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Citation

Sediq, A. S. (2017, May 9). *Analysis of sub-visible particles in complex injectable formulations*. Retrieved from <https://hdl.handle.net/1887/49076>

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Title: Analysis of sub-visible particles in complex injectable formulations

Issue Date: 2017-05-09

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 effects¹. 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 formulations². 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 particles⁵.

These methods are also used for characterization of particulate drug delivery systems (DDS) that are by design based on SVP⁶. 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 arena⁹. In this case the drug substances themselves, *i.e.* the cells, are SVP¹⁰. 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 molecules¹¹. During the past few decades, protein therapeutics have become increasingly 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 trials¹².

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 solution¹³. The presence of aggregated therapeutic protein greatly compromises product quality and potentially drug safety¹⁴. Presence of aggregates has been linked to reduced drug efficacy, serious adverse effects and even death¹⁵. 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 device 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 quality¹⁷ 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 syringes¹⁹. 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 products²². 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 aggregates¹⁶.

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 principles²³.

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 systems³⁰. 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 products³¹. The great advantage of PLGA is its biocompatibility and biodegradability³² and the ability to modify the hydrophobicity by changing the lactic acid / glycolic acid ratio of the polymer³³. 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 drug³⁷. Moreover, particle porosity has been reported to influence the loading and kinetics of the drug release³⁸. 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 tissue⁴⁰ and (2) release of cytokines, chemokines and growth factors to facilitate self-healing of an organ or region⁴¹. Currently a variety of products from multiple cell sources are approved for use⁴².

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 administration⁴³. 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 manufacturing⁴⁴.

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 administration⁴³. 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 confidence⁴⁷. 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 solution⁴⁷.

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 blue⁴⁸ (passes the membrane of dead cells) and propidium iodide (passes ruptured cell membranes and becomes fluorescent upon binding nucleic acid⁴⁹) 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 microscope⁴⁸. 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.

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