Pharmaceutical aspects of subvisible particles in protein formulations

Weinbuch, D.

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 valuably 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.

References

1. Narhi LO, Jiang Y, Cao S, Benedek K, Shnek D. A critical review of analytical methods for subvisible and visible particles. Curr Pharm Biotechnol. 2009 Jun;10(4):373–81.

2. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJA, Middaugh CR, Winter G, et al. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. J Pharm Sci. 2009 Apr;98(4):1201–5.

3. Ríos Quiroz A, Lamerz J, Da Cunha T, Boillon A, Adler M, Finkler C, et al. Factors Governing the Precision of Subvisible Particle Measurement Methods - A Case Study with a Low-Concentration Therapeutic Protein Product in a Prefilled Syringe. Pharm Res. 2016;33(2):450–61.

4. Hawe A, Hulse WL, Jiskoot W, Forbes RT. Taylor dispersion analysis compared to dynamic light scattering for the size analysis of therapeutic peptides and proteins and their aggregates. Pharm Res. 2011 Sep;28(9):2302–10.

5. Cipelletti L, Biron JP, Martin M, Cottet H. Measuring Arbitrary Diffusion Coefficient Distributions of Nano-Objects by Taylor Dispersion Analysis. Anal Chem. 2015;87(16):8489–96.

6. Latunde-Dada S, Bott R, Hampton K, Leszczyszyn OI. Application of the Exact Dispersion Solution to the Analysis of Solutes beyond the Limits of Taylor Dispersion. Anal Chem. 2015;87(15):8021–5.

7. Lubich C, Malisauskas M, Prenninger T, Wurz T, Matthiessen P, Turecek PL, et al. A Flow-Cytometry-Based Approach to Facilitate Quantification, Size Estimation and Characterization of Sub-visible Particles in Protein Solutions. Pharm Res. 2015;(2):2863–76.

8. Nishi H, Mathäs R, Fürst R, Winter G. Label-Free Flow Cytometry Analysis of Subvisible Aggregates in Liquid IgG1 Antibody Formulations. J Pharm Sci. 2013 Nov 11;103(1):1–10.

9. Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. J Pharm Sci. 2004 Jun;93(6):1390–402.

10. Molloy S, Fesinmeyer RM, Martinez T, Murphy PD, Pelletier ME, Treuheit MJ, et al. Optimized UV Detection of High-Concentration Antibody Formulations using High-Throughput SE-HPLC. J Pharm Sci. 2015;104(2):508–14.

11. Hawe A, Schaubhut F, Geidobler R, Wiggenhorn M, Friess W, Rast M, et al. Pharmaceutical feasibility of sub-visible particle analysis in parenterals with reduced volume light obscuration methods. Eur J Pharm Biopharm. Elsevier B.V.; 2013 Feb 27;85(3 PART B):1084–7.

12. Zölls S, Gregoritza M, Tantipolphan R, Wiggenhorn M, Winter G, Friess W, et al. How subvisible particles become invisible-relevance of the refractive index for protein particle analysis. J Pharm Sci. 2013 Mar 5;102(5):1434–46.

13. Yoshino K, Nakamura K, Yamashita A, Abe Y, Iwasaki K, Kanazawa Y, et al. Functional evaluation and characterization of a newly developed silicone oil-free prefillable syringe system. J Pharm Sci. 2014 Mar 18;103(5):1520–8.

14. Majumdar S, Ford BM, Mar KD, Sullivan VJ, Ulrich RG, D'souza AJM. Evaluation of the effect of syringe surfaces on protein formulations. J Pharm Sci. 2011 Jul;100(7):2563–73.

15. Krayukhina E, Tsumoto K, Uchiyama S, Fukui K. Effects of syringe material and silicone oil lubrication on the stability of pharmaceutical proteins. J Pharm Sci. 2015 Sep 24;104(2):527–35.

16. Gerhardt A, Mcgraw NR, Schwartz DK, Bee JS, Carpenter JF, Randolph TW. Protein aggregation and particle formation in prefilled glass syringes. J Pharm Sci. 2014 Jun;103(6):1601–12.

17. Thirumangalathu R, Krishnan S, Ricci MS, Brems DN, Randolph TW, Carpenter JF. Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution. J Pharm Sci. 2009 Sep;98(9):3167–81.

