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Origins of Amyloid Oligomers and Novel Approaches for their Detection

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Origins of Amyloid Oligomers and Novel Approaches for their Detection

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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List of Abbreviations

AD     Alzheimer’s disease
HEWL   Hen Egg White Lysozyme
CV     Crystal Violet
ThT    Thioflavin T
gOs    Small Globular Oligomers
CFs    Curvilinear Fibrils
RFs    Rigid Fibrils
Tos    Toxic Oligomers
TEM    Transmission Electron Microscopy
AFM    Atomic Force Microscopy
DLS    Dynamic Light Scattering
FTIR   Fourier Transform Infrared Spectroscopy
FPLC   Fast protein Liquid Chrom
Abstract

Alzheimer’s disease, type II diabetes, and other amyloid diseases are known to be associated with the formation of amyloid aggregates. It has been thoroughly researched whether amyloid fibrils or oligomers are the main culprit for these diseases, and recent evidence has connected oligomers as the most disease relevant aggregate species. However, many difficulties have arose in confirming this hypothesis. Techniques for oligomer detection are often limited in their sensitivity, and in many cases are unable to distinguish oligomers from rigid fibrils. Additionally, the role oligomer splay in fibril assembly is still unclear, and has led to the belief that different types of oligomers may exist with potentially differing biological relevance.

The first part of my research aimed to develop an assay for effectively screening fluorescent dyes for selectivity toward a specific amyloid aggregate species. Lysozyme was shown to undergo amyloid formation along two pathways, with a clear difference in their kinetics. Fibril growth had a clear lag period followed by sigmodial growth, while early oligomer growth instead transitioned the kinetics to biphasic. This transition was utilized to screen various dyes for oligomer sensitivity, and found a promising candidate for an oligomer selective dye in crystal violet. Additionally, concurrent kinetics of both Thioflavin-T and crystal violet allowed me to separate the biphasic kinetics into its oligomer and fibril components.

The second part of my researched shows the formation of two distinct types of oligomers formed by Lysozyme. These two types of oligomers formed at different time points
during amyloid fibril growth and showed a clear difference in their level of cytotoxicity. This difference highlights a main issue in determining the disease relevance of oligomers, as specific environmental conditions can lead to the formation of oligomers with differing levels of biological activity. Both oligomer types were characterized through a variety of techniques and were shown to share many similarities but were found to differ in secondary structure, and in their response to the oligomer selective antibody A11.
Chapter 1 - Introduction

Amyloid diseases come about from the propensity of certain proteins to assemble into long fibrils with a characteristic cross-β structure called amyloid fibrils (1-4). The most infamous amyloid diseases are Alzheimer’s and Parkinson’s Disease, but amyloid diseases are not limited to only being neurodegenerative in nature. They can be localized around other organs in the body or can even affect groups of organs, such as AL and Lysozyme amyloidosis. (3) These diseases represent a growing healthcare concern, with Alzheimer’s predicted to affect more than 150 million people by 2050. As of now essentially no amyloid diseases have a cure and in many cases only limited treatment methods.

Amyloid fibrils were first described by Virchow in 1854 using iodine staining. Followed by identification of their prominent birefringence in optical microscopy (6, 7). Their underlying characteristic cross-β sheet structure was obtained 40 years later using X-ray diffraction (8). Understanding the mechanisms of formation and the pathogenic action of amyloid aggregates is critical in order to suppress or cure amyloid diseases eventually. This objective, however, faces multiple obstacles. Many different proteins and peptides have the propensity to form amyloids, such as amyloid-beta 1-42, β2 microglobulin, and hen egg white lysozyme, with no discernable structural or functional similarities between them to explain it. There is even
growing evidence that all peptide chains may have some propensity to form amyloid fibrils, with some amyloids having functions beneficial to the human body (3-5). In addition, amyloid aggregates have been shown to assemble along multiple pathways resulting in aggregate populations with distinct morphologies, tectorial properties, stabilities, structures and toxicities (11-17). Namely, besides their characteristic late stage rigid fibril structures amyloid proteins will form early stage globular oligomers and intermediates. There has been progress made in the understanding of the growth kinetics for rigid fibrils. In particular, the role of secondary nucleation mechanisms dominating the early stages of fibril formation is now widely accepted (5). Much less is known about the origin of globular oligomers, which are metastable precursors of fibrils and have been implicated as perhaps the most toxic and disease-relevant amyloid aggregate species (1-5). Unraveling the role oligomer intermediates play in both fibril assembly and pathophysiology, would likely provide valuable insight into the origins of, and potential cures for amyloid diseases. However, generating and characterizing pure oligomer samples, in particular, is challenging since they are inherently metastable, their size distributions can vary widely, they can polymerize into so-called curvilinear fibrils, and there are indications that their underlying structures are sensitive to the specific growth conditions chosen to generate them.

The first part of my thesis work is focused on work towards identifying novel fluorescent indicator dyes for in situ detection of oligomers vs. fibrils. Towards that end I will exploit a reliable method developed in our lab for growing amyloid aggregates along two distinct assembly pathways. The first is the commonly observed sigmoidal fibril growth pathway with its distinct lag period, absent of any discernible aggregate growth, followed by rapid nucleation and growth of rigid fibrils. The second pathway is characterized by lag-free biphasic kinetics in
which global oligomers form immediately, followed by a second upswing upon fibril nucleation and growth. Using this convenient difference in aggregation kinetics, I suggest an assay to screen small fluorophores for potential selectivity towards either oligomers or fibrils. Some dyes, namely Thioflavin-T have already proven to be excellent indicators of fibril growth. This assay will allow us to identify candidates for dyes that can selectively monitor oligomer formation \textit{in vitro} and \textit{in vivo}.

In the second part of my thesis work I will study the formation and origin of oligomers. Oligomers are commonly believed to be metastable precursors of rigid fibrils that assembly from the monomers of a given amyloid protein. However, my lab has some recent findings that suggest that an additional species of oligomers may emerge during late stage fibril formation. If this conclusion is true, it could be an important step forward in understanding why fibrils have sometimes been found to be toxic to cells, despite the growing evidence that oligomers are the most problematic species in amyloid diseases. In this thesis I describe several experiments providing evidence that these fibril-associated aggregates are indeed oligomers, as well as some preliminary findings that fibril-associated oligomers can form from other amyloid proteins.
Chapter 2 - Screening Assay for Oligomer Selective Dyes

2.1 Background

Amyloid diseases are generally identified by deposits of insoluble plaques of amyloid fibrils in tissue (11, 12). These deposits have been shown to be birefringence, indicating they must have an organized structure. In addition, these plaques display Congo Red and Thioflavin T fluorescence (52). Initially the presence of these plaques in post-mortem histology were considered diagnostic of the underlying illness, and were widely believed to be the main underlining cause of amyloid diseases, as well.

2.1.1 From Amyloid Cascade Hypothesis to Toxic Oligomer Hypothesis

The amyloid cascade hypothesis was established as a possible explanation for the origin of the disease (53). It was believed that a variety of factors, such as genetic mutations and environmental effects, would increase ones propensity to have the toxic form of the amyloid beta peptide. Amyloid beta, in turn, would form amyloid plaques, which leads to tea tangles and neuronal loss, and results in the dementia associated with Alzheimer’s (53). This cascade hypothesis had a number of issues, mainly that the correlation of concentration of amyloid plaques in post-mortem brain pathology to severity of the disease is poor. Specifically, significant plaque loads can be found in persons without disease symptoms and vice versa (62).
Over the years the amyloid cascade hypothesis has been modified to the toxic oligomer hypothesis. This modification was due to at least three observations. First, as mentioned above, plaque densities did not correlate well with disease symptoms. Second, the presence of small and long-lived oligomeric precursors to fibril formation were detected that were perhaps even more toxic than fibrils (60, 61). In addition, oligomer populations, as detected with oligomer-selective antibodies, correlated better with pre-mortem dementia (1-5, 45-46). Early research led many to believe oligomers were pre-cursors to Fibril formation, but today oligomers are more widely accepted to form early and before plaque formation (33-35).

2.2 Introduction

As the field shifts toward studying oligomers as the source of neurodegenerative amyloid diseases, a whole new set of questions and complications have emerged. Compared to their much larger counterparts of amyloid fibrils, oligomers have proven to be significantly more elusive. Some techniques do exist to detect amyloid oligomers namely, atomic force microscopy (14), and immunostaining with conformation-dependent anti-oligomer antibodies (15,16). However, these techniques have low temporal resolution making them poor candidates for studying oligomer growth kinetics. A common assay for detecting amyloid fibrils and for monitoring their growth kinetics is to use Thioflavin-T (ThT). This dye dramatically increases its fluorescence upon binding to amyloid fibrils (6-8). While Thioflavin-T has some oligomer sensitivity, its fluorescence is typically dominated by its fibril response. Therefore, Thioflavin-T can only provide limited insights into oligomer formation in vitro, and the role they might play during fibril formation (9). Ideally one would like to identify dyes that have a strong selectivity for oligomers over fibrils to act as a companion to Thioflavin-T. With ThT’s selectivity
towards fibrils, a dye with selectivity towards oligomers would allow to identify which aggregate species form when during amyloid formation.

While monitoring oligomer growth kinetics is an important aspect of amyloid research, oligomer detection would be a critical step towards diagnosing and monitoring amyloid diseases. Early diagnoses of neurodegenerative amyloid diseases are particularly elusive as symptoms can be indistinct and often lead to misdiagnoses. If oligomers are indeed the main culprit for these diseases, finding a technique to detect them \textit{in vivo} would alleviate the challenges of diagnosing. Many of the current methods to detect oligomers, such as anti-oligomer antibodies, are unsuitable for \textit{in vivo} screening of oligomers. A potential oligomer selective fluorescent dye however, could be modified to work with already existing \textit{in vivo} detection methods such as PET-MRI.

\textbf{2.3 The basis for a dye screening assay: “Critical Oligomer Concentration”}

I proposed an efficient \textit{in vitro} assay for screening dyes for potential selectivity towards globular oligomers. As amyloid protein for this screening assay I used the readily available protein hen egg-white lysozyme (HEWL). HEWL is made of 129 amino acids and has a relatively small molecular weight of 14.3 kDa \cite{21,22}. HEWL has been used widely as a model amyloid, mainly for its structural similarities with human lysozyme, and for its readily availability and affordability. Both Human and chicken lysozyme are well known to form amyloid aggregates under acidic solution conditions. What makes HEWL ideal for our assay is that our lab has shown that HEWL amyloid assembly, upon crossing a well-defined “critical oligomer concentration” (COC), switches from rigid fibril formation in the absence of oligomer formation.
in the lag phase to one generating a rapidly increasing population of metastable globular 
oligomers (17, 18). Crossing this COC also correlates with a sharp transition in ThT kinetics from
the ubiquitous “sigmoidal” growth kinetics of nucleated polymerization to a lag-free bimodal 
kinetics. The initial phase of this bimodal growth is dominated by gO/CF formation while the
secondary upswing indicates RF nucleation and growth. Below the COC, HEWL grows RFS 
aggregates with a distinct lag phase before the start of growth. Growing fibrils in the absence
(sigmoidal) or presence (biphasic) of significant populations of oligomeric species by simple
changes to either protein or salt concentration forms the basis of our oligomer dye screening 
assay. My former lab member Tatiana Miti had mapped out the protein and salt concentration
space to clearly identify the transition between these two types of amyloid assembly regimes.
By monitoring dye kinetics to HEWL amyloid growth below and above the COC allows rapid
determination whether a given fluorescent dye responds only to the initial gO/CF or
subsequent RF phase of amyloid growth.

