Analysis of sub-visible particles in complex injectable formulations

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Chapter 7

Summary and perspectives

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Summary

Complex injectable formulations, such as protein therapeutics[1], controlled release systems[2] and cell therapy products[3], are gaining a paramount position in the therapy of many life-threatening and chronic diseases. Most of these products have in common that sub-visible particles (SVP), *i.e.*, particulate matter in the size range of about 1 – 100 µm, are critical quality attributes. Most protein therapeutics are liquid or freeze-dried formulations in which the presence of SVP is unwanted[4]; many injectable controlled release systems are based on particulate drug delivery systems in the sub-visible size range[5]; and cell concentration and viability are important characteristics of cell therapy products[6]. With the continuous improvement of existing and emerging particle analysis techniques, the potentials of these tools in addressing current characterization challenges in the field of complex injectable formulations have to be investigated. Therefore, the aim of this thesis was to develop methods, based on a set of state-of-the-art particle analysis techniques, for characterization of pharmaceutically relevant sub-visible particles and to study the value of these methods in the characterization of complex injectable formulations.

Stirring-induced protein aggregation is encountered at different stages of manufacturing of biologics. In *Chapter 2* we have investigated the cause of this particular type of aggregation due to mechanical stress. For this purpose we designed an overhead stirring set-up in order to manipulate the presence and magnitude of the contact between the stir bar and the container. It was found that contact sliding during stirring initially resulted in the formation of submicron protein aggregates (100 – 1000 nm) of tested protein, IgG, as measured by nanoparticle tracking analysis (NTA). Continuation of such stirring increased the number of aggregates and led to the growth of particles into sub-visible aggregates, as analyzed by Micro-Flow Imaging (MFI). Interestingly, no increase in oligomer content was seen in size-exclusion chromatography (SEC), suggesting a rapid particle formation. Our investigation showed that the adjustment of the contact area of stirring and pressure applied by the stir bar on the glass surface affected the extent of aggregation. Formation of particles during stirring could be prevented by either addition of polysorbate 20 or avoiding contact between the solid surfaces. Herewith we have provided new insight into the mechanism of stirring-induced protein aggregation, by pointing out its root cause and suggesting formulation and process strategies to inhibit this route of protein aggregation.

In *Chapter 3* we have used a combination of NTA and MFI to get new insights into the kinetics and mechanism of protein-polyelectrolyte complexation. In this study we used IgG and dextran sulfate (Mw = 5000) as the model protein and polyelectrolyte, respectively. A solution of each with equal mass based concentration was prepared, mixed with the other and the resulting mixture was homogenized gently. Samples were taken at different time points and analyzed for monomer content and quantity of nano- and microparticles.

SEC analysis showed that immediately after mixing all the protein was complexed into a high number of nanometer sized particles, which was detected, sized and counted by NTA. Thereafter, these nanoparticles were reduced in number and increased in size. After 1.5 hours microparticles were formed and also these particles continued to grow in size and be reduced in number, as observed by MFI measurements. With the help of the Smoluchowski's perikinetic coagulation model[7], we fitted the changes in the total particle concentration, which enabled us to derive the so-called sticking probability of IgG-dextran sulfate complexes. The sticking probability is a related to the interaction between the protein-polyelectrolyte complexes and can help determination of the growth rate of such complexes. We expect that this combination of particle analysis techniques could be used also for other systems, such as complexes between polymers and proteins, DNA and other biomacromolecules.

