“Aggregate Structure, Morphology and the Effect of Aggregation Mechanisms on Viscosity at Elevated Protein Concentrations”

Gregory V. Barnett¹, Wei Qi², Samiul Amin², E. Neil Lewis², and Christopher J. Roberts^{1,*}

¹ Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, DE 19716, USA

² Malvern Biosciences Inc., Columbia, MD 21046, USA

* Corresponding Author: tel. (1) 302-831-0838; fax. (1) 302-831-1048; email: cjr@udel.edu

Keywords: Protein Aggregation, Protein Structure, Aggregate Mechanism, Viscosity

Abstract

Non-native aggregation is a common issue in a number of degenerative diseases and during manufacturing of protein-based therapeutics. There is a growing interest to monitor protein stability at intermediate to high protein concentrations, which are required for therapeutic dosing of subcutaneous injections. An understanding of the impact of protein structural changes and interactions on the protein aggregation mechanisms and resulting aggregate size and morphology may lead to improved strategies to reduce aggregation and solution viscosity. This report investigates non-native aggregation of a model protein, α -chymotrypsinogen, under accelerated conditions at elevated protein concentrations. Far-UV circular dichroism and Raman scattering show structural changes during aggregation. Size exclusion chromatography and laser light scattering are used to monitor the progression of aggregate growth and monomer loss. Monomer loss is concomitant with increased β -sheet structures as monomers are added to aggregates, which illustrate a transition from a native monomeric state to an aggregate state. Aggregates grow predominantly through monomer-addition, resulting in a semi-flexible-polymer morphology. Analysis of aggregation growth kinetics shows that pH strongly affects the characteristic timescales for nucleation (τ_n) and growth (τ_g), while the initial protein concentration has only minor effects on τ_n or τ_g . Low-shear viscosity measurements follow a common scaling relationship between average aggregate molecular weight (M_w) and concentration (σ), which is consistent with semi-dilute polymer-solution theory. The results establish a link between aggregate growth mechanisms, which couple M_w and σ , to increases in solution viscosity even at these intermediate protein concentrations (less than 3 w/v %).

Introduction

Non-native protein aggregation is a common process in certain neurodegenerative diseases and is a widespread degradation route for proteins of interest in the biotechnology industry. For protein-based therapeutics, the presence of aggregates during drug administration has the potential to induce an immune response and possibly jeopardize drug effectiveness and patient safety [1]. Recently, high protein-concentration formulations have been a growing focus area due to the low-volume dosing requirement for subcutaneous administration [2,3]. At high protein concentrations, non-ideal protein-protein interactions may give rise to reversible, self-associated protein states and increases in solution viscosity [4]. High viscosity therapeutics may be painful for self-administered injections, or even may preclude self administration if the viscosity is sufficiently high [5]. In addition, irreversible non-native aggregation rates can be much faster at high concentrations than expected based on low concentration behavior [6].

Although the relationship between protein structure and morphology has been developed for low concentrations, it has not been established for intermediate to high concentrations where strong non-ideal protein-protein interactions are expected [7,8]. The behavior of protein solutions at intermediate to high concentrations is difficult to predict based on protein properties or measurements at lower concentrations [9]. Previous work has calculated dilute-solution interactions, such as second osmotic coefficients, *in silico* from protein crystal structures [10,11], but it remains a challenge to extend the calculations to higher protein concentrations where three-body and high order interactions may be expected [12]. Many biophysical techniques, such as circular dichroism, analytical ultra centrifugation, and HPLC based techniques, are limited to low protein concentration for a variety of reasons [2,13]. Traditionally, scattering techniques have been used to measure protein behavior at elevated concentrations; however, a common problem is scattering due to inter-protein correlations (i.e.,

“structure factor” effects) [14]. As such, there is a need for techniques to monitor protein structure, morphology, and solution viscosity *in situ* at higher protein concentrations with limited time and material.

During aggregation, structural changes may potentially occur for the constituent monomers because it is well documented that some degree of monomer unfolding facilitates nucleation of new aggregates for originally monomeric proteins, as well as facilitating monomer addition to existing aggregates. Spectroscopic techniques, such as infrared spectroscopy [15], dye binding [16,17], circular dichroism spectroscopy [7,18], and intrinsic fluorescence spectroscopy [19], can be used to monitor changes in protein secondary and/or tertiary structure. However, the signals may or may not correlate with aggregation monitored more directly by techniques such as chromatography, laser scattering, electron microscopy, atomic force microscopy, or small angle neutron/x-ray scattering [20]. An additional complication is that measurements often need to be performed ex-situ, so as to adjust sample conditions to allow for constraints such as the operating range of the instruments [21].

The resulting aggregate size and morphology is expected to play a role in determining the net solution viscosity in the system. To date, no direct link between the viscosity and aggregation mechanism(s) has been established under these conditions. Protein aggregation has been correlated with increases in solution viscosity in other systems. For example, previous work used aggregate morphology from SANS to relate the aggregate fractal dimensions to increases in solution viscosity for monoclonal antibodies [22]. However, it remains a challenge to predict how the size and concentration of protein aggregates will affect solution viscosity. Available techniques may provide weighted averages for characteristic dimensions (e.g., weight-average molecular weight, radius of gyration, and hydrodynamic radius), but these are biased towards larger particles in solution, and cannot directly provide information on the concentration of aggregates [23]. Additionally, the aggregation mechanism

will directly affect the size and concentration of aggregates in solution [24]. Interestingly, mass balance approaches have been developed that combine scattering measurements with population balances to quantify size and concentration(s) during irreversible protein aggregation. However, these have primarily been constrained to low-protein-concentration conditions, and/or have been used to interpret the effects on solution viscosity for only a small number of systems [25–28].

This study addresses questions of how changes in structure, aggregate size, concentration and viscosity are related at intermediate concentrations at acidic pH conditions where net protein-protein interactions are highly repulsive. It focuses on the aggregation behavior of a model aggregation-prone protein, α -chymotrypsinogen (aCgn), at acidic pH and elevated temperatures where it is known to form amyloid aggregates at low concentrations (~ 1 mg/mL). At low concentrations, strong inter-protein interactions have been shown to alter aggregation mechanism for aCgn [20] and other proteins [18,29,30]. The aggregate size and concentration is also expected to depend on aggregation mechanism, and therefore this may also be expected to alter the net solution viscosity [24]. More recent results highlight the presence of strong electrostatic intermolecular repulsions for aCgn at pH 3.5 when one considers higher protein concentrations (~ 10 -40 mg / mL) [12]. However, it remains unclear how aggregate structure and morphology will evolve at elevated protein concentrations under these acidic pH conditions, where large protein-protein interactions are expected.