2.4 Sigmoidal vs Biphasic HEWL growth kinetics

Using the above mentioned phase space, we picked out solution conditions that either
fell well below or well above the COC. Typically, four solution conditions would be prepared
with HEWL concentration varying across the COC and with NaCl concentration fixed at 400 mM
NaCl. 15 µM ThT was added to each sample. Solutions were placed in a 96-well plate to be
incubated at 52 °C, i.e. above the denaturation temperature for HEWL at pH 2 as partial
unfolding is necessary for amyloid aggregate formation.
Fluorescence was monitored in the plate reader for several days in order to record growth kinetics. At higher protein concentrations, above the COC (Figure 2.1C), we see an immediate onset of growth corresponding to formation of globular oligomers and curvilinear fibril (gO/CFs). Eventually ThT kinetics reaches an initial plateau which is followed by a second upswing indicating nucleation and growth of rigid fibril (RFs). This represents the aforementioned biphasic growth kinetics. At lower protein concentrations, i.e. below the COC (Figure 2.1a), we see a distinct lag phase. During this lag phase no metastable globular oligomers are detectable, giving us the previously mentioned sigmoidal shape growth kinetics. The morphologies of the associated aggregate species were confirmed with AFM imaging at a
few relevant time points during amyloid growth, mostly taken at 24 hour intervals. The gO/CFs observed during biphasic growth have the morphologies consistent with those of toxic oligomers by other groups (19). It is this sharp change from sigmoidal to biphasic kinetics, combined with the homogenous population of fibrils vs oligomers during sigmoidal vs. early-stage biphasic growth that serves as the basis for my dye screening assay.

2.5 Secondary structure of HEWL aggregate

Fourier transform infrared spectroscopy (FTIR) is a common technique to determine the secondary structure of proteins. It has been shown that certain absorption wavenumber peaks correspond to specific secondary structures in proteins. The limited set of secondary protein structures gives rise to regions with strong absorption commonly referred to as bands. In particular, the Amide I band (1600- 1700 cm$^{-1}$) has extensively studied in the amyloid field and has proven to be a good indicator of proteins' secondary structure. This band arises mostly from molecular vibrations of the double bond between a carbon and oxygen atom. While the exact locations of these peaks vary marginally between proteins, the Amide I band contains several peaks that correspond to anti-parallel or parallel β-sheets, β-turns, α-helices and disordered regions. It has already been shown that amyloid fibril and oligomers will display specific changes to their secondary structure from the monomeric form of the protein (55). Generally, a rather large uptick in beta sheets is observed as well as a smaller increase in anti-parallel beta sheets. This expected change in secondary structure is a strong indication that a protein aggregate formed is an amyloid.
To confirm that the above gO/CFs match the characteristics of toxic oligomers observed in amyloidoses, determined their FTIR spectra after isolating them from their monomeric background. Towards that end, RFs or gO/CFs samples were centrifuged at 15,000 g’s overnight and resuspended the pellet in their respective growth buffers. This process was repeated at least 3 times in order to sufficiently purify the aggregated protein from monomers. The underlying secondary structure of these isolated aggregate samples were then checked with FTIR. The Amide 1 bands visible in figure 2.2 show that both RFs and gO/CFs have a noticeable peak in the 1630 cm$^{-1}$ to 1610 cm$^{-1}$ region. This is the aforementioned expected diagnostic for amyloid beta sheet formation (20). While both RFs and gO/CFs have a peak in the characteristic amyloid band between 1620-1630 cm$^{-1}$, they are notably distinct from each other. This is particularly noticeable in Figure 2.2 B, where the FTIR spectrum of monomers was subtracted

Figure 2.2: FTIR spectra of HEWL monomers, CFs and RFs

(A) Peak-matched FTIR absorption spectra of the Amide-I band for HEWL monomers and isolated CFs and RFs at pH 2. CFs and RFs show a prominent peak in the characteristic amyloid band between 1630 and 1610 cm$^{-1}$. Yet their peak wavenumbers are slightly but reproducibly shifted with respect to each other. (B) CF and RF differences spectra obtain after subtraction of monomer spectrum in (A). Figure from Barton et al. Biomolecules Sep. 2019
from both of our aggregate FTIR spectrums. The resulting difference spectra nicely emphasize the prominent increases in amyloid beta sheet content in both fibril and oligomer aggregate species. But the spectra also show a small, but reproducible difference in the exact location of the beta sheet absorption peak between CFs and RFs: CFs have their amyloid beta sheet peak at 1618 cm\(^{-1}\) and RFs at 1624 cm\(^{-1}\).

### 2.6 Screening of dyes for amyloid aggregate response

The method of growing distinct, homogenous aggregate populations under very similar growth conditions allowed me to screen dyes for potential selectivity for oligomers over fibrils. Protein solutions were prepared with a fixed salt concentration and with protein concentrations varying across the Critical-oligomer-concentration boundary (COC), where this sharp transition from sigmodial to biphasic kinetics occurs.

As shown previously, below the COC only rigid fibrils grow, allowing us to measure a new dye's response to rigid fibril formation. Above the COC, gO/CFs initially grow to a plateau, providing us a region to observe a dye's response to oligomer and curvilinear fibril formation. Each of these protein solutions was divided into multiple test tubes and a fluorescent dye was added in preparation for fluorescent growth kinetic measurements in a plate-reader. Identical solutions, but with 15 µM ThT added, were used as a reference to confirmed the sigmodial kinetics below and biphasic kinetics above the COC. Typically, three other dyes in addition to ThT could be accommodated on a 96-well plate for screening under the same growth conditions. Typical concentrations for novel dyes were 5-10 \(\mu\)M. Growth solution were incubated at 52 °C in a 96-well plate for several days. Figure 2.3 shows an example of a plate.
where I measured kinetics responses of (A) thioflavin T, (B) the amyloid dye X-34, (C) acridine orange and (D) acid fuchsin.

2.7 A possible oligomer specific dye

Through our screening of dyes, we eventually identified the dye Crystal Violet as a promising candidate for an oligomer selective dye. Crystal violet has a fluorescence excitation and emission peak at 590 nm and 620 nm, respectively. We ran this dye through the same screening assay and plotted the fractional change in fluorescence \((F/F_0)\) in order to better compare its affinity for gO/CFs vs. RFs to that of ThT (Fig. 5A).
During the initial phase of biphasic growth, CV has a response to gO/CFs comparable to ThT. However, CV remained unchanged, or declined slightly, upon the onset of rigid fibril formation. Below the COC CV had a much weaker response to rigid fibril growth than ThT, likely explaining its lack of any noticeable increase to the onset of rigid fibrils when above the COC.

We also correlated the CV fluorescent signal to ThT’s under both biphasic and sigmodal growth conditions (Fig. 2.4 C). The linear correlation during sigmodal growth and the first phase of the biphasic kinetics indicate that the amyloid assembly kinetics reported by either dye are identical, albeit with different sensitivities. The kink in this correlation upon the onset of fibril formation is...
formation during biphasic growth, in turn, highlights the dramatic difference in the sensitivity to RFs by ThT vs CV.

One potential explanation for CV’s lack of biphasic kinetics above the COC could have been that CV actively inhibits rigid fibril formation. I checked for this by measuring CV and ThT in the same wells, while also using a series of different CV concentrations (Fig. 2.4 B). As shown, for concentrations comparable to our previous experiments, there is no discernable difference in ThT kinetics. At the larger CV concentrations ThT has a slightly reduced signal, however the

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**Figure 2.5: Evolution of Amyloid Aggregate Morphology during Biphasic Kinetics**

(A) Staggered incubation of 350 $\mu$M HEWL undergoing biphasic amyloid growth at pH 2, 52 C and 450 mM NaCl, recorded simultaneously with 15 $\mu$M ThT (●) and 5 $\mu$M CV (■). Fresh solutions were added to the 96 well plates at the moments indicated by the arrows. Corresponding total incubation periods are shown next to each arrow. (B) AFM images of aliquots imaged from wells incubated for the indicated time periods. While the initial phase of biphasic growth indicates the presence of gOs and increasing numbers of CFs, the late phase shows the simultaneous presence of RFs and CFs, often in direct contact with each other. The false color scale indicates aggregate heights. All images are 3 µm on a side. Figure from Barton et al. Biomolecules Sep. 2019
time scale of growth is unchanged. This decrease in ThT fluorescence is then likely explained by some level of competitive binding to fibrils between CV and ThT. In our biphasic kinetics, once rigid fibril formation begins, CV has mostly plateaued, and with its strong affinity to gO/CFs this likely means that gO/CFs persist during the nucleation and growth of RFs. To confirm this, we used AFM imaging on aliquots sampled from a 96-well plate incubated under growth conditions. Identical solutions were added every 24 hrs. For several days, and only a couple hours on the last day prior to stopping the experiment. This staggered incubation protocol allowed us to image samples at different stages of the reaction but at the same time point, with AFM (Figure 2.5). These images show the aggregate species expected for each stage of our biphasic kinetics. In addition they show that residual gO/CFs persist well into the second plateau phase. In related experiments we had previously observed that gO/CFs persist for weeks into the apparent plateau phase of fibril formation. This suggests that the second ThT "plateau" during biphasic growth is not an indication of the "reaction endpoint" of fibril formation. Instead, it indicates the depletion of free monomers by RFs and the subsequent very slow process of gO/CF depolymerization and subsequent incorporation of the released monomers into RFs.

2.8 Separating biphasic kinetics into its gO/CF and RF components

As our AFM images show, the two distinct aggregate species have some period of overlap during biphasic kinetics. In addition, we have shown that gO/CFs act as inhibitors of rigid fibril nucleation (22). The identification of the predominately gO/CF sensitive dye CV vs. the predominately RF-sensitive dye ThT opens up the possibility of decomposing amyloid growth kinetics into its underlying RF and gO/CFs components during biphasic region.. To do so
I used protein concentrations spanning across the COC to get biphasic and sigmoidal kinetics. In Figure 2.6C, when accounting for the difference in fluorescence enhancement of CV vs. ThT upon binding to RFs, it is clearly shown that CV and ThT have identical growth kinetics in the sigmoidal RF regime, but with of course significantly differences in sensitivity. Similarly, the difference in fluorescent intensity was accounted for in the biphasic regime and shows CV and ThT changes correlate perfectly until a sudden divergence. Fig. 2.6 D shows the ThT trace resulting after subtracting the amplitude-matched CV trace. This trace shows a clear lag period.
with the expected sigmoidal RF kinetics, given us a nearly perfect decomposition of gO/CF and RF growth kinetics during biphasic kinetics.

2.9 Screening of more fluorescent dyes

While crystal violet has shown selectivity towards oligomers it is possible that its selectivity is limited to the specific pH, temperature, and other specific solution condition used in our assay. It may be important to identify a number of other oligomer selective dyes in order to combat scenarios where specific solution conditions may render one oligomer dye more useful than another. I continued to screen for other possible candidates of oligomer selective dyes. Other dyes I had screened included the amyloid dye X-34, Ethidium Bromide, and Hoechst 33342. Ethidium Bromide and Hoechst 33342 are common dyes used for cell staining.

Our lab has run several cell toxicity experiments using these dyes and has observed them binding to the aggregates that were put on these cells. While several of these dyes had some possible selectivity toward and aggregate species none of them were quite as selective as crystal violet, except perhaps for methyl violet which is similar to crystal violet in both its level of selectivity as well as chemical structure. Our lab still continues to screen dyes to hopefully find more promising selective dyes.

