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Amyloid Protein Aggregation and Associated Toxicity

Chamani A. Niyangoda

University of South Florida

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Amyloid Protein Aggregation and Associated Toxicity

By

Chamani A Niyangoda

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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College of Arts and Sciences
University of South Florida

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Keywords: Deep-blue Autofluorescence, Amino Acids, Carbonyl Double Bond, Lysozyme,
Cytotoxicity, Amyloid β Aggregation

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Dedication

To my beloved husband, Kumarasiri

Thank you for being wind beneath my wings
Acknowledgement

It was gratifying and was an absolute privilege to have Dr. Martin Muschol as an adviser, without his mentorship and support this dissertation would not have been possible. I would like to thank my committee members, Dr. Zhimin Shi, Dr. Jianjun Pan, and Dr. Piyush Koria for their time and advice throughout this process. My sincere appreciation also goes to Dr. Laura Blair for taking time out of her busy schedule to chair my PhD committee. I also would like to extend my gratitude to Dr. Ghanim Ullah, for his support during my candidacy. My warm appreciation goes to my fellow graduate students over past several years. Their support over these years has been enormous. I could not have asked for a better team. Last but not least, I would like to express my heartfelt gratitude to my beloved family and friends for their love, support, and encouragement.
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<td>Alzheimer’s Disease</td>
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<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
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<td>PET</td>
<td>Positron Emission Tomography</td>
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<td>HEWL</td>
<td>Hen Egg White Lysozyme</td>
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<td>gOs</td>
<td>Globular Oligomers</td>
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<td>cNP</td>
<td>Nucleated Polymerization with Competing Off-pathway Oligomers</td>
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<td>dbAF</td>
<td>Deep-blue Autofluorescence</td>
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<td>ThT</td>
<td>Thioflavin T</td>
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<tr>
<td>BLG</td>
<td>β lactoglobulin</td>
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<tr>
<td>BSA</td>
<td>Bovin Serum Albumin</td>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
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<td>PLE</td>
<td>Poly-l-glutamic Acid</td>
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<tr>
<td>PLK</td>
<td>Poly-l-lysine</td>
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<tr>
<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<tr>
<td>CV</td>
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Abstract

Amyloidosis is a group of diseases in which amyloid fibrils accumulate and deposit into plaques and intracellular inclusions which lead to disruption of the tissue architecture and function. Most of the amyloid diseases are incurable due to a lack of understanding of the amyloid formation, as well as associated toxicity. My research work is focused on three different aspects of amyloid aggregation.

The aim of the first project is to investigate the potential use of deep-blue autofluorescence (dbAF) as an intrinsic optical probe to study amyloid self-assembly. This novel fluorescence signal is excited at the long wavelength edge of the UV and emits in the deep blue and believed to emerge as a result of protein aggregation. However, we are able to show that dbAF is present at the level of monomeric proteins and even poly- and single amino acids. We are interested in finding the molecular origin of this fluorescence signal and our data implicates the carbonyl double bonds in amino acids as the likely source. Furthermore, we have shown that dbAF is sensitive to both the chemical identity and solution environment of amino acids and has the potential to use as a tool for studying the structure and dynamics of amino acids, proteins and, by extension, DNA and RNA.

The amyloid aggregate species mediate the cytotoxicity associated with many amyloid disorders still remain elusive. In the second project, the focus is to investigate the cytotoxicity of oligomeric vs. fibrillar amyloid aggregates formed by hen egg white lysozyme (HEWL). Both of these amyloid aggregates form macroscopic structures once they are transferred to physiological
media from growth conditions. The HEWL oligomeric species form gel-like clusters similar to diffuse plaques in Alzheimer’s patients and show no significant toxicity in cell culture experiments. Conversely, fibrillar plaques with the ordered internal structure are analogous to neuritic plaques and induced toxic effects to the cells. However, interestingly, the most toxic effect is observed from the background solution of the fibrillary aggregates. Our data reveal that these background solutions contain amyloidogenic oligomeric species which differ from monomer derived oligomers in origin, structure, and toxicity.

The third project is focused on studying the mechanisms of amyloid β peptide aggregation associated with Alzheimer’s disease. Previously we have shown that amyloid aggregation occurs in two different pathways, oligomeric and oligomer-free for two different amyloid proteins. Using amyloid β 1-40 and 1-42 peptides, we show a sharp transition in the kinetics of amyloid assembly as a function of peptide concentration, from oligomer-free to oligomer-dominated rigid fibril formation under near-physiological conditions.
Chapter 1: Introduction

The diseases associated with amyloid formation have become a major healthcare problem in the modern world. These diseases arise from conversion of disease-specific proteins and peptides from their functional soluble state into highly organized fibrillary aggregates known as amyloid fibrils [3, 6-8]. Amyloid diseases can be categorized into neurodegenerative conditions, (Alzheimer’s disease, Parkinson’s disease) non-neuropathic systemic amyloidoses (AL amyloidosis, Lysozyme amyloidosis) and non-neuropathic localized diseases (type II diabetes) [8]. Interestingly, there is growing evidence indicating that amyloid formation is an intrinsic property of almost all peptide chains and can be beneficial to cells in some instances [8-12]. However, comprehensive understanding of amyloid aggregation on the molecular level remains incomplete due to the complex, heterogeneous and transient nature of the various types of aggregates forming inside the living organisms. Therefore, many scientific disciplines have come together to understand the phenomenon of amyloid aggregation and to develop treatments to suppress and reverse amyloid diseases.

An important step towards that goal is to investigate the underlying aggregation mechanisms and the pathogenic actions of these various aggregates. The most widely used techniques for studying amyloid self-assembly include fluorescence spectroscopy, immunostaining and high resolution imaging. Fluorescence spectroscopy is used to monitor the aggregation kinetics of amyloid aggregation in vitro while immunostaining and imaging are used to characterize structure and morphology of the amyloid aggregates. Although there is rapid
advancement in the amyloid field over the last couple of decades, it is still challenging to study the real time amyloid aggregation where monomeric proteins convert to highly ordered amyloid fibrils via different metastable intermediates *in vivo* as well as *in vitro*. Even Thioflavin T, an extrinsic amyloid fluorescence dye which is considered the “golden standard” for selectively staining amyloid aggregates can interfere with amyloid aggregation kinetics [13]. Therefore it is critically important to develop non-invasive, quantitative spectroscopic assays that can provide more insight into the aggregation process. Recently, an intrinsic fluorescence signal was reported which is excited near the long wavelength edge of UV and emits in the deep blue and was shown to be associated with amyloid formation [14]. This drew attention in the amyloid field as a potential candidate for a non-invasive, optical assay to monitor amyloid self-assembly kinetics.

The first part of my dissertation is focused on investigating the origin of this new fluorescence signal, which we named deep blue autofluorescence (dbAF), determine its photophysical/photochemical properties and explore its use for studying amyloid aggregation. We monitored amyloid aggregation of hen egg white lysozyme (HEWL), a model amyloid protein using this fluorescence signal. Interestingly, we discovered that dbAF is actually present at the monomeric level of proteins/peptides and is enhanced in the aggregated states. We further investigated the origin of dbAF by following this signal down to the single amino acids and their associated chemical moieties such as carbonyl, carboxyl and amide groups. At the end of this chapter, I will discuss potential utilities of dbAF for protein biophysics.

The second part of my dissertation aims to characterize the cytotoxicity of oligomeric vs. fibrillar amyloid aggregates. The toxicity of different amyloid aggregates and their molecular mechanisms of cell damage has been the subject of intense research for many years [15-18]. Identifying the predominant pathogenic species associated with amyloid diseases is challenging
due to the transient nature particularly of early stage intermediates during amyloid aggregation [19-21]. Initially, late-stage fibril plaques present in the affected tissues were considered to be the primary cause for cellular death [22, 23]. However, a growing body of research has implicated metastable oligomeric intermediates as the culprit of cytotoxicity while fibrils are often considered comparatively inert end products of amyloid assembly [15, 16]. As a result, the aggregate species involved in mediating the cytotoxicity associated with many amyloid disorders still remain elusive.

In this study, we investigated the cytotoxicity of both fibrillar and oligomeric amyloid aggregates formed by hen egg white lysozyme (HEWL). HEWL readily forms different amyloid aggregates under various destabilization conditions such as acidic pH and elevated temperatures. In this chapter, I discuss how the transfer of these aggregates from non-physiological growth conditions to physiological cell media affects their state of assembly and their subsequent effects on cell viability of the cells.

The third part of my dissertation is focused on studying the mechanisms of amyloid β peptide aggregation. The hallmark of Alzheimer’s disease is the accumulation of fibril plaques in the brain that predominantly consist of amyloid β, a peptide generated as a result of proteolytic cleavage of the membrane-bound amyloid precursor protein (APP) [24, 25]. Previously we have shown that the onset of oligomer formation in two different amyloid proteins induces a sharp transition from the sigmoidal kinetics of nucleated polymerization to biphasic kinetics with the early phase related to oligomer formation and the second phase indicating fibril nucleation and growth [5]. The main focus of this part of my dissertation is to confirm that amyloid assembly by Amyloid β obeys the same type of two-pathway oligomer vs. fibril assembly. To unravel these aggregation mechanisms, I measured aggregation kinetics as function of peptide concentration using fluorescence spectroscopy. A detailed characterization of different aggregate morphologies
emerging at different time points of the reaction was performed with high resolution imaging (transmission electron microscopy, atomic force microscopy).
Chapter 2: Background

2.1 Amyloidosis

Amyloidosis constitutes a large group of diseases caused by extracellular and/or intracellular deposition of insoluble, highly structured amyloid fibrils in tissues and organs [1, 26, 27]. Approximately 50 different proteins and peptides, each with a different primary sequence, structure and functions, are known to self-assemble into amyloid fibrils [1]. Amyloidosis can either be localized or systemic. In localized amyloidosis, the intracellular and/or extracellular amyloid deposits occur from proteins and/or peptides expressed by the cells at the deposition site [28]. The most common forms of localized amyloidosis are neurodegenerative disorders such as Alzheimer’s disease (AD), Parkinson’s disease and Huntington’s disease. In contrast, systemic amyloidosis are extracellular, and proteins systematically precipitate after being produced at a site distinct from the deposition site [27, 29]. In the case of systemic amyloidosis, multiple organ systems can be affected and most common examples are AL amyloidosis (immunoglobulin light chain amyloidosis) and familial amyloidosis.

Amyloidosis are challenging to study and diagnose due to the systemic nature and indistinct symptoms which can be misdiagnosed easily. Up to now, only transthyretin amyloidosis can be treated effectively while all amyloid diseases essentially being incurable and having a significant social, medical and economic impact [30]. According to the world Alzheimer report in 2018, it is
estimated that 152 million people will be affected by AD in 2050 [31]. There are approximately 4000 new cases of AL amyloidosis reported annually in the United States only and actual occurrence being much higher due to under-diagnosis [32]. Many of these diseases are no longer rare and it is critically important to understand the process of amyloid formation and to develop therapeutic interventions to prevent and cure amyloid diseases.

2.2 Amyloid Fibrils

It took about four centuries after the first observation of amyloid deposits which was described as lardaceous liver and ‘white stone’-containing spleen, for scientists to obtain the structural information of the amyloid fibril in atomic details [1]. The biophysical methods used for studying the structural features of mature amyloid fibrils include solid state nuclear magnetic resonance (NMR) [33, 34], X-ray fiber and powder diffraction [35-37], cryo-EM [38, 39], site-directed spin labeling [40, 41], and proline-scanning mutagenesis [42]. To date more than 50 different proteins and peptides have been identified to form amyloid fibrils associated with human diseases. Despite the structural differences between precursor proteins, amyloid fibrils share a remarkably similar underlying architecture. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) revealed that amyloid fibrils usually consist of typically 2-6 protofilaments twisted together to form rope-like fibrils with a diameter of 7 – 13 nm [43, 44]. Amyloid fibrils have a common core structure known as cross β sheet structure, where β strands aligned perpendicular to the fibril axis and hydrogen bonds between β strands running parallel/antiparallel to the axis [45, 46]. The overall structure of a mature fibril involves stacking of β strand into extended β sheets which come together to form a compact dehydrated interface named steric zipper. Due to the fixed length of the interstrand hydrogen bonds, the spacing between β strands within a β sheet is 0.47 – 0.48 nm.
This well-defined structure leads to the characteristic x-ray diffraction pattern which was first observed by biophysicist William Astbury in 1935 [35]. As shown in figure 2.1, the sharp reflection at 0.48 nm, directed along the meridional direction reflects the \( \beta \) strands running perpendicular to the fibrils. The additional diffuse reflection in the equatorial direction at 0.8 – 1.0 nm, is attributed to the \( \beta \) sheets spacing within the fibrils [7].
This highly ordered structure of amyloid fibrils gives rise to the apple-green birefringence under polarized light when stained with Congo red dye, which is considered the histological gold standard for confirming amyloids in vivo and in tissue sections [47].

**Figure 2.1 Structure of amyloid fibrils:** (a) The characteristic cross-\(\beta\) diffraction pattern observed when X-rays are directed on amyloid fibers. (b) Five layers of \(\beta\) strands of the steric zipper structure of the sequence segment GNNQQNY from the yeast prion Sup35. The arrow marks the fibril axis. (c) Two layers of \(\beta\)-sheets come together to create a compact dehydrated interface of steric zipper (image source Eisenberg and Jucker. Cell, (2012))

### 2.3 Amyloid Aggregation

The cause for the onset of amyloid formation is not fully understood. Recently scientists have identified a number of proteins that can form amyloid aggregates in bacteria, fungi, insects, invertebrates as well as in humans that perform physiological roles [9, 11, 48]. The amyloid fibrils formed by the curli protein which is used by *Escherichia coli* to colonize inert surfaces and mediate binding to host proteins is one of the examples of functional amyloids [2]. These observations suggest that formation of amyloids is not just a rare phenomenon associated with amyloid diseases, but an inherent property of proteins or peptides utilized by biological systems.
Understanding the molecular mechanisms of amyloid self-assembly and formation of potentially pathogenic aggregates in the process is critical for unraveling the ability of amyloids to act as either functional biological or pathogenic entities. This requires the identification of all the intermediate, conformational states assumed by monomeric proteins during the assembly process and determination of the thermodynamic and kinetic processes involved. Particularly, the identification and characterization of dynamic, metastable intermediates or oligomeric species has gained strong emphasis in the field due to the critical role these species play in the pathogenesis of amyloidosis.

