Analysis of sub-visible particles in complex injectable formulations

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Chapter 1

General introduction and thesis outline

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Since the early encounters of humans with snakebites, it was obvious that the parenteral route is a very pronounced and probably efficient way of drug administration. This observation and the use of poisoned arrows by ancient tribes were followed by the experiments of Sir Christopher Wren in the 17th century. With the use of a bladder and quill for a syringe and needle, he injected wine, ale, opium and other substances into the veins of dogs and studied their effects[1]. Over the following centuries, technological improvements and increasing knowledge of physiology and pharmacy resulted in the development of injections and infusions as routine medical practices in hospitals.

Direct injection and infusion into the blood provide rapid action compared with other routes of administration where the drug has first to be absorbed. These methods allow for achievement of optimum levels of the drug in the blood accurately and immediately. Possibility of application of injections in case of unconsciousness or uncooperative state of the patient is another advantage of these methods. Additionally, drugs that undergo inactivation after oral administration are better off through injection or infusion. The latter is especially the case for therapeutic proteins and peptides, which are vulnerable to proteolytic degradation in the gastro-intestinal tract, besides being poorly absorbed.

Although the highly efficient protective barriers in other routes of administration (e.g., skin and mucous membranes) are circumvented with injections and infusions, the introduction of microorganisms, impurities and other toxic agents can be an accompanying risk for these methods of delivery. Therefore, parenteral preparations must be as impeccable as possible with respect to purity, freedom from toxicity and contamination. Specifically for therapeutic protein products, sub-visible particles (SVP) have received a lot of attention as impurities in protein formulations[2]. These impurities in therapeutic protein drug products are divided into two well-studied categories: protein aggregates and non-proteinaceous particles originating from packaging materials or excipients[3,4]. Due to importance of the issue, several methods have been developed that allow for quantification and characterization of SVP and visible particles[5].

These methods are also used for characterization of particulate drug delivery systems (DDS) that are by design based on SVP[6]. Many types of particulate DDS are available that differ by the site of drug action and the method of particle delivery (local vs. systemic). The performance of DDS depends on their size, shape and surface characteristics[7,8]. Therefore, characterization of these systems with high accuracy and detailed output is crucial in the formulation development and the overall safety and efficacy of the DDS in the clinic.

More recently, cell therapy products have entered the pharmaceutical arena[9]. In this case the drug substances themselves, *i.e.* the cells, are SVP[10]. As the clinical safety and effectiveness of these products depend on the concentration and viability of cells, it is of utmost importance to have quantification and characterization

methods in place during manufacturing, product distribution and prior to administration. This thesis deals with the characterization of SVP in the above-mentioned types of products. Below, these products are briefly discussed together with the role of analytical techniques used to characterize SVP in these products. The last section of this chapter provides a short description of the aims and outline of this thesis.

Therapeutic protein formulations

The highly specific and complex function of a protein cannot be mimicked by small molecules; therefore, therapeutic proteins have prominent advantages over small molecule drugs in terms of functionality. In principle, a protein's functionality is accompanied by a highly specific action and less adverse effects as compared to small molecules[11]. During the past few decades, protein therapeutics have becomeincreasingly important for the treatment of chronic and life-threatening diseases and conditions. In the year 2013, a total number of 338 monoclonal antibodies, 20 interferons, 93 other recombinant proteins and 250 vaccines were reported to be in various phases of clinical trials[12].