Using aggregated aCgn solutions as a model system, the relationships between protein structural changes, aggregate morphology and size, and increased viscosity are systematically explored during the process of non-native aggregation at “intermediate” concentrations (~ 1 to 3 w/v %). Structural changes during aggregation are monitored with Raman scattering and compared to circular dichroism. Analysis of aggregation mechanisms and the measured mass-to-size scaling behaviors confirm growth by chain-polymerization and polymer-like morphologies. In addition, a link between aggregation growth

mechanism(s) and increases in solution viscosity is illustrated for protein aggregation at intermediate concentrations, and is shown to be consistent with polymer solution theory.

Materials and Methods

Sample Preparation

aCgn (lyophilized powder, 5x crystallized, Worthington Biochemical Corp.) was purchased and used without further purification. All solutions were prepared using Milli-Q filtered water (Millipore, Billerica, MA), buffered with 10 mM citric acid monohydrate (Fisher Scientific), and titrated to the desired pH using sodium hydroxide (Fisher Scientific). The protein powder was dissolved in a small volume (<15 mL) of desired buffer and dialyzed against the buffer using Spectra/Por 7 tubing (10 kDa MWCO, Spectrum Laboratories, Santa Clara, CA) as previously described [29]. Following dialysis, a given protein solution was concentrated using an Amicon centrifuge tube (Millipore) and filtered with a 0.22 micron filter (Millipore). The resulting protein concentration was measured using the UV-Vis absorbance at 280 nm (Agilent 8453 UV-Vis, Agilent Technologies, Santa Clara, CA) with an extinction coefficient of 1.97 mL/mg cm [31]. All solutions were diluted gravimetrically with buffer solution to desired concentrations.

Size exclusion chromatography (SEC)

Samples were prepared at pH 2.9, 3.2, and 3.6 with initial protein concentrations of 15, 20, and 25 mg/mL. Protein samples were incubated in hermetically sealed HPLC vials (Waters, Millford, MA). All samples at a given pH were incubated isothermally and subsequently quenched on ice at different time points to arrest aggregation. The incubation temperature at a given pH was chosen such that

monomer loss kinetics would have half-lives of 4-5 hours. All samples before and after quenching were transparent and free from visible particles based on visual inspection.

After quenching, samples were diluted gravimetrically to 1.5 mg/mL and held at room temperature before injecting on the HPLC. For each sample, the monomer fraction remaining following aggregation was quantified using size exclusion chromatography (SEC). An Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA) was connected in-line to a Protein-Pak 7.8 x 300 mm size exclusion column (Waters). 100 μ L of solution were injected using an autosampler and mobile phase consisting of 0.5% phosphoric acid (Fisher Scientific) at pH 2.5 as previously reported [32]. The monomer fraction, defined as monomer peak area of a heated sample relative to an unheated protein solution, was determined by measuring absorbance at 280 nm using a variable wavelength detector (VWD Agilent technologies, Santa Clara, CA). Additional details are the same as previously reported [32].

Far-UV Circular Dichroism

Quenched solutions were gravimetrically diluted to 0.5 mg/mL, and far-UV circular dichroism (CD) spectra were measured at room temperature using a Jasco J-810 spectrophotometer (Jasco, Easton, MD). Spectra were measured from 200 to 250 nm at a scan rate of 20 nm/min using 1x10 mm Hellma cuvettes (Plainview, NY). Ten spectra were collected and averaged for each measurement. The buffer was subtracted and the mean residue ellipticity, $[\theta]$, was calculated using [Equation \[1\] as previously described \[33\]](#). M_0 is the molecular weight of monomer (25.7 kDa), c_0 is the known protein concentration, d is the cuvette path length, and n_r is the number of monomer amino acid residue in the [protein sequence](#).

$$[\theta] = \frac{\theta M_0}{10c_0dn_r} \quad [1]$$

The CD spectra had contributions from both monomer and aggregate species. As done previously [7,18,20,32], the mean residue ellipticity, $[\theta]$, was analyzed with Equations [2a-b]. Using the known monomer fraction from SEC, (m), the monomer contribution to a given CD spectrum was subtracted at a given wavelength by subtracting the product of m and the measured CD spectra for a pure monomeric solution, $[\theta_m]$. The contribution to mean residue ellipticity from the aggregate population in a given sample, $[\theta_{agg}]$, was calculated from Equation 2a. Doing so helps to distinguish between changes in spectra for different samples that are due only to the fact that different samples have different amounts of monomer and aggregate present. $[\theta_{agg}]$ is the mean residue ellipticity for proteins contained with aggregates, and can be compared directly with the analogous quantity for proteins that are monomeric. For each time point, the normalized CD spectra, defined as the measured ellipticity subtracted monomer contribution and normalized by the net aggregate contribution, was then calculated by Equation 2b.

$$[\theta] = [\theta_m]m + (1 - m)[\theta_{agg}] \quad [2a]$$

$$[\theta_{agg}] = \frac{[\theta] - m[\theta_m]}{(1 - m)} \quad [2b]$$

Combined dynamic light scattering with Raman spectroscopy (DLS-Raman)

DLS-Raman experiments were performed on a Zetasizer Helix system (Malvern Instruments, Malvern, UK), which combines dynamic light scattering (DLS) with Raman scattering. Raman scattering was excited by a 785 nm laser with approximately 280 mW power, while DLS was collected at the 173 degree backscattering angle from a 632 nm laser. For a typical experiment, $\sim 120 \mu\text{L}$ sample was loaded into a 3×3 mm quartz cuvette and placed into a Peltier temperature-controlled sample compartment. Raman and DLS data were acquired sequentially. To properly process the data, Raman

spectra of corresponding buffer were acquired under the identical conditions with the same experimental set up. Unless otherwise noted, Raman spectra were collected with 10 co-additions of a 40 s exposure. More detailed information could be found in prior work [34–36].