PLGA microparticle porosity is one of the critical parameters that affect the performance of these drug delivery systems with respect to the degradation of the particles and release of the drug. Our study in *Chapter 4* describes a novel method to derive microparticle porosity by tracking the sedimentation velocity of suspended particles using a flow imaging microscope. Two sedimentation based methods were investigated. In the first method we tracked sedimentation velocity of particles in liquids with different densities. Thereafter, we (intra- or) extrapolated to the point where the sedimentation velocity would be equal to 0. The fluid density value that matched that point was considered to be equal to the density of the particles under investigation. This value was then used to estimate the porosity with the given size and composition of the particles and the densities of the particle constituents. In the second method, we measured the size and sedimentation velocity of individual microparticles in a suspending fluid with known density. Then the density of the individual particles was calculated by using Stokes' law of sedimentation and porosity values were derived from density. We investigated the influential parameters and validated the methods using different sizes of polystyrene standard beads. Using the developed method, we determined microparticle porosity of different PLGA microparticle batches, and found similar results as the ones obtained by mercury intrusion porosimetry (MIP).

Chapter 5 introduces another method to derive PLGA microparticle porosity by using MFI. In this method, we suspended a known mass of microparticles in a fluid and measured the total microparticle volume accurately. The apparent density of particles was easily calculated based on the mass/volume ratio. Together with the known composition of the particles and the density of the components, we were able to derive the microparticle porosity. Parameters affecting the accuracy of the method, such as particle concentration, fluid density and particle adsorption to the container and instrument tubing, were investigated by using control PMMA beads. These influential parameters were optimized for the analysis of

7 different PLGA microparticle batches in order to derive the porosity of each. The results for all the 7 batches were fairly similar to the ones derived with MIP. The results indicate that this method can be used for a reliable assessment of PLGA microparticle porosity with only a few milligrams of powder.

In *Chapter 6* we have explored the applicability of flow imaging microscopy (MFI and FlowCAM) as a new technique to count cells in a suspension and determine cell viability. In this study, we have exposed B-lineage leukemia cells from two different donors to ambient conditions for several days, resulting in a reduction of cell viability. During this incubation study we measured samples from these cell suspensions with MFI, FlowCAM, hemocytometry and automated cell counting. The results from both flow imaging microscopy techniques showed changes in cell morphology that could be monitored with a number of parameters derived from each instrument. Using fluorescence assisted cell sorting (FACS) we were able to separate and collect dead and live cell populations. The morphological parameters of the separated dead and live cell populations were determined with FlowCAM and MFI, in order to develop morphological filters to discriminate dead and live cells in an unknown cell suspension. The filters were shown to be distinctive, as our analyses with flow imaging gave similar results for cell viability as the conventional methods (hemocytometry and automated cell counting). Also the cell concentration measurements delivered similar, if not better, results as hemocytometry and automated cell counting.

General discussion and perspectives

In the work described in this thesis we have investigated and developed new methods using either a combination of particle analysis techniques or a single particle analysis technique. All the methods described here have potential to be applied in pharmaceutical research and development.

In our study on protein aggregation induced by stirring (*Chapter 2*), the power of NTA to be able to track early stages of aggregation was shown. Although this method has its shortcomings with respect to reproducibility[8], it still remains one of the few methods that can size and quantify particulate matter in the nanometer size range. Another interesting observation from *Chapter 2* was the concentration dependent average size of the micron sized particles that were formed: at higher protein concentration (0.5 mg/mL) the particles were consistently more than 1 µm larger than microparticles formed at lower concentration (0.1 mg/mL), as shown by MFI. This observation suggests that MFI is clearly able to reveal differences in the low-micrometer range and can be used for studying the kinetics of protein aggregation. Ideally, for investigation of the aggregation kinetics one needs to derive mass transfer or aggregation rate in relation to the influential parameters[9], *i.e.,* for stirring induced aggregation the effect of the parameters such as surface area, protein concentration and

stirring rate (among others) on monomer/aggregate mass balance should be studied. One needs to recognize that converting aggregate size distribution to total aggregate mass is accompanied with a lot of assumptions with respect to the density of proteins and translation of the size to volume[10]. In this respect, the method for porosity assessment of microparticles described in *Chapter 4* might also be suitable to derive the density of protein aggregates (explained further in this section).