The complex and large structure of these macromolecules, however, make them susceptible to conformational changes in the structure of the protein. These changes may occur during the production of the bulk substance, the formulation, storage, transportation or other treatments that a protein goes through. Conformational changes can among others lead to aggregation of the protein in solution[13]. The presence of aggregated therapeutic protein greatly compromises product quality and potentially drug safety[14]. Presence of aggregates has been linked to reduced drug efficacy, serious adverse effects and even death[15]. One needs to clarify the currently accepted nomenclature for differently sized aggregates. Considering the fact that aggregates are particulate species we extend this nomenclature further into other injectable systems discussed throughout this thesis. Visible particles are classified as particles seen during visual inspection, typically above 100 µm. Particles in the micrometer range that are not detected during visible inspection are called SVP (1 – 100 µm). The nanometer range is divided into the submicrometer range (100 – 1000 nm) and the nanometer range (size-exclusion chromatography high-molecular-weight species) (< 100 nm)[16]. The particles that appear in therapeutic protein products in the SVP range can, in turn, be assigned into three categories: (1) extrinsic particles or contaminants (materials that are not part of the drug product, package and/or process); (2) intrinsic particles (undesirable, non-proteinaceous materials related to the manufacturing, packaging and/or devise itself); and (3) inherent particles (protein aggregates or formulation components). Particulate impurities other than protein aggregates, such as excipients and packing materials, can influence the product quality as well. For instance, sugars of pharmaceutical-grade quality[17] and surfactants (such as polysorbate 20)[18] have been

shown to be the source of particulate impurities in drug products. Another example of intrinsic particulate impurities are silicone oil droplets which leach into the protein solution from the glass barrel and the plunger of prefilled syringes[19]. These droplets can get coated with the protein from the solution and agglomerate to increase turbidity in the protein solution[20,21]. The latter example of the silicone oil droplet induced agglomeration in protein solution highlights the heterogeneous composition of particles in therapeutic protein products[22]. In addition, SVP are often a very heterogeneous population of micron sized aggregates, which in case of protein aggregates can consist of subpopulations of reversible, native aggregates and irreversible, unfolded aggregates[16].

The use of analytical tools to count, size and identify the type of particles in therapeutic protein products is the most crucial component of understanding and controlling the presence of these species. The complexity in composition and subpopulations, as sketched above, brings several analytical challenges to the process. In addition, the choice of the analytical method for characterization of SPV depends on the stage of development, which determines the available amount of the product under consideration. The measurement principle of the techniques used for characterization of protein aggregates, determines the boundaries and limitations of each particular technique. Many of these techniques are able to size and quantify SVP in solution, but not to identify the composition or nature of the particle (e.g., proteinaceous or non-proteinaceous). To overcome analytical limitations and boundaries for characterization of protein aggregates, it makes sense to combine methods with different underlying principles[23].

Controlled release formulations

With respect to DDS we limit our work and discussion in this thesis to controlled release formulations in the SVP range, in particular microspheres. Microspheres are here defined as particulate systems where the drug is dispersed in the matrix of a carrier material. The aim of these drug products lies in the improvement in safety and/or efficacy of an active pharmaceutical ingredient (API). Rate-controlled and targeted release is often used as a tool to avoid toxic levels of an API in plasma while maintaining therapeutic concentrations for prolonged periods of time.

The aim of the STW project number 12144 was to develop a novel process for the coating of dry protein-containing cores with a shell consisting of oppositely charged polyelectrolytes (polymers containing charged or ionizable groups), and subsequently analytical methods to characterize and evaluate the performance of these DDS. Therefore in this thesis, particle characteristics of DDS based on hydrophilic polyelectrolyte complexes and hydrophobic polymers are studied. In polyelectrolyte complexes, the (mainly) electrostatic driven interaction of the polyelectrolyte (polymers containing charged or ionizable groups) with

the drug can lead to formation of controlled release particulate systems[30]. In the category of hydrophobic drug carriers, poly lactic-co-glycolic acid (PLGA) is currently the most studied for small molecules and biologics, with almost 10 marketed drug products[31]. The great advantage of PLGA is its biocompatibility and biodegradability[32] and the ability to modify the hydrophobicity by changing the lactic acid / glycolic acid ratio of the polymer[33].
Particle characteristics are important quality attributes of microparticulate DDS that could affect the clinical performance of the product[37,39]. For instance, the size and shape of the particles affect important quality parameters of product, such as release rate of the drug[37]. Moreover, particle porosity has been reported to influence the loading and kinetics of the drug release[38]. The latter will also be influenced by the extent and strength of interactions between the polymer and the drug.
Different techniques are available for studying shape, surface morphology and porosity of microspheres. Optical microscopy and scanning electron microscopy (SEM) are probably the most employed methods for studying particle shape, size and surface morphology. With respect to the measurement of the average size of microspheres, laser diffraction (LD) techniques are widely used as well. For the measurements of porosity, techniques employing gas adsorption and mercury intrusion are commonly used. In addition, SEM can be used to determine porosity when cryo-cutting techniques are used as a pretreatment for the particle-containing sample.

