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Probing Submicron Aggregation Kinetics of an IgG Protein by Asymmetrical Flow Field-Flow Fractionation

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ABSTRACT

A lack of reliable analytical methods has hindered the quantification of submicron protein aggregates and a detailed understanding of their formation kinetics. In this study, a simple asymmetrical flow field-flow fractionation (AF4) method with good size selectivity (>0.5) is used to investigate nanometer (<0.1 μm) and submicron (0.1-1 μm) aggregates of heat-stressed anti-streptavidin (anti-SA) IgG1. The Lumry–Eyring nucleated polymerization (LENP) model for non-native protein aggregation is fit to the AF4 data, and kinetic analysis shows that aggregates are formed via slow nucleation and aggregate condensation at long stress times. Comparison of centrifuged and uncentrifuged heat-stressed anti-SA IgG1 AF4 results show the removal of high molar mass submicron aggregates and large material (>20 μm) and suggests that centrifugation may influence the aggregation kinetics. Furthermore, qualitative LENP model analysis of centrifuged and uncentrifuged samples suggests that significant aggregate–aggregate condensation occurs even at early stress times and highlights the potential of AF4 to determine aggregation kinetics for species greater than 1 μm.

Introduction

In the last 20 years, the use of therapeutic proteins has grown rapidly for the treatment of diseases including cancer, Crohn's disease, and rheumatoid arthritis, among others.[1] During production, transportation, and storage, therapeutic proteins experience a variety of stress conditions that may result in the formation of protein aggregates.[2] Aggregation of therapeutic proteins is a major concern because of the reduced product efficacy and potential immunogenicity.[3] However, developing stable therapeutic protein formulations and understanding aggregate formation is a challenge because of the complex nature of proteins.

Protein aggregates can range from small nanometer (<0.1 μm) to larger submicron (0.1-1 μm), micrometer (1-100 μm), and visible (>100 μm) particles making their characterization difficult by a single analytical technique.[4-9] The United States Pharmacopeia (USP) <788> specifies methods for counting subvisible particles greater than 10 μm in therapeutic formulations.[10] Visual inspection is commonly used to detect particles greater than 100 μm, and light obscuration (LO) and optical microscopy are described in the USP for quantifying particulates greater than 10 μm. In recent years, flow imaging techniques such as Microflow Imaging™ and FlowCam™ have become more common for quantifying particles from 1 to 10 μm.[7,11-14] Using a CCD camera, images are captured as the sample flows through the field of view of a microscope. Image analysis is then used to determine particle size, number, shape, and transparency. One significant advantage in flow imaging analysis is the discrimination between proteinaceous and nonproteinaceous particles such as silicone oil droplets.[15] Enabling technologies such as new ethylene tetrafluoroethylene polymer particle standards with a similar refractive index (1.40) as proteins have been developed to allow more accurate size analysis.[16,17] Despite these recent advances, the lower size limit of flow imaging techniques remains at approximately 1 μm.

The submicron (0.1-1 μm) aggregate size range is an area of particular interest because of the lack of reliable analytical

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techniques and potential immunogenicity of aggregates.[2,18,19] Dynamic light scattering (DLS) is commonly used for submicron analysis and relies on the correlation between fluctuations in light scattering intensity and the analytes' diffusion coefficients (D) and, in turn, their hydrodynamic diameters (d_h). DLS results are often biased toward larger sizes because of the higher scattering intensity ($I \propto {d_h}^6$) of large particles and contaminants such as dust.[7,20] Furthermore, DLS is a low-resolution technique and particle populations must differ in size by a factor of at least three to be resolved.[20] Nanoparticle tracking analysis (NTA) utilizes a CCD camera and laser illumination to capture the Brownian motion and light scattered by individual particles. Subsequent software analysis yields D and d_h values. By tracking individual particles, NTA is able to distinguish particles of similar sizes better than DLS and provides semiquantitative particles counts.[20] NTA analysis has an optimum aggregate concentration range between 10^7 and 10^9 particles per mL[20,21] and a lower size limit of approximately 40 nm (for low refractive index particles such as protein aggregates).

The upper size limit is often reported as 1 μm, although in practice, particles above approximately 0.5 μm are often re-moved by filtration or centrifugation to minimize scattering interference that can mask the signal from smaller particles. Resonance mass measurement (RMM) is another recently introduced method for quantifying protein aggregates.[22] It is performed by flowing analytes through a microchannel resonator that is suspended in a vacuum chamber. Changes in the resonant frequency as particles pass through the channel are correlated with their buoyant mass. Silicone oil droplets and protein particles can be distinguished by RMM, but submicron aggregate analysis is hindered by a lower size limit of approximately 0.4 μm, potential clogging of the microfluidic channel by large particles, and the need to assume protein aggregate density. Flow cytometry and Coulter counter have also been used to analyze the submicron aggregates region.[23,24] However, both techniques are limited to particles greater than 0.5 μm, and high conductivity electrolyte solutions required for Coulter counter analysis may alter aggregate populations, thereby limiting its utility for aggregate quantification in formulation buffer.[2,5,7,9]

