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Overlooking Subvisible Particles in Therapeutic Protein Products: Gaps that may Compromise Product Quality

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Therapeutic protein products provide unique and effective treatments for numerous human diseases and medical conditions. In many cases, these treatments are used chronically to slow disease progression, reduce morbidity and/or to replace essential proteins that are not produced endogenously in patients. Therefore, any factor that reduces or eliminates the effectiveness of the treatment can lead to patient suffering and even death. One means by which efficacy of therapeutic proteins can be compromised is by an immune response, resulting in antibody-mediated neutralization of the protein's activity or alterations in bioavailability.[1,2] For example, in the case of treatment of hemophilia A, neutralizing antibodies to Factor VIII can cause life-threatening bleeding episodes, resulting in significant morbidity and necessitating treatment with a prolonged course of a tolerance-inducing therapy to reverse immunity.[3,4] In other cases, drug-induced antibodies to a therapeutic version of an endogenous protein can cross-react with and neutralize the patient's endogenous protein. If the endogenous protein serves a non-redundant biological function, such an immune response can have devastating results. For example, pure red cell aplasia can result from neutralizing antibodies to epoetin alpha. [1,2]

It is well established that protein aggregates in therapeutic protein products can enhance immunogenicity[2], and such an effect is therefore an important risk factor to consider when assessing product quality. The purpose of this commentary is to accomplish the following:

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i. provide brief summaries on the factors affecting protein aggregation and the key aspects of protein aggregates that are associated with immunogenicity;

ii. emphasize the current scientific gaps in understanding and analytical limitations for quantitation of species of large protein aggregates that are referred to as subvisible particles, with specific consideration of those particles 0.1–10 μm in size;

iii. offer a rationale for why these gaps may compromise the safety and/or efficacy of a product;

iv. provide scientifically sound, risked based recommendations/conclusions for assessment and control of such aggregate species.

Causes of Protein Aggregation

Proteins usually aggregate from partially unfolded molecules, which can be part of the native state ensemble of molecules.[5] Even though product formulations are developed to maximize and maintain the fraction of the protein molecules present in the native state, significant amounts of aggregates can form, especially over pharmaceutically-relevant time scales and under stress conditions. For example, exposure to interfaces (e.g., air-liquid and solid-liquid), light, temperature fluctuations or minor impurities can induce aggregation. Such exposure can occur during processing steps, as well as in the final product container during storage, shipment and handling. Furthermore, protein particles (visible and subvisible) can be generated from protein alone or from heterogeneous nucleation on foreign micro- and nanoparticles that are shed, for example, from filling pumps or product container/closures.[6–8]

The levels and sizes of protein particles present in a given product can be changed by many factors relevant to commercial production of therapeutic proteins. Such factors include a change in the type of filling pump during scale-up to commercial manufacturing, changes in formulation or container/closure, and even unintentional changes in the manufacturing process such as alterations in filling pump mechanical parameters or other unforeseen factors.[8,9] Thus, unless appropriate quality controls are in place for subvisible particles, a product that was safe and effective in clinical trials may unexpectedly cause adverse events in patients after commercialization.

Effects of Aggregate Characteristics on Immunogenicity

From work on fundamental aspects of immunology and vaccine development, it is known that large protein assemblies with repetitive arrays of antigens, in which the protein molecules have native conformation, are usually the most potent at inducing immune responses.[2,10,11] Furthermore, efforts to develop more effective vaccines have shown that adsorbing antigenic proteins to nano- or microparticles comprised of other materials (e.g., colloidal aluminum salts or polystyrene) can greatly increase immunogenicity.[12,13] Applying these lessons to therapeutic protein products, it has been argued that large aggregates containing protein molecules with native-like conformation pose the greatest risk of causing adverse immune responses in patients.[2] Thus, for example, particles of therapeutic proteins formed by adsorption of protein molecules onto foreign micro- and nanoparticles might be particularly prone to cause immunogenicity. These particles contain numerous protein molecules, and in the two examples published to date, the adsorbed protein molecules were shown to retain their native conformations.[6,8]

