A Mechanistic Understanding of Self-Propagating Amyloid-β Oligomer Conformations in Alzheimer Disease

Dexter Nathanael Dean
University of Southern Mississippi

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A MECHANISTIC UNDERSTANDING OF SELF-PROPAGATING
AMYLOID-β OLIGOMER CONFORMATIONS IN
ALZHEIMER DISEASE

by

Dexter Nathanael Dean

A Dissertation
Submitted to the Graduate School,
the College of Science and Technology
and the Department of Chemistry and Biochemistry
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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ABSTRACT

Alzheimer disease (AD) is a fatal neurodegenerative disorder characterized by the widespread deposition of proteinaceous plaques abundant in amyloid-β (Aβ) aggregates. Although the plaques mainly contain high molecular weight, insoluble Aβ fibrils, the low molecular weight soluble aggregates called oligomers have been shown as the primary toxic species responsible for synaptic dysfunction and neuronal loss in AD. The process of aggregation is nucleation-dependent, but also highly stochastic and inhomogeneous resulting in biophysically diverse assemblies. Recent advances in the field indicate a potential correlation between the phenotypic diversity observed in AD subtypes and aggregate polymorphism. Therefore, understanding the molecular mechanisms which lead to the generation of diverse Aβ oligomer structures (strains), and their subsequent propagation to polymorphic fibrils is crucial in establishing structure-phenotype correlations in AD. Our laboratory has previously characterized a specific Aβ oligomer called large fatty acid-derived oligomers (LFAOs), generated in the presence of fatty acid micelles. The work presented here has two main objectives: i) to determine the biophysical and biochemical properties of LFAOs in the context of strain behavior, particularly in the propagation of their structure; and ii) to determine the mechanism of oligomer strain generation by a family of lipids that are known to interact with Aβ. This work details the mechanism of LFAO strain propagation, which occurs in three distinctive phases involving a key intermediate. Also detailed is how LFAOs affect neuronal cells and selectively induce cerebral amyloid angiopathy (CAA) in transgenic AD mice brains, cementing the idea that distinct oligomer strains can influence AD phenotypes. Lastly, this work reveals that a family of Aβ oligomer strains can be
generated in interfacial conditions, suggesting that lipids present in the AD brain may play a role in strain generation. Overall, this brings forth fundamental mechanistic paradigms involved in oligomer strain generation and propagation that has invoked substantial insights into AD pathology.
ACKNOWLEDGMENTS

I would first like to acknowledge and thank my mentor, Dr. Vijay Rangachari, for his immeasurable support and contribution to my success: for being patient with me through mistakes, believing in me as a research scientist, and challenging me to think deeper and reach higher than what I thought possible.

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DEDICATION

I dedicate this dissertation to my family, who have believed in and supported me each and every day throughout this long journey. I cannot claim this accomplishment solely for myself, because I truly would not have made it to this point without their encouragement and love. To my wife, Sara Beth; my mom and step-dad, Donna and Chuck; my dad and half-siblings, Doug, Allie, and Colby; my maternal grandparents, Faye and GC; my paternal grandparents, Verna Mae and Jack; my brother and his family, Drew, Destiny, and Dayleigh; my father-, mother-, and sister-in-law, Jim, Becky, and Meg: take pride in this document and know that without you, it would not be what it is. From the bottom of my heart, thank you for everything you have done for me. I am forever grateful for you and your investment in my life.

Lastly, none of this would be possible without my Lord and Savior Jesus Christ, in Whom I have placed my faith and life. Throughout this journey, God has encouraged me through His Word in Titus 2, “Show yourself in all respects to be a model of good works, and in your teaching show integrity, dignity, and sound speech that cannot be condemned.”
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LIST OF ABBREVIATIONS

AD          Alzheimer disease
CAA         cerebral amyloid angiopathy
Aβ          amyloid-β
LFAO        large fatty acid-derived oligomer
A           Aβ monomer
O           LFAO 12mer
L           LFAO 12-24mer
P           LFAO 48-84mer
F           LFAO-seeded fibril
F_{on}      on-pathway fibril
WT          wild-type
HMW         high molecular weight
CMC         critical micelle concentration
SEC         size exclusion chromatography
ME          monomer elongation
OA          oligomer association
OE          oligomer elongation
PA          P association
ODE         ordinary differential equation
EKS         ensemble kinetic simulation
ANS         8-anilinonaphthalene-1-sulfonic acid
DCVJ        9-(2,2-diicyanovinyl)julolidine
<table>
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<tr>
<td>ThT</td>
<td>thioflavin-T</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
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<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
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<td>AFM</td>
<td>atomic force microscopy</td>
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<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PG</td>
<td>phosphatidylglycerol</td>
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<td>LPG</td>
<td>lysoPG</td>
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<td>LPGO</td>
<td>LPG oligomer</td>
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<tr>
<td>GM1O</td>
<td>GM1 oligomer</td>
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Published


**In Preparation**


10. *Dean, D. N.,* and Rangachari, V. Cross-Propagation of Aβ Oligomers in Alzheimer Disease and Cerebral Amyloid Angiopathy.

*Indicates publications detailed in this work.
1.1 Alzheimer disease

Alzheimer disease (AD) is a fatal neurodegenerative disorder characterized by a progressive and irreversible decline in the cognitive functions of the brain, resulting in acute memory loss. Recent statistics reveal that AD affects over 5.5 million people in the United States, and is the fifth-leading cause of death in individuals age 65 and older.\textsuperscript{1,2} The AD-inflicted brain is characterized by two pathological hallmarks: intracellular tangles of hyper-phosphorylated tau protein and extracellular plaques composed of the peptide amyloid-β (Aβ; Figure 1.1).\textsuperscript{3} While both of these lesions are found in postmortem AD brains, it is believed that the deposition of Aβ is the primary trigger of disease, with tau accumulation occurring secondarily.\textsuperscript{4} This was first postulated nearly three decades ago as the “amyloid cascade hypothesis” of AD pathogenesis.\textsuperscript{5-7}

![Figure 1.1 Hallmarks of AD pathology](image)

Aβ plaques (a) and tau tangles (b) found in postmortem AD brains. Scale bars represent 125 (a) and 63 (b) μm, respectively. Reproduced from LaFerla et al.\textsuperscript{8}
1.2 Cerebral amyloid angiopathy and AD

While the majority of AD cases are sporadic, a fraction of cases (5-10%) are familial, which occur in individuals having a genetic predisposition. The majority of these autosomal dominant missense mutations lead to increased production of Aβ, resulting in an early onset of disease symptoms. However, some mutations, such as the Arctic (E22G) and Italian (E22K) types, are known to induce a severe cerebrovascular disorder known as cerebral amyloid angiopathy (CAA). Affecting 80-90% of all AD patients, CAA is caused by Aβ deposition within the cortical and leptomeningeal vessels of the brain, which can lead to intracerebral hemorrhage and ischemic stroke. Taken together, it is clear that understanding the role of Aβ in the pathogenesis of both AD and CAA may lead to successful therapeutic intervention in disease progression.

1.3 Aβ generation

In 1984, Glenner and Wong were the first to isolate the subunit of proteinaceous plaques from postmortem AD brains. At the time, they reported what is now known as Aβ to be found in two isoforms with nearly identical sequences, suggesting one to be a proteolytic cleavage product of the other. Later on, it was discovered that Aβ is produced by the sequential proteolysis of a larger transmembrane protein, called the amyloid precursor protein (APP), by β- and γ-secretases, respectively. APP is localized to the endoplasmic reticulum during translation, then post-translationally trafficked through the secretory pathway to the cell membrane. The processing of APP to produce Aβ can occur during intracellular trafficking, resulting in Aβ production in the lumen, or after trafficking to yield extracellular Aβ. While β-secretase cleavage occurs on the C-terminus
of position 671 in APP, the γ-secretase complex (minimally consisting of the proteins presenilin 1 or 2, nicastrin, presenilin enhancer 2, and anterior pharynx defective 1) lacks specificity and depending upon the cleavage site, produces various isoforms of Aβ ranging between 38 and 43 amino acids in length (Scheme 1.1). Among these, the predominant isoforms found in extracellular plaques of AD brains are Aβ40 and Aβ42, similar to what Glenner and Wong first reported. APP is ubiquitously expressed and processed in both neuronal and non-neuronal cells, producing Aβ constitutively in the plasma and cerebrospinal fluid of both healthy and diseased individuals throughout life.

This leads to question the mechanism that Aβ undergoes which leads to toxicity in the AD-inflicted brain.

![Scheme 1.1 Proteolytic processing of APP by β- and γ-secretases](image)

Mutations in the Aβ sequence at position 22 involved in CAA are indicated in bold.

### 1.4 Aggregation of Aβ

The processing of APP releases Aβ as a single (monomeric) 38–43 amino acid long peptide. It has been well documented that monomeric Aβ has no organized secondary structure (randomly coiled), placing it in the class of intrinsically disordered proteins (IDPs). Additionally, Aβ is particularly prone to a process of self-aggregation.
association, or aggregation, in which monomeric units interact to form small multimers (oligomers) and eventually higher order aggregates (fibrils) that possess a distinct cross-β-sheet structure. The aggregation and deposition of Aβ fibrils as senile plaques constitutes one of the hallmark pathologies observed in AD brains. In fact, protein aggregation and amyloid formation has been implicated in over 30 diseases, including Creutzfeldt-Jakob disease (CJD) and Parkinson disease (PD). Although these pathologies involve different amyloid-forming proteins, the parallel, in-register β-sheet structure of the amyloid fibril is conserved among them all. Aβ amyloid formation follows a sigmoidal growth pattern similar to crystal growth with three distinct phases: the lag phase, growth phase, and saturation phase (Scheme 1.2). The lag phase is characterized by the formation of a nucleus, followed by exponential growth via elongation and association of protein aggregates, and lastly saturation of high molecular weight (HMW), insoluble protein fibrils. The lag phase can be abrogated by ‘seeding’, which involves the addition of a pre-formed aggregate (seed) to the monomeric protein, resulting in augmentation of aggregation. While much has been revealed about the mechanism of protein aggregation, there still remains the question of which aggregate species (size/conformation) gives rise to toxicity in the AD brain.
Scheme 1.2 A schematic representation of Aβ aggregation

*Inset,* a typical aggregate growth curve representing the lag, growth, and saturation phases. Adapted from Gosh et al.\(^\text{35}\)

### 1.5 Aβ oligomers and toxicity

Initially it was believed that Aβ fibrils were the toxic species in AD, as they constituted the plaques observed in postmortem AD brains. However, the onset of AD symptoms in transgenic mice has been shown to occur well before the detection of fibrillar Aβ deposits,\(^\text{36,37}\) suggesting that senile plaques might be ‘tombstones’ of the principle toxic events which occur in the etiology of disease.\(^\text{3}\) Along these lines, Fritschi et al. have recently reported that Aβ seeding potency in transgenic mice is highest at early stages of aggregation, and decreases with the onset of Aβ deposition.\(^\text{38}\) Erten-Lyons et al. further suggested the contribution of Aβ fibrils in AD toxicity to be insignificant when they revealed the presence of Aβ plaques in healthy individuals that had no signs of memory impairment.\(^\text{39}\) On the other hand, numerous reports have revealed that levels of soluble, oligomeric Aβ correlate closely with cognitive decline in AD.\(^\text{40-42}\) Additional reports have revealed that Aβ oligomers impair long term memory,\(^\text{43}\) target synapses and disrupt plasticity,\(^\text{44,45}\) inhibit neuronal signal transduction,\(^\text{46,47}\) and induce other
deleterious electrophysiological effects. Collectively, this suggests that Aβ oligomers are the primary cause of neuronal dysfunction and contribute significantly in the etiology of AD. This has resulted in an adapted version of the amyloid cascade hypothesis, termed the amyloid oligomer hypothesis.

1.6 Polymorphism in Aβ aggregation

Although protein aggregation follows a conserved mechanism of growth, it is not a homogenous process by any means. In fact, protein aggregation involves a significant extent of heterogeneity, giving rise to a diversity of aggregate sizes and structural conformations, otherwise known as polymorphic structures. Polymorphism among Aβ fibrils has been shown to depend upon the growth conditions in vitro, such as temperature, pH, ionic strength, and even protein concentration. However, other reports have revealed the formation of polymorphic fibrils within a single in vitro reaction. Furthermore, fibril polymorphism has also been observed within the same tissue section of postmortem AD brains, as well as in Aβ derived from AD brain tissue. Lu and co-workers generated fibrils from brain-derived Aβ in two AD patients and found that each case gave rise to a single predominant fibril structure, but those structures varied between each patient. Along similar lines, Cohen et al. have reported structural differences in Aβ from patients with varying phenotypes. They found that Aβ aggregates in patients who suffered from rapidly progressive AD (rpAD) were conformationally-distinct from Aβ aggregates in patients suffering from slowly progressing AD (spAD). This has been recently supported by others, which collectively suggest that the heterogeneous nature of Aβ aggregation gives rise to a
multitude of unique and distinct conformations, which may lead to clinical and pathological phenotypes in AD.

1.7 Prion protein propagation

While the concept of structural polymorphism leading to phenotypic diversity has recently emerged in AD, it has long been observed in other neurodegenerative diseases, particularly prion diseases. In CJD, the prion protein (PrP) can adopt a multitude of misfolded, toxic conformations (scrapie prion, PrPSc) which recruit natively folded non-toxic PrP (cellular prion, PrPc) to adopt the same PrPSc conformation through a templated mechanism of aggregation. The propagation of distinct PrPSc conformations, or ‘strains’, from one host to another occurs with a high degree of structural fidelity and can be characterized by certain physiochemical traits such as incubation periods and rates of progression, as well as by specific patterns of neuropathological targeting and spreading (Scheme 1.3). Along with cognitive decline and memory impairment, PrP strains can also be characterized by certain clinical manifestations, such as myoclonus, cerebellitis, and various psychiatric disorders. This leads to question if Aβ behaves similar to PrP in the induction of physiochemical, neuropathological, and clinical variances observed in AD.

1.8 Prion-like propagation of Aβ

Unlike PrP, Aβ is not known to be transmissible and infectious under normal conditions. However, Aβ does display several prion-like characteristics. These similarities include: i) Aβ and PrP both form misfolded aggregates rich in β-sheets
and display resistance to inactivation by heating, detergents, and proteases.\textsuperscript{69}

Furthermore, \textit{ii}) both Aβ and PrP propagate their structures by seeding both \textit{in vitro} and \textit{in vivo}, and \textit{iii}) can induce widespread pathology in the brain from a single inoculation site.\textsuperscript{70} Lastly, \textit{iv}) Aβ and PrP both form polymorphic structures, which in the case of PrP clearly results in distinct phenotypes.\textsuperscript{49, 65} While polymorphic Aβ aggregates are hypothesized to be involved in such a phenotypic behavior in AD, as described earlier, it is not as well understood. To this point, Condello and Stohr have proposed that the initial self-propagating Aβ assembly (i.e., strain) formed in the AD brain behaves as a prion, undergoing faithful propagation which governs the rate and pattern of neuropathology (i.e., phenotype; Scheme 1.3).\textsuperscript{71} \textit{If so, this would suggest that Aβ oligomer strains can propagate with high structural and biochemical fidelity to induce distinct phenotypic traits in the AD brain.} Additionally, the important question of what factors influence or promote the generation of Aβ oligomer strains still remains.

![Scheme 1.3 Aβ strain propagation](image)

**Scheme 1.3 Aβ strain propagation**

Factors influencing the generation of conformeric strains and a schematic representation of the physiochemical and neuropathological consequences of strain propagation. Figure inspired from similar review articles.\textsuperscript{71, 72}
1.9 Interplay in Aβ strain generation

Condello and Stohr suggest that the interplay of varying isoforms of Aβ (ranging in amino acid length) might be responsible for the generation of conformer strains (Scheme 1.3). Others have shown that mutant isoforms of Aβ involved in CAA (such as Artic and Italian types) form distinct aggregate structures, suggesting that mutant isoforms of Aβ may play a role in strain generation (Scheme 1.3). While these genotypes do contribute to strain generation, the complexity is rather large considering that each isoform of Aβ (having the same amino acid sequence and length) can form a multitude of conformations depending on the environment and growth conditions (Scheme 1.3, shaded box). It is daunting to decipher the possibilities within the Aβ interactome, especially when considering hybrid strains formed via the interplay between strains. However, delineating the mechanisms underlying strain generation and propagation is paramount in understanding AD pathogenesis.

1.10 Aβ strain generation within the cerebral microenvironment

One area of particular interest is identifying the factors that contribute to strain generation that are not genotypic in origin (Scheme 1.3, shaded box). As discussed already, it is well known that reaction conditions can give rise to Aβ polymorphism in vitro; however, understanding how this occurs within the cerebral microenvironment of the AD brain (in vivo) is substantially more complex. While a wide range of factors may contribute to Aβ polymorphism in the AD brain, this work is focused on the role of membrane surfaces in generating polymorphs of Aβ. The plasma membrane contains certain subdomains, called lipid rafts, which are made up of a distinct composition of
proteins and lipids enriched in cholesterol and glycosphingolipids.\textsuperscript{79} Altered lipid metabolism, including lipid raft composition, has been shown to occur in the AD brain;\textsuperscript{80-82} and it has been revealed that lipid deposits co-localize with Aβ plaques.\textsuperscript{83} This suggests that membrane lipid interfaces may play a role in catalyzing the generation of self-propagating Aβ assemblies in the etiology of AD.\textsuperscript{84}

Scheme 1.4 Aβ misfolding and aggregation at GM1-rich lipid rafts

Reproduced from Yagi-Utsumi et al.\textsuperscript{85}

1.10.2 Interfacial interactions \textit{in vivo}

In several reports by Yanagisawa and co-workers, a novel membrane-bound Aβ species was isolated from postmortem AD brains exhibiting early stages of disease pathology.\textsuperscript{86, 87} They revealed this Aβ species tightly bound to the ganglioside GM1, one of the most abundant gangliosides in the brain constituting a primary component of lipid rafts.\textsuperscript{88} It was later revealed that GM1 bound to Aβ with micromolar (μM) affinity\textsuperscript{89, 90} and induced a β-sheet secondary structure transition.\textsuperscript{91-94} Collectively, this suggests that
Aβ may bind to GM1-rich lipid rafts and form a β-sheet template that can act as an endogenous seed to initiate aggregation (Scheme 1.4).\textsuperscript{86, 95-97}

1.10.3 Interfacial interactions \textit{in vitro}

In a broader sense, others have shown \textit{in vitro} that Aβ binds and undergoes a structural transition to β-sheet in the presence of negatively-charged lipid bilayer vesicles (liposomes),\textsuperscript{98-100} and that binding is enhanced when membrane curvature is high.\textsuperscript{101} Micelles, made up of spherical monolayers, have also been shown to stimulate Aβ aggregate formation. Rangachari et al. have shown the folding of Aβ42 into β-sheet rich oligomers in the presence of the micelle forming anionic surfactant, sodium dodecyl sulfate (SDS).\textsuperscript{102} Using another SDS-derived Aβ oligomer called globulomers, Barghorn et al. immunized mice to generate monoclonal antibodies that specifically recognized the oligomeric epitope.\textsuperscript{103} Using this monoclonal antibody, they detected the same epitope to be present in both transgenic mice and AD brains,\textsuperscript{103} indicating that Aβ oligomer conformations generated from \textit{in vitro} reactions are relevant \textit{in vivo} and may be viable models for investigating Aβ aggregation and strain generation in interfacial environments.

