Characterizing the in-vitro morphology and growth kinetics of intermediate amyloid aggregates

Shannon E. Hill
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Characterizing the *In-Vitro* Morphology and Growth Kinetics of Intermediate Amyloid Aggregates

by

Shannon E. Hill

A thesis submitted in partial fulfillment of the requirements for the degree of
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Characterizing the In-Vitro Morphology and Growth Kinetics of Intermediate Amyloid Aggregates

Shannon E. Hill

ABSTRACT

The mechanisms linking deposits of insoluble fibrils of amyloid proteins to the debilitating neuronal cell death characteristic of neurodegenerative diseases remain enigmatic. Recent findings suggest that transiently formed intermediate aggregates, and not the prominent neuronal plaques, represent the principal toxic agent. Evaluating the neurotoxicity of intermediate aggregates, however, requires unambiguous characterization of all aggregate structures present, their relative distributions, and how they evolve in time. Hen-egg white lysozyme represents an attractive model for studying intermediate aggregate formation since it is an extensively characterized globular protein, and its human variants can lead to systemic amyloidosis.

Combining in-situ dynamic light scattering (DLS) with atomic force microscopy (AFM), we have characterized the morphologies and growth kinetics of intermediate aggregates formed during lysozyme fibrillogenesis. Upon incubation at elevated temperatures, small uniform oligomers form with their numbers increasing for several hours. After a variable lag period protofibrils spontaneously nucleate. The heights and widths of protofibrils closely match those of oligomers. This match in physical dimensions, combined with the delayed onset of protofibril nucleation vs. the continuous
formation of oligomers, suggest that protofibrils both nucleate and grow from oligomers. Protofibril morphologies and structures, visualized with AFM, are quite distinct from subsequently emerging mature fibrils. Overall, the evolution of aggregate morphologies during lysozyme fibrillogenesis follows a clear hierarchical pathway: amyloid monomers initially coalesce into oligomers of uniform size. Their steadily increasing numbers eventually induce nucleation and growth of protofibrils. Protofibrils, in turn, nucleate and grow via oligomer addition until they start to self-assemble into micron-sized double-stranded fibrils.
I. INTRODUCTION

1.1 Motivation and Background

Alzheimer’s and Parkinson’s disease are familiar representatives for a category of human disorders called amyloidoses. Amyloidoses are characterized by prominent extracellular protein plaques, composed predominately of amyloid protein fibrils, and extensive tissue damage in their immediate vicinity. A group of twenty-five different proteins or proteolytic protein fragments have been implicated in various forms of amyloidoses (1, 2). Recent findings have strongly implicated small soluble aggregates, formed transiently during the early phases of fibrillogenesis, as the main toxic species mediating cell death (3, 4). However, the specific identity of the toxic intermediates, their relative populations under different solution conditions and at different times, as well as their specific mode of action have remained uncertain. This uncertainty is reflected in the ambiguous terminology used to refer to intermediate aggregates varying from (prefibrillar or fibrillar) oligomers (3-5) micelles (6), amorphous aggregates (7), protofibrils (8), protofilaments (9), to ADDL's or A-derived diffusible ligands (10).
These distinct labels imply markedly different aggregate structures, but it is often unclear whether they are used to refer to the same or distinct soluble aggregate species. Part of the problem appears to be the specific choice of biophysical, biochemical or histopathological methods used to characterize intermediate aggregates. Individual techniques can provide only partial information (MW, hydrodynamic radius, overall morphology, antibody reactivity) which does not necessarily discriminate between the distinct structural or morphological features of different aggregates. These difficulties are further aggravated since aggregates sizes and morphologies can be dramatically altered by subtle changes to solution conditions or sample preparation (11). Hence, *insitu* and quantitative characterization of the sizes and morphologies of different soluble amyloid intermediates, and of changes to their growth kinetics in response to various interventions, represents an essential first step toward unraveling the elusive relationship between different types of intermediate aggregates and their cellular toxicity.
1.2 Hen Egg White Lysozyme: An Attractive Model Protein

We have investigated amyloid fibrillogenesis of the small globular enzyme, hen egg white lysozyme (HEWL). With a molecular weight of 14,388 g/mol and a single chain of 129 amino acids, HEWL’s biophysical and biochemical properties have been extensively characterized. Essentially upon incubation in acidic solutions below pH = 4 and at elevated temperatures, native HEWL has also been shown to grow amyloid fibrils (13). Commercial lysozyme stock can be prepared that is essentially devoid of pre-assembled aggregates which often interfere with nucleation and growth studies, including those using the Alzheimer Aβ peptides. With variants of human lysozyme being implicated in hereditary systemic amyloidosis, HEWL provides an attractive and biomedically relevant model system for characterizing the transition of an ordered protein monomer with a well-defined secondary structure to the amyloid fibril state (14).
1.3 Dynamic Light Scattering (DLS) Theory

Dynamic Light Scattering is a technique used for measuring the size of submicron particles by measuring fluctuations in intensity due to particles undergoing Brownian motion. In a typical experiment, a beam of light is passed through a suspension of particles which scatter light. If the particles are comparatively small to the wavelength of light, the intensity of scattered light is uniform in all directions and is termed Rayleigh scattering. However if the particles are large compared to the wavelength of light, the intensity is angle dependent or called Mie scattering.

