Subvisible (2 um to 100 um) Particle Analysis During Biotherapeutic Drug Product Development: Part 2, Experience with the Application of Subvisible Particle Analysis

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Abstract

Measurement and characterization of subvisible particles (including proteinaceous and non-proteinaceous particulate matter) is an important aspect of the pharmaceutical development process for biotherapeutics. Health authorities have increased expectations for subvisible particle data beyond criteria specified in the pharmacopeia and covering a wider size range. In addition, subvisible particle data is being requested for samples exposed to various stress conditions and to support process/product changes. Consequently, subvisible particle analysis has expanded beyond routine testing of finished dosage forms using traditional compendial methods. Over the past decade, advances have been made in the detection and understanding of subvisible particle formation. This article presents industry case studies to illustrate the implementation of strategies for subvisible particle analysis as a characterization tool to assess the nature of the particulate matter and applications in drug product development, stability studies and post-marketing changes.

Introduction

The compendial method for subvisible particle testing, based on USP <788> and <787>, uses light obscuration for monitoring particles having an equivalent circular diameter (ECD) of $\geq 10 \mu\text{m}$ and $\geq 25 \mu\text{m}$ [1,2]. As discussed in a previous manuscript [3], health authorities often request testing beyond the limitations of the compendial method and investigation of particles smaller than $10 \mu\text{m}$ for more complex biotherapeutic parenteral drug products, which may have the propensity to form proteinaceous particulate matter. Consequently, the application of particle analysis across the entire subvisible particle range has become a key consideration during the development of a biotherapeutic drug product [3-17]. In this article, we provide examples demonstrating how a variety of subvisible particle analysis techniques can be applied in practice during clinical and commercial development. The term “subvisible” applies to particulate matter of the size range defined as $2\text{-}100 \mu\text{m}$, which may be proteinaceous or non-proteinaceous.

Subvisible particle analysis requires significant long-term data under actual conditions of drug product storage, with material made at different times and from different facilities, to understand the drug product and method variability. The key to understanding such variability in the particles and amounts seen is to have sufficient data to understand product trends in subvisible particulate matter and the ability to determine if the particulate matter is protein-based or originates from other intrinsic or extrinsic species that are also counted in quantitative subvisible

particle assessments [18-20]. Collection of information on particle morphology and composition may be additional components of particle analysis and trending during development.

The case studies presented are a compilation of the selected experiences of the companies that participated in the preparation of this manuscript, and illustrate ways in which this strategy can be applied. The case studies are organized into two sections: (1) Particle Characterization and (2) Applications of Particle Characterization in Drug Product Development, Stability Studies and Post-Marketing Changes.

While the information presented is illustrative of the types of approaches being taken to evaluate subvisible particles also below 10 μm , the examples are not intended to prescribe strategies or the application of any specific techniques considered necessary to meet requirements in relation to regulatory applications for product development or licensure.

Section 1: Particle Characterization

Introduction

Light obscuration particulate matter analysis has been a key analytical tool for development of a suitable injectable drug product consistent with current pharmacopeia requirements. The development of new particle analysis techniques has led to a better understanding of particle characteristics. In particular, morphology information can facilitate deeper understanding of the origin and differentiation of the types of subvisible particles for characterization and identification of particles in samples [11,21-29].

The ability to characterize particle morphology with flow imaging technologies has led to efforts to differentiate specific particle types using a variety of image processing filters [24,27-29]. Silicone oil microdroplets and air bubbles are examples of particles that have morphological features (e.g. shape, contrast pattern) that make them relatively straightforward to identify, with reasonable accuracy. Other types of particles, including both proteinaceous and non-proteinaceous ones, can be much more challenging to identify because a unique set of morphological features is difficult to define. Flow cytometry is another technique which is being explored for its ability to differentiate between particle populations. One major difficulty that remains to be addressed is the assessment of the accuracy of the different classification procedures. The latter is typically performed using artificially generated pure samples which makes it challenging to evaluate the relevance to the mixtures present in "real life" and which so far have precluded their wide application.

In some cases further particle characterization is needed, and it can be performed with microspectroscopy techniques such as Fourier Transform Infrared (FTIR) microspectroscopy and Scanning Electron Microscopy with Energy Dispersive Spectroscopy detectors (SEM-EDS). These investigational tools can provide information on chemical composition of particles and, in some cases, give insights into their structure [2,4,5]. Identification and analysis of particles by

such techniques can help to classify particles more definitively (e.g. inherent, intrinsic, and extrinsic) [5]. Particle composition and structure information can be useful for understanding root causes of particle formation [7,8] in process optimization studies.

The case studies in this section show the application of particle characterization in the following areas: (1) minimization of artifacts in particular matter analysis via optimization of a sample preparation technique [1,2,21,22], (2) classification and differentiation of particles and (3) identification with further characterization [14,16,23,30,31].

Case Study 1.1: Optimization of Sample Preparation for a Lyophilized Drug Product

Experimental

Three recombinant proteins rP 1, rP 2, and rP 3 (lyophilized drug product presentations) and formulation buffers 1, 2, and 3 (liquids) were used in the study. Lyophilized products were slowly reconstituted with sterile water for injection (SWFI) to avoid foam and bubble formation. Ten vials of each were pooled for analysis and analyzed immediately after pooling (initial), 2 hours later, and after 5 min or 10 min exposure to a 20 kPa vacuum. Samples were analyzed in one replicate using a Liquid Particle Counting Systems Hiac/Royco 9703 with HRLD-400 liquid sensor, following the harmonized light obscuration particle count procedures [2,20]. Samples were also analyzed in two replicates using a Microflow Imaging™ DPA4100 instrument (Protein Simple). The MFI View Analysis Suite (MVAS) software filter screen was used to obtain separate count of subvisible particles and air bubbles.

Results and Discussion

The selective results from an optimization study of sample preparation techniques for light obscuration and flow imaging analysis using a 2 hour sample hold, and 5 min and 10 min of vacuum application for rP 3 are shown in Figure 1 (A, B). As has been observed by others, the number of particles counted by flow imaging was significantly higher than that obtained by light obscuration [9,16]. When the frequency counts were allocated into 1 μm bins, the highest particle count was observed in the particle range of 2 μm to 3 μm for rP 3. The number of particles was significantly reduced after application of vacuum or 2 hour sample hold in rP 3, while it remained unchanged in rP 2 and rP 1 (not shown). There were also no significant differences between particle counts for all samples of rP 1, rP 2, and rP 3 exposed to vacuum for 5 min or 10 min. In some cases, vacuum application was more efficient in removing the air bubbles than the 2 h sample hold. The high particle count of the initial samples of rP 3 was due to the subvisible air bubbles, which was confirmed by light microscopy analysis of liquid

samples immediately after reconstitution and after application of different sample preparation techniques (not shown).

Based on the sample optimization data discussed above, two techniques, the 10 min vacuum application and the 2 h sample hold, were chosen for technique optimization, with a study performed by one analyst on three days for one lot of each rP 1, rP 2, and rP 3 using flow imaging. In addition to size and count, this technology provides particle images and allows particle characterization based on their unique morphology. The classification of particles in the studied samples showed that they were mostly attributed to protein particles and air bubbles with limited presence of oil droplets. Therefore, the particle count data obtained by flow imaging for the three rP 1, rP 2 and rP 3 drug product presentations were analyzed using the MVAS soft filter screen, which allows segregation of particle and air bubble counts from the total particle count. The results obtained for the rP 3 presentation are shown in Figure 2 (A,B). After application of 10 min of vacuum, the total number of particles in the 2 μm to 5 μm range was reduced by about 83% compared to that of the initial sample and was attributed to a significant reduction of the subvisible air bubbles. Similar data were obtained for 2 hour sample hold (not shown). Samples of rP 1 and rP 2 did not show a significant reduction in subvisible air bubbles for all studied conditions (not shown).