1.11 Fatty acid-derived Aβ oligomers

Similar to detergents, non-esterified fatty acids (NEFAs) also associate to form micelles at critical concentrations, which may provide a more physiological micellar system for analyzing Aβ aggregation and strain generation in interfacial environments. In fact, Kumar et al. have investigated this in detail over several reports, which has yielded
useful insight into this area of interest.\textsuperscript{104-106} Initially, Kumar and co-workers investigated the aggregation of Aβ42 in the presence of medium chain (C9:0 – C12:0) NEFAs at concentrations below, near, and above the critical micelle concentration (CMC).\textsuperscript{104} They found that below the CMC, NEFAs had no effect on Aβ42 aggregation. However, Aβ oligomers ranging in size from 12-18mers were observed by immunoblotting at concentrations near (just below) the CMC of each respective NEFA; while oligomers of 4-5 monomer units were observed at concentrations well above (3-5 fold) the CMC. Both the 4-5mers and the 12-18mers persisted even after 10 days of incubation with NEFAs, as evident by immunoblotting. Furthermore, the 12-18mers formed by C12:0 NEFA could be isolated free of micelle association by size exclusion chromatography (SEC),\textsuperscript{104,106} and were stable as an oligomeric species for up to 10 days. These 12-18mer oligomers were thusly-dubbed large fatty acid-derived oligomers, or LFAOs. Collectively, the data suggests that these oligomers are formed along an alternative pathway (“off-pathway”) that does not easily proceed toward fibrils (Scheme 1.5), which is similar to what has been reported previously for SDS-derived oligomers.\textsuperscript{102,107} Given that oligomers are reported to be the primary neurotoxic species in the AD brain, this may have significant implications in AD pathology and warrants additional investigation.
1.12 Unique properties of LFAOs

A more detailed characterization of the morphology and size of isolated (free of micelle association) LFAOs by atomic force microscopy (AFM) revealed them to be spherical punctate dots with a bimodal height distribution ranging from 7-10 nm and 16-19 nm. Analytical ultra-centrifugation experiments similarly revealed a bimodal distribution of disperse oligomers ranging from 56-110 kDa (12-24mers) in molecular weight. But perhaps the most interesting aspect of LFAOs was their behavior when seeded upon Aβ42 monomers. Rather than seeding towards fibril deposition, as what is typically observed, LFAOs underwent a self-propagative mechanism of replication that resulted in a quantitative amplification of oligomers, not fibrils. Illogically, the
efficiency of this process was inversely proportional to the seeding percentage; whereas increasing the amount of seeds from 0.2 to 20% (mole fraction) of LFAOs decreased the overall fold-increase of amplified oligomers. While the reasons for this are yet unanswered, this work revealed a novel self-propagating mechanism among certain Aβ oligomers. Kumar et al. proposed that these unique properties may arise due to the off-pathway nature of LFAO formation, which arrests them as a kinetically-trapped species within a local energy minimum. Oligomers formed along such a pathway have an increased half-life as compared to their on-pathway counterparts, which results in a window where fibril-free, monomer-oligomer reactions can occur leading to oligomer amplification (Scheme 1.6). Such a mechanism of templated corruption could explain several of the prion-like behaviors observed for Aβ, such as the spreading of toxicity from a single inoculation site in transgenic mice brains. Furthermore, faithful propagation of such oligomeric structures in vivo may be responsible for physiochemical and phenotypic differences in AD. Investigating the molecular details of this process may yield novel insight into the mechanisms of Aβ oligomer strain propagation and proliferation in AD.
Scheme 1.6 A schematic representation of LFAO replication

The kinetically-trapped, off-pathway nature of LFAOs and its implications for mechanisms of self-propagative replication.

Reproduced from Kumar et al.105
1.13 Rationale and Hypotheses

As presented above, understanding the role of Aβ oligomers in the etiology and pathogenesis of AD has become one of the focal points in this field of research. A large body of evidence suggests that polymorphic Aβ fibrils are responsible for the phenotypic diversity observed among AD cases. Fibrils are assembled from oligomeric building blocks, suggesting that polymorphic Aβ oligomers may govern phenotypic diversity in AD. However, the role(s) of conformational Aβ oligomer strains, and the molecular details and physiological consequences thereof, are lacking. Previous work by Kumar and others have shown that interfacial aggregation of Aβ along alternative pathways results in the generation of soluble Aβ oligomers with prion-like self-propagative properties.

Therefore, we hypothesize the following:

(i) LFAOs are a unique oligomer strain capable of undergoing a structurally and biochemically faithful process of propagation with distinct physiochemical and neuropathological effects.

(ii) LFAOs of wild-type Aβ42 can cross-propagate to induce oligomers of mutant isoforms of both Aβ40 and Aβ42 to generate novel hybrid Aβ oligomers.

(iii) Unique Aβ strains can be generated using other micelle- and liposome-forming surfactants.

In the ensuing chapters, these hypotheses will be tested in order to provide insight into the molecular details of Aβ strain generation and propagation. First, factors governing the self-propagative replication (amplification) of LFAOs will be investigated in Chapter II in an effort to better understand the inverse relationship between replication efficiency and LFAO amount. Next, the prion-like faithful propagation of LFAOs and
consequences thereof will be examined in Chapter III, with insight into the kinetics of the process following in Chapter IV. In Chapter V, the ability of LFAOs to cross-propagate leading to hybrid oligomer formation will be evaluated. And lastly, the generation of other oligomer strains in micellar and liposomal conditions will be explored in Chapter VI before presenting the overall conclusions and future directions in Chapter VII. Chapter VIII will contain an exhaustive list of all materials and experimental procedures herein.
CHAPTER II – LFAO CONFORMATIONAL DYNAMICS

2.1 Introduction

As mentioned previously, LFAOs undergo a self-propagative replication in the presence of Aβ monomers that results in a quantitative increase in the amount of LFAOs. However, the efficiency of replication displays an inverse correlation to the amount of LFAO seeds.\textsuperscript{105} This leads to the question the molecular mechanism governing the efficiency of LFAO self-propagative replication. In this chapter, this was explored by investigating the concentration-dependent dynamics of LFAOs. The results obtained were initially disseminated by Dean et al. in the journal, \textit{Biochemistry},\textsuperscript{109} and are reproduced here with permission.

2.2 Replication efficiency is inversely proportional to the initial concentration of LFAO seeds

Replication efficiency as a function of initial LFAO seed concentration was investigated by initiating reactions with 0.5, 1, 3, 10, or 14 μM LFAO (O) seeds in the presence of a constant amount (50 μM) of freshly-purified Aβ monomer (A) at 25 °C in quiescent conditions. Replication efficiency was qualitatively determined using SDS-polyacrylamide gel electrophoresis (PAGE) with immunoblotting (Figure 2.1f, inset) and quantitatively determined using SEC (Figure 2.1a-e) after 72 h of incubation. To determine the quantitative fold-increase (replication efficiency) of Os at each respective seed concentration, integration of the seeded reactions (Figure 2.1a-e, solid lines) were normalized against that of the seed alone (Figure 2.1a-e, dashed lines). Like previously observed,\textsuperscript{105} this yielded an inverse correlation with the seed concentration (Figure 2.1f).
The replication efficiency exponentially decreased from 6.1 to 2.6 as the O seed concentration increased from 0.5 to 3 μM, then reached a plateau of ~1.5 at 10 and 14 μM, respectively (Table 2.1). An immunoblot of the corresponding reactions (Figure 2.1f, inset) revealed that upon replication, Os form disperse species ranging from roughly 56-60 (single arrow) and 80-110 (double arrow) kDa, which corresponds to 12 and 24mers, respectively. The O seeds alone (odd-numbered lanes) interestingly revealed a concentration-dependent banding pattern. At low concentrations (0.5 and 1 μM), O seeds were predominantly 12mers (single arrow), while disperse 12-24mer bands (single and double arrow) were observed at concentrations above 1 μM. This suggests that LFAOs display concentration-dependent dynamics between 12mer and 12-24mer formations, which may influence the efficiency with which they replicate.
Figure 2.1 $O$ replication efficiency

(a-c) SEC chromatograms of $O$ seeds alone (dashed) or after 72 h of incubation with 50 μM $A$ (smooth) for 0.5, 1, 3, 10, and 14 μM seeds, respectively. (f) Quantitative fold-increase of $O$s at varying seed concentrations. *Inset* A representative immunoblot of $O$ replication reactions. For each $O$ concentration, the left (odd) and right (even) lanes represent the seed and seeded reactions, respectively. Lane C represents 50 μM $A$ alone after 72 h. The amount loaded into each well was kept constant at 34 ng for $O$ seeds and seeded samples (based on the initial $O$ concentration), while 3.4 μg was loaded for the $A$ control (lane C).

2.3 Detailed quantitative analysis of LFAO replication

In order to more accurately quantify replication reactions, a simple equilibrium model was considered involving competing on- and off-pathways. The on-pathway was considered to lead to fibril ($F_{on}$) formation via self-nucleation of Aβ monomers not influenced by $O$ seeds. The off-pathway was considered to be the pathway leading to replication of $O$s, as this has been shown previously to occur along an alternative
In a seeded reaction such as the one considered here, these reactions compete for $A$ as such:

$$\text{O} \rightleftharpoons \text{A} \rightleftharpoons \text{F}_{\text{on}}$$

Therefore, accounting for the amount of $O$, $A$, and $F_{\text{on}}$ species should reveal more insight into these competing reactions. Upon incubation of $O$s with $A$s after time $t$, the following reaction was utilized to quantify the amount of $A$:

$$A_0 = A_c + A_f - A_d$$

Where $A_0$ is the initial concentration at time 0, $A_c$ is the concentration of monomer consumed by the on- and off-pathways, $A_f$ is the concentration of free monomer not consumed by either pathway, and $A_d$ is the amount of monomer which partially dissociates from $O$s during SEC. To differentiate between $A_c$ consumed along the on- and off-pathways, the following was considered:

$$A_c = \sum (O, F_{\text{on}}) = A' + A''$$

Where $A'$ represents the on-pathway leading to $F_{\text{on}}$ and $A''$ represents the off-pathway leading to amplified $O$s. To account for $A_d$, the linear proportionality between $A_d$ and the $O$ concentration (Figure 2.2m) was considered as follows:

$$A_d = f ([O]) = (s)[O] + b$$

Where $s$ is the slope and $b$ is the $y$ intercept, respectively. Combining the above equations yielded:

$$A_f = A_0 - (A' + A'') + [(s)[O_0] + b]$$

In a control reaction in the absence of $O$ seeds (Figure 2.2k), this equation solved for $A'$ was defined as:

$$A' = A_0 - A_f$$
Which upon analysis by integrating the SEC peaks (Figure 2.2k) yielded a value of ~6 μM (Table 2.1) dedicated to the on-pathway in the absence of O seeds. In order to experimentally determine $A''$ for seeded reactions, the following was considered:

$$A'' = O_f - O_0$$

Where $O_f$ is the final concentration of Os determined by SEC chromatograms of seeding reactions at 72 h of incubation (Figure 2.2a, c, e, g, and i). Plotting $A''$ (also defined as the change in $O$, $\Delta O$) as a function of $O_0$ yielded a positive exponential correlation (●; Figure 2.2l and Table 2.1). Not considering the contribution of on-pathway aggregation, calculating $A_f(A_{fi,c})$ from the given values became:

$$A_{f(c)} = A_o - A'' - A_d$$

Using the SEC results (Figure 2.2a, c, e, g, i) from replication experiments, the actual $A_f (A_{fi,a})$ values were determined, which are shown in Table 2.1. Given this, $A'$ was calculated for seeding reactions as:

$$A' = A_{f(c)} - A_{f(a)}$$

Which was found to be 5-6 μM committed toward the on-pathway, in good agreement with the experientially-determined value. Furthermore, plotting the net change in A ($\Delta A$) yielded an exponential decrease reciprocal to that of the net increase in Os (□, Figure 2.2l; Table 2.1). Considering that a constant amount of As were consumed along the on-pathway, this suggests that as the amount of O seeds increased, more As were expended toward the off-pathway. Lastly, it is interesting that the amount of Os ($\Delta O$, Figure 2.2l) increased as a function of seed concentration, while replication efficiency ($O_f / O_0$; Figure 2.1f) decreased. This may be due to conformational dynamics between 12 and 12-24mer formation, which requires more investigation.
Figure 2.2 Quantitative analysis of LFAO replication

(a–k) Representative SEC chromatograms of reactions that were used to derive the parameters in Table 2.1. (a, c, e, g, and i) O seeds alone (dashed) or after 72 h of incubation with 50 μM A (smooth) for 0.5, 1, 3, 10, and 14 μM seeds, respectively. (b, d, f, h, and j) Reactions of 50 μM A alone at 0 h (smooth) or with 0.5, 1, 3, 10, and 14 μM O seeds, respectively (dashed). (k) A reaction of 50 μM A alone at 0 h (smooth) and 72 h (dashed) of incubation. (l) Net increase in O concentration upon replication (ΔO; •) and net change in free A concentration (ΔA; □) as a function of initial O concentration. The data represent an average of three independent trials. (m) Quantitation of O dissociation to A during SEC. The integrated A peaks (fractions 22-27) were normalized against a known A concentration (50 μM).
Table 2.1 Quantitative analysis of LFAO replication

<table>
<thead>
<tr>
<th>Monomer (μM)</th>
<th>LFAO Seed (μM)</th>
<th>Replication Efficiency</th>
<th>Replicated LFAO (μM)</th>
<th>Δ LFAO (μM)</th>
<th>Calculated Monomer (μM)</th>
<th>Actual Monomer (μM)</th>
<th>Δ Monomer (μM)</th>
<th>Monomer On Pathway (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₂</td>
<td>O₂</td>
<td>RE</td>
<td>O₇</td>
<td>A'</td>
<td>A₆₉₁</td>
<td>A₆₉₂</td>
<td>ΔA</td>
<td>A'</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>6.1 (±1.3)</td>
<td>3.0 (±0.66)</td>
<td>2.5</td>
<td>48</td>
<td>41 (±1.8)</td>
<td>-9</td>
<td>6*</td>
</tr>
<tr>
<td>50</td>
<td>1.0</td>
<td>3.8 (±0.81)</td>
<td>3.8 (±0.81)</td>
<td>2.8</td>
<td>47</td>
<td>42 (±1.9)</td>
<td>-8</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>3.0</td>
<td>2.6 (±0.47)</td>
<td>7.7 (±1.4)</td>
<td>4.7</td>
<td>45</td>
<td>40 (±1.8)</td>
<td>-10</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>1.7 (±0.10)</td>
<td>17 (±1.0)</td>
<td>7.0</td>
<td>43</td>
<td>37 (±2.7)</td>
<td>-13</td>
<td>6</td>
</tr>
<tr>
<td>50</td>
<td>14</td>
<td>1.5 (±0.15)</td>
<td>22 (±2.1)</td>
<td>7.6</td>
<td>42</td>
<td>37 (±1.7)</td>
<td>-13</td>
<td>5</td>
</tr>
</tbody>
</table>

Data derived from SEC chromatograms of three individual replication experiments (denoted as *) shown in Figures 2.1 and 2.2 are summarized along with their standard deviations in parentheses. The results calculated from the experimental results are denoted as “*”, while the known parameters are denoted as “**”. The symbols A₆₉₁, O₆₉₁, P_f, A', A₆₉₂, A₆₉₃, ΔA, and A' are defined within the equations above and are described in the text.

2.4 Insights into O conformations

In order to better understand the unique conformational properties of Os, the extrinsic fluorescence dyes ANS (8-anilinonaphthalene-1-sulfonic acid) and DCVJ (9-(2,2-diicyanovinyl)julolidine) were utilized to investigate O binding affinity. It has been well documented that ANS preferentially binds to solvent-exposed hydrophobic surfaces of proteins, which results in an increased fluorescence intensity and a blue shift in the emission maxima. The less established probe, DCVJ, has been recently reported to bind strongly to amyloid oligomers, resulting in increased fluorescence emission.

Therefore, fluorescence experiments were conducted with As, Os, and F_on in the presence of either ANS or DCVJ, respectively (Figure 2.3). Neither ANS nor DCVJ fluorescence was increased in the presence of As, as expected (□, Figure 2.3a-b). Both dyes bound to F_on species (△), albeit to a lesser degree as compared to Os (●). ANS binding was marginally increased in the presence of Os, suggesting more solvent-exposed
hydrophobic surfaces. However, DCVJ binding to Os was roughly twice the amount as compared to $F_{on}$ aggregates, which agrees with previous reports suggesting DCVJ to preferentially bind to amyloid oligomers.\textsuperscript{111} Collectively, these data indicate that the structure of $O$ varies from that of the fibril structure, and warrants further investigation.

![Figure 2.3 Biophysical characterization of Os](image)

(a-b) ANS and DCVJ binding fluorescence, respectively, for $A$s (□), Os (●), and $F_{on}$ (△). ANS and Aβ concentrations were kept constant at 100 μM and 6 μM, respectively. DCVJ and Aβ concentrations were kept constant at 10 μM and 5 μM, respectively. (c) FTIR of Os (smooth), $F_{on}$ (dashed), and BSA (dash-dot). A total of 4096 accumulations were collected by scanning 1750 – 1550 cm$^{-1}$ at a resolution of 2 cm$^{-1}$.

Previous reports by Kumar et al. have revealed that Os have a β-sheet secondary structure,\textsuperscript{104} however the characteristic of the β-sheets, either parallel or antiparallel, was not distinguished. As stated in the introduction, amyloid fibrils form a characteristic in-
register parallel β-sheet structure,\textsuperscript{28, 29} and it is important to know if Os have a similar or dissimilar orientation. Fourier transform infrared spectroscopy (FTIR) has been shown to distinguish between parallel and antiparallel β-sheets due to subtle variations in amide bond stretching frequencies.\textsuperscript{112} Therefore, Os and $F_{on}$ FTIR spectra were collected (Figure 2.3c), as well as a control sample of the known α-helical protein bovine serum albumin (BSA). As expected, the FTIR spectra of BSA contained an amide stretching at 1655 cm\textsuperscript{-1} (dash-dot, Figure 2.3c), indicative of α-helical structure. In addition, stretching at 1626 cm\textsuperscript{-1} was observed in $F_{on}$ aggregates (dashed line, Figure 2.3c), indicative of parallel β-sheets, similar to what has been reported previously.\textsuperscript{113, 114} The FTIR spectra of Os was similar to that of fibrils, suggesting that the oligomeric assembly is made up of parallel β-sheets (smooth line, Figure 2.3c). Interestingly, the presence of antiparallel β-sheets at 1690 cm\textsuperscript{-1} was not observed in the FTIR spectra of Os, which is contrary to what has been observed for other amyloid oligomers.\textsuperscript{115-117} However, this is in agreement with our previous results that Os are detected by the fibril-specific conformational antibody, OC,\textsuperscript{105} suggestive of fibrillar oligomers.\textsuperscript{118, 119}

\subsection*{2.5 Concentration-dependent assembly of Os}

In order to further investigate the observed concentration-dependent size distribution of Os, immunoblotting was performed (Figure 2.4a). As observed previously, two distinct size distributions centered at 12 and 24mer Aβ species were observed at high concentrations (Figure 2.4a, 4-10 μM). Also as observed prior, the upper (double arrow) 24mer band faded with decreasing O concentrations, suggesting a concentration-dependent assembly of two individual 12mer (O) units. In order to eliminate the
possibility that this may be due to the presence of the SDS denaturant, a 10 μM Os sample was electrophoresed under native conditions (lane N, Figure 2.4a), which revealed two oligomer distributions similar to what was observed in the presence of SDS. To investigate this further, the samples were subjected fractionation by SEC on a Superdex-75 column. This revealed that 10 μM Os elute from the column at 8.67 mL, while 0.5 μM Os elute at a more inclusive volume of 8.79 mL (Figure 2.4b). In fact, plotting the elution volume of Os against the molar concentration revealed a decreasing sigmoidal trend, suggesting the formation of smaller sized oligomers at lower concentrations (Figure 2.4b, inset).

