novel analytical approaches to characterize particles in biopharmaceuticals

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Chapter 1

General introduction and thesis outline

Biopharmaceuticals as a quickly developing and expanding class of medicines

Biopharmaceuticals are a still emerging class of therapeutics, with insulin being the first recombinant human protein product approved in 1982[1]. Their origin dates back to 1890 when Emil Behring and Shibasaburo Kitasato demonstrated on guinea pigs the curing effects of sera from mammals previously exposed to sub-lethal doses of *Clostridium tetani* or *diphtheria toxin*[2]. Despite this early research on serum therapies, translation into successful human trials was difficult to achieve because of the inconsistencies between produced therapeutic sera. The breakthrough came in the mid-1890s, when Paul Ehrlich developed standardized methods for the production of high-quality anti-diphtheria serum in larger animals, which became the first international standard reference preparation[3]. Since then, the advancement in our understanding and technological progress within the fields of medicine and pharmaceutics has allowed for optimization of the purity of protein drug products and for a more detailed characterization of the active pharmaceutical ingredients (APIs).

With the advent of recombinant DNA technologies and emergence of other new technologies, the portfolio of biopharmaceuticals has expanded rapidly during the past few decades. Today the class encompasses a range of therapeutic modalities, such as hormones, cytokines, fusion proteins, monoclonal antibodies (mAbs), antibody-drug conjugates, viral vectors and living cells. Currently, mAbs are the dominant class within biopharmaceuticals with a market value of approximately $115 billion as of 2018[4]. However, the elaborate manufacturing process, high susceptibility to degradation and difficult analytical characterization of biopharmaceuticals result in ongoing concerns with respect to quality, safety and efficacy of these drug products[5,6]. These concerns escalate for advanced therapy medicinal products (ATMPs), which include gene therapy products, cell-based products and tissue-engineered products. The intrinsic complexity of viruses, cells and tissues pose even greater technical challenges in delivering safe and effective medicinal products[7].

Protein-based biopharmaceuticals and their formulation aspects

Therapeutic proteins are usually formulated in a liquid or lyophilized state, most commonly designed for parenteral administration[8]. The therapeutic efficacy of proteins is mostly dependent on their native three-dimensional structure. However, the inherent conformational instability of proteins poses a threat to their therapeutic activity[9]. Protein (partial) unfolding and decline in colloidal stability leads inevitably to aggegation[10]. Aggregation of proteins may follow different types of pathways and lead to the formation of a variety of aggregates, which may differ in several aspects, such as size, morphology and reversibility[10-13]. Formation of protein aggregates can occur during protein expression and purification, formulation and filling, storage, transportation and upon administration to patients[14].

In order to achieve sufficient stability of native protein structures, formulation development is essential during the development of these drug products. The goal of designing a formulation for a drug candidate is to prevent instability, achieve a sufficient shelf-life and make the drug product convenient for administration, e.g., formulating as a subcutaneous instead of an intravenous injeciton[15]. The conformational integrity of protein molecules is maintained by three main stabilizing forces: hydrophobic interactions, electrostatic interactions and hydrogen bonding[16,17]. Each of these factors can be weakened or strengthened by altering the formulation variables, such as pH, ionic strength and inclusion of specific excipients in protein formulations[18,19]. The substances used for stabilization of proteins include, among others, surfactants, buffering agents, amino acids, salts, polyols and sugars. Despite the availability of a large number of chemical compounds from each category, only a few dozens of different substances are usually considered during formulation development[20]. Four groups of excipients frequently used in drug products formulations are briefly described below.

- To successfully control the pH of protein formulations, and other pH dependent physical properties of the solution (e.g., solubility, viscosity and phase separation[21-23]), buffering agents are typically used. The excellent buffering capacity of some amino acids (e.g., histidine) or multivalent salts (e.g., phosphate

salts) make them popular excipients used in mAb-based formulations. Optimization of solution pH according to the specific mAbs formulation increases the stability of the liquid drug product. The highest conformational and colloidal stability of protein is achieved within a narrow range of pH at which the overall surface charges guarantee favorable electrostatic interactions[24-27]. Furthermore, in low pH solutions proteins are more susceptible to cleavage or isomerization, whereas at higher pH values deamidation and oxidation rates of proteins increase and lead to formation of chemical degradants[5,28,29].