Given that the incident light is coherent and monochromatic (such as a laser) it is possible to detect the time-dependent fluctuations in the scattered intensity by using a photomultiplier and recording the number of scattered photons. These fluctuations in scattered intensity arise from the particles undergoing random thermal Brownian motion resulting in constructive and destructive interference. By analyzing the time dependence of these intensity fluctuations, the diffusion coefficient of the particle and the hydrodynamic radius can be determined.

The time dependence of the intensity fluctuation is commonly analyzed using a digital correlator which calculates the intensity autocorrelation function given by:

\[ g_2(\tau) = \frac{\int I(t)I(t+\tau)dt}{\int I(t)I(t)dt} \]
The intensity-intensity correlation coefficient is then used to calculate the size and polydispersity of the various populations by numerical algorithms relating the exponential decay of the correlation function to the translational diffusion coefficient.

In order to convert the intensity-intensity correlation function into a particle size distribution, the intensity correlation function is first changed into the field correlation $g_1(\tau)$ by the using the Siegert relationship:

$$g_1(\tau) = \sqrt{g_e(\theta)} - 1$$

This in turn is the sum of the exponential decay rates for the different size particles given by:

$$g_1(\tau) = \sum \exp(-Dq^2\tau)$$

where $D$ is the translational diffusion coefficient and $q$ is the scattering wave vector. The scattering wave vector is given by:

$$q = \left( \frac{4\pi n}{\lambda_o} \right) \sin(\theta/2)$$

where $n$ is the refractive index of the scattering medium, $\lambda_o$ is the wavelength of incident light in a vacuum, and $\theta$ is the angle of scattering.

Finally, from the translation diffusion coefficient, $D$, the hydrodynamic radius for a spherical particle can be calculated given by the Stokes-Einstein equation,

$$R_h = \frac{k_B T}{6\pi\eta D}$$

where $k_B$ is the Boltzman constant, $T$ is the absolute temperature, and $\eta$ is the viscosity of the solution.
The typical sampling time is 100ns to several seconds depending upon the particle size and viscosity of the medium. The size range for dynamic light scattering is typically submicron to a few microns in diameter. The lower limit of the particle size is highly dependent on the scattering properties of the particles concerned, incident light intensity, and detector. For instance, the Malvern Zetasizer Nano, quotes a lower limit as low as 0.6 nm and an upper limit equal to 6 microns.

**Figure 1** *Diagram of Dynamic Light Scattering Set-up from Malvern Instruments.* As the laser illuminates the sample, scattered light is measured by the detector at a specified angle and passed to the digital correlator for processing by software algorithms to calculate the size and polydispersity of the suspension of particles. (30)
1.4 Atomic Force Microscopy (AFM) Background

The atomic force microscope is a mechanical imaging technique capable of measuring the three dimensional topography of a surface on the order of fractions of nanometer. The AFM consists of a sharpened probe or tip usually made of silicon on the end of a cantilever. The silicon tip is used to scan the surface of a sample by measuring the forces between the sample and tip. As the tip approaches the surface, the forces between the tip and sample lead to a deflection of the cantilever which is measured by using a laser spot reflected from the top of the cantilever to an array of photodiodes. The electronic output of the photodetector is then sent to a feedback controller that drives a Z motion generator to maintain a constant force or distance between the tip and sample.

Figure 2 Diagram of Atomic Force Microscope. As the cantilever approaches the surface, the cantilever bends, changing the light path of the laser reflected from the top of the cantilever to the photodetector. (31)
AFM imaging methods can be divided into static (contact) modes and dynamic (non-contact) modes. In contact mode, the deflection or force between the tip and sample is kept constant by adjustments in the z direction from the feedback mechanism. The voltage that the feedback amplifier applies to the piezo to raise or lower the sample is a measure of the height of the sample’s features as a function of the lateral position. Contact mode is usually chosen for hard or rough samples where lateral forces will not damage the sample due to scraping across the surface.

