treatment with H₂O₂ is consistent with previous reports,²⁰⁻²³ but it was essential to establish reliable reaction parameters for further experimentation. Note, the oxidation of other residues within A β has been observed; however, these oxidation events require transition state metals.^{19,21}

To verify the selectivity of oxidation at Met35 under experimental conditions, 20 μ M samples of A β ₄₀ were incubated with an excess of H₂O₂ (980 mM) for 3 h and 24 h. After a disaggregation step, these A β ₄₀ samples were analyzed by ¹H NMR spectroscopy (Figure. 2.1C).

The ^1H NMR spectrum of unoxidized $\text{A}\beta_{40}$ shows a singlet peak at 1.99 ppm which belongs to ϵCH_3 of Met. The ^1H NMR spectra reveal two relatively downfield singlet peaks at 2.55 ppm and 2.95 ppm, respectively and were assigned directly to the oxidized species of Met35 (Met-sulfoxide and Met-sulfone). Oxidation of other residues was not observed at these harsher conditions.

2.3.2 Methionine oxidation decreases total fibril content associated with $\text{A}\beta_{40}$ aggregation. To establish if the presence of H_2O_2 would alter the aggregation process of $\text{A}\beta_{40}$, the rate of fibril formation was monitored with a ThT assay, which detects β -sheet formation associated with fibrils (Figure 2.2A). Amyloid formation occurs via a characteristic two-step process consisting of a lag phase and growth phase. The lag phase is associated with the slow formation of a critical nucleus that triggers fibrillization. The growth phase is associated with fibril elongation. For all concentrations of H_2O_2 , the lag-phase of $\text{A}\beta_{40}$ aggregation was similar (Figure. 2.2B). The relative slope of the growth phase, R_{slope} (h^{-1}), were similar at lower H_2O_2 concentrations, but interestingly, the increase of oxidation conditions to that of 3.0 mM and 6.0 mM H_2O_2 significantly reduced slopes of the growth phase, from $0.62 \pm 0.041 \text{ h}^{-1}$ and $0.24 \pm 0.018 \text{ h}^{-1}$ respectively, when compared to the control $0.85 \pm 0.032 \text{ h}^{-1}$ (Figure 2.2C). However, once steady state was achieved (see Figure 2.3 for all individual ThT curves), the magnitude of total fibril content (as measured by the relative fluorescence at steady state) was reduced as the amount of H_2O_2 present was increased with a statistical significance starting at 1.0 mM H_2O_2 compared to control (Figure 2.2D).

Figure 2.2. Impact of H_2O_2 on $A\beta_{40}$ aggregation (A) Average ThT assays of 20 μM $A\beta_{40}$ (green) aggregation performed at 37 °C with the addition of 0.1 mM (blue), 1.0 mM (purple), 3.0 mM (orange), and 6.0 mM H_2O_2 (red). Continuous lines represent non-linear best fits (Equation 1) to the average experimental traces. The lag-phase was calculated from non-linear fits of three independent experiments represented by bar-graphs as the mean $\pm$ SEM. Kinetic parameters include the (B) lag-phase, t_{lag} , (C) relative slope of the normalized ThT curves, R_{slope} (h^{-1}), and (D) normalized steady-state maximum fluorescent response, and relative fluorescence at steady state. P-values are indicated as follows (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$, and (****) $P \leq 0.0001$.

2. Hydrogen peroxide modifies $A\beta$ -membrane interactions with implications for $A\beta_{40}$ aggregation

Figure 2.3. Individual curves for ThT assays. Assays performed at a concentration of 20 μ M $A\beta_{40}$ (green) aggregation performed at 37 °C in the absence of H_2O_2 (green) or with the addition of 0.1 mM (blue), 1.0 mM (purple), 3.0 mM (orange), and 6.0 mM H_2O_2 (red). Three independent plates were run.

2.3.3 *Increased oxidation modifies the distribution of $A\beta_{40}$ aggregate species.* It is well-known that variations in the chemical environment can induce polymorphic aggregates in $A\beta$,^{60–64} that is, fibrils of unique morphology and structure. To determine if oxidation could result in polymorphic $A\beta$ aggregates, incubations of $A\beta$ with various concentrations of H_2O_2 were sampled every hour for analysis by atomic force microscopy (AFM) to determine the impact of oxidative environments on aggregate species and morphology (Figure 2.4). To determine populations of different aggregate species, oligomers were defined as particles larger than 1 nm in height with an aspect ratio (defined as long axis/short axis) of less than 2.5, indicating a globular morphology. Due to the high density of crossing fibrils in some images, the number of fibrils were manually counted. To prevent bias due to overcounting, individual fibrils were marked by tracing along the contour of each fibril in Photoshop. As fibril length can vary greatly, the total fibril load at any given time point was also determined by percent area of the mica surface covered by fibril aggregates (Figure 2.4B). At time point 0 h, no observable oligomer or fibrils were observed for every condition (H_2O_2 concentration). Within the first-hour, the number of oligomeric aggregates grew similarly for all conditions to higher than ~20 oligomers per μ m²; however, these oligomer populations dramatically dropped after 2 h (a 2 to 4-fold reduction). This decrease in oligomers under each condition coincided with the appearance of fibrils. The appearance of fibril after 2 h is consistent with the lag phases determined from the ThT assays (Figure 2.2A). The number of oligomers continued to decrease as the fibril load increased for each condition. With that being stated, the decrease in oligomers (and subsequent increase in

fibrils) for the 6.0 mM H_2O_2 condition was significantly less pronounced compared with the other conditions, with a noticeable oligomer population still observed into the 3rd h of incubation. The number of fibrils observed at any given time point as a function of H_2O_2 concentration was consistent with the total fibril load associated with the ThT assays, that is, the number of fibrils and the surface area covered by fibrils decreases as the concentration of H_2O_2 increases.

Figure 2.4. Atomic force microscopy analysis of $\text{A}\beta_{40}$ aggregation as a function of H_2O_2 concentration. (A) Representative AFM images of $20\ \mu\text{M}$ $\text{A}\beta_{40}$ incubated at $37\ ^\circ\text{C}$ with the addition of 0.1 mM, 1.0 mM, 3.0 mM, and 6.0 mM H_2O_2 taken at several time points. The color scale is the same for each image. (B) Determination of the number of oligomers and fibrils per unit area and the percent area of the mica surface covered by fibrils as observed in AFM images as a function of time. (C) Height histograms of oligomers observed in AFM images.

Under all conditions, the distribution of oligomer size was consistent with a mode of oligomer size of 2.8-3.2 nm in height (Figure 2.4C). In contrast, there was a distinct polymorphic fibril structure formed in the presence of 6.0 mM H_2O_2 (Figure 2.5). With no H_2O_2 and H_2O_2 concentrations up to 3.0 mM, the average height along the contour of the fibril was ~3 nm. At these conditions, there were often regions where fibrils were bundled or crisscrossed, and these regions were thicker. When H_2O_2 concentration was 6.0 mM, the height along the contour of the fibrils was only ~2 nm, suggesting that the fibrils formed under this condition were structurally different. Interestingly, this is also the condition for which the elongation rate was significantly different and that the amount of Met35 oxidation can reach 50% within 2 hours of incubation.

Figure 2.5. Comparison of $A\beta_{40}$ fibrils formed in the presence of varying concentrations of H_2O_2 . (A) AFM images of fibrils formed by $A\beta_{40}$ in the presence of H_2O_2 concentrations ranging from 0-6.0 mM. The color scale is the same for each image. The black lines in each image corresponds to the height profile to the right of the image. In the height profile, a red dashed line is provided at a height of 3 nm for visual reference. (B) Histograms of the average height along the contour of fibrils observed in AFM images.

2.3.4 Secondary structural transitions of $A\beta_{40}$ are altered by H_2O_2 . To determine if oxidative environments altered secondary structural changes in $A\beta_{40}$ associated with aggregation, circular dichroism (CD) spectra were collected of $A\beta_{40}$ exposed to various concentrations of

H₂O₂ as a function of time (Figure 2.6). In the absence of H₂O₂, A β ₄₀ underwent a clear structural transition between 2 and 3 h of incubation. Specifically, during the first 2 h of incubation, A β ₄₀ was predominately random coil. At 3 h, there was a significant increase in α -helical content, suggesting the appearance of an α -helical intermediate that has been proposed.^{29,65–67} After 3 h, the parallel β -sheet content of A β ₄₀ increased, consistent with extensive fibril formation.

Figure 2.6. Secondary Structural Transitions Altered by H₂O₂. Time-dependent changes in CD spectra recorded from 190-260 nm over 5 h, discontinuously, at 25°C from 20 μ M A β ₄₀ samples incubated continuously at 37 °C in a 10 mM K₂HPO₄ 50 mM Na₂SO₄ buffer (pH 7.23) containing (A) 0.0 mM H₂O₂, (B) 1.0 mM H₂O₂, and (C) 6.0 mM H₂O₂. Changes in secondary structure as a function of time from CD deconvolution presented as percent contribution of helical content (red), antiparallel β -sheet (blue), parallel β -sheet (purple), and turn/random coil regions (green) for A β ₄₀ in the presence of (D) 0.0 mM H₂O₂, (E) 1.0 mM H₂O₂, and (F) 6.0 mM H₂O₂. Each spectra was deconvoluted using the online program BeStSel (Beta Structure Selection, <http://bestsel.elte.hu/>).

Adding H₂O₂ to the A β ₄₀ incubations at concentration of 1.0 mM and 6.0 mM altered the development of secondary structural elements as a function of time. Neither condition caused a

significant change in secondary structure of A β ₄₀ for the first 2 h of incubation, that is, random coil was the dominant structural element. At 3 h, the rise of α -helical structure in A β ₄₀ was still apparent with 1.0 mM H₂O₂. The transition from the α -helical intermediate to β -sheet structure associated with fibrillization still occurred in the presence of 1.0 mM H₂O₂ after 3 h of incubation, but this α -helical content persisted at the 4 and 5 h time points. Similar to aggregation in the absence of H₂O₂, there was an increase in parallel β -sheet content. At the 6.0 mM concentration of H₂O₂, the transition away from random coil structure was not apparent over the time course of the experiment, which is consistent with previously published results for A β ₄₂ and A β ₄₂-Met35[O]²⁰ and further supports the notion that fibrils observed under this condition represent a polymorph when compared to A β fibrils formed in the absence of H₂O₂.

2.3.5 The presence of TBLE LUVs impedes aggregation of A β ₄₀. To understand how the presence of lipid membranes alter A β ₄₀ aggregation, and how oxidation may modulate that interaction, we evaluated A β ₄₀ aggregation in the presence of large unilamellar vesicles (LUV) of TBLE with ThT assays. Due to inconsistent results in the literature demonstrating the increase^{68,69} or decreases^{70,71} of lag-phase behavior for A β aggregation in the presence of lipids, first performed to understand how A β aggregates as a function of lipid mass for the specific lipid system and A β preparation protocols used here (Figure 2.7). These experiments were performed in the absence of H₂O₂ and at an A β ₄₀ concentration of 20 μ M. As the lipid to peptide ratio was increased, the lag phase associated with A β ₄₀ aggregation (in the absence of H₂O₂) extended, increasing by ~3.5 h when the lipid to peptide ratio exceeded 50:1 (Figure 2.7B). These extended lag phases are attributable to the effect of interactions between A β ₄₀ and the LUVs. The slope of the growth phase and extent of β -sheet formation at steady state varied extensively without a clear trend in the presence of TBLE.

Figure 2.7. Presence of TBLE extends lag-phase of $A\beta_{40}$ aggregation (A) Average ThT assays of 20 μM $A\beta_{40}$ performed at 37°C in the absence (green) and presence of TBLE vesicles various lipid-to-peptide (L:P) ratios; 10:1 (blue), 20:1 (purple), 30:1 (orange), 50:1 (red), and 110:1 (pink). Equation 1 was utilized to provide a non-linear best fit to the average of triplicate traces represented as continuous lines. (B) The lag-phase, t_{lag} , was calculated from non-linear fits of experiments represented by bar-graphs as the mean $\pm$ SEM. (C) The relative slope of the normalized ThT curves, R_{slope} (h^{-1}), and (D) relative fluorescence at steady state, RF_{ss} , were calculated of the linear fits of the growth phase and plateau region of the ThT curves, respectively.