Unfortunately, lacking are published studies that comprehensively investigate the range of parameters that could influence immunogenicity of aggregates. Because each protein may differ in aggregate formation and consequences, factors that need to be investigated include

but are not limited to type, amount and size of aggregates, as well as protein conformation in aggregates, on a case by case basis. Of course, other factors, particularly pertaining to patient status and treatment protocol, are also critical in determining the propensity to generate immune responses. These include immune competence of the patients, route of administration, and dosing frequency and duration. Given the consequences of aggregate-induced immunogenicity in patients, it is important to understand these issues and to reduce the risk to product quality for every therapeutic protein product.

Because the exact characteristics and levels of protein aggregates that lead to an enhanced immune response are unclear and may differ among proteins, it is not possible to predict, *a priori*, the *in vivo* effects of different sizes, types or quantities of aggregates for therapeutic protein products. In such situations, careful analysis of the relationship between clinical performance and the presence of protein aggregates in relevant clinical trial material may help in the design of suitable control strategies that ensure product quality. However, the validity and utility such correlations are only optimized when the full spectrum of protein aggregate species are thoroughly characterized by multiple and orthogonal techniques.

Critical Gaps in the Analysis and Control of Subvisible Particles

Protein aggregates that can be quantified based on the mass percentage for each aggregate size are usually classified as soluble and insoluble. There is mass balance between the amount of protein in the aggregates and the loss of the monomeric protein. However, subvisible particles usually do not constitute a sufficient mass fraction of the protein population to be quantified based on mass of protein in the particles or by loss of monomeric protein. Typically, these particles are quantified by counting the number of particles in given size ranges. Subvisible particles are usually defined as particles that are too large for analysis by size exclusion chromatography (SEC) (e.g., ~ > 0.1 μm), but too small to be visible to the unaided eye (e.g., < 100 μm). Subvisible protein particles are thus relatively large assemblies (e.g., 0.1–10 μm) that contain thousands to millions of protein molecules.

Historically, analysis of subvisible (and visible) particles has been required for final product release testing to mitigate the risk associated with the presence of extraneous particles in injectable solutions, particularly the risk of blood vessel occlusion for intravenously administered solutions of small molecule parenteral drug products. Consequently, USP requirements for the light obscuration test <788>, the standard test for sub-visible particulate analysis, specifies that particulates >10 μm in size are controlled at or below 6000 particles/container and particles >25 μm are limited to at or below 600 particles/container Although ICH quality guidance Q6B states that the requirements set forth by pharmacopoeias pertaining to analytical procedures and acceptance criteria for particulate matter are applicable to biotechnological products[14], the risks associated with the administration of large aggregated protein particles were never considered in establishment of the USP light obscuration test <788>. However, ICH Q6B clearly states that specifications “should focus on those molecular characteristics found to be useful in ensuring the safety and efficacy of the product”[14] and is potentially applicable to the control of large protein aggregates.

In USP 30 monograph <788>, there is an exclusion of injections intended solely for intramuscular or subcutaneous administration. This exclusion may be appropriate for the risk of blood vessel occlusion but would not be appropriate for the risk of immunogenicity. Since subcutaneous and intramuscular routes are often more immunogenic than intravenous administration, it is appropriate to consider particulates for all parenterally administered proteins. The revised harmonized version of <788> published in USP 31 does not contain this exclusion and thus appears to cover all parenteral routes. However, subvisible particles less then 10 μm are still not evaluated in the USP test.