Figure 2.4 Molecular size of Os
(a) An immunoblot of 0.5, 1, 2, 4, 6, and 10 μM Os, respectively. The amount loaded onto each lane was maintained at 45 ng. Lane N represents a 10 μM (113 ng) O sample electrophoresed under non-denaturing conditions (without SDS). Single and double arrows represent O 12mer and 24mer band, respectively. (b) SEC elution profiles for 0.5 (smooth) and 10 (dashed) μM Os. Inset) Elution volumes for 0.5, 1, 3, 10, and 14 μM Os, respectively.

To investigate the possibility of two individual 12mer Os associating to form a disperse 12-24mer, ANS binding was performed at varying concentrations of Os (Figure
As the concentration of Os increased from 0.5 to 8 μM, an increase and blue shift in the emission maxima was observed (Figure 2.5a). The same data was collected for As and \( F_{on} \) as controls, which was processed by integration and normalization as shown in Figure 2.5b. ANS did not bind to As, similar to what has been presented above, and therefore did not show any concentration-dependent changes (○, Figure 2.5b). \( F_{on} \) aggregates did bind ANS, but did not show any concentration-dependent changes upon dilution from 8 to 0.5 μM (△, Figure 2.5b). However, Os did display concentration-dependent ANS binding (●, Figure 2.5b). Below 1 μM, Os bound ANS to a lesser degree, which then increased above the level of \( F_{on} \) aggregates upon increasing concentration. A transition such as this suggests conformational dynamics involving the solvent-exposed hydrophobic regions of oligomer assemblies. Upon fitting the Os ANS data to a monomer-dimer model (ie, 12mer to 24mer), a dissociation constant (\( K_d \)) of 0.1 uM was determined.

Far-UV circular dichroism (CD) spectroscopy was then used to determine whether the observed conformational changes resulted in secondary structure changes (Figure 2.5). Os were evaluated at 0.5 and 8 μM, concentrations where they showed the greatest degree of variation in ANS binding. The minima observed at 217 nm, indicative of \( \beta \)-sheets, was identical for both 0.5 and 8 μM Os (Figure 2.5c). This is similar to the spectrum observed for fibrils (Figure 2.5d). However, since the solvent exposed hydrophobic content increased with an increase in LFAO concentration, which was not observed for fibrils, one could conclude that the dynamics involve tertiary or quaternary structure changes.
Figure 2.5 Conformational changes involving O dynamics

(a) ANS scans of 0.5, 1, 2, 4, 6, and 8 μM Os along with 100 μM ANS blank (dashed). (b) ANS fluorescence scans from (a) were integrated and normalized against Aβ concentrations, which were then plotted for A (□), O (●), and F (Δ). The data were fitted with a monomer-dimer equation given in the methods section. (c-d) CD spectra for 0.5 (smooth) and 8.0 (dashed) μM O (c) and F (d) samples. The perpendicular dotted line indicates 217 nm.

2.6 Os also display concentration-dependent effects in cell culture

*In vitro* cell culture assays were employed to assess the neurotoxicity of Os in human neuroblastoma SH-SY5Y cells. To investigate the ability of Os to induce apoptosis, the TdT-mediated dUTP nick-end labeling (TUNEL) assay was employed, which has been previously used to evaluate Aβ toxicity.\(^{120-122}\) Interestingly, the toxicity of Os was highest at 0.01 μM, and decreased with increasing concentrations of 0.1 and 1 μM (Figure 2.6a and c). This inverse proportionality suggests that conformational dynamics may play a role in cellular activity, as it correlates well with the ANS binding and replication efficiency data.
To further investigate this unique activity in cell culture, the ability of Os to activate caspase enzymes was probed using a fluorescent inhibitor of caspases (FLICA) technique. In contrast to the apoptosis results, caspase activation was not found to be inversely proportional to Os concentration. Rather, both 0.1 and 1 μM Os activated caspase to a high degree, whereas caspase activation by 0.01 μM Os was significantly decreased. These results suggest that Os induced caspase activation may trigger non-apoptotic pathways, as caspases are known to have a multitude of non-apoptotic cellular activities. In fact, three of the caspases utilized in this experiment are regulators of inflammatory immune response, not apoptosis.  Regardless, it is clear from the
apoptosis data presented here that LFAO conformational transitions play a role in cellular activity.

Figure 2.7 O induced caspase activation

(a) Nuclear Hoechst (blue) and FLICA-labeled active caspase (green) stained images of SH-SY5Y cells following 24 h treatment with 0.01, 0.1, 1 μM Os along with (b) negative (CONT, buffer equivalent) and positive (2 U/μL TNF-α) controls. Images are representative of 3–5 independent experiments. (c) Cellular caspase activation quantified using MATLAB functions applied to single channel fluorescence microscope images. Results are reported as the fraction (FLICA/Hoechst) of caspase activated cells. ***p < 0.001 and **p < 0.01 vs negative control; +p < 0.05 vs 0.01 μM O. Error bars represent SEM, n = 3-5.

2.7 Conclusions

Based on the results described above, we conclude that LFAOs are dynamic oligomeric species and show a concentration-dependent association of 12mers (56 kDa) to form diffuse 12-24mers (56-110 kDa). ANS binding revealed that LFAOs undergo a conformational reorganization upon dilution that does not result in β-sheet secondary structure reorganization, as shown by CD spectroscopy. Interestingly, this conformational
reorganization profoundly affected the ability of LFAOs to self-replicate in the presence of Aβ monomers. This is best shown by comparing the replication efficiency data from Figure 2.1f (△, Figure 2.8) and the ANS binding data from Figure 2.5b (●, Figure 2.8), which display near identical concentration-dependent responses. This further solidifies the relationship between conformational reorganization and replication efficiency.

![Figure 2.8 LFAO dynamics and replication efficiency show similar trends](image)

**Figure 2.8 LFAO dynamics and replication efficiency show similar trends**

Comparison of replication efficiency (△, derived from Figure 2.1f) and %12mer (●, derived from Figure 2.5b) as a function of LFAO concentration.

LFAO conformational dynamics also manifests in cellular activity, particularly in their ability to induce apoptosis in human neuroblastoma cells. Considering these aspects of LFAO behavior together, this data reveals the hypothetical model depicted in Scheme 2.1. At low concentrations, LFAOs are primarily 12mers and display maximum replication efficiency and apoptotic activity (Figure 2.8). However, the association of 12mers to 12-24mers at increased concentrations leads to negligible replication and apoptotic behavior, which poses to question if 12-24mer LFAOs may undergo a more
conventional prion-type propagation leading to HMW fibrillar aggregates. This has been considered in detail and is the topic of Chapter III. Regardless, it is clear from the data presented here that concentration-dependent conformational dynamics between LFAO 12mer and 12-24mer formation governs their ability to both replicate and induce apoptosis.

Scheme 2.1 A schematic representation of LFAO dynamics revealed by this work and its consequences
CHAPTER III – LFAO PROPAGATION

3.1 Introduction

As described in the previous chapter, LFAO 12mers (O) undergo replication when seeded upon Aβ monomers (A). However, only a modest level of replication occurs at high concentrations of LFAO seeds, where they exist as disperse 12-24mers (L). Considering Ls are ineffective seeds for replication, this leads to question the end result of seeding A with L. Particularly, do L seed HMW fibrils, and if so, will L manifest in the fibril structure and function? Investigation of this has yielded novel insights into the strain-like behavior of Aβ oligomers, which are reproduced herein from the published report by Dean et al. in *Scientific Reports*.124

3.2 Inoculation of LFAO 12-24mers in TgCRND8 mice

Inoculation of Aβ aggregate seeds in transgenic animals has been previously shown to undergo prion-like propagation, leading to widespread deposition throughout the brain.70 We were interested to know if introduction of LFAOs into the neonatal brain of an AD transgenic mice would yield similar results. Therefore, Ls (10 μM) were injected into the cerebral ventricles of newborn TgCRND8 mice (Figure 3.1), which overexpress the Swedish and Indiana mutants of human APP leading to amyloid pathology three months after birth.125 Parallel injection of A and F aggregates to the litter-mates were used as controls. Mice were euthanized at three months, and brains were extracted and analyzed for Aβ pathology. One hemibrain was used for visualization of Aβ plaques via immunostaining (Figure 3.1a-c), while the other hemibrain was used to quantify total Aβ amounts (Figure 3.1d). Compared to the buffer only control, L and F

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administration resulted in an increased number of amyloid plaques throughout the cortex and hippocampus, as well as diffuse deposits along the anatomical structure of the hippocampus and the performant path (Figure 3.1a and d). Image analysis was used to quantify the total amyloid burden (Figure 3.1b), which was highest among mice injected with $F_{on}$, followed by $Ls$ and $As$. However, a large number of $A\beta$ positive vessels in the meninges and choroid were observed in mice injected with $Ls$ (Figure 3.1a and c), leading to a profound amount of CAA pathology in these mice. This suggests that the unique structural conformation (i.e., strain) of LFAOs selectively induces the CAA phenotype in TgCRND8 mice.

To quantify the total amount of $A\beta$ from the other hemibrain, sequential fractionation was performed with SDS (2%) and formic acid (FA, 70%), and $A\beta$ levels were measured using the enzyme-linked immunosorbant assay (ELISA). Compared to the buffer only control, $A$, $L$, and $F_{on}$ injected mice had an increase in both $A\beta40$ and $A\beta42$ levels in both the SDS and FA fractions (Figure 3.1d). The increase was highest in the brains of mice injected with $F_{on}$ aggregates, followed by $Ls$ and $As$. Overall, the data reveal a 3-4 fold-increase in $A\beta40$ and $A\beta42$ in SDS, and a 7-8 fold-increase in FA fractions, following neonatal administration of $F_{on}$ and $L$ seeds. Together, the data suggest that LFAOs are capable of inducing propagation and widespread deposition of $A\beta$ aggregates, and specifically induce CAA in TgCRND8 mice brains.
Figure 3.1 LFAOs selectively induce widespread amyloid deposition and CAA in TgCRND8 mice

(a) Newborn CRND8 mice were injected with 4 μL Aβ F18, Ls, As (10 μM), or PBS buffer (control) in the cerebral ventricles. Three months post-injection, brains were extracted and one hemibrain fixed and stained with anti-Aβ antibody. Amyloid staining of plaques and CAA in the representative paraffin sections is shown in the cortex, meningeal vessels and choroid plexus, and in the hippocampus of injected mice. Scale bars are 500 μm in cortex and CAA images, while 250 μm in hippocampus images.

(b) Quantification of Aβ positive immunostaining. Data represent mean ± sem. n = 6–10/group. ***p < 0.01, unpaired two-tailed t test.

(c) Quantification of CAA in blood vessels throughout the brain, done in a blind manner. Data represents mean ± sem. n = 6–10/group. ***p < 0.01, unpaired two-tailed t test.

(d) Biochemical analyses of sequentially extracted Aβ42 and Aβ40 levels by end-specific sandwich ELISA in SDS soluble and formic acid extractable insoluble fractions. Data represents mean ± sem. n = 6–10/group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, 2-way Anova with Tukey’s multiple comparison test.
3.3 Seeding of monomer with 12-24mer LFAOs shows marked deviation from other seeding regimes

After identifying that the propagation of LFAOs within mice brains specifically lead to CAA (Figure 3.1), the kinetics of L seeding in vitro was investigated to obtain mechanistic insights into LFAO propagation. Seeding was initiated on As (50 μM) with Ls (10 μM) and the kinetics of aggregation was monitored by the widely utilized fluorescence dye, thioflavin-T (ThT; Figure 3.2). Control reactions of As only (unseeded, Un) and As seeded with F_on aggregates (10 μM) were also monitored. Sigmoidal growth with a lag time of 8 days was observed in the Un sample (Figure 3.2a, □), which is expected for Aβ monomer in the absence of seeds. By seeding As with F_on aggregates (sFib), the lag time was abolished, resulting in immediate growth towards fibrils (Figure 3.2a, ✿). Seeding As with Ls (sLFAO) displayed a biphasic growth curve (Figure 3.2a, ●). Over the first 6 days of incubation, sLFAO samples displayed a slow incremental increase in growth followed by a rapid growth phase and saturation thereafter. This growth curve suggests that L and F_on seeding pathways differ, which warrants further investigation.

Samples were analyzed by PAGE at the saturation point of growth (14 days, Figure 3.2b-c). Under denaturing conditions (SDS-PAGE), HMW fibrillar aggregates were observed in all samples (Figure 3.2b, lanes 1-3). In addition, sLFAO samples contained a significant amount of soluble oligomers (Figure 3.2b, lane 3), which are likely dissociated species generated during electrophoresis under denaturing conditions. To decipher if this is the case, samples were also analyzed in non-denaturing (native PAGE) conditions without SDS treatment (Figure 3.2c). A, F_on, and L were used as
controls (Figure 3.2c, lanes 4-6). The immunoblots showed that \( F_{on} \) seeds migrated the least, followed by \( Ls \) and \( As \). Analyzing the samples after 14 days of incubation revealed HMW aggregates in all samples (Figure 3.2c, lanes 7-9). However, a second species with a lower mass/charge (m/z) was observed in the sLFAO sample (lane 9), confirming the presence of soluble species even after the saturation point of aggregation. This oligomeric species migrated with a higher m/z as compared to the \( L \) control, indicating it may be a larger species.

Figure 3.2 12-24mer LFAOs seed fibrils in a unique fashion with distinct biophysical characteristics

(a) ThT fluorescence aggregation kinetics of \( A \) (50 \( \mu \)M) in the absence of a seed (Un, □) or seeded with 10 \( \mu \)M \( Ls \) (sLFAO, ●) or \( F_{on} \) seeds (sFib, △). (b) Denaturing (SDS) PAGE of Un (1), sFib (2), and sLFAO (3) at 14 days of incubation. The single and double arrows represent \( A\beta \) 12mer (56 kDa) and 24mer (110 kDa), respectively. (c) Non-denaturing (native) PAGE of \( A \) (4), \( F_{on} \) (5), and \( L \) (6) along with Un (7), sFib (8), and sLFAO (9). The lower, middle, and upper dashed lines represent native migration of LFAO 12mer, 24mer, and HMW oligomeric species, respectively. 540 ng was loaded into each well for all samples. (d) Percentage of solubility, as determined by centrifugation and ThT fluorescence, at 28 days of incubation (14 days at 25 °C + 14 days at 4 °C). (e-f) Quantitative SEC of sLFAO (e) and sFib (f) at 4, 5, 6, and 7 days of incubation, respectively. The arrows represent the trends from 4-7 days. (g-h) Representative AFM images of fractions 19 (g) and 16 (h) from sLFAO at 7 days of incubation. Scale bar represents 1 \( \mu \)m.
To investigate this further, the solubility of aggregates after four weeks of growth (14 days at 25 °C + 14 days at 4 °C) was analyzed by ThT fluorescence after centrifugation at 18,000g for 20 min (Figure 3.2d). The solubility (%) was determined by normalizing the ThT fluorescence in the supernatant against that of the total sample before centrifugation. This indicated that Un and sFib samples have 30-40% of soluble aggregates, while sLFAO samples have ~65%, in agreement with electrophoresis experiments (Figure 3.2b-c). In order to learn more about the size of species populated during the sLFAO reaction, SEC was utilized (Figure 3.2e). Aliquots of seeding reactions from Figure 3.2a were taken at 4, 5, 6, and 7 days of incubation and fractionated on a Superdex-75 column. Initially, the sLFAO (Figure 3.2e) sample fractionated into two peaks corresponding to soluble oligomers (fraction 18) and unreacted monomers (fraction 25). As the seeding reaction proceeded, the monomer and oligomer peaks decreased as a third peak, eluting at fraction 16, emerged. This indicates that a transient oligomeric species is populated during the initial slow growth phase of biphasic sLFAO aggregation. This was not the case with sFib samples (Figure 3.2f), revealing a distinct seeding property of LFAOs. AFM was utilized to investigate the morphology of the transiently formed oligomeric species (Figure 3.2g), which revealed punctate dots similar to what has been reported previously for Ls<sup>106</sup> as well as larger, elongated aggregates. These elongated species may be the transient species formed during the initial slow growth phase of sLFAO aggregation, and warrant further investigation. Finally, AFM was used to confirm that the peak near the void volume (fraction 16) contained larger fibrils of 1-2 μm in length (Figure 3.2h). Together, these data reveal that Ls seed monomers toward fibrils via a pathway differing from that of F<sub>on</sub> seeding.
3.4 sLFAO fibril morphology reveals mechanistic insights into propagation

In order to examine if sLFAO aggregation leads to fibrils with a distinct morphology, samples were analyzed using AFM 10 days after initiating seeding reactions (Figure 3.3). AFM images of the Un (Figure 3.3a-c) and sFib (Figure 3.3d-f) reactions revealed long fibrils with average cross-sectional heights of 6.3 and 6.1 nm, respectively. Analysis along the surface of the aggregates (Figure 3.3k-l) showed a smooth morphology void of any distinctive patterns. While a relatively similar average cross-sectional height of 5.8 nm was observed for sLFAO aggregates (Figure 3.3g-i), further analysis indicated a unique repeating morphology along the fibril axis (Figure 3.3m). To ascertain if such a morphology could be due to the association of LFAO seeds, AFM of Os was performed, which revealed punctate dots with an average height of 6.3 nm (Figure 3.4j and n). Overlaying the cross-sectional analysis of Os (dashed line) with the sLFAO surface analysis (solid line) showcased that each peak of the repeating pattern represents an O (12mer) assembly (Figure 3.3m). After analyzing 30 fibril segments from multiple images and fields, this morphology was observed in 67% of sLFAO aggregates, while in only 23% of Un and sFib aggregates, respectively (Figure 3.3o). Overall, this reveals that LFAOs faithfully propagate to form morphologically-unique fibrils containing repeating oligomer assemblies.
Figure 3.3 sLFAO fibril morphology reveals mechanistic insight

(a-j) AFM images of Un (a-c), sFib (d-f), and sLFAO (g-i) aggregates at 10 days of incubation, along with Os (j). Scale bars represent 200 nm. (k-n) Surface morphology analysis of Un (k), sFib (l), sLFAO (m, solid), and Os (n), as indicated by the black arrows. The dashed lines in panel (m) represent data from Os in panel (n). (o) Statistical analysis of AFM images.