Having established how TBLE alters A β ₄₀ aggregation in the absence of H₂O₂, a series of ThT assays were run with increasing amounts of H₂O₂ (from 0.1 mM to 6.0 mM) and a lipid to peptide ratio of 30:1 (Figure 2.8). At the 30:1 lipid to peptide ratio, the lag phase was 3.61 ± 0.39 h in the absence of H₂O₂. Increasing H₂O₂ concentration systematically reduced the lag phase associated with A β aggregation. The effect was significant enough that at the highest concentration of H₂O₂, 6.0 mM, the lag-phase was roughly equivalent to the lag-phases of A β ₄₀ observed in Figure 2.2 in which no lipid was present (1.10 ± 0.51 h and 1.27 ± 0.06 h, respectively), suggesting that increased H₂O₂ concentration decreases the interaction between A β ₄₀ and lipids. Once nucleation occurred, the slope of the growth phase, associated with fibril elongation, remained unchanged as H₂O₂ concentration was increased up to a concentration of 3.0 mM. At 6.0 mM H₂O₂ the slope of the growth phase abruptly dropped to 0.21 ± 0.015 h⁻¹. The magnitude of ThT signal once steady state A β aggregation was reached also diminished with increased exposure to H₂O₂. Collectively, these observations suggest that the oxidative environment provided by H₂O₂ reduced the affinity of A β for the lipid bilayer surface, causing the characteristics of A β aggregation to shift toward those associated with the absence of lipids.

Figure 2.8. Impact of H₂O₂ on Aβ₄₀ aggregation in the presence of TBLE vesicles (A) Average ThT assays of 20 μM Aβ₄₀ (green) aggregation in the presence of TBLE vesicles (30:1 lipid to peptide ratio) performed at 37 °C with the addition of 0.1 mM (blue), 1.0 mM (purple), 3.0 mM (orange), and 6.0 mM H₂O₂ (red). Continuous lines represent non-linear best fits (Equation 1) to the average experimental traces. The lag-phase was calculated from non-linear fits of three independent experiments represented by bar-graphs as the mean ± SEM. Kinetic parameters include the (B) lag-phase, t_{lag} , (C) relative slope of the normalized ThT curves, R_{slope} (h⁻¹), and (D) normalized steady-state maximum fluorescent response, and relative fluorescence at steady state. *P*-values are indicated as follows (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$, and (****) $P \leq 0.0001$.

2.3.6 Oxidation reduces A β /lipid interaction and lipid oxidation plays a large role. As the ThT assays suggested a reduced interaction between the lipid vesicles and A β_{40} , a polydiacetylene (PDA) lipid binding assay was performed to measure the total interaction between A β_{40} and lipid membranes (Figure 2.9A). Polydiacetylene is a lipid moiety that can be incorporated into vesicles and photo-crosslinked to create vesicles that are sensitive to mechanical perturbations associated with peptide binding and aggregation, resulting in both a color change (blue to red) and enhanced fluorescence. Here, the PDA was incorporated into TBLE vesicles, and the response upon exposure to A β_{40} was measured fluorescently. Controls were performed demonstrating that exposure to various concentration of H₂O₂ did not invoke a fluorescent response in the PDA assay in the absence of A β and used for background correction. Upon exposure of TBLE/PDA vesicles to A β_{40} , the fluorescent signal steadily increases as the peptide binds and aggregates on vesicles. While the presence of 0.1 mM and 1.0 mM H₂O₂ did not significantly alter the interaction between A β_{40} and the vesicles, H₂O₂ concentrations of 3.0 mM and higher resulted in a significant decrease in peptide/lipid interaction, which correlates with the ThT data presented in Figure 2.8.

Figure 2.9. Aβ₄₀ binding assays performed with TBLE/PDA vesicles. (A) PDA fluorescence response (λ_{Ex} 485 nm, λ_{Em} 560 nm) as a function of time when exposed to 20 μ M Aβ₄₀ (green) incubated at 25°C and in the presence of 0.1 mM (blue), 1.0 mM (purple), 3.0 mM (orange), and 6.0 mM H₂O₂ (red). (B) Isolated effects of varied oxidation states of TBLE and Aβ₄₀ on peptide-membrane interactions measured by PDA fluorescence incubated at 25°C. PDA assays were performed with the following combinations; unoxidized TBLE/PDA and Aβ₄₀ (green, circle), TBLE/PDA and Aβ₄₀ oxidized (orange, triangle), TBLE_{ox}/PDA and Aβ₄₀ (purple, diamond), lastly TBLE_{ox}/PDA and Aβ₄₀ oxidized (red, square).

H₂O₂ can oxidize not only Aβ but also lipids. To elucidate if oxidation of Aβ₄₀, TBLE LUVs, or both was the modulator of the observed decreased peptide/lipid interaction, additional lipid binding assays were performed in which the oxidation of the varying components was controlled. Briefly, Aβ₄₀ and TBLE were oxidized separately and lyophilized to remove residual H₂O₂. These pre-oxidized materials were then used for a series of TBLE/PDA assays. The pre-oxidized stock of Aβ₄₀ was 76% oxidized (as measured by MS), and the pre-oxidized stock of

TBLE was 62% oxidized (as determined by an LPO Assay Kit). The oxidized form of TBLE was used in conjunction with PDA to create an oxidized-TBLE/PDA vesicles (TBLE_{Ox}). Due to concerns about changes in solubility upon oxidation of the lipids, it was necessary that the total lipid mass delivered to each sample be standardized. Standardization was carried out through the use of optical density measurements at 470 nm conducted before photoactivation of TBLE/PDA vesicles. Maximum responses of positive controls were also compared and used to standardize the responses across different TBLE/PDA batches; maximum fluorescent intensities were found to be directly comparable even before normalizing across batches.

When both oxidized TBLE (TBLE_{Ox}) and A β ₄₀ (A β ₄₀Ox) were used in the PDA assay, a significant decrease in peptide/lipid interaction was observed compared with using un-oxidized TBLE and A β ₄₀, which is consistent with the PDA assay results from Figure 2.9B. However, when only A β ₄₀ was oxidized, the interaction of the peptide with un-oxidized TBLE vesicles increased in comparison with un-oxidized A β ₄₀ (Figure 2.9B), which is the opposite effect that was observed when H₂O₂ was just added directly to the interaction. When TBLE_{Ox} was used in the PDA assay, exposure to un-oxidized A β ₄₀ resulted in an even greater decrease in the measured peptide/lipid interaction compared with oxidized A β ₄₀. These observations suggest that oxidation of A β and lipid both play a role in their co-interaction, but with opposite effects. That is, oxidation of A β enhances the affinity for TBLE based membranes, while lipid oxidation inhibits the binding of A β to membranes. Of the two, lipid oxidation appears to have a larger impact on the peptide/lipid interaction.

2.4 Discussion

A number of studies have investigated the impact of Met35 oxidation on A β aggregation in the absence of lipids with varying results. Some studies suggest that Met35 oxidation enhances aggregation to fibrils;²⁶ meanwhile, others suggest it impedes aggregation.^{23,25,72} A complicating factor in evaluating studies of A β aggregation and structure is divergent preparatory conditions greatly influence experimental outcomes.⁷³ Of these reports, the study by Gu and Viles is most directly comparable to our results, as the same A β preparation protocol was used, oxidation was performed with a similar range of H₂O₂, and the aggregation occurs with mixtures of oxidized and unoxidized A β .²³ In fact, the extent of oxidation in their study and ours under similar concentrations of H₂O₂ were comparable. While Gu and Viles observed a H₂O₂ concentration dependent reduction in the lag phase,²³ we observed no statistically significant effect on the lag phase. A key difference between the two studies is that we elected to use small borosilicate beads in each well to mix our aggregation reactions during the ThT assays. This is known to enhance A β aggregation and provide more reproducible aggregation rates.⁷⁴ As a result, our aggregation reactions proceeded much more rapidly in comparison with Gu and Viles. We elected to use this protocol as without the beads our rates of aggregation were too variable to perform a statistical analysis of kinetic parameters, even without the addition of H₂O₂.

In these studies, the similarities in the lag-phase associated with the presence of up to 3.0 mM H₂O₂ suggest that oxidation of Met35 is not pivotal in the initial formation of the critical nucleus associated with fibril formation under our experimental conditions. The critical nucleus associated with A β fibrillization is often considered to be a small oligomer.^{75–77} While cross-linking methods to stabilize small A β oligomers have demonstrated that A β ₄₀ forms an equilibrium distribution of monomers, dimers, trimers, and tetramer formation, isolated

A β ₄₀Met35[O] only formed dimers, as determined by ESI-MS.^{78,79} However, co-incubation of A β ₄₀ and A β ₄₀Met35[O] resulted in trimer and tetramer formation with statistically random incorporation of A β ₄₀Met35[O] into oligomers. This heterogenic mixing is the scenario that is applicable to our experimental setup as increasing H₂O₂ concentration resulted in a gradual increase in A β ₄₀ oxidation at Met35, maxing out at ~50-65%. Taken together, it appears that for homogenous systems of oxidized A β Met35 plays a large role in the initial oligomerization, but under heterogenous systems of A β ₄₀ and A β ₄₀Met35[O] oligomerization on-pathway to fibril formation can still proceed.

Despite the lag phase of aggregation being predominately unaltered in the absence of lipids, at large enough H₂O₂ concentrations (at least 6.0 mM) the relative slope of the growth phase, associated with fibril growth of A β ₄₀, was significantly reduced indicating that the addition of monomeric A β ₄₀ to the terminal ends of fibrils is altered. Since the extent of oxidized A β ₄₀ varies with increases in the oxidative environment, representing a higher population of A β ₄₀-Met35[O] to A β ₄₀, the reduction in fibril growth could reflect a lower affinity of A β ₄₀-Met35[O] for fibril ends. This is consistent with experiments in which the aggregation of monomeric A β ₄₀-Met35[O] was less efficiently seeded by the addition of pre-formed fibrils.²³ The addition of monomeric A β to fibril ends has been proposed before to proceed through a two-step “dock-lock” mechanism⁸⁰⁻⁸² with the C-terminal hydrophobic residues having a conformational preference for the initial docking stage.⁸³ Since Met35 is the only observable modification to A β ₄₀ determined by MS and ¹H NMR, it is possible that oxidation of Met35 is inhibiting the ability of A β ₄₀ to dock to fibril ends successfully. However, this is difficult to ascertain as the reduction of fibril growth at 6.0 mM H₂O₂ may also reflect the underlying fibril structure, as our AFM and CD analysis indicated that a distinct, polymorphic fibril was observed

under this condition. That is, the docking and locking mechanism may be distinct as the underlying fibril structure is likely different as discussed below.

The altered morphology observed by AFM and CD spectral differences associated with fibrils formed with 6.0 mM H₂O₂ suggest a unique fibril structure. Several lines of evidence support that oxidation of Met35 could promote a fibril polymorph. Intermolecular interactions between Met35-Met35 found in hydrophobic domains have been observed for two-fold⁸⁴ and three-fold symmetric fibrils.⁸⁵ Cryo-EM resolved structures demonstrating an *LS-shaped* motif placed Met35 in one of three stabilizing hydrophobic clusters. Gremer et al. proposed that these Met35-Met35 interactions occur along the fiber axis instead of across the fibril axis as previously thought.⁸⁶ Therefore, A β ₄₀-Met35[O] with significant alterations to its dipole moment and hydrophobicity^{72,87} would likely not be incorporated into these types of fibril structures as easily. NMR structures of pure A β ₄₂-Met35[O] fibrils have been obtained in which Met35[O] is facing away from the hydrophobic core making it solvent exposed.³⁵ In addition, solution state NMR studies suggest that the more solvent exposed Met35[O] of A β ₄₀ or A β ₄₂ reduces hydrophobic and electrostatic associations involved in fibril formation.²⁹

The presence of a distinct fibril polymorph might suggest unique oligomeric precursors. Just as structural changes of A β are hypothesized to be the driving force for aggregation and subtle environmental factors can result in unique fibril structures,^{84,88} one could envision that the ratio of A β ₄₀-Met35[O] to A β ₄₀ would influence the formation of both off-pathway and on-pathway oligomers. However, evaluation of oligomer morphology by AFM did not result in any observable differences at a morphological level. Time resolved CD spectroscopy did suggest that transitory secondary structural elements associated with A β ₄₀ aggregation was altered at 6.0 mM H₂O₂. From the absence of H₂O₂ to 1.0 mM H₂O₂, evidence of an α -helical intermediate was

observed, but this was not apparent at 6.0 mM H₂O₂ despite the similarities in the lag phase. A β α -helical intermediates on-pathway to fibril formation have been observed via Raman⁸⁹ and CD spectroscopy.⁹⁰ The NMR study of monomeric A β ₄₀ in SDS micelles²⁸ and aqueous⁶⁵ environments suggested that the structure of monomeric A β ₄₀ adopts helical conformations. Met35 oxidation specifically disrupts a helical region of A β spanning from residues 28-36.²⁸ In light of the unique fibril structure associated with fibrils formed in the presence of 6.0 mM H₂O₂, it is possible that the oligomers observed under these different conditions were structurally different despite being similar in size.