For biological or soft samples, non-contact modes or AC modes are used by oscillating the cantilever near its resonance frequency without constant contact to the sample. The changes in the oscillating frequency or the amplitude of the cantilever provide the signal for imaging. For instance, in tapping mode the amplitude of the cantilever’s oscillation decreases as the tip gets closer to the surface extending into the repulsive regime where the tip intermittently touches the surface.
2. MATERIALS AND METHODS

2.1 General Approach

As mentioned above, one experimental concern are potential distortions of aggregation kinetics or disruptions to aggregate structures by characterization techniques (gel electrophoresis, liquid chromatography, AFM, TEM, mass spectroscopy etc) that require aggregates to be removed from their solution environment and/or separated prior to analysis (see e.g. ref. (15). For example, imaging of amyloid aggregates using atomic force microscopy alone can be problematic since deposition on different surfaces might alter the very aggregate structures one is trying to characterize (16). DLS distinguishes itself from alternative characterization techniques since it is essentially non-invasive, is highly sensitive to aggregate formation, and provides an *in-situ* read-out of particle distributions and growth kinetics (17). These significant advantages of DLS are mitigated by its limited ability of resolving aggregates whose hydrodynamic radii $R_h$ differ by less than a factor of two or three. AFM, in contrast, has the ability to resolve height differences much smaller than typical protein radii. Hence, we used dynamic light scattering to follow the in-vitro aggregation kinetics of lysozyme amyloid fibrillogenesis while we visualized and characterized aggregate morphologies off-line with atomic force microscopy (AFM).
By calibrating the sizes of our AFM scanning tips we were able not just to visualize aggregate morphologies but to obtain quantitative measurements of aggregate dimensions. Combining quantitative measurements of individual aggregate morphologies obtained from AFM with *in-vitro* particle size distributions derived from DLS provided a self-consistent and more detailed characterization of intermediate aggregates than either technique can yield on its own. Our combined approach provides a detailed description of oligomer and protofibril morphologies and their mutual assembly that challenges several currently prevailing views.
2.2 Preparation of HEWL Amyloid Fibrils

Solutions of lysozyme (17 mg/mL) at pH = 2.0 with 175 mM NaCl were prepared by dissolving lyophilized lysozyme in distilled water at twice its final concentrations and mixing it 1:1 with a NaCl/water solutions, also at twice its final concentration. Prior to mixing, lysozyme solutions were warmed to 45°C in order to remove any preformed clusters. All samples were centrifuged at 9,500 g for 5 min, and filtered consecutively through a 220 nm and a 20 nm pore size syringe filter. Solution pH was readjusted to pH 2.0 with 1N HCl. Lysozyme concentrations in solution were determined from UV absorption measured at λ=280 nm (α_{280}=2.64 ml mg^{-1} cm^{-1}) (27). Lysozyme solutions incubated for 4-5 days at 50°C started to form a soft gel. Fibers taken from the gelled-out samples induced a red shift in the absorption spectrum of congo red, a feature diagnostic of amyloid fibril formation (data not shown).
2.3 Dynamic Light Scattering (DLS) Apparatus

DLS measurements were performed with a Zetasizer Nano S (Malvern Instruments Ltd., UK) containing a 3 mW He–Ne laser ($\lambda = 633$ nm) and with built-in temperature control for sample cuvettes. After thermal equilibration of the samples (typ. less than 5 min), autocorrelation functions were collected every 10 minutes using acquisition times of 60 s. Autocorrelation functions were converted into particle size distributions using the “narrow modes” algorithm provided with the Zetasizer Nano S. Particle size distributions obtained from alternative inversion algorithms yielded comparable results.
2.4 Atomic Force Microscope (AFM) Set-up

Amyloid samples were imaged with an Asylum Research MFP-3D atomic force microscope using silicon tips (MikroMasch NSC36/NoAl or Nanosensors PPP-FMR-50) with nominal tip radii of 10 nm and 7 nm respectively. The cantilever was driven at 60 to 70 kHz in AC mode and a scan rate of 0.5 Hz acquiring images at 1024 x 1024 pixels resolution. Raw image data were corrected for image bow and slope.
2.5 AFM Tip Calibration with Gold Colloids

AFM tip radii were determined by imaging 5nm gold colloid standards (BBI International, cat# GC5). Poly-d-lysine (1%) was deposited onto freshly cleaved mica for 10 seconds, rinsed with deionized water and dried with nitrogen. The gold colloids were diluted 1:20 with deionized water and deposited for 1 minute, rinsed with deionized water, and dried with nitrogen. As indicated in Fig. 3, the apparent width $W_{app}$ of an incompressible object imaged with an AFM tip is considerably larger than its physical width $W$.