- Salts are also frequently included in formulations, e.g., to achieve tonicity, to increase protein stability, and/or as viscosity-reducing agent[30,31]. One of the types of interactions of salts with proteins are long-range protein-protein electrostatic interactions. In a solution where the pH is close to the isoelectric point (pI value) of a protein, the overall surface charge is close to neutral, reducing the electrostatic interactions between molecules. This may be favorable for reducing the viscosity of a protein solution, but also promotes short-range hydrophobic interactions leading to protein unfolding and non-native aggregation[32]. A charged protein state exhibits greater colloidal stability due to the protein-protein electrostatic repulsion. However, addition of salt leads to charge-screening effects upon which hydrophobic intermolecular interactions become enhanced[32,33].
- Nonreducing sugars, such as sucrose and trehalose, are another class of commonly used excipients in protein formulations. The commonality of using these excipients relates to their excellent stabilizing properties in liquid and lyophilized products. Preferential exclusion of sucrose from protein surfaces via hydrogen bonding increases the hydration shell of protein molecules, which in turn promotes a more compact native state and thereby increases their molecular conformational stability[34,35]. However, the quality of sugars must be also considered, as even pharmaceutical-grade sucrose may contain nanoparticulate impurities which have been shown to destabilize mAbs[36-38].

- Surfactants are often included in protein formulations to minimize the damage induced by interfacial stress[39-43]. The most commonly used surfactants in protein formulations are polysorbate 20 (Tween 20) and polysorbate 80 (Tween 80), and to a lesser extent poloxamer 188 (Pluronic F-68). Their relatively low toxicity profile and excellent stabilizing properties at low concentrations contribute to their popularity[44,45]. Polysorbates (PS) are non-ionic amphiphilic molecules comprising a sorbitan (hydrophilic) head group linked to four polyethylene glycol (PEG) chains. Each of these chains is esterified with a fatty acid side chain which vary among different types of polysorbates[46]. However, the complex manufacturing process, challenges associated with purification, and chemical instability of PS result in products of a highly heterogeneous chemical mixture[46]. Surfactants are believed to have a dual stabilizing effect on protein molecules[40,46]. Competitive adsorption to hydrophobic interfaces is believed to be the prevalent stabilization mechanism[39-41]. The other stabilization mechanism is the direct binding of PS to protein molecules[40,41,47]. It has been suggested that PS acts as a chaperone, which can catalyze the correct folding of proteins and shield their hydrophobic patches[47,48]. Despite the excellent stabilizing properties of PS, numerous studies have also reported destabilizing effects of PS degradants[43,49,50]. The main degradation pathways for PS are oxidation and (enzymatic) hydrolysis[51]. The latter yields free fatty acids that can form insoluble hydrophobic particles. Improved characterization methods are currently being developed to achieve greater selectivity for impurities in PS, which would allow for better evaluation of their stability and batch-to-batch variability.

Advancing analytical tools for characterization of particles in biopharmaceuticals

Monitoring the stability of protein-based therapeutics can be performed by a plethora of analytical techniques intended for particle characterization. The hydrodynamic diameter of a mAb monomer is ca. 5 – 12 nm, depending on the hydration shell[52]. However, protein instability leads to physical and chemical changes of the native monomers, resulting in their self-association and the formation of proteinaceous particles[5]. The risks associated with the formation of protein aggregates in therapeutic drug products is not limited to the loss of clinical efficacy. Unwanted immunogenicity is an additional concern, as perturbed protein structures have been shown to be more immunogenic compared to native protein monomers[53-56]. Nano-meter and micro-meter sized protein aggregates are also potentially more immunogenic than the native monomer. However, *in vitro* and *in vivo* immunogenicity studies have so far delivered conflicting results[57-61]. Nevertheless, the presence of aggregated interferon and human growth factor in drug products has been linked with adverse immunological responses in human patients[62-65]. In addition to proteinaceous particles, nonproteinaceous particles originating from excipients, primary packaging material, manufacturing processes and production environment can also pose threats to the integrity of drug products[66]. Silicone oil droplets deriving from the coatings of internal surfaces of pre-filled syringes or rubber particles shed from vial stoppers may increase the kinetics of protein aggregation[67,68], or lead to blockage of small (micro-) blood vessels within the lungs[69,70].

