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Pharmaceutical aspects of subvisible particles in protein formulations

Weinbuch, D.

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Author: Weinbuch, D.

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CHAPTER 9

SUMMARY AND PERSPECTIVES

Summary

Biopharmaceuticals have been highly successful in treating severe diseases and disorders that could not be treated by classical pharmaceutical compounds. A major obstacle for current research and development programs of biopharmaceutical drug products is the instability of the therapeutic protein, which may compromise safety and efficacy. For instance, the formation of aggregates, especially in the nm- and μm -size range, has been linked to immune reactions in patients, also known as unwanted immunogenicity. In the light of challenges regarding the analytical characterization of nm- and μm -particles, the aim of this thesis was to evaluate and improve established and emerging analytical techniques in this size range. These analytical techniques were then applied to characterize particles in the nm- and μm -size range present in protein formulations and to study the effect of nanoparticulate impurities on the stability of therapeutic proteins.

Chapter 2 introduced the concept of protein formulation development, which aims to assure the quality, safety, efficacy of a therapeutic protein product throughout the intended shelf life. Furthermore, various formulation strategies were outlined and challenges that can be encountered during the different stages of research and development for biopharmaceutical drug products are discussed.

Chapter 3 introduced the concept and underlying mechanisms of unwanted immunogenicity and gave guidance on how to select a suitable set of currently available immunogenicity prediction models during the different stages of research and development of biopharmaceutical drug products.

In **Chapter 4**, an improved version of the already established light obscuration technique was successfully applied to determine subvisible particle concentrations in formulations with high protein concentrations. It could further be shown how currently applied systems are limited in the analysis of viscous samples and that exceeding those limits could lead to an underestimation of particle counts.

Chapter 5 comparatively evaluated Micro-Flow Imaging (MFI) and Resonant Mass Measurement (RMM) as emerging techniques for the differentiation of protein particles and silicone oil droplets in biopharmaceutical formulations. The data showed that a customized morphological filter, developed specifically for this study, greatly improved the results delivered by the MFI instrument and enabled reliable discrimination of particles with a size as low as 2 μm . RMM showed highly accurate discrimination in the size range of about 0.5–2 μm . Therefore, it is recommended applying both techniques for a comprehensive analysis of biotherapeutics potentially containing silicone oil droplets and protein particles in the submicron and micron size range.

Chapter 6 compared four of the most relevant flow-imaging microscopy instruments and identified their differences, benefits and shortcoming to enable researchers the employment of the most suitable system for a given application. Based on the results, the systems were categorized into high-resolution systems, obtaining detailed morphology parameters enabling an accurate particle classification, and high-efficiency systems, delivering particle counts and sizes with high accuracy and precision.

In **Chapter 7**, it was shown that the interference of sugar-containing formulations with light scattering based analytical techniques is caused by the presence of a so far unknown type of nanoparticulate impurity in pharmaceutical-grade sugars. The results suggested them to be agglomerates of a variety of impurities (dextran, ash and aromatic colorants) not fully removed by the sugar refinement processes.

Chapter 8 investigated the effect of the nanoparticulate impurities discovered in Chapter 7 on the stability of four therapeutic monoclonal antibodies currently on the market. The stability of all antibodies was impaired by the presence of the nanoparticulate impurities resulting in the formation of aggregates, and nm- and μm -sized particles, however, to different extents among the antibodies. Furthermore, it was shown that the nanoparticulate impurities themselves contain immunomodulatory molecules potentially able to elicit immune responses in patients.

Perspectives

The work presented in this thesis aimed to support scientific efforts in making future biopharmaceutical products safer, by increasing the scientific understanding on the proper employment, strengths and limitations of crucial analytical techniques and by providing new insights into the nature and criticality of nm- and μm -sized particles. Future investigations and scientific studies should aim to improve particle characterization analytics, increase the fundamental understanding and optimize the prevention of aggregation, and to deliver further insights into the relationship between aggregate properties and immunogenicity.

Characterization of particles in biopharmaceutical products

The demand for novel and improved analytical techniques for the characterization of particles in the nm- and μm -size range has been expressed by many research groups in the past and is still valid (1–3). The “subvisible size gap” has been closed in part by the development of novel analytical techniques, some of which were evaluated in this thesis. In general, orthogonal methods employing truly different measurement principles are needed and should then be applied to overcome weaknesses and biases of instruments

relying on the same measurement principle. As an example, light-scattering based techniques NTA and DLS may be supported by emerging promising methods such as tailor dispersion analysis and flow cytometry, providing true orthogonality to commonly applied techniques (4–8).