Lipid membranes are known to influence the aggregation of A β ,⁷¹ and this is highly sensitive to membrane composition.^{43,71,91} As a result, we aimed to determine how oxidative environments influenced A β aggregation in the presence of lipid membranes of TBLE. In the absence of H₂O₂, the addition of TBLE LUVs increased the lag-phase as a function of peptide/lipid ratio. We then introduced oxidizing environments to a standardized ratio of TBLE LUVs and A β ₄₀. Our observations established that increasing H₂O₂ concentration reduced TBLE LUV's effect on A β ₄₀ fibrillogenesis, resulting in similar aggregation profiles for A β in 6.0 mM H₂O₂ in the presence and absence of TBLE. A potential explanation is that binding of A β to TBLE LUVs effectively retards aggregation, but the affinity of A β is reduced as Met35 is oxidized. As the affinity of A β for the TBLE is reduced due to oxidation, the impact of TBLE LUVs on aggregation would decrease, and aggregation would proceed in a manner similar to the condition when the LUVs are absent. To test this assertion, PDA assays were performed that confirmed that increased H₂O₂ concentration directly correlated with decreased A β ₄₀ interaction with TBLE LUVs.

Due to hydrogen peroxide's ability to oxidize A β ₄₀ and TBLE-LUVs simultaneously, we sought to distinguish the relative contribution of peptide and lipid oxidation on the reduced binding interaction by systematically performing PDA assays with varying combinations of oxidized and un-oxidized components. This is important as A β binding to cellular membranes represents a key phenomenon in assessing cellular susceptibility to A β -induced toxicity.⁴¹ Interestingly, Met35 oxidation increased the affinity of A β ₄₀ for TBLE membranes that were not oxidized; whereas, lipid peroxidation reduced the ability of A β ₄₀ to bind lipids. There are several physicochemical possibilities as to why Met35 oxidation increased the affinity of A β ₄₀ for lipid membranes. The methionine sulfoxide side chain is stiffer and more polar compared to methionine, and the hydropathy index of methionine sulfoxide is similar to that of lysine,⁹² which is known to play a role in membrane protein anchoring,⁹³ cell-penetrating peptides,⁹⁴ and antimicrobial peptides.⁹⁵ These chemical changes could enhance the affinity of A β ₄₀-Met35[O] with membranes via stronger interactions with polar and charged lipid head groups. Regarding the role of lipids, lipid peroxidation is known to rigidify membranes. Interestingly, alteration in membrane rigidity correlated with the ability of A β ₄₀ to bind and damage supported TBLE bilayers.^{96,97} Collectively, our results suggest that the affinity of A β for cellular membranes would depend not just on intrinsic factors associated with the peptide, but also environmental factors.

2.5 References

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3. Acetylation of A β ₄₀ alters aggregation in the presence and absence of lipid membranes

One characteristic of Alzheimer's disease (AD) pathology is the formation of extracellular senile plaques comprised of the peptide β -amyloid (A β). Aggregation of A β is a complex nucleation-dependent polymerization process involving the creation of a variety of metastable intermediates that can be on-pathway towards fibril formation or off-pathway towards amorphous or annular aggregates. An essential feature of the aggregation of A β to fibril aggregates is the formation of a β -sheet hairpin which is stabilized by a salt bridge between D23 and K28. Recent evidence has demonstrated that the forming of the salt bridge is a critical step in the nucleation process. Lysine-16 has also been implicated in participating in the intermolecular formation of salt bridges between fibril strands. Lysine's in general are involved in protein-membrane interactions between the cationic lysine and anionic lipid headgroups, and it is this general interaction of A β with membranes that is proposed to be a toxic mechanism of AD. In this work, we explored the role of lysine's positive charge in the aggregation of A β ₄₀ in the presence and absence of membranes through its removal via acetylation. Here, we report that lower levels of acetylation, primarily affecting K16, has minor effects on the fibril formation but reduces oligomerization of A β ₄₀ while higher levels of acetylation involving K16 and K28 inhibits fibril formation and further reduces oligomerization. However, in the presence of total brain lipid extract (TBLE) acetylated A β was observed to interact less with the membrane but was still able to form fibrils and under extensive levels of acetylation promoted the formation of annular aggregates. Our results suggest that in the absence of membranes the aggregation process is highly dependent on the electrostatic interactions of K16 and K28, and in the presence of

membranes lipids promote the formation of the critical nucleus towards fibril formation in addition to promoting secondary aggregation pathways.

3.1 Introduction: The importance of lysine in the aggregation and membrane binding of A β

Two prominent pathological features associated with Alzheimer's disease (AD), a fatal neurodegenerative disorder, are cerebrovascular, diffuse, and neuritic plaques composed predominantly of the amyloidogenic peptide amyloid- β (A β) and neurofibrillary tangles comprised of the protein tau. These proteinaceous deposits of tau and A β are predominantly comprised of β -sheet-rich fibrous aggregates termed amyloid fibrils. A β aggregation can be a multifaceted and complex process that involves a variety of metastable, intermediate species that may or may not be directly on-pathway to fibril formation.¹ The aggregate species that can be formed include a variety of soluble A β oligomers (A β Os), insoluble protofibrils, and annular aggregates.¹⁻³ Beyond the heterogeneity of smaller intermediate aggregate structures, A β fibrils can form a variety of distinct structure and morphologies, often termed polymorphs, as a result of preparatory and/or environmental conditions during the aggregation process.⁴⁻⁶

Specific domains or amino acids within the A β peptide appear to play a prominent role in dictating the rate of aggregation and the resulting aggregate morphologies.⁷ For example, specific regions within the A β peptide have been implicated as being particularly amyloidogenic.⁷⁻¹⁰ Using site-directed spin labeling electron paramagnetic resonance, two β -strand forming domains in A β were identified (residues 11–21 and 29–39, respectively) that were separated by a β -turn.¹¹ NMR studies further support the two β -strand regions separated by a β -turn motif in a number of fibril structures.¹²⁻¹⁴ Various fragments of A β display different propensities to aggregate.⁷ For example, the first β -strand forming stretch of A β also contains a

hydrophobic core (residues 16–21) with enhanced amyloidogenic properties.¹⁵ Furthermore, a number of point mutations associated with familial forms of AD clearly alter aggregation rates and aggregate morphologies.¹⁶

Lysine is of interest as it contains a cationic charge at the end of an aliphatic chain. There are two lysines in A β : lysine-16 (K16) and lysine-28 (K28). Electrostatic interactions facilitated by these two lysines in A β have been suggested by many to play an essential role in A β folding, nucleation, oligomerization, fibril formation, and toxicity.^{17,18} Specifically, a salt bridge forms between D23 and K28 that stabilizes a β -turn in a variety of fibril structures.^{18–20} K16 is the first amino acid of the previously mentioned hydrophobic core. Additionally, K16 can be involved in intermolecular salt bridge formation in some fibril structures of A β ;^{18,21} however, K16 is solvent exposed in other fibril structures.²² Beyond fibril formation, lysine residues are involved in oligomer formation, as substituting alanine for each lysine altered A β assembly and highlighted a role of K16 in A β toxicity.²³

In addition to playing a role in A β aggregation, lysines play a prominent role in the interaction of A β with lipid membranes. In general, lysine plays an important role in facilitating a variety of peptide-membrane interactions, as it contains the ability to electrostatically interact with charged lipid headgroups while the alkyl chain allows for hydrophobic interactions within the lipid tails.²⁴ Interaction with cellular membranes, comprised predominately of lipid bilayers, can heavily influence A β aggregation,²⁵ potentially promoting unique aggregation pathways,^{26–28} nucleating aggregation,^{29–34} and/or promote/stabilize specific A β aggregate species.^{26,32,35,36} It is even suggested that the binding of A β to the plasma membrane is a key step in A β toxicity.^{37,38} A variety of model lipid membranes alter A β conformation and exert enormous influence on the aggregation state, but the exact impact of lipids on A β aggregation is specific to membrane lipid

composition.³⁹ In addition to influencing A β aggregation^{40,41} A β can directly damage cellular membranes, resulting in membrane dysfunction through altered mechanical properties⁴² and/or the formation of discrete pores.^{32,35,43} While lipid composition and biophysical properties of membranes play a role A β /lipid interactions, physicochemical properties of the residues within A β also play a role in the surface interactions.^{16,44} For example, disease-related point mutations within A β alter the aggregate species observed on model lipid membranes, with hydrophilicity and electrostatic properties of specific amino acids playing a key role.¹⁶

Here, our aim was to understand how acetylating lysine alters A β_{40} aggregation in the presence and absence of lipid membranes. A β_{40} was acetylated by exposure to sulfo-N-hydroxysulfosuccinimide acetate (NHS) producing acetylated K16 (AcK16) and K28 (AcK28). The lipid system used was total brain lipid extract (TBLE), which contain a physiologically relevant mix of lipid components. In the absence of lipid vesicles, acetylation of A β_{40} reduced both oligomerization and fibrillization, with extensive acetylation resulting in complete inhibition of fibril formation. However, the presence of TBLE lipid vesicles appeared to invoke a distinct aggregation mechanism, resulting in fibril formation even when A β_{40} was extensively acetylated.

3.2 Materials and Methods

3.2.1 Peptide Preparation — Synthetic A β_{40} (Invitrogen, 1 mg) was equilibrated to room temperature for 30 mins. A β_{40} was dissolved in 10% NH₄OH(w/v) to acquire an alkaline stock solution of A β_{40} (0.5 mg/mL). The stock solution was unperturbed for 10 min followed by 5 min of bath sonication. The A β_{40} stock solution was aliquoted and snap frozen. Aliquots were lyophilized overnight and stored at -80 °C until the day of experimentation. For experiments,

individual aliquots of A β ₄₀ were equilibrated to room temperature. An alkaline solution, 60 mM NaOH(aq), was used to solubilize A β ₄₀ (target concentration of 1.5 mg/mL), creating what is referred to as the A β ₄₀•NaOH solution. The A β ₄₀•NaOH solution was incubated at room temperature for 10 min, after which it was gently pipette mixed. The actual concentration of the A β ₄₀•NaOH solution was measured (NanoDrop, ThermoScientific) via absorbance at 280 nm with an extinction coefficient of 1440 M⁻¹ cm⁻¹. The pH of the A β ₄₀•NaOH solution was controlled to be $\geq$ 11.23. For all experiments, the A β ₄₀•NaOH solution was diluted into a 20 mM HEPES 150 mM NaCl buffer, pH = 7.23.

3.2.2 Mass Spectrometry — For the MS experiments, the A β ₄₀•NaOH solution was divided into 10 μ g aliquots. These aliquots were diluted in the HEPES buffer and then treated with NHSA at increasing molar ratios of NHSA:A β ₄₀. Samples were allowed to incubate at room temperature for 1 h before the NHSA reaction was quenched with 1 M TrizmaTM (Sigma Aldrich, St. Louis, MO). All samples were snap-frozen and lyophilized overnight. The following day, samples were dissolved in 1 M acetic acid containing 0.5 mg/mL of pepsin (10:1 pepsin: A β ₄₀). Samples were left overnight protected from light and incubated at room temperature. On the 3rd day samples were analyzed via LC-MS (ThermoFisher Scientific Q Exactive Mass Spectrometer, an LC-MS/MS with orbitrap). To remove residual salts, chromatography utilized a C18 column (ZORBAX RRHD Eclipse XDB 80Å C18, 2.1 x 100 mm, 1.8 μ m; Agilent, Santa Clara, CA) and a mobile phase of 0.1% formic/water (v/v) (%A) and 0.1% formic acid/acetonitrile (v/v) (%B) with a gradient of 5%B for 0-2 min, 40%B for 5 min, 50%B for 6 min, 95%B for 7-8 min, and 5% B for 8.5-10 min with a flowrate of 300 μ L/min. A full scan was performed for the mass charge range of 200-2000 m/z with a resolution of 70,000 and a resolution of 17,500 for the Top 5 method that was used. Mass spectrum analysis was performed

using Proteome DiscoveryTM to determine the percent abundance of AcK16 and AcK28. This was carried out by comparing lysine specific fragments of A β ₄₀ to the same peptide fragments containing a 43.05 Da mass shift, for all charge states.