2.3.1 Amyloid Formation Occurs via a Nucleated Growth Mechanism

It is widely accepted that amyloid fibril formation occurs via nucleated polymerization [49, 50]. The classic nucleated polymerization reaction has a sigmoidal reaction time course which includes a lag phase that is followed by a rapid exponential growth phase. At the end, a plateau phase develops owing to the decline in reaction rate by the depletion of reactants. In the case of amyloid formation, a spontaneous nucleation process (primary nucleation) is required to form a stable nucleus from partially denatured monomeric proteins. However, the formation of a nucleus is energetically unfavorable. The lag phase in the amyloid formation kinetics reflects the time required for sufficient numbers of primary nuclei to form. The rapid upswing, in turn is related to secondary nucleation processes such as fibril fragmentation and secondary nucleation on surfaces [3, 51-53]. The fibril fragmentation increases the number of fibrils ends which lead to rapid growth through adding monomers to the ends, and can therefore result in accelerated growth. Similarly, secondary nucleation in which fibril surfaces act as nucleation sites leads to an exponential proliferation of fibrillar species. It is also possible to significantly shorten or completely abolish the lag period by “seeding”, i.e. by adding pre-formed fibrillar species to a monomer sample [54].
The process of seeding eliminates the requirement to overcome the primary nucleation barrier, with the rate of the reaction only limited by fibril elongation.

2.3.2 Oligomeric Intermediates of Amyloid Assembly

For years, scientists believed that extracellular amyloid fibrils were the main toxic species associated with amyloid disorders [4, 23]. More recent data suggested the idea of pre-fibular aggregates or metastable, transient oligomeric species mediating the toxicity in amyloidosis [15, 55]. Significant efforts have been directed towards identifying, characterizing and isolating intermediate oligomeric species not only because of their likely toxicity but also to understand the basic mechanisms of amyloid fibril formation [15, 56].

Many amyloid systems including α-synuclein [57], amylin [56], the immunoglobulin light chain [58], transthyretin [59], polyQ-containing proteins [56], β2-microglobulin [60], equine lysozyme [61], and hen egg white lysozyme (HEWL) [62] are known to form oligomers with globular structure of 2-5 nm (gOs) and polymeric assemblies of oligomers termed protofibrils or curvilinear fibrils (CFs). These oligomeric and protofibril structures can bind Thioflavin T and Congo red dyes and also contains extensive β sheet structure. Obtaining structural information of gOs by NMR and spectroscopy and X-ray crystallography is difficult due to the heterogeneity in their structure, their transient nature and variable stoichiometry. However, Laganowsky et.al. were able to elucidate the X-ray crystallographic structure of oligomers formed by an 11 residue fragment of αβ. It is composed of 6 β-strands that form a twisted antiparallel β-sheet that closes back on to itself to form a cylinder [63]. In more recent work, Sangwan et.al. also determined the structure of oligomers assembled by an 11-residue fragment of superoxide dismutase 1 [64]. So
far, scientists have not being able to elucidate high resolution structure of gOs formed by full-length amyloid proteins or peptides.

Generally, small gOs and CFs precede the assembly of late stage amyloid rigid fibrils [21, 65]. But how these metastable early stage aggregates are replaced by the late stage amyloid fibrils is still not clear. Oligomeric aggregates could affect RF formation in several ways; oligomers could be on-pathway precursors that are obligatory for RF assembly; oligomers could be off-pathway precursors that are incapable of converting into RF and act as competitors to RF nucleation and growth; or oligomers could have an inhibitory effect on RF assembly. Distinguishing these scenarios is critical for understanding amyloidogenesis and to reverse or prevent amyloid formation. However these scenarios are difficult to distinguish experimentally since they result in identical temporal sequences of aggregate populations and growth kinetics.
Chapter 3: Carbonyl Based Autofluorescence of Proteins and Amino Acids

3.1 Introduction

Intrinsic protein fluorescence is among the most wildly used technique in protein characterization and research due to its non-invasiveness, high sensitivity, and ease of application [66, 67]. However, until recently, intrinsic protein fluorescence was limited to the near-UV autofluorescence arising from aromatic amino acids, mainly tryptophan and, to a lesser extent, tyrosine and phenylalanine [68]. The molecular origin of this fluorescence signal is the delocalized electron states of indole, phenyl and benzene residues in these three amino acids respectively. The molar absorptivity and high quantum yield, combined with the intrinsic sensitivity of tryptophan fluorescence to its environment, has made tryptophan a widely used optical probe for measuring numerous aspects of protein structure and dynamics [66, 69]. Its applications range from routine determinations of protein concentrations via UV absorbance, [70] over monitoring of protein unfolding from conformation dependent Stokes shifts, [71] to recent investigations of peptide dynamics from tryptophan triplet-state quenching rates. [72, 73]. However, this fluorescence probe is limited to the proteins and peptides that contains aromatic amino acids.

Over the past decade, there have been reports of a different protein autofluorescence, independent from aromatic residues and emerging upon formation of protein aggregates such as crystals, spherulites, and amyloid fibrils [74-77]. This fluorescence signal is excited near the edge of the UV (370 nm) and emits in the visible range (450 nm), which we refer to as deep-blue
autofluorescence (dbAF). The first observation of dbAF was reported by Shukla et al [77] from gamma-II crystalline precipitates and hen egg white lysozyme crystals. Later on del Mercado et al [74] showed a similar fluorescence signature arise from amyloid-like nano-fibrils made of elastin-related polypeptide poly(ValGlyGlyLeuGly). Measured lifetimes in the nanosecond range established that this signal arose from a resonant fluorescent emission instead of some inelastic scattering process [75]. A few different hypotheses were set forth to understand the molecular origin of this novel dbAF as it could be a promising optical tool to study protein structure and aggregation. Shukla et al [77] hypothesized that electron delocalization through intramolecular or intermolecular hydrogen bond formation during aggregation resulted in these long-wavelength electronic transitions. This hypothesis was supported by the fact that all the protein aggregates exhibiting dbAF contained high proportions of β sheet structures.

Recently, few groups have shown that dbAF is also present in amyloid fibrils formed by proteins such as amyloid β, α-synuclein, tau and lysozyme [14, 75, 78-80]. Despite differences in the native structure and the amino acid sequence of the constituent proteins, all amyloid fibrils are composed of an array of β sheets with strands running perpendicular to the fibril axis [7, 81]. This universal “cross-β” core structure consists of hydrogen bonds along the β sheets and parallel to the fibril axis [82]. It has been suggested that dbAF arises from the electron delocalization of intermolecular hydrogen bond network along the fibril [75, 79].

This observation drew attention in the amyloid field as dbAF could be used to monitor amyloid aggregation in a non-invasive manner. An intrinsic indicator of amyloid assembly could avoid some of the limitations of extrinsic amyloid dyes such as ThT or Congo red, since the latter dyes can interfere with the very aggregation process they are meant to monitor [13]. The presence of dbAF upon assembly of native proteins into protein crystals, however, casts some doubt on the
purported one-to-one relation between amyloid formation and dbAF. We therefore considered both the origin of this dbAF and the molecular processes that dbAF reports to be open questions. The main focus of this project was to investigate the molecular origin of this novel fluorescence signal and critically evaluate its potential as intrinsic indicator of amyloid fibril assembly.

3.2 Deep-blue Autofluorescence (dbAF) in Globular Proteins

Our preliminary findings suggested that precursors to dbAF already existed prior to protein aggregation. Specifically when we monitored the amyloid assembly by hen egg white lysozyme (HEWL) using dbAF, we noticed a weak intrinsic fluorescence was already present prior to the onset of aggregation. We therefore investigated whether this weak fluorescence was a feature common to monomeric proteins other than HEWL and, if so, what the underlying molecular substrate of this signal might be. Figure 3.1 shows the fluorescence excitation and emission spectra for HEWL (Fig 3.1 (a)), β-lactoglobulin (BLG, Fig 3.1 (b)) and bovine serum albumin (BSA, Fig 3.1 (c)) protein monomer solutions. Each of these globular proteins is known to form amyloid-like aggregates [83-85]. Interestingly, excitation and emission spectra for all three proteins exhibit similar spectral features, including an emission peak around 440 nm and excitation maxima ranging from 335 nm to 390 nm. Protein-specific differences included the intensity of dbAF for different proteins, the wavelengths for peak emission and peak excitation as well as the overall width of the excitation and emission spectra. To confirm that the observed fluorescence indeed arose from proteins, we checked background fluorescence from all buffer solutions (Fig 3.1 (a)). We excluded the potential contamination of our protein samples from small fluorophores by extensively dialyzing protein samples in Millipore water and comparing dbAF spectra of concentration matched samples before and after dialysis. As shown in figure 3.2, both before and
after dialyzing dbAF spectra remained within a few percent of each other. It is also possible that this dbAF signal is actually a measurement artifact from second-order diffraction peaks of the spectrofluorometer gratings or Rayleigh light scattering of protein molecules in the samples. To exclude these possibilities we placed high-Q optical filters in both excitation (360/20 nm) and emission (395 nm long pass) paths and were able to preserve the dbAF spectra.

![Deep-blue autofluorescence (dbAF) spectra of globular proteins](image)

**Fig 3.1. Deep-blue autofluorescence (dbAF) spectra of globular proteins.** Fluorescence excitation and emission spectra for deep-blue autofluorescence (dbAF) from (A) hen egg-white lysozyime (HEWL) (B) β-lactoglobulin (BLG) and (C) bovine serum albumin (BSA), all at 50 mg/ml. The dashed line near the bottom in (A) is the buffer background spectrum with its associated Raman peak. DbAF peak positions reported for the weaker HEWL emissions are likely distorted by contribution from the buffer Raman peaks we couldn’t fully correct for. Spectra in (A-C) used identical spectrofluorimeter settings and all spectra were collected at pH 7 (20 mM HEPES).(adapted from Niyangoda et al 2017)

### 3.3 Does deep-blue Autofluorescence (dbAF) arise from Protein Monomers?

Since dbAF emission is known to be present in protein aggregates, we took precautions to minimize aggregation in our experiments. First, all the solution conditions we used for these experiments are within the solubility limits of a given protein. For example hewL at pH 7 and ionic strength at 50 mM is soluble to at least 200 mg/ml, a value that increases even further at pH 2 used
for measurements in fibril growth buffers [86]. All the samples were filtered through 200 nm and 50 nm pore size syringe filters prior to all measurements to avoid any large non equilibrium clusters in the lyophilized protein solutions. Dynamic light scattering was used to further confirm the absence of any large aggregates in the samples used for the experiments. Figure 3.2 (b) shows the particle size distribution of the HEWL samples used to measure dbAF spectra. Folded proteins require heating past the onset of thermal denaturation to form amyloid aggregates [87]. Therefore to further reduce the risk of aggregation under our measurement conditions, all the measurements were performed at room temperature (with the exception of the thermal denaturation experiment).

**Fig 3.2. Comparison of dbAF spectra for HEWL before and after dialyzing.** (a). HEWL was dialyzed in 2 liters of Millipore water with 4 changes during a 2 day period. The dbAF spectra was measured before (red solid line) and after (black dashed line) at near identical concentrations. The concentrations of before and after dialyzing samples were 10mg/ml and 10.3 mg/ml respectively. (b) Particle size distribution of both before (red solid line) and after (black dashed line) dialyzing HEWL samples shows a single peak centered around 2 nm which corresponds to the expected radius of the HEWL monomers. (adapted from Niyangoda et al 2017)

The dbAF emission as a function of monomer concentration was mapped out for HEWL and BLG (Fig 3.3 (a, b)). As shown in Fig 3.3 (c), both proteins showed a linear increase of dbAF
intensity over two orders of magnitude in protein concentration. The lowest concentration used for BLG was 0.5mg/ml and it was five times above the buffer background. Similarly, dbAF of 2 mg/ml of HEWL solution was three times higher than the background. Importantly, the observed linear relationship of dbAF as a function of monomer concentration argues against the formation of small oligomers as the possible origin of the dbAF. The presence of oligomers in the monomer solutions would result in a sublinear increase in dbAF due to the associated binding equilibria.

![Fig 3.3. dbAF as a function of monomer concentration: dbAF emission of (a) BLG and (b) HEWL dissolved in 20 mM HEPES buffer at pH 7. Excitation was fixed at 356 nm and 389 nm respectively. Buffer background is represented in dashed line. (c) Log-log plot of the concentrations dependence of dbAF peak emission for BLG and HEWL, expressed as multiples above their buffer background $F_0$. The solid lines are fits with a linear function through the data. (adapted from Niyangoda et al 2017) (a) (b) (c)](image)

To determine whether minor populations of small protein aggregates, if present, could explain the observed dbAF emission, we determined dbAF intensities from fully aggregated HEWL amyloid fibrils. HEWL fibrils were formed by incubating HEWL monomer sample at elevated temperatures (50$^\circ$C) under acidic conditions (pH 2). HEWL fibril growth was confirmed using ThT and transmission electron microscopy (TEM). Fibrils were isolated from their monomer
background by three consecutive centrifugation/resuspension steps. As shown in Fig 3.4 A, isolated HEWL fibrils generated a significant but still moderate 25-fold increase in dbAF emission over their equivalent monomeric background. If aggregates are indeed responsible for dbAF emission, to account for the observed fluorescence, at least 4% of our monomer sample should be contaminated with aggregates. DLS confirmed that our monomer samples does not contain large aggregates. However, it is possible that dimerized monomers, which are not detectable using DLS, can give rise to dbAF. When considering the lower number of intermolecular bonds in dimers vs. fibrils, which are believed to underlie fibrillar dbAF emission, the fraction of required preexisting aggregates to account for dbAF emission from monomeric solutions would increase even further. In a previous publication we have shown that static light scattering from the same hewL stock, over the same range of concentrations, extrapolates to the proper molecular weight for lysozyme monomers (14.3 kDa) and dynamic light scattering yielded polydispersity indices as low as 0.15 or less [62]. We therefore estimate that our solutions could contain at most one percent of protein dimers, with even lower values when allowing for larger oligomers. For the above reasons, preexisting aggregates can not account for the dbAF emissions from our monomeric hewL solutions. Figure 3.4 B shows the superposition of dbAF spectra for HEWL fibrils and monomers. The similarities of these two spectra suggest that the molecular moiety underline dbAF is already present in the monomer state of the proteins.