3.5 Sonication of sLFAO fibrils generates smaller propagating units

Considering sLFAO fibrils are made up of O units leads to question if LFAO seeds can be regenerated upon the breakdown of fibril samples, as has been previously observed for PrP. Therefore, sonication was utilized to generate fragments of Un, sFib, and sLFAO aggregates, which were then purified via SEC and analyzed using immunoblotting (Figure 3.4). Sonication of all samples resulted in predominantly two peaks when fractionated on a Superdex-75 column (Figure 3.4a-c), one peak eluting near the void volume ($V_0$, fractions 17-19) corresponding to oligomeric species, and another at a more inclusive volume (fractions 24-27) corresponding to monomeric species. The

<table>
<thead>
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<th>Sample</th>
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<th># with repeat morphology</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Un</td>
<td>30</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>sFib</td>
<td>30</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>sLFAO</td>
<td>30</td>
<td>20</td>
<td>67</td>
</tr>
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</table>
oligomeric peak for sonicated samples (solid lines) eluted at a more inclusive volume as compared to the samples before sonication (dashed lines), indicating a reduction in the size of aggregates upon sonication. Fractionation after the sonication of Un samples (Figure 3.4a) revealed a smaller oligomeric peak (and corresponding larger monomeric peak) as compared to sFib (Figure 3.4b) and sLFAO (Figure 3.4c) samples. This suggests that sonication of Un not only generates oligomeric seeds, but also some monomers. Samples before and after sonication, as well as SEC fractions of sonicated samples, were then analyzed by immunoblotting under both denaturing (Figure 3.4d) and non-denaturing (Figure 3.4e) conditions. This showed the presence of ~160 kDa oligomers in fraction 18 from Un and sFib samples, as well as some fibrils and monomers. However, fraction 18 of sLFAO samples showed a differing banding pattern. In the denaturing immunoblot, oligomers corresponding to Ls were observed, which could be due to sonication or also due to dissociation in the presence of 1% SDS. In the non-denaturing immunoblot (Figure 3.4e), fraction 18 of sLFAO samples (lane 6) migrated with a larger m/z as compared to Ls (lane 3), indicating SDS to be the cause of the 12-24mers observed in the denaturing gel. This suggests that sonication and isolation of sLFAO samples (herein referred to as iLFAOs) produces fragments larger than Ls.
Figure 3.4 Sonication of sLFAO fibrils generates smaller propagating units

(a-c) SEC chromatograms before (dashed) and after (solid) sonication of Un, sFib, and sLFAO samples, respectively. The downward arrow represents the void volume \(V_0\) of the column. Inset) Chromatograms showing the full elution profile including monomers. (d) Immunoblot of aggregates under denaturing conditions before (B) and after (A) sonication as well as fractions (17–19) from SEC after sonication. Single and double arrows represent Aβ 12 and 24mer, respectively. (e) Non-denaturing (native) immunoblot of A (1), Fmn (2), and L (3), along with Un (4), sFib (5), and sLFAO (6) samples before and after sonication, as well as fraction 18 from fractionation after sonication. The lower, middle, and upper dashed lines represent the native migration of LFAO 12mer, 24mer, and HMW oligomeric species. 540 ng was loaded into each well for all samples.

3.6 ‘Repeat morphology’ is also observed in iLFAO fragments

AFM and dynamic light scattering (DLS) were used to further characterize iLFAO fragments generated by sonication of sLFAO fibrils (Figure 3.5). AFM images of sonicated and isolated Un (Figure 3.5a-b) and sFib (Figure 3.5c-d) fragments (termed iUn and iFib, respectively), revealed a uniform size ~120 nm in length. Furthermore, the
morphology along the surface of these samples (Figure 3.5g-h) appeared to be smooth, as observed prior. While iLFAO fragments were of a similar length (Figure 3.5e-f), the morphology again showed a unique repeating pattern (Figure 3.5i) in which the peaks seem to correlate with the height of individual \(O\) assemblies (dashed line). Analyzing multiple images revealed a consistent pattern of 4-6 peaks in iLFAO fragments, suggesting them to be 4-6 times larger than the dodecameric \(O_s\). This was further corroborated using DLS, which yielded a diameter of \(\sim 38\) nm for iLFAOs (Figure 3.5j), roughly 4 times larger than the previously reported size of \(O_s\). Like observed in the length analysis from AFM images, DLS revealed all fragments to be of a similar size, suggesting that sonication parameters (power level, sonication time, number of cycles, etc.) may play a role in the size of fragments produced.

The varying LFAO species (\(L_s\), iLFAOs, and sLFAOs) were further characterized by ANS binding and CD spectroscopy (Figure 3.6). ANS binding (Figure 3.6a) revealed that \(L_s\) contain more solvent exposed hydrophobic regions, as evidenced by increased binding. No significant difference between iLFAO (●) and sLFAO (△) ANS binding was observed. Using far-UV CD, it was shown that all samples have a \(\beta\)-sheet secondary structure (minima at 217 nm), of which the extent was less in \(L_s\) (Figure 3.6b).
Figure 3.5 Repeat morphology is retained within iLFAO fragments

(a-f) AFM images of sonicated and isolated (fraction 18) Un (iUn, a-b), sFib (iFib, c-d), and sLFAO (iLFAO, e-f). Inset) AFM images used for surface analysis. Scale bars for (a, c, and e) represent 1 μm, while (b, d, and f) and insets represent 200 nm. (g-i) Surface morphology analysis, as indicated by black arrows. The dashed line in panel (i) represents O analysis from Figure 3.3n. (j) DLS of fractions 18 of iUn, iFib, and iLFAO samples. **represents p < 0.05.

Figure 3.6 Biophysical comparison of Ls, iLFAOs and sLFAOs

(a) ANS (100 μM) fluorescence scans of 10 μM sLFAO (∆), iLFAO (●), and L (○), respectively. Samples were equilibrated 1 min before measuring fluorescence using an excitation of 388 nm and scanning emission between 405 and 650 nm. (b) CD spectra for 10 μM sLFAO (dashed), iLFAO (solid), and L (dotted). The vertical line indicates 217 nm.
3.7 Conclusions

The data presented here, together with the data presented in Chapter II and previously by Kumar et al.,\textsuperscript{105, 106} reveals a holistic picture of Aβ dodecamer (LFAO) propagation, which proceeds in three distinct macroscopic phases (Scheme 3.1). In the initial replication phase, Os (12mers) undergo self-propagative replication in the presence of As, as reported previously.\textsuperscript{105} Upon reaching a threshold concentration, Os associate to form Ls as indicted in Chapter II. As shown here, Ls then seed toward morphologically-unique Fs, but do so in a biphasic manner suggesting the formation of at least one intermediate. Using sonication and AFM, it was revealed that smaller fragments of Fs, made up of 4-6 O units, can be generated and isolated. These novel LFAO intermediate species, which we are denoting as Ps, are the rate-limiting species formed during the second phase of LFAO propagation. Upon formation of Ps, Fs are rapidly formed (propagation phase) via reactions which are not yet defined. Interestingly, inoculation of Ls in transgenic mice specifically induced CAA, supporting the idea that the faithful propagation of conformationally-unique Aβ oligomers to polymorphic fibrils contributes to the observed phenotypic diversity in AD. Overall, this reveals molecular-level insight into the propagation mechanism of one such conformational Aβ oligomer strain.
Scheme 3.1 A schematic representation of the mechanism of LFAO propagation
CHAPTER IV – DETAILS OF LFAO PROPAGATION PROBED BY MASS ACTION KINETICS

4.1 Introduction

In the previous chapter, the faithful propagation of Os to morphologically-unique fibrils containing repeating oligomer units was established. It was revealed that O propagation occurs in three macroscopic phases: replication, an intermediate phase involving P generation, and propagation (Figure 4.1a). However, the microscopic reactions defining the macroscopic phases have yet to be established. In this chapter, the microscopic reactions were investigated by dissecting the macroscopic phases and evaluating the kinetics of each reaction individually. The rate constants derived from these in vitro reactions were used to build an ensemble kinetic simulation (EKS) model. Collectively, this has yielded novel insight into the reaction mechanism of LFAO-strain propagation, which is discussed below. The results presented herein were originally disseminated by Dean et al. in the Biophysical Journal, and are reproduced here.

4.2 LFAO propagation at varying seed concentrations

As described in Chapter II, LFAOs display concentration-dependent dynamics between O and L assemblies. In Chapter III, the biphasic behavior involved in the propagation of Ls toward Fs was described (Figure 4.1b, ◊). To investigate if conformational dynamics plays a role in the biphasic seeding behavior, seeding reactions were initiated at 0.1 (○) and 1 (▲) μM LFAOs (Figure 4.1b), where the O/L fraction is increased (more Os, less Ls). As expected, seeding of O/Ls resulted in a decreased lag time as compared to the unseeded A control (Figure 4.1b, ■). However, the biphasic
curve observed in 10 μM O/L seeding was not observed at 10- and 100-fold lower concentrations (Figure 4.1b). This may be due to an increase in the competing on-pathway aggregation (self-nucleation of As) in low seeding conditions, which is elaborated below.

Figure 4.1 O/L propagation at varying seed concentrations

(a) A schematic representation of the reactions and rate constants considered in LFAO strain-specific propagation. These include monomer elongation (ME), oligomer elongation (OE), and oligomer association (OA) reactions, respectively. Adapted from 124 with permission. (b) A (50 μM) seeded with 0.1 (○), 1 (▲), or 10 (□) μM O/Ls, respectively. The unseeded A control is represented by (●).

4.3 Monomer elongation predominates in the replication and intermediate phases

The microscopic reactions considered in the replication and intermediate phases, along with the corresponding rate constants obtained, are listed in Table 4.1. The elongation of Os by As (monomer elongation, ME) was considered in the replication phase, while both ME and oligomer association (OA) were considered in the intermediate phase involving the generation of Ps.

To investigate the kinetics of these reactions, the initial rate method and ThT fluorescence were employed, which have both been previously used to analyze other
amyloid proteins. To investigate the rate of ME, the reactions were initiated at varying concentrations of As (1 (■), 10 (○), 25 (▲), and 50 (▼) μM) with a constant seed concentration (O/Ls) of 5 μM (Figure 4.2a). The data revealed that increasing the concentration of A increased the rates of the reactions (Figure 4.2a). Initial rates of each reaction were obtained from the slope of the linear fit of the initial time points (t₀-t₃₀₀ s; Table 4.2). Plotting the initial rates against the initial A concentration revealed a linear correlation (Figure 4.2d, □), suggesting the reaction to be first order with respect to A. Additionally, the slope of this plot represented the rate constant ($k'_{O/LME}$), which was 0.43 x 10⁻³ s⁻¹. To determine if O/L conformational dynamics played a role in reaction kinetics, similar experiments were performed at 1 (○) and 10 (▲) μM seed concentrations, which yielded $k'_{O/LME}$ values of 0.018 x 10⁻³ s⁻¹ and 0.42 x 10⁻³ s⁻¹, respectively (Figure 4.2d). Plotting the rate constant values against the initial O/L concentration revealed a deviation from linearity at 1 μM (Figure 4.2b), supporting the model (Figure 4.1a) that Os do not seed toward P formation, but rather undergo replication to form Ls.

Table 4.1 A comprehensive list of reactions and their corresponding rate constants

<table>
<thead>
<tr>
<th>Monomer Elongation (ME)</th>
<th>Oligomer Association (OA)</th>
<th>Oligomer Elongation (OE)</th>
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<td><strong>A (μM)</strong></td>
<td><strong>P (μM)</strong></td>
<td><strong>Rate Constant</strong></td>
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50
To determine the reaction order with respect to the $O/L$ concentration, varying amounts of $O/L$s (0.1 (■), 1 (○), 5 (▲), 7.5 (▽), and 10 (◆) μM) were seeded with a constant concentration (50 μM) of As (Figure 4.2c). Similar to what was observed prior, increasing the seed concentration lead to increased rates. A linear relationship was found by plotting the initial rates against the initial $O/L$ concentration (Figure 4.2d, ■), suggesting a first order reaction with respect to the seed. Together with the previous data, this reveals an overall second order reaction for ME in the intermediate phase. The rate constant for the varying $O/L$ reactions ($k''_{O/L ME}$) was found to be $5.48 \times 10^{-3} \text{ s}^{-1}$, which is ~13 fold higher than $k'_{O/L ME}$. This suggests that the amount of seeds, not As, drives the reaction toward the formation of $P$s.

Table 4.2 Experimentally determined initial rates of ME, OA, and OE reactions detailed in this work
Figure 4.2 Kinetics of replication and intermediate phases

(a) ThT fluorescence of 1 (■), 10 (○), 25 (▲), and 50 (▼) μM A seeded with O/L (5 μM), respectively. (b) The rate constant ($k'_{O/L}$) for reactions at 1, 5, and 10 μM O/L, respectively. (c) ThT fluorescence of A (50 μM) seeded with 0.1 (■), 1 (○), 5 (▲), 7.5 (▼), and 10 (●) μM O/L, respectively. (d) Initial rates, as calculated by the slope of the linear line from the first 300 s of the reactions in panels (a) and (c). (e) ThT fluorescence of 1 (■) and 10 (○) μM O/L, respectively. Inset) Initial rates from the first 150 s of the reactions.

The symbol * represents p<0.005 from statistical t test.
The conversion of $L$s to $P$s in the intermediate phase may also occur through the association (OA) of $O/L$s in the absence of $A$s. To investigate this, kinetics were monitored at 1 (■) and 10 (○) μM $O/L$s upon the addition of 100 mM NaCl (Figure 4.2e), which has been shown previously to promote lateral association of Aβ protofibrils. The initial rates of these reactions indicated that, compared to 1 μM, association is enhanced at 10 μM where LFAOs primarily exist as $L$s (Table 4.2). Comparing the rate of OA against that of ME reactions at similar concentrations revealed that ME is preferred over OA (Table 4.2), suggesting that the conversion of $O$s to $P$s primarily occurs through ME.

4.4 Oligomer elongation predominates in the propagation phase

The conversion of $P$s to $F$s in the propagation phase may occur through ME, $P$ association (PA), or a third pathway where $P$s serve as a template for $O/L$s to bind and elongate (oligomer elongation, OE). Investigating these reactions in vitro is challenging due to the transient nature of $P$ formation. However, as shown in Chapter III, short fragments of 4-6 $O$ units can be generated and isolated via sonication of $F$s. Therefore, this method was employed to generate apparent $P$ units, which were then investigated for ME, PA, and OE kinetics (Table 4.1). ME reactions were investigated by seeding 1 (■), 10 (○), 25 (▲), 50 (▼), and 70 (◆) μM $A$s with a constant concentration (5 μM) of $P$s (Figure 4.3a). As observed with previous reactions, increasing concentrations of $A$s resulted in an increase in the rate of the reaction. However, plotting the initial rates against the initial $A$ concentration revealed a Michaelis-Menten type plot (Figure 4.3c). At low $A$ concentrations, the rate increased linearly with the $A$ concentration, suggesting
a first order reaction. However, at increased A concentrations there was a plateau of the rate, suggesting a zero order reaction. Overall, this suggests that there are a limited number of seeding ends in Ps, which upon saturation by As results in no further increase in the rate of the reaction. The initial first order reaction (1, 10, and 25 μM points) were used to determine the rate constant ($k'_{PME}$), which was $2.6 \times 10^{-3}$ s$^{-1}$.

To investigate whether the saturation of the initial rate at increased A concentrations is due to the limited number of binding sites on P, reactions were initiated with increasing amounts of Ps (0.1 (■), 1 (○), 5 (▲), 7.5 (▽), and 10 (●) μM) and a constant amount (50 μM) of As (Figure 4.3b). We hypothesized that increasing the amount of Ps will likewise increase the number of seeding ends, which would not result in a plateau of the initial rate. Plotting the initial rates against the initial P concentration (Figure 4.3c) resulted in a linear relationship, confirming this hypothesis. The rate constant ($k''_{PME}$) for this reaction, $24.4 \times 10^{-3}$ s$^{-1}$, was ~10 fold higher than $k'_{PME}$, suggesting that the amount of Ps in the system, not As, drives the reaction forward.

To investigate the lateral association of Ps, experiments were initiated in the presence of 100 mM NaCl (Figure 4.3d), similar to what was described above. PA was monitored at 1 (■) and 10 (○) μM, which as expected yielded an increase in the rate of association at the increased concentration. Comparing these initial rates with those of the ME reactions at the same P concentrations (Table 4.2) reveled a 5-6 fold-increase of ME over PA (Table 4.2), similar to what was observed for O/Ls. To investigate the kinetics of OE in the propagation phase, Ps (5 μM) were mixed with 1, (■), 3 (○), 5 (▲), or 7 (▽) μM O/Ls, respectively (Figure 4.4a). Plotting the initial rates against the initial O/L concentration revealed a linear, first order, correlation (Figure 4.4b) with a rate constant
$(k'_{\text{POE}})$ of $7.4 \times 10^{-3} \text{ s}^{-1}$. Comparing this to $k'_{\text{PME}}$ indicated a 3-fold higher rate of OE to ME, suggesting that $P$s primarily form $F$s by addition of $O/L$s, not $A$s.

Figure 4.3 ME and PA kinetics in the propagation phase

(a) ThT fluorescence of 1 ( ), 10 ( ), 25 ( ), 50 ( ), and 70 ( ) μM A seeded with $P$ (5 μM), respectively. (b) ThT fluorescence of A (50 μM) seeded with 0.1 ( ), 1 ( ), 5 ( ), 7.5 ( ), and 10 ( ) μM $P$, respectively. (c) Initial rates, as calculated by the slope of the linear line from the first 300 s of the reactions in panels (a) and (b). (d) ThT fluorescence of 1 ( ) and 10 ( ) μM $P$, respectively. **Inset** Initial rates from the first 150 s of the reactions. The symbol ** represents $p<0.001$ from statistical t test.
Figure 4.4 OE kinetics in the propagation phase

(a) ThT fluorescence of 1 (■), 3 (○), 5 (▲), and 7 (▽) μM O/Ls seeded with Ps (5 μM), respectively. (b) Initial rates, as calculated by the slope of the linear line from the first 150 s of the reactions in panel (a).