3.2.3 Thioflavin-T Fluorescence Assay (ThT) — Monitoring fibril formation was achieved with thioflavin T (ThT; Sigma Aldrich, St. Louis, MO) assays. Assays were carried out in a Costar 96-well black clear flat bottom plate. The A β ₄₀•NaOH solution was added to 20 mM HEPES 150 mM NaCl solutions containing 3 molar equivalents of ThT to A β ₄₀ and varied molar equivalents of NHSA to A β ₄₀ at a pH of 7.23. The resulting solutions contained 20 μ M A β ₄₀ and the addition of 0.16, 0.32 0.50 1.00 1.50 mM NHSA. Additionally, a 3 mm borosilicate bead was introduced to facilitate homogeneous mixing across wells.⁴⁵ The assay plate was sealed with plate tape, and incubated at 37 °C for 24 h on a SpectraMax M2 Multi-Mode plate reader (Molecular Devices, Sunnyvale, CA). Fluorescence intensities were measured in 5 min intervals at excitation and emission wavelengths of 440 nm and 484 nm, respectively, with shaking in between reads. Assays performed in the presence of TBLE were performed in the same manner with the addition of TBLE vesicles prepared as described below.

Data analysis was performed utilizing GraphPad Prism 6 was to average replicates, perform baseline corrections, and error analysis reported as the standard error mean (SEM). Additionally, through the use of a Boltzmann sigmodal function (Equation 1) kinetic parameters such as the nucleation time, commonly referred to here as lag-phase (t_{lag}), was empirically obtained by fitting the individual traces of ThT curves.^{46,47}

$$Y = (y_i + m_i x) + \frac{y_f + m_f x}{1 + e^{-\left(\frac{x - x_0}{\tau}\right)}} \quad (\text{Eq. 1})$$

Utilizing GraphPad Prism 6 the initial lag-phase and plateau regions of the curves are defined by linear fits where the initial fluorescence and slope are defined by, y_i and m_i , and the final

fluorescence and slope as, y_f and m_f , respectively. The time to reach the half maximum of the curve is defined by x_o and the elongation time constant of the growth phase is defined as τ . As a nonideal sigmodal function, no constraints were applied. A least squares fitting operation was then performed for a maximum iteration limit of 1000. The lag-phase time was calculated from the kinetic parameters, $t_{lag}=x_o-2\tau$. The slope of the growth phase is associated with the growth of fibrils. The slope was calculated by normalizing the ThT traces to the mean maximum response of control group (no NHSA added) within each independent experiment for comparison across experiments. To obtain the relative slope of the growth phase, $R_{slope}(h^{-1})$ the linear fits of the growth phase were performed by defining the boundaries of the fit to only include the linear region between the lag-phase and the plateau region as visually determined for each ThT curve. The maximum signal of fluorescence at steady state was calculate using a linear fit over the plateau region of the sigmodal curve. This was accomplished by adjusting the starting point of the data to be fit and moving it further back to optimize the r^2 value. Initial starting point points were visually determined to be near the inflection point where the sigmoidal curve enters the plateau region. The fits were performed from these starting points to the end of the data set. Once the boundaries were defined, the average fluorescence signal within this plateau region was determined. For comparison between trials, these were converted to the relative fluorescence at steady state (RF_{ss}) by dividing each value by the control experiment (no NHSA for that experiment). All kinetic parameters were then averaged from triplicates of independent experiments to determine the mean $\pm$ SEM. Significance was reported as greater than 95% confidence calculated using a standard Student's T-test to determine variations in parameters across varied NHSA concentrations.

3.2.4 Lipid Vesicle Preparation — Total lipid brain extract (TBLE; Avanti Polar Lipids, Alabaster, AL) was dissolved in chloroform at 25 mg/mL and divided into 5 mg aliquots. Chloroform was removed in vacuo, producing a transparent film, and each aliquot was stored at -20°C. A 5 mg amount of TBLE was hydrated with a 20 mM HEPES 150 mM NaCl buffer, pH = 7.23, and allowed to incubate for 30 mins at 60°C and 1400 rpm in a Thermomixer. Lipid samples were vigorously pipetted and scraped to remove all traces of TBLE from the walls of the centrifuge tube, after which the samples were put through 7 freeze-thaw cycles and 30 min of bath sonication at 37°C. Lipid stocks were again heated to 60°C before being extruded through a 100 nm polycarbonate membrane filter. Resulting lipid solutions were kept at 37°C before being diluted into a HEPES solution at a final concentration of 0.432 mg/mL within a 96-well plate for a protein to lipid ratio of 30:1.

3.2.5 Ex situ Tapping Mode Atomic force microscopy (TMAFM) — Incubations of A β ₄₀ (20 μ M) were performed in a similar manner to previous ThT assays with the same concentrations and ratios of A β ₄₀ to NHSA. Solutions were incubated at 37°C and shaken and read at the same intervals as previous ThT assays, however at the time points of 0, 1, 3, 5, 7, 8, 9, 11, and 24 h the 96-well plate was removed from the plate reader and two 2 μ L samples were taken from each solution. The experiment was done in this manner in order to track the progress of A β aggregate formation. Once samples were taken, they were allowed to incubate for 1 min before being washed with 200 μ L of 18 M Ω water for one sample and 100 μ L of 18 M Ω water for the other sample of the same variable. Samples were dried with compressed air immediately after being washed. After taking samples, the plate was returned and incubated until the next time interval. Samples were imaged via atomic force microscopy (AFM) under tapping mode on a Nanoscope V Multimode (Veeco, Santa Barbara, CA) with a closed-loop vertical engage J-

scanner. Cantilevers used for images were a diving-board-shaped silicon oxide cantilever with a resonance frequency of ~300 kHz and a spring constant of 40 N/m (Budget Sensors).

3.2.6 TMAFM Image Analysis — Images were processed utilizing Matlab equipped with the image processing toolbox (MathWorks, Natick, MA).⁴⁸ To correct for background curvature, height images were flattened using a 2nd polynomial flattening algorithm. To aid in automatically identifying individual aggregates, AFM images were converted into binary maps after establishing a height threshold of 1.0 nm. Individual aggregates were determined from the binary maps using a recognition algorithm. Physical features of individual aggregates (e.g., height, volume, diameter, shape factor, length, aspect ratio, length, and area covered) were measured. Average heights of fibrils were determined by measuring the fibrils along their contour length while excluding any cross over regions of overlapping fibrils. The range of fibril contour length, or the spread of fibril heights, was calculated as the interquartile range which involves taking the median of the lower and upper of a given dataset. Populations of oligomers and fibrils, along with the percent area covered by fibrils, were plotted using GraphPad Prism 6 to average across replicates and perform error analysis reported as a standard error mean (SEM).

3.2.7 Preparation of TBLE/Polydiacetylene Vesicles (PDA) — Separately 8.4 mg of 10,12-tricosadiynoic acid (PDA) (GFS Chemicals, Columbus, OH) and 5.0 mg of TBLE was dissolved in a 1:1 chloroform: ethanol solution. The two were combined in a glass vial at a 60:40 mass ratio of PDA to TBLE, and solvents were removed in vacuo to produce a TBLE/PDA film. This film was hydrated with 8 mL of the same 20 mM HEPES 150 mM NaCl buffer, pH = 7.23, and temperature of 75°C. The solution was probe sonicated at 180 W continuously for 10 min. The resulting solution was stored protected from light in a 4°C fridge overnight. The following day the TBLE/PDA solution was allowed to reach ambient temperature before transferring it to a

100-mL beaker. The solution was stirred at 300 rpm for 10 min under cover of a black box adapted with a TLC lamp that irradiated the solution at 254 nm for the full length of time. PDA assays were performed in a Costar 96 black clear flat bottom well plate with the addition of 3 mm borosilicate glass beads. Peptide-lipid interactions were observed fluorescently at room temperature with excitation and emission wavelengths of 485 nm and 560 nm utilizing a SpectraMax M2 Multi-Mode plate reader (Molecular Devices, Sunnyvale, CA). Data analysis was performed by averaging triplicates and performing baseline-corrections all within the GraphPad Prism 6 software suite

3.3 Results

3.3.1 Lysine-16 is preferentially acetylated over lysine-28. To determine the extent at which K16 and K28 were accessible for acetylation and determine experimental conditions under which the impact of acetylation on aggregation could be studied, A β ₄₀ (20 μ M) was exposed to NHSA, an established agent for acetylating primary amines.^{49,50} A β ₄₀ was treated with varying concentrations of NHSA ranging from 0.02 mM to 2.5 mM (resulting in a range of peptide to NHSA molar ratios of 1:1 to 1:125). As there are two lysines in A β ₄₀ that can be acetylated (K16 and K28), A β ₄₀ was digested with pepsin to generate peptide fragments containing only one of the lysines so that the extent of acetylation of each discrete lysine could be determined by MS.

The percent of acetylated K16 (AcK16) and K28 (AcK28) was determined by comparing the abundance of these peptide fragments containing just one lysine that were acetylated or not (Figure 3.1). When no NHSA was added to A β_{40} , no acetylation of lysine was observed. At lower concentrations of NHSA, ranging from 0.02 mM to 0.16 mM, K16 is preferentially acetylated over K28 with K16 acetylation being 5-3 $\times$ more abundant than K28 (Figure 3.1B). With the 0.16 mM treatment of NHSA, AcK16 and AcK28 only represented 47% and 14% of the total A β acetylated respectively. Effectively, these lower concentration treatments of NHSA resulted in conditions at which K16 was the predominately acetylated lysine; however, most of the available lysines (both K16 and K28) were not acetylated.

Figure 3.1. Acetylation of K16 occurs more readily than K28 (A) MS analysis of K16 and K28 specific peptide fragments reported as the percent abundance of acetylated to unacetylated lysine. (B) Ratio of AcK16 to AcK28 over increasing ratios of NHSA to A β_{40} .

At NHSA concentrations of 0.50 mM, 75% of K16 was acetylated, while 35% of K28 was acetylated, resulting in a condition in which the vast majority of A β was acetylated with AcK16 being the most abundant form ($\sim 2\times$). Further increasing the concentration of the acetylating agent to 1.0 mM and 1.5 mM produced an increase in the acetylation of K28 to about 60-75% and reduced the ratio of AcK16:AcK28 to

about 1:1 at 1.5 mM NHSA. While AcK16 and AcK28 was observable with all treatments with NHSA, the NHSA concentrations of 0.02 mM to 0.50 mM represent a regime in which K16 is predominantly more acetylated than K28; whereas, concentrations of 1.0 mM and above of NHSA both K16 and K28 are acetylated to similar and significant extents.

3.3.2 Acetylation of $A\beta_{40}$ extends the lag phase and extent of aggregation. Having established the extent of acetylation of K16 and K28 exposed to varying amounts of NHSA, we next determined the impact of AcK16 and AcK28 on the aggregation of $A\beta_{40}$. ThT assays were performed with 20 μ M $A\beta_{40}$ that had been exposed to increasing concentrations of NHSA (Figure 3.2).

Figure 3.2. ThT assays investigating the impact of acetylation on Aβ₄₀ aggregation. (A) Representative ThT curves for one of three independent experiments performed at 37°C, all at concentrations of 20 μM Aβ₄₀ (green), with increases in NHSA concentrations to 0.16 mM (blue), 0.32 mM (purple), 0.50 mM (pink), 1.0 mM (orange), and 1.5 mM (red). Non-linear sigmoidal lines of best fits represented as continuous lines calculated using Equation 1. Kinetic parameters of (B) lag-phase time, t_{lag} (h), and (C) slope of growth phase Slope(h⁻¹) were extracted from Equation 1 for all experiments and shown as the mean ± SEM. (D) Normalized maximum response of the steady state region of ThT curves, RF_{ss} , calculated by averaging the plateau region then normalizing all variables to their respective control. Data shown as mean ± SEM of the normalized raw fluorescence signals of three independent experiments; (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$, and (****) $P \leq 0.0001$.