Two prior reports provide compelling evidence of dbAF emission from monomeric proteins. Kallman et al. showed the presence of dbAF from collagen [88]. More recently, Guptasarma reported similar dbAF fluorescence from γ crystalline and lysozyme [89]. Both agree well with our observations. Therefore, there is mounting evidence from multiple laboratories that monomeric proteins of various types and structures emit a deep-blue autofluorescence.
3.4 DbAF is present in poly-amino acids and single amino acids

So far dbAF was believed to arise as a result of aggregation. However, the presence of dbAF emission of the monomeric proteins raised the question, what is the moiety underlying dbAF? Are native fold or are well-defined secondary structure elements such as α-helices or β-sheets prerequisites for dbAF emission? We wanted to explore how far below the structure of folded proteins one could go while preserving dbAF emission. To answer these questions, we investigated the dbAF emission of two poly-amino acids, poly-l-glutamic acid (PLE) and poly-l-lysine (PLK) which are known for assuming random-coil conformations at neutral pH [90, 91]. As figure 3.5 (a) shows, despite the slight differences in peak emission wavelength and with significantly different amplitudes at the same concentrations, both PLE and PLK emitted dbAF when excited at 370 nm. This observation suggest that dbAF is intrinsic to polypeptide chains themselves and does not require a native fold or a well-defined secondary structure. Also dbAF emission from these simple polypeptides devoid of any aromatic residues reconfirms that dbAF is not dependent on aromatic ring structures which typically are associated with protein autofluorescence.

The presence of dbAF in polyamino acids suggests the weak electron delocalization from peptide bonds of polypeptides as another possible source of dbAF. It has been shown that conjugated systems with alternating double and single bonds support fluorescence of synthetic polymers and aromatic compounds in the visible range [92]. The partial double-bond character of peptide bonds might therefore provide the substrate for dbAF emissions. If so, one would expect to observe the absence of dbAF from substances without peptide bonds such as single amino acids. Nevertheless, to our surprise, dbAF emission was present in all of a series of amino acids we investigated. This included charged, hydrophobic and aromatic amino acids which all showed
weak but clearly detectable dbAF emissions of varying intensities and with emission peaks near 440 nm (Fig 3.5 (b)). The significantly stronger dbAF signal from tryptophan and phenylalanine (Fig 3.5 (c)) further suggests that side chains can couple into and enhance emission from the excited state relevant for dbAF.

We also find that crystals of amino acids emit bright dbAF when visualized in epifluorescence using standard 4’, 6-diamidino-2-phenylindole (DAPI) fluorescence filters. Figure 3.6 shows bright field and epifluorescence images of 8 amino acid crystals. Interestingly, the intensity emitted from different amino acids in solution strongly correlates with the relative dbAF fluorescence emitted from the corresponding amino acid crystals (Fig 3.7). The prominent variation in dbAF emission intensities and wavelengths with amino acid identity, whether measured in solution or in the solid state, suggests that dbAF emission is predominately sensitive

![Image](https://via.placeholder.com/150)

**Fig 3.4. Comparing dbAF emission from lysozyme fibrils and monomers.** (a) Quantitative comparison of dbAF emission spectra from hewL monomers and purified hewL fibrils in pH 2 buffer, 100mM salt measured at room temperature. Spectra are shown on a semi-log scale to facilitate comparison of relative enhancement. The control monomer spectrum was taken with hewL stock freshly dissolved in fibril growth buffer and concentration matched to the pure fibril sample. (b) Superposition of dbAF emission spectra for hewL amyloid fibrils and monomers, measured at room temperature in fibril growth buffer. Spectral amplitudes were normalized to their peak emission values. (adapted from Niyangoda et al 2017)
to the amino acid’s chemical structure (residue), irrespective of the specific environment they are in.

3.5 Carbonyls are the Likely Source of dbAF Autofluorescence

The presence of dbAF in single amino acids indicates that the chemical structure responsible for dbAF is not the peptide bond. Therefore we considered the carbonyl bond in amino acids as a possible source of electron delocalizing underlying autofluorescence. To explore this possibility we looked for dbAF fluorescence from simple organic compounds with carbonyl groups: formaldehyde (H₂CO), acetone ((CH₃)₂CO) and acrylamide (C₃H₅NO). All three compounds do contain the carboxyl groups present in amino acids. Intriguingly, all three chemical compounds showed similar fluorescence signatures to dbAF (Fig 3.8). The excitation and emission peaks for acetone and formaldehyde were blue-shifted between 40–70 nm compared to those of

Fig 3.5. DbAF from polyamino acids and individual amino acids. (a) DbAF emission spectra (370 nm ex.) for (A) poly-l-lysine (PLK) and poly-l-glutamic acid (PLE) at 10 mg/ml each and at pH 7 (50 mMHEPES). (b) DbAF emissions from eight different non-aromatic amino acids (indicated by their standard single-letter code) at 20 mg/ml each and at pH 2 (25 mM phosphate buffer). All spectra in (b) were buffer subtracted. (c) Same as (b) but for the aromatic amino acids tryptophan (W) and phenylalanine (F), which display much larger dbAF (see scale in b vs. c). (adapted from Niyangoda et al 2017)
amino acids and proteins. However fluorescence spectra from acrylamide, which contains an amide group next to its carbonyl group, were noticeably more similar to the previously observed dbAF fluorescence. This suggests that the addition of the amide group near the carbonyl has the ability to shift fluorescence properties of the carbonyl groups significantly.

At a minimum, the above data indicate that small organic molecules with carbonyl groups, at high concentrations, are capable of fluorescence emission in the deep blue. In addition, the details of the molecular structure surrounding the carbonyls clearly affected fluorescence intensities and associated spectral properties. This is likely to contribute to the large difference in emission from formaldehyde vs. acetone, particularly when accounting for their difference in molar concentrations (170 mM for acetone vs. 10 M for formaldehyde). To confirm the important role of carbonyls as the structural element underlying this autofluorescence, we looked for intrinsic fluorescence from methanol (H$_3$COH) and isopropanol (CH$_3$)$_2$CHOH, in which the carbonyl in formaldehyde and acetone is replaced by a hydroxyl group. This nearly abolished autofluorescence from either compound. These observations indicate that one should expect to find intrinsic fluorescence in the deep blue from any molecule containing carbonyl groups, including proteins and amino acids. At the same time, the residues surrounding the carbonyl groups in these small organic molecules have significant effects on the absorption and fluorescence excitation spectra and their spectral overlap. This matches well with the variable dbAF intensities from amino acids, as shown in Fig 3.5 B, C.

Interestingly, the intrinsic fluorescence of acetone and formaldehyde are well established and have been utilized for some time for remote sensing in nonreactive and reactive flows [93, 94]. Corresponding quantum-mechanical calculations of acetone absorbance and fluorescence properties, particularly in solution, have become available recently, as well [95].
Fig 3.6 Brightfield (top row) and epifluorescence (bottom row) images of eight different amino acid crystals: Amino acids are identified by their single-letter code added to their brightfield images. Exposure times for epifluorescence images decreased from 20 s (A) to 5 s (G, V, P, R), 2 s (I) and 0.5 s (W, F). For display, grayscales of fluorescence intensities were adjusted. Amino acid stock was identical to that used for measuring dbAF spectra in solution. Scale bar in all images: 50 μm. (adapted from Niyangoda et al 2017)
Fig 3.7 Correlation of average dbAF intensities from amino acid crystals vs. corresponding solution spectra: Amino acids are indicated by their standard single-letter code. (adapted from Niyangoda et al 2017)

Fig 3.8 dbAF of organic solvents with and without carbonyl groups. Absorption, fluorescence excitation and emission spectra of (a) formaldehyde (CH$_2$O) vs. methanol (CH$_3$OH) at 10 M concentrations. (b) 170 mM acetone (CH$_3$)$_2$CO vs. undiluted (13.1 M) isopropanol (CH$_3$)$_2$CHOH and (c) Fluorescence excitation and emission spectra of 700 mM acrylamide (C$_5$H$_8$NO) (d) Absorption, excitation and emission spectra from 1.3 M proline at pH 7 (20 mM HEPES), buffer subtracted. Excitation spectrum of acetone was distorted due to strong inner filtering from UV absorption. Gaps in spectra in (a) are due to removal of large solvent Raman peaks. Large water Raman peak was subtracted in (c) (adapted from Niyangoda et al 2017)
Autofluorescence of polyacrylamide gels with similar characteristics as those in Fig 3.8 C have also been noted. We are not aware of any comparable theoretical or experimental reports of dbAF emission from amino acids without aromatic residues. Following our identification of carbonyls, we found one reference 50 years back implicating carbonyls as source of collagen fluorescence, but without proof of its origin at the single amino acid level or any detailed physiochemical characterization [88]. This earlier observation was apparently discredited as artifact of UV-induced photoproducts of collagen that can be phosphorescent [96]. As we point out above, the wide variety of proteins, polypeptides and single amino acids are exhibiting this fluorescence, and its close association with both carbonyl double bonds and its congruence with aggregation-induced fluorescence suggest that this earlier observation was likely correct. The spectra in Fig 3.8 reveal another feature of dbAF that seems to be shared by proteins, amino acids and organic solvents with carbonyls: there is a noticeable offset between the optimal wavelength for photon absorption vs. fluorescence excitation, as shown here for proline (Fig 3.8 (d)) formaldehyde (Fig 4.8 (a)) and acetone (Fig 3.8 (b)). This suggests that the most favorable wavelength for photon absorption excites electrons into states above the lowest excited state and, therefore, might undergo non-radiative energy loss prior to emission. Hence, fluorescence emission will depend on the spectral overlap between the fluorescence excitation spectra with the molar absorptivity. In the case of proteins, the weak absorbance associated with dbAF does not result in a distinct peak but seems buried underneath residual absorbance from aromatic residues (Fig 3.9). Such displacements between maximal absorbance and optimal fluorescence excitation are common for other small molecules, such as the indole moiety of tryptophan [97]. At the same time, strong fluorophores such as quinine only have a minimal shift between their (fluorescence-associated) absorbance and fluorescence excitation.
Hence, this spectral feature of dbAF provides some additional support for carbonyls as the shared molecular substrate for dbAF across these otherwise very different molecules.

3.6 Quantum-yield and molecular extinction coefficients of dbAF

As step toward further characterization of the photophysical properties of dbAF from proteins and amino acids, we determined the molar extinction coefficient and quantum yield for proline and for BLG. As reference standard for quantum yield in the wavelength range near dbAF we used quinine sulfate with a quantum yield of 0.54 [98]. To account for the shift in maximal absorption vs. excitation of dbAF spectra in the determinations of quantum yields, we used the molar extinction coefficients ε at the peak of the fluorescence excitation spectrum which, for proline in pH 7 buffer, occurs at 350 nm and for BLG occurs at 357 nm. Plots of fluorescence emission vs absorbance for BLG and proline used to extract the slopes for BLG and proline are shown in Fig 3.10 (a), B. The dbAF quantum yields QY we obtained for proline and BLG at pH
7, listed in Table 3.1, are very similar and in a range only about one order of magnitude below common fluorophores.

Table 3.1. Molar extinction coefficient $\varepsilon$ and quantum yield (QY) for dbAF from BLG and proline at pH 7

<table>
<thead>
<tr>
<th></th>
<th>$\varepsilon$ (mol$^{-1}$cm$^{-1}$)</th>
<th>Quantum Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-lactoglobulin (BLG)</td>
<td>43.3 (357 nm)</td>
<td>0.011</td>
</tr>
<tr>
<td>Proline (Pro)</td>
<td>0.036 (350 nm)</td>
<td>0.012</td>
</tr>
</tbody>
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In contrast, the molar extinction coefficient of both BLG and proline are significantly smaller than for typical fluorophores (e.g. tryptophan’s molar extinction coefficient is 5,690 M$^1$cm$^{-1}$). Yet, the small molar extinction coefficients and the fluorescence emission in the visible would be compatible with nonbonding to antibonding n-$\pi^*$ transition. Guptasarma [89] reported that dbAF excitation in $\gamma$ crystallin was associated with a CD signal. While this author guardedly associated the chirality with hydrogen bonding within the protein’s secondary structure, it might instead arise from the chirality of n-$\pi^*$ transitions in carbonyl compounds [99]. Such n-$\pi$ transition have also been implicated in the fluorescence from various aldehydes [100]. There is a noticeable difference in the molar extinction coefficients of BLG and proline, even after multiplying $\varepsilon$ for proline by 162, i.e. the number of amino acids in BLG. However, uncertainty in the absolute values of amino acid concentrations and variability in dbAF emission intensities might contribute to the observed discrepancies. Additional considerations compel us to classify the EC/QY measurements as estimates. These include the difficulty in separating the fluorescence-associated absorbance from overlap with other non-dbAF related absorbance, as well as the strong sensitivity of our values to subtle changes in solution conditions (pH, salt, temperature).
A detailed investigation of the relation between the dbAF of carbonyls and its modulation by the vicinity to different amino acid residues, its sensitivity to neighboring residues, its relation to the molecular structure of the protein and the origin of its aggregation-related augmentation need to be addressed in the future.