2.10 Shifting of dye screening assay to near-physiological conditions

2.10.1 Amyloid Beta

While CV has proven to be fairly reliable oligomer indicator under the acidic growth conditions for HEWL amyloids it would be helpful to find an oligomer selective dye that works
with a disease relevant protein under near-physiological conditions. Dyes are well known to have varying dependences on solutions conditions and testing dyes under conditions similar to physiological means the dyes are more likely to hold up their oligomer selectivity as an in vivo detector of oligomer. With that in mind there are a number of possible ways to improve this dye screening assay. Namely, an attempt to shift it towards a more disease relevant protein, as well as a more biological pH.

One of the changes I made to the assay was to use amyloid beta peptide instead of HEWL as the protein in the assay. Aβ is intrinsically disordered and has two main variants Aβ 1-40 and Aβ 1-42. It is believed to be the underlying or initial culprit for Alzheimer’s disease (23-25). Our lab has shown that the same transition from sigmoidal to biphasic kinetics upon increasing protein concentration, is present in amyloid beta peptide (Aβ). This illustrates how the same methodology of using sigmoidal vs biphasic kinetics to screen dyes for potential selectivity toward oligomers would work for amyloid beta as well would work.

The in vitro growth conditions for this transition were pH 7.4 with either no salt or physiological NaCl concentration, minimizing the risk that a dye identified in this assay is unresponsive in vivo. Ideally I would check all of our potential oligomer selective dyes under these conditions, as dyes that hold up as oligomer selective under these conditions would be very promising candidates for in vivo detection of oligomers. However, this protein is expensive and using it for our dye screening assay would likely be reserved for only our most promising dye candidates.
Figure 2.7. Growth kinetics for Aβ1-40 and Aβ1-42
Transition from sigmoidal to biphasic kinetics and associated gO/CF formation for Aβ1-40 and Aβ1-42. (a) Transition in Aβ40 growth kinetics from pure sigmoidal (orange) to biphasic (blue) kinetics (pH 7.4, no salt). (b) Same as (a) but for Aβ1-42. Semilog plot emphasizes weak ThT response during gO/CF phase. (c) ThT fractional change during Aβ1-40 growth in physiological saline. Notice the significant increase in gO/CF amplitude relative to panel (a). (d–f) TEM images of samples of (a)—Aβ1-40 RFs following sigmoidal growth at 50 μM (d) versus biphasic growth at 150 μM, with gO/CFs formed within 1.5 days, and (e) mixtures of gO/CF and RFs after 6 days (f). Figure from Barton et al. Biomolecules Sep. 2019

2.10.2 pH 5 HEWL Assay

For more preliminary testing of dyes, I planned to move the HEWL assay closer to physiological pH thereby preserving the convenience and cost effectiveness of our current dye screening assay, versus the much more expensive Aβ, but still improving the similarity to physiological conditions. This meant finding sigmoidal and biphasic growth conditions for HEWL when approaching physiological pH. I started this process by increasing the pH from 2 to 5, as a stepping stone for the eventual goal of pH 7. The denaturation temperature of HEWL at pH 5
was found to be roughly 63°C. A plate was prepared with several protein and salt conditions and was incubated at 65°C overnight with continuous agitation at 700 rpm.

(A)                                                                                  (B)

Figure 2.8: pH 5 HEWL Fibril Growth

HEWL amyloid fibril formation (65 °C, 700 rpm, 1 mg/ml, 200 or 400 mM NaCl, at pH 5. Figure (A) shows the ThT growth kinetics for 1 mg/ml of HEWL with varying salt concentrations. Figure (B) is a TEM image taken at the endpoint of one of the 1 mg/ml,400 mM NaCl traces in (A). (A,B) Show the expected sigmoidal growth behavior and morphology of amyloid fibrils, albeit with the latter significantly more bundled than the fibrils grown at pH 2.

Finding conditions at this pH that would give the expected biphasic behavior, or finding it at pH 7, proved to be challenging and is an ongoing goal of this dye screening project. If we find the transition of kinetics for HEWL at pH 5 or pH 7 we can continue to use our cheap model protein for initial dye screening. At the same time we would be improving the relevance of any of the oligomer dyes we find to detecting amyloid disease oligomer in vivo.

2.10.3 Human Lysozyme

Another potential improvement attempted to be made to the dye screening assay was to use human lysozyme, the amyloid protein behind lysozyme amyloidosis, instead of HEWL.
Lysozyme amyloidosis is a disease in which large amounts of plaques of lysozyme accumulate in organs within the body. These amyloid structures often result in organ dysfunction leading to a variety of symptoms depending on the affected organ. Since HEWL has roughly 60% of the same homology and nearly identical secondary structure to human lysozyme, exploring lysozyme amyloidosis and applying our dye assay to HuLys, in theory, is a convenient avenue to make the dye assay more disease relevant.

The first step was checking HuLys for the same sigmoidal and biphasic behavior that was observed in HEWL under acidic conditions. A plate was prepared with similar solution conditions to one of the HEWL plates that would cross the COC by varying protein concentration at a fixed salt concentration. I noticed however that HuLys did not behave as similar to HEWL as expected. Most notably, I was unable to get HuLys to display the same biphasic kinetics that we observed in both amyloid beta and HEWL. Once suitable conditions for

![Figure 2.9: Human Lysozyme pH 2 Fibril growth kinetics](image)

**HuLys** (from Sigma) was prepared at varying protein concentrations with salt concentration fixed at 500 mM NaCl. (A) HuLys solutions were incubated at 52 °C and had their ThT Fluorescence (15 uM) monitored in a plate reader for 2 days. The plate was lightly agitated with pulse shaking. Figure (B) TEM images of HuLys fibrils grown at 2 mg/ml in 500 mM NaCl solution after incubation.

The first step was checking HuLys for the same sigmoidal and biphasic behavior that was observed in HEWL under acidic conditions. A plate was prepared with similar solution conditions to one of the HEWL plates that would cross the COC by varying protein concentration at a fixed salt concentration. I noticed however that HuLys did not behave as similar to HEWL as expected. Most notably, I was unable to get HuLys to display the same biphasic kinetics that we observed in both amyloid beta and HEWL. Once suitable conditions for
this assay are found that do display biphasic kinetics one could begin checking the most promising dye candidates from the HEWL assay with HuLys. Ideally, we would like to show that any oligomer sensitive dyes we find, like Crystal Violet, are generally selective to oligomers formed by various amyloid proteins, and not just those by HEWL

2.11 Discussion

Using the clear transition from sigmoidal to biphasic growth kinetics in HEWL, I developed a straightforward assay for screening dyes for their fluorescence responses to different aggregate species. Utilizing this assay I screened a number of dyes and identified the oligomer sensitive dye crystal violet. However, it is known that dye fluorescence can have strong dependence on pH, temperature, ionic strength, ion identity and, perhaps, oligomer identity. For example, I have observed that the amyloid dye ThT loses its fluorescence after about 2 days upon incubation at elevated temperatures (> 40 C) at pH 7. Ideally, one would investigate dyes for multiple proteins under physiological growth conditions.

I’ve provided evidence that transitioning this assay to more physiological conditions is possible. The model protein HEWL itself can undergo amyloid formation at elevated pH, albeit at a much higher denaturation temperature. Additionally, Human Lysozyme was shown to form amyloid aggregates under similar conditions to HEWL. Most importantly, Aβ40 and Aβ42 were shown to have this same transition from sigmoidal to biphasic kinetics at physiological pH and ionic strength. This suggests that one could readily extend this assay to amyloid beta peptides, or other disease relevant proteins. Dyes that appear oligomer selective under physiologically growth conditions with amyloid beta peptide would be excellent candidates for an oligomer detector in both ex and in vivo
A more challenging question is whether the early-phase oligomers grown with HEWL above the COC are the most disease-relevant oligomer species. The second part of my thesis research is focused on this question.

2.12 Materials and Methods

2.12.1 Preparation of HEWL

Two times recrystallized, dialyzed and lyophilized HEWL (Worthington Biochem. Corp., Lakewood, NJ) was dissolved in 25 mM KH$_2$PO$_4$ pH 2 buffer and then placed in a water bath at 42 °C for a few minutes in order to completely dissolve the protein. Protein solutions were filtered with a 220 nm syringe filter and mixed with the appropriate proportions of 0 mM NaCl and 1 M NaCl buffer solutions to reach specific NaCl conditions for our assay. By fixing the NaCl concentration, typically at 400 or 450 mM, we could cross the COC for oligomer formation by increasing protein concentration. This approach minimized changes in dye fluorescence due to ionic strength or ion-specific effects. Concentrations of HEWL samples were determined from UV absorption measurements at 280 nm ($\alpha_{280} = 2.64$ ml mg$^{-1}$ cm$^{-1}$). UV absorption measurements were taken with a DeNovix DS-11-FX UV spectrophotometer.

2.12.2 Preparations of Thioflavin-T and other dye stock solutions

Thioflavin-T stock was prepared by dissolving it in DI water to 1-2 mM and filtering it with a 220 nm syringe filter. Other dyes stocks were prepared similarly unless they were not soluble in water, in which case they were dissolved in DMSO. Concentrations were measured using the same spectrophotometer and the dye’s published absorption coefficients. For dyes dissolved in DMSO, an additional control was incorporated into the dye screening protocol by
adding the largest DMSO concentration to a HEWL growth solution together with ThT. This indicated whether DMSO itself would alter the assembly behavior.

2.11.3 Fluorescent spectra measurements

A FluoroMax-4 spectrofluorometer (Horiba Yobin-Ivon, Piscataway, NJ) was used for measurements of fluorescent spectra of dyes. For a given dye excitation and emission spectra were taken to determine the appropriate wavelengths for fluorescent measurements. Typically, an optimal excitation and emission peak was determined for any dye under three conditions: in pH 2 buffer, at pH 2 with monomeric forms of protein present, and at pH 2 with isolated HEWL rigid fibrils present.

2.12.4 Fluorescence measurements of growth kinetic

Fluorescent kinetic measurements were performed using one of two plate readers: a SpectraMax M5 grating based fluorescence plate reader (Molecular Devices, San Jose, CA) or a Fluorostar Omega plate reader (BMG Labtech, Cary, NC). Typically, 270 µL of a growth solution was added to a well in 96-well glass bottom plates (Cellvis, Mountain View, CA) and monitored at growth temperatures. In most cases three identical wells were prepared for each solution condition. Typically, fluorescent readings were acquired every 15-20 minutes for several days in order for growth kinetics to reach a plateau. To accelerate the growth kinetics, plates were gently agitated using pulse shaking at 100 rpm for 3 seconds before each measurement.
2.12.5 Atomic Force Microscope.

Samples were typically diluted 10-100 fold depending on solution conditions. Then 40 μl of the diluted sample was deposited onto a freshly cleaved mica sheet for 3-5 minutes. Droplets of water were added to mica and then removed in order to wash away excess salt. These mica sheets were then dried using nitrogen gas. Aggregates deposited on mica imaged were imaged in air with a MFP-3D atomic-force microscope (Asylum Research, Santa Barbara, CA) using PFP-FMR-50 (Nanosensor, Neuchatel, Switzerland) silicon tips with nominal tip radii of 7 nm.

