NOTE

Technical Decision-Making with Higher Order Structure Data: Detecting Reversible Concentration-Dependent Self-Association in a Monoclonal Antibody and a Preliminary Investigation to Eliminate It

JULIE Y. WEI, GEORGE M. BOU-ASSAF, DAMIAN HOUDE, ANDREW WEISKOPF

Protein Pharmaceutical Development, Biogen Inc., Cambridge, Massachusetts

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ABSTRACT: Protein self-association or aggregation is a property of significant concern for biopharmaceutical products due to the potential ability of aggregates to cause adverse toxicological and immunological effects. Thus, during the development of a protein biopharmaceutical, it is important to detect and quantify the level and nature of aggregate species as early as possible in order to make well-informed decisions and to mitigate and control potential risks. Although a deeper understanding of the mechanism of aggregation (i.e., protein-protein interactions) is desirable, such detailed assessment is not always necessary from a biopharmaceutical process development point of view. In fact, the scope of characterization efforts is often focused on achieving a well-controlled process, which generates a product that reliably meets established acceptance criteria for safety and efficacy. In this brief note, we evaluated the utility of size-exclusion chromatography, dynamic light scattering, and analytical ultracentrifugation in their simplest forms, to effectively reveal and confirm the presence of concentration-dependent reversible self-association (RSA) in a monoclonal antibody in the early stages of formulation development. Using these techniques, we also initiated preliminary work aimed at reducing the occurrence of this RSA behavior by varying the pH of the formulation buffer. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:3984–3989, 2015

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INTRODUCTION

The propensity for a protein to aggregate with itself (or self-associate) is a function of its physicochemical properties (which is governed by its primary structure and three-dimensional folded structure), its chemical and physical environment (including solution matrix or formulation buffer, container closure, and storage conditions), and its prior history of production and purification. In most cases, the mechanisms associated with protein self-association are tied to the protein's colloidal stability and/or its conformational stability. Such self-association can be concentration-dependent involving covalent and/or non-covalent interactions. In the latter case, protein association can also be reversible or irreversible. Consequently, protein aggregation is a complex phenomenon.[1]

When placed in certain physicochemical conditions, protein biopharmaceuticals can exhibit intrinsic concentration-dependent reversible self-association (RSA) properties.[2] Such physicochemical characteristics have been observed in many biological molecules and can play a critical role across an array of biological activities.[3] However, in the case of recombinant monoclonal antibodies (mAbs), such behavior can give rise to irreversible aggregation that can lead to adverse effects.[4] At relatively low concentrations, mAbs are normally well-behaved and exhibit a low propensity to self-associate. However, at higher concentrations, molecular crowding and other effects can cause mAbs to aggregate and can lead to unwanted physical properties such as high viscosity, precipitation, poor manufacturability, lower bioactivity, and/or immunogenicity.[5,6] Because protein aggregation has been linked to adverse toxicological and immunological responses,[7,8] distinguishing the levels of various modes of aggregation in a protein biopharmaceutical drug is key to its successful development. Size-exclusion chromatography (SEC), the standard workhorse method used to measure protein aggregation, can easily miss the presence of RSA or erroneously invoke it.[9] A major reason for this is that SEC is often conducted under fairly low concentrations using mobile phase conditions that are different from the formulation of the biopharmaceutical product, in order to prevent adsorption of the protein to the chromatographic media. This difference in solution conditions can significantly weaken or eliminate RSA that is normally present when the biopharmaceutical is in its formulation buffer at vialed concentration (and under rare conditions might even induce RSA that was not present in the biopharmaceutical's formulation[9]. Another factor that can make the correct assessment of RSA difficult via SEC is the impact of increasing sample concentration on the retention time and shape of the eluting sample peak due to sample overloading. This factor can give rise to chromatographic effects (resulting in poor mass transfer between the SEC's mobile phase and its stationary phase) that can mimic the chromatographic behavior used to detect RSA. Hence, unless orthogonal characterization work is conducted, RSA behavior of proteins can easily go undetected or falsely implicated.[9]

Correspondence to: Andrew Weiskopf (Telephone: +6179147904; Fax: +6176792308; E-mail: andy.weiskopf@biogen.com)

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In assessing and characterizing aggregation of a protein biopharmaceutical, two key pieces of information are required. The first is to determine the levels of aggregation that are inherently present in the sample, and the second is to understand their nature. In the case of RSA, a native monomeric protein reversibly forms an oligomer or oligomers as a result of noncovalent intermolecular interactions. Oligomers may increase in quantity and size with increasing concentration. However, the long-term stability of such aggregates may not necessarily compromise the safety and efficacy of these drugs. Assessment of this question partly depends on the reversibility of protein–protein interactions during long-term storage, the nature of the final dosing solution, its mode of delivery, and its response within the *in vivo* environment. On the other hand, RSA may lead to non-reversible aggregation which is more problematic. Therefore, simple processes for detecting RSA behavior and simple approaches to minimize or eliminate it can be crucial to ensuring successful development of mAb-based therapies and expediting their development.