Cell therapy products

This field of pharmaceutical product development has its roots in human stem cell therapy and tissue and organ transplantations. Two main principles by which cells facilitate therapeutic action are recognized: (1) engraftment, differentiation and long term replacement of damaged tissue[40] and (2) release of cytokines, chemokines and growth factors to facilitate self-healing of an organ or region[41]. Currently a variety of products from multiple cell sources are approved for use[42].
An important quality attribute of cell therapy products (CTP) is the concentration of viable cells, which in general are required to obtain the desired effect. These attributes (together with a purity and surface marker evaluation) are characteristics to be tested during different stages of CTP development. Moreover, in clinical practice the viability of the product needs to be attained before administration[43]. As the manufacturing of CTP is becoming more sophisticated and complex, certain institutions, such as the US-based National Institute of Health (NIH) and National Heart, Lung and Blood Institute (NHLBI) have designed a so-called Production Assistance for Cellular Therapies (PACT) program to support researchers with the manufacturing[44].
From a formulation point of view there is currently very limited knowledge about what additives

to use for improving stability of the therapeutic compound (the cells), except for addition of osmotic agents such as NaCl. Several procedures during the production, transport, storage and even administration to the patient can potentially harm the cells and trigger cell death[45,46]. Therefore, stability, consistency and comparability tests are performed to ensure that product potency is preserved under different circumstances encountered from production up to bedside administration[43]. Many different cell assay analysis methods exist for cell viability determination. Nevertheless, the greatest challenge in CTP development is the inability to reliably characterize critical cell attributes. National Institute of Standards & Technology (NIST) has published an article on a number of strategies that could be used to ensure measurement confidence[47]. Techniques employed for cell counting and viability determination vary in the nature of the test sample (e.g., starting material, in-process sample and final drug product) as well as in the required performance of the test. An assessment of viability without counting will be likely of little use and therefore a single method that provides both parameters will in most situations be the most efficient solution[47].

Methods to count cells mostly depend on the ability of the method to distinguish a cell from other particulates, such as visualization (e.g., microscopy), light scattering (e.g., flow cytometry) and electrical impedance. For measuring the concentration of viable cells, labeling with a fluorescent dye is normally required. Trypan blue[48] (passes the membrane of dead cells) and propidium iodide (passes ruptured cell membranes and becomes fluorescent upon binding nucleic acid[49]) are frequently used dyes for this purpose. Two well-known cell viability assays are hemocytometry and flow cytometry. Hemocytometry is a very fast method to determine the total cell concentration and percent of (viable) cells in a sample that is spiked with a dye under a microscope[48]. In a flow cytometer single cells from a cell suspension pass through the designated fluorescence and light scattering detectors. A scatter plot of the scattering signal (which is related to the type and size of the cell) and fluorescence signal (representing the viability) is plotted, and with that the percentage of viable cells can be derived. Flow cytometry-based methods can be very accurate and reproducible; however, determination of the cell concentration is not easily attained. Therefore, the search for new techniques and methods for qualitative and quantitative analysis of CTP may contribute to improved quality control of this emerging group of pharmaceutical products.

Aim and outline of this thesis

The aim of this thesis is to explore novel applications and capabilities of a number of particle analysis techniques to characterize complex injectable formulations, including (aggregated) protein solutions, protein-polyelectrolyte complexes, PLGA microspheres and cells. The outcome of our research should lead to (further)

application of these tools for characterization of complex injectable formulations and therewith improve the quality of pharmaceutical products used in modern healthcare. The research described in Chapter 2 concerns an investigation of the cause of stirring induced protein aggregation, in order to unravel the mechanism behind this well-known mechanical source of particle formation. To this end, size-exclusion chromatography, nanoparticle tracking analysis and Micro-Flow Imaging (MFI; a flow imaging microscope) were used. In Chapter 3, the same combination of analytical techniques is utilized to study the kinetics of the formation and growth of protein-polyelectrolyte complexes driven by electrostatic interactions.