Static Laser Light Scattering

Aggregated samples for laser light scattering were prepared by diluting quenched samples to a final protein concentration of 0.5 to 1.5 mg/mL to minimize the effect of structure factor contributions at higher protein concentration. Static and dynamic light scattering were measured on each diluted sample using a multi-angle-light-scattering (MALS) Dawn Heleos II (Wyatt, Santa Barbara, CA) with a Microcuvette accessory (Wyatt Technologies). From static light scattering, the total molecular weight of the sample (M_w^{tot}) and average radius of gyration (R_g) were determined using the ASTRA VI software (Wyatt Technologies). Dynamic light scattering (DLS) was collected using the WyattQELS accessory (Wyatt Technologies) in the HELEOS II instrument. The autocorrelation function was exported and analyzed using non-linear regression (in Matlab) to a cumulant expansion given by Equation [3] [37].

$$g_2(\tau) = \alpha + \beta \exp(-2Q^2 D_0 \tau) \left(1 + \frac{\mu_2}{2!} \tau^2\right)^2 \quad [3]$$

α is a constant for the short delay-time baseline, β is an instrument specific constant, D_0 is the self diffusion coefficient (when protein concentrations are low), Q is the magnitude of the scattering vector defined in Equation [4], n is the refractive index of solvent, λ is the laser wavelength (658.9 nm), and θ is the scattering angle, τ is the decay time, and μ_2 is the second cumulant and is related to the sample polydispersity index.

$$Q = \frac{4\pi n}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad [4]$$

The average hydrodynamic radius, R_h , was calculated from measured D_0 values using the Stokes-Einstein relation (Equation [5]) where k_b is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity.

$$R_h = \frac{k_b T}{6\pi\eta D_0} \quad [5]$$

Microcapillary-Viscometry

The viscosities of quenched aggregated samples and unheated monomer solutions were measured using a Viscosizer 200 (Malvern Instruments, Malvern, UK). By combining a dual window capillary configuration and an UV area-imaging detector at 280 nm, the instrument is capable of detecting real-time concentration profiles within protein solution as the solution travels past the capillary windows. The time for the protein sample to pass between the two windows was calculated and compared to that from the buffer. Provided the buffer viscosity, (η_{ref}) is known, the sample viscosity is then determined from Equation [6].

$$\eta_{sample} = \left(\frac{\Delta t_{sample}}{\Delta t_{ref}} \right) \eta_{ref} \quad [6]$$

Typically, $\sim 100 \mu L$ samples were loaded and approximately $10 \mu L$ was consumed for a single run. Unless specified otherwise, triplicates were measured at the specified temperature (25 °C) with 30 minutes equilibrium times prior to each measurement.

Results and Discussion

aCgn structural changes during aggregation

Figure 1A shows illustrative far-UV CD spectra for aCgn at pH 2.9 and 15 mg/mL. As the protein solution is held isothermally at elevated temperature, the spectra in Figure 1A shift systematically with increasing incubation time. Previous work indicated that protein unfolding in the absence of aggregation results in minimal secondary structure changes but significant tertiary structure changes (i.e., a molten-globule unfolded state). However, when monomers form stable aggregates the CD spectra showed an increase in beta sheet signatures, presumably as a result of intermolecular β -sheet formation [7,38].

The protein structural changes correspond to an ensemble average structure and have contributions from native and non-native states. Previous work with this protein [20] and others has shown that structural techniques monitoring protein aggregation show a concomitant loss in monomer and increase in aggregates (on a mass basis) [18]. If this is the case, then it may be reasonable to expect that there are two predominate protein states in solution after a measureable extent of aggregation has occurred, and samples have been cooled to arrest further aggregation: folded monomers, and non-native protein chains that have been incorporated into aggregates. The CD spectra were normalized assuming this two state parsing between folded monomers and non-native aggregate (cf., Methods). Figure 1B illustrates the net aggregation contribution to the CD spectra, $[\theta_{agg}]$, as a function of wavelength. Each spectrum corresponds to an incubation time point for aCgn at pH 2.9 and 15 mg/mL, and was determined via Eq. [2b] using the monomer fraction from SEC. Inspection of Figure 1B shows that $[\theta_{agg}]$ remained constant for different incubation time points, as previously reported for this protein [20] and others[18].

The fact that $[\theta_{agg}]$ vs. wavelength is the same for each sample suggests that the two-state approximation for the CD spectra was reasonable even when created at intermediate to high protein concentrations. In this case, previous work showed that one could use the CD signal as a quantitative

surrogate for m or $1-m$ (i.e., the fraction of monomer converted to aggregate) [20]. However, caution should be used in assuming that this case will always hold. For example, once aggregate growth occurs via additional mechanisms such as aggregate coalescence, techniques such as CD spectroscopy and related methods may not provide accurate monitors of the progress of aggregation because the signal may be influenced by scattering and absorption from aggregation, which may be pronounced at lower wavelengths [33,39].

Figure 2A shows the Raman second derivative spectra of the Amide I region over time for aCgn pH 2.9, 20 mg/mL, heated at 49.5 °C. The shift in the peak position of the Amide I region, assigned predominantly to the C=O stretching mode of the peptide backbone, is an indicator of a change in the protein secondary structure; an effect which is strongly influenced by hydrogen bonding [40,41]. The secondary structure deconvolution was determined from a partial least squares algorithm utilizing a database of 18 proteins of known secondary structure [34]. Figure 2B compares $(1-m)$, determined from SEC, to the β -sheet content from a deconvolution of the Raman scattering. $(1-m)$ was determined from quenched samples, and the Raman scattering was collected on a sample from an identical stock solution heated isothermally at 49.5 °C over the same range of incubation times.

Overall, aCgn aggregates displayed an increase in β -sheet content compared to monomeric samples. Secondary structure predictions using CD spectra were attempted, but the low wavelength CD signal (below ~205 nm) was unreliable due to increased detector voltages, and the CD data were therefore insufficient for reliable secondary structure predictions. At lower wavelengths, absorbance from the citrate buffer and protein and scattering from larger sized aggregates may convolute the CD signal [33]. Increased β -sheet content was expected as aCgn has been shown to form intermolecular β -sheets during aggregation [38]. However, those measurements were for only pH 3.5 solution conditions, and with much lower protein concentrations than what was considered here.

Raman scattering also has additional markers that may change during aggregation. One marker reported here is the ratio of the scattering intensity of the tyrosine peaks at 830 and 850 cm^{-1} , which has been attributed to hydrogen bonding around the tyrosine residue [40,41]. Changes in the microenvironment around tyrosine are apparent in the Raman spectra here, and may or may not occur concomitantly with secondary structural changes. The inset of Figure 2B shows the ratio of the Raman scattering intensity of the tyrosine peaks as a function of incubation time for the same conditions indicated in the main panel. This ratio is sensitive to the hydrophobic or hydrophilic nature of the phenoxyl side chain of the tyrosine residue. Lower ratios (e.g. 0.3) tend to be hydrogen donors (hydrophobic), while higher ratios (e.g. 2) tend to be hydrogen acceptors (hydrophilic) [41].