2.12.6 Electron Microscope images

Samples were diluted in DI water to concentrations ranging between 10-0.1 μM depending on the specific sample. A 5 uL drop was then added to Formvar/carbon film coated, 200 mesh copper grids (Electron Microscopy Science). This droplet was left on the grid for 2-10 minutes and then blotted from the grid with filter paper. The grid was washed repeatedly, mostly to remove excess salt, by adding a 5 μL drop of water to the grid and blotting it with filter paper after a few seconds, repeating this process for 3-10 times depending on amount of salt in the sample solution. A stock of 8% w/v stock solution of uranyl acetate was used to negatively stain these grids for imaging. A 5 μL droplet of our uranyl acetate solution would be added to the grids and blotted away after 30 seconds. The DI water washing technique used to remove salt was then repeated to remove any remaining uranyl acetate from the grid. Grids were imaged using a FEI Morgani transmission electron microscope at 60 kV with an Olympus MegaView III camera.
2.12.7 Fourier Transform Infrared Spectroscopy Measurements

Fourier Transform infrared Spectroscopy (FTIR) measurements were taken on a Bruker Optik Vertex 70 (Ettlingen, Germany) spectrometer. Typically, 27 μL of solution were added to the top of silicon crystal used for the ATR setup of this instrument. Background scans of the solvent solution were taken over 1200 scans at 2 cm⁻¹ resolution. Sample spectra would be measured from 3000 to 1000 cm⁻¹ wavenumbers, with 600-1000 scans taken depending on the intensity of the signal. FTIR spectra in the Amide I Band between 1600 to 1700 cm⁻¹ were normalized to either their peak values or their total area under the peak. Difference spectra for aggregate samples were created by subtracting the normalized monomer IR spectrum from the spectrum of the amyloid aggregate sample.

2.12.8 Fast protein liquid chromatography

A Superdex 75 10/300 GL column (GE Healthcare) was connected to an ATKA-pure purification protein system pump (GE Healthcare) and was washed for 2 column lengths with water, and then washed with the same buffer as the sample is in. Both of these wash steps were done with column down flow. Sample were typically injected at 1-2 mg/ml with a total volume of 300-500 μL into the system. UV absorption was monitored and any observed peaks were fractionated and collected into test tubes. The column was then cleaned by running two column volumes of water upward through column followed by 1-2 column volumes of ethanol. Column was kept in FPLC unit while filled with ethanol until next uses.
2.12.9 Human Lysozyme sample preparation

Recombinant and lyophilized Human Lysozyme was purchased from Sigma-Aldrich (St. Louis, MO). Typically, 100-200 mg of protein was dissolved into DI water and dialyzed with 20 kDa membrane tubing overnight. Water was typically replaced at the 2, 4, 18 and 20 hour time points of dialysis. The protein was then lyophilized in FreeZone™2.5 Plus (LabConco) Freeze Dryer. Samples were typical left over 2-3 before collecting the lyophilized powder. This purified powder was then used to prepare samples in a manner identical to our HEWL sample preparation.

2.12.10 Amyloid Beta peptide sample preparation.

Roughly 1 mg of Amyloid β peptide (rPeptide) was dissolved in 500 µl of 100 or 150 mM NaOH and passed through and FPLC with a Superdex 75 10/300 GL column (GE Healthcare). This column was washed with water and 35 mM Na$_2$HPO$_4$, 5 mM NaOH, 50 mM NaCl at pH 11. The monomers were fractionated and collected from the FPLC. Final monomer concentrations were measured using UV absorption using $\varepsilon_{280} = 1490$ cm$^{-1}$. Aβ samples then had their pH adjusted from 11 to 7.4 by adding 1.5%, by volume, of 1 M NaH$_2$PO$_4$. 
Chapter 3 - Toxic Oligomers originating in the Rigid Fibril assembly pathway

3.1 Background

The original amyloid cascade hypothesis for Alzheimer’s disease states that the formation of amyloid fibrils and plaques is the root cause of subsequent neurodegeneration and clinical symptoms (26). However, this is at odds the observation that the severity of clinical symptoms bears little correlation to the amount of amyloid plaques present in the brain post mortem (27, 28). As previously mentioned, there is now growing evidence that precursors or intermediate states of fibril formation, such as globular oligomers, may be the main disease-causing culprit. It is known that many disease relevant amyloid proteins have the ability to form oligomers, such as α-synuclein, tau and amyloid beta peptide (49, 50, 54, 55). Yet, there is still no agreement on the disease relevance of oligomers and results in the field often disagree which aggregate species are cytotoxic. The heterogeneous nature of amyloid aggregation has made it difficult to determine which aggregate species do form, what their mechanisms of cytotoxicity or, and which of those mechanisms are most relevant for AD.

Our lab is uniquely equipped to explore how growth conditions affect which types of amyloid species do emerge during amyloid formation. The ability to aggregate HEWL amyloid aggregates along one of two distinct assembly pathways gives us the ability to selectively generate gO/CFs or RFs and study their cell toxicity separately. In addition, by isolating either gO/CFs or RF from the monomers present via centrifugation we can better characterize the
spectroscopic and tinctorial properties of these aggregates. By better characterizing our aggregate species, and forming homogenous aggregate populations, we were able to quantify the cell toxicity of specific amyloid aggregate species, which has been a shortcoming of similar studies in the past. While performing these cell toxicity experiments, we also uncovered yet another type of oligomer that displayed the highest level of toxicity of any amyloid species we have studied (Figure 3.2). We therefore set out to elucidate the origin of this new species and provide an at least preliminary characterization of its biophysical and immunological properties.

3.2 Preliminary Toxicity Data

3.2.1 Preparing and isolating HEWL aggregate species for cell toxicity assay

In order to conduct cell toxicity experiments gO/CFs and RFs were prepared in DI water, adjusted to pH 2 by the addition of small amounts of hydrochloric acid. This differs from my previous described method of preparing aggregates in pH 2 Potassium Phosphate buffer because preliminary cell toxicity results showed that this buffer was toxic to cells. While RFs, were grown at 50 mM NaCl concentration (below COC), gO/CFs were generated by incubating monomers with 250 mM NaCl (above COC). For both above and below COC conditions HEWL concentration was 20 mg/ml and solutions were incubated at 52°C in a heat block. RF samples were incubated between 6-9 days while gO/CF samples were incubated for only 2-4 hours, with their growth monitored with dynamic light scattering. These aggregates were then isolated from monomers by spinning them at 15,000 g overnight and resuspending their pellets in pH adjusted water. This process was repeated 2-4 more times until the supernatant contained a negligible amount of residual monomers. Morphologies of these samples were then checked.
with TEM (Figure 3.1). Once isolated, aggregates were transferred to pH 7.4 cell media. Upon transfer these aggregates precipitated into distinct structures: rigid fibrils showed a strong tendency to bundle into highly organized fibril sheets, as seen in TEM (Fig. 3.1, top row). In contrast, gO/CFs formed diffuse, fractal-like clusters. We also checked for standard characteristic of amyloid plaques using optical microscopy (Figure 3.1, bottom two rows). Rigid fibrils showed strong Thioflavin S (ThS) fluorescent and birefringence, a staple diagnostic of fibril amyloid plaques. In contrast CFs show only weak ThS staining and no birefringence, indicating a lack of long-range order within the CF precipitates.

These distinct differences between RF and CF precipitates under optical microscopy has an interesting correlation with the types of plaques found in Alzheimer’s patients. Amyloid beta plaques, present in patients, are typically described as either being neuritic and dense core, or diffusive. Neuritic/ dense core plaques have strong ThS fluorescence and birefringence, while diffusive plaques have a weak ThS fluorescence and no birefringence. The origins and relevance’s of these two types of amyloid plaques are still widely debated, but our results suggest these different types of plaques may originate from different amyloid aggregate species.

3.2.2 Cell Toxicity Results

Cell toxicity of various amyloid aggregate species was evaluated using an imaging live-dead cell viability assay. This assay used two fluorescent dyes: Ethidium Homodimer, which is a membrane-impermeable dye that can bind to the nucleus of dead cells, and NucBlue which is cell permeant and can therefore bind to the nuclei of living cells. Ethidium Homodimer and
NucBlue fluorescence are excited at 528, and 360nm, and fluorescence collected at 617 nm and 460 nm respectively.

**Figure 3.1: Images of Lys amyloid aggregates before and after transferring to pH 7**
TEM images before and after transferring isolated RFs and CFs from pH 2 to pH 7 cell media (top two rows). It also shows bright field, ThS fluorescence and Congo Red birefringence of the same isolated aggregate species, imaged using optical microscopy (bottom two rows). These optical images resemble the types of plaque neuritic and diffusive plaques associated with Alzheimer’s Disease. Images of neuritic and diffuse plaques are taken from Hayne, David & Lim, Sinchun & Donnelly, Paul. (2014). Chemical Society reviews. 43. 10.1039/c4cs00026a.
Once aggregates were added to cells, the cells were incubated for three days and cell viability was determined from the average number of live cells under a given incubation conditions. Several control solutions are used as reference. Namely, cell media is used as a negative control, and the apoptotic peptide KLAK as a positive control. In addition, plain growth buffer (pH 2 buffer + NaCl), and growth buffer with HEWL monomers, as well as the respective supernatants of each of our isolated aggregates species were used as controls to eliminate any possible effects on cell viability not originating from the added aggregate species.

**Figure 3.2: Cell toxicity data of HEWL aggregates**
The above shows the cell viability for both HEWL RFs and CFs, obtained via a live-dead fluorescence microscope assay. In addition to these two aggregate types, several control conditions are shown, including the supernatant of the first centrifuge spin to isolate RFs or gO/CFs from smaller aggregates. Notably, the RF supernatant appeared to be the most toxic condition, while CFs showed only marginal levels of toxicity.
The cell toxicity data in Figure 3.2, shows that isolated rigid fibrils were somewhat toxic to cells. However, it was the supernatant of RFs that was the most toxic. This was surprising as we assumed that the supernatant of these samples only contained monomeric HEWL, with perhaps small amounts of fibrils that had not been properly spun down. Since the fresh monomer control had no discernable toxicity this led us to believe that some other small aggregate must be present in our RF supernatant. We speculated that these small aggregates could perhaps be oligomers. Since the gO/CF oligomers grown in conditions above the COC were marginally toxic, at best, this implied that we had generated an oligomer species different from those prevailing above the COC. I therefore set out to characterize this new oligomer species, to determine when and how it emerged during what we had believed was "oligomer-free" fibril growth, and performed some biophysical characterization of these new oligomers.

3.3 Checking for Oligomers in RF SN with DLS and FPLC

I explored a number of techniques to confirm the existence of an oligomeric aggregate species in the RF supernatant. Initially these experiments focused on detecting assemblies with a molecular weight or size different from that of the HEWL monomers present in solution. The supernatant was first checked with dynamic light scattering (DLS) to determine whether it contained small fibrils that had not been spun down, or some other larger aggregates that centrifugation didn’t separate from the supernatant. Dynamic light scattering is particularly well suited for differentiating between monomers and rigid fibrils, since they are several orders of magnitude different in size. In addition, larger aggregates contribute roughly in proportion to the square of the molecular weight to the DLS signal and are therefore particularly prominent in the resulting particle size distribution. The particle size distribution in Figure 3.3a shows the RF
supernatant as only having a broad peak around 2 nm hydrodynamic radius, which is characteristic of HEWL monomers. Occasionally a larger peak was observed in DLS measurements of the RF supernatant, but typically the samples were overwhelmingly monomeric and any large aggregates that were seen in dynamic light scattering could be removed by filtering the sample through a 50 nm syringe filter.

Figure 3.3: Size characterization of Rigid Fibril supernatant
The left (A) shows the DLS trace of the rigid fibril supernatant. There is one peak localized around 2 nm, typical of our HEWL monomers, this suggests no fragmented rigid fibrils are present. On the right (B) is UV vs elution volume trace of the same supernatant run through our FPLC size exclusion column, as well as a pure monomeric sample. The supernatant in FPLC shows tow peaks, one that lines up with the monomeric peak, and another slightly larger one that we believe corresponds to our fibril derived oligomers.