Overall, the study showed that some of the reconstituted lyophilized drug product presentation samples might have an excessive amount of subvisible bubbles, which can be identified by flow imaging MVAS soft screen and removed by application of vacuum (10 min at 20 Kpa) or sample hold (2 h) prior to light obscuration and flow imaging analysis.

Case Study 1.2: Application of Flow Imaging for Classification of Particles

Experimental

Several batches of prefilled syringe and cartridge presentations containing drug product formulations were tested using flow imaging (Protein Simple Microflow ImagingTM). Flow imaging was used to characterize the particles by applying morphology filters to distinguish between particles. A sample volume of 0.65 mL was analyzed following a 0.20 mL sample purge in a 400 μL flow cell. The results are provided as averages of three injections and are expressed as number of particles per milliliter registered at different size ranges based on equivalent circular diameter (ECD). The particle classification results obtained after applying morphology filters are indicated in terms of percentage of each different sub-population with respect to the total particles detected.

Results and Discussion

Figure 3 shows the results of the flow imaging analysis for the prefilled syringe presentation for particles greater than 10 μm using a histogram (particle concentration vs. size by ECD) and a

scatter plot (aspect ratio vs. object intensity mean). The particles $> 10\ \mu\text{m}$ (about 5% of the population) are attributed to a mixture of non-proteinaceous particles, proteinaceous particles, air bubbles, and silicone oil, while the particles greater than $20\ \mu\text{m}$ were mainly identified as fibers. The overall comparison of the two presentations (prefilled syringe and cartridge) in terms of subvisible particle population distribution is summarized in Table 1. Comparing the prefilled syringe and cartridge presentations, it was observed that: (1) A higher relative amount of particles having $\text{ECD} > 10\ \mu\text{m}$ was detected in the samples in the cartridge presentation compared to the prefilled syringe presentation. (2) Silicone oil droplets are more evident in prefilled syringes than in cartridges. (3) A higher amount of small air bubbles was detected in prefilled syringes compared to the cartridge presentation.

Overall, flow imaging software is capable of classifying the detected subvisible particles into different sub-populations by grouping particles having similar geometric and optical characteristics. For example, particles that have an aspect ratio < 0.8 , i.e. not circular, and have an intensity mean > 700 are likely to be non-proteinaceous or proteinaceous particles. Particles with an aspect ratio > 0.8 and an intensity mean < 700 are likely to be air bubbles or silicone oil droplets (see classification criteria detailed in Table 1). Also, fibers that may be present in the sample from the manufacturing process can be distinguished from other particles, as they are usually large, opaque, and variably shaped. The categorization of particles was confirmed by visual assessment of the images greater than $10\ \mu\text{m}$. The efficacy of the morphology filter for particle classification has been verified by spiking silicon oil droplets in the placebo solution and verifying the increasing level of silicon oil related sub-population (not shown).

Case Study 1.3: Use of SbVP Morphology Assessments to Study Impact of Container Closure Systems

Experimental

In this study, three lots of drug product and one lot of placebo contained in siliconized prefilled syringes (PFS) were analyzed by flow imaging (Protein Simple DPA 5200, Ottawa, Canada). A sample volume of $0.40\ \text{mL}$ was analyzed following a $0.20\ \text{mL}$ sample purge in a $100\ \mu\text{L}$ flow cell. The non-spherical particle concentration was determined by applying a filter of $\text{ECD} \geq 5\ \mu\text{m}$ and aspect ratio (AR) < 0.70 . An additional proprietary filter (PSA) was also used to determine the non-spherical particle concentration. Additionally, samples were analyzed using flow cytometer (FACScanTM) analysis on a Becton-Dickinson FACS Aria IIu. Forward Scatter (FSC) vs. Side Scatter (SSC) plots were generated using $488\ \text{nm}$ laser excitation and displayed using logarithmic amplification.

Results and Discussion

Figure 4A shows the variability of non-spherical particle count data between three PFS drug product lots. Filtering of the results to remove spherical particles decreased the particle counts relative to the un-filtered data (not shown), though a significant amount of particles remained. However, in this case analysis of placebo PFS indicates the presence of significant amounts of non-spherical particles in the placebo syringes (not shown) as well, suggesting these are not from proteinaceous particles. When the morphology of these non-spherical particles in the placebo was examined visually, a significant number of images appeared to consist of doublets or triplets of associated spherical silicone oil droplets (Figure 4B). Figure 4C shows the subvisible particle data comparing the data filtered with the simple algorithm to a refinement of these data by visual inspection. This removes clusters of silicone oil spheres that show coincidence, and refinement using a more complex proprietary software filter designed for the same application for one lot of PFS drug product and placebo. Overall, refinement of the filter by manual visualization or software modification resulted in an improved identification of silicone oil complexes which allowed comparison of both the effect of container closure system on the amount of silicone oil present, and comparability in terms of amount of subvisible particles made up of protein and other aggregate.

Flow cytometry was also used as an orthogonal technique to evaluate the population of subvisible particles in the PFS drug product [32,33]. As shown in Figure 5, the population of subvisible particles can be subdivided into silicone particles and other particles which are non-proteinaceous, proteinaceous, or a mixture of these based on the forward and side scatter of the particles. The population shown in red was attributed to particles (including both proteinaceous and non-proteinaceous), and the population shown in green was attributed to silicone oil droplets. The coloration of red and green are intended to show areas of highest particle concentration that may be classified. The blue population likely contains some combination of all particle types, and could also include heterogeneous particles. The magnitude of laser light scatter in the forward direction is an estimate of the size of the molecules doing the scattering. Silicone oil and non-proteinaceous and proteinaceous particles span comparable size ranges and exhibit comparable forward scatter values. Side scatter magnitude is representative of the internal complexity of the particle, or the ability of the particle to scatter laser light in multiple directions. Silicone oil droplets have a smooth, regular surface and interior, resulting in relatively lower side-angle laser light scatter than other particles of comparable size which are more irregular. This type of analysis can provide an orthogonal characterization technique to assess the relative contribution of silicone oil to the subvisible particle count for a PFS or other siliconized container closure systems, for comparability assessments, etc.

Case Study 1.4: Assessment of Subvisible Particulate Matter after Filling into a Prefilled Syringe and Actuation of a Device

Experimental

The instruments used in this study were a light obscuration liquid particle counting system (HIAC 9703) and two flow imaging systems (Protein Simple, Microflow Imaging™ MFI 5100 or DPA 4100). Particle concentrations were determined with a lower size limit of 2 µm for both instruments. The following drug products were evaluated in the study: (1) High concentration formulation at 100 mg/mL of an IgG solution, as formulated filtered bulk or after filling into 1 mL siliconized glass syringes (BD Hypak™) at nominal 1 mL volume; syringes were also studied in an autoinjector device. (2) Placebo formulation filled into the same 1 mL siliconized glass syringes (BD Hypak™) at nominal 1 mL volume as the IgG formulation. The same batch of BD Hypak™ syringes were used for both active and placebo.

Results and Discussion

A high concentration IgG formulation was analyzed by flow imaging to assess particle concentration in the size range ≥ 2 µm, before and after filling into 1 mL siliconized syringes. The results show that particle concentrations from 2-45 µm increased significantly after filling due to the contribution of silicone oil droplets especially in the 5-10 µm size range for this syringe/product combination (Table 2) [24].

