1 The Critical Need for Robust Assays for Quantitation and Characterization of Aggregates of Therapeutic Proteins

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1.1 INTRODUCTION

Since the commercialization of monoclonal antibodies and recombinant therapeutic proteins in the 1980s, millions of lives have been saved or improved by these unique medicines. As with all therapeutics, assurance of product quality is key to providing a consistent clinical performance related to both safety and effectiveness. These assurances are more challenging with therapeutic proteins than with small molecular entities because of their heightened susceptibility to degradation via physical or chemical means, the dependency for their activity on often complex three-dimensional conformation, the complicated manufacturing processes needed for their production, and their propensity to induce immune responses, relative to small molecular entities. Indeed, extensive development and formulation studies to obtain a product that has appropriate stability during production, shipping, storage, and delivery to the patients are undertaken for each protein therapeutic. Robust, high resolution analytical methods are essential to meet the requirement to ensure product quality and for the development of the appropriate means to stabilize the protein.
Degradation of therapeutic proteins by one or multiple means (e.g., heat, light, agitation, and long-term storage in aqueous solution) causes a loss of product quality and, critically, may cause adverse effects on safety and efficacy. Among degradation products of therapeutic proteins that have adverse effects on safety and efficacy are protein aggregates [1,2]. Aggregates include assemblies of protein molecules ranging from dimers to those large enough (e.g., ≥0.5 μm) to be classified as subvisible particles to larger, visible particles. Typically, oligomeric protein aggregates that are small enough to remain in solution are referred to as soluble aggregates and/or high molecular weight species. Assemblies of protein molecules large enough to be pelleted during centrifugation or filtered out of solution are often termed insoluble aggregates. Aggregate assemblies large enough to be detected and quantified with particle-counting instruments are usually called subvisible particles. If they are large enough to be seen with the unaided eye, particles are referred to as visible.

There are many challenges regarding the choice of analytical methods for assessment of protein aggregates and evaluation of the data from such methods. First, the methods employed must cover the extremely large size range of aggregates, usually requiring several methods to provide rigorous data across this size range [3,4]. Second, analysis of aggregates is challenging because the mass fraction of the aggregates in a sample may be extremely low (e.g., 0.1–1.0%), thus requiring highly sensitive methods. Third, the process of conducting the analysis may change the aggregate composition in a sample. For example, sample dilution during size-exclusion chromatography may cause disaggregation [3]. Thus, it absolutely essential to use orthogonal methods to assure the accuracy and robustness of a given analytical method for protein aggregates [5]. Orthogonal methods are those that use a different operating principle to obtain corroborating data on a given analyte. For example, sedimentation velocity analytical ultracentrifugation (SV-AUC) is routinely used to confirm results from size-exclusion chromatography [2–5].

Besides presenting challenges for analytical methods, aggregates pose a particular concern for patient safety in that they may be potent inducers of immune responses with varying manifestations [1,2]. The spectrum of clinical effects induced by aggregates pertains to a multiplicity of factors including but not limited to their size and valency, whether the key epitopes in the protein are in the native state or degraded, and whether the therapeutic protein product has an endogenous counterpart or is a foreign protein [1,2]. On one end of the spectrum are mild effects including minor alterations of pharmacokinetics, while on the other end of the spectrum are serious clinical effects including frank anaphylactic reactions (IgG or IgE mediated), neutralization of product activity with loss of efficacy, and, for therapeutic counterparts of endogenous proteins, neutralization of both product and endogenous counterpart [1,2]. The latter may result in a factor or cellular deficiency caused by loss of activity of the endogenous protein, if it has a unique function. Immune responses triggered by aggregates may target aggregate-specific (i.e., denatured or cryptic) epitopes, which do not cross react on the native protein, such as was the case in older studies of human serum
albumin, intravenous immunoglobulin, and human growth hormone [1,2]. Alternatively, immune responses triggered by aggregates in which the native protein conformation is preserved may neutralize the critical native domains that mediate activity, as is the case for example, for a percentage of patients taking type I interferon therapy [1,2].

Indeed, advantage has been taken of the capacity of a protein, in its native state, to elicit an immune response by constructing or formulating target proteins as particulates in vaccines. Typically, in vaccines, the particulates are formed by adsorbing the protein antigen onto the surface of another material such as a colloidal aluminum salt or other experimental microparticles [6]. Heterogeneous particles may also be present among protein aggregates in therapeutic protein products [6] arising from adsorption of proteins to particles originating from filling pumps, such as stainless steel particles, or from the container closure, such as glass particles [7–9].

