novel analytical approaches to characterize particles in biopharmaceuticals

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Chapter 8

Summary, conclusions and perspectives

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The establishment of analytical methods for characterization of particles in biopharmaceutical products is of critical importance during drug product development. Early assessment of particulate impurities and degradation pathways in protein-based formulations during (accelerated) storage and forced degradation conditions can decrease the chances of clinical failures at later stages of development and support the quality assessment of drug products. Furthermore, cell-based medicinal products (CBMPs), an emerging class of therapeutics, are comprised of heterogeneous mixtures of particulates and require analytical methods with advanced data processing approaches for comprehensive product characterization.

In **Chapter 2**, we investigated the advantages and limitations of a recently developed nanoparticle characterization technique – microfluidic resistive pulse sensing (MRPS). The single particle analysis technique was compared to other more established particle characterization techniques, including dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and resonant mass measurement (RMM), with respect to particle sizing and counting. To this end, samples comprising polystyrene beads, liposomes, bacteria and protein aggregates were measured. MRPS was shown to have the widest measurable concentration range amongst all tested techniques. This would make MRPS superior in quantification of drug products with high particle loads, such as virus- or liposomal-based products, as no dilution of the sample is required. MRPS, RMM and NTA are single particle counting techniques, thus their sizing resolution of samples with heterogeneous particle populations is superior to that of DLS. More in-depth examination of size resolution revealed that MRPS slightly outperforms RMM, whereas NTA demonstrated the poorest capabilities in resolving two distinct particle populations. However, the requirement of a relatively high electrical conductivity for samples measured by using MRPS can be a limitation of the technique, as shown in the next chapter.

Chapter 3 describes a follow-up study on nanoparticle characterization techniques for the analysis of proteinaceous particles within a mAb formulation. In particular, the impact of spiking electrolytes into mAb formulations was investigated. Measurements with tunable

resistive pulse sensing (TRPS) and MRPS require samples to have sufficient electrical conductivity. For commonly used protein formulations with low electrical conductivity, samples must be spiked with electrolytes prior to analysis. However, by using RMM and NTA we found a substantial increase in nano-meter sized particles in heat stressed monoclonal antibody (mAb) formulations that were spiked with sodium chloride or histidine. Such a change upon spiking was not observed for unstressed protein samples. Therefore, the impact of adding electrolytes to protein formulations for resistive pulse sensing measurements requires prior assessment with respect to the stability of protein monomers and aggregates.

In **Chapter 4,** we investigated the immunogenicity of previously discovered nanoparticle impurities (NPIs) present in sugars of pharmaceutical grade[1,2]. NPIs isolated from pharmaceutical-grade sucrose, a commonly used formulation excipient, were found to contain β-glucans that can act as adjuvants in the presence of immunogenic agents (e.g., protein aggregates). In contrast to previous studies, trastuzumab formulations incubated for several hours in presence of NPIs showed only a minor increase in the concentration of nano-meter sized particles and no substantial increase in the levels of micro-meter sized particles. NPIs alone at a high concentration (10^{10} p/ml) or in the presence of trastuzumab at 1 mg/ml did not activate monocyte-derived dendritic cells (moDC). These results suggest that, in contrast to our expectations, NPIs in the presence or absence of protein are not immunogenic in the tested *in vitro* model.

Chapter 5 focused on the role of the grade polysorbate 80 (PS80), another important excipient, on its protein-stabilizing effect during mechanical stress. Exposure of protein formulations to mechanical stress is known to lead to particle formation[3,4]. Two grades of PS80, i.e., specified by the United States Pharmacopeia (USP) and the Chinese Pharmacopeia (ChP), were tested for their stabilizing properties towards a model mAb under three mechanical stress conditions: shaking, free-fall and pumping. UV spectroscopy, DLS, backgrounded membrane imaging (BMI) and flow imaging microscopy (FIM) were used for assessing the stability of the mAb under these conditions. Despite the higher purity of ChP PS80 (content of oleic acids >98%) compared to USP PS80 (content of

oleic acids 50-60%), no clear differences in terms of functionality were observed between the two grades. Taking into consideration the lower chemical stability of ChP PS80 compared to USP PS80[5], high purity grade polysorbate may not provide greater stability towards mAbs upon long-term storage.