A proprietary morphological filter (based on shape and apparent intensity properties) was applied post-data acquisition using optimized morphological settings for the different particle sizes to reduce the contribution of silicone oil droplets. After morphological filtering, the reported particle concentrations for the syringe fill, although lower, were not consistent with the initial levels measured in the bulk (Table 2). This could be due to incomplete removal of the silicone oil droplets by the morphological filter because (1) some of the silicone oil may have a non-spherical shape (e.g., droplets partly coincident or clustered together) or (2) the optical resolution (number of pixels) is not sufficient in the 2-3 µm size range to differentiate between particles based solely on morphological parameters. When the same sample was analyzed using the S-factor filter [27] similar results were obtained for the ≥ 3 µm sized particles (not shown). Increased particle concentrations due to silicone oil droplets were also observed when analyzing the same samples by the light obscuration method (not shown). Additionally, it cannot be ruled out that the filling process of the protein solution into the syringe or interaction with silicone oil may cause an increase in the number of particles. It is therefore not possible to consistently and accurately differentiate between all particles originating from the primary packaging components (i.e., silicone oil), those related to the formulation (i.e., protein particles), and other non-proteinaceous particles. The use of morphological filtering does not completely remove the contribution of silicone oil particles especially in the size range of 2 to 10 µm.

The presence of silicone oil droplets in biotech products filled into syringes not only increases the measured particle counts, but can also result in higher variability of the particle assay, particularly < 10 µm due to variable amounts of silicone oil in individual syringes. A placebo

solution was filled into 1 mL glass syringes (to exclude the contribution of product-related particles), and the particle concentrations were determined for each individual syringe by flow imaging (Table 3). The relative standard deviation (RSD% , n=10) of the particle concentration ranged from 29 to 45% for the particle size ranges below 10 μm . Statistically, it would be expected that higher particle concentrations should have provided better precision (lower RSD%). However, the experimentally derived RSD% was significantly higher than the assay variability of 3.0 % (n=5), as determined by spiking prefilled syringes with certified polystyrene particles at 20,000 particles per syringe.

The impact of silicone oil is even more pronounced when analyzing the particle concentrations of an IgG formulation from syringes assembled in an autoinjector. The particle concentrations associated with drug product solutions expelled with an autoinjector increased by a factor of 2 to 3 compared to those of naked syringes when analyzed by a small volume light obscuration test (Figure 6). The particle concentration in the 2-10 μm size range significantly exceeded the operating range of the sensor of the light obscuration instrument (18,000 particles per mL). This increase in measured particle concentration was potentially due to the higher amounts of 2-10 μm silicone oil droplets that were expelled from the syringes due to the action of the autoinjector. Flow imaging showed the same trend, and evaluation of the particle morphologies confirmed the presence of substantial amounts of silicone oil droplets from samples expelled with the autoinjector device (not shown). It should be mentioned that degassing of the expelled solutions at 20 mbar for 2 hours did not reduce the measured particle concentrations indicating that air bubbles were most likely not causing the observed particle increase for the autoinjector device.

Case Study 1.5: Characterization and Identification of Particles in a Monoclonal Antibody Product

Experimental

Flow imaging (FlowCAM[®] 3, Fluid Imaging Technologies, Yarmouth, ME, USA) was used for the characterization of subvisible particles. A semi-quantitative in-house visual appearance method was used to monitor levels of visual particles. An FTIR microscope (Spotlight 400, PerkinElmer, Waltham, MA, USA) and SEM-EDS (Hitachi TM 1000 with Swift ED EDS detector, Angstrom Scientific, Ramsey, NJ, USA) were used to characterize particle composition. For FTIR microscopy and SEM-EDS analysis, particles were captured on aluminum oxide filters with 0.1 μm pore size (Anopore, Whatman). Second derivative spectra of the Amide I band signal from FTIR microscopy were used to evaluate the protein structure of individual particles. Assignments of bands to secondary structural elements were performed [8].

Results and Discussion

Particles appeared shortly after MAb X was filled into vials in early clinical development. The particles grew in counts and size during storage at 4 °C. To understand the root cause of particle formation and to prevent their formation, a thorough characterization of particles was performed. Characterization testing included sizing and counting of particles, as well as analysis of particle composition.

Particle analysis data for MAb X samples monitored over a period of 4 months are summarized in Table 4. Initially, subvisible particles of greater than or equal to 10 µm were present at 3000 particles/mL, with apparent counts declining to less than 1000 particles/mL at later time points. At the same time, visible particles significantly increased over time, and their presence was readily evident upon visual inspection of vials with MAb X product (Figure 7). It is possible that the subvisible particles either combined to form visible particles, or their presence caused a partial blockage of the 100 µm flow imaging cell, resulting in lower subvisible particle counts at later time points.

At the four-month time point, analysis of particle composition by FTIR microscopy showed multiple particles of various sizes and shapes, including particles that were greater than 20 µm (Figure 8). Twenty of these large particles were randomly selected and analyzed in IR transmittance point mode. The presence of Amide I and Amide II bands in the acquired IR spectra confirmed that these particles were proteinaceous (Figure 9A), indicating that these were inherent particles to MAb X. Some particles also exhibited weak bands at 2960 cm⁻¹, 1410 cm⁻¹, and 1260 cm⁻¹ which are characteristic of silicone oil (Figure 9B). Analysis of elemental composition of the same preparation of particles from MAb X solution by SEM-EDS confirmed this result: several of the examined particles contained some level of Si in addition to elements corresponding to organic material (C, O, H) (not shown).

Second derivative spectra of the Amide I band from FTIR were used to evaluate the protein structure of individual particles [23]. The position of the dominant band in the 1700-1600 cm⁻¹ region was used to assess the secondary structure of protein in the particles [34]. The second derivative spectra of protein from individual particles were compared to the spectrum obtained for a reference MAb X sample. Only the major band in the second derivative spectra of particles was used for the analysis. Positions of minor side bands were not reliable due to a relatively low signal-to-noise ratio of IR spectra of individual particles (see Figure 10). From this analysis, it was found that most of the particles did not exhibit the native β-sheet band around 1641-1636 cm⁻¹, which is usually characteristic of IgG molecules and observed for the reference MAb X sample at 1640 cm⁻¹. Instead, most particles contained denatured protein with their main bands shifted towards either random coil (1649-1644 cm⁻¹) or intermolecular β-aggregate regions (1626-1622 cm⁻¹) of the spectra (Figure 10). This observation pointed to the possibility of at least two different particle formation pathways in the drug product. In some cases, formation of intermolecular β-sheet aggregates has been linked to nucleation and rapid growth of protein aggregates.¹⁰ After process improvements were implemented to minimize particle formation, particles with Amide I bands shifted towards intermolecular β-sheet aggregate structures were no

longer observed. One possible explanation is that a specific population of subvisible particles was nucleating growth of larger visible particles during storage, and control of these subvisible particles afforded benefits for control of visible particles, as well.

Based on the results of the initial investigation MAb X was manufactured by an improved process and yielded materials with relatively low levels of subvisible and visible particles. A systematic study was conducted to characterize particle types during storage. Samples of the improved MAb X product were analyzed by flow imaging, visual appearance, and FTIR microscopy every 3 months during storage at 4 °C for 24 months. As shown in Table 5, MAb X samples remained practically free or free from visible particles during the entire testing period. Subvisible particles in the $\geq 10\ \mu\text{m}$ and $\geq 25\ \mu\text{m}$ ranges were less than 1500 and 50 particles/mL, respectively. FTIR microscopy detected very few discernible particles from MAb X solution retained on the filter. Most particles were smaller than $20\ \mu\text{m}$, and, in general, less than 10 particles per preparation were suitable for analysis in the entire filter area (Figure 11 and Table 5) [21], unlike multiple large $>20\ \mu\text{m}$ particles observed in samples of particle-forming Mab X lot prior to process optimization (Figure 7).