Dynamic light scattering however, has very poor resolution for distinguishing between molecules of comparable hydrodynamic radius, including any potential toxic small oligomers (<10 monomers) that may be present. I therefore performed fast protein liquid chromatography (FPLC) on the RF supernatant to check for the presence of any small oligomers.
The UV trace in figure 3.3b of the eluent shows that the RF supernatant contained no large aggregates but did show two peaks, a broadened monomer peak near 20 ml and a prominent oligomeric peak at 16 ml. This confirmed that the supernatant of our fibril samples did indeed contain a population of small oligomers that had remained undetected using the kinetic assays (ThT, light scattering) to monitor RF growth below the COC.

3.4 What are the size/molecular weight of these toxic oligomers?

There is some discussion in the field about the importance of the molecular weight of oligomers regarding their role in Alzheimer’s. Some labs have proposed to separate Aβ oligomers into two species: essentially nontoxic low molecular weight species and highly toxic high-molecular weight oligomers (56 kDa oligomers) (31-32,64). Therefore, it was of interest of us to establish the molecular weight of these toxic oligomers. I therefore calibrated the elution volume of our FPLC column using a series of reference proteins. A solution was prepared of equal concentrations of hen-egg white lysozyme (14.3 kDa), trypsin (23.3 kDa), pepsin (34.5 kDa), and bovine serum albumin (66.5 kDa), dissolved in pH 2 adjusted water. The corresponding elution profile is shown in Figure 3.4. The UV trace peaks of each monomer protein have different heights, because they were added at the same protein concentration but have different absorption coefficients. In addition, I have observed that different proteins have different affinities for sticking to the injection site, the entry filter to the column, or in the case of lysozyme have a high affinity to interact with the agarose/dextran matrix within the column itself.
Figure 3.4: FPLC of protein with varying molecular weight
Above (A) is the FPLC UV trace of a sample mixed with roughly 0.5 mg/ml of HEWL, trypsin, pepsin and albumin at pH 2 50 mM NaCl (red) and our RF SN (blue) whose largest peak is believed to be our toxic oligomers. B shows the MW vs elution volume calibration curve, on log-log scale, for the size-exclusion column under these conditions. The table below lists the molecular weight, in kilo Dalton of each of the respective proteins.

Table 3.1 Molecular weights of Proteins used in FPLC size-calibration
Shows the molecular weights of the different proteins used in the experiment shown in Figure 3.4

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hen-egg white Lysozyme</td>
<td>14.3 kDa</td>
</tr>
<tr>
<td>Trypsin</td>
<td>23.3 kDa</td>
</tr>
<tr>
<td>Pepsin</td>
<td>34.5 kDa</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>66.5 kDa</td>
</tr>
</tbody>
</table>
This calibration of molecular weight in our column provided a rough read-out of the molecular weight of the oligomers in the RF supernatant. In Figure 3.4, I showed that the RF supernatant toxic oligomer peak is at roughly 16 mL elution volume, i.e. between that for pepsin and BSA. This suggests that these toxic oligomers are about the size of a trimer. This also explains why DLS was unable to separate them from the monomer peak in those particular size distribution measurement. It is worth noting that elution volume shares a logarithmic relationship with molecular weight. Using this relationship, I plotted molecular weight vs log volume of our mixed molecular weight sample and fitted a linear trend to it. This plot shows that our toxic oligomers are likely trimers with a molecular weight estimated to be ~38 kDa.

3.5 Techniques to Isolate Toxic Oligomers from Monomers

For further characterization of this species of small, toxic oligomers, I needed a way to isolate them from monomers, similar to how centrifugation has been used to isolate RFs and CFs from monomers. I was able to use FPLC as a method to fractionally collect the toxic oligomers from the monomers in the RF supernatant in some of my experiments. However, this approach has limited yield due to the overall limitation on loading volume for our analytical FPLC column. Furthermore, for many of my experiments I needed to separate several samples at once, making FPLC a time-consuming approach.

I therefore tested centrifugal cut-off filters as means for isolating toxic oligomers from monomers. After the initial "standard" centrifugation to remove rigid fibrils, the resulting supernatant was passed through a 50 kDa molecular weight cut-off filter at 14,000 g for 15 minutes. Buffer was repeatedly added to the top of the filter, and again spun at 14,000 g for 15
This wash step was repeated three times. I tested this protocol on both the RF supernatants and fresh monomer samples and measured the concentration of protein that came through the filter after each spin, as well as what remained at the top of the filter after the final spin.

**Table 3.2: Effectiveness of cut-off filters to isolate TOs**

Progressive protein concentration measurements of filtrate during 50kDa cut-off filter centrifugation protocol. Shown are the residual protein concentrations using either fresh monomers or the RF SN (toxic oligomer sample). The order of magnitude of more protein collected using this method. In the RF Sn vs monomer sample shows how well toxic oligomers are collected and isolated via this protocol.

<table>
<thead>
<tr>
<th></th>
<th>Monomer</th>
<th>RF SN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial Conc.</strong></td>
<td>7.6 mg/ml</td>
<td>7.6 mg/ml</td>
</tr>
<tr>
<td>1st spin (bottom filtrate)</td>
<td>7.1 mg/ml</td>
<td>6.7 mg/ml</td>
</tr>
<tr>
<td>2nd spin (bottom)</td>
<td>1.5 mg/ml</td>
<td>2.1 mg/ml</td>
</tr>
<tr>
<td>3rd spin (bottom)</td>
<td>0.2 mg/ml</td>
<td>0.5 mg/ml</td>
</tr>
<tr>
<td>(top residue) Collected after third wash</td>
<td>0.2 mg/ml</td>
<td>2.1 mg/ml</td>
</tr>
</tbody>
</table>

Both monomer and toxic oligomer samples started this separation protocol at a concentration of 7.6 mg/ml. The concentration left at the top of the filter after three washes was 0.2 mg/ml for monomers and 2.1 mg/ml for the toxic oligomer sample. As expected, significantly more protein remained at the top of the cut-off filter for the toxic oligomer sample.
This method gives us roughly a 10:1 ratio of toxic oligomers to monomers after three spins. For all following experiments samples were therefore washed no less than three times to isolate toxic oligomers from monomers.

As mentioned above, the transfer of toxic oligomers from pH 2 to pH 7 for cell experiments caused noticeable precipitation. If this precipitation was solely due to toxic oligomers, then pH 7 transfer would provide another way to isolate toxic oligomers from monomers. However, I needed to determine whether hydrolyzed monomers, which were also present in solution, could contribute to the precipitates at pH 7.

![Precipitation of Rigid fibril supernatant](image)

**Figure 3.5: Precipitation of Rigid fibril supernatant**
The above shows a picture of an RF SN (toxic oligomers still mixed with hydrolyzed monomers) and isolated hydrolyzed monomers of the same sample, after being transferred to pH 7. Similar levels of precipitation are also observable with the other two aggregate types (RFs and CFs). Hydrolyzed monomers had no visible precipitates. This proposes pH transfer as a possible method of isolation of these toxic oligomers to the monomeric background.

To check whether my precipitate was formed by toxic oligomers, monomers or a mixture of the two, toxic oligomers and monomers were separated using the above mentioned
cut-off filter separation protocol. Once separated, the concentration of both monomer and isolated toxic oligomer sample was measured, and diluted to the same concentration in pH 2 buffer. My monomer and toxic oligomer samples were then diluted 10 fold into pH 7 sodium bicarbonate buffer. After pH 7 transfer visible precipitation had formed in the toxic oligomer sample while the sample containing just hydrolyzed monomers displayed no visible precipitation. Samples were checked periodically for several days to make sure that precipitation had not simply occurred at a slower rate with the monomer sample. Some time-dependence of precipitation was often observed for even the toxic oligomer samples, in particular if the concentration of toxic oligomers was low. Later experiments however, would show that while my toxic oligomer sample is isolated from monomers using this method, a small concentration of toxic oligomers may still exist within the “hydrolyzed monomer” sample.

With confirmation that the precipitates at pH 7 were predominately due to the precipitation of oligomers, transferring toxic oligomers to a higher pH could serve as a method of isolating the aggregates from the hydrolyzed monomers. Albeit, precipitation did not appear to be reversible upon transfer back to pH 7. Hence this approach is only useful for concentrating toxic oligomers at pH 7. Other amyloid aggregates showed similar precipitation behavior upon transfer to pH 7 and were characterized during the previously described cell culture experiments. For subsequent experiments, the protocol used for isolation of TOs was the above described use of 50 kDa molecular weight, since it was often preferable to have the TOs still under growth conditions and for them to still be soluble in solution.
3.6 Amyloid Characteristics of Toxic Oligomers

3.6.1 TOs Secondary Structure

The above experiments provided solid evidence that some small aggregates were present in the RF supernatant. To confirm that these aggregates were in fact amyloids I investigated the secondary structure content of these aggregates using FTIR. Specifically I explored whether the FTIR spectra from toxic oligomers, like other amyloid aggregate species, have a peak in the characteristic amyloid beta-sheet range between 1610 and 1630 cm\(^{-1}\). I prepared toxic oligomers using the standard RF growth and separation protocol and measured their FTIR absorbance spectrum (Figure 3.6). Samples were also diluted ten-fold in pH 7 sodium bicarbonate buffer, and measured the absorbance spectrum after pH transfer.

![Figure 3.6: FTIR of Rigid Fibril Supernatant](image)

(Left) Amide I IR spectrum for fresh monomers, and toxic oligomers before and after transferring to pH7. (Right) Difference spectra obtain by subtracting monomers spectrum from oligomer spectrums at either pH. Peaks were normalized by area under the amide I band prior to subtraction. Spectrums show expected characteristic cross-beta sheet peak (~1620 cm\(^{-1}\)) in RF SN, as well as possible enhancement in anti-parallel beta sheet structure (~1690 cm\(^{-1}\)).
The toxic oligomers show a characteristic amyloid beta sheet peak near 1620 cm\(^{-1}\) in the Amide I band, both at pH 2 after transfer to pH 7. Perhaps even more importantly, the toxic oligomers have an additional peak in the anti-parallel beta sheets regime around 1680 cm\(^{-1}\). I had measured this anti-parallel structure in our globular oligomers before but not as strongly as it appears here. In addition, I believe the reason transferring the oligomers to pH 7 strengthens this signal is because the oligomers precipitate (as shown in Figure 3.5 and more heavily concentrate on our crystal.

3.6.2 ThT Fluorescence

As previously discussed, fluorescent dyes are commonly used to detect amyloid aggregates, most commonly with Thioflavin-T However, there are some oligomer species that show little to no ThT fluorescence (63). Even if responsive to ThT, I wanted to determine their ThT fluorescence relative to the gO/CFs formed above the COC. ThT fluorescence was determined from 100 \(\mu\)M solutions of all three amyloid species at pH 2. In addition, the cut-off filters during TO isolation was used as hydrolyzed monomer sample. Figure 3.7 shows the ThT (15 \(\mu\)M) fluorescence excitation and emission spectra in the presence of RFs, TOs, CFs, hydrolyzed and native monomers. Hydrolyzed monomers induce a weak (not resolved in Fig. 3.7) two-fold enhancement in ThT fluorescence over native monomers. TOs result in a 14-fold, CFs in a 24-fold and RFs in a nearly 150-fold enhancement of ThT fluorescence. The ThT enhancement induced by the toxic oligomers further supports the idea that these toxic aggregates are indeed amyloid oligomers. Comparing ThT fluorescence from isolated toxic
oligomers to that of hydrolyzed monomers also indicates that the ThT fluorescence of the tOs is distinct from hydrolysis-induced changes in ThT responses to monomers.