Developments of new instruments for particle characterization should furthermore aim to address challenges presented by future biopharmaceutical drug products. The current trend, especially for monoclonal antibody products, goes towards highly concentrated preparations (e.g., above 100 mg/mL) for subcutaneous administration, due to the necessity of high doses (several mg/kg) with frequent dosing regimens (9). These products create new demands on current and future analytical technologies, such as small scale methods with low sample volume requirements and the ability to measure samples of high viscosity and high refractive index without the necessity of sample preparation (10–12). Some currently applied techniques would require a sample dilution step because of analytical limitations, which could alter a protein's aggregation state through a change in solvent composition and protein concentration, thereby affecting the reliability of test results (12).

Another trend for future biopharmaceutical drug products is the development of dedicated application devices and the use of prefilled syringes. These developments aim for a quicker and more accurate dosing, while enabling administration by non-professionals or self-administration (13). However, these developments come with new challenges. For example, the commonly applied process of siliconization of syringe surfaces for lubrication may lead to the presence of subvisible silicone oil droplets in some products (14–16). This creates the necessity for differentiation and identification of particles and demands novel analytical technologies and methodologies, some of which were evaluated during this thesis. While particles originating from primary packaging are not always harmful themselves, they can negatively affect the stability of the therapeutic protein (17,18). It is furthermore important to develop novel surface modification techniques that overcome the weaknesses of current container closure systems (13,19).

The combination of different measurement principles within one analytical device should also be in the focus of future development programs. For example, a device applying imaging microscopy or dynamic light scattering in liquid samples alongside Raman spectroscopy could establish a direct link between particle size and morphology and particle origin (20–23). Such insights would be highly valuable during biopharmaceutical development and troubleshooting.

Understanding and prevention of aggregation

A highly active field of research aims to understand the fundamental mechanisms underlying protein aggregation and the formation of nm- and μm -particles. Many different aggregation mechanisms have been identified, but it is not yet possible to predict which pathways will be predominant for a certain protein in a particular formulation (24). Furthermore, different pathways can exist in parallel and their occurrence depends on the molecular nature of the protein, the protein environment (e.g., formulation and primary container) and the applied stress conditions. If the molecular nature makes a protein prone to aggregation because of the presence of potential aggregation hot-spots, one could attempt to change the protein's sequence and structure by protein engineering (24,25). This, however, may not eliminate the formation of aggregates, since factors other than primary and secondary structure are important in this context. For some proteins, aggregation pathways in relation to pH and ionic strength have been identified (26–29). Unfortunately, these can in most cases not be directly applied to other proteins. Furthermore, it is currently not fully understood how proteins aggregate when exposed to solid-liquid and liquid-air interfaces (30,31). Thus, formulation developers still rely mostly on empirical data and scientific experience to find suitable formulation conditions and the (or a) right combination of stabilizing excipients. A correlation of protein characteristics to a range of potentially optimal formulation conditions, including suggestions for type and concentration of excipients, would enable a faster and more focused formulation-, and thereby product development.

Relationship between aggregate properties and immunogenicity

It is clear that the presence of protein aggregates, especially in the nm- and μm -size range, can dramatically increase the risk for unwanted immunogenicity and the occurrence of adverse effects in patients. Still, there is currently little understanding as to which specific properties of aggregates and particles are involved in immunogenicity (32). Studies have shown that the amount of aggregates and particles determined in drug products does not necessarily correlate to the presence, type, or severity of immunological reactions in patients (33). Thus, besides number and size of aggregates and particles, there must be many other attributes important for immunogenicity, such as the arrangement and content of T-cell and B-cell epitopes on the aggregates' surface, protein conformation within the aggregate, type and extent of chemical modifications accompanied with aggregation, and aggregate density and morphology. It is an active field of research to understand the contribution of each of those attributes to the overall immunogenicity of a biopharmaceutical drug product. These efforts, however, are often impaired by the availability of clinical data and the ability to compare quality attributes among the

different products, related to the lack of standardized particle analytics (34,35). Thus, improved techniques for the analysis of aggregates and particles, utilized in a standardized way, will contribute to the investigation of unwanted immunogenicity.

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