The heterogeneity of particulate impurities in protein-based formulations prompt for their classification in several different ways, e.g., based on size (nano-, micro-meter or larger than 100 µm sized particles) or origin (extrinsic, intrinsic or inherent particles according to USP <1790> and extrinsic or intrinsic according to Ph. Eur.5.17.2)[12,66,71]. Specifications set by pharmacopeial monographs for parenteral biopharmaceuticals are primarily focused on particle sizes and provide acceptable limits of particulates sized above 10 and 25 µm within a specified unit of drug product[72-74]. The commentary from Carpenter et al. in 2009 was one of the first to address the safety concerns arising from sub-visible (1 – 100 µm in size) particles in biopharmaceutical formulations, as well as the challenges associated with

characterization of these impurities[14]. One year later, Demeule et al. published a critical evaluation of three characterization techniques for micro-meter sized particles for characterization of protein aggregates: light obscuration (LO), flow imaging microscopy (FIM) and electric sensing zone (ESZ)[75]. The authors addressed several challenges associated with these techniques, and other research groups further explored limitations of these techniques with respect to protein formulations[66,76-82]. For example, the small difference between the refractive index (RI) of proteinaceous particles and (protein-containing) formulation buffers results in underestimation of recorded particle concentrations when using optical-based techniques. The underestimation is augmented in case of formulations with high protein concentration or presence of sugars, where the refractive index differences between protein aggregates and the matrix are particularly small[78]. Furthermore, the evaluation of statistical significance of measured particle concentrations in samples with low particle load should be performed. Even when considering a well homogenized sample and high precision measurements, experimental data of particle concentrations within single digits determined by using LO comprise high statistical variances[83]. One must also note the variability of determined particle concentration and size between different techniques (or even between different instruments of the same technique[84]) for a single sample[75,85]. Such discrepancies are to be expected due to the different measuring principles of each technique for detection and characterization of particles as well as differences in the size range covered. For instance, LO is known for underestimation of particle concentrations compared to FIM due to different sensitivities of the two techniques[85,86]; and samples with translucent particles will have higher particle counts and sizes reported by ESZ compared to optical-based techniques[81].

Currently, the content of particles within the lower micro-meter and nano-meter size range present in drug products is not regulated. However, regulatory authorities recommend the assessment of these particles within drug products[87]. In comparison to the characterization of micro-meter sized particles, quantification and sizing of particles within the nanometer-size range is associated with many more challenges. Nonetheless, substantial advancements in the fields of microscopy, nanotechnology and microfluidics

have delivered promising techniques for characterization of nano-meter sized particles. Transmission electron microscopy, scanning electron microscopy and atomic force microscopy are powerful techniques for visualization and sizing of particles over a broad size range. However, the low throughput of these methods, their high costs and laborious sample preparation requirements limit their usage for formulation screening and protein aggregate characterization[88]. Light-scattering based techniques are currently the methods of choice for evaluating the formation of nanoparticles in protein-based formulations. Dynamic light scattering (DLS), static light scattering (SLS), laser diffraction (LD) and nanoparticle tracking analysis (NTA) are techniques which utilize the events of particle-light interactions (i.e., Mie scattering, Rayleigh scattering, diffraction) for measurements of particle size and counts. DLS and NTA measure the scattered light of particles under Brownian motion in a low-Reynold number liquid and determine their diffusion coefficients[89-91]. The hydrodynamic diameter of particles can then be calculated from the Stokes-Einstein equation, assuming that the measured particles are of spherical shape and the viscosity, RI and temperature are known[92]. Orthogonal to light scattering-based techniques are resonant mass measurement (RMM) and resistive pulse sensing (RPS). RMM measures the size and concentration of particles passing near a resonating cantilever suspended within a microfluidic system[93,94]. If the density of particles is known, the volumetric diameter can be obtained. Instruments measuring particles by using RPS, operate on the basis of the Coulter principle that was originally employed in cell counters for sizing and quantifying cells using impedance measurements[95-97]. In microfluidic RPS (MRPS), sample material is loaded into chips with microfluidic passages and particles are directed through the orifice of a nanoconstriction. Each passage of particle induces a change in the electric current and its magnitude is directly related to the volumetric diameter of the particle[98].