![Figure 3.7: ThT Spectra of isolated aggregates](image)

**Figure 3.7: ThT Spectra of isolated aggregates**
ThT excitation and emission spectra for RFs, TOs, CFs, as well as native and hydrolyzed monomers. All spectra were collected using 100 µM of protein and 15 µM ThT. TOs show Thioflavin-T fluorescence and notably at a comparable level to that of CFs.

### 3.6.3 Immunostaining with the oligomer sensitive antibody A11

A common assay for detection of oligomers is immunostaining with oligomer-selective antibodies (44,45). These antibodies are formed by immunizing animals with amyloid oligomers and extracting their immune serum. It has been shown that these antibodies formed against amyloid oligomers of one protein can be used to detect amyloid oligomers from different proteins. However, there are some contradictory reports as to whether A11 binds to HEWL oligomers (29, 30). We used dot-blots with two different variants of A11 to determine their
response to the three HEWL amyloid aggregate species RFs, CFs and TOs. Preliminary experiments by my fellow graduate student Nabila Bushra indicated that RFs did bind to the nitrocellulose membranes used for dot blots. Ms. Bushra used Ponceau staining to determine the differences in deposition and retention rates of RFs relative to all the other amyloid aggregates. These experiments indicated that RF concentrations had to be reduced by a factor of 4 relative to the other amyloid aggregates in order to retain equivalent amounts of protein on the membrane prior to antibody application (Figure 3.8 Left). The difficulty of getting oligomeric HEWL aggregates to adhere to the membranes might explain some of the discrepancies in the immunostaining from earlier work.

Dot blots with either A-11 (Novus Biologicus) or A11-19 (gift from Rakez Kayed) antibodies were performed with concentrations adjusted to result in comparable amounts of total protein for each aggregate type. Figure 3.8 (Middle) shows the Novus A-11 antibody reacting to both RFs and CFs. While A11-19 also reacted to RFs, it reacted with TOs instead of CFs. This suggests that both variants of A11 do have a rather prominent affinity to hewL RFs. At the same time, the select reactivity of A11 and A11-19 to the two different HEWL oligomer species serves as further evidence that the underlying conformations of these two oligomer species are distinct. Unfortunately, the cross-reactivity of both A11 variants with RFs means immunostaining is not a feasible method of oligomer detection for this system. Additionally, many attempts to replicate these results have been made and Ms. Bushra has found the immunostaining of Novus A11 body to be highly reproducible, reactivity towards RFs and TOs, but some has found some variability with A11-19 immunostaining where reactivity towards both oligomer types may occur.
Figure 3.8: A11 antibody immunostaining
Immunostaining of amyloid aggregates with A11 and A11-19 anti-oligomer antibody. (Left) Ponceau staining of aggregates blotted onto membrane. (Middle) Reactivity of A11 from Novus Biologicus with RFs (top row) and late-stage oligomers (middle row) (Right) Reactivity of A11-19 with RFs (top row), toxic oligomers (2nd row) and native monomers (bottom row). The concentrations used for the various aggregate were RFs: 2, 1 and 0.5 mg; toxic oligomers: 4, 2 and 1 mg; and for every other sample 12, 8 and 4 mg. These concentrations were used as they were found to yield equal protein deposition on the membrane for each aggregate species

3.7 Origin of Toxic Oligomer and Time-course of their Formation

3.7.1 Are Toxic Oligomers Fibril Derived?

As shown in Figure 3.9, tOs do induce ThT fluorescence comparable in intensity to that generated by early-stage gO/CFs. Yet, our kinetic measurements of fibril growth below the COC lacked any sign of TO formation during the lag-phase of RF growth. In addition, we were only able to isolate TOs at the late-stages of fibril formation. One possible explanation was that tOs were actually directly derived from rigid fibrils, perhaps by dissociating off of RFs, or RFs being necessary to catalyze TO formation.
Figure 3.9: Seeded Rigid fibril growth
Above (left) shows ThT growth kinetics of fresh monomers, isolated fibrils and monomers seeded with rigid fibrils, incubated at 52°C. Seeded rigid fibril sample shows immediate RF formation with no lag phase (templated growth). Right shows the FPLC traces of the seeded sample after 2 days along with UV traces of native monomers and typical RF Sn to compare. No oligomeric peak was found from seeded grown rigid fibrils.

As a result, the dominant light scattering and ThT fluorescence from fibril formation would mask any minor contributions from concurrent tO formation. This would also agree with the observations of "oligomeric clouds" surrounding dense core plaques in Alzheimer’s disease (59). I therefore investigated two possible mechanisms by which fibrils could generate toxic oligomers. Firstly, rigid fibrils could be the immediate source of toxic oligomers by shedding them over time. Secondly, fibrils might act as catalysts promoting the formation of toxic oligomers from monomers.

If isolated fibrils "shed" toxic oligomers they should do so in the absence of any monomers and, therefore, in the absence of fibril growth. To test this, isolated RFs were
generated using our typical centrifugation protocol. The 4th fibril pellet was resuspended in pH 2 buffer at 25 C. After several days of incubation, RFs were removed from solution by centrifugation at 15,000 g overnight, and the protein concentration of the supernatant was determined. This did result in an elevated protein concentration in the supernatant. However, further inspection revealed that centrifugation had “fragmented” some fibrils which, therefore, had remained in the supernatant after one centrifugation cycle. After filtering out these fragmented fibrils with a 50 nm syringe filter, the protein concentration in the supernatant was at a level indistinguishable from that prior to the incubation of the isolated fibrils. Incubation of isolated fibrils at the growth temperature used for fibril growth yielded comparable negative results.

Alternatively, I checked whether rigid fibrils "catalyzed" the formation of toxic oligomers from monomers. If so, seeding of monomers with preformed rigid fibrils should accelerate the formation of toxic oligomers. To test this idea, we incubated three different solutions containing either monomers only, isolated RFs only, or monomers seeded with isolated RFs in the presence of 15 μM ThT, and measured ThT fluorescence over time. Incubating the seeded sample at the usual growth temperature of 52 C induced immediate RF growth, as indicated by a rapid increase in ThT fluorescence. Both the monomer and fibril controls showed no increase in fluorescence and remained unchanged for 24 hours. Hence neither of the two control conditions (monomers only, fibrils only) generate toxic oligomers with in this time frame. Therefore, any toxic oligomers detected in the seeded solution would have to be the result of fibril-catalyzed oligomer formation.
The observed increase in ThT fluorescence of the seeded sample in Figure 3.9, by itself, does not provide any information about possible toxic oligomer formation since it is dominated by rigid fibril elongation. To check for toxic oligomer formation, the sample was spun overnight at 15,000 gs and the supernatant collected. This supernatant was then passed through the size exclusion column in FPLC to check for the presence of oligomers. The UV trace in Figure 3.9 indicates that no toxic oligomers appeared as a result of seeded fibril growth, as only the single monomer peak is present in this trace.

### 3.7.2 Time-course of Toxic Oligomer Formation

These experiments indicated that toxic oligomers were neither shedding from rigid fibrils, nor did they form as a result of fibril-mediated catalysis from monomers. This implied that toxic oligomers formed along a pathway distinct from fibril formation, possibly even competing with the latter for monomers.

The first step in exploring this hypothesis was to determine when exactly during RF formation toxic oligomers did form. In contrast to the globular oligomers that form above the COC, neither DLS nor ThT fluorescence indicated oligomer formation below the COC during the lag phase of rigid fibril formation. Since tOs do have ThT responses similar to those of the gO/CF formed above the COC (see section 3.6.2. above), it implied that toxic oligomers were late-stage aggregates formed during or after fibril nucleation and growth. However, this prevented us from detecting toxic oligomers using DLS or ThT as both of these signals were dominated by RF formation at that point.
In order to measure the onset of toxic oligomer formation a series of solutions identical to those used for generating TOs during RF growth: 20 mg/ml of HEWL, 50 mM NaCl, adjusted to pH 2 were incubated at 52 C. I stopped their incubation at different time points and using physical separation from both fibrils and monomers was able to determine their TO content at these respective time points. The series of solutions was incubated both in 15 mL test tubes, with 3 mL of solution, and in 96 well plates, with 250 µL of solution each. Firstly, incubating samples in a 96 well plate allowed me to monitor RF formation by measuring ThT (15 µM) fluorescence. Additionally, we had noticed that the smaller volume of the well plates accelerated RF formation compared to the larger volume incubated in my 15 mL test tubes. This is most likely due to the preferential nucleation of RFs at the air/water interface, combined with the larger surface to volume ratio of the smaller wells versus our other containers used for incubation such as test tubes. These two separate incubation conditions thus alter the time scale for RF formation, while still keeping solution conditions constant. Hence, following TO formation under these two different conditions allowed me to determine whether the time course of RF and TO formation are correlated or not.

Toxic oligomer concentration under both growth conditions (96 well plate and test tubes) was determined by isolating toxic oligomers at various time points, roughly every 24 hours, via centrifugal cut-off filters (this protocol was described in Section 3.5). Solutions from several wells of the 96 well plates were pooled in order to use identical solution volumes for the subsequent TO isolation and measurement. Toxic oligomer concentration measurements were then taken using DeNovix UV spectrometer
Figure 3.10: Time course of TO formation
HEWL was incubated in 15 ml tubes (orange) or in 96 well plates (blue) at 20 mg/ml hewL, pH 2, 50 mM NaCl (below COC conditions). Toxic oligomers were physically separated and their concentration was measured with UV absorption daily. ThT fluorescence was monitored continuously for the solutions incubated in the plate reader. Toxic oligomers were found to have a delay before formation, and additionally accelerating rigid fibril formation (via smaller incubation volume in 96-well plate) delayed toxic oligomer formation.

The concentration of toxic oligomers, measured with UV absorption, along with the ThT fluorescence of the samples in the plate reader is plotted versus time in Figure 3.10. Measured toxic oligomer concentrations clearly show an extended lag period before the onset of toxic oligomer formation. The 3 mL solutions indicate rapidly forming toxic oligomers after a 4 day lag period. This closely matches the lag periods for RF formation under these growth conditions. In contrast, the plate reader samples only showed toxic oligomer formation after 6 days while the RF lag period is shortened to two days. Hence, accelerating fibril formation deaccelerated toxic oligomer formation. This implies not only that toxic oligomers are off-
pathway to rigid fibril growth but, at a minimum, are competing with each other for the same pool of monomers for their respective growth.

### 3.7.3 Monomeric Composition of Toxic Oligomers

One possible explanation for the delayed onset of toxic oligomer formation could be that they require at least partial hydrolysis of HEWL monomers which is progressing quite slowly under the pH and temperature conditions (pH 2 and 52°C) used for RF growth (51). This does, in turn, imply two distinct scenarios for TO formation. For one, TOs could form preferentially from a fully hydrolyzed fragment of HEWL monomers. Alternatively, TO formation from HEWL monomers requires the additional flexibility that comes from partial hydrolysis of the monomers, In this case, partially hydrolyzed but still full-length monomers (with their disulfide bonds holding them together) should form the building block for TOs.