**4.5 Temporal simulation of LFAO propagation**

As shown above, Ls grow toward Fs in a biphasic manner, suggesting the formation of a larger intermediate, termed Ps. While *in vitro* experiments in Chapter III have revealed that sonication of Fs generates LFAO fragments 4-6 times greater than O 12mers, similar sized fragments were also observed in other samples. In order to bring clarity to this issue and unambiguously determine the size of Ps, *in silico* numerical simulation analysis was performed using ordinary differential equations (ODEs) to build an EKS model to define LFAO propagation. Similar methods have been employed previously to yield novel insight into Aβ aggregation. However, attempts to fit the biphasic 10 μM seeding reaction using previously defined platforms, such as the widely utilized ‘AmyloFit’, were unsuccessful (Figure 4.5a-c), suggesting the need to develop a model specific for LFAO propagation.
To develop such a model, the results from the \textit{in vitro} kinetic experiments were used in the ODE simulated rate constants (e.g., $k_{O/LME} > k_{O/LA}$ and $k_{POE} > k_{PME}$). The complete list of parameters and the equations considered can be found in Chapter VIII. Overall, the model considered that the replication and intermediate phases occur through ME, and not by OE, as supported by experimental observations. Also, simulations were initiated at varying ratios of Os and Ls based on the seeding concentration (0.1, 1, or 10 μM) and the $K_d$ for $O/L$ dynamics (0.1 μM, see Chapter II). The size of $P$ was floated from 36-120 Å units ($O_3 - O_{10}$) and evaluated for the best fit to determine the $P$ size. Reasonable fits were obtained for $P$ sizes of $A_{48} - A_{84}$ ($O_4 - O_7$; Figure 4.6b), which is in good agreement with the sonication and AFM data in Chapter III. While ME reactions were exclusively considered in the replication and intermediate phases, all reactions (ME, PA, and OE) were considered in the propagation phase. Overall, this EKS model shows good agreement with the experimental LFAO seeding reactions (Figure 4.6a), which can be defined by ODEs with a single set of global rate constants (Figure 4.6c). Additionally,
this model incorporates the contribution from the competing on-pathway reaction ($F_{on}$), which increased with decreasing seeding concentrations (Figure 4.6d).

Figure 4.6 EKS Model of LFAO propagation

(a) Aggregation profiles from the EKS model (solid lines) with the experimental unseeded (■) and 0.1 (○), 1 (▲), and 10 (◇) μM O/L seeded reactions, respectively. $P$ size = 48mer. (b) RMSE and least $R^2$ values at each respective gateway ($P$) size. (c) Estimated rate constants for $P$ = 48mer. (d) $F_{on}/F$ for 0.1, 1.0, and 10 μM seeding reactions.

Figure 4.7 Flux analysis from the EKS model

The concentration (μM) flux of $A$, $O$, $L$, $P$, $F$, and $F_{on}$ $Aβ$ species during the 0.1 (a), 1 (b), and 10 (c) μM O/L seeding reactions for the EKS model described in the text.
The EKS model is also able to quantitatively simulate the temporal evolution of the various species (As, Os, Ls, Ps, Fs, and Fon) during the seeding reaction (Figure 4.7). These quantitative concentration values were compared to that of experimental values calculated from SEC data at 4, 5, 6, and 7 days of 10 μM LFAO seeding (Figure 4.8a). The SEC peaks were considered to be either fibrils (F + Fon, ▲), oligomers (Os + Ls + Ps, ○), or monomers (As, ■), and the fraction of each species was plotted against time (Figure 4.8b). Comparing these results with that from the simulation revealed a good agreement between the EKS model and experimental data, further validating the model.

Figure 4.8 EKS and experimental quantitative analysis

(a) SEC fractionation of 10 μM LFAO seeding at 4, 5, 6, and 7 days of incubation, respectively. The dashed arrows represent the trends from days 4-7. Reproduced from Chapter III. Fractions 16-17 (▲) represent fibrils, fractions 17-20 (○) represent oligomers, while fractions 23-27 (■) represent monomers. (b) Quantification of the fraction of fibrils, oligomers, and monomers from experimental (symbols) or simulated (P=48mer, lines) data. (c) RMSE values for panel (b).
4.6 Conclusions

This chapter presents mass action-based kinetic insight into the LFAO propagation mechanism. The overall seeded aggregation was dissected and individual reactions such as ME, OE, and OA were investigated for each phase of propagation. The data suggest that the conversion of $L$s to $P$s primarily occurs through ME reactions, and that the amount of $O/L$s drive the reaction to completion. It was similarly observed that the conversion of $P$s to $F$s is driven by the amount of $P$s, however the primary mechanism for conversion is by OE, not ME. The kinetic insights from these reactions were used to build an EKS model, which was validated quantitatively by comparing the flux of Aβ species from experimental results. Overall, this has yielded unique and novel insights into the molecular events defining the strain-specific propagation of Aβ dodecamers in vitro, which involved a three-step seeded process. Such a seeding mechanism has not been reported before, making this potentially significant in understanding strain propagation, which is essential in order to develop strategies that target and inhibit the process in the AD brain. This work provides a platform for characterizing Aβ strains by their rates of propagation, which may be used to better establish and characterize distinct phenotypes among AD patients.
CHAPTER V – LFAO CROSS-PROPAGATION

5.1 Introduction

The previous chapters have established that LFAOs are an Aβ oligomer strain capable of undergoing both replication and propagation with Aβ42 WT monomers. As discussed in Chapter I, CAA is a cerebrovascular pathology that emanates from deposition of Aβ, particularly mutant isoforms, within the cerebral vessels of the brain.11-13 A high incidence of CAA exists among AD patients,12,13 which leads to questions if an interplay exists between WT and mutant isoforms of Aβ, leading to the co-existence of both pathologies. Additionally, the interplay of various Aβ isoforms may lead to novel hybrid oligomer strains with distinct biophysical properties and pathological consequences. Therefore, in this chapter the cross-propagation of Aβ42 WT LFAOs (herein referred to as LFAOs) with other Aβ isoforms was explored to yield insight into this process.

5.2 LFAO cross-propagation with WT and Arctic-Aβ40

Initially, the cross-propagation of LFAOs with Aβ40 WT monomers was explored, as Aβ40 is the predominant isoform of Aβ found in the brain and thus may play a significant role in AD pathology. To do this, LFAOs (5 μM) were seeded upon Aβ40 WT monomer (30 μM) and allowed to interact for 72 h at 25 ºC in quiescent conditions (seeded; Figure 5.1a). Aβ40 WT monomer in the absence of a seed (C) and the LFAO seed alone (seed) served as controls. Compared to the seed alone, the immunoblot showed an amplification of ~56 kDa Os in the seeded sample (Figure 5.1a). The monomer control sample had a single band at 4.5 kDa corresponding to As, indicating
that no oligomers were formed by the self-nucleation of Aβ40 WT in these reaction conditions. To confirm that the amplification of the 56 kDa oligomer was due to incorporation of Aβ40 WT, similar reactions were initiated with N-terminally labeled fluorescein isothiocyanate Aβ40 (FITC-Aβ40 WT). It was hypothesized that amplification can occur either by the self-association of Aβ40 WT on the LFAO template, or by insertion within the LFAO assembly (Figure 5.1b). In either condition, the presence of FITC in the isolated oligomer would suggest the incorporation of Aβ40 WT in the cross-propagated oligomer. SEC fractionation of the LFAO seeded reaction with FITC-Aβ40 WT (Figure 5.1c) yielded a 1.5-fold amplification over the seed alone. Immunoblotting analysis confirmed the isolation of 56 kDa oligomers in fractions 17 and 18 (Figure 5.1c, inset). Analyzing the fractions for FITC fluorescence revealed the presence of FITC-Aβ40 WT in fractions 16-18, as evident by the emission maxima at 525 nm upon excitation at 490 nm (Figure 5.1d). The maximum FITC fluorescence was found in fraction 17, which was in agreement with the band intensities in the immunoblot (Figure 5.1c). Overall, this suggests that LFAOs can cross-propagate with Aβ40 WT monomers, leading to an amplification of novel hybrid oligomers containing both Aβ40 and Aβ42 WT isoforms.

To investigate whether this can occur with Aβ40 E22G (Arctic-Aβ40) monomers, similar reactions were initiated by seeding Arctic-Aβ40 monomers (25 μM) with LFAOs (2 μM, Figure 5.1e). The immunoblot revealed the amplification of 56 kDa Os in the seeded sample, although the fold-increase from the seed alone seemed diminished as compared to cross-propagation with Aβ40 WT. Regardless, this unveils an additional mechanism of LFAO cross-propagation leading to hybrid oligomer formation.
Figure 5.1 Cross-propagation of LFAOs with WT and Arctic Aβ40 monomer

(a) Immunoblot of LFAOs (1.5 μM) alone (seed) or after incubation with Aβ40 WT monomer (30 μM) for 72 h at 25 °C (seeded). Aβ40 WT monomer in the absence of a seed served as a control (C). (b) A schematic representation of FITC-labeled reactions in panels (c) and (d). (c) SEC fractionation of LFAO seeds (2.5 μM) alone or seeded with 50 μM Aβ40 WT monomer (20% FITC-labeled). Inset, an immunoblot of the seeded reaction before SEC (B), and of fractions 16-18, respectively. (d) FITC fluorescence of fractions 16-18 of SEC from panel (c). (e) Immunoblotting analysis of LFAOs (2 μM) alone (seed) or after incubation with Arctic-Aβ40 monomer (25 μM) for 72 h at 25 °C (seeded). Arctic-Aβ40 monomer in the absence of a seed served as a control (C).
5.3 LFAO cross-propagation with Arctic-Aβ42

As shown in Figure 5.1e, cross-propagation of LFAOs with Arctic-Aβ40 produced hybrid oligomers roughly 56 kDa in size. To investigate if the Arctic-Aβ42 isoform showed similar trends, LFAOs (5 μM) were seeded upon Arctic-Aβ42 monomer (50 μM) and allowed to incubate at 25 °C for 24 h. The immunoblot (Figure 5.2a) revealed the formation of a disperse cross-propagated oligomer containing predominantly two size distributions at 56 (single arrow) and 110 (double arrow) kDa. This is analogous to what was observed when seeding LFAOs with Aβ42 WT monomers, as shown in Chapter II. To confirm that the cross-propagation was due to incorporation of Arctic-Aβ, FITC-labeled Arctic-Aβ42 monomers were used (Figure 5.2b). SEC fractionation yielded a two-fold increase in the cross-propagated species eluting at fraction 17 in the seeded sample, and the immunoblot confirmed the oligomers to be 56-110 kDa in size (Figure 5.2c). The presence of FITC in the fractions (Figure 5.2d) confirmed that the oligomer amplification was due to incorporation of Arctic-Aβ42. The highest FITC fluorescence was found in fraction 17, followed by fraction 18 and 16, which corroborates with the immunoblotting data. Overall, this reveals an additional mode of cross-propagation among LFAOs leading to the formation of hybrid oligomers.
Figure 5.2 Cross-propagation of LFAOs with Arctic-Αβ42 Monomer

(a) An immunoblot of LFAOs (5 μM) alone (seed) or after incubation with Arctic-Αβ42 monomer (50 μM) for 24 h at 25 °C (seeded). Arctic-Αβ42 monomer in the absence of a seed served as a control (C). (b) A schematic representation of FITC-labeled reactions in panels (c) and (d). (c) SEC profiles of LFAO seeds (2 μM) alone or seeded with 40 μM FITC Arctic-Αβ42 monomer (100% FITC-labeled). Inset, an immunoblot of the seeded reaction before SEC (B), and of fractions 16-18. (d) FITC fluorescence of fractions 16-18 of SEC from panel (c).
5.4 Reverse cross-propagation of Arctic-Aβ42 oligomers with Aβ42 WT monomer

To explore the possibility of mutant Aβ oligomers cross-propagating with WT Aβ monomer (reverse cross-propagation), conditions for generating and isolating Arctic-Aβ42 oligomers were optimized. As discussed in Chapter I, Kumar et al. have shown previously that incubation of C12:0 NEFAs near or above their CMC promotes oligomer formation with Aβ42 WT monomers. Therefore, Arctic-Aβ42 monomer (50 μM) was allowed to aggregate in the presence of C12:0 NEFA micelles (15 mM) for 24 h at 37 ºC in quiescent conditions. Analyzing the samples by SDS-PAGE with immunoblotting showed the formation of 30-40 kDa oligomers (Figure 5.3a, lane B), which was not observed in the control reaction without C12:0 (lane C). Fractionation of the oligomers using a Superdex-75 column (Figure 5.3b) resulted in three predominant peaks; fibrils (fraction 15), oligomers (fraction 18), and monomers (fraction 26), which is in agreement with the immunoblotting data (Figure 5.3, lane B). Furthermore, the immunoblot confirmed the presence of 30-40 kDa (~8-9mer) oligomers in fractions 17 and 18 (Figure 5.3a, lanes 17 and 18).

To explore the propensity of Arctic oligomers to reverse cross-propagate, Aβ42 WT monomers (25 μM) were seeded with Arctic-Aβ42 oligomers (1 μM) and allowed to incubate at 25 ºC for 72 h in quiescent conditions. Immunoblots of reactions revealed the formation of a larger, 40-80 kDa oligomer in the seeded sample (Figure 5.3c). As expected, the control reaction of unseeded Aβ42 WT monomer (lane C) did not show formation of any oligomers within these reaction conditions. Fractionation of the seed and seeded samples revealed a 4.4 fold-increase of reverse cross-propagated oligomers in the seeded sample (Figure 5.3d). Overall, these results showcase that Arctic-Aβ42
oligomers can be generated in interfacial reaction conditions similar to what has been reported previously for Aβ42 WT LFAOs, and the reverse cross-propagation of Arctic oligomers with WT monomers suggests additional modes of hybrid oligomer formation within the complex Aβ interactome.

Figure 5.3 Reverse Cross-propagation of Arctic-Aβ42 Oligomers with Aβ42 WT Monomer

(a-b) A representative immunoblot (a) and SEC profile (b) of Arctic-Aβ42 oligomers formed upon incubation of monomer (50 μM) with 15 mM C12:0 fatty acid at 37 °C for 24 h. Lane B represents the sample before SEC, while 17 and 18 represent fractions from SEC in panel (b). Monomer in the absence of C12:0 served as a control (C). (c-d) A representative immunoblot (c) and SEC profile (d) of Arctic-Aβ42 oligomers (1 μM) alone (seed) or after incubation with Aβ42 WT monomer (25 μM) for 72 h at 25 °C (seeded). Aβ42 WT monomer in the absence of a seed served as a control (C).
5.5 Conclusions

In this chapter, the formation of hybrid oligomers derived from the interplay of WT and Arctic (E22G) Aβ isoforms has been demonstrated. LFAOs of Aβ42 WT were able to cross-propagate with Aβ40 WT, Arctic-Aβ40, and Arctic-Aβ42 monomers to form hybrid oligomers of varying sizes. Furthermore, reverse cross-propagation of Arctic oligomers with Aβ42 WT monomers lead to hybrid oligomers unique from that of LFAO/Arctic hybrid oligomers. These preliminary results shed light into the potential complex interplay of Aβ species within the AD brain, and reveal the seemingly unlimited possible reactions present within the Aβ interactome. Further investigation into the structure-phenotype relationship of hybrid oligomers is necessary to delineate their physiochemical and neuropathological consequences, as discussed in Chapter VII.
CHAPTER VI – PHOSPHATIDYLGLYCEROL- AND GANGLIOSIDE-DERIVED OLIGOMERS

6.1 Introduction

The majority of the work presented thus far has shown that LFAOs propagate toward fibrils in a biphasic mechanism resulting in the selective induction of CAA in transgenic mice. As hypothesized in Chapter I, a family of Aβ oligomer strains may be generated using different micelle- and liposome-forming lipids. This hypothesis will be explored in this chapter by evaluating the ability of the anionic phospholipid, phosphatidylglycerol (PG), to induce Aβ oligomerization. Oligomer formation in the presence of the ganglioside GM1 will also be investigated, as it has been widely implicated in AD pathology (discussed in Chapter I). The results presented here showcase that both micellar and liposomal lipids generated by PG and GM1 modulate Aβ aggregation pathways toward Aβ oligomer formation.

6.2 Micelle-derived oligomer strains

6.2.1 Generation and isolation of Aβ oligomers in the presence of anionic micelles

As shown previously by Rangachari et al., as well as others, the anionic surfactant SDS is capable of inducing the generation of stable, off-pathway Aβ oligomers. More recently, Kumar et al. have shown that anionic NEFAs also promote off-pathway Aβ oligomer formation under a narrow regime of NEFA concentrations. Since both SDS and NEFAs are micelle-forming surfactants, it warrants investigation into whether anionic phospholipids, which are primary components of cellular membranes, could also behave similarly in inducing the formation of off-pathway oligomers. Lyso-PG (LPG) is
similar to both SDS and NEFAs in that it contains a single acyl chain and forms micelles as opposed to bilayer-containing liposomes. Aβ oligomer formation was investigated in the presence of LPG micelles containing saturated acyl chains of 14 (C14:0 LPG), 16 (C16:0 LPG), or 18 (C18:0 LPG) carbons in length (Figure 6.1). Aβ monomer (50-60 μM) was added to C14:0, 16:0, or 18:0 LPG micelles buffered in 20 mM Tris (pH 8.0) containing 50 mM NaCl and allowed to incubate at 37 °C in quiescent conditions for 48 h. While the final concentration of LPG in each reaction varied based on the lipid species (see Table 8.1 in Chapter VIII), the concentration was kept at least two-fold above the reported CMC to ensure the lipids were in a micellar form.\textsuperscript{132} After incubation, the reactions were electrophoresed and visualized via immunoblotting, which revealed the formation of soluble, 30-60 kDa oligomers in the presence of LPG micelles (Figure 6.1a). The control reaction in the absence of LPG (Figure 6.1a, lane C) contained HMW fibrils, a broad HMW oligomer, and monomers, respectively. This suggests that the 30-60 kDa oligomers generated in the presence of LPG micelles was due to the presence of the micelles. Furthermore, the size of the LPG-derived oligomers (LPGOs) seemed to correlate with the acyl chain length of the micelle, with longer carbon chains inducing larger oligomers (Figure 6.1a).

Fractionation of LPGO samples on a Superdex-75 column (Figure 6.1b-d) revealed predominantly three Aβ species, mainly monomers, oligomers, and fibrils, which corroborates with the immunoblotting data. Similar fractionation of the control reaction (Figure 6.1b-d, dashed lines) showed only fibrils and oligomers, further indicating that the LPGOs are formed due to the presence of the micelles, not the reaction conditions. Immunoblotting of the fractionated LPGOs (Figure 6.1f) revealed that the
least inclusive peak (fraction 17) contained fibrils, as expected. The more inclusive peak eluting at fractions 18-20 contained the soluble oligomers generated in the presence of the LPG micelles, while the fractions at the most included volume (fraction 25) represented monomeric Aβ (Figure 6.1f). The predominant species generated in the presence of C14:0 and C16:0 LPG micelles were the soluble oligomers (C_{14} LPGOs and C_{16} LPGOs, respectively). However, this was not the case for Aβ in the presence of C18:0 micelles, which contained more fibrils (fraction 17) than oligomers (Figure 6.1b-d). This was clear from the presence of fibril bands in fractions 18-20 of the immunoblot (Figure 6.1f, C_{18} LPGO), which was reflected in the poor resolution of the two species during SEC (Figure 6.1d). Nevertheless, the collective data indicate that LPG micelles promote the formation of Aβ oligomers which, in the case of C_{14} and C_{16} LPGOs, can be isolated via SEC.