Robust and accurate characterization of protein drug products requires development and qualification of the applied analytical methods[99]. For example, the particle characterization methods must be proven to reliably detect and/or quantify protein degradants. Thus, forced degradation studies to induce the formation of degradants in protein formulations are essential in the development of biotherapeutics[100,101]. These

studies mimic (exaggerated) real-life conditions to which drug products can be exposed to and usually involve freezing, thawing, thermal stress, mechanical stress, light exposure, oxidative stress or interaction with specific components present in the primary packaging. Evaluation of mechanical stress, such as stirring, pumping or shaking, on protein stability is necessary, as this type of stress is the most common one to which biopharmaceutical products are exposed to during processing and handling[102]. In solution under quiescent storage, monomeric proteins exist in an equilibrium between native folded and unfolded structures[103]. However, the balance can be disrupted upon exposure to interfaces, such as liquid-liquid, air-liquid and solid-liquid[104,105]. Manufacturing and transportation induce a plethora of interfacial stresses, which may cause perturbation of the native protein structure, leading to protein aggregation[106,107]. The main degradants formed in protein formulations exposed to mechanical stress have been shown to be proteinaceous particles[100,108-110]. Consequently, methods for reliable detection of particulate impurities in drug products are required for selection of the most optimal surfactant (concentrations) and evaluation of the stability of protein formulations against these stress conditions.

The increasing number of available characterization techniques and the exponential growth of collected data requires improved data processing and analysis approaches to gain a better understanding of the outcome of performed analytics. Artificial intelligence and its subfield machine learning are becoming integrated in the biopharmaceutical field for evaluation of the large quantities of generated data[111]. The main objective of machine learning is to discover patterns and trends in collected data in order to obtain relationships between variables and set predictions. Depending on the needs and available data, machine learning models can be based on linear regression algorithms or utilize deep convolutional neural networks (CNNs). Logistic regression and decision tree models require modest processing power to perform predictions or weighed selections. In contrast, application of high-performance processor cores (i.e., graphics processing units [GPUs]) becomes necessary when parsing data with CNNs. These models consist of sophisticated architectures with multiple numbers of hidden layers and interconnected nodes for extraction of features from highly structured data. The teaching of machine learning algorithms can be done via two different approaches: unsupervised or supervised

learning. In unsupervised machine learning, principal component analysis (PCA) is commonly used for data clustering and segregation of different patterns[112]. Simplification of high-dimensional data by feature elimination and extraction, while retaining trends and patterns, allows for projection of correlations between certain variables, such as particle morphology, Raman spectrum and polymorphic states of a compound[113]. On the contrary, the supervised learning approach requires knowledge on the input fed into the model for learning. Labelled datasets must be provided to the model for training during which specific relationships between the input and output data are being recognized. For the testing of the model, new (i.e., not used during training) unlabeled data is inputted and the predictions (results) are made based on the previous learning process. Models based on artificial neural networks (ANNs) are particularly favored for the supervised learning approach. These networks consist of a group of interconnected nodes in which algorithms apply non-linear transformations to learn specific features or patterns in the received data[114]. To further improve model performance, ANNs were developed into CNNs in which the input and output layers are connected to multiple locally linked hidden layers[111]. The connectivity and distribution of units within each layer allows for generating simple local features and hierarchically combining them into complex high dimensional objects. The intricacy of CNNs makes them extremely useful for image classification where extraction of complex patterns allows for, e.g., identification of proteinaceous particles formed by different types of stress factors[115,116]. Similarly, CNNs were used for discrimination between types of particles, such as silicone oil droplets and protein aggregates[117].