In general, increasing acetylation of Aβ₄₀ inhibited aggregation. That is, as the concentration of NHSA increased, the lag phase extended and the total extent of fibrillization decreased. The addition of 0.16 mM and 0.32 mM NHSA did not significantly change the lag phase associated with Aβ₄₀ compared to control (Aβ₄₀ that was not exposed to NHSA). Aβ₄₀ in the absence of an NHSA treatment, with 0.16 mM, or 0.32 mM treatment of NHSA exhibited similar lag-phases of

6.8 ± 0.16 h, 6.4 ± 0.22 h, and 7.0 ± 0.17 h respectively (Figure 3.2B), and the relative slope of the growth phase, R_{slope} (h^{-1}), was also not significantly changed ($0.40 \pm 0.058 \text{ h}^{-1}$, $0.33 \pm 0.037 \text{ h}^{-1}$, and $0.27 \pm 0.046 \text{ h}^{-1}$ respectively, Figure 2C). Treatment with 0.16 mM, 0.32 mM, and 0.50 mM NHSA reduced the total extent fibrillization as the relative fluorescent unit signal of the steady-state plateau region, RF_{ss} , of the ThT curve by 23%, 45%, and 53% respectively (Figure 3.2D). Increasing the concentrations of NHSA to 0.50 mM resulted in a statistically significant extension of the lag phase, $7.7 \pm 0.13 \text{ h}$. In addition, the slope of the growth phase at 0.50 mM NHSA significantly deviate from the control, $0.15 \pm 0.040 \text{ h}^{-1}$ (Figure 2C), and the total extent of fibril formation (measured as relative fluorescence at steady state) was reduced by 58%. Finally, treatment with 1.0 mM NHSA and 1.5 mM NHSA completely suppressed fibril formation over the 20 h ThT assay; although, there is a hint of a potential fibril nucleation event after ~ 18 h. As such, accurate lag phase and growth phases could not be determined; however, the relative fluorescence at the end of experiment is less than 20% of the control for both conditions.

3.3.3 Acetylation of $A\beta_{40}$ inhibits fibril formation and oligomerization. While the ThT assays demonstrate that acetylation of lysine via exposure to NHSA reduce fibrillization of $A\beta_{40}$, these assays do not provide information concerning the potential of acetylation to promote polymorphic fibril structure or A β O formation. In light of previous observations that substituting lysine in A β with alanine results in altered fibril morphology,²³ we incubated $A\beta_{40}$ under the exact same conditions as used in the ThT assays and sampled these solutions at multiple time points for AFM analysis. Beyond looking at fibril morphology, this has the added benefit of allowing for the assessment of A β O formation. To analyze the population and dimensions of A β fibrils and A β Os, criteria based on aggregate morphologies were used for automated image analysis.⁴⁸ A β O were defined in AFM images as being larger than 1 nm in height, having an

aspect ratio of between 1.0 and 2.5 (indicating a non-fibrillar morphology), and occupying a surface area less than 9500 nm². The surface area criteria were based on our observation that the smallest fibrils occupied a surface area of ~ 10,000 nm² and that oligomers occupied an area typically 5 times smaller than this. Fibrils were identified as aggregates that were at least 1 nm in height with an aspect ratio greater than 2.5 and occupying a surface area or at least 10,000 nm². Upon solubilization, an initial aliquot of A β ₄₀ was sampled for all conditions (effectively time point 0 h), and no aggregates were observed.

Figure 3.3. Atomic force microscopy images of $A\beta_{40}$ aggregates as a function of increasing acetylation and time. Representative *ex situ* AFM images sampled at multiple time points over 20 h of $A\beta_{40}$ (20 μ M) at NHSA concentrations of 0.0 mM, 0.16 mM, 0.32 mM, 0.50 mM, 1.0 mM, and 1.5 mM. Depositions of $A\beta_{40}$ imaged at 5x5 μ m scan size on a mica substrate, shown here as 2.5x2.5 μ m zoomed in images. Color scale ranges from -5 nm to 10 nm.

Figure 3.4. AFM image analysis of the impact of acetylation on $A\beta_{40}$ aggregate populations and morphologies. (A) Population of oligomers per μm^2 over time determined by image analysis. Oligomer counts divided by the total area of the image analyzed. (B) Percent area covered by fibrils determined by filtering out aggregates with an area less than 95 nm^2 , converting images to binary maps, and dividing by the total image area. (A, B) Bar graphs shown as mean $\pm$ SEM (C) Histogram of oligomeric heights measured over the entire 20 h time course of the experiment for $A\beta_{40}$ ($20 \mu\text{M}$) at NHSA concentrations of 0.0 mM, 0.16 mM, 0.32 mM, 0.50 mM, 1.0 mM, and 1.5 mM. Red dashed line represents the oligomeric height of unacetylated $A\beta_{40}$ at 2.5 nm.

Based on AFM images and consistent with the ThT assays, increasing acetylation of A β ₄₀ impaired aggregation (Figure 3.3). A β O growth was quantified by counting the number of discrete oligomers per unit area observed on mica (Figure 3.4A); fibril growth was quantified by the percent of the mica surface covered by fibrils (Figure 3.4B). This approximation was used to quantify the extent of fibril formation due to fibril bundling and a heterogeneity in length, making it difficult to manually, or with an algorithm, determine the exact numbers of fibrils. When non-acetylated A β was incubated, a large population of oligomers were observed within 3 h with a peak population appearing between 5-7 h, as measured by the number of A β Os per unit area (Figure 3.4A). Fibrils appeared after 7 h of incubation, and the fibril density on the mica surface steadily increased, as measured by the % area of the surface occupied by fibrils (Figure 3.4B). The appearance of fibrils coincided with a steady decline in the A β O population.

When treated with NHSA concentrations of 0.16 mM, 0.32 mM, and 0.5 mM, A β Os also appeared after 3 h of incubation; however, the A β O population was significantly reduced in an acetylation-dependent manner (Figure 3.3 and Figure 3.4A). With all three of these NHSA treatments, fibrils were still observed at later time points. For both 0.16 mM and 0.32 mM concentrations of NHSA, fibrils could be observed after 7 h of incubation, but fibrils were not observed by AFM for A β ₄₀ treated with 0.5 mM NHSA until after 9 h of incubation (Figure 3.3 and Figure 3.4B). The density of fibrils observed on mica was also decreased as a function of acetylation (Figure 3.4B). Consistent with the ThT assays, treatment with NHSA concentrations of 1.0 mM and 1.5 mM resulted in no observable fibrils over the 11 h incubation time. In addition, the number of A β Os observed at any given time point was greatly reduced (~20 times), suggesting that extensive acetylation arrests A β ₄₀ aggregation at the oligomeric phase. It should

be noted that the detection limit of A β Os associated with this AFM analysis is likely on the order of octamers. That is, smaller A β Os may still have formed, but would be difficult to observe with this assay. Hexameric and smaller A β Os have been observed for A β ₄₀ in which the lysines have been substituted for with alanines.²³ With that being said, size analysis demonstrates that reaching a condition in which A β ₄₀ is significantly acetylated results in smaller and fewer A β Os. Specifically, A β Os formed without NHSA treatment or with NHSA treatments of 0.32 mM or less resulted in oligomers of with a broad height distribution with a mode of ~1.5-3.5 nm (Figure 3.4C). NHSA treatments of 0.5 mM or greater resulted in oligomers that had a mode height of ~1-1.5 nm (Figure 3.4C). As the size of A β Os observed under a specific condition did not change as a function of time and so few oligomers were observed at large NHSA treatments, the height histograms presented were compiled over all time points.

To determine if acetylation promoted unique fibril polymorphs, morphological features of fibrils grown under the different treatments of NHSA were determined by AFM image analysis (Fig 5). Height profiles were taken perpendicular to the axis of each fibril for A β ₄₀ fibrils formed at NHSA concentrations of 0.0 mM , 0.16 mM, 0.32 mM, and 0.50 mM (Figure 3.5A). The 1.0 mM and 1.5 mM NHSA condition were not analyzed as fibrils were not observed. Profiles were taken in a manner to avoid fibril crossings in order to better represent the height scale of single fibril species. The fibril height profiles of A β ₄₀ at 0.0 mM , 0.16 mM, 0.32 mM, and 0.50 mM NHSA concentrations all had a mode fibril heights of ~2.5 nm (Figure 3.5A), suggesting that the fibrils formed under these conditions were not polymorphic. As fibril height can vary along the contour of the fibrils, the average height along fibril contour of every individual fibril was determined. As fibril heights did not appear to vary as a function of time, data time points of 7 h and beyond were compiled for each variable to produce histograms of the

average fibril contour height (Figure 3.5B). The median fibril contour height for NHSA concentrations of 0.0 mM , 0.16 mM, 0.32 mM, and 0.50 mM were 2.4 nm, 2.2 nm, 3.1 nm, and 2.5 nm, respectively; with an interquartile range of 1.0 nm, 0.8 nm, 0.4 nm, and 0.6 nm, respectively (Figure 3.5B), confirming the absence of any polymorphic fibril structures for A β ₄₀ under these acetylating concentrations.

Figure 3.5. Comparison of fibril aggregates of A β ₄₀ as a function of acetylation. (A) Height profiles of representative *ex situ* AFM images of A β ₄₀ (20 μ M) at 0.0 mM, 0.16 mM, 0.32 mM, and 0.50 mM NHSA concentrations at 11 h. Colored lines of images correspond to adjacent height profiles. Conditions that did not produced fibril were excluded (B) Histogram of the average fibril height as measured along the contour length of each fibril for time points of 7 h and greater. (A, B) Red dashed-line represents height of 2.5 nm.

3.3.4 Exposure to TBLE membranes promotes fibrillization of acetylated A β ₄₀. As the presence of lipid membranes has been implicated as a modifier of A β aggregation,^{39,51} and lysines play a prominent role in peptide/lipid interactions,^{52–54} we next wanted to determine how the presence of lipid membranes modifies the aggregation of A β ₄₀ under the various acetylating conditions. ThT assays were performed in the presence of large unilamellar vesicles (LUV) of TBLE. TBLE was chosen as a model system due to its physiologically relevant mixture of membrane lipids and its extensive use in studying A β membrane interactions.^{7,16,55,56} In short, A β ₄₀ (20 μ M) was incubated in the presence of TBLE LUVs at a lipid to peptide ratio of 30:1 (0.5 mg/mL) under the same experimental and instrumental conditions as the assays performed in the absence of lipids. There are examples in the literature of the presence of lipids both accelerating^{57–59} or slowing^{39,60} A β aggregation. While the lipid composition of the membrane plays a role,^{39,61,62} the preparatory history of A β may also influence the A β /lipid interaction.⁵⁶ With the A β preparation protocol used here, TBLE membranes slowed aggregation compared to aggregation in the absence of lipids (compare the ThT assay curves of A β ₄₀ without NHSA treatment in Figure 3.2 and Figure 3.6).⁶³

Figure 3.6. ThT assays investigating the impact of acetylation on $A\beta_{40}$ aggregation in the presence of TBLE LUVs. (A) Representative ThT curves of three independent experiments of $A\beta_{40}$ (20 μ M) (green) in the presence of TBLE LUVs at 30:1 lipid-peptide ratio (~ 0.5 mg/mL) at 37°C for all other variables. $A\beta_{40}$ was also incubated under increasing concentrations of NHSA, 0.16 mM (blue), 0.32 mM (purple), 0.50 mM (pink), 1.0 mM (orange), and 1.5 mM (red). Lines represent the sigmodal fit of calculated from Equation 1. Bar-graphs of kinetic parameters of the (B) lag-phase, $t_{lag}(h)$, and (C) the slope of the growth phase, $R_{slope}(h^{-1})$, was determined for all variables by extracting values from the line of best fit. Values of $t_{lag}(h)$ and $Slope(h^{-1})$ were extracted from three independent experiments plotted as the mean value. (D) Normalized maximum response of the steady state region of ThT curves, RF_{ss} , calculated by averaging the plateau region then normalizing all variables to their respective control. Data shown as mean $\pm$ SEM of the normalized raw fluorescence signals of three independent experiments; (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$, and (****) $P \leq 0.0001$.

For example, the presence of TBLE extended the lag phase of $A\beta_{40}$ aggregation ~ 5 h. While acetylation associated with NHSA treatment of 1.0 mM or greater completely prevented fibrillization in the absence of TBLE vesicles, $A\beta_{40}$ formed fibrils under all acetylating conditions based on the ThT assay curves (Figure 3.6), and this was later confirmed by AFM images of these solutions at the end of the ThT assay (Figure 3.7). In the presence of TBLE, the lag-phase decreased with increasing acetylation, with the following lag-phase times exhibited;

3. Acetylation of $A\beta_{40}$ alters aggregation in the presence and absence of lipid membranes

A β ₄₀, 9.8 ± 0.67 h; 0.16 mM NHSA, 7.8 ± 0.40 h; 0.32 mM NHSA, 7.2 ± 0.31 h; 0.50 mM NHSA, 6.8 ± 0.34 h; 1.0 mM NHSA, 7.1 ± 0.36 h; and 1.5 mM NHSA, 6.8 ± 0.99 h (Figure 3.6B). This trend suggests that A β acetylation promotes the formation of the critical nucleus associated with fibril initiation. This is in stark contrast to impact of acetylation in the absence of TBLE, in which the lag phase was extended. In addition to the shortened lag phase in the presence of TBLE vesicles, acetylating conditions of 0.5 mM, 1.0 mM, and 1.5 mM NHSA all exhibited a statistical significance decrease in the slope of the growth phase compared to the control; A β ₄₀, 0.43 ± 0.042 h⁻¹; 0.5 mM NHSA, 0.26 ± 0.026 h⁻¹; 1.0 mM NHSA, 0.17 ± 0.027 h⁻¹; 1.5 mM NHSA, 0.18 ± 0.031 h⁻¹ associated with A β ₄₀ acetylation in the presence of TBLE. Concomitant with this shortened lag phase, the overall β -sheet load, as measured by relative fluorescence at steady state, systematically decreased with increasing acetylated lysine content. The reduction in β -sheet formation started with ~20-35% reduction at 0.16 mM NHSA and 0.32 mM NHSA concentrations to ~60% reduction for 1.0 mM NHSA and 1.5 mM NHSA (Figure 3.6D).