The anionic glycosphingolipid, GM1, is also a micelle forming lipid species with a CMC in the low micromolar range.\textsuperscript{133,134} As discussed in Chapter 1, several reports indicate GM1 as a significant contributor to Aβ aggregation in the AD brain.\textsuperscript{86, 87, 95, 96} To investigate the connection between GM1 and Aβ further, Aβ monomer (50-60 μM) was incubated in the presence of GM1 micelles buffered in 20 mM Tris (pH 8.0) containing 50 mM NaCl and the reaction was allowed to incubate at 37 °C in quiescent conditions for 48 h. Immunoblotting analysis of the reaction (Figure 6.1e, GM1) show the formation of an Aβ oligomer with a bimodal molecular-weight distribution centered at ~40 and ~56 kDa, respectively. Unlike LPGO reactions, no HMW fibrils were observed in the immunoblot of GM1 derived oligomers (GM1Os). This was confirmed by SEC fractionation (Figure 6.1e), which yielded just two peaks corresponding to the oligomers
The absence of HMW fibrils was further established by immunoblotting analysis of fraction 17, in which no fibril bands were observed (Figure 6.1f). Overall, this suggests that GM1 micelles catalyze the formation of distinct Aβ oligomers without detectable fibril formation.

Figure 6.1 Generation and isolation of micelle-derived oligomers

(a) A representative immunoblot of samples before fractionation. The amount of Aβ loaded into each well was kept constant at 1 μg.

(b-e) SEC of Aβ (50 μM) incubated alone (dashed) or with C14:0 LPG (b), C16:0 LPG (c), C18:0 (d), or GM1 (e) micelles (solid) after 48 h of incubation at 37 °C in quiescent conditions. (f) Immunoblotting analysis of fractions 17-20 after fractionation as shown in (b-e). The volume of each fraction loaded onto the gel was kept constant at 15 μL.

6.2.2 Biophysical characterization of LPGOs and GM1Os reveals similarities

To characterize the size and structure of micelle-derived oligomers, LPGO and GM1O samples were analyzed by DLS, CD, and FTIR spectroscopy (Figure 6.2). DLS size analysis of C14 and C16 LPGO samples before SEC isolation (Figure 6.2a-b) revealed
a significant degree of polydispersity present, with ~10 nm diameter species as well as larger species ranging from 0.5-5 μm observed. Fractionation of C_{14} and C_{16} LPGOs via SEC eliminated the larger aggregates, indicating the oligomers to be ~10 nm in diameter (Figure 6.2e-f). The C_{18} LPGO sample prior to SEC isolation contained ~60 nm and ~2 μm sized aggregates (Figure 6.2c). Attempts to collect DLS spectra of the isolated C_{18} LPGO were unsuccessful due to poor yields, as well as poor resolution of fibril and oligomer peaks in SEC, as shown above. Unlike LPG-containing samples, GM1Os prior and after SEC isolation had similar DLS spectra containing a single peak at ~10 nm (Figure 6.2d and g). This further supports that fibril formation is minimal in the presence of GM1, and that the oligomers observed could be formed along an alternative, off-pathway. Overall, the data suggest that C_{14} and C_{16} LPGOs, as well as GM1Os, have a similar hydrodynamic diameter of 10 nm, which is consistent with the SEC and immunoblot data above indicating all oligomers have roughly similar sizes. This is also consistent with the size of LFAOs, which has been previously reported to be ~10 nm in diameter by Kumar et al.°°

To investigate the structural conformation of LPGOs and GM1Os, CD and FTIR were employed (Figure 6.2h-j). Prior to SEC fractionation, a similar CD spectra containing a single minima at 217 nm was observed for all samples (Figure 6.1h), indicating all aggregates to have β-sheet secondary structure in the presence of micelles. Similar spectra were obtained upon isolation (Figure 6.1i), with the exception of C_{18} LPGOs, which could not be collected due to poor yield of the oligomeric species. However, increased signal intensity was observed for GM1Os (Figure 6.2i), which may be due to association of the lipid in the SEC-fractionated oligomer. This possibility was
explored and is elaborated in detail below. Regardless, this reveals that LPG and GM1 micelles promote the formation of β-sheet rich oligomers.

Figure 6.2 Biophysical characterization of LPGOs and GM1Os

(a-d) DLS spectra of Aβ monomer (50–60 μM) in the presence of C14:0 LPG (a), C16:0 LPG (b), C18:0 LPG (c), and GM1 (d) after 48 h of incubation at 37 °C. (e-g) DLS spectra of isolated C14 (e) and C16 (f) LPGOs, as well as GM1Os (g). (h-i) CD spectra of micelle-derived oligomers prior to (h) and after (i) SEC isolation. (j) FTIR of micelle-derived oligomers after isolation (solid lines), as well as purified F-on (dashed) and LFAO (dotted) samples. A total of 64 accumulations were collected by scanning 4,000 – 650 cm⁻¹ at a resolution of 4 cm⁻¹. DLS and CD spectra were collected as described in Chapter VIII.

To ascertain the type of the β-sheets (parallel or anti-parallel), FTIR was employed (Figure 6.2j). As initially described in Chapter II, FTIR is able to differentiate parallel and anti-parallel β-sheets based on their respective amide stretching frequencies. FTIR spectra of F-on aggregates (dashed line) and LFAOs (dotted line)
were collected as controls (Figure 6.2j), which both gave the expected absorbance maxima at 1625 cm\(^{-1}\) indicative of parallel \(\beta\)-sheets. Similar results were obtained for C\(_{14}\) and C\(_{16}\) LPGOs, as well as GM1Os. In all cases, the presence of anti-parallel \(\beta\)-sheets at 1690 cm\(^{-1}\) was not observed, suggesting that both LPGOs and GM1Os form parallel \(\beta\)-sheets. While this is contradictory to recent reports that A\(\beta\) oligomers are anti-parallel in nature,\(^{115-117}\) it does bring into focus a unifying characteristic among surfactant-derived oligomers.

6.2.3 Fractionated GM1-derived oligomers (GM1Os) are not free of GM1, unlike LPGOs

The increased CD signal intensity of fractionated GM1Os, as well as the lack of fibril formation observed, may suggest association of GM1 with the oligomer. To investigate this possibility, electrospray ionization mass spectrometry (ESI-MS) in negative ion mode was utilized (Figure 6.3). Initially, controls of C14:0 LPG, C16:0 LPG, and GM1 in the absence of A\(\beta\) were collected (Figure 6.3a-c), which revealed the presence of the \([M - 1H]^{-}\) ion for both C14:0 and C16:0 LPGs at 455 and 483 m/z, respectively (Figure 6.3a-b). In the case of GM1, multiple signals were observed (Figure 6.3c) corresponding to the varying sphingosine (denoted ‘d’) and acyl moieties present. This primarily included the 1545 (\([d18:1, C18:0] - 1H]^{-}\)) and 1573 (\([d20:1, C18:0] - 1H]^{-}\)) m/z ions, which have been observed previously.\(^{135-137}\) Also observed were the 1612 and 1680 m/z ions, which were unassigned derivatives of GM1, consistent with the manufactures warning of multiple GM1 isoforms present in the product.
Figure 6.3 ESI-MS of LPGOs and GM1Os
(a-h) ESI-MS of 10 μM C14:0 LPG (a), C16:0 LPG (b), GM1 (c), Aβ monomer (d), LFAOs (e), C14 (f) and C16 (g) LPGOs, as well as GM1Os (h), respectively. Spectra were collected as described in Chapter VIII.

Control Aβ reactions of monomer or LFAO were also examined in negative ion mode, which yielded a 1503 m/z signal corresponding to the [M - 3H]3 Aβ ion (Figure 6.3d-e). This was also observed in the isolated C14 and C16 LPGO samples, respectively (Figure 6.3f-g). Moreover, the C14:0 LPG (455 m/z) and C16:0 LPG (483 m/z) ions were absent from the isolated C14 and C16 LPGOs, respectively. This suggests that the two species (lipid and Aβ oligomer) are separated upon fractionation in the Superdex-75 column. However, this was not observed with the isolated GM1O sample (Figure 6.3h), in which only ions corresponding to GM1 were present. This suggests that GM1Os are tightly bound to GM1 and elute as a co-complex, and the two do not separate during SEC.
fractionation. This is unlike LPGs, which are able to dissociate from the oligomer (LPGO) upon fractionation, similar to what has been reported by Kumar et al. for LFAOs. In the case of GM1Os, this may explain why fibril formation is not observed upon incubation of Aβ monomer with GM1, as well as be reason for the increased intensity of the CD signal in GM1Os.

6.2.4 Micelle-derived oligomers seed Aβ fibril formation

One important question to address is whether LPGOs and GM1Os are capable of acting as seeds for Aβ monomer elongation and fibril formation. To investigate this, reactions were initiated by seeding Aβ monomer (25 μM) with 3 μM of either C14 LPGO (▲), C16 LPGO (○), or GM1O (▼) seeds, respectively (Figure 6.4a). Aβ in the absence of seeds served as a control (■, Figure 6.4a). Reactions were buffered in 20 mM Tris (pH 8.0) with 50 mM NaCl and were maintained in quiescent conditions at 37 °C for 7 days. ThT measurements were taken periodically to monitor aggregate growth, which in the case of the control reaction (■, Figure 6.3a) revealed a typical sigmoidal growth curve with a lag time of ~24 h. Seeding Aβ monomer with micelle-derived oligomers resulted in a decreased lag time, as evidenced by the incremental increase in ThT fluorescence beginning at 18 h. While this does suggest that both LPGOs and GM1Os are capable of seeding Aβ monomer toward fibril deposition, it remains to be understood if they lead to distinct fibril polymorphs. Also, it is important to bear in mind that since GM1 is present in the fractionated GM1O (as shown above), it is therefore also present in the seeding reaction. Thus, the observed fibril formation in the GM1O seeding reaction seems contradictory to what was observed above upon incubation of GM1 micelles with Aβ.
monomer. However, it is important to remember that the concentration of GM1 in the SEC fractionated GM1O would be significantly decreased as compared to the GM1 concentration prior to SEC (75 μM), which is due to the dilution that occurs upon passage through the Superdex-75 column. Therefore, GM1 at low concentrations may promote fibril formation as oppose trapping Aβ monomers in an oligomeric state.

Figure 6.4 Micelle-derived oligomers seed Aβ fibril formation
(a) Aβ monomer (25 μM) alone (■) or seeded with 3 μM C14 LPGOs (▲), C16 LPGOs (○), or GM1Os (△). (b) The percent solubility after 7 days of incubation, as determined by ThT fluorescence and centrifugation (18000g for 20 min).

To shed insight into whether the seeded reactions lead to unique fibril polymorphs, the percentage of soluble Aβ at the end of the reactions were monitored by ThT fluorescence and centrifugation (Figure 6.4b). As shown in Chapter III for LFAO propagation, LFAO-seeded fibrils displayed an increased solubility as compared to F_on aggregates. The same analysis was therefore performed for LPGO- and GM1O-seeded fibrils, which in the case of the control reaction revealed only ~25% of soluble Aβ remaining at the end of the reaction. This was increased to ~40% in the case of LPGO-
and GM1O-seeded fibrils, which is consistent with what has been observed previously in Chapter III for LFAO-seeded fibrils. While this does not reveal a faithful reproduction of the oligomer assembly within the fibril structure, it does suggest the possibility of polymorphic fibril structures from LPGO- and GM1O-seeding reactions.

6.3 Anionic liposomes accelerate Aβ aggregation

The results presented above showcase that micelles of anionic LPG and GM1 are capable of inducing the formation of Aβ oligomers with unique biophysical signatures and seeding behavior. However, it remains to be understood if liposomal systems are similarly able to give rise to distinct Aβ oligomer conformers. Therefore, this was investigated using the anionic phospholipid, 1,2-dimyristoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DMPG), which contains two saturated acyl chains of 14 carbons (C14:0) each (Figure 6.5). Small unilamellar vesicles (SUVs) were prepared in 20 mM Tris (pH 8.0) buffer by extrusion through a 50 nm Whatman® nuclepore polycarbonate membrane, as described in Chapter VIII. Upon extrusion, the SUV size was confirmed by DLS, which revealed the successful formation of uniform liposomes having a hydrodynamic diameter of ~50 nm (Figure 6.5a).

To investigate the effect of DMPG SUVs on Aβ aggregation, Aβ monomer (25 μM) was incubated alone (C) or with increasing concentrations of SUVs from 12.5 (1:0.5), 25 (1:1), 50 (1:2), 250 (1:10) or 500 (1:20) μM, respectively. Samples were buffered in 20 mM Tris (pH 8.0) with 50 mM NaCl and 10 μM ThT and prepared in 96 well microplates. Reactions were kept at 37 °C inside of a plate reader and aggregate growth was monitored by ThT fluorescence every 15 min for 8 h (Figure 6.5b). The Aβ
control reaction in the absence of SUVs displayed typical sigmoidal growth kinetics with a lag time of ~45 min (Figure 6.5b). Incubation of Aβ monomer with a sub-stoichiometric concentration of SUVs (1:0.5, Figure 6.5b) did not result in an observable change in the lag time or the final ThT intensity in the saturation phase, which can be used as a rough indicator of fibril polymorphs as shown by Spirig et al. For samples incubated with a 1:1 and 1:2 ratio of Aβ:SUVs, a decrease in the lag time and increase in the ThT plateau intensity was observed, although the two reactions were indistinguishable from each other (Figure 6.5b). A concentration dependent decrease in the lag time was observed for the 1:10 and 1:20 reactions, however the ThT plateau intensity did not vary between the two reactions (Figure 6.5b). Overall, this reveals that stoichiometric and super-stoichiometric concentrations of DMPG SUVs increase the rate of Aβ aggregation. Moreover, based on ThT plateau intensities of the fibrils, low ratios (1:1 and 1:2) and high ratios (1:10 and 1:20) of Aβ:SUVs seem to form fibrils along varying pathways, potentially leading to varying fibril polymorphs. However, it remains to be investigated if DMPG SUVs promote the formation of Aβ oligomers, or simply accelerate the formation of Aβ fibrils. Therefore, further experiments should be done to bring clarity in this regard.
6.4 Conclusions

The results presented above reveal that anionic surfactants promote the formation of Aβ oligomers along distinct pathways, supporting the hypothesis that varying lipid environments are a governing factor in the generation of conformeric oligomer strains. Anionic micelles, composed of either LPG or GM1 lipids, promoted the formation of 30-60 kDa soluble Aβ oligomers, similar to what has been reported in the presence of SDS as well as NEFA micelles.102-104 Interestingly, increasing the chain length of LPG micelles from C16:0 to C18:0 promoted fibril formation, suggesting that both lipid composition (in this example, charge) and size (or surface area) may factor into how lipids modulate Aβ aggregation pathways. This is supported by the fact that anionic liposomal SUVs, which are much larger than LPG micelles, accelerated Aβ aggregation toward fibril formation, as evidenced by ThT fluorescence. No fibrils were observed upon incubation of Aβ monomer with GM1 micelles, suggesting that the unique surface
created by the oligosaccharide moiety traps Aβ in an oligomeric state. Das et al. have reported similar observations with synthetic GM1 glycopolymer mimics, which revealed the selective induction of Aβ oligomers by glycopolymers containing purely glucose pendant groups.\textsuperscript{138}

GM1Os were also unique in that they did not dissociate from the lipid upon fractionation, which is consistent with earlier reports of a tightly-bound Aβ:GM1 species isolated from postmortem AD brains.\textsuperscript{86,87} However, LPGOs could be isolated from the LPG micelle and remained as a stable oligomeric species, which is similar to what Kumar et al. have reported for fatty acid-derived LFAOs.\textsuperscript{106} Investigation into the conformation of the micellar oligomers by CD and FTIR revealed they are composed of parallel β-sheets, identical to what was reported in Chapter II for LFAOs. This commonality among oligomers derived from anionic micelles suggests they may be part of a larger family (or class) of oligomer strains, which is discussed in detail below (Chapter VII). While further work is needed to unambiguously define LPGOs and GM1Os as oligomer strains (as elaborated in Chapter VII), this work does reveal that distinct lipid environments can in fact modulate Aβ aggregation pathways towards the formation of conformationally-unique Aβ oligomers.
CHAPTER VII – CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Propagation of Aβ oligomer strains: LFAOs as model oligomers

Chapters II-IV showcase a mechanistic insight into the self-propagation of an Aβ oligomer strain (LFAOs), which seem to selectively induce CAA in transgenic mice brains. We now know that the mechanism of LFAO propagation involves three distinct phases of replication, generation of an intermediate, P, followed by propagation to mature Fs. At low concentrations, Os undergo replication, resulting in a 5-7 fold-increase of oligomers (Chapter II). While this may seem modest, in a physiological scenario, even small amplifications from a numerically large number of LFAO-like replicating oligomer seeds could result in widespread dissemination of such oligomers with significant consequences. The fact that LFAOs induce maximum toxicity at low concentrations (10 nM), where they primarily exist as Os (Chapter II), further provides credence to this hypothesis. Furthermore, the increase in the local concentration of oligomers results in the conformational conversion from Os to Ls in vitro (Kd = 0.1 µM, Chapter II). This dynamic also manifest in the kinetics of ME reactions, whereby a significant deviation from the linear rate constant occurs at low seeding concentrations (Chapter IV), suggesting that Os do not directly seed toward Ps. It is interesting that the conformational conversion from Os to Ls is the ‘switch’ which initiates propagation towards Fs. While L formation seems to be the rate-initiating factor for propagation, they do not directly seed Fs, but rather undergo elongation by As to form a key intermediate, Ps, which are 4-7 times larger than the initial dodecamer seed (Chapters III and IV). This type of mechanism seems to parallel recent reports of multiple nucleation events in amyloid aggregation, although it should be borne in mind the investigations presented here
are of seeded aggregation, where nucleation events are thought to be significantly suppressed or eliminated altogether. As evidenced in this work, this may not necessarily be the case and may vary based on the size and conformation (i.e. strain) of the propagating seed. In summary, this work presents a case wherein a self-propagating oligomer strain constitutes the foundation of a phenotypic variant in AD (CAA), cementing the idea that polymorphic Aβ oligomers govern phenotypic diversity in AD.

7.2 Future directions

7.2.1 Molecular details of the LFAO structure

High concentrations of LFAOs show parallel β-sheets by FTIR, which is in conflict with some recent reports that predict anti-parallel β-sheets for amyloid oligomers.\textsuperscript{115-117, 142} This suggests that LFAOs have a structure distinct from other reported oligomers, which could explain their ability to replicate. Furthermore, other micelle-derived oligomers (LPGOs and GM1Os) have similar parallel β-sheet amide stretching FTIR spectra, suggesting it may be a unifying characteristic of interfacially-derived oligomers. To probe this further, the structure of LFAOs should be investigated at the atomic level using techniques such as solid and solution state nuclear magnetic resonance (NMR) spectroscopy, isotope-edited FTIR spectroscopy, molecular dynamics (MD) simulations, among others. Kotler et al. have shown that high-resolution structural elucidation of Aβ40 oligomer conformations can be obtained using $^1$H-$^1$H dipolar couplings from NMR.\textsuperscript{143} Similar experiments, as well as solid-state NMR measurements, have been initiated in collaboration with Dr. Ramamoorthy at The University of Michigan, which will yield more details on the molecular organization of LFAOs. The
distance constraints derived from the NMR experiments, along with other size and conformational insights from the biophysical experiments presented here, will be considered in MD simulations conducted by Dr. Hansmann at The University of Oklahoma, which has been described previously for Aβ oligomers. Collectively, this will yield an atomic resolution structure of LFAOs. Using this platform, similar NMR experiments and MD simulations will be done using LPGOs and GM1Os, and the respective structures will be compared to that of other oligomers reported in the literature to identify structural characteristics unique to interfacially-derived oligomers. The structural details of the oligomers will also allow for the rational design and development of inhibitors (small molecules, peptides, etc.) of LFAO replication, which is the primary and most toxic phase of LFAO propagation in vitro.