Recently, Esfandiary et al.[10] have combined dynamic light scattering (DLS), concentration-gradient multi-angle light scattering, and analytical ultracentrifugation (AUC) to comprehensively characterize protein RSA. The complementarity of these techniques allowed elucidation of detailed aspects of RSA behavior in order to develop a detailed model for aggregation. Here, we employ similar techniques (SEC, DLS, and AUC) to rapidly detect the presence of RSA behavior in an immunoglobulin G1 (IgG1) mAb (mAb1) when studied in its initial formulation buffer containing phosphate and sucrose at pH 7.2. Our objective was to approach the problem through the lens of product development, in order to accelerate formulation optimization rather than elucidating the detailed nature of the self-association mechanism. This tactic enabled us to simply and rapidly identify and confirm the occurrence of RSA, and initiate preliminary steps to reduce or eliminate it. In so doing, we were able to demonstrate our ability to support appropriate decision-making steps that could be taken early in the drug development process in a timely manner.

MATERIALS AND METHODS

Reagents and Antibody

All chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted. The drug substance IgG1 antibody (mAb1) discussed in this note was expressed in mammalian cells and initially formulated in phosphate buffer containing sucrose at pH 7.2.

Size-Exclusion Chromatography

SEC analysis was performed on a Waters Alliance HPLC system (Milford, Massachusetts) using a TSKgel G3000SWXL column (TOSOH Bioscience, San Francisco, California) operating under isocratic conditions. Up to 100 μg of mAb1 was injected onto the column (200 μL injection at 0.5 mg/mL) with a flow rate of 1.0 mL/min. The SEC buffer was 50 mM sodium phosphate, 100 mM sodium chloride, pH 6.0.

Dynamic Light Scattering

Diffusion coefficients were measured as a function of protein concentration on a DynaPro PlateReader Plus (Wyatt, Santa Barbara, California) at a laser wavelength of 828.88 nm. Aliquots of the filtered (0.22 μm) mAb1 sample were transferred into sterile, 96-well, clear-bottom plate Greiner Sensoplates (Greiner Bio-One, Monroe, North Carolina). Wyatt Technology Dynamics software (v. 7.3.1) was used to schedule and automate 20 independent 60 s scans for each sample. Three replicates ($n = 3$) were averaged to reduce systematic error in the sample preparation and analysis. Measurements were performed at 20°C. The average mutual translational diffusion coefficients, D_{m}, determined for each mAb solution at protein concentrations between 0.5 and 10.0 mg/mL were plotted as a function of concentration. In these plots, the concentration-dependent interaction term, k_{D}, was obtained from the slope of a simple linear regression analysis, while the y-intercept provided the value for D_{m} at zero concentration.

Analytical Ultracentrifugation

AUC experiments were performed on a Beckman-Coulter XL-I centrifuge operated at 161,300 × g and 20°C. 12 or 3 mm double sector charcoal-filled epon centerpieces were used with sapphire windows. UV data were collected at 280 nm, and the data spacing was 0.003 cm (radius). Sedimentation coefficients were determined by processing the data with SEDFIT (v14.1).[11] AUC experiments were performed only once for each sample.

RESULTS AND DISCUSSION

Assessing Self-Association by SEC

Figure 1a shows the normalized SEC profiles (280 nm) of five different constant volume (10 μL) injections of mAb1 at concentrations of 0.5, 1, 2, 5, and 10 mg/mL in a mobile phase that is different from the mAb1 formulation buffer. The right-most chromatogram corresponding to mAb1 at 0.5 mg/mL shows an ideal monomer peak shape that broadens and appears less symmetrical with increasing concentration (from right to left) as a result of gradual tailing on the backside of the peak. In addition, as the mAb1 concentration increases, the peak maximum elutes at increasingly earlier retention times. However, no detectable high molecular weight species (as a peak or shoulder on the front side of the main peak) were observed for any of the concentrations analyzed.

Although this type of chromatographic behavior is consistent with the presence of RSA, it can also be consistent with a chromatographic effect that is due to sample overloading, and/or concentration-dependent secondary interactions between the protein and the SEC stationary phase. The overloading effect arises when injecting samples of such high protein concentration that one may impact both the kinetics and thermodynamics of macromolecular transfer between the mobile and stationary phases in SEC. Such on-column behavior can result in artificially premature retention times, as well as peak tailing[9,12] that could effectively mimic the presence of RSA behavior, which would lead to erroneous conclusions about mAb1. As a result, SEC's ability to detect the presence of RSA may be limited, and the chromatographic behavior observed by SEC leaves unanswered questions.[13,14] Furthermore, differences between formulation buffer and SEC mobile phase composition, along with interactions between the injected protein sample and the surface of the chromatographic media, can perturb, exacerbate, or remove aggregates and render the SEC data unrepresentative of what was present in the initial sample. Therefore, techniques

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