![Figure 3 Dilation of Lateral Particle Dimension During AFM Imaging](image)

**Figure 3** *Dilation of Lateral Particle Dimension During AFM Imaging.* The finite size of the AFM scanning tips (shown here as parabola) increases the apparent width $W_{app}$ of a particle well beyond its actual width $W$. For an ellipse, $W$ can be derived using the measured height $H$ and $W_{app}$ together with the parabolic tip radius $R_{tip}$ (28).
Apparent width $W_{app}$ and height for a given gold colloid were determined from individual scan lines across the maximal height of a given gold colloid (see Fig. 4B). Following ref. (28), the apparent width $W_{app}$ and height of a sphere was used to obtain the radius $R_t$ of the scanning tip. As assessed from the measured heights, the radii of individual gold colloids vary slightly. Therefore, we determined the radius $R_t$ using a series of gold colloids. This approach yielded reproducible and self-consistent values for $R_t$ (Fig 4C). Repeating tip calibrations during extended imaging sessions on mica substrates indicated that tip radii remained unaltered.

**Figure 4** Calibration of AFM Tip Radius Using Colloidal Gold Standards. (A) AFM image of 5 nm gold colloids deposited on mica. (B) Height vs. position profile across the center of a single gold colloid which yield the apparent width $W_{app}$ and diameter (height) for a colloidal gold sphere. (C) Tip radii $R_{tip}$ obtained for several gold colloids with slightly different diameters. As indicated, the tip radius was independent of the specific diameter of the gold colloid used for calibration.
2.6 AFM Imaging of Amyloid Aggregates

During DLS measurements of amyloid fibrillogenesis, 10µL aliquots of solution were taken from the DLS cuvette for subsequent AFM imaging. Aliquots were diluted 100-fold with 175 mM NaCl/pH = 2.0 salt solutions. 75 µL of solution was deposited on freshly cleaved mica for 5 minutes, rinsed with deionized water and dried with dry nitrogen. Images of amyloid aggregates were acquired in air. Using DLS, we confirmed that cooling the lysozyme samples from 50°C to room temperature essentially arrested any further aggregation (data not shown). Hence, AFM images faithfully represented the aggregate distribution at the time of aliquot collection.
2.7 Correction of AFM Images for Tip Dilation

To correct for the dilation in apparent particle width induced by a calibrated scanning tip (see Fig. 3), line-profiles of apparent particle width vs. height were taken across the center of a given particle. The direction for these line profiles was chosen perpendicular to the scanning direction of the AFM tip during image acquisition. The shape of the profiles was highly symmetric around the peak height, suggesting that mechanical tip distortion were small. Assuming that aggregates had ellipsoidal cross-sections and using the AFM tip radius determined during tip calibration, the actual particle width was calculated (28).
2.8 Prediction of Hydrodynamic Radii from AFM Particle Dimensions

Assuming that particles were oblate ellipsoids, particle dimensions for lysozyme monomers and oligomers were extracted from AFM line scans. Perrin’s formulas then predict their (18) expected hydrodynamic radii. For an oblate ellipsoid with major axis \( a \) and minor axis \( b \), the hydrodynamic radius is given by (29)

\[
R_h = \frac{b}{\tan^{-1}(x)}, \text{ where } x \left(\frac{a}{b}\right)^2 - 1\right)^{1/2}
\]

Similarly, the straight protofibrils present shortly after nucleation were approximated as cylindrical rods. The hydrodynamic radius for a cylinder of length \( L \) and diameter \( d \), is given by

\[
R_h = \frac{L}{2} \left\{ \left(1-x\right)^{1/2} / \ln\left(1+(1-x)^{1/2}/x\right) \right\}, \text{ where } x \frac{d}{L} \left[ 1 + 0.37(L-d)/L \right]
\]
3. RESULTS AND DISCUSSION