In Chapter 4 and 5 novel applications of flow imaging microscopy techniques for the characterization of PLGA microspheres are introduced. The focus of Chapter 4 lies in investigating the ability of FlowCAM (a flow imaging microscope) to be used as a tool to analyze the sedimentation behavior of these particles, in order to deduce their porosity. In Chapter 5 MFI is used to measure the total volume of microspheres in a suspension with known microsphere concentration in order to determine the batch porosity of PLGA microspheres.

In Chapter 6 MFI- and FlowCAM-based label-free methods are presented for counting and assessing the viability of B-lineage acute lymphatic leukemia cells. Chapter 7 summarizes the main findings and conclusions of the work described in this thesis. In addition, the future of particle analysis techniques in the field of pharmaceutical formulation development is discussed.

References

1. Feldmann H 2000. History of injection. Laryngorhinootologie 79(4):7.

2. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJ, Middaugh CR, Winter G, Fan YX, Kirshner S, Verthelyi D, Kozlowski S, Clouse KA, Swann PG, Rosenberg A, Cherney B 2009. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. Journal of pharmaceutical sciences 98(4):1201-1205.

3. Carpenter J, Cherney B, Lubinecki A, Ma S, Marszal E, Mire-Sluis A, Nikolai T, Novak J, Ragheb J, Simak J 2010. Meeting report on protein particles and immunogenicity of therapeutic proteins: filling in the gaps in risk evaluation and mitigation. Biologicals 38(5):602-611.

4. Singh SK, Afonina N, Awwad M, Bechtold-Peters K, Blue JT, Chou D, Cromwell M, Krause HJ, Mahler HC, Meyer BK, Narhi L, Nesta DP, Spitznagel T 2010. An Industry Perspective on the Monitoring of Subvisible Particles as a Quality Attribute for Protein Therapeutics. Journal of pharmaceutical sciences 99(8):3302-3321.

5. Zölls S, Tantipolphan R, Wiggenhorn M, Winter G, Jiskoot W, Friess W, Hawe A 2012. Particles in therapeutic protein formulations, Part 1: overview of analytical methods. Journal of