The inset in Figure 2B shows a decrease in the tyrosine marker from ratio of 1.6 to ~ 1.0 . Interestingly, this trend does not seem to correlate well with β -sheet content or monomer fraction in the main panel of Figure 2B. However, the trend suggests that the average tyrosine microenvironment is becoming more hydrophobic and may be expected as aggregates grow and bury many of these side chains within the aggregates, shielding them from the influence of the solvent. While this differs from the simple models and data interpretation that were used previously with aCgn, it is consistent with more recent results that indicated that aCgn aggregates can “anneal” or change structure over time after they have initially formed [42]. Those changes were difficult to detect in prior work, but Raman spectra were not available in that case. This suggests one potential benefit of Raman data, compared to other techniques such as CD and intrinsic fluorescence [42], when monitoring protein aggregation. Compared to other structural techniques such as CD, fluorescence spectroscopy, or FTIR, Raman scattering can investigate protein structure at high protein concentrations, larger particle sizes, and low transmittance. See Supporting Information for $[\theta_{agg}]$ and β -sheet content of aCgn aggregates at pH 3.2 and 3.6.

Effect of pH and initial protein concentration on aggregation kinetics

Following isothermal incubation of protein solutions over a given time course, quenched samples were diluted and the monomer fraction remaining, m , was determined for each time point using SEC. Additionally, the total molecular weight (M_w^{tot}) and hydrodynamic radius (R_h) were determined by static and dynamic light scattering after diluting to lower concentration to minimize structure-factor artifacts (cf., Methods). Figure 3 plots M_w^{tot}/M_0 as a function of $(1 - m)^2$, where M_0 is the monomer molecular weight (25.7 kDa). Each value for M_w^{tot} and m are for a given incubation time. Prior work has shown that M_w^{tot} is expected to scale with the square of $(1-m)$ because M_w^{tot} is the second moment of the size distribution (as opposed to the first moment of the size distribution, which scales linearly with $1-m$) [43].

Plotting parametrically in this fashion allows one to directly compare M_w^{tot} versus the extent of monomer consumption for a wide range of timescales and different values of pH and C_0 . Figure 3 illustrates aggregation profiles for pH 2.9 (panel A), pH 3.2 (panel B), and pH 3.6 (panel C) for 15 mg/mL (grey closed symbols), 20 mg/mL (open black symbols), and 25 mg/mL (closed black symbols).

Prior work has shown that aCgn aggregates grow through a chain polymerization mechanism at pH 3.5 in low ionic strength. Based on simple mass-balance or population-balance arguments, this results in a linear increase of M_w^{tot}/M_0 when plotted versus $(1-m)^2$ [25,26]. Non-linear increases in M_w^{tot}/M_0 indicate additional growth mechanisms, such as aggregate-aggregate coalescence [25,26]. Visual inspection of Figures 3A-C indicates aggregate growth occurs predominately through chain polymerization. However, for a few conditions it is apparent that M_w^{tot}/M_0 vs $(1-m)^2$ has slight upward curvature at later stages of growth, indicating growth by coalescence is occurring.

Values of M_w^{tot} and m as a function of incubation time for each data set in Figure 3 were non-linearly regressed with the Lumry-Eyring nucleation polymerization (LENP) model that was used previously with aCgn aggregation kinetics [25,26]. Data that exhibited signs of aggregate coalescence

(see above) were excluded from the fitted data because once aggregate coalescence occurs, many possible model solutions will fit the data equally well [20]. At pH 3.6, aggregates with a protein concentration of 20 mg/mL and 25 mg/mL have only three and one data point(s) respectively, because at later incubation times formed a gel-like state and this made SEC and light scattering results unreliable (data not shown). LENP model equations are given below in Equation [7a-c], as previously described [26]. x is defined as the aggregate nucleus size, and t is the incubation time.

$$\frac{dm}{dt} = \frac{xm^x}{\tau_n} - \frac{m\sigma}{\tau_g} \quad [7a]$$

$$\frac{d\sigma}{dt} = \frac{m^x}{\tau_n} \quad [7b]$$

$$\frac{d(M_w^{tot} - m)}{dt} = \frac{x^2 m^x}{\tau_n} + \frac{m}{\tau_g} (\sigma + 2(1 - m)) \quad [7c]$$

The characteristic timescales, or inverse rate coefficients, for nucleation (τ_n) or for growth via monomer addition (τ_g) are fitted parameters from the model. Additionally, the model provides an estimate for the total concentration of aggregates in solution (scaled by the initial protein concentration), denoted as σ , as well as how σ changes with incubation time. Illustrative fits of Eq. [7a-c] to the experimental data are provided in Supporting Information.

Figure 4A shows fitted parameter values and 95 percent confidence intervals for τ_n and τ_g as a function of pH for each initial protein concentration. The values for τ_n are 2-5 orders of magnitude larger than τ_g , which indicates nucleation is a much slower event compared to growth, and is in keeping with previous results [7,20,44,45]. As pH increases, the difference between τ_n and τ_g increase. Prior analysis has shown that at a given value of m , if the ratio of τ_n to τ_g increases (decreases), aggregates grow to larger (smaller) M_w^{tot} , and the aggregate concentration (σ) is smaller (larger). Physically, this follows because a larger ratio of τ_n to τ_g means the nucleation of new aggregates is much slower than

growth of those aggregates after they are nucleated. Therefore, each new aggregate will rapidly consume additional monomers as it grows, and this more rapidly depletes the monomer concentration. Therefore, fewer aggregates will nucleate over a given period of time, but those that do nucleate will grow to larger M_w^{tot} . Figure 4B displays the curves for $\sigma(t)$ that follow from the fitted parameters in Figure 4A and solution of Eq. [7a-c].

Changing pH had a pronounced effect on aggregate growth, as lower pH favored smaller ratios of τ_n to τ_g . pH is expected to alter electrostatic inter-protein interactions and the energy barrier for aggregation based on previous work with this protein [20] and other proteins [18,29,30]. aCgn has an isoelectric point ~ 9 and has a positive charge in acidic conditions. As the value of the pH is decreased from 3.6 to 2.9, the net charge on the protein surface is expected to increase from 14 to 18 (based PropKa calculation) and the ionic strength from citrate buffer will decrease from 8.6 to 3.9 mM (based on published pK_a values for citrate buffer) [46–49]. Both effects are expected to increase the net electrostatic repulsions felt by neighboring proteins, and presumably also increase energy barriers for aggregation. Additionally, aggregation at pH 3.6 with 0.1 M NaCl resulted in gel-like aggregates at concentrations lower than 15 mg/mL. Prior work has shown strong protein-protein repulsions are present in the pH range of 2.5 to 3.5 at lower protein concentration (0-10 mg/mL) [20] and at pH 3.5 at higher concentrations (10-40 mg/mL) [12].