The complexity (size, shape, and density) of protein aggregates necessitates the incorporation of a separation stage into the analysis of these complex samples. Size-exclusion chromatography (SEC) is the most widely used technique to separate and quantify protein monomer and nanometer (<0.1 μm) aggregates.[9] However, well-known limitations exist for the SEC analysis of protein aggregation.[25] Interaction of analytes with the column packing can lead to changes in elution times, peak shapes, and sample recoveries. Adjusting the mobile phase ionic strength and using additives, such as arginine or organic modifiers, can reduce interactions, but may also affect the aggregate subpopulations present in the original formulation.[26] Protein formulations often include excipients, stabilizers, and additives and loading these samples onto the SEC column can influence column stability and reduce the column lifetime.[27] The inherent shear forces experienced during SEC analysis especially under high pressures (>100 bar) can also lead to changes in aggregate populations, especially for large and weakly bound species. In one SEC study, increased amounts of aggregates were generated when using separation conditions of 410 bar compared with 125 bar.[28] In addition to sample changes caused by SEC columns, aggregates are often removed by precolumn frits or centrifugation during sample preparation to avoid plugging the column.[29] These sample preparation steps can eliminate submicron sample components of interest. Analytical ultracentrifugation is a complementary technique to SEC for nanometer size aggregates, but is limited by low throughput and complicated data analysis.[9]

The limitations of SEC and other techniques outlined above can be addressed by asymmetrical flow field-flow fractionation (AF4).[30,31] The open AF4 channel (no packing material) translates to reduced undesirable interactions and sample loss, low pressures (<15 bar), and low shear rates that allow weakly bound protein complexes and aggregates to be characterized. Pollastrini et al.[32] showed that AF4 was able to quantify weak binding (K_d >1 μM) of an IgG/FcRn complex that was not measurable by SEC. Additionally, there is more flexibility in the choice of carrier liquid in AF4 (aqueous and organic channels are commercially available) and the formulation buffer can of-ten be used.[26,31,33] This is an important advantage as the carrier liquid composition can dramatically affect both protein structure and aggregate formation.[34]

Asymmetrical flow field-flow fractionation has been used to investigate the effects of protein formulation stability and accelerated protein aggregation.[31,34] A variety of accelerated stress conditions including pH, freeze-thaw, and heat can cause aggregate formation. Despite its many advantages, AF4 has not been used to determine submicron non-native aggregation kinetics. Non-native aggregation (from here on referred to simply as aggregation) involves a change in conformation of the native protein and is a major source of degradation products in processing, packaging, transport, storage, and administration of protein therapeutics.[4,35-38] Understanding aggregation kinetics is important for controlling and eliminating aggregate species to ensure product safety.[3] A number of models exists that incorporate aggregate nucleation and growth steps to describe protein aggregation kinetics.[39-45] Many of these models are included as limiting cases in a general model for protein aggregation called the Lumry–Eyring nucleated polymerization (LENP) (Fig. 1).[46-50] This model, developed by Roberts and coworkers,[47,48] is highly suited for this work because experimentally relevant quantities such as monomer fraction (m) and molar mass (M) are measurable by AF4.

A simplified schematic of the LENP model[51] is shown in Figure 1. Aggregate formation involves a series of processes beginning with conformational changes in native monomer (N) proteins to form reactive monomer (R) conformers that subsequently self-assemble to form reversible oligomers (R_x) composed of x number of monomers. These reversible oligomers rearrange to form the smallest (essentially) irreversible aggregate nucleus (A_x). The LENP model considers the nucleation step to be irreversible if significant stabilizing interprotein interactions occur and/or if aggregate growth is much faster than nucleation. Aggregate growth can occur by two mechanisms. Chain polymerization aggregate growth occurs via addition of one or more reactive monomer species (R_δ), where δ is the number of monomers added per growth event. Growth may also occur by aggregate condensation where aggregate species (A_j), where j is some number of monomers greater than x, associate to form larger aggregates and eventually insoluble aggregates that phase separate. The description of the LENP model above is brief as existing publications discuss the model in detail.[48,51]

The LENP model consists of a system of coupled differential equations that can be solved numerically (Eqs. 1-3). Regression of these solutions against experimental data, m and M with respect to time (t), yield characteristic time constants for nucleation (τ_n), chain polymerization growth (τ_g), and aggregate condensation growth (τ_c). These time constants are inversely proportional to rate constants for each stage. The change in m with respect to t is described by Eq. 1:

$$\frac{dm}{dt} = -x\frac{m^x}{\tau_n} - \frac{\delta}{\tau_g}m^\delta\sigma \quad (1)$$

where σ is the total aggregate concentration over the initial monomer concentration. As A_x species form via nucleation, they