Fig 3.10 Quantum yield measurements for BLG and Proline Plots of the absorbance vs. molar concentration for (a) BLG and (b) proline. Plot of fluorescence peak emission intensity vs. absorbance for (c) BLG and (d) proline used to extract the slopes $S_{BLG}$ and $S_{Pro}$, respectively. (adapted from Niyangoda et al 2017)
3.7 Potential Utility of DbAF for Studying Protein Structure and Function

To explore the utility of dbAF as intrinsic probe of protein properties, we investigated whether dbAF could substitute for tryptophan fluorescence for tracking thermal protein unfolding. Upon denaturation of natively folded proteins, tryptophans in the interior of proteins become exposed to water which induces a red-shift in their fluorescence emission wavelength [101]. Fig 3.11 (a) documents this red-shift of tryptophan fluorescence during the cooperative unfolding of lysozyme at pH 2. Superimposed to the red shift in tryptophan fluorescence is the peak dbAF emission wavelength recorded under the same conditions. As shown, dbAF emission undergoes a red-shift of near identical magnitude to that of tryptophan upon lysozyme unfolding.

However, dbAF detection required relative high HEWL concentrations, which could have induced aggregation and could have confounded any dbAF changes. By comparing both light scattering intensities (within noise, 0.2% difference) and particle size distributions of HEWL solutions prior to and following thermal denaturation (see insert, Fig 3.11 (a)) we confirmed that the thermal denaturation cycle was short enough to avoid any discernible aggregation. Hence, similar to intrinsic tryptophan fluorescence, the energetics of the excited states underlying dbAF are sensitive to the protein’s conformation.

Our data cannot distinguish whether the observed dbAF red-shift results directly from changes in protein conformation or, alternatively, responds to solvent or electrostatic effects on dbAF that are a consequence of protein unfolding. Guptasarma [89] had previously reported an increase in dbAF emission upon chemical denaturation of lysozyme, but without discernible red-shift. This difference in dbAF response to lysozyme denaturation might arise from differences of solvent effects on dbAF during chemical vs. thermal denaturation, and from differences in solution pH. As additional confirmation of the fluorescence properties and potential applications of dbAF,
we established that dbAF fluorescence is quenched, albeit weakly, by acrylamide. Care must be taken to subtract acrylamide spectra at each concentration from protein spectra due to the presence of dbAF-like fluorescence in acrylamide, as well. A Stern-Volmer plot of the data yields a quenching coefficient of $K_{sv} = 0.3 \text{ M}^{-1}$ (Fig 3.11 (b)).

Fig 3.11 dbAF emission tracks lysozyme denaturation, is sensitive to quenching. (a) The peak wavelength $\lambda_{max}$ of the fluorescence emission for dbAF (■, left axis) and Trp (○, right axis) during thermal denaturation of heWL at pH 2. Lines are sigmoidal fits through the dbAF (—) and Trp (- -) data, respectively. The insert shows the particle size distribution derived from dynamic light scattering of the dbAF solution prior to and after heat cycling. (b) Stern-Volmer plot for quenching of dbAF emission from BLG (2.5 mg/ml) by increasing concentrations of acrylamide. Here, dbAF represents the dbAF emission from BLG without acrylamide. DbAF from acrylamide at a given concentration was subtracted from dbAF values for BLG/ acrylamide mixtures. (adapted from Niyangoda et al 2017)

### 3.8 Discussion

Our investigation implicates carbonyls as the likely source of a weak and previously overlooked autofluorescence from individual amino acids and proteins in the visible range. Our
observations with multiple amino acids, several polyamino acids and different proteins from various suppliers extend earlier findings with individual proteins [88, 89], protein crystals [75, 77], polypeptide spherulites [76], and amyloid fibrils [75]. When including not just inter- and intramolecular hydrogen bonding but allowing for hydrogen-bonding with water, our observations are for the most part consistent with previously suggested models of dbAF. At the same time, our measurement do not specifically address the role of hydrogen bonding. The fluorescence emission from other carbonyl-containing compounds (formaldehyde, acetone, acrylamide) suggest that carbonyls could sustain dbAF emission even in the absence of hydrogen bonding.

The strong dependence of dbAF intensities on the amino acid identity, irrespective of whether recorded in solution or in the crystalline phase, further suggests that amino acid residues play an important role in modulating dbAF intensities. We cannot exclude, though, that some of the emissions described here are due to phosphorescence instead of fluorescence. For example there are reports of blue phosphorescence from BSA in powder form [102]. The same authors also described phosphorescence emissions similar to those of BSA from the solid but not solution states of carbonyl-rich starch and cellulose.

One intriguing question is why this intrinsic fluorescence from amino acids and proteins has essentially remained unnoticed or unexploited. We are not aware of prior theoretical work suggesting the existence of carbonyl-based fluorescence from individual proteins or amino acids even though carbonyl fluorescence from organic solvents is well established. Among potential reasons for this lack of recognition of dbAF in the protein community might be the weak molar extinction coefficient for dbAF excitation, the blue-shift of its absorption spectrum compared to its fluorescence excitation spectrum and the lack of a discernible shoulder in the absorption spectrum at the optimal excitation wavelength for proteins. The original findings by Kallman et
al. of dbAF in collagen were believed to be contaminated by photochemically induced fluorescent byproducts of collagen [88]. It is worth noting that this investigation into potential dbAF from proteins was prompted only by prior reports of significant dbAF from amyloid fibrils [74]. Lifetimes in the nanosecond range established that fibrillar dbAF, measured from amyloid fibrils, resulted from a resonant fluorescence emission instead of some inelastic scattering process [75]. The strong similarities of dbAF spectra from HEWL fibrils and monomers (Fig 3.4 B) bolster our hypothesis that the previously identified dbAF from protein aggregates and dbAF from monomeric proteins and amino acids detailed here both arise from their shared carbonyl moieties.

The presence of carbonyl autofluorescence in proteins opens up the possibilities for a wide range of novel applications for studying protein structure and dynamics—particularly at biologically relevant high protein concentrations often present in cellular environments. We have already shown that dbAF emission underwent a prominent red-shift upon thermal denaturation of lysozyme, similar to the well-known behavior of tryptophan fluorescence (Fig 3.11 A). Improved understanding of the sensitivity of dbAF signals to protein structure, state of aggregation, chemical modifications, and various solvent effects is likely to significantly expand the utility of this novel autofluorescence.

Our measurements of extinction coefficients and quantum yields, while preliminary, are a first step towards identifying the electronic transitions underlying dbAF. There are clearly many questions regarding the specific role of carbonyls in dbAF, and how amino acid structure, protein structure, and the extent of hydrogen bonding (in aggregates and in solution) alter dbAF properties. We hope that these results will inspire band structure calculations of amino acids underlying dbAF, thereby providing a solid theoretical understanding of amino acid fluorescence.
3.9 Materials and Methods

3.9.1 Proteins and Chemicals

Two times recrystallized and lyophilized hen egg white lysozyme (hewL) was purchased from Worthington Biochemicals. To exclude presence of small fluorophores, we dissolved lyophilized HEWL at 40 mg/ml in deionized water, placed it into a 6–8 kD MWCO dialysis tube, and dialyzed against 2 L of deionized water for two days with four changes of water. Lyophilized \( \beta \)-lactoglobulin (BLG), Poly-L-lysine hydrobromide (PLK), L-lysine, poly-L-glutamic acid (PLE), L-glutamic acid, L-cysteine, glycine, quinine sulfate, isopropanol and acetone were obtained from Sigma-Aldrich and used without further purification. Bovine serum albumin (BSA) and L-tryptophan were from MP Biomedicals. Remaining amino acids were purchased from Anaspec. All other chemicals were purchased from Fisher Scientific. Solvents and chemicals were reagent grade or better. Solutions were prepared using 18 MΩ water from a reverse osmosis unit (Barnstead E-pure).

3.9.2 Preparation of samples and solutions

Proteins and polyamino acids were dissolved in pH 7 buffer (20 mM HEPES, no salt or 50 mM HEPES and 50 mM NaCl, respectively) and typically filtered through 220 nm and 50 nm pore size filters. Protein concentrations were determined from UV absorption at 280 nm using the appropriate UV absorption coefficient. Amino acids were dissolved in either pH 2 potassium phosphate buffer or pH 7 HEPES buffer and concentrations were determined from the weight to solvent volume ratios. Amyloid fibrils of hen egg-white lysozyme (HEWL) were grown by incubating 20 mg/ml of HEWL in 25 mM phosphate buffer at pH 2, 100 mM NaCl at 52°C for 3–
5 days. For determination of the dbAF increase upon HEWL fibril formation, amyloid fibrils were separated from their monomeric background via three rounds of centrifugations (24 hours, 16,000 rpm) and resuspensions of the fibril pellet in buffer. Based on the monomer concentration in the supernatant vs. the total protein concentration in the fibril pellet after the 3rd round, the residual monomer content in the purified fibril suspension was estimated to be 1%.

3.9.3 Fluorescence spectroscopy

A FluoroMax-4 spectrofluorometer was used for all fluorescence measurements. Unless specifically stated, instrument settings were kept constant to permit comparison of relative intensities across samples. DbAF was typically excited near 370 nm and emission spectra were measured between 400 and 600 nm. With the exception of the thermal denaturation data, dbAF spectra were collected at room temperature. Buffer/salt solutions or deionized water were routinely checked for background fluorescence that might interfere with dbAF signals. Tryptophan fluorescence during thermal denaturation was excited at 280 nm using HEWL concentrations of 5 μg/ml. Fluorescence quenching data were collected using 2.5 mg/ml BLG at pH 2 and 25°C at the indicated concentrations of acrylamide. For each BLG/acrylamide mixture, background spectra without BLG but at the same acrylamide concentration were collected. The buffer spectrum (dominated by the water Raman peak) was measured and subtracted from both sets of spectra before subtracting acrylamide from BLG/acrylamide spectra. The Stern-Volmer constant was determined from a plot of the peak dbAF fluorescence of BLG before adding acrylamide (dbAF) over its corresponding values in the presence of the stated concentrations of acrylamide (dbAF), after correcting these spectra for their buffer and acrylamide background.
3.9.4 Epifluorescence Images of Amino Acid Crystals

Microscope fluorescence images were acquired on an inverted microscope (Olympus IX-70) using a 40x, 1.15 NA water immersion objective (Olympus UApo/340) and a 1.5x tube lens. Images were recorded with a high-sensitivity camera (Andor Ixon, DV-885K) cooled to -60°C with preamplifier gain set to 3.6x, but without EM gain. Epifluorescence illumination was provided by a 385 nm LED (Thorlabs M385L2, 750 mA drive current) passing through a standard DAPI filter cube (Chroma 31000: AT350/50 ex; 400 dclp dichoroic; D460/50 em). Fluorescence images were processed to provide intensity distributions and contrast suitable for visualization. For quantification of relative dbAF intensities from different crystals the average fluorescence intensities was measured over a large “region of interest”, thereby integrating over the noticeable variations in dbAF intensities. Background fluorescence measured over a comparable area off the crystals was subtracted and the resulting difference values were divided by the exposure time used to collect the images. The resulting relative dbAF intensities for amino acid crystals were plotted against the peak dbAF intensity of the same amino acid measured in solution.

3.9.5 Molar Extinction Coefficient and Quantum Yield Determination

Absorption spectra were recorded on a Lambda 950 UV/vis Spectrometer using 10 mm pathlength quartz cuvettes. Beer’s Law relates absorbance A, molar extinction coefficient ε, molar concentration c and the pathlength l of the cuvette via A = ε c l. Molar extinction coefficients of BLG (18.4 kD) and proline (115.1 Da) were determined from linear fits to the absorbance A at 350 (Pro) or 357 nm (BLG) vs. their corresponding molar concentrations. Quantum yields QY for dbAF of proline (Anaspec) and BLG (Sigma) were determined via comparison of their dbAF
emission amplitudes with the quantum yield standard quinine sulfate. Quinine sulfate was dissolved in 0.1 M sulfuric acid and its fluorescence measured at concentrations between 1 to 5 μg/ml. Quinine sulfate emission was excited at 350 nm, while BLG and Proline dbAF were excited at 357 and 350 nm, respectively. Quinine sulfate fluorescence intensities were corrected for the use of an ND1 neutral-density UV filter in the excitation path and a 395 LP Schott filter in the emission path with a measured attenuation of 88% and 21%, respectively. Solvent backgrounds were subtracted from all fluorescence emission spectra. Peak fluorescence emissions were plotted against absorbance at the optimal excitation wavelength for a series of different concentrations of quinine sulfate, BLG and proline. These plots were linear, as required to exclude inner filtering, aggregation or other systematic measurement errors. The slopes $S_X$ (were the subscript $X$ stands for either proline or BLG) of the fluorescence emission vs. absorbance plots and for the quinine sulfate standard ($S_{QS}$) were determined. The corresponding quantum yield $QY_X$ was then determined from the ratio of the slopes $S_X/S_{QS}$, multiplied by the quantum yield $QY_{QS}$ of quinine sulfate of 0.54, i.e.

$$QY_X = QY_{QS}(S_X/S_{QS})$$
Chapter 4: HEWL Amyloid Aggregates and Cell Toxicity

4.1 Introduction

Despite significant advances in the protein aggregation field, the exact in vivo mechanisms of cytotoxicity, and which type of amyloid aggregates cause them, remain elusive. The original “amyloid hypothesis” presumed that toxicity originated from amyloid fibrils and their associated plaques [4]. This conflicted with the observation that clinical symptoms poorly correlated with post-mortem plaque load. More recent studies have shown that even metastable intermediates of amyloid assembly of many different proteins can be equally or more cytotoxic than mature fibrils [103, 104]. These intermediates are not readily detectable by classical pathological stains, but immunostaining techniques have found these intermediates in post-mortem brains of patients. Similarly, no clear agreement exists regarding the mechanisms of cell death caused by fibrils or their precursors. For amyloid fibrils proposed mechanisms of cell toxicity include lipid rafts clustering which trigger apoptotic pathways [22], acting as a infectious particle in prion disease [105] or mere physical damage of cell structures. Metastable intermediates, in turn, have been shown to induce oxidative stress [106], to alter cellular calcium ion signaling [107, 108], and to cause membrane permeabilization [109]. Taken together, these studies suggest that the heterogeneous nature of the amyloid aggregation and the multitude of cellular processes they can affect has prevented the clear identification of the most relevant toxic aggregates species in amyloid diseases and their dominant mechanism of cell toxicity. One inherent shortcoming of
many of these studies is the lack of careful controls of the actual composition of aggregate species applied to cells, and whether they maintain their characteristics once they are transferred from *in vitro* growth conditions to physiological conditions for toxicity studies. The main goal of this project is to study the cell toxicity associated with the model amyloid protein, HEWL while addressing some of the shortcoming of existing toxicity experiments.