However, if this were the only effect of changing pH, then one would expect both τ_n and τ_g to increase with decreasing pH – i.e., net charge increases for both monomers and aggregates. Perhaps surprisingly, τ_n instead decreases with decreasing pH. While the exact reason for this is unclear, one possible explanation is that decreasing pH also greatly reduces the unfolding free energy (ΔG_{un}) for aCgn[20]. Therefore, decreasing pH would increase the net rate of nucleation because τ_n scales with the inverse of $\exp(-\Delta G_{un})$ [50].

Changing the initial protein concentration appeared to have little effect on aggregation rates or τ_n and τ_g , but the protein concentration was only varied over a relatively small range. Surprisingly, at pH 2.9, varying concentration had no effect on the aggregation kinetics, which is in contrast to previous work with aCgn at lower protein concentrations that would have caused an almost ten-fold change in nucleation rate even the small range of protein concentration tested here [50]. Based on the assumptions of the LENP model or other population-balance models, increased protein concentrations are expected to decrease τ_n relative to τ_g , and τ_n decreases more so if nucleation involves more than two protein monomers. Earlier work indicated that the nucleation involved trimer and tetramers (or larger species) [32]. Even with the relatively small range of concentrations tested here (factor of only approximately 2), an oligomer nucleus would be expected to show stronger changes in τ_n and τ_g than what was observed here [7]. The reasons for this apparent discrepancy are not known, but one possible explanation is that LENP and analogous models assume mass-action kinetics that are expected to hold most accurately at low (“dilute”) concentrations. The results here may indicate that these types of models will be limited in their quantitative utility when dealing with higher protein concentrations, and/or large non-idealities due to strong protein-protein interactions [51].

Aggregate morphology

Scaling of the average aggregate molecular weight with respect to the size (e.g., R_g or R_h) gives an indication of morphology [7,23,30]. Prior work [25,26] has shown that a mass balance on all protein species allows one to determine M_w^{agg} from M_w^{tot} and m via Equation [8].

$$\frac{M_w^{agg}}{M_0} = \frac{\left(\frac{M_w^{tot}}{M_0} - m\right)}{1 - m} \quad [8]$$

Figure 5 shows the scaling between average M_w^{agg} and R_h , where values of R_h were determined from dynamic light scattering. Symbols correspond to the same conditions of pH and initial protein concentration as Figure 3. Two linear $\log M_w^{agg}$ vs $\log R_h$ regimes appear in Figure 5. The smaller- M_w^{agg} regime includes aggregates created at pH 2.9 (all conc.) and pH 3.2 (20 and 25 mg/mL), and has a scaling exponent of 1.90 ± 0.12 . The larger- M_w^{agg} regime includes aggregates created at pH 3.6 (all concentrations) and pH 3.2 (15 mg/mL), and has a scaling exponent of 2 ± 0.2 . Interestingly, while the two regimes have similar slopes, they are offset with respect to each other. For a given R_h , the values of M_w^{agg} can be significantly different depending on which conditions were used to create aggregates.

As mentioned above, aggregate growth followed a chain-polymerization mechanism and the aggregate M_w^{agg} vs R_h scaling is consistent with previous results for semi-flexible polymer morphology of aCgn aggregates [7]. The slope (scaling exponent) was approximately 2, and was common for all samples. This suggests that the morphology is similar for aggregates created at various pH and initial protein concentrations. The aggregate morphology and mechanism at much higher concentrations are similar to those at low concentration, suggesting a similar growth process. As such, the mechanism is expected to involve highly specific interaction and assembly, and is reasonably independent of solution pH or protein concentration within the ranges investigated here.

Aggregate growth affects viscosity

The viscosities of quenched samples were measured with a Viscosizer at multiple values of shear rates, and the low shear viscosities (in first Newtonian regime) were reported and utilized. For samples that had very large increases in viscosity or formed a gel (e.g. pH 3.6, 25 mg/mL) the viscosity was not determined. Figure 6A shows the relationship of the zero-shear viscosity and M_w^{agg} . From inspection of Figure 6, two separate regimes are apparent. They follow similar groupings, in terms of pH and initial

protein concentration, as the M_w^{agg} to R_h scaling in Figure 5. The first regime at smaller values for M_w^{agg} includes pH 2.9 (at all concentrations), and pH 3.2 (20 mg/mL). The second regime at larger M_w^{agg} includes the aggregates created at pH 3.6 (15 and 20 mg/mL) and pH 3.2 (15 mg/mL).

The viscosity increases for aggregated samples ranged from 0.1 to ~2 cP, which is a small viscosity increase compared those observed elsewhere: (i) due to non-native aggregation of monoclonal antibodies [22]; (ii) solution non-idealities due to monomer protein-protein interactions of native antibodies at high concentrations (~ 15 w/v %) [3]; (iii) at high concentrations (~ 30 w/v %) for other globular proteins [52].

Based on polymer theory, one might expect solution viscosity to be influenced by the concentration, molecular weight, and morphology of protein aggregates [23,53]. The aggregate molecular weight is experimentally accessible through light scattering and an overall mass balance (cf., Eq. 8 above), and the total concentration of aggregates in solution can be estimated from the value of σ , which follows from the model regression of M_w^{tot} and m versus incubation time. As noted above in reference to Figure 5, the aggregate morphology remains similar for all conditions investigated here. As aggregates grow, they may potentially overlap and become entangled. This would lead to different viscosity behavior when compared to aggregates that are in dilute regime. In the present case, the overlap concentration for each data point was calculated based on polymer theory [53] and is reported in Supporting Information. In all cases here, the overlap concentration was much smaller than σ , which is consistent with viscosity behavior expected in the semi-dilute regime [54].

M_w^{tot} and σ contributions to the solution viscosity were determined by fitting the viscosity data to Equation [9], where K , α , β are fitting parameters. Eq. [9] has been used in polymer solutions and it has been shown to superimpose the effects of M_w and concentration on solution viscosity [55].

$$\eta = K \sigma^\alpha (M_w^{agg} / M_0)^\beta \quad [9]$$

Figures 6B-6C show the result of rescaling the viscosity by the contribution from σ (panel B) and M_w^{agg} (panel C) respectively. Fitted values for K , α , β and 95 percent confidence intervals are 0.025 ± 0.022 , 0.52 ± 0.12 , and 0.71 ± 0.15 respectively. Viscosity data were also fit by separating the data into smaller groups that formed the two regimes in Figures 4 and 5, but there was not a significant difference in the coefficients from fitting the data separately or all together.

While two viscosity regimes appear in Figure 6A, in this form there does not appear to be a clear trend. However, renormalizing the viscosity by the values for M_w^{tot}/M_0 and σ show two linear regimes. This suggests the viscosity is determined by not only how large aggregates grown, but also by the concentration of aggregates. Both of these quantities are coupled through mass balances, as a result of the growth mechanism (specifically, the ratio τ_n/τ_g) and they are not easy to predict how they will change with pH and protein concentration.