3.3.5 TBLE promotes unique aggregate morphologies of acetylated A β ₄₀. While not resulting in unique fibril morphologies in the absence of lipids, point mutations in A β associated with familial forms of AD aggregate into unique polymorphic structures in the presence of TBLE bilayers.¹⁶ This suggests that acetylation could promote polymorphic fibrils in the presence of TBLE, despite not producing polymorphs in the absence of lipids. To determine if acetylated forms of A β ₄₀ produced polymorphic fibrils, A β ₄₀ (20 μ M) was incubated in the presence of TBLE LUVs at a lipid to peptide ratio of 30:1. After 20 h samples were deposited on a mica substrate and AFM image analysis was performed for all conditions. Height profiles of fibrils

demonstrated a fibril height of 2.5-3.0 nm (Figure 3.7A), like that observed in the absence of TBLE (Figure 3.5A). However, there are several regions along the contour of the fibrils formed in the presence of TBLE that appeared thicker. That is, the morphology along the contour of the fibril deviated to a greater extent.

Figure 3.7 Comparison of fibril morphologies of $A\beta_{40}$ as a function of acetylation. (A) Height profiles of representative *ex situ* AFM images of $A\beta_{40}$ (20 μM) at 0.0 mM, 0.16 mM, 0.32 mM, 0.50 mM, 1.0 mM, and 1.5 mM NHSA concentrations at 20 h. Colored lines of images correspond to adjacent height profiles. (B) Histogram of the average fibril height as measured along the contour length of each fibril. (A, B) Red dashed-line represents height of 2.5 nm.

As a result, the height along fibril contours had a much broader distribution when compared to fibrils in the absence of lipids. Median heights of fibrils in the presence of TBLE were 3.6 nm (0.0 mM NHSA), 3.3 nm (0.16 mM NHSA), 3.3 nm (0.32 mM NHSA), 3.9 nm (0.50 mM NHSA), 2.8 nm (1.0 mM NHSA), and 3.3 nm (1.5 mM NHSA), and the distribution of heights defined by the interquartile range were 2.6 nm (0.0 mM NHSA), 2.0 nm (0.16 mM NHSA), 1.9

nm (0.32 mM NHSA), 2.0 nm (0.50 mM NHSA), 2.1 nm (1.0 mM NHSA), and 2.1 nm (1.5 mM NHSA) (Figure 3.7B). An example of this broadening can be observed for A β ₄₀ and 0.50 mM NHSA in Figure 3.8B. Collectively, it appears that TBLE does not promote a fibril polymorph in acetylated A β ₄₀; however, the increased variation in morphology along the fibril contour may indicate that lipids are being incorporated into or decorating the observed fibrils. This does not appear to be dependent on acetylation. Rather, it appears to be a consequence of fibril formation in the presence of TBLE vesicles.

While the morphology of fibrils formed in the presence of TBLE did not appear to be altered by acetylation, increased acetylation promoted the formation of annular aggregates in the presence of TBLE (Figure 3.7A and Figure 3.8). For conditions of 1.0 and 1.5 mM NHSA, a large population annular aggregates formed (2.6 ± 0.4 to 6.4 ± 0.6 per $25 \mu\text{m}^2$, respectively) that were not readily observed in the absence of lipid or with lower levels of acetylation. These annular aggregates were considerably thicker than fibrils with heights ranging from ~ 5 -10 nm (Figure 3.8B). The inner diameter of these annular aggregates ranged from ~ 60 -200 nm with a median size of ~ 114 nm (Figure 3.9B). Many annular aggregates appeared to be strongly associated with fibril aggregates as they were co-localized on the mica surface, with some fibrils appearing to protrude directly from annular aggregates (Figure 3.9A).

Figure 3.8 Acetylation of $A\beta_{40}$ in presence of TBLE produces annular aggregates. (A) $5 \times 5 \mu\text{m}$ representative ex situ AFM images of $A\beta_{40}$ (20 μM) at 1.0 mM NHSA and 1.5 mM NHSA concentrations in the presence of TBLE. Boxes illustrating regions of zoomed in images (B) Zoomed in $0.5 \times 0.5 \mu\text{m}$ images of annular aggregates for 1.0 mM and 1.5 mM NHSA. Colored lines correspond to height profiles with the red dashed line representing 8.0 nm height. Color scale constant for all images.

Figure 3.9. Fibrils and annular aggregates often colocalize. (A, B) Zoomed in $1.0 \times 1.0 \mu\text{m}$ images of annular aggregates and fibrils of $A\beta_{40}$ ($20 \mu\text{M}$) at 1.5 mM NHSA. (A) Height profiles of annular and fibril aggregates. Colored line corresponds to colored line of image. (B) Representative *ex situ* AFM image showing the range of pore sizes for annular aggregates. Color scale is the same for all images.

Figure 3.10. Polydiacetylene binding assay of $A\beta_{40}$ at increasing states of acetylation. PDA fluorescence response (λ_{Ex} 485 nm , λ_{Em} 560 nm) time-course of $A\beta_{40}$ (green) interacting with PDA/TBLE vesicles, incubated at 25°C . $A\beta_{40}$ was additionally incubated under increasing concentrations of NHSA, 0.0 mM NHSA (green), 0.16 mM NHSA (blue), 0.32 mM NHSA (purple), 0.50 mM NHSA (pink), 1.0 mM NHSA (orange), and 1.5 mM NHSA (red).

3.3.6 Acetylation reduces the affinity of $A\beta_{40}$ for TBLE membranes. As the presence of TBLE vesicles clearly influenced the aggregation of acetylated $A\beta_{40}$, a polydiacetylene (PDA) lipid binding assay was performed to measure the total interaction between $A\beta_{40}$ and TBLE

3. Acetylation of $A\beta_{40}$ alters aggregation in the presence and absence of lipid membranes

membranes (Figure 3.10). Polydiacetylene is a lipid moiety that is easily incorporated into vesicles and photo-crosslinked, resulting in hybrid vesicles with enhanced fluorescence in response to mechanical stress associated with peptide binding and aggregation. By monitoring fluorescence of these vesicles as a function of time, the total interaction of A β with the TBLE/PDA vesicles can be monitored. Control experiments were performed with the various doses of NHSA, and a fluorescent response was not invoked in these control experiments. Upon exposure of TBLE/PDA vesicles to A β_{40} , the fluorescent signal steadily increases as the peptide binds and aggregates on the vesicles. As the extent of A β_{40} acetylation was increased, the total interaction between the peptide and vesicles gradually was reduced. As binding to and aggregation on the vesicle can both induce PDA response, it is important to interpret this assay with the results of the ThT assay in mind. As the shortest lag phase for any condition was ~ 7 h as determined by the ThT assay, the PDA response for the first 7 h is dominated by the initial binding of A β to the membrane. It appears that acetylation reduces the overall affinity of A β for TBLE vesicles. As the steady state phase of fibril formation is reached for all conditions by 11 h of incubation (as determined by the ThT assay), the total fibril load has its largest contribution to the total PDA signal. In this regard, the magnitude of the PDA decreases with increasing acetylation in a similar fashion in comparison with the ThT assay. Lastly, at ~ 17 h the PDA/TBLE fluorescence signal associated with exposure to A β_{40} that had been not been treated NHSA or treated with 0.16 mM NHSA began to decrease instead of maintaining a plateaued maximum response. This drop-in signal eventually reaches that of the maximum response of other experimental conditions. Extensive fibrillization on lipid vesicles can lead to PDA vesicle instability leading to flocculation, and these two conditions were associated with the largest fibril

load. As a result, this likely reflects a loss in signal to the instability of the PDA/TBLE vesicles upon prolonged exposure to late-stage aggregates that alter the colloidal suspension.

3.4 Discussion

Understanding how individual amino acids of A β affect aggregation and peptide-membrane interactions are significant as it provides the possibility for focused, therapeutic targeting and may provide more effective treatment strategies. In this work, we explored the role of lysine's cationic charge in the aggregation of A β_{40} in the presence and absence of membranes through its removal via acetylation. In summary, we demonstrate that 1) acetylation reduces the aggregation propensity of A β_{40} , eventually abolishing fibril formation; 2) acetylation impedes the formation of A β Os; 3) under our experimental conditions, TBLE membranes slow A β_{40} aggregation; however, acetylation of A β_{40} promotes aggregation in the presence of TBLE compared to control; 4) acetylation of A β_{40} reduces the total A β /membrane interaction; 5) the combination of acetylation and the presence of TBLE can promote the formation of annular aggregates of A β_{40} . This work suggests that the A β aggregation process (in both the presence and absence of lipid bilayers) is highly dependent on the charge state of lysine residues and that the A β aggregation mechanism is unique on TBLE bilayers.

Through increasing the concentration of NHSA, both K16 and K28 could be acetylated, but K16 was more readily acetylated. The preferential acetylation of K16 is consistent with structural studies of monomeric A β demonstrating that K16 is solvent exposed while K28 is partially protected.^{22,64} Our experimental conditions created two regimes of A β acetylation: one in which AcK16 predominated (0.16 mM and 0.32 mM NHSA) and the other in which the

abundance of AcK16 to AcK28 were similar (1.0 mM and 1.5 mM NHSA). As the complete absence of fibrils was observed only when K28 was appreciably acetylated, it is tempting to associate K28 acetylation with this observation. However, as substitution of either K16 or K28 with alanine impacted A β aggregation,²³ it is likely that acetylation of both lysines plays a role in the observed inhibition of aggregation. There are plausible mechanisms associated with acetylation of either lysine. K16 is associated with an amyloidogenic core sequence in A β that plays a key role in fibril formation,^{9,10} and as a result, altering this region is likely to impact aggregation. There is precedent for such a notion as the Flemish mutation in A β , which changes an alanine to a glycine and is at the end of this amyloidogenic core sequence, significantly reduces the fate of A β fibrillization.^{65–67} Indeed, low concentration treatments of NHSA, when K28 is not appreciably acetylated, exhibited significant (although not absolute) reduction in fibril and oligomer formation. A potential mechanism for AcK16 inhibition of aggregation is provided by computational studies of A β ₄₂[K16A] demonstrating that the substitution of lysine results in a loss of intrapeptide contacts between the hydrophobic core and C-terminus of A β , therefore, reducing the propensity of β -sheet formation required for aggregation.⁶⁸ With regard to K28, acetylation would disrupt its ability to participate in a salt bridge with D23 or E22. This salt bridge is associated with the turn region of a β -hairpin observed in an A β fibril structure.¹⁷ The removal of K28's cationic charge through acetylation would, therefore, destabilize A β fibrils. Support for this mechanism can be seen in the success of a variety of A β fibril inhibitors directly targeting this salt bridge.^{69–73} Furthermore, artificially creating the salt bridge between D23 and K28 via the addition of a lactam linker increases the rate of fibril formation by removing the entropic barrier imposed by the establishment of the salt bridge and effectively removing the lag-phase.⁷⁴ Changes in the net charge of A β ₄₀ from -3 to -4 or -5 may also play a role in impeding

fibril formation as increases in the solvation energy would reduce the strength of the hydrophobic collapse of A β that is associated with the initial stages of aggregation.^{75,76}

Beyond inhibiting fibril formation, acetylation of A β reduces oligomerization. This finding appears to be in contradiction to the oligomerization of A β ₄₀[K16A], and A β ₄₀[K28A] which displayed similar oligomerization distributions to wild-type A β ₄₀.²³ However, the detection limitations of the AFM analysis used here would not allow for the detection of the small oligomers (hexamers or smaller) observed by Sinha et al. With regard to the oligomers observable by AFM, these tend to be classified as high molecular weight (HMW) oligomers, and a number of toxic HMW A β Os have been reported.^{77–80} The smaller, low molecular weight (LMW) A β Os tend to be less toxic or even benign compared with their HMW counterparts.^{77,79–}
⁸¹ Our analysis suggests that even if acetylation does not alter the formation of LMW A β Os, the more toxically-relevant HMW oligomers are reduced by acetylation. With regards to potential mechanisms for this inhibition of A β O formation, molecular dynamics suggest that A β oligomerization is governed through electrostatic interactions⁴⁰ and that reducing electrostatic attraction promotes smaller ordered oligomers.⁴¹ Such a scenario is consistent with our observation that high levels of acetylation resulted in fewer and smaller A β Os, as acetylation would reduce the electrostatic attraction between A β peptides.