4.2 Hen Egg White Lysozyme as a Model Protein

The amyloid protein used in my cell toxicity studies is hen egg white lysozyme (HEWL). It is a small enzyme (molecular weight 14.3 kDa) consists of 129 amino acid residues. The three-dimensional structure, conformational stability, folding-unfolding mechanism of HEWL are all well understood [110-112]. The crystallographic structure of HEWL reveals that 41% of its secondary structure is alpha-helical while only 10% assumes a beta sheet structure. The rest of the secondary structure is comprised of disordered portions, beta turns or remains unassigned (Figure 4.1(A)).

In addition to its easy availability, HEWL is structurally nearly identical to human lysozyme, which is associated with fatal hereditary systemic amyloidosis [113]. Lysozyme systemic amyloidosis is characterized by the deposition of large amounts of lysozyme amyloid aggregates which leads to multi-organ dysfunction including kidneys, liver and the gastrointestinal system [114]. To date, eight point mutations in human lysozyme are known to induce amyloid aggregation *in vivo*: Ile56Thr, Asp67His, Phe57Ile, Trp82Arg, Trp64Arg, Thr70Asn, Asp68Gly, Trp112Arg [115]. The primary sequence of human lysozyme has 60% homology with HEWL and its secondary and tertiary structures are almost identical [116]. HEWL’s propensity to form
amyloid fibrils under denaturing conditions was first shown by Krebs et al. using full length, unreduced HEWL [117]. Since then, HEWL has become a model protein to study amyloid aggregation. In our lab, we have extensively studied the amyloid aggregation mechanisms of HEWL [65, 118, 119]. Upon changing protein and/or salt concentration, HEWL can generate two distinct aggregate species; long unbranched rigid fibrils (RF) vs. metastable globular oligomers (gOs) and curvilinear fibrils (CFs) [118]. The ability to generate highly homogeneous populations of different amyloid aggregates under defined solution conditions makes HEWL an ideal starting point for our investigation of distinct cellular toxicity.

4.3 Preparation of Distinct Amyloid Aggregate Species

Both rigid fibrils (RFs) and globular oligomers (gOs) and their curvilinear fibrils (CFs) were formed under strongly destabilizing acidic pH. The pH was adjusted to pH 2 by adding a small amount of hydrochloric acid to aqueous solutions of HEWL. Samples were incubated at 50 °C, slightly above the onset temperature for thermal denaturation of HEWL since partially unfolded species are required for aggregation. Different concentrations of HEWL or sodium
chloride were used to alter aggregate composition. At low ionic strength, HEWL forms long unbranched RFs via nucleated polymerization. The corresponding sigmoidal aggregation kinetics, as monitored using ThT, is characterized by a long lag period followed by exponential growth which eventually levels off into a plateau (Fig 4.2 (c)). These RFs are a few nanometers in diameter and reach several tens of micrometers in length (Fig 4.2(a)). Increasing the ionic strength leads to a sharp transition to biphasic assembly kinetics with the initial phase generating well-defined populations of gOs and CFs (Fig 4.2 (d)). These gOs have a globular morphology and polymerize to form worm-like CFs (Fig 4.2 (b)). The morphology of each aggregate species was confirmed using TEM and AFM imaging.

Interestingly, HEWL RFs and CFs have distinct particle size distributions in dynamic light scattering (DLS) [62]. Measuring particle size distribution during RF growth shows near-simultaneous appearance of two aggregate peaks with distinctly different hydrodynamic radii around 30 nm and 300 nm, respectively (Fig 4.2 (e)). In contrast, the early stage of biphasic growth (Fig 4.2 (f)) only generates one aggregate peak indicating the presence of CFs, with its hydrodynamic radius varying between 20 nm and 50 nm. The initial formation of globular oligomers only results in a slight shift in the average “monomer radius” since DLS is not sensitive enough to separate the hydrodynamic radius of small oligomer populations from the hydrodynamic radius of the dominant monomeric background.

4.4 Changes in Aggregate Structure upon Transfer to Physiological Media

The HEWL RFs and gO/CFs grown at pH 2 typically exist as single stranded polymers that remain in suspension (protein concentrations exceeding several hundred μM could result in
volume-spanning gel, though). Before studying cell toxicity of these aggregates we investigated the effects of transferring HEWL aggregates from in vitro growth conditions (pH 2, varying salt concentrations) to physiological cell media (pH 7, 150 mM salt). Upon transfer, both RFs and CFs readily formed macroscopic precipitates. Figure 4.3 (a) and (b) show negatively stained TEM images of RF and CF precipitates shortly after transfer. Subsequent incubation at physiological temperatures (37°C) for another 3 days did not cause any further changes. Figure 4.3 (c) and (d) show corresponding optical microscopy images of equivalent aggregates indicating that both RF and gO/CFs formed precipitates spanning hundreds of micrometers. At both length scales, RFs assembled into distinct bundled sheets while gO/CFs turned into gel-like clusters. Equally striking, both of these distinct precipitates reflected the morphology of the underlying rigid RFs and flexible CFs they were assembled from. This raised the questions whether these two distinct precipitate structures might reflect the equally distinct characteristics of diffuse vs. neuritic amyloid plaques seen in Alzheimer’s disease pathology. Ex vivo, both of these plaques stain with the amyloid dye thioflavin S, while only neuritic plaques display green birefringence after staining with Congo Red. In addition only neuritic plaques are associated directly with neuronal death. As shown in figure 4.3 (e) and (f), both RF and gO/CF precipitates are fluorescent when stained with 10 μM of ThS. In contrast, only the highly organized RF sheets displayed birefringence, both with and without CR staining, while gO/CF precipitates showed no signs of preferential depolarization under crossed polarizers. (Figure 4.3 (g,h)).

4.5 Cell Line

The cytotoxicity of RFs and CFs was investigated using adenocarcinomic human alveolar basal epithelial cells (A549). This cell line was first developed through culturing cancerous lung
tissue of a 58 years old Caucasian male in 1972 by D. J. Giard et al. A549 cells can act as model epithelium and when cultured \textit{in vitro}, they grow as a monolayer, attaching to the culture flask.

\subsection*{4.6 Cell Viability Experiments}

Cell viability in the presence of the above two types of amyloid aggregate species was assessed using a Live/Dead fluorescence assay, supplemented with optical imaging of changes to cell morphology. The Live/Dead assay determines the viability of the cells based on plasma membrane integrity of cells by sequential fluorescence staining of dead and viable cells. Ethidium Homodimer-1 (Phenanthridinium, 5,5'-(1,2-ethanediylbis(imino-3,1-propanediyl))) bis(3,8-diamino-6-phenyl)-, dichloride, dihydrochloride) is a membrane-impermeable fluorescent dye that binds to nucleic acids of dead cells, as their membranes become permeable. It emits 40 times enhanced bright red fluorescence (excitation/ emission: 528 nm/ 617 nm) upon binding to the nucleus. Once the dead cells are stained, NucBlue live reagent (Hoechst 33342) is added to the cells. This fluorescence dye is a cell-permeant nuclear counterstain that emits blue fluorescence at 460 nm when excited at 360 nm. However, prior binding of ethidium homodimer prevents binding of NucBlue. Cell viability after treating with HEWL aggregates was assessed using fluorescence microscopy.

\subsection*{4.7 Cell Toxicity of Different Amyloid Aggregates}

We used the live-dead assay to assess the toxicity of HEWL RFs and CFs. The growth conditions of RFs and CFs are summarized in Table 4.1. No buffer solutions were used for the HEWL sample preparation. All the samples were prepared in DI water and added NaCl.
**Fig 4.2: Characterization of HEWL aggregates** Negatively stained TEM images of HEWL rigid fibrils (a) and curvilinear fibrils (b) grown in 50 mM and 250 mM NaCl solutions at pH 2.0 respectively. Scale bar represents 2 μm. HEWL aggregation kinetics monitored using ThT fluorescence for rigid fibrils (c) and gOs/CFs (d) growth at 50°C. Particle size distribution of rigid fibrils always yield two aggregate peaks (e) while only a single aggregate peak is observed with curvilinear fibrils (f) in dynamic light scattering measurements. The smallest peak near 2 nm represents HEWL monomers.
Figure 4.3: Distinct Precipitates of RFs and gO/CFs after transfer to cell media
(a) Negatively stained TEM images of precipitates of HEWL rigid fibrils (a) and curvilinear fibrils (b) after transferring from growth solution to cell media. (Scale bars are 20 µm and 2 µm, respectively). (c, d) Same as (a) and (b), but using optical microscope to visualize precipitates. (e,f) hewL RF and gO/CF precipitates after staining with 10µM ThS. (g,h) shows the same precipitates stained with Congo Red and visualized using crossed polarizers.
The pH of the solutions was adjusted to 2.0 by adding concentrated Hydrochloric acid (about 0.1 v/v %). The growth of RFs and CFs were monitored using DLS and ThT fluorescence and their morphologies were confirmed using TEM. Once a significant amount of aggregates was grown in solution, samples were diluted into cell media to the desired concentrations (less than 8 v/v %). Then these samples were incubated with A549 cells grown to about 70% confluence for 3 days.

**Table 4.1: The growth conditions of HEWL RFs and CFs at pH 2**

<table>
<thead>
<tr>
<th></th>
<th>HEWL Concentration</th>
<th>pH</th>
<th>NaCl concentration</th>
<th>Incubation Temperature</th>
<th>Length of Incubation Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFs</td>
<td>1.4 mM</td>
<td>2.0</td>
<td>50 mM</td>
<td>52 °C</td>
<td>4-5 days</td>
</tr>
<tr>
<td>gOs/CFs</td>
<td>1.4 mM</td>
<td>2.0</td>
<td>250 mM</td>
<td>52 °C</td>
<td>3-5 hours</td>
</tr>
</tbody>
</table>

As shown in figure 4.4, incubation of cells with HEWL RF for 3 days, resulted in reduction of cell viability in a concentration-dependent manner. 100 µM of RFs reduced cell viability to about 60% compared to control cells in medium. Surprisingly, gOs/CFs showed no statistically significant effect on cell viability even at 100 µM concentration. Incubating cells for different time intervals (3, 4 and 5 days) had no significant effect on cell viability. Using phase contrast imaging, though we observed noticeable differences in the cell morphology of A549 cells. Negative control cells kept in cell medium retained the healthy morphology of A549 cells observed during cell passage (Figure 4.5 (a)). Positive control cells exposed to the toxic peptide KLAK, in turn, showed the typical cell fragmentation associated with apoptosis (Figure 4.5 (b)). In contrast to both, exposure to rigid fibrils caused A549 cells to shrink and display somewhat corrugated cell
membranes (Figure 4.5 (c)). This suggests that the effects of RF on cell viability are closer to those of a chronic irritant than an acute toxin.

![Cell toxicity of HEWL RFs and CFs](image)

**Figure 4.4**: Cell toxicity of HEWL RFs and CFs cell viability of A549 cells using live/dead assay for 3 different concentrations (10, 50, 100 µM) of RFs and CFs.

### 4.8 Isolation of aggregates from monomeric background

The toxicity results we have obtained contradict the common understanding in which intermediate oligomers/CFs are toxic while RFs are relatively inert end products. In order to properly compare toxicity of these amyloid aggregates we repeated measurements by controlling the concentration of aggregate species applied to cells. Therefore we isolated the RFs and CFs from their monomeric background through a series of centrifugation steps. Particle size distribution of the initial aggregated samples were measured with dynamic light scattering. RF samples that were grown to late stages were not suitable as starting points as cross linking of fibrils
formed solid gels. Also the CF samples incubated for longer periods tended to form milky precipitates which could not be used for successful separation. Aggregated solutions were subjected to 2-3 successive cycles of centrifugation and separation until the monomer concentration of the supernatant reached less than 2% of the pellet concentration (see Methods for details). To confirm the absence of monomers from the aggregate pellet, particle size distribution of the resuspended and diluted pellet were measured. Fig 4.6 (a) and (b) shows the particle size distribution of RF and CF samples before and after centrifugation. The morphologies of the isolated aggregates were verified by transmission electron microscopy. The stability of the isolated RFs and CFs at room temperature was assessed by thioflavin T fluorescence, static light scattering and transmission scanning microscopy for 6 days and we confirmed that no significant morphological changes occurred.

![Figure 4.5: Differences in Cell Morphologies](image)

A549 cells incubated 3 days in (a) cell media (negative control) (b) in the presence of KLAK (Positive control) and (c) 100µM RFs. Scale bar represents 200 µm.

### 4.9 Cell Toxicity of Isolated HEWL Aggregates

The concentrations we used for these experiments were 1, 10, and 25 µM of isolated HEWL aggregates. As before, cells incubated with isolated CFs showed no statistically relevant reduction
in cell viability while cells exposed to isolated RFs showed about 30% reduction in cell viability at the highest concentration used, compared to control (figure 4.7). Both RFs and CFs precipitated after transfer to the cell media. As confirmation that the observed toxicity did arise from RFs instead of a minor oligomeric component, we studied the toxicity of the corresponding supernatant collected after the first centrifugation during isolation of the aggregates. Before incubating these samples with cells we used particle size distribution obtained with DLS to exclude the presence of large aggregates in the supernatant. To our surprise, though, the RF supernatant induced an about 40% reduction in cell viability while the CF supernatant remained near 90% viability.