Figures 6B and 6C illustrate the effect of aggregate growth on solution viscosity. Aggregates that growth larger may be expected to increase viscosity, but the concentration of aggregates also has a significant contribution to viscosity. Mechanisms of aggregation cause aggregate molecular weight and aggregate concentration to change in opposite directions. Rescaling the viscosity by σ or M_w^{agg}/M_0 helps to collapse the data to a reasonably linear trend, although the trend is only semi-quantitative in both cases. While the range of viscosity values is only approximately one order of magnitude, the results indicate that polymer solution principles combined with mechanistic analysis of protein aggregation kinetics can help with rationalizing the effects of protein aggregation on low-shear solution viscosity.

Conclusions

This report focused on the relationship between protein structural changes, aggregate morphology, aggregation mechanism(s), and increased viscosity for aCgn solutions at acidic pH and elevated protein concentrations. Protein aggregation is concomitant with increased β -sheet content, based on both CD and Raman spectroscopies. Changes in pH and initial protein concentration alter the ratio of nucleation to growth timescales within the aggregation process, and this in turn results in changes in the size to which aggregate growth, and the net concentration of aggregates. The aggregate mass-to-size scaling, or fractal dimension, supports polymer-like morphology as previously reported [7] that is similar across pH and concentration range investigated. Finally, combining the results with existing polymer solution theory provides a simple scaling relationship to captures the changes in sample viscosity as aggregation progressed. The results illustrate a direct connection between the aggregation mechanism(s) and the degree to which solution viscosity will increase at even low to intermediate protein concentrations.

Acknowledgements

GVB and CJR gratefully acknowledge support from the National Institute of Standards and Technology (NIST 70NANB12H239) and the National Science Foundation (CBET 0931173).

References

- [1] A.S. Rosenberg, Effects of protein aggregates: an immunologic perspective, *AAPS J.* 8 (2006) E501–E507.
- [2] S.J. Shire, Z. Shahrokh, J. Liu, Challenges in the development of high protein concentration formulations, *J. Pharm. Sci.* 93 (2004) 1390–1402.
- [3] M. Bowen, N. Armstrong, Y. Maa, Investigating high-concentration monoclonal antibody powder suspension in nonaqueous suspension vehicles for subcutaneous injection, *J. Pharm. Sci.* 101 (2012) 4433–4443. doi:10.1002/jps.23324.
- [4] B.D. Connolly, C. Petry, S. Yadav, B. Demeule, N. Ciaccio, J.M.R. Moore, et al., Weak Interactions Govern the Viscosity of Concentrated Antibody Solutions: High-Throughput Analysis Using the Diffusion Interaction Parameter, *Biophys. J.* 103 (2012) 69–78. doi:10.1016/j.bpj.2012.04.047.
- [5] F. Cilurzo, F. Selmin, P. Minghetti, M. Adami, E. Berton, S. Lauria, et al., Injectability Evaluation: An Open Issue, *AAPS PharmSciTech.* 12 (2011) 604–609. doi:10.1208/s12249-011-9625-y.
- [6] W. Wang, C.J. Roberts, Non-Arrhenius protein aggregation, *AAPS J.* 15 (2013) 840–851.
- [7] W.F. Weiss, T.K. Hodgdon, E.W. Kaler, A.M. Lenhoff, C.J. Roberts, Nonnative Protein Polymers: Structure, Morphology, and Relation to Nucleation and Growth, *Biophys. J.* 93 (2007) 4392–4403. doi:10.1529/biophysj.107.112102.
- [8] V. Iyer, N. Maddux, L. Hu, W. Cheng, A.K. Youssef, G. Winter, et al., Comparative signature diagrams to evaluate biophysical data for differences in protein structure across various formulations, *J. Pharm. Sci.* 102 (2013) 43–51. doi:10.1002/jps.23367.
- [9] A. Saluja, R.M. Fesinmeyer, S. Hogan, D.N. Brems, Y.R. Gokarn, Diffusion and Sedimentation Interaction Parameters for Measuring the Second Virial Coefficient and Their Utility as

Predictors of Protein Aggregation, *Biophys. J.* 99 (2010) 2657–2665.

doi:10.1016/j.bpj.2010.08.020.

- [10] A. Grünberger, P.-K. Lai, M.A. Blanco, C.J. Roberts, Coarse-Grained Modeling of Protein Second Osmotic Virial Coefficients: Sterics and Short-Ranged Attractions, *J. Phys. Chem. B.* 117 (2013) 763–770. doi:10.1021/jp308234j.
- [11] B.L. Neal, D. Asthagiri, A.M. Lenhoff, Molecular origins of osmotic second virial coefficients of proteins, *Biophys. J.* 75 (1998) 2469–2477.
- [12] M.A. Blanco, T. Perevozchikova, V. Martorana, M. Manno, C.J. Roberts, Protein–Protein Interactions in Dilute to Concentrated Solutions: α -Chymotrypsinogen in Acidic Conditions, *J. Phys. Chem. B.* 118 (2014) 5817–5831. doi:10.1021/jp412301h.
- [13] J.G. Barnard, S. Singh, T.W. Randolph, J.F. Carpenter, Subvisible particle counting provides a sensitive method of detecting and quantifying aggregation of monoclonal antibody caused by freeze-thawing: Insights into the roles of particles in the protein aggregation pathway, *J. Pharm. Sci.* 100 (2011) 492–503. doi:10.1002/jps.22305.
- [14] P.S. Sarangapani, S.D. Hudson, R.L. Jones, J.F. Douglas, J.A. Pathak, Critical Examination of the Colloidal Particle Model of Globular Proteins, *Biophys. J.* 108 (2015) 724–737. doi:10.1016/j.bpj.2014.11.3483.
- [15] W. Dzwolak, R. Ravindra, J. Lendermann, R. Winter, Aggregation of Bovine Insulin Probed by DSC/PPC Calorimetry and FTIR Spectroscopy, *Biochemistry (Mosc.)*. 42 (2003) 11347–11355. doi:10.1021/bi034879h.
- [16] S. Shi, A. Semple, J. Cheung, M. Shameem, DSF method optimization and its application in predicting protein thermal aggregation kinetics, *J. Pharm. Sci.* 102 (2013) 2471–2483. doi:10.1002/jps.23633.