The impact of acetylation on A β aggregation suggests that targeting K16 and K28 can be an effective way to manipulate aggregation for potential therapeutic purposes. Indeed, there are several indications that such a strategy may be effective. A molecular tweezer, CRL01, that selectively binds to lysine side chains inhibits the formation of A β Os and fibrils.⁸² Aspirin, which can act as an acetylating agent,^{83–85} can reduce the risk of AD⁸⁶ and protects HEK239

cells against cytotoxicity associated with exposure to A β fibrils.⁸⁷ More broadly, aspirin reduces protein aggregation in several amyloid-based, neurodegenerative diseases,^{88,89} and this protection was directly linked to its ability to acetylate proteins in *C. elegans* models of Huntington's Disease and AD.^{88,90} However, aspirin's ability to acetylate proteins is not lysine specific.⁹¹

Lipid membrane environments have been shown to influence the aggregation of a number of amyloid-forming proteins^{29,61,92–96} and can even induce unique aggregation pathways.^{26,94} Such a unique aggregation pathway would be consistent with our results with regard to A β aggregation in the presence and absence of TBLE vesicles. While acetylation extended the lag phase A β aggregation in the absence of TBLE vesicles, the opposite was observed in the presence of such vesicles, and A β was able to form fibrils even after extensive acetylation associated with high concentration treatments of NHSA. Despite the shortened lag phase associated with acetylation of A β in the presence of TBLE vesicles, the total fibril load was reduced, and polymorphic annular aggregates were observed. Collectively, this suggests that, in the presence of lipids, acetylation enhances the formation of the critical nucleus associated with initiating fibrillization but promotes secondary aggregation pathways. The enhanced nucleation could be related to enhanced diffusion along the membrane surface. That is, once bound to the lipid membrane, the removal of the cationic nature of lysine via acetylation could reduce the interaction with anionic head-groups, which may allow for freer diffusion of A β peptides along the membrane surface and increase the ability of peptides colliding and coalescing into a critical nucleus. A plausible scenario for the observed decrease in the slope of the growth phase, associated with the reduction in fibril elongation; and β -sheet load is that the inability to form a salt bridge between K28 and other residues increases the dissociation rate of monomers from the growing fibril ends relative to the association rate, resulting in an overall slower elongation.

References 3.5

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4. Outlook: Future directions for advancing the understanding of A β aggregation in Alzheimer's disease

The proposed studies presented here are focused on gaining actionable information about preparatory conditions of A β for studying aggregation, developing new tools for research of A β O dynamics and structure, and exploring the role of a protective mutation on peptide-membrane interactions. By extracting trends about A β preparatory protocols, insights can be gained into environmental changes that influence the self-assembly of A β , toxic mechanisms or promote more stable forms of oligomers. This information could be used for the advancement of BODIPY based oligomer specific dyes. The development of fluorescent small molecule oligomer specific dyes would allow for real-time tracking of A β Os formation, which is a current limitation in the field. The use of BODIPY oligomer specific dyes would also facilitate a high throughput methodology for screening therapeutics or stabilizing conditions for structural studies of A β Os. Also, we explore the idea of preserving isotope enriched A β in a heterogenic oligomer with a delayed transition towards fibril structures. The success of this technique could assist in the future development of more specific fluorescent dyes and provide knowledge of the three-dimensional structure of A β Os, which is still currently unknown. Lastly, we contemplate a study designed to explore a protective mutation in AD and understand how its protective effects are tied to aggregation, morphology, and peptide-membrane interactions. Collectively these future projects are designed to address issues within the field and provide new tools and knowledge to advance the treatment of AD.

4.1 Introduction

Alzheimer's disease is a neurodegenerative disease that currently affects 5.4 million Americans.

¹ The classical neuropathology of AD is characterized as the formation of extracellular senile plaques and intercellular neurofibrillary tangles.² A significant amount of effort has focused on the formation of senile plaques and earlier aggregate species of A β as the neurotoxic culprit, according to the amyloid cascade hypothesis.^{3,4} While there are indeed genetic links to AD, that cause early-onset AD, these are rare and only account for 5% of all AD-related cases.¹ To date no genetic linkage or biomarkers have surfaced within the other percentage of the patient group, and the cause of AD still remains elusive.⁵ Currently, the largest risk factor for AD is aging, and 95% of all cases occur within the elderly demographic.¹ Therapeutic strategies and clinical trials have focused on both reducing the expression of A β and breaking up the existing aggregates within the brain.³ So far these efforts have mainly been unsuccessful,^{3,6-11} and the search for a common cause or mechanism of sAD is ongoing.

A β is commonly post-translationally modified in the brain tissue and cerebrospinal fluid of AD patients,¹² As hyperphosphorylation of tau is a known trigger for tau's aggregation, it is likely that PTMs of A β trigger and modify amyloid formation. As an experimental methodology, PTMs represent valuable tools to probe the individual roles of specific amino acids on the self-assembly and other physiologically relevant interactions of A β .

One of the most well-known PTMs of A β is the oxidation of Met35 to its sulfoxide derivative. Due to the increase in reactive oxygen species associated with aging, oxidation of A β is elevated in patients of AD.^{13,14} In Chapter 2, the aim was to determine how changes in the oxidative environment associated with aging affected the aggregation of A β in the absence or presence of TBLE. This study demonstrated that exposing A β_{40} to H₂O₂ post-translationally

modified Met35 to its singly oxidized sulfoxide derivative, A β ₄₀Met35[O], as verified with MS and NMR. The percentage of A β ₄₀Met35[O] was found to be dependent on the H₂O₂ concentration with increases in the H₂O₂ producing a higher percentage of A β ₄₀Met35[O]. This post-translational modification of methionine in the absence of lipids showed reductions in the slope of the growth phase, indicating a decrease in the elongation rate of fibril ends, and a reduction in the magnitude of fibril formation, but no significant changes in the lag phase time. AFM analysis of A β aggregation under increasing concentrations of H₂O₂ also revealed that the oligomerization of A β was not inhibited, but structural transitions towards parallel β -sheet structures were inhibited. Further evidence from the AFM also demonstrated the presence of polymorphic fibrils in the presence of 6.0 mM H₂O₂. However, when kinetic assays were performed in the presence of membranes, A β ₄₀ exposed to higher concentrations of H₂O₂ correlated with less peptide-membrane interactions, and it was determined that the oxidation state of total brain lipid (TBLE) vesicles largely modulated the peptide-membrane interaction.

Acetylation of A β is not a physiologically observed PTM of A β ; however, the ability to chemically modify lysine makes it an attractive target for studying the role of that cationic character of lysine in A β aggregation. A β contains two lysine residues, K16 and K28, both of which are known to form intermolecular salt bridges in A β amyloid structures.^{15–18} In Chapter 3, we sought to determine how the removal of lysine's cationic charge through acetylation would alter A β ₄₀ aggregation in the presence and absence of lipid membranes. Here we demonstrated that acetylation of A β readily occurs at the more solvent exposed K16¹⁹ while K28 is acetylated at higher concentrations of NHSA. The acetylation of A β was shown to increase the lag-phase of A β ₄₀ while also reducing the magnitude of fibril formation to the point that at 1.0 mM NHSA and 1.5 mM NHSA fibril formation was completely inhibited. AFM analysis of acetylated A β ₄₀

also showed a marked reduction in the number of oligomers formed, demonstrating that fibril formation and oligomerization are both inhibited by acetylation. Morphological height analysis of A β fibrils formed under milder acetylating conditions indicated the absence of any polymorphisms suggesting fibril inhibition may be impart due to the prevention of A β to form salt bridges and participate in fibril formation. However, the presence of TBLE lipid vesicles promoted the aggregation of acetylated forms of A β_{40} with a decreased lag-phase and extent of fibril formation for all acetylating concentrations. The reduction in the lag-phase compared to the control of A β_{40} was found to arise from the decrease in the total A β -membrane interactions as assessed by PDA. Lastly, the presence of TBLE under NHSA concentrations of 1.0 mM and 1.5 mM NHSA promoted the formation of a distinct annular aggregate species. Collectively the work of Chapter 3, suggested that the oligomerization and fibril formation of A β is highly dependent on the cationic charge of lysines and that under the influence of membrane surfaces the mechanism of A β aggregation is alter.

As there is still no treatment for AD, continued research into the root causes of this disease are warranted. Here, several issues still facing the field will be discussed in context of the experimental results presented in this dissertation. These persisting issues in the field include: 1) the inconsistency of A β disaggregation and preparation protocols that complicate analysis across literature reports; 2) the inability to monitor oligomer formation in real-time in a high throughput manner; and 3) unraveling the structure of oligomeric A β species.

4.2 Meta-analysis of A β sample preparation and experimental outcomes

Preparation protocols of A β typically involves three steps; disaggregation, reconstitution, and dilution into buffering system, but the choice in the protocol used can alter experimental outcomes.²⁰ While several methods for the disaggregation of A β exists reproducibility across labs, manufactures, and batches of A β is poor.^{21,22} To overcome this many laboratories use hydrogen bond disrupting alcohols, strong acids, strong bases, or organic solvents such as hexafluoroisopropanol (HFIP), trifluoroacetic acid (TFA), ammonia, or dimethyl sulfoxide (DMSO). Next, A β is reconstituted in a secondary step before being diluted into a buffer. The disaggregation and solubilization protocols vary from laboratory to laboratory and complicate the ability to compare results.^{23,24} These differences in experimental outcomes can include changes in kinetic measurements,²⁵ morphology,^{26,27} secondary structural transitions,²⁸ membrane interactions,³⁸ and toxicity.^{25,28} Ultimately, the irreproducibility of results create controversy within the field, and attempts to standardize a methodology will not be applicable to all experiments.²⁹

In Chapter 2 A β was disaggregated with ammonium hydroxide and reconstituted with a 60 mM NaOH solution to obtain a monomeric stock of A β_{40} . This was a deviation from the typical A β preparation protocol that had been historically used in the Legleiter laboratory, which was based on the ‘Stine prep’ (disaggregation of A β with HFIP and solubilizing in DMSO).³⁰ As DMSO is a radical quencher of H₂O₂, the 'Stine prep' was not suitable for our experiments. The ‘Stine prep’ produces fibrils of ~6 nm thick and exhibited accelerated fibril formation in the presence of TBLE; whereas, the protocol used in Chapter 2 and 3 resulted in fibrils that were ~3 nm in width, and reduced rates of fibril formation in the presence of TBLE. Furthermore, the lag phase of control A β aggregation reactions increased in studies reported in Chapter 3 compared to

those reported in Chapter 2. This change was the result in differences in the synthesized A β peptides associated with different suppliers. The A β_{40} in Chapter 3 had a counterion of TFA as opposed to NH $_4^+$ in Chapter 2. Ultimately the problem lies in the lack of knowledge of how preparation protocols of A β influences results. Understanding how changes in the preparation of A β alters aggregation could provide insights into how multiple conditions affect aggregation and could allow for experimental designs that would probe variations in aggregation.

A possible solution to this issue could come in the form of a combinational approach utilizing data mining and establishing a convenient method for the field to compare experimental results. To discover connections between the varied preparations of A β and provide the ability to directly compare results across laboratories, a two-stage approach could be taken. The first stage, acquisition of data, would require the entry of research findings into a database that would catalog entries based on the preparatory steps used, kinetic parameters, toxicity results, structural observations, etc. Databases could be built by extracting relevant research articles from journal services and incorporating pdf into our own database. The biggest hurdle to overcome would be getting copyright permission from journal services as the methods sections containing A β preparation is not freely available like abstracts, however many journals have been amenable to such studies recently. In addition, data mining could then be performed to search our databases to look for similarities in results and trends that may not be readily observable between across disciplines. This may provide useful insights into the aggregation differences between expressed or synthetic A β and HFIP or alkaline disaggregated A β .