7.2.2 Cross-propagation of heterogeneous Aβ isoforms leads to hybrid oligomers

A recent review by Condello and Stohr introduced the hypothesis that the observed structural heterogeneity and phenotypic diversity in AD arises from a complex interplay of Aβ isoforms within what they term the ‘Aβ interactome’. However, a mechanistic understanding of such a phenomenon, specifically oligomer-to-oligomer cross-propagation of Aβ isoforms, is lacking. Chapter V of this document describes that seeding LFAOs with heterogeneous Aβ monomers results in the generation of hybrid oligomer species containing multiple isoforms of Aβ. Furthermore, Arctic-Aβ42 oligomers are able to seed WT monomers, which reveals that cross-propagation is bidirectional and need not originate from a specific Aβ isoform.
While this unambiguously confirms cross-propagation of Aβ isoforms in vitro, the physiological consequences of such mechanisms remain unknown. This is currently being explored and will be disseminated at a later date, but includes in vitro cell toxicity assays to address i) the overall toxicity of hybrid aggregates vs. their homogeneous seeds and ii) the specific mechanisms/avenues of toxicity (activation of inflammatory pathways, apoptosis, necrosis, etc). Additionally, inoculation of hybrid aggregates into transgenic mice have been initiated to explore differences in the neuropathological localization/spreading. Overall, this will yield much needed insights and provide a platform for further delineation of the complex mechanisms within the Aβ interactome.

7.2.3 A new perspective in classifying oligomer strains

While the interplay of Aβ isoforms may contribute to strain generation through the formation of hybrid oligomers (as described in 7.2.2), we believe that other, heterotypic interactions also influence this process. This is showcased in Chapter VI, which reveals that anionic lipid micelles act as catalysts to generate soluble oligomers of Aβ in vitro. Biophysical characterization of these LPGOs and GM1Os has revealed similarities to LFAOs, such as their size (~10 nm hydrodynamic diameter) and secondary structure (parallel β-sheets). The latter suggests that the macroscopic structure of oligomers derived from anionic micelles are shared, and leads to question if they also share physiochemical properties. Seeding reactions seem to suggest they do, revealing LPGOs and GM1Os seed fibrils with nearly identical ThT kinetic profiles. Also, the fact that LPGO-, GM1O-, and even LFAO-derived fibrils have increased solubility as compared to $F_{on}$ aggregates further supports this notion. This leads to hypothesize that
while oligomers may have varying atomic structures (thus can be categorized as conformational strains), they may also have shared traits (such as those listed above) which allow them to be grouped into certain families or classes. However, this should be read with caution as the mechanism of LPGO and GM1O propagation has yet to be explored, the atomic structures of these oligomers are currently unknown, and the phenotypic manifestation(s) of LPGOs and GM1Os in vivo remain to be understood. Furthermore, the cell-to-cell transmission of LFAOs via neuronal trafficking mechanisms has yet to be investigated, which is a well-defined feature of PrP strains and is considered a canonical characteristic of a bona fide strain. Thus, this leads to ambiguity in classifying these oligomers as distinct strains, and these points should be investigated before making definitive conclusions. Regardless, it is clear from the data presented that other anionic micellar lipid systems are capable of catalyzing Aβ oligomer formation, yielding insight into how oligomer strains may be populated within the complex cerebral microenvironment of the AD brain.
8.1 Materials

Lyophilized stocks of synthetic Aβ42 WT, Aβ40 WT, and Arctic-Aβ42 peptides were procured from the Mayo Clinic (Rochester, MN), while FITC-Aβ40 WT and FITC-Arctic-Aβ42 were obtained from the Yale School of Medicine (New Haven, CT). Dr. Yona Levites at the University of Florida (Gainesville, FL) kindly provided the monoclonal Ab5 antibody that was used. SEC columns (Superdex-75 HR 10/30) were purchased from GE Life Sciences (Marlborough, MA). The equipment and materials for gel electrophoresis and immunoblotting, including SuperSignal™ West Pico Chemiluminescent Substrate, were purchased from either Thermo Fisher Scientific, Inc. (Waltham, MA) or Bio-Rad Laboratories, Inc. (Hercules, CA). LPG (C14:0, C16:0, and C18:0), DMPG, and GM1 lipids were procured from Avanti Polar Lipids, Inc. (Alabaster, AL); while C12:0 NEFA was procured from NuCheck Prep, Inc. (Elysian, MN). Purchased from Sigma-Aldrich Corp. (St. Louis, MO) were Tris base, Tris hydrochloride, DCVJ, BSA, SDS, Triton X-100, as well as all cell culture media. Other essential chemicals, reagents, and consumables were purchased from either VWR, Inc. (Radnor, PA) or Thermo Fisher Scientific, Inc. (Waltham, MA).

8.2 General methods

8.2.1 Purification of Aβ monomers

To purify synthetic Aβ monomer in all isoforms, 0.5-2 mg of lyophilized peptide was dissolved in 490 μL of nanopure H2O (npH2O) and allowed to stand at 25 °C for 0.5-1 h. Ten minutes before loading onto a Superdex-75 HR 10/30 SEC column attached
either to an AKTA FPLC system (GE Healthcare, Buckinghamshire) or a BioLogic DuoFlow™ system (BioRad), NaOH was added to a final concentration of 10 mM. Monomer was then purified by loading the sample onto a pre-equilibrated (20 mM Tris, pH 8.0) column and fractionating at a flow rate of 0.5 mL/min at 25 °C. Fractions of 500 μL were collected, and monomeric Aβ was found to elute in the ~23-27th fraction. A Cary 50 UV-Vis spectrometer (Agilent Technologies, Inc.; Santa Clara, CA) was used to determine the molar concentration of Aβ in each fraction, which for non-FITC labeled samples was done using Beer-Lambert’s law (ε = 1450 cm⁻¹ M⁻¹ at 276 nm). In the case of FITC labeled samples, protein concentrations were determined by the following equation:

\[
\frac{A_{276\text{nm}} - (A_{494\text{nm}} \times 0.3)}{1450}
\]

Where \(A_{276\text{nm}}\) represents the absorbance from the Tyr residue in Aβ, \(A_{494\text{nm}}\) represents the absorbance from FITC, 0.3 represents the correction factor (provided by Thermo Fisher Scientific ‘Tech Tip #31’) and 1450 represents the extinction (ε) coefficient of Aβ.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to confirm the purity and integrity of the peptide. The purified monomers were stored at 4 °C and used to initiate experiments within 48 h of purification.

8.2.2 Purification of Aβ oligomers

Purification of Aβ oligomers was initiated by incubating freshly purified Aβ monomer with the specified lipid species in the conditions listed in Table 8.1.
Table 8.1 Conditions for generating Aβ oligomers

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aβ Monomer (μM)</th>
<th>Lipid Species</th>
<th>Lipid (mM)</th>
<th>NaCl (mM)</th>
<th>NaN₃ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O/Ls</td>
<td>50-60</td>
<td>C12:0 NEFA</td>
<td>5</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Arctic-Aβ42 O</td>
<td>50-60</td>
<td>C12:0 NEFA</td>
<td>15</td>
<td>50</td>
<td>0.01</td>
</tr>
<tr>
<td>C₁₄ LPGO</td>
<td>50-60</td>
<td>C14:0 LPG</td>
<td>0.4</td>
<td>50</td>
<td>0.01</td>
</tr>
<tr>
<td>C₁₆ LPGO</td>
<td>50-60</td>
<td>C16:0 LPG</td>
<td>0.15</td>
<td>50</td>
<td>0.01</td>
</tr>
<tr>
<td>C₁₈ LPGO</td>
<td>50-60</td>
<td>C18:0 LPG</td>
<td>0.075</td>
<td>50</td>
<td>0.01</td>
</tr>
<tr>
<td>GM10</td>
<td>50-60</td>
<td>GM1</td>
<td>0.075</td>
<td>50</td>
<td>0.01</td>
</tr>
</tbody>
</table>

All samples were kept at 37 °C in quiescent conditions for 48 h. The samples were then purified via SEC, as described above, after first pelleting the insoluble peptide by centrifugation at 18000g for 20 min. Fractions of 500 μL were collected, and Aβ oligomers were found to be in the 18-20th fraction. The molar concentration was determined by UV-Vis spectroscopy, as described above. Samples were either stored at 4 °C and used for experimentation within 72 h, or lyophilized and kept at -80 °C for extended storage prior to experimentation.

8.2.3 Lyophilization and resuspension of Aβ

For lyophilization, aliquots of either 1 or 5 μg were made and flash-frozen by plunging into liquid N₂ for 30 s prior to freeze-drying overnight using a FreeZone® Triad™ freeze dry system (Labconco Corp.; Kansas City, MO) maintained at 0.420 mBar. Lyophilized samples were then stored at -80 °C until use. To resuspend the lyophilized samples for experimentation, either npH₂O or Tris buffer (pH 8.0) was added.
to reach the desired molar concentration of peptide, and the sample was allowed to stand at 4 °C for at least 1 h prior to centrifugation at 18000g for 20 min. The supernatant was then carefully removed and used for experiments.

8.2.4 Generation of Aβ fibrils

Aβ fibrils were generated by either inducing the nucleation of Aβ monomer (F\text{on}) or by seeding with O/Ls (Fs). Aβ monomer (100–150 μM) was added to 150 mM NaCl and allowed to incubate at 37 °C in quiescent conditions for 4–5 days for F\text{on} generation. After incubation, the sample was subjected to centrifugation at 18000g for 20 min before carefully discarding the supernatant and resuspending the pellet in fresh 20 mM Tris (pH 8.0) with 0.01% NaN\textsubscript{3}. ThT fluorescence and centrifugation were used to determine percentage conversion to Aβ fibrils, which was typically 80–90%. Fibrils were stored at 4 °C and used within 30 days. Generation of Fs was done by seeding Aβ monomer (50 μM) with 10 μM of O/Ls in 20 mM Tris (pH 8.0) with 0.01% NaN\textsubscript{3}. ThT was used to monitor the reactions daily for a total of 14 days of quiescent incubation at 25 °C. The samples were then subjected to an additional 14 days of quiescent incubation at 4 °C, upon which they were stored at 4 °C until sonication to generate fibril fragments called Ps.

8.2.5 Generation of Ps

To generate Ps, Fs stored in 1.5 mL siliconized tubes were sonicated (on ice) using a Misonix (Farmingdale, NY) XL-2000 series unit with a 2.4 mm diameter microprobe, kept at a depth of 17 mm inside of the tube. Samples were subjected to 7 cycles of sonication at 80% power, with each cycle consisting of 5 s of sonication.
followed by 20 s of rest. The sample was then immediately fractionated using SEC, as described above, and the peak near the void volume (fractions 15-18) were collected. The concentration of each fraction was determined by UV-Vis spectroscopy, as described earlier, and the samples were stored at 4 °C to be used within 72 h of purification.

8.2.6 Oligomer seeding reactions

For O/L replication reactions, Aβ monomer (50 μM) was seeded with varying concentrations of O/Ls in 20 mM Tris (pH 8.0) and allowed to react in quiescent conditions at 25 °C for 72 h. Qualitative analysis of replication was done by subjecting the samples to electrophoresis and immunoblotting. Quantitative analysis was done by subjecting the samples to SEC analysis and normalizing chromatograms for the seeded reactions against the corresponding chromatograms for the seeds alone, after first pelleting the insoluble material by centrifugation at 18000g for 20 min. Due to the variability of cross-propagation reaction conditions, they are not listed here. However, they are provided for each individual reaction in the figure legends, which can be found in Chapter V. For C_{14} and C_{16} LPGO, as well as GM1O seeding reactions, Aβ monomer (50 μM) was seeded with 3 μM oligomer seeds in 20 mM Tris (pH 8.0) with 50 mM NaCl and 0.01% NaN_{3}. The reactions were kept in quiescent conditions at 37 °C and ThT fluorescence was monitored for 7 days.

8.2.7 Denaturing and non-denaturing PAGE with immunoblotting

Samples were diluted into 1X Laemmli loading buffer either with (denaturing) or without (non-denaturing) 1% SDS, and loaded, without boiling, onto either 4-12%
NuPAGE or 4-20% Bis-Tris BioRad TGX gels. Pre-stained molecular-weight markers (Novex Sharp Protein Standard, Life Technologies) were run in parallel for MW determination in denaturing PAGE. For immunoblotting, proteins were transferred onto a 0.2 μm nitrocellulose membrane (BioRad). Following the transfer, the immunoblot was boiled for 1 min in a microwave oven in 1X PBS, followed by blocking for 1.5 h at 25 °C in 1X PBS containing 5% nonfat dry milk with 1% Tween 20. Blots were then probed overnight at 4 °C with a 1:6000 dilution of Ab5 monoclonal antibody, which detects amino acids 1–16 of Aβ. Following primary incubation, blots were probed with a 1:6000 dilution of anti-mouse, horseradish peroxidase-conjugated secondary antibody for 1.5 h at 25 °C before being imaged using a SuperSignal™ West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific).

8.2.8 DMPG liposome preparation

DMPG SUVs were prepared by first transferring an aliquot of DMPG (10 mg/mL) suspended in chloroform:methanol (1:1) into a glass vial, followed by evaporating it to dryness using vacuum evaporation. Next, the dry lipid ‘cakes’ were resuspended at a final concentration of 1.67 mg/mL in warm (37 °C) 20 mM Tris (pH 8.0) buffer. The sample was then hydrated for 1-2 h by agitating on an orbital shaker (250 rpm) kept inside of a 37 °C incubator. Periodically (every 5-10 min), the sample was removed and vortexed for 30 s to decrease the time required for hydration. Once hydrated, the sample was transferred into a siliconized microcentrifuge tube and subjected to 10 freeze/thaw cycles. Each cycle consisted of first plunging the sample into liquid N₂ for 30 s, followed by a boiling water bath for an additional 30 s. Following the
10th freeze/thaw cycle, the sample was extruded by passing it a total of 19 times through a 50 nm Whatman® Nuclepore™ track-etch polycarbonate membrane using an Avanti mini-extruder kept on top of a 45 °C heating block. SUVs were then analyzed by DLS to confirm their size, followed by determining the concentration using Beer-Lambert’s law from the absorbance at 210 nm. The ε coefficient was experimentally determined to be 69.1 cm⁻¹ M⁻¹ using a standard absorbance curve from known concentrations of LPG. SUVs were stored at 4 °C and used for experimentation within 48 h of extrusion.

8.3 Biophysical methods

8.3.1 ThT fluorescence kinetics

Excluding section 6.3 of this dissertation, ThT fluorescence measurements were collected by monitoring emission at 482 nm while exciting the sample at 452 nm using a Cary Eclipse fluorometer (Agilent Technologies, Inc.) in kinetics mode. For ThT measurements of extended reactions (>24 h), measurements were collected after a 1 min equilibration upon mixing 70 μL of a ThT stock (10 μM) with 5 μL of each respective sample. For experiments monitoring the initial reaction kinetics (Chapter IV), ThT (10 μM) emission was collected every 30 s for 1 h immediately upon mixing samples.

For section 6.3 of this dissertation, samples were prepared in non-treated, Nunc™ MicroWell™ microplates (Thermo Fisher Scientific) and ThT measurements were collected in a Synergy H1 microplate reader (BioTek Instruments Inc., Winooski, VT) operated by Gen5™ version 2.07 software. Top optics position measurements were collected every 15 min at 37 °C with excitation and emission wavelengths of 452 and 482 nm, respectively. The gain was kept constant throughout the measurements at 70 (unit-
less as defined in the software). Prior to each measurement collection, the microplate was orbitally shaken for 1 s at a frequency of 205 cpm.

8.3.2 ANS, DCVJ, and FITC fluorescence

Using a Cary Eclipse fluorometer (Agilent Technologies, Inc.) in scan mode, ANS, DCVJ, or FITC fluorescence were measured. For ANS, fluorescence was measured by exciting the sample at 388 nm while scanning emission between 400 and 650 nm upon the addition of Aβ (6 μM) and ANS (100 μM). DCVJ fluorescence was collected by exciting the sample at 433 nm while scanning emission between 400 and 650 nm upon the addition of Aβ (5 μM) and DCVJ (10 μM). In titration experiments, samples were diluted to the desired concentration by adding the dilution buffer (100 μM ANS in 20 mM Tris, pH 8.0) directly to the cuvette, followed by a 1 min equilibration before measuring fluorescence. The area under the curve was normalized against each respective Aβ concentration, and then plotted against the Aβ concentration to give the curves represented in Chapter II. The data obtained for LFAO ANS binding were fit to the following monomer–dimer model using Origin 8.5 software.

\[
F = \frac{2}{M} \star \left\{ F_0 - (F_0 - F_f) \times \left( \frac{(4M + K_d) \pm \sqrt{(4M + K_d)^2 - 16M^2}}{8} \right) \right\}
\]

Here, \(F\) is the fraction of dimer (24mer), \(M\) is the total Aβ concentration, \(F_0\) is the initial fluorescence value, \(F_f\) is the final fluorescence value, and \(K_d\) is the apparent dissociation constant. For FITC measurements, cross-propagated oligomer fractions were excited at 490 nm while scanning the emission spectrum from 510-600 nm. The slit widths were kept constant at 10 nm.
8.3.3 Fourier transform infrared (FTIR) spectroscopy

For measurements presented in Chapter II, FTIR was performed in grazing angle attenuated total reflectance mode (GATR) using a Nicolet 8700 (Thermo Fisher Scientific) instrument equipped with a VariGATR accessory (grazing angle 65, germanium crystal; Harrick Scientific). For measurements in Chapter VI, a Nicolet 6700 instrument equipped with a ATR accessory was used. In both cases, Aβ was lyophilized using either vacuum evaporation or methods described above prior to collecting spectra. Aβ samples were then resuspended in D₂O to a final concentration of 1 mM before accumulating spectra as described in the text. Spectra were processed by blank subtraction (using D₂O as the blank) and baseline correction using OriginLab 8.0 software.

8.3.4 Circular dichroism (CD) spectroscopy

A Jasco (Easton, MD) J-815 spectropolarimeter was used to collect an average of 6-16 spectral scans in the far-UV region (260-190 nm) at a rate of 50 nm/min (8 s response time, 1 nm bandwidth, 0.1 nm data pitch). Averaged spectra were smoothed using the Savitzky-Golay algorithm with a convolution width of 15 using the Jasco spectrum analysis program.

8.3.5 Dynamic light scattering (DLS)

DLS was collected by averaging 12 runs of 10 s each with a pre-equilibration time of 30 s using a Zetasizer Nano S instrument (Malvern, Inc., Worcestershire, UK). The diameter was determined using the volume (%) function.
8.3.6 Electrospray ionization mass spectrometry (ESI-MS)

All samples were evaporated to dryness using vacuum evaporation followed by resuspension at a concentration of 10 μM in a water:acetonitrile:formic acid (1:1:0.2) solvent. Samples were analyzed by direct infusion into a Finnigan™ LQX™ ion trap mass spectrometer (Thermo Fisher Scientific) operated in negative ion mode. Signals were accumulated by scanning the spectrum from 150-2,000 m/z for a total of 3 min (~800 total scans) at a constant flow rate of 100 μL/min. Other instrument parameters were kept as follows: spray voltage (5 kV), capillary temperature (275 °C), capillary voltage (-42 V), and automatic gain control (1.5 x 10⁴).