The focus of the studies described in **chapters 6 and 7** was the analytical characterization of CBMPs. The research aimed to expand the currently limited analytical toolbox for the characterization of cell suspensions. In **chapter 6,** we developed a method based on FIM assisted with convolutional neural networks (FIM-CNN) for image analysis to detect and quantify particulate impurities in CBMPs. The focus was set on the identification of Dynabeads, antibody coated magnetic beads used for cell activation, in the presence of Jurkat cells at a concentration up to 500,000 cells per ml. The standard morphological parameters obtained from the instrument's software were not sufficient for discrimination between Dynabeads, cells, cellular debris and adducts (Dynabeads attached to cells). Thus, machine learning for image classification was implemented in this study. By using FIM-CNN, the error rate in classification of Dynabeads dropped by 50-fold compared to using the standard morphological parameter approach. Furthermore, a limit of quantification (LOQ) was determined at 50,000 beads/ml with a high recovery and low variability in concentration determination above the LOQ. The work performed in chapter 6 paved the way to study the stability of Jurkat cell suspensions submitted to forced degradation conditions. **Chapter 7** described the characterization of cell suspensions submitted to different stress conditions: thawing at various temperatures, freeze-thawing and shaking. Analysis of cells was performed by using the newly developed FIM-CNN alongside more established fluorescence-based cell characterization techniques. FIM-CNN was applied to determine the concentration of cells (viable and necrotic) and debris particles. The viability results obtained from FIM-CNN compared well to the read-outs of calcein-AM and propidium iodide assays. Thawing of frozen cell aliquots at low temperature (5 ^{o}C) showed to be detrimental to cell viability and count, compared to thawing at 20 and 37 ^{o}C. Furthermore, cells were formulated with different DMSO concentrations (0 - 10% [v/v]) and submitted to one freeze-thaw cycle (-20 ^{o}C – 37 ^{o}C). The lowest DMSO concentration tested (1% [v/v]) showed no protective effects upon freeze-

thawing, whereas the best cell-stabilizing properties of DMSO were achieved at 5% (v/v). Horizontal shaking of cell suspensions did not affect the cell viability at the tested conditions, but rather led to a substantial decrease in total cell counts. The drop in cell concentration after shaking stress was mitigated by addition of FBS (10% [v/v]) to the cell suspension. Our findings show the usefulness of the three types of forced degradation studies for CBMP formulation studies, as well as the importance of including orthogonal analytical techniques for cell characterization.

Perspectives

Characterization of particles within the nano- and micro-meter size range is an important aspect in the development of novel biopharmaceutical drug products. The work in this thesis aimed to strengthen the knowledge on current analytical techniques and methods used for the assessment of particle populations in protein- and cell-based formulations. New approaches utilizing artificial intelligence were introduced, which in future studies should be further developed in order to maximize the information obtained from data produced by particle characterization methods. Other objectives of upcoming research should attempt to further increase the fundamental understanding of the quality of current and prospective excipients in relation to their functionality in drug products.

Particulates in drug products

One of the main concerns with respect to the quality and stability of biopharmaceuticals is the presence of particulate impurities in drug products. The industry is currently showing great interest in the identification and characterization of impurities in raw materials in order to improve their quality through better manufacturing and purification processes. For example, Merck has recently released an improved multi-compendial grade of sucrose (Emprove Expert) that is low in nanoparticle content. The eliminated nanoparticulate impurities from sucrose have been shown to destabilize mAbs[1,2]. However, for excipients of a more complex chemical nature than sucrose, higher purity may not always translate into better performance and stability. Manufacturers of surfactants are keen to improve the quality of their products by producing polysorbate 20 (PS20) and polysorbate 80

(PS80) of higher purity grades. Unexpectedly, the increased content of lauric acid and oleic acid in PS20 and PS80, respectively, has shown to considerably exacerbate the risk of oxidative degradation[5]. The instability of polysorbates in protein formulations may result in the formation of insoluble fatty acid particles which pose a threat to protein stability[6]. Furthermore, as demonstrated in this thesis, the functionality of PS80 in mAbs formulations did not improve with increased content of oleic acid.