Cell-based medicinal products and new challenges in formulation and analytical characterization

Cell-based medicinal products (CBMPs) are therapeutics that are rapidly gaining importance in the treatment of chronic and life-threatening diseases, for which often no other treatment options are available. Several CBMPs have reached the market and a few thousand CBMPs are currently in clinical development[118]. Cell therapy products involve somatic cells of autologous (patient) or of allogenic (healthy donors) origin. The APIs of these products are (mainly) living cells which have been submitted to substantial

manipulation to achieve the desired therapeutic effect[119]. Irrespective of the class of therapeutics cells, manufacturing and administration of these products are associated with multiple processing steps that may include the addition/removal of various raw materials, several handling steps, cryopreservation and transportation. Cells as living units respond to their local environment and interact with each other[120]. The sensitivity of cells to external stimuli (stress factors) makes them uniquely fragile and susceptible to abrupt death (necrosis), resulting in a release of intracellular components and formation of debris particles. Formulation development for CBMPs may help to achieve maximal stability and efficacy of these therapeutics. Several of the current common additives used for stabilization of cells, such as dimethylsufoxide (DMSO) and human serum albumin (HSA), have multiple drawbacks and examining alternative excipients is required[121]. The complexity of CBMPs combined with the intricate features of living and dead cells pose greater challenges for analytical characterization compared to protein-based products. Therefore, gaining better understanding of the critical quality attributes of CBMPs, and the development of robust, low-volume and high-throughput analytical methods is essential in order to achieve safe, effective and high-quality formulations.

Subvisible particle analysis of CBMPs is very challenging because of the presence of cells in the size range of interest. Nonetheless, particulate impurities, whether process- or cell-derived, remain a concern and should be accurately characterized[118,122]. The concern is amplified by the fact that only large-pore filters (≥ 70 µm pore size) can be used during manufacturing to remove particles (if present). Process and product particle impurities and contaminants within the micro-meter size range, cannot be removed by filtration without compromising quality and potentially efficacy of the product. Consequently, control measures must be in place to prevent or minimize particulate contamination from sources like instruments, raw materials, processes, environment and people[123]. Verification of the particle load in CBMPs from a quality (e.g., to demonstrate batch-to-batch consistency and stability), safety and potentially efficacy perspective is prudent[118]. This involves micrometer-size particle characterization not only with respect to the cell concentration and cell viability, but also potential particulate impurities. FIM is emerging as an attractive tool for

characterization of particles in cell suspensions, owing to its capability of generating high-resolution images and processing samples with a high particle load[124].

Aims and outline of the thesis

The objective of this thesis is to investigate novel analytical approaches for the characterization of particulates in biopharmaceutical products, in particular therapeutic proteins and CBMPs. **Chapter 2** focuses on the comparison of the novel MRPS against the three other more established nanoparticle characterization techniques for biopharmaceutical product characterization, namely RMM, NTA and DLS. It includes an assessment of the applicability of each of these techniques and describes their advantages and limitations. **Chapter 3** continues to focus on nanoparticle characterization techniques, specifically tunable RPS and MRPS, which require a minimum electrical conductivity of the samples. The colloidal stability of a mAb formulation is assessed upon spiking histidine and sodium chloride. **Chapter 4** explores the *in vitro* immunogenicity of nanoparticulate impurities found in pharmaceutical-grade sucrose. **Chapter 5** describes the implementation of shaking, free-fall and syringe pump stress methods for the evaluation of mAbs stability and particle formation. Moreover, the performance of two grades of polysorbate 80 is compared with respect to their stabilizing properties towards a model mAb upon mechanical stress. Chapter 6 and 7 describe the characterization of particulate impurities in CBMPs. In **Chapter 6** a novel method, based on FIM and machine learning, was introduced to detect and quantify antibody-coated magnetic beads (Dynabeads) within cell suspensions. In **Chapter 7** the developed FIM method was used alongside other cell characterization techniques for the assessment of cell stability after exposure to different thawing temperatures, freeze-thawing and shaking stress. **Chapter 8** summarizes the work performed in this thesis and gives an outlook on further potential developments in the field of particle analysis within protein- and cell-based medicinal products.

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