pharmaceutical sciences 101(3):914-935.
6. Kohane DS 2007. Microparticles and nanoparticles for drug delivery. Biotechnol Bioeng 96(2):203-209.
7. He Y, Park K 2016. Effects of the Microparticle Shape on Cellular Uptake. Molecular pharmaceutics 13(7):2164-2171.
8. Patino T, Soriano J, Barrios L, Ibanez E, Nogues C 2015. Surface modification of microparticles causes differential uptake responses in normal and tumoral human breast epithelial cells. Sci Rep 5:11371.
9. Mason C, Brindley DA, Culme-Seymour EJ, Davie NL 2011. Cell therapy industry: billion dollar global business with unlimited potential. Regen Med 6(3):265-272.
10. Mount NM, Ward SJ, Kefalas P, Hyllner J 2015. Cell-based therapy technology classifications and translational challenges. Philos Trans R Soc Lond B Biol Sci 370(1680):20150017.
11. Leader B, Baca QJ, Golan DE 2008. Protein therapeutics: a summary and pharmacological classification. Nature reviews Drug discovery 7(1):21-39.
12. PhRMA A. 2013. Biologic Medicines in Development. ed., Washington DC: PhRMA. p 8.
13. Frokjaer S, Otzen DE 2005. Protein drug stability: a formulation challenge. Nature reviews Drug discovery 4(4):298-306.
14. Shekunov BY, Chattopadhyay P, Tong HH, Chow AH 2007. Particle size analysis in pharmaceutics: principles, methods and applications. Pharmaceutical research 24(2):203-227.
15. Services UDoHaH. 2009. Q4B Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions Annex 3 Test for Particulate Contamination: Subvisible Particles General Chapter. In Administration FaD, editor, ed., Rockville: USDHHS.
16. Narhi LO, Schmit J, Bechtold-Peters K, Sharma D 2012. Classification of protein aggregates. Journal of pharmaceutical sciences 101(2):493-498.
17. Weinbuch D, Cheung JK, Ketelaars J, Filipe V, Hawe A, den Engelsman J, Jiskoot W 2015. Nanoparticulate Impurities in Pharmaceutical-Grade Sugars and their Interference with Light Scattering-Based Analysis of Protein Formulations. Pharmaceutical research 32(7):2419-2427.
18. Tomlinson A, Demeule B, Lin B, Yadav S 2015. Polysorbate 20 Degradation in Biopharmaceutical Formulations: Quantification of Free Fatty Acids, Characterization of Particulates, and Insights into the Degradation Mechanism. Molecular pharmaceutics 12(11):3805-3815.
19. Chantelau E, Berger M, Bohlken B 1986. Silicone oil released from disposable insulin syringes. Diabetes Care 9(6):672-673.
20. Jones LS, Kaufmann A, Middaugh CR 2005. Silicone oil induced aggregation of proteins. Journal of pharmaceutical sciences 94(4):918-927.
21. Thirumangalathu R, Krishnan S, Ricci MS, Brems DN, Randolph TW, Carpenter JF 2009. Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution.

Journal of pharmaceutical sciences 98(9):3167-3181.
22. Ripple DC, Dimitrova MN 2012. Protein particles: What we know and what we do not know. Journal of pharmaceutical sciences 101(10):3568-3579.
23. Filipe V, Hawe A, Carpenter JF, Jiskoot W 2013. Analytical approaches to assess the degradation of therapeutic proteins. Trac-Trend Anal Chem 49:118-125.
24. Cao S, Jiang, Y., Narhi, L. 2010. A light obscuration method specific for quantifying subvisible particles in protein therapeutics. Pharm Forum 36(3).
25. Werk T, Volkin DB, Mahler HC 2014. Effect of solution properties on the counting and sizing of subvisible particle standards as measured by light obscuration and digital imaging methods. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 53:95-108.
26. Weinbuch D, Zölls S, Wiggenhorn M, Friess W, Winter G, Jiskoot W, Hawe A 2013. Micro-flow imaging and resonant mass measurement (Archimedes)--complementary methods to quantitatively differentiate protein particles and silicone oil droplets. Journal of pharmaceutical sciences 102(7):2152-2165.
27. Narhi LO, Jiang Y, Cao S, Benedek K, Shnek D 2009. A critical review of analytical methods for subvisible and visible particles. Curr Pharm Biotechnol 10(4):373-381.
28. Corvari V, Narhi LO, Spitznagel TM, Afonina N, Cao S, Cash P, Cecchini I, DeFelippis MR, Garidel P, Herre A, Koulov AV, Lubiniecki T, Mahler HC, Mangiagalli P, Nesta D, Perez-Ramirez B, Polozova A, Rossi M, Schmidt R, Simler R, Singh S, Weiskopf A, Wuchner K 2015. Subvisible (2-100 mum) particle analysis during biotherapeutic drug product development: Part 2, experience with the application of subvisible particle analysis. Biologicals 43(6):457-473.
29. Kliche W, Herre, A., Garidel, P. 2012. Microscopic methods for particle characterisation in protein pharmaceuticals Analysis of aggregates and particles in protein pharmaceuticals, ed., New Jersey: John Wiley & Sons.
30. Tong WJ, Song XX, Gao CY 2012. Layer-by-layer assembly of microcapsules and their biomedical applications. Chem Soc Rev 41(18):6103-6124.
31. Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM 2008. Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. J Control Release 125(3):193-209.
32. Freiberg S, Zhu XX 2004. Polymer microspheres for controlled drug release. International journal of pharmaceutics 282(1-2):1-18.
33. Anderson JM, Shive MS 2012. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliver Rev 64:72-82.
34. Patil SD, Burgess, D.J. 2010. Pharmaceutical development of modified-release parenteral dosage forms using bioequivalence (BE), Quality by Design (QbD), and In Vitro In Vivo Correlation (IVIVC) principles. In Shargel L, Kanfer, I., editor Generic drug product development speciality