In recent years, a manifold of new structural formats of therapeutic antibodies, as well as other proteins, nucleic acids, viruses and cells have been designed to exhibit potential therapeutic effects[7-10]. In contrast, the list of excipients used to maintain the stability and efficacy of drug products remained relatively constant. Out of the vast number of chemical entities available, only 57 are used as excipients in marketed biopharmaceutical products[11]. With the support from academia, industry and regulatory authorities, further research should look for novel excipients that are able to better stabilize biopharmaceuticals and/or are more stable themselves. In any case, the quality and stability of excipients should be carefully evaluated in future work where new and/or commonly used stabilizing agents are incorporated into formulations.

Particles in drug products can also serve a positive role and act as the active pharmaceutical ingredient (API), a drug delivery vehicle or a (viral) vector for gene therapies. For example, the recently approved mRNA-based COVID-19 vaccines use lipid nanoparticles (LNPs) to deliver the nucleic acids into the cells where the expression of the encoded virus spike protein can take place[12]. The efficacy of mRNA-LNP vaccines is heavily dependent on the composition, size distribution and quantity of the nanoparticle population[13]. The same holds true for gene therapy products utilizing recombinant viral vectors for achieving the desired therapeutic effect. For instance, unwanted immunotoxicity and altered biodistribution resulting in inconsistent *in vivo* functionality can result from instability of virus particles and formation of aggregates[14]. Furthermore, the cells in CBMPs are micro-meter sized API particles. Demonstrating the integrity and concentration of these particles requires robust and accurate particle (cell) characterization techniques.

Particle characterization techniques

Characterization of particles in biopharmaceutical products has been an active field of research in the past decade[15-19]. Despite the significant advancements made in technologies used for the detection, sizing, counting and characterization of particles in the nano- and micro-meter size range, several challenges remain.

Techniques based on particle-light interactions play a major role in the detection and analysis of nanoparticles. Examples include dynamic and static light scattering (DLS and SLS, respectively), and nanoparticle tracking analysis (NTA). These techniques can be used for early detection of the onset of protein aggregation and particle formation[20-22]. NTA has additionally been validated by several research groups for the sizing of polydisperse nanoparticles, showing high accuracy and precision in the determination of the mean size for distinct particle populations[23-25]. However, not all samples may be suitable for these techniques; high background noise arising from formulation components can disturb the analysis and the presence of larger particles scattering more light will overshadow particles of smaller size. Alternative techniques have been developed to cover the "sub-micron gap" and overcome some of the challenges encountered with light scattering-based techniques. Examples include resonant mass measurement (RMM) and resistive pulse sensing (RPS), both of which provide some advantages with respect to sizing resolution and elimination of artefacts arising from light scattering events. Nonetheless, the microfluidic systems employed within these techniques require laborious cleaning procedures or can result in blockages, which compromises sample throughput. Furthermore, the precision with respect to quantification of nanoparticles remains low, especially for inter-laboratory experiments (Benkstein et al., in preparation). The reason for the imprecise quantification is related to the minuscule volume analyzed per measurement. For example, a single measurement performed by NTA processes ca. 0.08 nl of sample, and RMM or RPS can process up to several hundred nanolitres. Given the absolute concentration is provided in particles per millilitre, the extremely high extrapolation factors lead to high deviations in cases where small differences in particle counts are measured. Technical improvements in design to increase the volume of analyte

measured would increase the reliability and robustness of nanoparticle characterization techniques.