In collaboration with my lab mate Palle K. W. Karunarathne, we tested the role HEWL hydrolysis in toxic oligomer formation, and determined whether they are formed from full-length but partially hydrolyzed monomers or from a hydrolyzed fragment. Towards that end, we ran reducing SDS-PAGE gels using native monomers as well as toxic oligomers and their corresponding monomer fraction, to compare monomeric composition between these samples. Using a reducing gel assures that we observe the full extent of hydrolysis of the sample. If some fully hydrolyzed fragments of HEWL were the main source of toxic oligomers, though, one would expect to see an accumulation of this hydrolyzed fragment in the toxic oligomers, relative to the composition of the hydrolyzed monomers present in solution at that time. Figure 3.11 shows that both hydrolyzed monomer and the toxic oligomer samples contain identical amounts of all hydrolyzed fragments. Hence, they are composed of the same partially
hydrolyzed monomers present in solution instead of selectively accumulating a particular HEWL fragment. This also supports that it is the aforementioned increased monomer flexibility from being partially hydrolyzed that is required for toxic oligomer formation, and not instead the need of some specific hydrolyzed HEWL fragment.

**Figure 3.11: SDS-Page gel of TOs and Hydrolyzed Monomers**
Reducing SDS PAGE gel of native monomers, hydrolyzed monomers and toxic oligomers. The gel indicate a noticeable amount of partial hydrolysis of the incubated HEWL monomers and toxic oligomers. Importantly, there is no discernable difference in the composition of hydrolyzed material between the hydrolyzed monomer and toxic oligomer sample. Specifically, no enhanced hydrolyzed fragment within TOs showing they are composed from partially hydrolyzed material and not from any specific hydrolyzed fragment.
3.8 Discussion

The specific role amyloid oligomers play in the pathogenesis of various amyloid diseases remains an area of active research (67). Recent findings have indicated that amyloid oligomers themselves display polymorphism when generated in vitro (65-67), as well as when isolated from patient post mortem (67-69). Furthermore, it was implicated that these different oligomer "strains" are associated with different disease pathologies (69-71). However, it has remained less clear what the mechanisms are that result in the formation of distinct oligomer species. In addition, most of the isolation and characterization of these distinct oligomer strains has relied on either immunohistochemistry or on some level of cell/tissue toxicity evaluation as means for their identification and characterization. Overall, my thesis research was to gain new insights into the mechanisms underlying the formation of distinct oligomer species, to provide a more detailed biophysical characterization of amyloid oligomer species and their interactions with amyloid fibrils, and to identify straight-forward fluorescence assays for the select detection of amyloid oligomers in vitro and, potentially, in vivo.

I have shown that HEWL can form two distinct types of amyloid oligomers. The first oligomer type, gOs, begin forming immediately upon incubation, but only under condition above a threshold concentration we called the critical oligomer concentration, or COC. The second, new type of oligomers, TOs, forms under conditions associated with gO-free fibril growth. It emerges on a time scale that typically overlaps with fibril formation in lysozyme and, therefore, was obscured in kinetic traces and high resolution images by the latter. I characterized this second type of oligomers and found several similarities but also several differences between them and their early-stage gO counterpart.
The most notably and relevant difference between these two oligomer populations is the dramatic difference in the level of cell toxicity induced by the toxic, fibril-associated oligomers compared to the early-stage oligomers. This is the reason I refer to the former as toxic oligomers (TOs). Another difference is the type of precipitates formed upon transfer to pH 7. GOs formed precipitates whose morphologies closely resembled that of diffusive plaques. Diffusive plaques are found in early stage AD patients but are shown to be relatively inert since they are also found in cognitively normal people. This finding, of negligible toxicity and resemblance to diffusive plaques, in gOs, is at variance with the hypothesis that oligomers are always the most disease relevant aggregate population. In addition, we have shown that gOs are off-pathway from fibril formation but strongly affect fibril growth. First of all, they compete with fibrils for the same monomer pool for growth, thereby retarding the late-stages of fibril elongation. In addition, I helped establish that gOs directly interact with rigid fibrils and thereby inhibit secondary fibril nucleation. Therefore, gOs might actually help retard the progression of amyloid diseases in vitro. These are, however, only hypotheses. For example, our German collaborator, Dr. Wolfgang Hoyer, showed that Aβ1-40 gOs generated in their lab, have minimal cell toxicity but did interfere significantly with synaptic transmission in neuronal cell cultures (72). In addition, the discovery of another type of highly toxic oligomers demonstrates that there are likely multiple distinct oligomer polymorphs with distinct biological activity. It is therefore important to develop an understanding of how these different oligomer polymorphs come about, how to readily identify them and how to relate their underlying structural characteristics to the corresponding difference in biological activity.
At first, the origin and mode of growth of this new species of toxic oligomers remained enigmatic. Prior to our cell toxicity experiments, neither our studies on the kinetics of amyloid formation in HEWL nor high-resolution imaging with TEM or AFM had turned up any evidence for their existence under sigmoidal fibril growth conditions. Therefore, one main focus of my thesis work was to elucidate the origin of this new oligomer species, determine its mode of formation, and to provide some biophysical and immunological characterization of its properties. After developing our assay for isolating tOs from fibrils and monomers, I established that tOs, under our growth conditions, start forming after the onset of rigid fibril nucleation and growth. This is in stark contrast to gOs, which immediately grow upon incubation and dominate during the lag phase of rigid fibrils formation. Since DLS and ThT signals are both decidedly biased in favor of RFs, the emergence of tOs during RF growth explained why we had previously missed the former in our kinetic studies of amyloid growth. It also implies that kinetic and imaging studies can readily miss admixtures of oligomers in fibril samples due to their select sensitivity to fibrils.

There are two previous studies that reported toxic oligomers forming after extended incubation under rigid fibril growth conditions of lysozyme (57,58). However, their growth conditions are somewhat distinct from ours and both studies lack a detailed characterization of oligomer morphologies which makes a clear assignment of what type of oligomer they observed difficult. The measured time-course of HEWL toxic oligomer formation provides evidence that these oligomers grow along a pathway competing with rigid fibrils, and are not on-pathway pre-cursors of fibrils. This is in contrast with a study of IAPP, which showed toxic oligomers forming in the lag phase of rigid fibril growth (63). In contrast to HEWL gOs, the IAPP
oligomers were also ThT negative. This suggests that both the type of amyloid protein and the specific growth conditions they are forming under play a role in the type of amyloid oligomers they form and the level of cell toxicity they display.

Based on our findings I theorized that the delayed onset of tO growth may be due a gradual increase in hydrolyzed HEWL. Since monomers could form gOs during the lag phase, i.e. when monomer hydrolysis was still minimal, this further suggested that it was the increase in structural flexibility with progressive hydrolysis that could play a role in the formation of different oligomer species. This also matched the prior observation on point mutations of Human Lysozyme associated with Lysozyme amyloidosis. These mutants were all structurally nearly indistinguishable from the native form but displayed lower thermal stability and higher overall flexibility (46, 81, 82).

When investigating the characteristic of both types of oligomers several similarities and important distinction between the two emerged. FTIR of both oligomers displayed a beta sheet peak around 1618 cm-1 which is indicative of their underlying amyloid-like structure. Notably, the beta sheet peak for both oligomers is shifted marginally but reproducibly from that for rigid fibrils, which is located at 1624 cm-1 (Figure 2 in Chapter 2). However, our preliminary data also indicate that there is an additional peak located in the 1700-1680 cm-1 range. This peak is typically associated with anti-parallel beta sheet content and is measurably larger for TOs than gOs. This anti-parallel beta sheet is a structural distinction between oligomers and amyloid fibrils observed in multiple systems, including Aβ (47, 73, 74). The extent of this anti-parallel beta sheet content might provide a structural basis for the degree of amyloid oligomer toxicity, and why it is distinct from that of amyloid fibrils. This hypothesis correlates well with the
distinct toxicity of these two oligomer species, where the oligomers with the most anti-parallel structure, TOs, are also by far the most toxic.

Overall, generating two distinct types of oligomers, with different levels of toxicity, is an important step towards understanding the mechanisms regulating oligomer formation in disease. In particular, the cellular compartments in which oligomers form not only affect oligomer formation by the inherent changes in the solution environment but might affect the type of oligomer species that emerge by modulating the structural flexibility of the monomers.

Identifying the distinctions of different type of amyloid oligomer species, and how those distinction translate to their relative toxicity, emphasizes the importance of an efficient approach to oligomer detection in amyloid research. A simple adaption to my previously described dye screening assay (Chapter 2), allows us to extend our dye screen to selective fluorescence dyes for HEWL TOs. As previously shown, pH 2 HEWL oligomers of either type, and fibrils precipitate when transferred to pH 7, with gO and RF precipitates resembling either diffusive or neuritic plaques, respectively (Figure 3.1). This suggests a modification to my original dye screening assay that takes advantage of this precipitation to look for oligomer-selective dyes, and to do so under more physiological solution conditions. Isolated amyloid aggregate samples could be transferred to pH 7 with dyes added to these precipitates at that point. Any oligomer-selective dye discovered under these conditions is automatically more likely to work under in vivo conditions. Figure 3.12 show a bar graph of preliminary measurements using this approach with ThT (10 uM) and CV (5 uM) fluorescence from HEWL aggregates. While some troubleshooting is required to transition the dye assay to this new
protocol, the results look reasonable with CV fluorescence from all amyloid aggregates comparable, while ThT fluorescence remains highly selective for RFs.

![Figure 3.12: ThT and Cv fluorescence of pH 7 transferred amyloid aggregates](image)

Above shows a bar graph of florescence of RFs, CFs, TOs, and Monos plotted as a normalized fold enhancement from buffer. Fluorescence was measured in a 96-well plate reader with pH 7 transfer accomplished by diluting pH 2 amyloid aggregates at a 10-fold dilution with pH 7 25 mM Sodium Bicarbonate buffer.

3.9 Materials and Methods

3.9.1 Aggregate Sample preparation

Lyophilized hen egg white lysozyme (HEWL) was purchased from Worthington Biochemicals. All aggregate species of HEWL, rigid fibrils (RFs), toxic oligomers (TOs) and globular oligomers/curvilinear fibrils (gOs/CFs), were grown at pH 2. The pH of DI water was adjusted to pH 2 by adding small amounts of HCL to aqueous solutions of both 50 mM and 250 mM NaCl. HEWL was dissolved in this solution to
prepare 20 mg/ml in the pH 2 50 mM NaCl solution (for formation of RFs and TOs) and 250 mM NaCl (for formation of gO/CFs). Samples were filtered through 220 nm PVDF (Fisherbrand, Fisher Scientific) and incubated at 52°C in a heat block. RF and TO samples were incubated for 7-9 days and gO/CF samples were incubated for 2-4hrs and monitored with dynamic light-scattering (DLS).

3.9.2 Isolating of amyloid aggregates

Distinct aggregate species were isolated from monomers via centrifugation. After incubation 2 mL of both RF (50 mM NaCl) and gO/CF (250 mM NaCl) samples were spun for 22-24 hours at 15,000g. The RF supernatant and both RF and CF pellets were collected. Aggregate pellets were re-suspended in 1.6 mL their respective solutions and spun again for 22-24 hour. This processes was repeated 3 times or until monomer concentration measured in supernatant was less than 1% of aggregate concentration in the pellet. The toxic oligomers present in the RF supernatant, were isolated from HEWL monomers via 50 kDa molecular weight centrifugal cut-off filters (Millipore Sigma Amicon, Fisher Scientific). Cut-off filters, with 500 uL RF SN, were spun at 15,000 gs for 15 min and filtrate was often collected to act as hydrolyzed monomer control in many experiments. Typically, sample was washed by adding 200 uL of 50 mM NaCl pH 2 solution to top of filter and spinning cut-off filter at 15,000gs for 15 min. After three washes filter was flipped and put in new centrifuge tube and spun at 1000g for 5 minutes to collect isolated toxic oligomers.