The method for determining particle growth and concentration presented in *Chapter 3* can be used to gain insight into the interaction strength between the polyelectrolyte-protein particles, which in turn affects the coagulation of complexes. The coagulation process continues until the aggregation and disintegration of complexes reach equilibrium, which in the end determines the final size of the complexes[11]. The charge on both components of the complexes can be tailored by changing the pH and/or ionic strength of solution[12]. In addition, concentration and ratio of the components have effects on the final size of the complexes[13]. The size of the final complexes determines their potential use in drug delivery[14]. Altogether, it can be concluded that the method presented in this thesis will serve as a valuable tool in the screening of the influential parameters on the size of protein-polyelectrolyte complexes. In addition, it is worth mentioning that in the field of vaccine product development protein-polyelectrolyte complexes form an important formulation platform[15]. The structural interplay between antigen and adjuvant in a vaccine formulation resembles same sort of interactions as mentioned above. Therefore, characterizing the interaction of these macromolecules is crucial for the vaccine potency[16].

Our application of the two different flow imaging microscopy techniques for drug delivery systems such as PLGA microparticles shows that the described methods can be of more value than porosity determination only. The counting and imaging of the particles in suspension brings an important advantage, namely getting information on the morphology and size distribution of PLGA microparticles. The high-resolution images of FlowCAM result in a high number of observable morphological parameters. In comparison with MFI, FlowCAM is a more flexible system, where the operator can practically change any setting or aspect of the instrument. The high imaging/analysis efficiency of the MFI can be advantageous when rapid and accurate size distribution of the particles is needed. MFI is more user-friendly when it comes to the execution of the analysis. Also the supported data acquisition mode, MVAS, has certain features (*e.g.*, removing stuck particles) that improve the counting accuracy.

In the development of drug delivery systems there is a need for exploratory studies to investigate if the platforms in development can be applicable to obtain a target product profile for the drug of interest. Quality-by-Design (QbD) approaches may facilitate development of controlled release products, by understanding the impact of the properties of material

components and manufacturing process on the product quality[17].One of the tools in QbD is the implementation of process analytical technologies (PAT) during the development. The latter concern systems that allow designing, analyzing and controlling the manufacturing process, through timely measurements during processing[18]. In this context, systems such as FlowCAM ES® can automatically extract, dilute and run samples from within the production or processing line[19]. This speeds up the production process and delivers continuous data to monitor the process and keep track of the quality of the (intermediate) product.

In addition to using flow imaging microscopy for PLGA microparticles, the sedimentation method itself may already be of interest for other types of complex injectable formulations, *e.g.*, for the determination of the density of the proteinaceous particles. The density of protein aggregates is frequently used to recalculate the number of micron size aggregates into mass of protein. In addition, resonant mass measurement (RMM) requires the density of aggregates in order to convert the buoyancy of the aggregate into an equivalent circular diameter[20]. Although attempts have been made to determine the aggregate density by RMM itself[21], the technique does not allow determination of the density of aggregates above 5 µm. With the sedimentation based method where we use fluids with different density, one could possibly attain the density of protein particles larger than 5 µm.

The last part of this thesis on counting and viability determination of cells (*Chapter 6*) opens a whole new field in the application list of flow imaging microscopy. In the cell therapy field knowledge about formulation development is very preliminary, and the availability of robust analytical tools that provide cell counts and various morphological characteristics can be of great value. In addition, the capability of flow imaging microscopy for cell product characterization may be broader than we have shown in our study. High-resolution images of FlowCAM may have added value for discrimination of different cells or perhaps a single cell at different stages of its differentiation. Moreover, certain FlowCAM models have a fluorescence detector that may open new opportunities to analyze fluorescently labeled cells. This option may increase the potential of flow imaging microscopy for the characterization of cell therapy products. The new application field for flow imaging microscopy that we introduced in *Chapter 6* is possibly useful directly in a clinical setting. In addition, flow imaging microcopy methods could be implemented to test the pharmaceutical quality of protein drugs as well as classical parenteral drug dosage forms that are manufactured aseptically in hospitals.

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