4.10 The Source of the Observed Toxicity of RF Supernatant

To better understand the toxicity of the RF-associated supernatant, we decided to scrutinize the HEWL supernatant incubated with cells. Unlike the HEWL monomer control or the CFs supernatant, the RF supernatant incubated with cells resulted in growth of macroscopic aggregates. Under the TEM, these aggregates exhibited CF like structure (figure 4.8(a)). Interestingly, cellular atrophy and special rearrangement of the cells was observed exclusively in the cells exposed to these CF-like aggregates from the RF supernatant (figure 4.8 (c) and (d)). The FTIR spectrum of these CF-like aggregates were studied to further understand their secondary structure. The underlying β-sheet structure of amyloid aggregates can be identified by the intense absorption between 1610 and 1630 cm\(^{-1}\) in the amide I region of the FTIR spectra [120]. As shown in Figure 4.8 (b), both isolated RFs and aggregates formed from the RF supernatant have absorption peaks in FTIR spectra around 1629 cm\(^{-1}\) which suggest, both aggregates have amyloid architecture. Interestingly, only the CF-like aggregates also exhibit a noticeable peak around 1680 cm\(^{-1}\) in FTIR spectrum considered indicative of anti-parallel β-sheets.
Figure 4.6 Isolating amyloid aggregates from monomeric background: Particle size distribution of RFs (a) and CFs (b) before (solid symbols) and after centrifugation (open symbols). Isolated aggregate samples do not contain monomer peak and we confirmed the monomer background in the supernatant to be less than 2% using uv absorption.

Figure 4.7: Cell toxicity of Isolated HEWL RFs and CFs cell viability of A549 cells using live/dead assay for few different concentrations (1, 10, 25 µM) of RFs, CFs and non-aggregated HEWL.
All these observations suggest that aggregates formed when we transfer the RF supernatant to cell media are amyloid in nature and toxic to the cells. We further studied the RF supernatant before transferring it to the cell media. Using DLS, we confirmed that there are no large aggregates present in the RF supernatant at pH 2 (figure 4.9 (a)). However, resolution of the DLS is not sensitive enough to resolve small differences in particle sizes. Therefore, dast protein liquid chromatography was used to get better insight into the size distribution of the particles present in the RF supernatant. As shown in figure 4.9 (b), two populations of particles were identified with different molecular weights; one particle size is corresponding to the size of the HEWL monomers, while the most prominent population is larger than the monomers. The enhancement of Thioflavin T fluorescence of the RF supernatant compared to fresh HEWL monomers suggest that these particles are indeed amyloidogenic (figure 4.9 (c)). The figure 4.9(d) shows about a 10 fold increase in the fluorescence intensity of Crystal Violet; another extrinsic amyloid dye sensitive to oligomeric amyloid aggregates, in the presence of RF supernatant. These data indicate that even before transferring RF supernatant to the physiological media, amyloidogenic oligomeric aggregates were present at pH 2.

4.11 Discussion

The toxicity of amyloid aggregates is yet to be understood. Whether the toxicity associated with amyloidosis is induced by intermediate oligomeric aggregates or rigid amyloid fibrils, the end product of amyloid assembly is not clear. This debate arises due to the complex, heterogeneous and transient nature of aggregates formed during amyloid assembly. For this study we used the amyloid aggregates formed by HEWL. Although HEWL is a model protein, it can provide a good platform to study toxicity of different amyloid aggregate species. We can successfully generate
isolated early stage aggregates such as gOs/CFs and end stage RFs under acidic conditions using HEWL. However, these aggregates form macroscopic precipitates when transferred to physiological media. The gel like clusters formed by gOs/CF, are analogous to diffuse plaques, commonly found in cerebellar cortex in early stage AD patients [121, 122]. These diffuse plaques are known to be inert and found in large numbers in cognitively normal people [123]. The observed non-toxic behavior of HEWL gOS/CFs, also suggest that aggregates formed from CFs have similar characteristics of diffuse plaques.

Figure 4.8: Changes in cells exposed to HEWL RF supernatant: (a) negatively stained TEM image of aggregates formed in cell media when RF supernatant was transferred. (b) Normalized FTIR spectra of fresh monomers (dashed line), RF supernatant transferred to cell media (solid line) and isolated RF aggregates in cell media (open circles). Phase contrast (c) and fluorescence (d) images of cells exposed to RF supernatant.
In comparison, macroscopic plaques formed from RFs, when transferred to cell media have well organized internal structure similar to neuritic plaques which are particularly abundant in areas of the AD brain showing substantial neuronal loss. The about 30% reduction in cell viability of cells exposed to RFs also suggest that these RF plaques have comparable characteristics to these neuritic plaques. However, the most significant reduction in cell viability is observed when cells
are incubated with RF supernatant. This toxic behavior corresponds to the presence of small amyloidogenic oligomeric species in the RF supernatant.

The molecular origin of these oligomeric species is still not clear. We are confident that the HEWL lysozyme itself is not forming any toxic oligomers as our freshly dissolved HEWL monomer control in cell toxicity experiments show comparable cell viability to the cell media control. One possible origin is hydrolyzed HEWL monomers which come together and form small clusters. We are using strong destabilizing conditions such as acidic pH and elevated temperature to generate amyloid aggregates. It has been shown that some of the HEWL monomers get hydrolyzed over time under these conditions [118]. However, once we applied pre-hydrolyzed (see Materials and Methods section for preparation method) HEWL monomers to the cells, no toxic effect was observed and no CF like aggregates were formed.

This leaves monomers shedding from RFs as the most probable origin of these toxic oligomers. However, confirming this possibility is challenging due to the very low solubility of RFs. There are two pieces of evidence supporting this hypothesis. We were able to collect shedded monomers using the centrifugation technique and once these shedded monomers were transferred to physiological media, ThT positive, CF like aggregates were formed (figure 4.10 (a)). The second evidence is decay of isolated RFs monitored using ThT fluorescence. As shown in figure 4.10 (b), initially ThT fluorescence decreased as expected in a decay experiment, but eventually, fluorescence signal starts to gradually increase suggesting a formation of amyloidogenic species. Although these evidence are suggestive, there are other reasons for this increase, such as fibril bundling. Further investigation is required to confirm depolymerization of RFs as the origin of toxic oligomers.
Based on our study, it is conceivable that amyloid oligomers exhibit polymorphism, and associated differences in toxicity, based on their origin of formation. A better understanding these different oligomeric intermediates can resolve many discrepancies in the field. Also, targeting most toxic and aggressive amyloid species, can leads to effective therapeutic approaches to prevent or limit the amyloid toxicity.

4.12 Materials and Methods

4.12.1 Protein and Chemicals

Two times recrystallized, dialyzed and lyophilized HEWL was obtained from Worthington Biochemicals (Lakewood NJ). Ultrapure grade thioflavin T was obtained from Anaspec (Freemont, CA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and were reagent grade or better. 18 MV RO purified water (Barnstead E-pure, Dubuque, IA) was used to prepare all the solutions.

Figure 4.10: Shedded monomers from RFs as the source of toxic oligomers: (a) Negatively stained TEM image of aggregates formed by transferring shedded monomers to cell media. (Scale bar 1µm) (b) The decay of isolated RFs monitored using ThT fluorescence, isolated fibrils (solid line), and fresh HEWL monomers (dotted line).
4.12.2 Hen Egg White Lysozyme Sample Preparation

Lysozyme solutions were prepared by dissolving lyophilized lysozyme at twice its final concentration in distilled water and was placed in a water bath at 45 °C for few minutes to remove preformed clusters. Then the sample was consecutively filtered through 200 nm and 50 nm pore size syringe filters. This sample was mixed 1:1 either with a 100 mM NaCl solution or with 500 mM NaCl solution (salt concentration is twice the final concentration). pH of the sample was adjusted by adding minimal volume (2-4% v/v) of concentrated HCl. Final pH of the sample was 2.0±0.2. Concentrations of the HEWL samples were determined from UV absorption measurements at 280 nm ($a_{280} = 2.64$ ml mg$^{-1}$ cm$^{-1}$). Samples were incubated at 50 °C for as little as 4 hours and as long as 5 days using a dry bath.

4.12.3 Thioflavin T Stock Solution Preparation

Thioflavin T stock solution was prepared by dissolving dye in distilled water and filtering through 200 nm pore size Nylon syringe filter. Actual Thioflavin T concentration was determined from absorption at 416 nm ($a_{416} = 26 620$ ml mg$^{-1}$ cm$^{-1}$).

4.12.4 Isolation of Aggregates from Monomer Background

The aggregated RF or CF sample was taken out of the dry bath and its particle size distribution was measured in the dynamic light scattering unit. Then the sample was transferred to a 2 ml centrifuge tube and centrifuged at 17,000 X g at 10 °C for 22-24 hours. The pellet and the supernatant were separated. The pellet was re-suspended in 2 ml of 50 mM NaCl (for RF) / 250
mM NaCl (for CF), pH 2 solution and centrifuged again for 22-24 hours. This cycle was repeated until the protein concentration of the supernatant was reduced to 1-2% of the concentration of the pellet. The resuspended pellet was measured with dynamic light scattering unit to confirm the particle size distribution.

4.12.5 Transferring Aggregates from pH 2 to Cell Media (pH 7.4)

Isolated rigid fibrils and curvilinear fibrils were diluted down to 100 uM, using 50 mM NaCl and 250 mM NaCl solutions at pH 2.0, respectively. 100 uL of each aggregate solution was mixed with 900 uL of Dulbecco’s modified Eagle’s medium supplemented with 1% of a Antibiotic-Antimycotic. Then the solution was centrifuged using a table top centrifuge for 10 minutes to separate precipitates from the solution. Both solution and the precipitate were imaged using transmission electron microscope. Then samples were incubated at 37 °C dry bath for 2-3 days. Again, solution and precipitates were separated using centrifuge and imaged.

4.12.6 Static and Dynamic Light Scattering

A Zetasizer Nano S (Malvern Instruments, Worchestershire, UK) equipped with a 4 mW He-Ne laser (λ = 633 nm) in back scattering geometry (θ = 173°) was used to perform static and dynamic light scattering (SLS and DLS) measurements. This instrument has a built in peltier temperature control unit. During in situ measurements, each correlation function was collected using signal acquisition times of 180 seconds and the time interval between subsequent measurements was typically 10-20 minutes. Autocorrelation functions were converted into particle size distributions using the built in Contin deconvolution analysis package.
4.12.7 Generating Hydrolyzed HEWL Monomers

5 mg/ml HEWL sample was prepared in DI water and final pH of the sample was adjusted to 2.0 by adding concentrated HCL. Then the sample was incubated at 52 °C for 5 days. Then Particle size distribution of the sample was measured using DLS to confirm the absence of aggregates.

4.12.8 Thioflaving T Fluorescence

Thioflavin T measurements were performed using a SpectraMax M5 grating based fluorescence plate reader (Molecular devices) with built in temperature controlled sample chamber. Thioflavin T fluorescence was excited at 440 nm and emission was collected at 485 nm. A 455 nm cut off filter was used in all the kinetic measurements to suppress any scattered excitation light leaking into the emission. HEWL samples we mixed with thioflavin T stock solution so that final dye concentration is 20 uM. 250 – 300 ul of HEWL/ thioflavin T mixtures were transferred into each well of 96-well glass bottom plates. All the measurements were done at 52 °C for 3-4 days.

4.12.9 Transmission Electron Microscopy

HEWL samples were diluted into distilled water before depositing onto the Formvar/carbon film coated, 200 mesh copper grids. 5 ul of the diluted sample was placed on the surface of the grid and allowed to dry in a vacuum oven at 40 °C. A 5 ul aliquot of distilled water was then added to the grid and blotted to remove salt crystals from the grid. This step was repeated
at least 3 times and then the grid was negatively stained with 5 ul of 8% (w/v) uranyl acetate for 1 minute, blotted and dry. Excess uranyl acetate was removed by repeated washing with distilled water and grid was left to air dry. All the grids were imaged using FEI Morgani transmission electron microscope at 60 kV with an Olympus MegaView III camera.

4.12.10 Cell Culture

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 in a 5% CO₂ humidified atmosphere at 37 °C. Cells were plated at a density of 10,000 cells/well in 96-well plates and incubated for 24 hours. Then the medium was replaced with Dulbecco’s modified Eagle’s medium without fetal bovine serum. The plate was incubated for 17-20 hours before treatment with HEWL aggregates. HEWL aggregates were initially diluted in the fetal bovine serum free cell medium and then added to the cells.

4.12.11 Live/Dead Cell Viability Assay

Cells were exposed to the indicated aggregate solutions for 3 days. After that cells were washed twice with PBS to remove HEWL aggregates and floating cells. Then ethidium homodimer-1 (Life Technologies (catalogue number E1169)) was added to each well to a final concentration of 4 uM. Cells were then incubated for 1 -2 hours at 37 °C. Then the medium was replaced with cell media containing NucBlue (Life Technologies (catalogue number R37605)) (2 drops of dye to 1 ml of cell media). The plate was then incubated for another 20 min. six random pictures were taken per well using the EVOS fluorescence microscope (10X magnification).
Pictures were taken in different channels (blue, red) and overlayed. Live/dead cell counting was performed using ImageJ.