The IR signals from these small particles were relatively weak. While it was still possible to identify the major particle components as protein due to the presence of characteristic Amide I and Amide II bands, analysis of protein structure by second derivative of the Amide I band was not practical due to the low signal-to-noise ratio (Figure 12). Overall, while IR data provided some identification information, detailed analysis of particle composition and structure were not possible due to their small size resulting in a low IR signal.

These two examples of particle characterization by FTIR and SEM-EDS microspectroscopy techniques illustrate their advantages and limitations. Analysis of particles can provide valuable information on composition and structure for investigations and process optimization, however, smaller $<20\ \mu\text{m}$, particles are often not amenable for probing by such tools.

Conclusions from Section 1: Particle Characterization

Flow imaging analysis and other techniques that provide information on morphology and particle structure offers the possibility to differentiate between diverse types of particles based on morphological parameters. This capability can be applied to the development of sample preparation technique to obtain accurate particle size distribution measurements to apply to other methods (e.g. light obscuration). Differences in morphology between protein particles and other particles can also be exploited to develop orthogonal techniques and mathematical filters which can remove a portion of the contribution that non-proteinaceous particles have on the total particle count (i.e., silicone oil). This can make the characterization and assessment of subvisible particulate matter more quantitative.

Beyond particle size measurements and morphology characterization, analysis of protein conformation and identification of particles larger than 20 μm may be achieved by sample filtration and analysis of particles retained on a membrane filter by FTIR and other microspectroscopic techniques. Options for identification of particles smaller than 20 μm can be limited due to constraints in resolution and sensitivity of most currently available instruments. Regardless of the techniques used, the expertise of the analyst and a deep understanding of the process and product are necessary in order to interpret the results.

Section 2: Applications of Particle Characterization in Drug Product Development, Stability Studies and Post-Marketing Changes

Introduction

As the candidate molecule progresses into clinical development, subvisible particle testing for characterization and quality control (consistent with current compendial requirements) becomes an important component to support the development of the drug product. Studies presented in this section using subvisible particle analysis include development of the formulation composition, manufacturing process, container closure and stability [11,35-45]. Additionally, during the post-marketing phase of a drug product's life cycle, changes to the container closure system may also require an approach that includes subvisible particle analysis to demonstrate the impact of the change.

Case Study 2.1: Application of Subvisible Particle Analysis to Manufacturing Process Development

Experimental

In this study, particle characterization was used to help optimize the fill and finish process of a therapeutic antibody. The drug product solution was recirculated through four filling technologies commonly used in biopharmaceutical manufacturing. The filling technologies evaluated were rotary piston, rolling diaphragm, time pressure (T/P) and peristaltic pumps. Samples were analyzed for subvisible particles initially and after each recirculation, through ten cycles. Subvisible particle analysis was conducted using flow imaging (Protein Simple DPA 5200, Ottawa, Canada) with a sample volume of 0.40 mL following a 0.20 mL sample purge in a 100 μL flow cell. The non-spherical particle concentration was determined by applying a filter of $\text{ECD} \geq 5 \mu\text{m}$ and aspect ratio (AR) < 0.70 ; minimization of this SbVP population was the goal of the study, since these particles were most likely due to protein aggregation as a result of the filling process.

Results and Discussion

As reported previously, there are always more 2 and 5 micron aggregate than 10 and 20, and monitoring the particles in these smaller sizes can often allow differentiation between samples that cannot be obtained by monitoring the 10 and 20 microns alone [21,46]. Figure 13 shows the results of the study indicating that rolling diaphragm and peristaltic filling technologies showed no significant propensity to form subvisible particles in the drug product solution. Rotary piston and time pressure filling technologies both showed an elevation in subvisible particles through each recirculation. These results revealed that this therapeutic protein was sensitive to the mechanical forces produced by these 2 fillers. Based on this screening study, the filling technologies that did not significantly impact the formation of subvisible particles for this drug product solution were then selected for further process optimization, minimizing the potential for generation of protein aggregates and the potential risks associated with in vivo administration of this drug product.

Case Study 2.2: Subvisible Particle Analysis of a Biotherapeutic Lyophilized and Solution Vial Drug Product on Stability

Experimental

Subvisible particles for two products have been monitored over approximately 2 years using light obscuration analysis. During this time frame, 91 measurements on 68 unique lots for both release and stability have been performed. These 68 lots utilized numerous different lots of excipients and packaging components. All packaging components were either autoclaved (stoppers) or depyrogenated (vials). All measurements were taken on a light obscuration liquid particle counting system (HIAC 9703, Pacific Scientific Instruments) using the experimental procedure outlined in USP <788>. In addition to the $\geq 10 \mu\text{m}$ and $\geq 25 \mu\text{m}$ particle counts, data from 2-10 μm was also collected. Samples were allowed to degas for 2 minutes at ambient conditions prior to testing. All values are presented as particles per milliliter, to allow for better comparison of data across configurations.

Product 1 is a lyophilized product with two configurations (fill volumes). For configuration 1, ten individual vials were pooled to perform one measurement. For configuration 2, eighteen vials were pooled to perform one measurement. Product 2 is a liquid product with a single configuration. Individual vials were used to perform a single measurement; however, the majority of the data was obtained by taking the average of three vials independently measured. Both products use siliconized stoppers.

Results and Discussion

Figure 14 provides the data for Product 1 across release and stability for lots manufactured over a 2-year window. At release, particle counts from both configurations (on a per milliliter basis) were similar. The average subvisible particle counts across samples ranging from 0 to 5 years were also similar, indicating no substantial change during storage. While no individual lot has at this point been monitored for an entire 5-year period, the pooling of data across multiple lots, despite the variability, allows a reasonable evaluation of the stability trajectory.

Figure 15 illustrates the data for Product 2 across release and stability for lots manufactured over a 2-year window. While there are fewer measurements for this product, similar observations can be made regarding the stability of subvisible particulate matter during storage. For Product 2, 11 of the 27 measurements were done by averaging three independent measurements from unique vials. The average coefficient of variance was 28 % (range from 14 % to 65 %). This parameter reflects both assay variance and vial to vial variance within a given lot. This analysis illustrates the relatively poor precision for this method, and is consistent with previously published precision data [9].

Figure 16 shows the correlation between the 2 μm to 10 μm data and the >10 μm data for Product 1. The resulting correlation is quite good ($r^2 = 0.73$), indicating that the >10 μm data is a good predictor for the relative amount of 2 μm to 10 μm subvisible particulate matter present. The correlation for Product 2 (not shown) was less conclusive ($r^2 = 0.42$), although still reasonably predictive given the overall variability of the method. The difference between product correlations demonstrates the dependency of the analysis to the specific product.

Expanding light obscuration measurements to include 2 μm to 10 μm size data is a relatively simple way to expand our knowledge around subvisible particulate matter, as the basic method is already routinely utilized for release and stability of biological products. However, the measurement variability makes it difficult to interpret meaningful differences until very large data sets are available. Relatively large differences (2-3 fold) between individual measurements cannot be considered significant in most cases. Despite these limitations, when sufficient data is combined, it does appear that global assessments of the stability trajectory can be made on a product (versus lot) basis. Hence, while the variability is high on an individual measurement, light obscuration can be used as a stability indicating assay and can provide useful information regarding lot-to-lot variability. The correlation between the 2 μm to 10 μm and > 10 μm data suggests that it may be feasible in some cases to utilize the > 10 μm data to routinely monitor lots as a surrogate for < 10 μm testing. However, this involves a trade-off between the noise and sensitivity associated with the 2 μm to 10 μm data, and requires demonstration through stressed product studies that the > 10 μm data sufficiently measures forced degradation.