3.1 DLS Measurement of Aggregate Size Distribution and Kinetics

Fig. 5A shows the evolution of the field correlation functions $g_1(\tau)$ of light scattered from lysozyme solutions undergoing fibrillogenesis at pH = 2.0 and T = 50 C. Initially, $g_1(\tau)$ decays nearly single-exponentially with a very narrow distribution of decay rates. After about 10 hours, $g_1(\tau)$ suddenly develops a prominent shoulder. The amplitude of this shoulder increases steadily while its decay rate continues to decrease. This indicates the formation of a second, larger population of aggregates with slower diffusion coefficients. All correlation functions were converted to distributions of decay rates and corresponding particle size distributions (PSDs), as described in Materials and Methods. The changes in the PSD with time indicate how aggregation in the solution progresses (Fig. 5B). As suggested by the raw correlation data, particle size distributions are dominated by two aggregate peaks with well-separated hydrodynamic radii. The single narrow peak prior to heating matches the mean hydrodynamic radius for lysozyme obtained at comparable lysozyme and salt concentrations but at pH = 4.5, i.e. for conditions under which lysozyme retains its native structure (18). Even after heating the samples to 50 ºC, the PSD remains monomodal for several hours. However, this monomodal peak does broaden slightly and becomes gradually more asymmetric, with its mean radius increasing from 1.9 nm to $r_h \sim 2.4$ nm (see Fig. 8A)
Figure 5 Light Scattering During Amyloid Fibrillogenesis of Hen Egg-White Lysozyme (HEWL). (A) Temporal evolution of the field autocorrelation function $g_1(\tau)$ of light scattered from HEWL solutions during amyloid fibrillogenesis at pH = 2.0 and T = 50 C. Around 10 hrs the correlation function develops a prominent shoulder which continues to grow in amplitude. (B) Semi-log plot of the particle sizes distribution (PSD) derived from $g_1(\tau)$. Initially, the PSD has a single, narrow (notice log axis) peak. Following an approx. 10 hr lag period a second peak emerges and starts to dominate the PSD. (C) Log-log plot of the center position of the two aggregate peaks as function of incubation period. While the small particle peak remains essentially stationary, the larger aggregates gradually and steadily increase in size. (D) Log-log plot of the static scattering intensity $I_{\text{scat}}$ vs. incubation period. The relative scattering intensity shown here was corrected for changes in attenuation used to avoid detector saturation.
Following a (sample specific) latency period of several hours, a second larger aggregate species nucleates with an initial $R_h$ around 25 nm (Fig 5C). As indicated by the growth of its integrated peak area (Fig. 5B) and the concurrent increase in the total intensity of scattered light (Fig. 5D), the total number of this larger aggregate species steadily increases.

In contrast to the nearly hundred-fold increase in scattering intensity (Fig. 5D), the mean hydrodynamic radius of the large aggregate species barely doubled over the course of 2-3 days (Fig. 5C). Following the nucleation of the larger aggregates, the size of the small aggregate peak starts to diminish until it becomes undetectable against the strong scattering background from the larger aggregates. Except for the expected variations in the latency period for nucleation, all of the above features in our DLS data were remarkably reproducible ($N_{total} = 12$).
3.2 AFM Imaging and Quantitative Analysis of Aggregate Morphology

We used AFM to address several ambiguities in the DLS data. In particular, we examined whether either the small or large aggregate peak (see Fig. 5B) were composed of several distinct aggregate species not resolved by DLS; determined the identity of the intermediate aggregates nucleating from solution; characterized the morphology for each of the intermediate aggregate species; and obtained quantitative measurements of aggregate dimensions.

For quantitative analysis of aggregate dimensions, the dilation of lateral particle dimensions introduced by the finite size of the AFM scanning tip needs to be corrected (see schematic in Fig. 3). Hence, we calibrated AFM scanning tips prior to imaging of aggregates morphologies. A typical result of scanning tip calibrations with colloidal gold standards is shown in Fig. 4 (for details, see Materials and Method). Scanning tip radii were typically at least twice as large as the tallest lysozyme aggregates we imaged. Therefore, we neglected higher-order distortions to lateral particle dimensions arising from contact of aggregates with the scanning tip shaft. In order to correlate DLS with AFM data, lysozyme fibrillogenesis was monitored using DLS while small aliquots were withdrawn for AFM imaging at various times during the incubation period.
AFM images of lysozyme before heating yield particles well approximated by oblate ellipsoids with dimension of $(3.0 \pm 0.2)$ nm height and $(3.8 \pm 0.8)$ nm equatorial width (see Fig. 6A & Table 1). We identify these particles as lysozyme monomers since their volume of $22.6 \text{ nm}^3$ is in close agreement with the volume of $21.2 \text{ nm}^3$ for lysozyme monomers in crystals (19). The aspect ratio of surface-absorbed lysozyme, however, is slightly different from its counterparts in solution (20). After raising sample temperatures to 50 C, solutions contained mixtures of lysozyme monomers and compact aggregates with narrow size distributions (see Fig 6B). For convenience sake, we will refer to these small aggregates as oligomers. The AFM observation, therefore, imply that the seemingly uniform "monomer" peak in DLS actually represents an evolving mixture of monomers and oligomers that form upon heating. It is worth noting that the apparent width vs. height ratios obtained for both oligomers and monomers are inconsistent with those for spherical particles but match those for oblate ellipsoids. Based on this ellipsoidal geometry, the volume of the oligomers was 8.1 times that for the lysozyme monomers, suggesting that oligomers contain eight monomers.