- [17] P. Arosio, S. Rima, M. Morbidelli, Aggregation mechanism of an IgG2 and two IgG1 monoclonal antibodies at low pH: from oligomers to larger aggregates, *Pharm. Res.* 30 (2013) 641–654. doi:10.1007/s11095-012-0885-3.
- [18] N. Kim, R.L. Remmele, D. Liu, V.I. Razinkov, E.J. Fernandez, C.J. Roberts, Aggregation of anti-streptavidin immunoglobulin gamma-1 involves Fab unfolding and competing growth pathways mediated by pH and salt concentration, *Biophys. Chem.* 172 (2013) 26–36. doi:10.1016/j.bpc.2012.12.004.
- [19] S.A. Abbas, G. Gaspar, V.K. Sharma, T.W. Patapoff, D.S. Kalonia, Application of second-derivative fluorescence spectroscopy to monitor subtle changes in a monoclonal antibody structure, *J. Pharm. Sci.* 102 (2013) 52–61. doi:10.1002/jps.23354.
- [20] Y. Li, B.A. Ogunnaike, C.J. Roberts, Multi-variate approach to global protein aggregation behavior and kinetics: effects of pH, NaCl, and temperature for alpha-chymotrypsinogen A, *J. Pharm. Sci.* 99 (2010) 645–662. doi:10.1002/jps.21869.
- [21] S.I.A. Cohen, M. Vendruscolo, C.M. Dobson, T.P.J. Knowles, From Macroscopic Measurements to Microscopic Mechanisms of Protein Aggregation, *J. Mol. Biol.* 421 (2012) 160–171. doi:10.1016/j.jmb.2012.02.031.
- [22] M.M. Castellanos, J.A. Pathak, W. Leach, S.M. Bishop, R.H. Colby, Explaining the Non-Newtonian Character of Aggregating Monoclonal Antibody Solutions Using Small-Angle Neutron Scattering, *Biophys. J.* 107 (2014) 469–476. doi:10.1016/j.bpj.2014.05.015.
- [23] W. Burchard, Solution properties of branched macromolecules, in: *Branched Polym. II*, Springer, 1999: pp. 113–194. http://link.springer.com/chapter/10.1007/3-540-49780-3_3 (accessed July 21, 2014).

- [24] S. Amin, G.V. Barnett, J.A. Pathak, C.J. Roberts, P.S. Sarangapani, Protein aggregation, particle formation, characterization & rheology, *Curr. Opin. Colloid Interface Sci.* 19 (2014) 438–449. doi:10.1016/j.cocis.2014.10.002.
- [25] J.M. Andrews, C.J. Roberts, A Lumry–Eyring Nucleated Polymerization Model of Protein Aggregation Kinetics: 1. Aggregation with Pre-Equilibrated Unfolding, *J. Phys. Chem. B.* 111 (2007) 7897–7913. doi:10.1021/jp070212j.
- [26] Y. Li, C.J. Roberts, Lumry–Eyring Nucleated-Polymerization Model of Protein Aggregation Kinetics. 2. Competing Growth via Condensation and Chain Polymerization, *J. Phys. Chem. B.* 113 (2009) 7020–7032. doi:10.1021/jp8083088.
- [27] P. Arosio, S. Rima, M. Lattuada, M. Morbidelli, Population Balance Modeling of Antibodies Aggregation Kinetics, *J. Phys. Chem. B.* 116 (2012) 7066–7075. doi:10.1021/jp301091n.
- [28] L. Nicoud, M. Owczarz, P. Arosio, M. Morbidelli, A multiscale view of therapeutic protein aggregation: A colloid science perspective, *Biotechnol. J.* 10 (2015) 367–378. doi:10.1002/biot.201400858.
- [29] R.K. Brummitt, D.P. Nesta, L. Chang, S.F. Chase, T.M. Laue, C.J. Roberts, Nonnative aggregation of an IgG1 antibody in acidic conditions: Part 1. Unfolding, colloidal interactions, and formation of high-molecular-weight aggregates, *J. Pharm. Sci.* 100 (2011) 2087–2103. doi:10.1002/jps.22448.
- [30] G.V. Barnett, V.I. Razinkov, B.A. Kerwin, T.M. Laue, A.H. Woodka, P.D. Butler, et al., Specific-Ion Effects on the Aggregation Mechanisms and Protein-Protein Interactions for Anti-streptavidin Immunoglobulin Gamma-1, *J. Phys. Chem. B.* (2015). doi:10.1021/acs.jpcb.5b01881.

- [31] W.M. Jackson, J.F. Brandts, Thermodynamics of protein denaturation. Calorimetric study of the reversible denaturation of chymotrypsinogen and conclusions regarding the accuracy of the two-state approximation, *Biochemistry (Mosc.)*. 9 (1970) 2294–2301.
doi:10.1021/bi00813a011.
- [32] J.M. Andrews, C.J. Roberts, Non-Native Aggregation of α -Chymotrypsinogen Occurs through Nucleation and Growth with Competing Nucleus Sizes and Negative Activation Energies [†], *Biochemistry (Mosc.)*. 46 (2007) 7558–7571. doi:10.1021/bi700296f.
- [33] S.M. Kelly, T.J. Jess, N.C. Price, How to study proteins by circular dichroism, *Biochim. Biophys. Acta BBA - Proteins Proteomics*. 1751 (2005) 119–139. doi:10.1016/j.bbapap.2005.06.005.
- [34] E.N. Lewis, W. Qi, L.H. Kidder, S. Amin, S.M. Kenyon, S. Blake, Combined dynamic light scattering and Raman spectroscopy approach for characterizing the aggregation of therapeutic proteins, *Mol. Basel Switz*. 19 (2014) 20888–20905.
doi:10.3390/molecules191220888.
- [35] C. Zhou, W. Qi, E. Neil Lewis, J.F. Carpenter, Concomitant Raman spectroscopy and dynamic light scattering for characterization of therapeutic proteins at high concentrations, *Anal. Biochem*. 472 (2015) 7–20. doi:10.1016/j.ab.2014.11.016.
- [36] S. Amin, S. Blake, S.M. Kenyon, R.C. Kennel, E.N. Lewis, A novel combination of DLS-optical microrheology and low frequency Raman spectroscopy to reveal underlying biopolymer self-assembly and gelation mechanisms, *J. Chem. Phys*. 141 (2014) 234201.
doi:10.1063/1.4903785.
- [37] B.J. Frisken, Revisiting the method of cumulants for the analysis of dynamic light-scattering data, *Appl. Opt*. 40 (2001) 4087–4091.