Case Study 2.3: Subvisible Particle Analysis of a Biotherapeutic Prefilled Syringe Drug Product on Stability

Experimental

In this study, 0.5 mL of either a protein drug product (dp) or placebo solution were filled into 1 mL long, BD sterile clean fill (SCF) syringes siliconized by the standard process, 0.4 mg (1X silicone), and by a modified process targeting two times the standard silicone oil quantity, 0.8 mg (2X silicone), as well as control syringes containing no silicone oil (0X silicone). The filled syringes were placed on stability. Data are reported for samples stored at 25°C. The samples were tested for particulate matter by light obscuration (HIAC 9703) using 30 pooled syringes and testing four replicate tests of a 3 mL sample size, in which the first replicate was discarded and the last three replicates were averaged. Flow imaging (Protein Simple Microflow Imaging™ 5200) analysis was conducted using six pooled syringes, testing three replicates of a 1 mL sample size, and averaging the three replicates. For both particle analysis techniques, the size ranges tested were ≥ 2 , ≥ 5 , ≥ 10 and ≥ 25 μm .

Results & Discussion

Figures 17 and 18 show the subvisible particle data for 0X, 1X and 2X siliconized syringes stored at 25 °C for 24 weeks. Drug product and placebo solutions of like silicone oil content exhibited similar particle counts based on light obscuration analysis, and did not show a trend over time when considering the variability in counts between time points. Flow imaging analysis shows some differentiation between drug product and placebo at ≥ 2 μm in the 2X silicone oil syringes and a similar overall profile to light obscuration analysis. Figure 19 displays representative images of ≥ 5 μm particulate matter from the flow imaging analysis of drug product and placebo solutions in syringes siliconized 1X after storage at 25 °C for 24 weeks. The images show the highly circular nature of the subvisible particles in the solution which is indicative of silicone oil. The particle images from syringes siliconized at the 2X level are consistent with analysis in Figure 19 (not shown). Although image analysis of ≥ 2 μm particles is not possible with this apparatus, the highly circular nature and lack of non-circular particles at ≥ 5 μm are likely indicative of particulate matter at ≥ 2 μm . The differentiation in the ≥ 2 μm flow imaging data between drug product and placebo is likely associated with silicone oil given the overall variability in the stability profile and related image analysis.

Control syringes containing no added silicone oil still have silicone oil present, due to the siliconization of the plunger which is required for insertion into the syringe barrel. The particle counts for the control syringes (0X) are consistent for both drug product and placebo solutions, and they are generally lower than syringes siliconized to the target 1X silicone oil level. This data also demonstrates that the drug product solution is not sensitive to silicone oil induced particle formation, even when exposed to double the quantity of silicone oil expected to be present in the syringe barrel.

Overall, the silicone oil coating on the syringe barrel of the prefilled syringe was the primary contributor of particulate counts detected at the ≥ 2 and 5 micrometer size range. The drug product has been shown to be stable with respect to subvisible particles at exposures up to twice the target silicone oil level for the prefilled syringe.

Case Study 2.4: Post-marketing Change of Primary Packaging of a Prefilled Syringe Product

Experimental

A new product version filled in sprayed-on siliconized, staked-in needle PFS (SIN-PFS) was developed for an already marketed biotech product, which was previously filled into luer-type baked-in siliconized PFS. The SIN-PFS configuration would allow for use with an autoinjector or needle-safety device. The changes related to the product are summarized in Table 6.

To assess and compare the pre- and post-change configuration of Product X, the stability of both configurations was evaluated at intended and accelerated storage conditions, using a range of analytical methods to test for pharmaceutical quality (pH, osmolality, sterility, endotoxins, color, clarity), protein content, content of polydimethylsiloxane (silicone), protein degradation (oxidation, deamidation, clipping, disulfide scrambling), soluble aggregates, subvisible and visible particles [47]. The analytical methods used were the same ones used for the currently marketed product configurations, partly augmented by further extended characterization analyses.

Subvisible particle analysis was performed for drug products using the compendial light obscuration (LO) testing methodology. The measurements described in this study were performed in compliance with Ph. Eur. 2.9.19 and USP <788> requirements. Measurement of subvisible particle levels of ≥ 2 and ≥ 5 μm sizes have been performed as an additional product characterization during stability testing of some SIN-PFS fill/finish batches. To further characterize the subvisible particles analyses were performed using flow imaging (Microflow Imaging™, FlowCAM®) and light microscopy.

Results and Discussion

There were no differences in the protein characteristics and no data suggested any increase in protein aggregation or other degradation pathways due to the change in syringe type or by increased amount of free silicone oil. No differences were found in the visible particles during inspection of the pre- and post-change product configurations; the product remained “essentially free of visible particles.”

Expected differences were found as follows: (1) The content of polydimethylsiloxane (PDMS) by inductively coupled plasma-atomic emission spectrometry (ICP-AES) was found to be higher in SIN-PFS compared to the luer-type syringes. ICP-AES determines silicone levels based on conversion to PDMS values, which is the most widely used silicone-based polymer. This finding was anticipated for a SIN syringe system and was attributed to the difference in siliconization processes, i.e., “baked” vs “free” silicone oil. (2) Subvisible particles were increased in the SIN-PFS compared to the luer-type syringe, both at release, as well as during the stability studies. This finding was also expected and attributed to the silicone oil itself, resulting from the difference in siliconization processes, i.e., “baked” vs “free” silicone oil [9].

During release and stability testing of the SIN-PFS, the subvisible particle counts of the ≥ 10 and ≥ 25 μm sizes were found to be higher than the corresponding values determined in the “luer type” syringes (Tables 7 & 8). Despite this expected difference (i.e., “baked” vs “free” silicone [8]), the values were still far below the compendial specifications of USP <788>. Nevertheless, extended characterization of the subvisible particles was performed and is described below.

As shown in Table 9, the number of droplet-like particles was significantly lower in luer-type syringes compared to SIN-PFS. Based on the corresponding reference images, the detected particles were not suggestive of protein particles, but rather were attributed to silicone oil or air bubbles. Using the FlowCAM[®] methodology (with 10x magnification), more than 95% of the subvisible particles detected were classified as silicone oil droplets by the statistical filter integrated into the instrument's software. The population of particles which were not classified as silicone oil droplets based on their morphological characteristics was negligible. Silicone oil droplets were also found in the currently marketed “luer type” syringes; however, the number was significantly lower as compared to that of a SIN-PFS. No particles with morphology typical of protein particles (i.e., irregular shape) were found in either syringe presentation.

When analyzing SIN-PFS compared to luer type syringes by the means of light microscopy, an increased number of droplet-like particles having a round shape were observed (Table 10). Since these particles appear as bright circles surrounding a darker interior, their optical properties allow them to be attributed with high certainty to silicone oil and are likely not related to air bubble.

As pointed out earlier it was not unexpected to observe differences in the silicone oil droplet content between syringes produced using different siliconization processes. More specifically, it was anticipated that the luer type syringe (pre-change) siliconized using a baked-in siliconization process may contain lower amounts of free silicone as compared to the staked in needle syringe (post-change) which was siliconized using a spray-on siliconization process. The latter process produces a silicone layer that is more readily mobilized from the wall of the syringe and therefore likely results in higher amounts of free silicone in the syringe as compared to syringes produced using the former process.