The nucleation of a second particle species detected by DLS (Fig 5C) coincides with the appearance of short fibrils in AFM images (Fig. 6C). After nucleation, these short fibrils increase in length over a time period of about two to three days. At the late stages of our experiments a second population of fibers emerges. These late-stage fibers are much longer and stiffer, with heights 1.5 times those of the smaller and shorter fibrils. We will refer to these distinct fibril populations as protofibrils and double-stranded fibers. The heights and widths of all intermediate amyloid aggregates are summarized in Table 1.
Figure 6 AFM Images of Intermediate Amyloid Aggregates formed by Lysozyme.

Progression of amyloid aggregate morphologies grown at pH = 2.0 and T = 50ºC deposited on mica and imaged in air. (A) Before heating (T = 20ºC), AFM images of HEWL samples show a uniform population of monomers (B) AFM images of HEWL samples after 3 hours of incubation at 50ºC predominately yield oligomers, which apparently adhere more readily to mica. (C) AFM image of protofibrils appearing shortly after the nucleation event around 10 hrs. (D) AFM image of protofibrils after 25 hours incubation. (E) AFM image of long and straight fibrils formed after approx. 100 hours of incubation, against a background of protofibrils. The tip radius for AFM images (A-B) was 16 nm while for the images (C-E) it was 12.5 nm. The dimensions for monomers, oligomers, protofibrils and mature fibrils derived from such AFM measurements are summarized in Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Height (nm)</th>
<th>Actual Width (nm)</th>
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<tbody>
<tr>
<td>Monomer (0 hrs)</td>
<td>3.0 ± .2</td>
<td>3.8 ± .8</td>
</tr>
<tr>
<td>Oligomer (3 hrs)</td>
<td>3.9 ± .1</td>
<td>9.5 ± 1.0</td>
</tr>
<tr>
<td>Prototibril (10.5 hrs)</td>
<td>3.9 ± .3</td>
<td>9.5 ± 1.7</td>
</tr>
<tr>
<td>Prototibril (55 hrs)</td>
<td>3.9 ± .2</td>
<td>9.6 ± 1.0</td>
</tr>
<tr>
<td>Mature Fibril (100 hrs)</td>
<td>6.0 ± .2</td>
<td>13.9 ± 1.0</td>
</tr>
</tbody>
</table>

Table 1 Height and Width of Intermediate Aggregates. Average of heights and actual widths measured from AFM height vs. tip position profiles for various intermediate aggregates formed during HEWL fibrillogenesis.
The AFM images of protofibrils reveal several intriguing features. First of all, protofibrils have noticeable and rather regular height undulations. In principle, this variegated appearance is compatible with the presumption that any polymeric precursor of mature fibrils already display the helical twists known to exist in mature fibrils (14). However, the maximum height and width of protofibrils are identical to those of oligomers seen prior to protofibril nucleation (see Table 1 and Fig. 7). This quantitative agreement between the heights and widths of protofibrils and oligomers implies that oligomers are not just nucleation centers for protofibrils (21) but also represent their basic growth unit. This conclusion is further supported by the distinct growth kinetics of oligomers vs. protofibrils. AFM imaging indicates that oligomers form essentially upon heating of our solutions. The dimensions of the oligomer are essentially constant, only their numbers increase over time. In contrast, no protofibrils are detected in AFM images prior to the well-defined nucleation event in our DLS data (see Fig. 5C and D). If protofibrils required oligomers only as nucleation sites but grew via monomer addition, protofibril growth should commence simultaneously with oligomer formation. This is not observed. Instead, the combination of AFM and DLS data strongly suggest that oligomer formation is the rate-limiting step for both the nucleation and growth of protofibrils.
Figure 7 AFM Surface Topography of Oligomers and Protofibrils. (A) AFM topography of a HEWL oligomer after 3 hours of incubation at 50º C. (B) AFM topography of HEWL protofibrils shortly after the nucleation event. Both images are 125 nm on a side and have not been corrected for tip dilation in the x-y plane.