The second stage would be to make the data freely available to scientist via a web-based interface. This web portal would allow for scientist to search based on a broad range of experimental outcomes, preparations, cellular models, etc. In addition to providing this service,

other scientist would be freely able to upload their results with links to their research to grow the datasets. The entries from the point of view of the scientist would be convenient/simple and would be comprised of a simple questionnaire that allows the researcher to enter in the disaggregation, reconstitution, and miscellaneous protocols used for their experiment. This would be beneficial over requiring the uploading of raw data into which can be time-consuming.

Lastly, once this information becomes more available and widely used the expansion of the database could include other amyloidogenic proteins such as α -synuclein and Huntingtin, providing a more significant impact beyond that of just Alzheimer's disease. Many scientific databases have proven useful to the scientific community such as the Spectral Database for Organic Compounds and the Protein Data Bank. Ultimately, it is with this aggregation of data into a centralized location that would allow for the extraction of information and trends among A β preparations that may provide actionable information that could further be experimentally tested in the laboratory.

4.3 Real-Time tracking of A β oligomerization

Environmental conditions, preparation, and the presence of membrane surfaces all affect the oligomerization of A β . Atomic force microscopy (AFM) and electron microscopy (EM) provide the capability to track A β O formation. In particular, AFM is a particularly useful technique in studying A β O formation and morphology^{31–41} as it can be operate at physiologically relevant conditions. Immunohistostaining of toxic A β O_s also appear to react with oligomer-specific antibodies like A11 (generic for amyloid oligomers in general).⁴² In Chapter 2 and 3 we used the state of the art technique of AFM to track oligomer formation, but our methods and others are limited by their ability to finely resolve the kinetics of A β O formation. Therefore, new tools that can allow us to track the kinetics of A β O_s in real-time would fill a current gap in

understanding the dynamics of oligomer formation. A promising method for achieving real-time tracking of A β O is the use of BODIPY base derivatives.

Specifically in Chapter 3 AFM was also used to visualize the inhibition of A β O formation when A β was acetylated, however our results differed from Sinha et al who observed the oligomerization of A β ₄₀[K16A], and A β ₄₀[K28A] which displayed similar oligomerization distributions to wild-type A β ₄₀.⁴³ One possible reason for the discrepancy comes from detection limitations of the AFM which does not allow for the detection of oligomers smaller than hexamers, as observed by Sinha et al. In addition, using cross-linking methods, e.g. PICUP, or imaging with AFM limits the speed at which one can assess the dynamics of A β O formation. In developing a tool that would allow for the detection of LMW and HMW A β O in real-time would be a vitally useful tool to all scientist studying A β O formation. A promising strategy to develop this capability is using BODIPY based fluorescent dye that are selective for A β O.

Several straightforward spectroscopic assays are well-established to track formation and kinetic parameters of fibrils (e.g., thioflavin-T), but methods to easily track A β O formation have been lacking. Recently, the use of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) dyes have been utilized to detect the A β O in vitro fluorescently.^{44,45} These BODIPY fluorescent probes have high quantum yields with the capability of selectively binding to A β O.⁴⁶ Importantly, increases and decreases in the “BD-Oligo” dye fluorescence also correlated with increasing and decreasing intensities of the A11 antibody (generic for amyloid oligomers in general staining), directly relating this signal to an established method to detect A β O.⁶⁷ In addition, the BD-Oligo dye can be used in parallel with ThT assays that detect fibril formation, allowing for the direct investigation of the correlation between oligomer and fibril formation.⁶⁷ Rational design of BODIPY based dyes also allows for the the ability to selectively target

different aggregates of A β or other amyloidogenic proteins. The design of a BODIPY-base dye (BAP-1) was successful in facilitating the direct visualization of A β plaques in transgenic mice,⁴⁶ and rational modifications of BAP-1 also allowed for near-infrared selective detection of tau neurofibrillary tangles.⁴⁷ The ability to rationally modify BODIPY dyes for fluorescent detection of specific aggregate species, the tunability of their spectroscopic properties, and their insensitivity to solvent and pH changes have made BODIPY dyes a recently promising avenue for high throughput studies.

Ultimately, the applications of a BODIPY dye derivative that is selective for A β Os are broad. BODIPY could replace expensive oligomeric antibodies providing the ability to stain gels, dot blots, slot blots, and tissue samples cheaply. BODIPY can also be applicable to studying the dynamics of A β O formation which is limited in AFM and photo-cross-linking studies presented in Chapter 2, 3, and the work by Sinha et al. Modification of BODIPY dye derivatives may also prove useful in the application of distinguishing LMW and HMW A β Os, though the failure to selectively distinguish these species would instead also provide an experimental overlap between HMW A β O measured in AFM studies, as presented in Chapter 2 and 3, and photo-cross-linking studies which focus more on LMW. Additionally, spectroscopic techniques such as super-resolution microscopy and total internal reflection fluorescence microscope can utilize BODIPY probes to resolve accumulations of A β Os at the membrane interface of cells through FRET pairing or track the dynamics of A β O aggregation, transitions, and possible even trafficking and cerebral imaging in biological systems. Broadly, the development of a fluorescent molecule that selectively binds A β O oligomers would be applicable to the discovering a fluorescent molecule like ThT which has proven to be a gold-standard in the development of assess A β fibril formation, dynamics, and aggregation pathways.

4.4 Using reverse-phase micelles for structural studies of oligomeric A β

Enormous efforts have been expended in identifying the most toxic/disease-relevant A β O species and the relevant underlying structure.^{48–50} This is a daunting endeavor due to the transient nature and extensive heterogeneity associated with A β O.^{51–53} It is possible that different A β O species may activate different deleterious changes associated with AD;^{48–50} however, there may also be relatively benign A β O species or even experimental artifacts.^{24,51,54} Unraveling the physiological activity of A β O may require separate analysis of these different species, which are altered by preparatory protocols.³² In practice performing varied preparatory protocols can yield changes in rates of aggregation, polymorphism, and toxicity of A β O.^{25,27,32}

To facilitate structure/activity analysis, significant efforts have been made to obtain structural details of A β O using a variety of methods (Table 4.1). This is required to fully elucidate the modes of interaction of A β O with other, fluorescent molecules e.g. BODIPY, biomolecules, and related toxic mechanisms.^{55–60} Structural characterization of specific A β O is challenging due to their transient nature and heterogeneity. A β O can exhibit conformational plasticity that can be heavily influenced by environmental factors, further complicating such analysis.

Table 4.1. Methods used for analyzing A β O_s.

Technique	Features of A β O _s Obtained
Atomic Force Microscopy (AFM)	Morphology, population distributions
Electron Microscopy (EM)	Morphology, population distributions
Ion-Mobility Mass Spectrometry (IM-MS)	Secondary structure, multimeric configurations
Nuclear Magnetic Resonance 2D (NMR)	Secondary structure, multimeric configurations, atomic resolution structure
Electron Paramagnetic Resonance (EPR)	Secondary structure, multimeric configurations, atomic resolution structure
Powder X-ray Diffraction (PXRD)	Atomic resolution structure
Small Angle X-ray Scattering (SAXS)	Atomic resolution structure
Single Crystal X-ray Diffraction (SCXD)	Atomic resolution structure
SDS-Page	Size distribution of multimers

In Chapter 2 we observed that increasing the ratio of oxidized to unoxidized A β had the effect of delaying the oligomer transition to protofibril or fibril species. This transition was roughly delayed by 3 h (Figure 2.3B). This 3 h window would provide ample time to perform other separation steps that would enable the isolation of A β O_s. This isolation of A β O in the absence of cross-linking and covalent linkages is more advantageous as it is less likely to induce peptide structural modifications. Capitalizing on this observation, it may be possible to use a mixture of oxidized and unoxidized A β to delay the oligomer transition to fibrils and further encapsulate them through reverse phase micelles (RM) to structurally study A β within an oligomeric aggregate.

First, determining the ideal ratio of A β ₄₀ to A β ₄₀Met[O] would be essential in optimizing the extension of the A β O phase. Once established, isotope enriched A β ₄₀ could be mixed with A β ₄₀Met[O] in a buffer to produce oligomeric aggregates with extended stability. While A β O_s are often heterogeneous in size, additional separation steps may be required to obtain more

homogenous samples that are amenable to structural analysis. This can be accomplished by size-exclusion chromatography (SEC) or a sucrose gradient and centrifugation. However, this may dilute samples below concentrations required for structural analysis, and A β O s , given time beyond the stabilizing window provided by oxidation, may further associate producing larger aggregates. Both issues could be solved via encapsulating the A β O s in RMs, which would prevent further aggregation and allow for concentrating samples. To encapsulate A β O s within our aqueous solution we first need create an emulsion of hexane and H $_2$ O to promote the formation of sodium bis(2-ethylhexyl)sulfosuccinate (AOT) RMs.^{61,62} After encapsulation centrifugation would be utilized to concentrate samples and be resuspended RM-A β O s in hexane at the desired concentration. The three-dimensional structure of A β within the A β O structure could then be determined through solution-state NMR. Previously, encapsulation of monomeric A β for structural studies and solution NMR structures of A β in water-micelle environments have been effective in determining structural elements of A β .^{63,64} By suspending the unoxidized isotope label A β ₄₀ within an A β O structure we might be able to determine the structure of A β within an oligomer.

Due to the lack of structural information of A β O s , insights into their structure would be paramount. This would allow for a more rational design of A β O fluorescent probes e.g. BODIPY which would allow for high throughput determination of oligomer dynamics. Additionally, the 3D structure of A β O s would also benefit computational chemistry by providing a relevant starting point for simulations overall reducing the requirement of computational resources required instead of starting with monomeric forms of A β . Finally, structural knowledge of A β O s would also allow for the development of more effective small molecule or antibody therapeutics helping to provide a treatment option where none are currently available.

4.5 Protective Mutations

In Chapter 3 we acetylated K16 and K28 to explore the role of lysine's electrostatic interactions that are associated with aggregation and membrane binding. We determined that in the absence of lysine's cationic charge oligomerization and fibrillization were inhibited, and while membrane interactions were reduced the membrane facilitated the formation of the critical nucleus promoting fibril formation alongside promoting secondary aggregation pathways. Initially we would have expected that removing the cationic character of lysine would diminish A β -membrane affinity. Instead other regions of A β , specifically the hydrophilic N-terminus, may also be involved in the peptide-membrane interaction. However, the role of the N-terminal region of A β in oligomerization and polymorphism has remained unexplored largely due to its unstructured nature.^{63,65} Recently, a structural study has proposed that the N-terminus is responsible for stabilizing the central hydrophobic core (CHC) in its monomeric state,⁶⁶ and may prevent the hydrophobic collapse of the CHC to the C-terminus which may initiate the aggregation process. More recently, the charge state of the N-terminal region is found to regulate A β -membrane interactions with the removal of anionic glutamic acid (E11) and cationic lysine's (K16) exhibiting the slowest deposition rates on POPC bilayer.⁶⁷

Recently, a familial mutation in A β , A2T, has been discovered and is the only mutation known to exert a protective effect in Alzheimer's disease. Also, the A2V mutation has been discovered but instead results in early-onset Alzheimer's disease. When mixing N-terminal fragments of the 1-7 A2T variant with A β fibrillization and cytotoxicity of A β was reduced, however when repeated with the addition of the A2V mutation the opposite effect was observed.⁶⁸ Furthermore, AFM imaging of A β ₄₀, A β ₄₀(A2T), and A β ₄₀(A2V) fibrils in the absence of membranes have demonstrated the presence of polymorphic fibrils,⁶⁹ and in the

presence of a zwitterionic membrane, A2T fibril formation is increased compared to wild-type A β .⁷⁰ From our experiments in Chapter 3 and these recent findings, it is possible that the polar A2T mutation will result in reduced oligomerization; whereas, the hydrophobic A2V will increase oligomerization. In addition, the A2T mutation should exhibit increased association with the membrane while disallowing the insertion of A β into the membrane; the A2V mutation should have the opposite effect due to the hydrophobic nature of valine. This could be tested experimentally using the same methods and techniques used in Chapters 2 and 3. Ultimately, this would clarify how changes in the N-terminus of A β effect the oligomerization and membrane binding of A β . If the more polar A2T mutant exhibited greater oligomerization and membrane binding but reduced membrane disruption this would show that the toxic mechanism of the A β -membrane interaction is not just connected with A β 's association to the membrane but also requires the insertion into the membrane, a finding that would mirror toxicity studies of oxidized A β and results of Chapter 2.⁷¹⁻⁷³

4.6 References

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