In summary, subvisible particle levels measured by light obscuration were increased in the post-change material (SIN-PFS) compared to the luer-type configuration. The specified subvisible particle counts (≥ 10 and ≥ 25 μm) were below the compendial and drug product specifications, and no significant increase in particles were observed during stability testing (up to 24 months). The higher levels of subvisible particles observed in the SIN-PFS were shown to be related to “free” silicone oil, and no evidence was found to indicate that these particles are proteinaceous. The latter conclusion was confirmed by the investigations related to the characterization of the subvisible particles and by determination of the PDMS content being significantly higher in the SIN-PFS as compared to the “luer type” syringes. To a lesser extent, “free” silicone oil was also found in the “luer type” syringes. The increased silicone oil levels in the SIN-PFS do not affect the product quality. The product was considered equivalent with regard to potency, purity and safety. Subsequently, it was concluded that no further preclinical or clinical evaluations were required to be performed. The post-change product configuration has been approved.

Conclusions from Section 2: Applications of Particle Characterization in Drug Product Development, Stability Studies and Post-Marketing Changes

Expanding light obscuration measurements to include 2 μm to 10 μm size data is a relatively simple way to expand our knowledge around subvisible particulate matter, as the basic method is already routinely utilized for release and stability of biological products. The addition of orthogonal techniques to further characterize the subvisible particles provides insight into the origin of the particles. Application of these techniques can be used successfully for process development and product stability.

The interpretation of subvisible particulate matter stability data can be challenging because the measurement variability makes it difficult to interpret meaningful differences until very large data sets are available. Relatively large differences (2-3 fold) between individual measurements cannot be considered significant in most cases. Despite these limitations, when sufficient data is combined, it does appear that global assessments of the stability trajectory can be made on a product (versus lot) basis.

Finally, in the case of a post-marketing change for a container closure system subvisible particle characterization provided insights which contributed to the assessment of the suitability of the product change.

Overall Conclusions

In recent years, regulatory expectations for product characterization of more complex biotherapeutic parenteral drug products, which may have the propensity to form proteinaceous

particulate matter through self-association, have expanded to extend subvisible particle testing beyond the compendial limits into the 2 to 10 µm range and to include orthogonal methods for characterization. This regulatory expectation includes collection of data on supporting development studies, drug product clinical supplies, stability batches including stress conditions, and sponsors may be requested to include these lower subvisible testing ranges as part of comparability studies and drug product validation.

From a quality control testing perspective, light obscuration remains the primary method for testing subvisible particulate matter. The 2 to 10 µm range can be characterized by extending the light obscuration testing range, in conjunction with other techniques available (i.e., flow imaging allowing for morphological characterization), that are needed to distinguish proteinaceous particles from other particulates (i.e., silicon oil droplets).

The case study examples in this paper demonstrate the importance of characterizing subvisible particles to provide technical support for formulation and manufacturing process development, and product stability. Additionally, the variability in particle count requires that sufficient data is combined to assess subvisible particle formation rather than relying on individual time points.

The overall goal for using subvisible analysis throughout development is to acquire a set of data which justifies the final drug product design and its control strategy. As illustrated in the case studies herein, a thorough understanding of subvisible particle properties and stability profiles, can support the use of current compendial requirements for routine drug product quality control purposes. For drug products where this type of knowledge is not as available, a more extensive strategy may be required.

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References

- [1] US Pharmacopeia. 2015. USP/NF General Chapter <788>. Particulate matter in injections. In US Pharmacopeia, National Formulary, USP38-NF-33, 37(6). Rockville, MD: United States Pharmacopeial Convention.
- [2] US Pharmacopeia. 2015. USP/NF General Chapter <787>. Subvisible particulate matter in therapeutic protein injections. In US Pharmacopeia, National Formulary, USP38-NF-33, 39(2). Rockville, MD: United States Pharmacopeial Convention.
- [3] Narhi LO, Corvari V, Ripple D, Afonina N, Cecchini, DeFelippis MR, et al. 2015. Subvisible (2 μm to 100 μm) particle analysis during biotherapeutic drug product development: part 1, considerations and strategy. *J Pharm Sci*, 104(6): 1899-1908.
- [4] Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJA, Middaugh CR, Winter G, et al. 2009. Overlooking subvisible particles in therapeutic protein products: Gaps that may compromise product quality. *J Pharm Sci* 98(4):1201-1205.
- [5] Luo Q, Joubert MK, Stevenson R, Ketchem RR, Narhi LO, Wypych J. 2011. Chemical modifications in therapeutic protein aggregates generated under different stress conditions. *J Biol Chem* 286(28):25134–25144.
- [6] Joubert MK, Luo Q, Nashed-Samuel Y, Wypych J, Narhi L O. 2011. Classification and characterization of therapeutic antibody aggregates. *J Biol Chem* 286(28), 25118-25133.
- [7] Filipe V, Jiskoot W, Basmeleh AH, Halim A, Schellekens H, Brinks V. 2012. Immunogenicity of different stressed IgG monoclonal antibody formulations in immune tolerant transgenic mice. *mAbs* 4(6):740-752.
- [8] Scherer TM, Leung S, Owayang L, Shire SJ. 2012. Issues and challenges of subvisible and submicron particulate analysis in protein solutions. *AAPS J* 14(2):236-243.
- [9] Singh SK, Afonina A, Awwad M, Bechtold-Peters K, Blue JT, Chou D, et al. 2010. An industry perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. *J Pharm Sci* 99: 3302-3321.
- [10] Ripple DC, Dimitrova MN. 2012. Protein Particles: What we know and what we don't know. *J Pharm Sci* 101(10):3568-3579.
- [11] European Pharmacopoeia (7.0). 2010. 2.19.19 Particulate contamination: Sub-visible particles. 7th ed, Supplement 7.2:3333-3335.
- [12] The Japanese Pharmacopoeia. 2006. Insoluble particulate matter test for injections. 15th ed. 6.07:111-113.
- [13] Das TK. 2012. Protein particulate detection issues in biotherapeutics development-current status. *AAPS Pharm Sci Tech* 13(2):732-746.