Following nucleation, the contour length of protofibrils increases significantly (Fig 6D) compared to the rather modest change in their hydrodynamic radius observed with DLS (Fig. 5C). AFM images, however, also show that protofibrils are soft and flexible polymers, as apparent from the many rapid twists and the small radius of curvature displayed by protofibrils that have grown out to 200 nm or longer. This is particularly apparent when comparing late-stage protofibril shapes to the much longer but straighter double-stranded fibrils seen in Fig. 6E. Clearly protofibrils do increase their contour length significantly during their 2-3 day growth period. Due to their short persistence length, however, the solution structure of protofibrils switches from that of short rods seen immediately after nucleation to random gaussian coils for the longer protofibrils predominant beyond the initial nucleation phase. Since the radius of gyration for random gaussian coils increases with the square-root of their contour length, the hydrodynamic radius measured in DLS would be expected to increase at a much lower rate than the corresponding contour length of protofibrils.
After the contour length of protofibrils has grown to about 300 - 400 nm, they begin to assemble into much longer double-stranded fibrils. Direct comparison of protofibril heights and widths with those of double-stranded fibrils indicates that the latter are about 1.5 times as high and wide as protofibrils. Both the radius of curvature of double-stranded fibrils and their overall length is significantly increased when compared to those of protofibrils (see Fig. 6E). This dramatic increase in persistence length indicates that mature fibrils are mechanically much stiffer than protofibrils. While we did not analyze this feature quantitatively, it is in good qualitative agreement with AFM images of Aβ-40 protofibrils vs. mature fibrils (e.g. Fig. 1 in ref (22)) and suggests yet another feature common to amyloid fibrils formation by different proteins.
3.3 Quantitative Comparison of DLS with AFM Data

A priori, it is not obvious whether the sizes and shapes of amyloid aggregates in solution should match those dried and deposited on mica substrate. The morphology of aggregates has been shown to change with the surface properties (e.g. charge and hydrophobicity) of the substrate they are deposited or grown on (23). Hence, we set out to test whether the particle sizes and morphologies derived from these two distinct measurement approaches yield quantitatively self-consistent results. In particular, we wanted to address two potential discrepancies between these data sets. First, prior to protofibril nucleation, the initial peak in the particle size distribution is nearly stationary, with only a subtle increase in its asymmetry and mean hydrodynamic radius (see Fig. 8A). AFM images, in contrast, indicate the presence of octomeric aggregates (see Fig. 6B). The total number of these octomeric aggregates steadily increased in time while their dimensions stay fixed. This raises the question whether the subtle changes in the "monomer peak" obtained from DLS are consistent with the obvious increase in octomer population detected with AFM. The second potential discrepancy relates to the apparent size of protofibrils shortly after nucleation. Protofibrils formed soon after the initial nucleation event appear as rather sizeable elongated rods of 100 nm length or more while DLS indicates the nucleation of particles with hydrodynamic radii of 20 - 30 nm.
To address these potential discrepancies we predicted hydrodynamic radii for either oligomers or short protofibrils, based on particle dimensions derived from calibrated AFM measurements, and compared them to the average hydrodynamic radii measured with DLS.

**Figure 8** Analysis of Oligomer Formation in DLS. (A) The PSD of the “small aggregate peak” prior to protofibril nucleation becomes increasingly skewed, with its center position shifting slightly from 1.9 nm to 2.4 nm. These changes coincide with the growth of uniformly-sized oligomers (Fig. 4B). (B and C) Comparison of the residual errors when fitting $g_1(\tau)$ either to a single-exponential function with adjustable radius or a double-exponential function with decay rates matched to the 1.8 nm monomer- and 3.8 nm oligomer- hydrodynamic radii estimated from AFM data. While the single-exponential fit suffers from clear systematic errors (C), the bimodal distribution observed with AFM provides an excellent fit.
We approximated the shape of lysozyme monomers and octomers as oblate ellipsoids, with their lateral dimensions corrected for AFM tip dilation (see Fig. 3). The morphology of short protofibrils, formed soon after nucleation (see Fig. 6C), was approximated by straight rods with the initial contour length of protofibrils after nucleation of 125 ± 28 nm and diameters close to 6 nm. Using Perrin's formulas (see Materials and Methods and ref. (24)), we calculated the corresponding hydrodynamic radii for oblate ellipsoids and long, thin cylinders. Table 2 summarizes the hydrodynamic radii for lysozyme monomers, oligomers, and the initially rod-like protofibrils as predicted from dry aggregate dimensions (AFM) vs. measured average hydrodynamic radii (DLS). Despite the various simplifying assumptions about particle geometry and the neglect of hydration layers, the hydrodynamic radii estimated from AFM measurements agree remarkably well with those measured using DLS. This self consistency implies that aggregate morphologies and sizes are not significantly altered by surface deposition onto mica, drying and the mechanical distortions intrinsic to AFM imaging.