8.3.7 Atomic force microscopy (AFM)

AFM was conducted by Pradipta K. Das in Dr. Sarah Morgan’s lab at The University of Southern Mississippi. Briefly, the mica was cleaved using a razor blade and taped to a magnetic sample holder. The mica stub was then covered with 150 μL of 3-aminopropyltriethoxysilane solution (500 μL of 3-aminopropyltriethoxysilane in 50 mL of 1 mM acetic acid) for 20 min. The solution was decanted, and the mica was rinsed 3 times with 150 μL of deionized water. After rinsing, the mica stub was dried with compressed N₂ gas and stored in a desiccator for 1 h. Next, 150 μL of 0.9 μM Aβ sample in 20 mM Tris buffer (pH 8.0) was added to the mica and allowed to adsorb for 30 min. The sample was then decanted, and the mica stub was rinsed three times with 150 μL of deionized water. Finally, the mica stub was dried with compressed N₂ gas and stored in a desiccator until imaging. The surface topography of each sample was explored by
imaging the peptide via a Dimension Icon atomic force microscope (Bruker).

Measurements were taken under ambient environmental conditions at a constant scan rate of 0.5 Hz in tapping mode using a ScanAsyst Air silicon tip (Bruker) on a nitride lever (cantilever length, 115 mm; nominal force constant, 0.4 N/m; and resonance frequency, 70 kHz). The scan size ranged from 5 μm × 5 μm to 1 μm × 1 μm and the resolution was kept constant at 512 × 512 data points. AFM scanning was performed using Nanoscope 5.30r2 software and the images were analyzed using Nanoscope Analysis 1.50 image analysis software (Bruker). Multiple areas were imaged for each sample, and whereas height, phase, and amplitude data were collected simultaneously, amplitude images were used most often in the text to discuss morphology variation between samples.

8.4 Ensemble kinetic simulation (EKS) modeling

EKS modeling was conducted by Pratip Rana in Dr. Preetam Ghosh’s lab at Virginia Commonwealth University.

Replication phase:

\[ O_{12} + A \rightleftharpoons O_{13} \]
\[ k_{n u} \]
\[ k_{n u} \]

\[ O_{13} + A \rightleftharpoons O_{14} \]
\[ k_{n u} \]
\[ k_{n u} \]

\[ \ldots \ldots \]

\[ O_{23} + A \rightleftharpoons L_1 \]
\[ k_{n u} \]
\[ k_{n u} \]
Intermediate phase:

Elongation of $L$

\[
L_1 + A \underset{k_{la-}}{\overset{k_{la}}{\rightleftharpoons}} L_2
\]

\[
L_2 + A \underset{k_{la-}}{\overset{k_{la}}{\rightleftharpoons}} L_3
\]

\[
\ldots
\]

\[
L_{n-23} + A \underset{k_{la-}}{\overset{k_{la}}{\rightleftharpoons}} P
\]

Propagation Phase:

Oligomer elongation with $L$ and $A$

\[
P + L_i \underset{k_{fb1-}}{\overset{k_{fb1}}{\rightleftharpoons}} F; i = 1, 2, \ldots, (n - 1 - 24)
\]

\[
F + L_i \underset{k_{fb1-}}{\overset{k_{fb1}}{\rightleftharpoons}} F; i = 1, 2, \ldots, (n - 1 - 24)
\]

\[
P + A \underset{k_{fb2-}}{\overset{k_{fb2}}{\rightleftharpoons}} F
\]

\[
F + A \underset{k_{fb2-}}{\overset{k_{fb2}}{\rightleftharpoons}} F
\]

On Pathway:

Nucleation phase:
\[ A + A \rightleftharpoons A_2 \]

\[ A_9 + A \rightleftharpoons F_{on} \]

Elongation phase:

\[ F_{on} + A \rightleftharpoons F_{on} \]

The reaction flux of the above reactions can be written as follows (considering \( F_0 \) and \( F \) are the same species; \( O_{24} \) and \( L_i \) are the same species; \( L_{n-23} \) and \( P \) are the same species):

Elongation of \( O \)

\[ G_i = k_{nu} [O_{12+i-1}] [A] - k_{nu} [O_{i+12}] ; \forall i \in (1, \ldots , 12) \]

Elongation of \( L \)

\[ I_i = k_{la} [L_i] [A] - k_{la} [L_{i+1}] ; \forall i \in (1, \ldots , n-24) \]

Formation/elongation of \( F \)

\[ J_i^1 = k_{fb1} [L_i] [P] - k_{fb1} [F] ; \forall i \in (1, \ldots , n-24) \]

\[ J_i^2 = k_{fb1} [L_i] [F] - k_{fb1} [F] ; \forall i \in (1, \ldots , n-24) \]

\[ J_i^3 = k_{fb2} [A] [P] - k_{fb2} [F] \]

\[ J_i^4 = k_{fb2} [A] [F] - k_{fb2} [F] \]

On pathway

\[ G_i^{on} = k_{nuon} [A_i] [A] - k_{nuon} [A_{i+1}] ; \forall i \in (1, \ldots , 9) \]
\[ I_{i}^{on} = k_{fbon}[F][A_i] - k_{fbon}[F] ; \forall i \in (1, \ldots, 9) \]

Based on this, the ODE formulation of each oligomer will be:

\[
\frac{dO_{12}}{dt} = -G_1 
\]

\[
\frac{dO_{12+i}}{dt} = G_i - G_{i+1} ; \forall i \in (1, \ldots, 11) 
\]

\[
\frac{dL_1}{dt} = G_{12} - I_1 - J_1^1 - J_1^2 
\]

\[
\frac{dL_{i+i}}{dt} = I_i - I_{i+1} - J_i^1 - J_i^2 ; \forall i \in (1, \ldots, n-2) 
\]

\[
\frac{dP}{dt} = I_{n-1} - \text{sum}(J_i^1) - J^3 
\]

\[
\frac{dF}{dt} = \text{sum}(J_i^1) + J^3 
\]

\[
\frac{dA}{dt} = -\text{sum}(G) - \text{sum}(I) - J^3 - J^4 - \text{sum}(G_{i}^{on}) - G_{i-1}^{on} - I_{9}^{on} 
\]

\[
\frac{dA_{i}}{dt} = G_{i-1}^{on} - G_{i}^{on} ; \forall i \in (2, \ldots, 9) 
\]

\[
\frac{dF_{on}}{dt} = G_{9}^{on} 
\]
Table 8.2 A list of parameters used in the EKS model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Physical Meaning of the Parameter</th>
<th>Parameter</th>
<th>Physical Meaning of the Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>$O_i$</td>
<td>Oligomer made of $i$ number of monomers</td>
<td>$k_{b2}$</td>
<td>$F+A$ direct association forward rate constant</td>
</tr>
<tr>
<td>$L_i$</td>
<td>Oligomer with additional $i$ monomers over $O_{23}$</td>
<td>$k_{b2^{-}}$</td>
<td>$F+A$ direct association backward rate constant</td>
</tr>
<tr>
<td>$P$</td>
<td>Intermediate</td>
<td>$k_{nuon}$</td>
<td>On pathway nucleation forward rate constant</td>
</tr>
<tr>
<td>$F$</td>
<td>LFAO-seeded fibril</td>
<td>$k_{nuen^{-}}$</td>
<td>On pathway nucleation backward rate constant</td>
</tr>
<tr>
<td>$A$</td>
<td>Monomer</td>
<td>$k_{bon}$</td>
<td>On pathway elongation forward rate constant</td>
</tr>
<tr>
<td>$A_d$</td>
<td>On-pathway oligomer</td>
<td>$k_{bon^{-}}$</td>
<td>On pathway elongation backward rate constant</td>
</tr>
<tr>
<td>$F_{on}$</td>
<td>On-pathway fibril</td>
<td>$p$</td>
<td>Map simulation concentration to ThT intensity for fibril</td>
</tr>
<tr>
<td>$k_{uu}$</td>
<td>$O_i+A$ association forward rate constant</td>
<td>$signal$</td>
<td>ThT intensity of oligomer and fibril</td>
</tr>
<tr>
<td>$k_{ux}$</td>
<td>$O_{x}+A$ association backward rate constant</td>
<td>$signal'$</td>
<td>ThT intensity of oligomer</td>
</tr>
<tr>
<td>$k_{la}$</td>
<td>$L_i+A$ association forward rate constant</td>
<td>$signal''$</td>
<td>ThT intensity of fibril</td>
</tr>
<tr>
<td>$k_{lx}$</td>
<td>$L_x+A$ association backward rate constant</td>
<td>oligomer_fraction</td>
<td>Total oligomer fraction in the system</td>
</tr>
<tr>
<td>$k_{hi}$</td>
<td>$P+L_i$ association forward rate constant</td>
<td>monomer_fraction</td>
<td>Total monomer fraction in the system</td>
</tr>
<tr>
<td>$k_{bl}$</td>
<td>$P+L_i$ association backward rate constant</td>
<td>fibril_fraction</td>
<td>Total fibril fraction in the system</td>
</tr>
</tbody>
</table>

**Parameter estimation:** Rate constant estimation for the EKS model was done following established methods. The above-mentioned differential equations were solved using MATLAB, and $R^2$ as well as root mean squared error (RMSE) values between the simulated curve and experimental data were calculated for different rate constant combinations ranging from $10^{-5}$ to $10^9$ in multiples of 10. Next, the estimated rate constants were fine-tuned to obtain a better correspondence with the experimental
data (see Chapter IV for a list of final rate constants). The size of the intermediate \( P \)
was similarly varied from 36-120mers and corresponding \( R^2 \) and RMSE values were
calculated.

To achieve global fits for both the ThT data and monomer/oligomer/fibril
concentration data, a two-step method was used. Here, the minimum \( R^2 \) values and
maximum RMSE values for the fitted curves were considered, instead of an average,
which can erroneously give higher confidence in the results. To ensure both a global fit
with ThT data, and a good correspondence with quantitative data on the species during
propagation, cutoffs on the allowable minimum \( R^2 \) and maximum RMSE values were
defined. A lower RMSE cut-off ensures a better global fit with ThT and concentration
plots. Hence, an RMSE cut-off of 0.11 with the ThT curves for gateways 48-84 was used.
It is worth noting that the same cut-off did not produce allowable rate constant estimates
for gateways 36 and 96-120. In the latter cases, the RMSE cut-offs were increased to
generate the global fitting parameters reported in Chapter IV. Next, all rate constant
combinations that achieved the cutoffs for the ThT fits were considered. These rate
constant combinations were then used to fit with the concentration curves to identify the
‘goodness-of-fit’. Overall, this two-step model first finds allowable rate constants that
meet the cutoff requirements for fitting the ThT data and then identifies the best rate
constants from this range to fit with the quantitation plots.

Mapping concentration values from simulation to experimental plots: The contributions
of oligomers to the ThT intensity was considered to be proportional to their size, whereas
the fibril contribution to the ThT intensity was taken as \( p \). Thus, the ThT signal contribution from the final fibril (signal') could be written as:

\[
signal' = [F] * p
\]

where, \([F]\) is the concentration of final fibril.

Similarly, the ThT signal contribution from the intermediate fibrils (signal'') could be written as:

\[
signal'' = \sum_{i=2}^{n-1} ([L_i])
\]

Hence, the total simulated ThT signal contribution for both final and intermediate fibrils became:

\[
signal = signal' + signal''
\]

For the fits with concentration data, monomer and oligomer concentration fractions from the simulation data were defined as:

\[
monomer\_fraction = [A]/[protein\_total]
\]

\[
oligomer\_fraction = (i * \sum_{i=12}^{23} O_i + (23 + i) * \sum_{i=1}^{n-24} L_i + n * P + i * \sum_{i=2}^{9} A_i^{on})/[protein\_total]
\]

However, predicting the concentration of fibrils from simulation data was challenging as the fibrils are considered a single species \((F)\) without distinguishing their individual sizes; this was done to keep the models tractable. Hence, the concentration of \( F \) was calculated in an indirect way as follows:

\[
[F] = 1 - monomer\_fraction - oligomer\_fraction
\]
8.5 Cell culture methods

Cell culture experiments were conducted by Dr. Kayla M. Pate in the lab of Dr. Melissa A. Moss at The University of South Carolina. Human neuroblastoma SH-SY5Y cells (American Type Culture Collection, Manassas, VA) were maintained in a 1:1 mixture of Ham’s F12K medium and DMEM (F12K/DMEM) containing 10% FBS. Medium was supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. All cultures were maintained at 37 °C in a humid atmosphere of 5% CO₂ and 95% air.

8.5.1 Cellular apoptosis

To assess the ability of LFAOs to induce apoptosis, a TUNEL system (Promega, Madison, WI) was used. Prior to experimentation, SH-SY5Y cells were seeded at a density of $1 \times 10^6$ cells/well onto 22 × 22 mm glass coverslips and maintained for 24 h in F12K/DMEM media containing 10% FBS. For cell treatment, 10 μM Aβ was diluted in F12K/DMEM containing 1% FBS to final concentrations of 0.01, 0.1, and 1 μM and added to cells for 24 h. Cells treated with buffer equivalent or 25 μM H₂O₂ served as negative and positive controls, respectively. Following 24 h incubation (37 °C, 5% CO₂), treatment was removed, and cells were fixed with 4% paraformaldehyde in PBS (10 min, 25 °C). Cells were then permeabilized with 0.1% Triton X-100, 0.01 M glycine in PBS (10 min, 25 °C) and washed twice with PBS (10 min, 25 °C). Cells were then placed in 100 μL of equilibration buffer (10 min, 25 °C) before incubation in 50 μL of rTdT incubation buffer (1 h, 37 °C), which labels the DNA strand breaks with fluorescein-12-dUTP. After incubation, labeling was halted by adding 17 mM saline-sodium citrate (pH 7.0) containing 150 mM NaCl (15 min, 25 °C), and cells were washed three times with...
PBS (5 min, 25 °C). Labeled cells were mounted onto glass slides using Fluoroshield containing DAPI for nuclear staining. Cells were imaged under a Nikon Eclipse 80i fluorescent microscope using a 40X objective. For each slide, three or four different fields were captured for analysis. For each field, both a DAPI image and TUNEL image were acquired. Custom MATLAB (MathWorks, Natick, MA) functions were developed to determine the fraction of apoptotic cells present for each sample. From DAPI images, the total number of cells was assessed using MATLAB to identify pixel intensity regions. The boundary of these regions was then increased in a stepwise manner until the pixel intensity of the boundary fell below the threshold, indicating that the boundary of the nuclei had been reached. Once all nuclei boundaries were determined, the total number of nuclei present was recorded by MATLAB. Using the nuclei boundaries determined from the DAPI images, the average TUNEL pixel intensity within each nucleus was determined from the corresponding TUNEL image. Cells exhibiting an average TUNEL pixel intensity above 20 were deemed apoptotic. The fraction of apoptotic cells was defined as the number of apoptotic cells divided by the total number of cells. For each treatment 300-600 cells were analyzed.

8.5.2 Caspase activation

An Image-iT LIVE Green Poly Caspases detection kit (Life Technologies, Carlsbad, CA) was used to determine if caspase activation was the driving force behind LFAO-induced cellular apoptosis. SH-SY5Y cells were prepared and treated for 24 h with 0.01, 0.1, and 1 μM LFAOs, as described above. Cells treated with buffer equivalent or 2 U/μL TNF-α served as negative and positive controls, respectively. Following 24 h
incubation (37 °C, 5% CO₂), treatment was removed, and cells were stained using a FLICA reagent for detection of a wide range of active caspase enzymes (caspase-1, -3, -4, -5, -6, -7, -8, and -9) and using Hoechst 33342 for labeling of nuclei. Cells were rinsed with phenol red free F12K/DMEM containing 1% FBS (PRF F12K/DMEM, 1% FBS) and incubated (37 °C, 5% CO₂) for 1 h with 1X FLICA stain diluted in PRF F12K/DMEM, 1% FBS. After incubation, the stain was removed, and cells were washed twice with PRF F12K/DMEM, 1% FBS before 10 min incubation (37 °C, 5% CO₂) with Hoechst 33342 stain (diluted 1:1000 in PRF F12K/DMEM, 1% FBS). The cells were then washed twice and mounted to slides using a fixative solution. Slides were imaged 2 h after staining to avoid signal loss using a Nikon Eclipse 80i fluorescent microscope with a 40X objective. For each slide, three to five different fields were captured for analysis. For each field, both a Hoechst image and a FLICA image were acquired. Custom MATLAB functions were developed to determine the fraction of caspase active cells. From Hoechst images, the total number of cells was assessed as described above for DAPI images. From corresponding FLICA images and using the nuclei boundaries determined from the Hoechst images as a reference, MATLAB was used to determine the average FLICA pixel intensity within a region that included both the nucleus and a specified region outside the nucleus, which accounts for caspase staining that can reside within both the nucleus and cytoplasm. Cells exhibiting an average FLICA pixel intensity above 5 were deemed caspase active. For each treatment, 200-600 cells were analyzed.
8.6 Transgenic mice methods

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida, and experiments were conducted by Franklin Burg in Dr. Yona Levites’ lab. All methods and procedures were performed in accordance to the guidelines and regulations. APP transgenic TgCRND8\textsuperscript{125} were bred in-house. Neonatal ICV injections with 1–4 μL of Aβ monomers, \( F_{on} \) aggregates, or \( O/Ls \) (10 μM) were performed similarly to described before.\textsuperscript{151}

8.6.1 Aβ ELISA

Mouse brains were sagittally dissected and left hemisphere was used for protein extraction using a sequential extraction protocol of RIPA buffer, 2% SDS and 70% formic acid as described previously.\textsuperscript{152} All ELISA results were analyzed using SoftMax Pro software (Molecular Device).

8.6.2 Immunohistochemical imaging and image processing

Following tissue harvesting, the right hemisphere was formalin fixed, paraffin embedded and used for immunohistochemistry. Immunohistochemical staining was done using anti-pan-Aβ mAb5Biotin antibody (1:1500)\textsuperscript{152}, (biotinilated antibody was chosen in order to eliminate mouse IgG background staining interference). Immunohistochemically stained sections were captured using the Scanscope XT image scanner (Aperio) and analyzed using ImageScope program. Aβ plaque burden was calculated using the Positive Pixel Count program (Aperio). At least three sections per sample, 30 μm apart, were averaged by a blinded observer to calculate plaque burden. For evaluation of CAA,
sections stained with anti-pan-Aβ mAb5Biotin antibody were evaluated by a blind 
observer and blood vessels scored for presence of positive staining. 0 – no staining, 1–
25% stained, 2–50% of the vessel has amyloid, 3 – entire vessel is stained. Vessels that 
received a score of 2 or 3 were counted in three sections per sample.
REFERENCES

with neurofibrillary tangles and cerebral hemorrhage in an aged wolverine (Gulo gulo), *Neurobiology of aging* 17, 243-247.