Thus, even though large protein aggregates that are classified as subvisible particles are potentially the most immunogenic class of protein aggregates, subvisible particles smaller than 10 μm are not currently monitored and recommendations for such testing for therapeutic proteins products are lacking. Furthermore, unlike the concern regarding extraneous particles in small molecule parenteral products, protein particles can accumulate over time during storage of the final presentation. Subvisible protein particles in the 0.1–10 μm range, as well as protein particles > 10 μm, have the potential to impact the safety and efficacy of the product over its shelf life, a criteria described in ICH Q6B. As with any product attribute, the level of control necessary should reflect the potential risk to product quality and could involve monitoring at release and on stability. Risk-mitigating factors are important in assessing the impact of a product attribute. For aggregates, such a factor could be the reversibility of the aggregate under the intended route of administration.

The need for manufacturers of pharmaceutical protein products to evaluate and control this risk to product quality is underscored by published case studies with therapeutic proteins in which subvisible particles were measured.[8,15,16] For example, during filling pump operations, it was found that an IgG formed hundreds of thousand of particles per ml in the 1.5–3 μm size range, but less than 1000 particles per ml in the 8–15 μm size range.[8] In a formulation study of a therapeutic cytokine formulated with human serum albumin, more than 90% of subvisible particles were in the 1–2 μm size range, 7–9% were 2–10 μm and less 0.01% were larger than 10 μm.[15] Similarly, subvisible particle counts in a recombinant hemoglobin product documented that particles smaller than 10 μm were approximately two orders of magnitude greater in number than particles larger than 10 μm.[16] As demonstrated by these examples, protein products that contain extremely large numbers of particles smaller than 10 μm might meet the current USP requirement for subvisible particles. If only particles > 10 μm were quantified in a given product, there could be gaps in understanding of important degradation products and in product quality assessment.

Also, there are important technical issues related to counting protein particles, especially those smaller than 1 μm, which is close to the lower size limit of detection of particle counting instruments operating by light obscuration or electrical current sensing zones. Hence, is not clear if the results obtained for the smaller size range of particles (e.g., ≤ 1 μm) are of sufficient accuracy and precision to allow for method qualification and, more stringently, validation of the method for use in pharmaceutical quality assurance. Particle counters that operate by laser light scattering can quantify particles as small as 0.1 μm, but there have not yet been published studies documenting the utility of these systems for protein particles. Another alternative method is microflow digital imaging, which can be used for particles as small as 0.75 μm. For each of these types of instruments, there are important sample handling issues including: a) the potential requirement for a sample volume exceeding the unit dose volume (e.g., 1 ml) for a given protein product; b) interference from air bubbles; c) potential need to dilute a sample; and d) difficulties in handling high viscosity samples. Furthermore, because protein particles may be translucent and loosely packed, compendial light obscuration techniques and the other methods may not work as well to quantify protein particles as they do for particles from extraneous materials. More research is needed to rigorously assess the capabilities of current instruments to quantify protein particles as small as 0.1 μm and to develop appropriate low volume sampling methods. Microscopic techniques such as atomic force microscopy and electron microscopy may be valuable, but these are low throughput methods and also would need extensive method validation. Finally, considering these technical issues, using multiple and orthogonal methods may currently be the most prudent means for evaluating subvisible particulates.

Conclusions

1. Subvisible protein particles have the potential to negatively impact clinical performance to a similar or greater degree than other degradation products, such as soluble aggregates and chemically modified species that are evaluated and quantified as part of product characterization and quality assurance programs.
2. Current USP particulate testing is not designed to control the potential risk of large protein aggregates to impact protein immunogenicity. Analytical methods that can assess particulate characteristics (including composition, amount and reversibility of the protein aggregate) are critical for developing scientifically sound approaches for evaluating and mitigating risk to product quality caused by large protein aggregates.
3. Pharmaceutical and academic researchers and instrument manufacturers should work together to help define the quantitative capabilities of current particle counting instruments for particles as small as 0.1 μm and develop new instruments as needed.
4. The impact of protein aggregates on immunogenicity needs to be elucidated and should include studies of the role of protein class, amount of aggregate, size of aggregates, and protein conformation in aggregates. These investigations should be published in peer reviewed journals.

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