- [14] 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.
- [15] Demeule B, Messick S, Shire SJ, Liu J. 2010. Characterization of particles in protein solutions: Reaching the limits of current technologies. *AAPS J* 12(4):708-715.
- [16] Zöls S, Tantipolphan R, Wiggenghorn M, Winter G, Jiskoot W, Friess W, et al. 2011. Particles in therapeutic protein formulations, Part 1: Overview of analytical methods. *J Pharm Sci* 101(3):914-935.
- [17] Singh SK, Toler MR. 2012. Monitoring of subvisible particles in therapeutic proteins. *Methods Mol Biol* 899:379-401.
- [18] Groves MJ. 1991. Particulate contamination in parenterals: Current issues. *Boll Chim Farm* 130:347-354.
- [19] Narhi LO, Schmit J, Bechtold-Peters K, Sharma D. 2012. Classification of protein aggregates. *J Pharm Sci* 101:493-498.
- [20] Liu L, Randolph TW, Carpenter JF. 2012. Particles shed from syringe filters and their effects on agitation-induced protein aggregation. *J Pharm Sci* 101:2952-2959.
- [21] Cao S, Jiang Y, Narhi L. 2010. A Light Obscuration Method Specific for Quantifying Subvisible Particles in Protein Therapeutics. *Pharm Forum* 36:3020.
- [22] European Pharmacopeia 2011, Ph. Eur. 2.9.19 Particulate Contamination : Sub-Visible Particles.
- [23] Kumru OS, Liu J, Ji JA, Cheng W, Wang YJ, Wang T, et al. 2012. Compatibility, physical stability, and characterization of an IgG4 monoclonal antibody after dilution into different intravenous administration bags. *J Pharm Sci* 101(10), 3636-3650.
- [24] Sharma DK, King D, Oma P, Merchant C. 2010. Micro-flow imaging: Flow Microscopy applied to sub-visible particulate analysis in protein formulations. *AAPS J* 12(3):455–464.
- [25] Barnard JG, Singh S, Randolph TW, Carpenter JF. 2011. Subvisible particle counting provides a sensitive method of detecting and quantifying aggregation of monoclonal antibody caused by freeze-thawing: Insights into the roles of particles in the protein aggregation pathway. *J Pharm Sci* 100(2):492-503.
- [26] Wuchner K, Buchler J, Spycher R, Dalmonte P, Volkin DB. 2010. Development of a Microflow imaging assay to characterize protein particulates during storage of a high concentration IgG1 monoclonal antibody Solution. *J Pharm Sci* 99(8):3343-3361.
- [27] Strehl R, Rombach-Riegraf V, Diez M, Egodage K, Bluemel M, Jeschke M, et al. 2012. Discrimination between silicone oil droplets and protein aggregates in biopharmaceuticals: a

novel multiparametric image filter for sub-visible particles in microflow imaging analysis. *Pharm Res* 29(2): 94-602.

[28] Zölls S, Gregoritz M, Tantipolphan R, Wiggernhorn M, Winter G, Friess W, et al. 2013. How subvisible particles become invisible-relevance of the refractive index for protein particle analysis. *J Pharm Sci* 102(5):1434-1446.

[29] Sharma DK, King D, Oma P, Pollo JJ, Sukumar M. 2010. Quantification and Characterization of subvisible proteinaceous particles in opalescent mAb formulations using micro-flow imaging. *J Pharm Sci* 99(6):2628-2642.

[30] Garidel P, Boese M. 2007. Non-Invasive Fourier Transform Infrared Microspectroscopy and Imaging Techniques: Basic Principles and Applications. Modern Research and Educational Topics in Microscopy. Méndez-Vilas and J. Díaz (Eds.).

[31] Liu W, Swift R, Torraca G, Nashed-Samuel Y, Wen ZQ, Jiang Y, Freund E, Davis J, Narhi, LO. 2010. Root cause analysis of tungsten-induced protein aggregation in pre-filled syringes. *PDA Journal of Pharmaceutical Science and Technology*, 64(1):11-19.

[32] Mach H, Bhambhani A, Meyer BK, Burek S, Davis H, Blue JT, et al. 2011. The use of flow cytometry for the detection of subvisible particles in therapeutic protein formulations. *J Pharm Sci* 100:1671-1678.

[33] Ludwig DB, Trotter JT, Gabrielson JP, Carpenter JF, Randolph TW. 2011. Flow cytometry: a promising technique for the study of silicone oil-induced particulate formation in protein formulations. *Anal Biochem* 410:191-199.

[34] Dong A, Huang P, Caughey WS. 1990 Protein secondary structures in water from second-derivative amide I infrared spectra. *Biochemistry (NY)* 29:3303-3308.

[35] 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. *J Pharm Sci* 98(9):3167-3181.

[36] Tyagi AK, Randolph TW, Dong A, Maloney K, Hitscherich JR, Carpenter JF. 2009. IgG particle formulation during filling pump operation: A case study of heterogeneous nucleation on stainless steel nanoparticles. *J Pharm Sci* 98(1):94-104.

[37] Nayak A, Colandene J, Bradford V, Perkins M. 2011. Characterization of subvisible particle formation during the filling pump operation of a monoclonal antibody solution. *J Pharm Sci* 100(10):1-7.

[38] Chi EY, Weickmann J, Carpenter JF, Manning MC, Randolph TW. 2005. Heterogeneous nucleation-controlled particulate formation of recombinant human platelet-activating factor acetylhydrolase in pharmaceutical formulation. *J Pharm Sci* 94(2):256-274.

- [39] Kalonia C, Kumru OS, Kim JH, Middaugh CR, Volkin DB. 2013. Radar chart array analysis to visualize effects of formulation variables on IgG1 particle formation as measured by multiple analytical techniques. *J Pharm Sci* 102(12):4256-4267.
- [40] Mahler HC, Friess W, Grauschopf U, Kiese S. 2009. Protein aggregation: Pathways, induction factors and analysis. *J Pharm Sci* 98(9):2909-2934.
- [41] Jones LS, Kaufmann A, Middaugh CR. 2005. Silicone oil induced aggregation of proteins. *J Pharm Sci* 94(4):918-27.
- [42] Kiese S, Pappengerger A, Friess W, Mahler HC. 2008. Shaken, not stirred: mechanical stress testing of an IgG1 antibody. *J Pharm Sci* 97(10):4347–4366.
- [43] Auge K, Blake-Haskins A, Devine S, Rizvi S, Li Y, Hesselberg M, et al. 2011. Demonstrating the stability of albinferon alfa-2b in the presence of silicone oil. *J Pharm Sci* 100:5100-5114
- [44] Majumdar S, Ford BM, Mar KD, Sullivan VJ, Ulrich RG, D'Souza AJM. 2011. Evaluation of the effect of syringe surfaces on protein formulations. *J Pharm Sci* 100:2563-2573.
- [45] Cromwell ME, Hilario E, Jacobson F. 2006. Protein aggregation and bioprocessing. *AAPS J* 8:E572-E579.
- [46] Cao S, Jiao N, Jiang Y, Mire-Sluis A, Narhi LO. 2009. Subvisible particle quantitation in protein therapeutics. *Pharm Bio Sci Notes*. 1:73-9.
- [47] US Pharmacopeia. 2015. USP/NF General Chapter <790>. Visible particulates in injections. In US Pharmacopeia, National Formulary, USP38-NF-33, 38(6). Rockville, MD: United States Pharmacopeial Convention.

A.

B.

Figure 1. Results of light obscuration (A) and flow imaging (B) analysis for rP 3 drug product presentation: Initial, 5 min and 10 min kept under vacuum and 2 hours (no vacuum).

A.

B.

Figure 2. Particle and air bubble count in rP 3 samples using flow imaging analysis with MVAS soft filter screen: Initial (A) and 10 min kept under vacuum (B)

Figure 3. Classification of particles > 10 μm for drug product contained in prefilled syringes using flow imaging analysis, Overlay of sub-populations (see Table 1 for the sub-population classification criteria)

A.

B.

C.

Figure 4. Differentiation of particle types based on morphological analysis using flow imaging: (A) variability in the non-spherical sub-visible particle counts $\geq 5 \mu\text{m}$ observed amongst drug product (DP) units derived from 3 lots; (B) examination of the non-spherical particles from placebo demonstrated the presence of silicone oil doublets in this formulation, which were misclassified by the original algorithm; (C) application of a proprietary filter resulted in comparable improved analysis.

Figure 5. Flow cytometry analysis of particles in drug product (DP). Data from syringes with DP were pooled together and sorted. The collected particles were re-analyzed by flow cytometry. Two distinct populations were observed, the silicon oil sorts as the green particles while the other particles including proteinaceous and non-proteinaceous subvisible particles are highlighted in red.

Figure 6. Subvisible particle analysis by light-obscuration of drug product (DP) in syringes or in autoinjector device. Particle concentration of an IgG formulation in DP syringes manually expelled (left bars) or in same syringes expelled by an autoinjector device (right bars).