- [38] A. Zhang, J.L. Jordan, M.I. Ivanova, W.F. Weiss, C.J. Roberts, E.J. Fernandez, Molecular Level Insights into Thermally Induced α -Chymotrypsinogen A Amyloid Aggregation Mechanism and Semiflexible Protofibril Morphology, *Biochemistry (Mosc.)*. 49 (2010) 10553–10564. doi:10.1021/bi1014216.
- [39] B.J. Litman, Effect of light scattering on the circular dichroism of biological membranes, *Biochemistry (Mosc.)*. 11 (1972) 3243–3247. doi:10.1021/bi00767a018.
- [40] A. Barth, C. Zscherp, What vibrations tell us about proteins, *Q. Rev. Biophys.* 35 (2002) 369–430.
- [41] Z.-Q. Wen, Raman spectroscopy of protein pharmaceuticals, *J. Pharm. Sci.* 96 (2007) 2861–2878. doi:10.1002/jps.20895.
- [42] R.W. Maurer, A.K. Hunter, A.S. Robinson, C.J. Roberts, Aggregates of α -chymotrypsinogen anneal to access more stable states: Annealed Aggregates Are More Stable, *Biotechnol. Bioeng.* 111 (2014) 782–791. doi:10.1002/bit.25129.
- [43] E. Sahin, C.J. Roberts, Size-exclusion chromatography with multi-angle light scattering for elucidating protein aggregation mechanisms, *Methods Mol. Biol. Clifton NJ*. 899 (2012) 403–423. doi:10.1007/978-1-61779-921-1_25.
- [44] R.K. Brummitt, D.P. Nesta, L. Chang, A.M. Kroetsch, C.J. Roberts, Nonnative aggregation of an IgG1 antibody in acidic conditions, part 2: Nucleation and growth kinetics with competing growth mechanisms, *J. Pharm. Sci.* 100 (2011) 2104–2119. doi:10.1002/jps.22447.
- [45] J.M. Andrews, Weiss, C.J. Roberts, Nucleation, Growth, and Activation Energies for Seeded and Unseeded Aggregation of α -Chymotrypsinogen A [†], *Biochemistry (Mosc.)*. 47 (2008) 2397–2403. doi:10.1021/bi7019244.

- [46] V.S. Stoll, J.S. Blanchard, Chapter 6 Buffers, in: *Methods Enzymol.*, Elsevier, 2009: pp. 43–56.
<http://linkinghub.elsevier.com/retrieve/pii/S0076687909630068> (accessed February 20, 2015).
- [47] D.C. Bas, D.M. Rogers, J.H. Jensen, Very fast prediction and rationalization of pKa values for protein–ligand complexes, *Proteins Struct. Funct. Bioinforma.* 73 (2008) 765–783.
doi:10.1002/prot.22102.
- [48] H. Li, A.D. Robertson, J.H. Jensen, Very fast empirical prediction and rationalization of protein pKa values, *Proteins.* 61 (2005) 704–721. doi:10.1002/prot.20660.
- [49] M.H.M. Olsson, C.R. Søndergaard, M. Rostkowski, J.H. Jensen, PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical pKa Predictions, *J. Chem. Theory Comput.* 7 (2011) 525–537. doi:10.1021/ct100578z.
- [50] C.J. Roberts, Non-native protein aggregation kinetics, *Biotechnol. Bioeng.* 98 (2007) 927–938.
doi:10.1002/bit.21627.
- [51] P.S. Sarangapani, S.D. Hudson, K.B. Migler, J.A. Pathak, The Limitations of an Exclusively Colloidal View of Protein Solution Hydrodynamics and Rheology, *Biophys. J.* 105 (2013) 2418–2426. doi:10.1016/j.bpj.2013.10.012.
- [52] V. Sharma, A. Jaishankar, Y.-C. Wang, G.H. McKinley, Rheology of globular proteins: apparent yield stress, high shear rate viscosity and interfacial viscoelasticity of bovine serum albumin solutions, *Soft Matter.* 7 (2011) 5150. doi:10.1039/c0sm01312a.
- [53] R.H. Colby, Structure and linear viscoelasticity of flexible polymer solutions: comparison of polyelectrolyte and neutral polymer solutions, *Rheol. Acta.* 49 (2009) 425–442.
doi:10.1007/s00397-009-0413-5.

- [54] Y. Heo, R.G. Larson, The scaling of zero-shear viscosities of semidilute polymer solutions with concentration, *J. Rheol.* 1978-Present. 49 (2005) 1117–1128. doi:10.1122/1.1993595.
- [55] S. Onogi, T. Masuda, N. Miyanaga, Y. Kimura, Dependence of viscosity of concentrated polymer solutions upon molecular weight and concentration, *J. Polym. Sci. Part -2 Polym. Phys.* 5 (1967) 899–913. doi:10.1002/pol.1967.160050508.

Figure 1. (A) Illustrative ex situ far-UV circular dichroism spectra of aCgn pH 2.9 at 15 mg/mL over time at 49.5 °C. Arrows show the direction of spectra changes for increasing time. (B). aCgn net aggregate spectra $[\theta_{agg}]$ over incubation time for pH 2.9 at 15 mg/mL. See also main text.

Figure 2. (A) Illustrative 2nd derivative Raman spectra at pH 2.9 at 20 mg/mL over time at 49.5 °C. Arrows show the direction of spectra changes over time. (B) Raman β -sheet content (grey circles) for pH 2.9 at 20 mg/mL heated at 49.5 °C. In both panels, black squares correspond to $(1-m)$ determined from SEC. (Inset) Ratio of Raman intensity of the tyrosine peak at 850 to 830 cm^{-1} as a function of time.

Figure 3. M_w^{tot} / M_o vs $(1-m)^2$ plots. (A) pH 2.9 at 49.5 °C, (B) pH 3.2 at 51°C, and (C) pH 3.6 at 53 °C. Symbols correspond to protein concentrations of 15 mg/mL (closed grey), 20 mg/mL (open black), and 25 mg/mL (closed black).

Figure 4. (A). Characteristic timescales for nucleation (τ_n) and growth, (τ_g) as a function of pH and initial protein concentration. Symbol types correspond to the same pH and initial protein concentration as in Figures 3-5. (B) σ , total concentration of aggregates as function of $(1-m)$ based on LENP fits for data at pH 2.9 (solid lines), pH 3.2 (dashed lines), and pH 3.6 (dotted lines). Initial protein concentrations correspond to 15 mg/mL (grey), 20 mg/mL (black), and 25 mg/mL (black).

Figure 5. Scaling of M_w^{tot} and R_h . Symbols correspond to the same conditions of pH and initial protein concentration as in Figures 3-6.

Figure 6. (A) Zero shear viscosity as a function of aggregate molecular weight. Scaling of zero shear viscosity normalized by (B) σ and (C) M_w^{agg} . Symbols correspond to the same conditions of pH and initial protein concentration as in Figure 3-6.

Figure1A

Figure1B

Figure2A

Figure2B

Figure3A

Figure3B

Figure3C

Figure4A

Figure4B

Figure5

Figure6A

Figure6B

Figure6C