Several orthogonal techniques for characterization of micro-sized particles were developed within the past few years and include holographic video microscopy (HVM)[26], backgrounded membrane imaging (BMI)[27] and imaging flow cytometry (IFC)[28] . One advantage of HVM and BMI, compared to light-based techniques, is that results are unaffected by the differences in refractive indices between measured particles and formulation buffer. Both techniques can therefore be considered for measurements of formulations with high protein or sugar concentrations. In addition, BMI results should not be affected by air bubbles or silicone oil droplets, because these will pass through the porous membrane. BMI and IFC techniques include fluorescence detection systems offering chemical identification of particles based on the selectivity of fluorescent probes used for staining particles of interest. The currently established light obscuration and flow imaging microscopy (FIM) techniques have been compared and critically evaluated with respect to size and concentration determination by several research groups[29-36]. More recently, advanced computational methods, such as machine learning (ML), have been utilized in processing images derived from FIM[19,37-39]. In this way, the limitations of standard particle classification approaches, which are based on morphological particle parameters derived from the instrument´s software, were overcome[40,41]. The intrinsic morphological features extracted from bright-field images by using machine learning allowed for recognition of minute differences in particle morphologies. Thereby, discrimination of particles highly similar in appearance, but of different origins, was possible to achieve. Integration of machine learning for image segmentation in a newly developed FIM instrument was also suggested by Krause et al.[42]. An oil-immersed objective embodied into the FIM device allows for detection of particles in the sub-micron and low micron range (0.3 – 10 µm). The current limitations of this method include low image contrast of particles and light-scattering, both of which can be potentially resolved by application of more sophisticated thresholding algorithms. Advancements in computer vision and image processing algorithms should be applied in future work on characterization of particles in the nano- and micro-meter size range in order to derive

more information from captured images as well as from other parameters (e.g., fluorescence data).

Development of cell-based medicinal products (CBMPs)

The current preliminary stage of cell therapy approaches as well as the limited number of analytical techniques applied for the characterization of CBMPs makes this field highly attractive for future research. Application of new analytical techniques, such as FIM, for the characterization of cells and particulate impurities in cell suspensions is necessary to help ensuring the quality of CBMPs[43]. Some of the analytical methods being examined for cell characterization have been originally developed for small molecules or protein-based therapeutics. Therefore, applying them to cells may not be straightforward and multi-analytical based approaches may be required for accurate product characterization[19,44-46]. In contrast to mAbs and other therapeutic proteins, therapeutic cells exhibit high levels of heterogeneity originating from patient/donor, harvesting and processing methods and storage/transportation conditions[47-49]. Therefore, repeatable and robust analytical methods are essential for defining quality attributes and decision making at all stages of a CBMP´s life cycle. On the one hand, especially for autologous products, for which the amount of material usually is very limited and the time available for testing is short, it is crucial to develop analytical methods that are fast and require very low sample volumes. On the other hand, comprehensive analysis of CBMPs requires the assessment of numerous attributes such as sterility, cellular or process impurities, cell viability, cell concentration, potency, and functionality. For this reason, the applicability of statistical methods should be explored in experiment design and data interpretation, where the impact of multiple parameters can be individually assessed and correlated with the product's CQAs[50].

Present formulations of CBMPs are at an early stage of development and are usually limited to several isotonic multi-electrolyte solutions with few types of cryoprotectant agents (CPAs)[51]. Currently the most widely used CPA with best cell stabilizing properties upon freezing is dimethyl sulfoxide (DMSO). However, alternative plausible CPAs are needed, as DMSO at the used concentrations is toxic to cells in a non-frozen state and has

the potential to cause adverse effects in patients[52]. Thus, further research is required in this arena in order to understand cell-excipient interactions, demonstrate cell-stabilizing properties of novel (preferably not of human or animal origin) excipients and recognize degradation pathways occurring at different stress conditions. An important consideration is that the active ingredients in CBMPs are living cells that secrete various cytokines, metabolites or growth factors into the media, adding an additional layer of complexity. Selection of primary packaging materials is another consideration for CBMPs due to the potential interactions of cells with the primary packing material[53]. Some CBMPs are submitted to extreme environmental conditions (i.e., freezing at ultra-low temperatures) during product processing, transportation and storage. Such conditions not only may affect the cells but also can alter the properties of primary packaging materials, such as vials, stoppers and (cryo)bags, consequently compromising container closure integrity[54]. Taking advantage of the lessons learned and experiences gained during development of protein-based products, further research should focus on establishing comprehensive analytical techniques and formulation strategies for CBMPs.

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