There is a preponderance of data in the literature that indicate that aggregates are culprits in causing immune responses to protein therapeutics and vaccine antigens (reviewed in 1,2). Therefore, for protein therapeutics, it is critical to assure that aggregates are at the lowest level practical and to minimize, in particular, the higher MW aggregates with multiple repetitive units, implicated in immune response induction. Meeting this standard requires that manufacturing processes, formulations, storage and shipping conditions, and education of patients and medical personnel administering these drugs are optimized for minimizing aggregate formation and that there are proper, robust, and high resolution assays developed to quantify and characterize protein aggregates for each therapeutic protein.

Developing such assays properly requires substantial knowledge and expertise—with respect to the specific challenges of the assays as well as to the properties of the given protein—because each therapeutic protein has unique physicochemical properties giving rise to different degradation pathways that engender aggregation. For example, even minor sequence changes (e.g., point mutations) or chemical degradation of a few residues in therapeutic monoclonal antibodies can cause different stability and aggregation behaviors [10,11]. For these reasons, it is generally not sufficient to rely on so-called platform analytical methods for a given class of products such as monoclonal antibodies. Of course, the experience and expertise gained with analytical method development for similar proteins can be extremely valuable in guiding work on a new therapeutic protein, and a standard algorithm to method development can guide and substantially shorten the development cycle. But ultimately, analytical methods for each protein product must be “customized” for that particular protein. Shortcuts could lead to problems with reliability and accuracy of analytical methods that could compromise product quality and patient safety.

Therefore, appropriate time and resource investment, in the areas of process understanding and validation, personnel training, equipment, facilities, raw materials qualification, and analytical methods development and validation are required because these are ultimately essential to development of successful
manufacturing and commercialization processes. The main methods for analysis of protein aggregates, their development and applications, challenges with their implementation, and the critical technical issues affecting their performance are expertly discussed in this book. These methods fall into the broad categories described below.

1.2 METHODS FOR SIZING AND QUANTIFYING SOLUBLE AGGREGATES

The main analytical method used to quantify and size soluble aggregates is high performance size-exclusion chromatography (HP-SEC) [3–5]. This method is used to characterize protein aggregates during process development for bulk drug substance and for release and stability assessments for drug product as well as during formulation development. Therefore, it is critically important that the values generated by HP-SEC for a given therapeutic protein precisely and accurately reflect the actual values for aggregates in a sample. Meeting this goal is challenging because of the potential for aggregates to dissociate during HP-SEC runs and/or to adsorb to the column media [3–5]. Owing to these problems, orthogonal methods should be employed to assess and assure the accuracy of results from HP-SEC and to guide development of robust HP-SEC methods for a given protein [3–5]. Currently, SV-AUC is a method used for this purpose, but this method also has its own challenges for obtaining robust, reproducible results for protein aggregates. There are also efforts to develop field-flow fractionation as a method to quantify and size soluble aggregates but because of its own particular challenges, this approach has not yet been as widely adopted as SEC-HPLC and SV-AUC.

1.3 METHODS FOR SIZING AND QUANTIFYING PARTICLES

Protein aggregates that are large enough to be considered as particles often constitute a minute mass fraction of the protein molecules in the drug product. Therefore, typically, the amount of protein in particles cannot be quantified based on loss of monomer. Instead, the particles are counted and sized by methods such as light obscuration, microflow imaging, and Coulter counting [12]. Each of these methods has its benefits and drawbacks, and there is a substantial amount of new research in this area [12]. Among the critical points is the need to differentiate between microparticles containing both foreign materials (and to identify the foreign material) and protein and those containing protein alone, as this is essential for identifying causative factors and precluding aggregate formation in subsequent lots. Of course, because proteins readily adsorb to foreign microparticles arising from materials involved in product manufacture and storage (e.g., steel particles from piston pumps and glass particles for storage vessels) [9], it
is expected that essentially all particles analyzed will contain at least a fraction of protein molecules.