4.12.12 FTIR Spectroscopy

FTIR measurements were taken on a Bruker Optik Vertex 70 (Ettlingen, Germany) spectrometer. 30 uL of samples were deposited on the silicon crystal. Baseline scans of the buffer solution were taken over 1000 scans at 2 cm\(^{-1}\) resolution, and sample spectrums were taken over 700 scans. Typically FTIR spectrums were acquired over 3,000 to 1,000 cm\(^{-1}\) wavenumber. FTIR spectrums were normalized to their peak value, and our aggregate spectrums had the normalized monomer spectrum subtracted from it to show the peak differences in the Amide I band for our aggregate species vs monomers.
Chapter 5: Alzheimer’s Disease and Amyloid β Aggregation

5.1 Introduction

5.1.1 Alzheimer’s Disease

Alzheimer’s disease (AD) is the most common age-associated form of dementia. According to the world Alzheimer report in 2018, about 50 million people are affected by Alzheimer’s disease at the present time and this number is expected to reach 152 million in 2050 [31]. Currently, direct and indirect costs for healthcare related to this disease is estimated at one trillion US dollars a year and forecasted to double by 2030 [31]. With continuing expansion of life expectancy, AD has become a major healthcare issue in the twenty-first century. To date, despite all the scientific efforts, there is no effective treatment for AD.

5.1.2 History of AD

“We must not be satisfied to force it into the existing group of well-known disease patterns.”

Alois Alzheimer November 3, 1906 [124]

The German clinical psychiatrist and neuroanatomist, Dr. Alois Alzheimer is credited with the report of the first case of AD. On November 3, 1906, he presented “A peculiar severe disease process of the cerebral cortex” to the 37th Meeting of South-West German Psychiatrists in
In his lecture, Alzheimer described a patient named Auguste Deter, a 50 year old woman, who presented with cognitive and language impairment, disorientation, behavioral symptoms such as hallucinations, delusions, paranoia, and psychosocial incompetence [126]. Her post-mortem autopsy revealed severe cerebral atrophy and, at the cellular level, the presence of amyloid plaques and neurofibrillary tangles. Even today, after a century, diagnosis of AD is based on post-mortem brain pathology, just as the original findings of Dr. Alzheimer in 1906.

5.1.3 AD pathology and Diagnosis

AD is a progressive neurodegenerative disorder which is characterized by a slow and irreversible loss of neuronal functions in expanding portion of the brain and eventually leads to severe cerebral atrophy and death [124, 128]. The progression of AD symptoms is divided into three stages: mild, moderate and severe (Table 2.1) [129]. AD begins in the entorhinal cortex and spreads to the hippocampus, the brain region responsible for learning and converting short-term to long-term memories. Then neurodegeneration spreads to the cerebral cortex which involves language, reasoning and attention. In the last stage of AD, neurodegeneration spreads throughout the brain resulting in atrophy and enlargement of ventricles.

The pathological hallmarks of AD are extracellular deposits consisting mostly of amyloid β fibrils and intraneuronal flame-shaped neurofibrillary tangles of hyperphosphorylated tau protein [24, 130] (Figure 5.1). The pathological diagnosis of AD can be attempted through cerebrospinal fluid (CSF) and positron emission tomography (PET) biomarkers [131, 132]. However, definitive diagnosis of AD requires autopsy based pathological evaluation of patient’s brain tissues [126, 132].
The current therapeutic approaches for AD provide a moderate symptomatic relief in the early phases of disease progression. The current treatments available include acetylcholinesterase inhibitors (rivastigmine, galantamine, donepezil) for patients at any stage of AD and N-methyl d-aspartate receptor antagonist (memantine) for patients with moderate to severe AD [132]. Antipsychotic and antidepressant treatments are also used for the behavioral symptoms of the disease. But researchers are not able to reverse or prevent disease progression. Understanding the underlying pathogenic mechanisms of Amyloid β aggregation has been a major focus of research as it can provide rational therapeutic approaches to treat AD.

![Fig 5.1 Amyloid plaques present in Alzheimer’s brain tissues. (a). Spherical extracellular amyloid β deposits. (b). intraneuronal flame-shaped neurofibrillary tangles of hyperphosphorylated tau protein (image source: https://alzheimersdiseaz.weebly.com/)](image)

### 5.2 Amyloid Precursor Protein and Amyloid β Peptide

The senile plaques present in the cerebral cortex of AD patients are primarily composed of aggregated forms of amyloid β peptide, derived from proteolytic cleavage of amyloid precursor protein (APP) [24, 133, 134]. APP is a single-pass transmembrane protein and, based on the enzymatic activity, APP can be processed in two different pathways. About 90% of the APP enters
the non-amyloidogenic pathway, where APP is cleaved first by \(\alpha\)-secretase to yield a soluble N-terminal fragment (sAPP\(\alpha\)) and a membrane retained C-terminal fragment (CTF\(\alpha\)).

**Table 5.1 Clinical symptoms of AD patients at different stages of the disease**

<table>
<thead>
<tr>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Memory loss</td>
<td>Increasing memory loss and confusion</td>
<td>Weight loss</td>
</tr>
<tr>
<td>Trouble handling money</td>
<td>Shortened attention span</td>
<td>Seizures</td>
</tr>
<tr>
<td>Poor judgment</td>
<td>Inappropriate outbursts of anger</td>
<td>Skin infections</td>
</tr>
<tr>
<td>Mood and personality changes</td>
<td>Hallucinations, delusions, suspiciousness or paranoia, irritability</td>
<td>Difficulty swallowing</td>
</tr>
<tr>
<td>Loss of spontaneity and sense of initiative</td>
<td>Loss of impulse control</td>
<td>Lack of bladder and bowel control</td>
</tr>
</tbody>
</table>

Subsequently, CTF\(\alpha\) is processed by \(\gamma\)-secretase to produce a soluble N-terminal fragment (p3) also known as amyloid \(\beta\) 17-42 peptide and a membrane-bound C-terminal fragment (AICD, or APP intracellular domain). The remaining 10% of APP is processed along the amyloidogenic pathway, where APP is cleaved by \(\beta\)-secretase, yielding a soluble N-terminal fragment (sAPP\(\beta\)) and a membrane-bound C-terminal fragment (CTF\(\beta\)). CTF\(\beta\) is then acted upon by \(\gamma\)-secretase, generating a membrane-bound C-terminal fragment (AICD) and a soluble N-terminal fragment (amyloid-\(\beta\)) [134]. Due to the non-specificity of \(\gamma\)-secretase proteolytic cleavage [135], the length of the amyloid \(\beta\) peptide can vary from 27 to 49 amino acids [136].

The amyloid \(\beta\) peptide is an intrinsically disordered peptide [24, 137]. The amino acid sequence of amyloid beta peptide is amphipathic (Fig 5.2(C)). The amino acid terminal, residues
1-28, are mostly polar, as it is derived from the extracellular domain of the APP while residues 29-40/42 are hydrophobic [138, 139]. Among the two main variants, amyloid β 1-40 and amyloid β 1-42, the latter is believed to be more toxic due to its high aggregation propensity [140].

![Diagram](image)

**Fig 5.2 Amyloid β peptide structure and amino acid sequence**: (a) The solution structure of Amyloid-β 1-42 peptide (image source: Protein Data Bank). (b) Amyloid fibrils generated from Amyloid-β 1-42 peptide (5µM amyloid β peptide in 50 mM HEPES buffer, pH 7.4). (c) Primary amino acid sequence of amyloid-β 1-42 and 1-40 peptides.

### 5.3 Amyloid β Aggregation

Studying aggregation behavior of amyloid β 1-42 and 1-40 peptides has been the focus of research for several decades. Amyloid β 1-40 is the most common isoform present in the brain [141] while amyloid β 1-42 is believed to be the isoform closely related to the neuropathology of AD [142, 143]. As shown in Figure 5.2(C), amyloid β 1-42 has two additional C terminal amino acids (Isoleucine and Alanine), compared to 1-40. Although the difference between these two peptides is only two amino acids at the C terminal, their aggregation behavior, physiological
functions, metabolism and toxicities are significantly different [144, 145]. Amyloid β 1-42 is more hydrophobic and highly aggregation prone, while 1-40 is comparatively stable.

Both amyloid β 1-42 and 1-40 can form amyloid fibrils under physiological conditions (Fig 5.2(B)). As all other amyloid fibrils, amyloid β fibrils have a cross-β sheet structure with β-strands oriented perpendicular to the fibril axis [21, 38]. These fibrils consist of stacked β strand – turn – β strand units oriented parallel and in-register. Significant progress has been made in modelling the aggregation mechanisms of fibril formation in the absence of metastable intermediates [51]. However, knowledge about the pre-fibriller intermediates or oligomeric species, which are believed to be responsible for neurotoxicity associated with AD, remains elusive. Therefore, it is vital to explore the conditions and mechanisms of intermediate aggregate formation of these amyloid peptides as these latter intermediates are believed to underlie AD progression.

5.4 Amyloid Cascade Hypothesis and Associated Problems

The two main pathological hallmarks of AD are the extracellular deposits of amyloid-β and intracellular neurofibrillary tangles of hyperphosphorylated tau [24]. However, gene mutations that lead inevitably to AD involve either the amyloid precursor protein (APP) or presenilin-1 and presenilin-2 which are the catalytic subunits of γ-secretase in amyloid β proteolysis. This suggests that amyloid-β accumulation is the causative event in AD neuropathology [4, 24, 25]. Further supporting this conclusion is the fact that trisomy 21 (Down’s syndrome patients with three copies of the APP gene) leads to the formation of amyloid-β plaques as early as age 40 [4, 146]. Based on these findings and transgenic mice experiments, many hypothesis have been proposed to explain how amyloid β aggregation underlies the etiology and pathogenesis of AD.
The most widely accepted hypothesis is the amyloid cascade hypothesis which was proposed by Hardy and Allsop in 1992. This hypothesis postulates that accumulation of amyloid \( \beta \) plaques acts as a pathological trigger to a cascade of events such as neuritic injury, neuronal dysfunction, cell death and dementia [4, 147] (Figure 5.3). There are four pieces of evidence/observations strongly supporting the amyloid cascade hypothesis. First, tau protein causes frontotemporal dementia with parkinsonism with fatal neurodegeneration in the absence of amyloid plaque formation [4, 148-151]. This observation suggest that formation of neurofibrillary tau tangle itself is not sufficient to induce amyloid-\( \beta \) plaque formation. Therefore formation of tau tangles is likely a consequence of amyloid \( \beta \) aggregation. The second piece of evidence comes from transgenic mice overexpressing both APP and tau which produce more tau tangles compared to mice expressing tau alone, while formation of amyloid plaques is not affected [152]. This observation support the idea that amyloid plaque formation occurs before tau alterations. The third piece of evidence comes from crossing APP transgenic mice with apolipoprotein E (apoE)–deficient mice which significantly reduced amyloid deposition in the offspring, suggesting that the human apoE locus is required for amyloid \( \beta \) formation [153]. Forth, the genetic variability in amyloid \( \beta \) metabolism and clearance can modify the risk of AD [154-158]. This hypothesis significantly influenced academic and clinical research carried over the last 25 years.

While there is substantial evidence supporting the amyloid cascade hypothesis, there are several unresolved issues. Chief among them is the weak correlation between amyloid plaque load even in healthy individuals and clinical manifestation of the disease [159]. Another criticism is that, while gene mutations of APP and presenilin increase the deposition of amyloid plaques, there is no direct relationship between a particular mutation and the age at which it first produces symptoms [160, 161]. Another concern has arisen from the recent finding of neurotoxicity of pre-
fibrillar oligomeric species of amyloid-β peptide instead from late stage amyloid fibrils [17, 162]. Moreover, negative outcomes from a number of phase III clinical trials targeting amyloid β deposits have made researchers question this hypothesis.

As a result, the amyloid cascade hypothesis has been modified to incorporate the various concerns and questions raised [163, 164]. According to the modified hypothesis, the allostatic load which includes aging of the brain, risk factors such as head trauma, vascular and systemic disease, and gene mutations lead to synaptic and neuronal dysfunction and degeneration, upregulation of genes determining various reactive and breakdown products, results in the formation of amyloid-β and tau. [164, 165]. Furthermore, secondary neurodegeneration and synapsis loss is believed to be mainly caused by small soluble amyloid oligomeric species not from late stage amyloid plaques. This recent shift in the amyloid cascade hypothesis redirected scientists to study the growth kinetics and formation of early stage amyloid aggregates of amyloid β as well as tau protein.

5.5 Two Model Amyloid Proteins and Kinetics

Multiple amyloid assembly pathways based on different intermediates have been reported for different proteins and peptides such as amyloid beta 1-42 [13, 166], β2 microglobulin [60, 167], human serum albumin [168] and hen egg white lysozyme (HEWL) [118, 119, 169]. The early stage amyloid aggregates, globular oligomers (gOs) (Fig 5.4 (a)) have a globular morphology with few nanometers in diameter and, in the case of HEWL, have been shown to be composed of 8-12 monomers. These metastable intermediates are considered the dominant amyloid aggregates responsible for the pathologies associated with amyloidoses [55, 56, 162]. How these gOs induce cell toxicity and how they spread within tissues is still an open question.
Missense mutations in APP, PS1 or PS2 genes

Increased amyloid-β 1-42 production and accumulation

Amyloid-β 1-42 oligomerization and deposition as diffuse plaques

Subtle effects Amyloid-β oligomers on synapses

Microglial and astrocytic activation (complement factors, cytokines, etc.)

Progressive synaptic and neuritic injury

Altered neuronal ionic homeostasis; oxidative injury

Altered kinase/phosphatase activities: tangles

Widespread neuronal/neuritic dysfunction and cell death with transmitter deficits

Dementia

**Fig 5.3 Amyloid Cascade Hypothesis** The sequence of pathogenic events leading to AD proposed by the amyloid cascade hypothesis [4].
Another common intermediate aggregate population are protofibrils or curvilinear fibrils (CFs), which have a worm-like morphology (Figure 5.4 (b)) [170]. We have shown that they represent polymeric assemblies of gOs [170]. End-stage amyloid aggregates are long unbranched rigid fibrils (RFs), which can grow up to several tens of micrometers in length (Figure 5.4 (c)). These amyloid fibrils are inherently stable and composed of extended networks of parallel β-sheets with their characteristic cross-β structure. [7, 171, 172].