3.9.3 Cell Toxicity Experiment

Human lung carcinoma cells (A549) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum and 2 g/L NaHCO₃. Cells were kept in a 5% CO₂ humidified atmosphere at 37°C. Cells were then plated with 10,000 cells/well in 96-well plates and incubated for 24 hours. Then the medium was exchanged with Dulbecco’s modified Eagle’s medium without fetal bovine serum. The plate was incubated for 20 more hours. After this amyloid
aggregates were added to the cell. Isolated aggregates were diluted in cell media to desired concentrations before adding to the cells. Once added cell plates were incubated for 72 hours. Before beginning fluorescence imaging cells were washed with PBS twice. 4 µM of Ethidium homodimer-1 (Life Technologies (catalogue number E1169)) was added to each well. Plate was then incubated another hour before exchanging the cell media with media containing NucBlue (Life Technologies (catalogue number R37605)) (2 drops of dye to 1 ml of cell media). Five or 6 images were then taken per well using the EVOS fluorescence microscope (10X magnification), and live cells were counted using ImageJ software.

3.9.4 Dynamic Light Scattering

A Zetasizer Nano S (Malvern Instruments, Worchestershire, UK) with a 4 mW He-Ne laser (λ = 633 nm) in back scattering geometry (θ = 173°) was used to perform dynamic light scattering (DLS) measurements. Correlation functions were collected over three minutes, and were converted in particle size distributions via the built in deconvolution analysis package in the Zetasizer software.

3.9.5 Fast protein liquid chromatography

A Superdex 75 10/300 GL column (GE Healthcare) was connected to an ATKA-pure purification protein system pump (GE Healthcare) and was washed for 2 column lengths with water, and then washed with the same buffer as the sample is in. Both of these wash steps were done with column down flow and a flow rate of 0.5 mL/min. Sample were typically injected at 1-2 mg/mL with a sample volume of 300-500 µL into the system. Column pressure was monitored for any fluctuations corresponding to bubbles within the system. UV absorption
was also measured and any observed peaks were fractionated and collected into test tubes.

The column was then cleaned by running two column volumes of water upward through column followed by 1 - 2 column volumes of ethanol, both steps again at 0.5 mL/min. Column was kept in FPLC unit while filled with ethanol until next uses.

3.9.6 Fourier Transform Infrared Spectroscopy Measurements

Fourier Transform infrared Spectroscopy (FTIR) measurements were taken on a Bruker Optik Vertex 70 (Ettlingen, Germany) spectrometer. Typically, 27 µL of solution were added to the top of silicon crystal used for the ATR setup of this instrument. Background scans of the solvent solution were taken over 1200 scans at 2 cm⁻¹ resolution. Sample spectra would be measured from 3000 to 1000 cm⁻¹ wavenumbers, with 600-1000 scans taken depending on the intensity of the signal compared to background. FTIR spectra in the Amide I Band between 1600 to 1700 cm⁻¹ were normalized to either their peak values or their total area under the peak. Difference spectra for aggregate samples were created by subtracting the normalized monomer IR spectrum from the normalized spectrum of the amyloid aggregate sample.

3.9.7 Thioflavin-T Excitation and Emission Spectra

A FluoroMax-4 spectrofluorometer (Horiba) was used for all fluorescence measurements. 15 µM of ThT was added to the sample and was typically excited at 445 nm and emitted at 480 nm. Spectra were measured at room temperature with an average of 5 accumulations taken for each spectra measurement.
3.9.8 Immunostaining

2 µL of sample were spotted on nitrocellulose membranes (Thermo fisher pierce). Membranes were blocked with 7% milk in 1X TBS at room temperature for 1h. Membranes were washed three time by swapping TBS buffer after five minutes each wash. Then membranes were incubated for 18 hours at 40°C with 1:1000 dilution of primary antibody, A11 (Novus biological) A11-19 antibody (gift from Dr. Rakez Kayed). Membrane were then washed again and were incubated in 1:1000 dilution of Ig goat anti-rabbit antibody (Southern biotech) for 1 h at room temperature. After 1 h, membranes were again washed and ECL western blotting substrate (Thermo fisher pierce) were added. Membranes were imaged using the Chemidoc imager (Bio-Rad).

3.9.9 Fluorescence measurements of growth kinetic

Fluorescent kinetic measurements were performed using one of two plate readers: a SpectraMax M5 grating based fluorescence plate reader (Molecular Devices, San Jose, CA) or a Fluorostar Omega plate reader (BMG Labtech, Cary, NC). Typically, 270 µL of sample were added to a well in 96-well glass bottom plates (Cellvis, Mountain View, CA) and monitored at growth temperatures. Typically, fluorescent readings were acquired every 15-20 minutes for several days.

3.9.10 Reducing SDS-Page Gel

Isolated amyloid aggregate samples were prepared. All samples were analyzed using 10–20% gradient Tris-tricine gels (Criterion, Bio-Rad) and a sodium dodecyl sulfate (SDS) running buffer without glycine. 2 mg/ml of sample were mixed with Tricine sample buffer (Bio-Rad) in the
presence of 2% (v/v) 2-Mercaptoethanol (Fisher Scientific) in 1:1 ratio and heated for 5 minutes at 90 °C. 30 µL of each sample was loaded onto precast gels (Criterion 10%-20% Tris-Tricine) and run for about 1.5 hours at 105 V. The gel was fixed in a solution of 40% methanol and 10% acetic acid for 30 minutes, stained for 2 hours using Coomassie blue (G-250 stain, Bio-Rad) for 1 hour, and was washed overnight with DI water.
Chapter 4 - Summary

In Chapter 2, I describe the formation of HEWL oligomers above a concentration threshold, referred to as the “critical oligomer concentration”. This onset of oligomer formation led to a transition from sigmoidal growth kinetics to biphasic. Using these two pathways of aggregation I developed an assay to screen fluorescent dyes for potential selectivity toward oligomers.

Fluorescent dyes are an excellent method of detection for oligomers ex vivo and with possibilities of development for in vivo detection. Some dyes have been reported to be oligomer selective, but in many cases the results were inconclusive, or the dyes required custom synthesis and thus were not readily commercially available (75-77). Using the above kinetic approach, I found a good candidate for an oligomer selective dye in Crystal Violet (CV). Crystal Violet is commonly used in bacterial staining and is readily available. Crystal Violet has similar fluorescence responses to oligomers as the “industry standard” amyloid dye Thioflavin-T, but is significantly less fluorescent when bound to rigid fibrils. This suggests that dual ThT and CV staining would provide complimentary information to help differentiate between rigid fibrils and oligomers. As an example, in ex-vivo staining one could use the membrane permeant variant of ThT, Thioflavin-s (ThS) and CV to stain amyloid plaques in brain tissue. Sections with strong ThT fluorescence but marginal CV fluorescence would likely be mostly composed of rigid fibrils, while sections where the two dyes fluorescence are comparable would likely be composed of oligomers.
Furthermore, I showed that amyloid beta peptide undergoes both sigmoidal and biphasic growth conditions when crossing a concentration threshold in the same manner as HEWL. This implies that the same dye screening assay I performed with HEWL in chapter 2, could instead be extended to amyloid beta and be performed under physiological solution conditions. This is crucial as dye fluorescence is known to have significant solution condition dependence. Fluorescent dyes that appear oligomer selective under this amyloid beta dye screening assay would be excellent candidates for oligomer selective dyes \textit{in vivo}.

Finding methods for oligomer detection, as described in chapter 2, remains linked to the question whether that specific oligomer species is the most disease-relevant polymorph. In chapter 3, I referred to a previously performed cell toxicity experiment by Chamani Niyangoda. She compared the toxicity of HEWL rigid fibrils to the oligomers formed above the COC. In this assay she found a surprising level of toxicity in one of her controls, the rigid fibril supernatant. Based on this toxicity result I confirmed the presence and characterized another distinct type of oligomers forming from HEWL than the previously described oligomers emerging above the COC.

This new polymorph of oligomers differed from those studied in chapter 2 by its level of cytotoxicity but also in its immunostaining response with the oligomer selective antibody A11. Another difference between these oligomers is the type of precipitates formed upon transfer to pH 7. GOs formed precipitates with morphologies that resembled diffusive plaques. Diffusive plaques are found in AD patients but are considered to be relatively innocuous. This is at odds
with the hypothesis that oligomers are always the most disease relevant aggregate population. But the second type of oligomers I characterized had high levels of cell toxicity.

I found this new type of oligomer to be likely composed of dimers and trimers. Previous studies have implicated amyloid beta dimers as the building blocks for toxic aggregates in AD (78-80) and have drawn an inverse correlation between size and an oligomers deleterious effect (67). The size of the species of HEWL oligomers I identified therefore lines up with this hypothesis. The underlying size differences of the two HEWL oligomer species may be a possible reason for the different levels of their cytotoxicity.

In contrast to the oligomers described in chapter 2, this new type of oligomer formed below the COC. I therefore investigated the possible origins of these oligomers. Initially I believed the oligomers were somehow fibril derived, perhaps dissociating off of the fibrils. Several experiments described in Chapter 3 provide evidence that this is not the case. Instead I suggest these oligomers form as monomers reaching a threshold in flexibility. In this system this flexibility threshold is reached due to the progressive hydrolysis of HEWL under our growth conditions. This hypothesis emerged as the measured time delay before oligomer formation matched with the slow progression of HEWL hydrolysis. Additionally, the idea of flexibility as an underlying threshold for amyloid formation is not a new idea for lysozyme amyloidosis. The propensity for human Lysozyme mutants’ propensity to aggregate to the increased levels of flexibility present in disease-relevant point mutants versus their native counterpart (46, 81, 82). Identifying different mechanisms resulting in the formation of distinct oligomer polymorphs is an important step towards understanding the role of oligomers in amyloid assembly and disease.
While other studies showing different types of oligomers have been performed previously (65-70), these often only characterized their aggregates via cell toxicity or in some cases immunostaining. To my knowledge, few studies have characterized these oligomers biophysically or explored the underlying mechanism behind distinct oligomer formation as I’ve done in Chapter 3. Overall, generating different oligomer species, with varied cell toxicity, is an important step towards understanding the mechanisms regulating oligomer formation in the body. Specifically, the solution conditions in cellular compartments not only affect oligomer formation but might affect the type of oligomer species that emerge by modulating the structural flexibility of the monomers. These findings further emphasize how proper characterization, and consequentially proper detection, of oligomer types is necessary if we are to understand the role oligomers play in amyloid disease.
References


17. Mocanu, M.M., et al., Polymorphism of hen egg white lysozyme amyloid fibrils


system neurotoxins. PNAS May 26, 1998 6448-6453; https://doi.org/10.1073/pnas.95.11.6448


73. Yuan Gao, Cong Guo, Jens O. Watzlawik, Elizabeth J. Lee, Danting Huang, Huan-Xiang Zhou, Terrone L. Rosenberry, Anant K. Paravastu. Out-of-register parallel β-sheets and antiparallel β-sheets coexist in 150 kDa oligomers formed by Aβ(1-42). bioRxiv 2020.03.03.974394; doi: https://doi.org/10.1101/2020.03.03.974394.


76. Younan N.D., Viles J.H. A Comparison of Three Fluorophores for the Detection of Amyloid Fibers and Prefibrillar Oligomeric Assemblies. ThT (Thioflavin T); ANS (1-Anilinonaphthalene-8-sulfonic Acid); and bisANS (4,4’-Dianilino-1,1’-binaphthyl-5,5’-disulfonic Acid) Biochemistry. 2015;54:4297–4306. doi: 10.1021/acs.biochem.5b00309.


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