Table 2: Comparison of measured (DLS) and estimated (AFM)

<table>
<thead>
<tr>
<th>Sample</th>
<th>DLS $R_h$ (nm)</th>
<th>AFM $R_h$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer (0 hrs)</td>
<td>1.9</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>Oligomer (3 hrs)</td>
<td>2.3</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>Protofibril (10.5 hrs)</td>
<td>21.6</td>
<td>18.2 ± 3.0</td>
</tr>
</tbody>
</table>

Table 2: Comparison of DLS and AFM Hydrodynamic Radii (2nd column) Mean hydrodynamic radii for lysozyme monomers (0 hrs), weighted average of monomers and oligomers (3 hrs) or protofibrils shortly after nucleation (10.5 hrs) measured with DLS. (3rd column) Predicted hydrodynamic radii for lysozyme monomers, oligomers and protofibrils calculated from measured AFM dimensions.
3.4 HEWL Aggregation Similarities to Aβ-40 Peptide

Lysozyme amyloid fibrillogenesis displays remarkable similarities with the aggregation kinetics and aggregate morphologies of the Alzheimer amyloid peptide Aβ-40. AFM images indicate the presence of "oligomeric" lysozyme aggregates of uniform size during the early stages of fibrillogenesis. In the case of Aβ amyloids, such oligomers have been implicated as a major toxic species mediating Alzheimer disease (3, 4). Aside from the intrinsic difference in monomer size and the lack of a well-resolved lag-period, the aggregation kinetics of Aβ-40 in acidic solutions obtained with DLS (Fig. 1 in ref. (6)) closely resembles that of lysozyme in this work (Fig. 5B). Finally, the relative sizes and appearance of lysozyme protofibrils and double-stranded fibrils (see Fig. 6E) agree surprisingly well with AFM images of comparable Aβ-40 samples (22).

These similarities support the emerging consensus that important aspects of amyloid fibrillogenesis are shared among many different types of amyloid proteins, including the formation of oligomeric species, the aggregation pathway from monomers over oligomers and protofibrils to double-stranded fibrils, the shared cross-sheet structure of the mature fibrils, and the binding of specific small ligands to amyloid fibrils (1). At the same time, combining in-situ DLS with off-line AFM measurements yields important new insights into the kinetics and the progression of morphological changes associated with amyloid fibrillogenesis. This is particularly true for the relation between small oligomers and the nucleation and growth of protofibrils.
3.5. Oligomers: The Building Blocks of Protofibril Growth

Oligomers grew from solutions without apparent lag time upon heating of the solutions. They had well-defined elliptical shapes and a very narrow distribution of particle dimensions containing about eight lysozyme monomers. While oligomer sizes were essentially constant, oligomer populations increased steadily prior to the nucleation of protofibrils. DLS and AFM measurements do not provide us with detailed information about the internal organization of these oligomers. Their tight size distribution and compact shape, however, are consistent with either "classical" oligomers (3, 4) or proteinaceous micelles (6).

Our data provide three distinct lines of evidence that protofibrils not just nucleate from oligomers but also grow via oligomer addition. First, the quantitative agreement of the heights and widths of oligomer and protofibril and, to a lesser extent, the periodic undulation in protofibrils height strongly support this interpretation. In addition, both DLS and AFM data indicate that oligomers begin to form essentially upon heating, albeit at small numbers. If protofilaments required oligomers only as nucleation centers but grew via monomer addition, their growth should commence without time-lag and simultaneous with the onset of oligomer formation. Instead, protofibrils only nucleated after the oligomer population has increased significantly.
Finally, protofilament growth seems to consume oligomers, with the latter becoming increasingly difficult to detect as protofibrils proliferate. This is consistent with our interpretation of protofibril growth via oligomer addition. Closer inspections of the AFM images for short protofibrils, i.e. those observed soon after nucleation, suggest that oligomers coalesce into linear aggregates along their highly curved perimeters. This model appears rather different from predictions of protofibrils with spiral geometries (25) typical for the cross β-sheet structure of mature fibrils (14, 26). AFM images of protofibrils next to double-stranded fibrils further boost our assertion that protofibrils do not yet share the helical cross β-sheet structure with mature fibrils. Macromolecules with helical structure tend to be mechanically stiff, with the large persistence length of double-stranded DNA as a classical example. The long, straight shapes of double-stranded fibrils certainly follow that expected relation between internal helical structure and mechanical stiffness. Protofibrils, in turn, are highly flexible and lack the mechanical stiffness of double stranded fibrils (see Fig 6E).
4. CONCLUSION

In summary, under acid growth conditions, lysozyme amyloid growth proceeded along a clearly defined and hierarchical aggregation pathway. Amyloid monomers aggregate into small oligomers of uniform size. These oligomers provide both the nucleation sites and the basic growth unit for protofibrils. Protofibrils grow as polymeric aggregates of oligomers. After reaching a contour length of few hundreds of nanometers, they begin to self-assemble into much longer and stiffer fibrils.
REFERENCES