dosage forms, ed., New York: Informa Healthcare USA, Inc. p 26.
35. Zidan AS, Habib MJ, Khan MA 2008. Process analytical technology: nondestructive evaluation of cyclosporine A and phospholipid solid dispersions by near infrared spectroscopy and imaging. Journal of pharmaceutical sciences 97(8):3388-3399.
36. idan AS, Sammour OA, Hammad MA, Megrab NA, Habib MJ, Khan MA 2008. Process analytical technology: non-destructive assessment of anastrozole entrapment within PLGA microparticles by near infrared spectroscopy and chemical imaging. J Microencapsul 25(3):145-153.
37. Kumar R, Palmieri MJ 2010. Points to Consider when Establishing Drug Product Specifications for Parenteral Microspheres. Aaps Journal 12(1):27-32.
38. Klose D, Siepmann F, Elkharraz K, Krenzlin S, Siepmann J 2006. How porosity and size affect the drug release mechanisms from PLIGA-based microparticles. International journal of pharmaceutics 314(2):198-206.
39. Martinez M, Rathbone M, Burgess D, Huynh M 2008. In vitro and in vivo considerations associated with parenteral sustained release products: a review based upon information presented and points expressed at the 2007 Controlled Release Society Annual Meeting. J Control Release 129(2):79-87.
40. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK, Goodell MA 2001. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 107(11):1395-1402.
41. Yagi H, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N, Yarmush ML 2010. Mesenchymal stem cells: Mechanisms of immunomodulation and homing. Cell transplantation 19(6):667-679.
42. Administration UFaD. 2016. Marketed cellular, tissue and gene therapy products. ed.: US Department of Health and Human Services.
43. Stroncek DF, Jin P, Ren J, Feng J, Castiello L, Civini S, Wang E, Marincola FM, Sabatino M 2010. Quality assessment of cellular therapies: the emerging role of molecular assays. Korean J Hematol 45(1):14-22.
44. Lindblad RW, Ibenana L, Wagner JE, McKenna DH, Hei DJ, Hematti P, Couture LA, Silberstein LE, Armant M, Rooney CM, Gee AP, Welniak LA, Mondoro TH, Wood DA, Styers D 2015. Cell therapy product administration and safety: data capture and analysis from the Production Assistance for Cellular Therapies (PACT) program. Transfusion 55(3):674-679.
45. Moviglia GA, Vina RF, Brizuela JA, Saslavsky J, Vrsalovic F, Varela G, Bastos F, Farina P, Etchegaray G, Barbieri M, Martinez G, Picasso F, Schmidt Y, Brizuela P, Gaeta CA, Costanzo H, Brandolino MM, Merino S, Pes ME, Veloso MJ, Rugilo C, Tamer I, Shuster GS 2006. Combined protocol of cell therapy for chronic spinal cord injury. Report on the electrical and functional recovery of two patients. Cytotherapy 8(3):202-209.

46. Leverett LB, Hellums JD, Alfrey CP, Lynch EC 1972. Red blood cell damage by shear stress. Biophysical journal 12(3):257-273.
47. Simon CG, Lin-Gibson S, Elliott JT, Sarkar S, Plant AL 2016. Strategies for Achieving Measurement Assurance for Cell Therapy Products. Stem Cell Transl Med 5(6):705-708.
48. Strober W 2001. Trypan blue exclusion test of cell viability. Current protocols in immunology / edited by John E Coligan [et al] Appendix 3:Appendix 3B.
49. Riccardi C, Nicoletti I 2006. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nature protocols 1(3):1458-1461.