Special attention is given to visible particles because each lot of parenteral dosage forms (e.g., vials, syringes, or more rarely ampoules) is subjected to 100% visual inspection after manufacturing. This approach requires highly skilled and trained operators. There are now efforts to automate “visible inspection,” which presumably could increase throughput.

1.4 METHODS FOR CHARACTERIZING CONFORMATION OF PROTEIN MOLECULES IN AGGREGATES

The conformation of protein molecules in aggregates can affect their biological activity as well as the consequences of immune responses directed to such of aggregates. Characterization of conformation of protein molecules in soluble aggregates can be studied in a mixture or after separation of a given aggregate population. Analysis by methods such as fluorescence spectroscopy (intrinsic, with fluorescence dyes or quenching), UV absorbance and near-UV circular dichroism spectroscopy, binding to conformationally dependent antibodies for tertiary structure, and infrared and far-UV circular dichroism spectroscopy for secondary structure should be considered. A potency assay for biological function may be used, although some proteins aggregated in their native state may lose the capacity to interact productively through their cognate receptors. For protein molecules in particles, in some cases, the particles can be separated from the solution and studied with these same spectroscopic methods [8].

1.5 REGULATORY ISSUES

It is a general principle that product quality attributes that contribute to clinical safety and efficacy must be identified, their levels correlated with clinical experience in patient populations, and specifications set for them to ensure that a favorable clinical performance profile is maintained with each lot of product produced. Of course, consistent with the principles established in the “Quality by Design Initiative,” enhanced knowledge of the attribute’s impact on the safety and efficacy profile of the product may allow more flexibility in setting specifications. Aggregates are considered a critical attribute in terms of their potential to elicit immune responses and affect product activity, enhancing or diminishing potency. Thus, even in phase I clinical trials, the aggregate content must be characterized and routinely measured for each lot by well-qualified assays and provisional specifications set.

Exploring process and formulation modifications to minimize protein aggregation is crucial during product development. Of course, for products that pose a higher risk to patient safety, such as those for which neutralizing antibodies can neutralize endogenous proteins with unique biological functions, special care
must be taken at the earliest stages of product development to accurately detect protein aggregates and to minimize their formation. For licensed products, it is important to ensure that the preferred aggregate assay for routine aggregate assessment, such as HP-SEC, can detect all the aggregate species that are likely to be present in the product, based on a full understanding of the process as it affects aggregation and on product degradation pathways. Therefore, orthogonal methods such as SV-AUC, field-flow fractionation, or other potential methods should be used to verify that any method or set of methods proposed for routine detection of aggregate species is capable of detecting and quantifying the desired range of aggregate species. It may be necessary to evaluate the robustness of the HP-SEC assay by demonstrating its ability to detect all protein aggregates generated under relevant stress conditions. If so confirmed, then HP-SEC may be the sole tool utilized for aggregate detection for routine assessments. However, following significant changes in manufacture, as is routinely done for nearly all protein therapeutics in the course of development, a more extensive comparability study must be performed in which the critical product quality attributes of the post-manufacturing change product are compared with those of the pre-change product by using well-qualified and robust assays. In such cases, aggregate assessment may again warrant orthogonal techniques to evaluate the levels and types of aggregates present in the post-manufacturing change product.

Regarding particulate assessment, the light obscuration test, as defined by USP 30 monograph <788>, requires analysis of particles >10 and >25 μm, leaving a gap in assessment of particles in the 0.1–10 μm subvisible range [6]. Although light obscuration can be used to quantitate particles that are between 2 and 10 μm, other methods such as Resonant Mass Measurement or Nanoparticle Tracking Analysis are currently being developed and evaluated for quantitation of particles that are <2.0 μm in size. The use of novel methods for evaluation of protein particles in the GMP environment will require a concerted effort.

1.6 IMPORTANCE OF THE CURRENT BOOK

Protein aggregates are a critically important class of degradation products in therapeutic proteins. Therefore, robust analytical methods for aggregates are essential for assuring the safety and efficacy of these products and for guiding their development. The current book is an invaluable resource for researchers and managers working on therapeutic proteins. It provides expert reviews of the state-of-the art for the range of analytical methods used for assessment of protein aggregates and the numerous challenges that are unique to each method. Furthermore, the book provides insight into the future of method development and regulatory issues for protein aggregates. With comprehensive coverage of the key issues, this book will be a critical reference for the field for many years.
REFERENCES