![Fig 5.4 Distinct morphologies of Amyloid Aggregates of HEWL](image1)

Our lab has recently published a collaborative study of two model amyloids: HEWL and a single-chain dimeric variant of Amyloid β 1-40 [5]. HEWL is a small enzyme which is very similar to human lysozyme associated with fatal hereditary systemic amyloidosis. The single chain Amyloid β dimer was constructed by connecting two amyloid β 1-40 monomers through a flexible glycine–serine-rich linker in a head-to-tail fashion. The kinetics of amyloid formation of these two model systems were monitored using the amyloid indicator dye thoflavin T, which shows augmented fluorescence emission upon binding to various amyloid aggregates. In both amyloid
systems, a sharp transition in kinetics was observed upon crossing a specific protein concentration. Below this threshold protein concentration, RF assembly followed the sigmoidal kinetics of nucleated polymerization, including a long lag period without discernible populations of metastable intermediates and a subsequent rapid growth phase (Figure 5.5). Above this threshold concentration, biphasic kinetic was observed with an initial immediate increase indicating gOs/CFs formation, followed by a second upswing indicating RF formation. Due to the similarity of this threshold with the abrupt onset of micelle formation, this threshold concentration was named “critical oligomer concentration” or COC.

5.6 Results

5.6.1 Preparation of Monomeric Amyloid β Stock

The above results prompted us to investigate whether the monomeric amyloid β 1-40 variant displayed a similar transition from (oligomer-free) sigmoidal to (oligomeric) biphasic fibril assembly. Studying Aβ aggregation kinetics, however, has been shown to be challenging due to its rapid aggregation propensity under physiological conditions. Due to the autocatalytic nature of amyloid β aggregation, a very small amount of pre-formed aggregates can have a significant effect on the subsequent aggregation kinetics. Therefore, it is crucial to initiate kinetic studies of aggregation with an aggregate-free peptide sample. Most of the protocols available in the literature to dissolve amyloid β peptide involve highly acidic [173] or organic solvents [174]. Dissolving peptide in acidic conditions can be problematic; when solutions are titrated to the physiological pH (7.4), since the peptide crosses its isoelectric point (pI = 5.3), where the it has the lowest
solubility [175]. Similarly, residual concentrations of organic solvent such as dimethyl sulfoxide (DMSO) or hexafluoro-2-propanol (HFIP) can alter the aggregation behavior of the peptide.

Fig 5.5 Transition from sigmoidal to biphasic growth kinetics upon oligomer formation. Transition from sigmoidal (orange) to bimodal (blue) amyloid growth kinetics of dimAb (A, B) and hewL (C–E), as monitored by ThT fluorescence. Concentration dependent time traces of (A, B) dimAb assembly in 50 mM Na-phosphate, 50 mM NaCl, pH 7.4, 37 C, and (C–E) hewL assembly in 25 mM K-phosphate, pH 2.0, 52 C, with 450 (C, E) and 500 (D) mMNaCl, respectively. Typical sigmoidal (D) and bimodal (B, E) growth kinetics correlated to AFM images of aggregate morphologies at the indicated time points (I–V) and concentrations of 20 mM dimAb (B), 21 mM hewL (D), and 280 mM hewL (E), respectively [5] (Reproduced by permission of The Royal Society of Chemistry)

https://pubs.rsc.org/en/content/articlelanding/2018/sc/c8sc01479e#!divAbstract
In order to overcome these difficulties and obtain a clean monomer sample, a series of precautions were taken. First the lyophilized amyloid β stock was dissolved in highly alkaline solutions (100 mM NaOH) where the peptide has its highest solubility. Then fast protein liquid chromatography was used to remove any remaining aggregates. The size exclusion column used was a Superdex-75 10/300 column which optimally separates proteins with molecular weights ranging from 3,000 to 75,000 Da. The running buffer used in the size exclusion column was 35 mM Na₂HPO₄, pH 11 buffer. Using a running buffer at pH 11 minimizes the aggregation of amyloid β peptide during elution. The monomer peak eluted at 14 ml and monomers were kept in an ice bath during sample preparation to avoid aggregation.

The concentration of the amyloid β monomer sample was measured using UV absorption at 280 nm based on the single tyrosine residue in amyloid β peptide (extinction coefficient: 1490 cm⁻¹ M⁻¹) [176]. By using this protocol, and reliable peptide supplies (r-peptide), we were able to obtain reproducible amyloid β kinetic traces for both Aβ 1-40 and Aβ1-42 peptides

![Fig 5.6 Amyloid β purification elution profiles of amyloid β 1-42 (a) and amyloid β 1-40 (b) Monomers are eluted at 14 mL. Both peptides were dissolved in 100 mM NaOH and the size exclusion column was equilibrated with 35 mM Na₂HPO₄ buffer at pH 11. Two blue vertical lines shows the monomer fraction we collected for our experiments.](image-url)
5.6.2. Amyloid β 1-40 Peptide Kinetics and Critical Oligomer Concentration (COC)

Amyloid β 1-40 monomers were prepared for kinetic experiments by diluting purified monomer solutions into 35 mM Na₂HPO₄ pH 11 buffer to the desired concentrations. The pH of the samples was brought down to physiological pH (pH 7.4) by adding 1.5% (v/v) of a 1M NaH₂PO₄ solution. The growth kinetics of the samples were monitored using Thioflavin T dye at 15 μM final concentration.

A sharp transition between two distinct types of assembly kinetics as function of peptide concentration was identified. At low concentrations, assembly kinetics of amyloid β displayed an extended lag period followed by a dramatic upswing and subsequent saturation (Fig 5.7 orange curves). This sigmoidal assembly kinetic is typical for nucleated polymerization growth. Beyond a well-defined threshold concentration the kinetics becomes biphasic. Slightly above this concentration, ThT displays a small but steady increase with no lag period followed by a prominent upswing. Further increasing monomer concentration results in a sublinear increase and plateau in the initial phase reaching before the second prominent upswing and saturation.

AFM imaging was used to study which type of aggregates form under these two different growth kinetics. AFM images of aliquots sampled for amyloid β 1-40 growth during biphasic kinetics indicated the formation of gOs which subsequently assembled into CFs during the initial phase, and RF nucleation and growth during the secondary phase (figure 5.8). AFM images taken at the end of the reaction contained RFs and significant amount of residual gOs/CFs (Figure 5.8). AFM images of aggregate populations sampled during sigmoidal kinetics indicated the formation of RF in the absence of gOs/CFs. Similar to the two amyloid systems discussed above: HEWL and a single chain dimeric variant of Amyloid β 1-40; we were able to observe a sharp transition between
two distinct forms of assembly kinetics. This threshold concentration is referred to as “critical oligomer concentration” or COC. The COC for amyloid β 1-40 incubated at 27 °C is near 60 µM.

![Kinetics of amyloid β 1-40 aggregation](image)

**Fig 5.7 Kinetics of amyloid β 1-40 aggregation:** Aggregation kinetics of different concentrations of amyloid β 1-40 monitored using thioflavin T fluorescence under near physiological conditions (50 mM sodium phosphate buffer with 150 mM NaCl pH 7.4 at 27°C).

### 5.6.3 Amyloid β 1-42 Kinetics and COC

The aggregation behavior of Aβ1-42 is significantly faster compared to Aβ1-40 (Figure 2.6(A)). The transition from sigmoidal to biphasic kinetics for Aβ 1-42 occurs at a much lower concentration, indicating a COC of only about 10 µM. However, unlike for Aβ 1-40, we cannot observe a clear plateau region above the COC as rigid fibril formation rapidly takes over.

### 5.7 Discussion

AD has recently been labeled as the ‘twenty-first century plague’, due to rapidly increasing number of patients and incurable nature. The main reason for this prevalence is the expansion
**Fig 5.8 Sigmoidal to biphasic growth kinetics correlated to AFM imaging**: The sigmoidal (yellow) and biphasic (blue) amyloid growth kinetics of two amyloid β 1-40 concentrations, 10 µM (a) and 85 µM (b), respectively in 50 mM Na-phosphate, 150 mM NaCl, pH 7.4, 27°C. The sigmoidal and biphasic growth kinetics correlated to AFM images of aggregate morphologies at the indicated time points (I–VI).

**Fig 5.9 Kinetics of Aβ 1-42 aggregation** (a) Amyloid β 1-42 aggregation monitored by Thioflavin T fluorescence for different concentrations (T =27 °C in 50mM sodium phosphate buffer, 50mM NaCl, pH 7.4 with 10uM Thioflavin T). (b) Negatively stained TEM image of 50 µM Aβ 1-42 incubated at 27 °C for 7 days. The scale bar represents 2µm.
Of life expectancy as a result of dramatic improvements in public health over the past century. Finding effective therapeutic strategies to combat onset and progression of AD is hampered by the limited knowledge and understanding of the molecular origin of this disease. The amyloid plaques present in brain tissues of AD patients mainly consists of amyloid fibrils. However, the modified amyloid hypothesis, based on a growing set of evidence, suggests that early stage amyloid oligomers are responsible for the neurodegeneration and synapsis loss associated with AD. Understanding the relationship between end stage amyloid fibrils and these toxic small oligomers is essential for targeting the molecular origins of AD.

Using amyloid β 1-40 and 1-42, we have confirmed the sharp transition in kinetics of amyloid assembly, from oligomer-free to oligomer-dominated rigid fibril formation under near physiological conditions. The sigmoidal kinetics below the threshold concentration (COC) represents nucleated polymerization of rigid fibril formation in the absence of early stage gOs/CFs. The well-defined transition from sigmoidal to biphasic kinetics above COC reflects the onset of formation of small gOs/CFs. This observation along with our data on kinetic of HEWL and dimeric amyloid β, suggest that biphasic kinetics represent a generic mode of amyloid assembly associated with oligomer formation. Also, the observed increase in RF nucleation lag periods with increasing amounts of gOs/CF and the absence of any small aggregates during the lag period of sigmoidal growth both suggest that these gOs/CFs are not precursors to the RFs.

This observation of two amyloid assembly pathways associated with amyloid β peptides allows us to address concerns associated with the amyloid cascade hypothesis. One of the major problems with the amyloid cascade hypothesis is the week correlation between plaque loads present in the tissues and the severity of the disease. This issue was addressed in the modified version of the amyloid hypothesis by introducing the concept of toxic early stage oligomers as the
toxic species not the end stage fibril plaques. However, if oligomers are on pathway precursors to
the RF formation then, again, the final plaque load should correlate with the neurodegeneration
and synapsis loss in AD.

Our observation of two distinct amyloid assembly pathways; oligomeric and oligomer free,
is providing an alternate explanation to this issue. The end stage RFs can form and accumulate in
the brain tissues either in the presence or/and absence of the toxic oligomers. Therefore it is
possible to observe amyloid plaques present in healthy individuals as well as modest amounts of
post-mortem plaques in patients who suffered from severe AD.

5.8 Materials and Methods

5.8.1 Protein and Chemicals

Amyloid beta 1-40 and 1-42 was purchased from GL Biochem (Shanghai, China). Ultrapure grade thioflavin T was obtained from Anaspec (Freemont, CA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and were reagent grade or better. 18 MV RO deionized water (Barnstead E-pure, Dubuque, IA) was used to prepare all the solutions.

5.8.2 Preparations of Amyloid β Peptides

1mg of amyloid β peptide was dissolved in 500 µl of 100 mM NaOH and subjected to gel filtration on a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with 35 mM Na₂HPO₄, 5 mM NaOH, 50 mM NaCl pH 11. The monomer peak eluted at 14 ml and was collected on ice. Peptide concentrations were measured using UV absorption at 280 nm (ε₂₈₀ = 1490 cm⁻¹ M⁻¹). 1.5% of 1 M NaH₂PO₄ was added to the samples to reduce the pH down to 7.4.
5.8.3 Amyloid β Kinetic measurements

All the kinetics measurements were performed using a plate reader (Fluostar Omega, BMG Labtech). The Thioflavin T fluorescence was measured using excitation filter 448 nm and emission filter 482 nm. 100 µL of each sample was pipetted into 96 well half area plate (Corning 3881) and covered with a transparent polypropylene film (Fisher 08-408-240). Final Thioflavin T concentration was 10 µM.

5.8.4 Thioflavin T Stock Solution Preparation

Thioflavin T stock solution was prepared by dissolving dye in distilled water and filtering it through a 200 nm pore size Nylon syringe filter. Actual Thioflavin T concentration was determined from absorption at 416 nm ($\alpha_{416} = 26\,620\,\text{ml\,mg}^{-1}\text{\,cm}^{-1}$).

5.8.5 Atomic Force Microscopy

Amyloid β samples were diluted 10-50 folds into the 50mM sodium phosphate, 50 mM NaCl, pH 7.4 buffer. Then 40 µl of the diluted sample was deposited onto freshly cleaved mica for 3-5 minutes. Mica surfaces were rinsed with deionized water and dried with dry nitrogen. Amyloid aggregates 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. The cantilever had a typical spring constant and resonance frequency of 2 nN/nm and 70 kHz, respectively. It was driven at 60–70 kHz in alternating current mode and at a scan rate of 0.25-0.5 Hz. Images were acquired at $512 \times 512$ pixel resolution. Amplitude, phase, and height images were collected for the same sample area.
5.8.6. Transmission Electron Microscopy

Amyloid β samples were diluted into distilled water right before depositing them onto the Formvar/carbon film coated, 200 mesh copper grids. 5 ul of the diluted sample was placed on the surface of the grid and allowed to air dry. A 5 ul aliquot of distilled water was then added to the grid and blotted to remove salt crystals from the grid. This step was repeated at least 3 times and then the grid was negatively stained with 5 ul of 8% (w/v) uranyl acetate for 1 minute, blotted and dried. Excess uranyl acetate was removed by repeated washing with distilled water and the grid was left to air dry. All the grids were imaged using a FEI Morgani transmission electron microscope at 60 kV with an Olympus MegaView III camera.
References


32. *Amyloidosis.* National Organization for Rare Disorders (NORD)


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