18. Jones LS, Kaufmann A, Middaugh CR. Silicone oil induced aggregation of proteins. J Pharm Sci. 2005 Apr;94(4):918–27.

19. Depaz R a, Chevolleau T, Jouffray S, Narwal R, Dimitrova MN. Cross-linked silicone coating: A novel prefilled syringe technology that reduces subvisible particles and maintains compatibility with biologics. J Pharm Sci. 2014 Mar 18;103(5):1383–93.

20. Li B. A Comprehensive Technology of Particle Characterization that Automatically Measure Particle Size, Shape and Chemical Identity in One Single Platform. In: Cai X, Heng J, editors. Particle Science and Engineering. Cambridge: Royal Society of Chemistry; 2014. p. 126–30.

21. Saggu M, Liu J, Patel A. Identification of Subvisible Particles in Biopharmaceutical Formulations Using Raman Spectroscopy Provides Insight into Polysorbate 20 Degradation Pathway. Pharm Res. 2015;2877–88.

22. Lewis EN, Qi W, Kidder LH, Amin S, Kenyon SM, Blake S. Combined dynamic light scattering and raman spectroscopy approach for characterizing the aggregation of therapeutic proteins. Molecules. 2014;19(12):20888–905.

23. Zhou C, Qi W, Lewis EN, Carpenter JF. Characterization of Sizes of Aggregates of Insulin Analogs and the Conformations of the Constituent Protein Molecules: A Concomitant Dynamic Light Scattering and Raman Spectroscopy Study. J Pharm Sci. Elsevier Ltd; 2016;105(2):551–8.

24. Roberts CJ. Therapeutic protein aggregation: mechanisms, design, and control. Trends Biotechnol. Elsevier Ltd; 2014 Jul;32(7):372–80.

25. Wu SJ, Luo J, O'Neil KT, Kang J, Lacy ER, Canziani G, et al. Structure-based engineering of a monoclonal antibody for improved solubility. Protein Eng Des Sel. 2010;23(8):643–51.

26. Li Y, Ogunnaike BA, Roberts CJ. Multi-variate approach to global protein aggregation behavior and kinetics: effects of pH, NaCl, and temperature for alpha-chymotrypsinogen A. J Pharm Sci. 2010 Feb;99(2):645–62.

27. Brummitt RK, Nesta DP, Chang L, Chase SF, Laue TM, Roberts CJ. Nonnative aggregation of an IgG1 antibody in acidic conditions: part 1. Unfolding, colloidal interactions, and formation of high-molecular-weight aggregates. J Pharm Sci. 2011 Jun;100(6):2087–103.

28. Sahin E, Grillo AO, Perkins MD, Roberts CJ. Comparative effects of pH and ionic strength on protein-protein interactions, unfolding, and aggregation for IgG1 antibodies. J Pharm Sci. 2010 Dec;99(12):4830–48.

29. Kim N, Remmele RL, Liu D, Razinkov VI, Fernandez EJ, Roberts CJ. Aggregation of anti-streptavidin immunoglobulin gamma-1 involves Fab unfolding and competing growth pathways mediated by pH and salt concentration. Biophys Chem. Elsevier B.V.; 2013 Feb;172:26–36.

30. Campioni S, Carret G, Jordens S, Nicoud L, Mezzenga R, Riek R. The presence of an air-water interface affects formation and elongation of α-synuclein fibrils. J Am Chem Soc. 2014 Jan;136(7):2866–75.

31. Perevozchikova T, Nanda H, Nesta DP, Roberts CJ. Protein Adsorption, Desorption, and Aggregation Mediated by Solid-Liquid Interfaces. J Pharm Sci. 2015 Jun;104(6):1946–59.

32. Filipe V, Schellekens H, Jiskoot W. Aggregation and Immunogenicity of Therapeutic Proteins. In: Wang W, Roberts C, editors. Aggregation of Therapeutic Proteins. 2010.

33. Moussa EM, Panchal JP, Moorthy BS, Blum JS, Joubert MK, Narhi LO, et al. Immunogenicity of Therapeutic Protein Aggregates. J Pharm Sci. Elsevier Ltd; 2016;105(2):417–30.

34. Hermeling S, Crommelin DJ a, Schellekens H, Jiskoot W. Structure-immunogenicity relationships of therapeutic proteins. Pharm Res. 2004 Jun;21(6):897–903.

35. Jiskoot W, Kijanka G, Randolph TW, Carpenter JF, Koulov A V., Mahler HC, et al. Mouse Models for Assessing Protein Immunogenicity: Lessons and Challenges. J Pharm Sci. Elsevier Ltd; 2016;105(5):1567–75.