Figure 7. MAb X particle-forming lot after 4 months at 4 °C. Multiple translucent visible particles were observed (several individual particles are circled)

Figure 8. Representative particles in particle-forming MAb X lot stored for four months at 4 °C observed in a visual mode of the FTIR microscope.

Figure 9. Transmittance IR spectra of particles from particle-forming MAb X lot acquired by FTIR microscope. (A) Overlay of spectra from individual particles shows presence of dominant Amide I and Amide II bands characteristic of proteins. (B) Several particles exhibited faint bands consistent with presence of trace amounts of silicone oil.

Figure 10. Second derivative IR spectra of Amide I band signal acquired with FTIR microscope for individual particles isolated from particle-forming MAb X lot solution.

Figure 11. Representative image of particles in post-process optimization MAb X lot sample stored at +4 °C. Particles were captured on an aluminum oxide filter and observed in a visual mode of the FTIR microscope.

Figure 12. IR spectra of particles from a representative post-process optimization MAb X lot sample acquired by FTIR microscope. IR spectra of individual particles shows presence of weak Amide I and Amide II bands characteristic of protein.

Figure 13: Effect of drug product filling technology on the formation of subvisible particles analyzed using flow imaging analysis.

Figure 14. Release and stability data for Product 1 using light obscuration analysis. Solid lines represent the $\pm 3SD$ values of the data sets at each time point. Dashed lines represent the average value of the data set from each time point. The diamonds and triangles for the release data represent the two different configurations (fill volume) at time 0. The remaining colorized data point symbols (circle, square, diamond and triangle) represent stability data from those lots placed on stability. The $\pm 3 SD$ result in a negative Y axis, however all particle measurements were positive values.

Figure 15. Release and stability data for Product 2 using light obscuration analysis. Solid lines represent the $\pm 3SD$ values of the data sets at each time point. Dashed lines represent the average value of the data set from each time point. The diamonds represent the release data at time 0. The remaining colorized data point symbols (square, circle, triangle and square) represent stability data from those lots placed on stability. The $\pm 3 SD$ result in a negative Y axis, however all particle measurements were positive values.

Figure 16. Scatter plot of 2-10 μm and > 10 μm light obscuration data for Product 1. Solid line represents the best linear fit.

Figure 17. Effect of silicone oil on particulate matter by light obscuration at 25°C, error bars indicate 3σ . (Data plotted on y-axis is particles per container ≥ 2 , ≥ 5 , ≥ 10 and $\geq 25 \mu\text{m}$)

Figure 18. Effect of silicone oil on particulate matter by flow imaging at 25°C, error bars indicate 3σ. (Data plotted on y-axis is particles per container ≥ 2 , ≥ 5 , ≥ 10 and ≥ 25 μm).

Figure 19: Representative flow images of ≥ 5 μm particulate matter from drug product and placebo syringes siliconized 1X after storage for 24 weeks at 25 °C. (Figure A: Drug Product 1X, B: Placebo 1X)

Table 1. Summary of the subvisible particles classification of drug product contained in prefilled syringes and cartridges by flow imaging

Sample	ECD (µm)	Percent of the entire population (observed range)	Aspect Ratio	Intensity Mean	Sub Population	Percent of the entire population (observed range)
Samples in syringe	<10	95 % to 97 %	≥0.80	<750	Air bubbles	13 % to 20 %
				≥750	Spherical non-proteinaceous and proteinaceous particles	49 % to 55 %
			<0.80	n.a.	Non-proteinaceous and proteinaceous particles	23 % to 29 %
	≥10	3 % to 5 %	<0.85	<550	Fibers	0.3 % to 1 %
				≥550 and <700	Dark non-proteinaceous and proteinaceous particles	1 % to 3 %
				≥700	Non-proteinaceous and proteinaceous particles	1 % to 2 %
			≥0.85	<600	Silicone oil droplets	0.3 % to 1.3 %
				≥600	Air bubbles	1 % to 2.3 %
Samples in cartridge	<10	88 % to 92 %	≥0.80	<700	Air bubbles	2 % to 6 %
				≥700	Spherical non-proteinaceous and proteinaceous particles	57 % to 73 %
			<0.80	n.a.	Non-proteinaceous and proteinaceous particles	17 % to 25 %
	≥10	8 % to 12 %	≥0.85	<680; ≥680 and <800 + circularity <0.5	Fibers	1 % to 7 %
				≥800	Non-proteinaceous and proteinaceous particles	2 % to 5 %
			>0.85	n.a.	Air bubbles	2 % to 5 %

Table 2: Impact of primary packaging on measured particle concentration (IgG formulation) determined by flow imaging analysis

Number of particles (ECD) per mL							
≥2 <3 µm	≥3 <5 µm	≥5 <10 µm	≥10 <25 µm	≥25 <45 µm	≥45 <70 µm	≥70 µm	

IgG before filling into syringes	1255	260	123	28	1.4	0.0	0.0
IgG after filling into syringes	8488	7651	16020	1746	8.7	0.9	0.0
Reported particle levels after morphological filtering for IgG filled into syringes	7928	504	323	149	2.5	0.0	0.0

Table 3: Impact of silicone oil droplets on the assay precision of 10 single placebo syringes measured individually by flow imaging

	Number of particles (ECD) per mL						
	≥ 2 <3 μm	≥ 3 <5 μm	≥ 5 <10 μm	≥ 10 <25 μm	≥ 25 <45 μm	≥ 45 <70 μm	≥ 70 μm
Average number of particles	18535	10908	9469	2140	71	1.3	0.0
RSD%	45%	35%	29%	20%	29%	110%	NA
Minimum	11935	6622	6900	1542	43	0.0	0.0
Maximum	41185	20717	15674	2855	113	4.3	0.0

NA: the RSD% calculation is not applicable to this data

Table 4. Subvisible and visible particles in particle-forming MAb X lot detected by flow imaging, semi-quantitative visual appearance, and FTIR microscopy methods

Time at 4 °C (months)	Subvisible particles (particles/mL)		Visible particles per container*	Protein particles detected by FTIR microscopy
	$\geq 10 \mu\text{m}$	$\geq 25 \mu\text{m}$		
0	2955	268	1-3 visible particles	Not performed
0.5	188	13	5-10 visible particles	Not performed
1	308	13	5-10 visible particles	Not performed
2	540	210	>10 visible particles	Not performed
2.5	693	39	>10 visible particles	Not performed
4	143	87	>10 visible particles	>>20 particles (only 20 particles were analyzed)

* Semi-quantitative visual appearance method involved an approximate assessment of number of visual particles observed in each vial

Table 5. Subvisible and visible particles in post-process optimization MAb X lot samples stored at 4 °C detected by flow imaging, visual appearance, and FTIR microscopy

Time at 4 °C (months)	Subvisible particles (particles/mL)		Visible particles per container*	Protein particles detected by FTIR microscopy
	$\geq 10 \mu\text{m}$	$\geq 25 \mu\text{m}$		
0	238	6	No visible particles*	0
3	42	9	No visible particles*	1
6	97	8	No visible particles*	6
9	448	20	1-2 visible particles*	3
12	224	0	No visible particles*	1
15	227	0	No visible particles*	1
18	1232	17	No visible particles*	2
24	1195	33	No visible particles*	Not performed

* Semi-quantitative visual appearance method involved an approximate assessment of number of visual particles observed in each vial

Table 6. Comparison of product changes

	Pre-Change	Post-Change	Comparison
Description	Luer-type PFS	Staked-in needle PFS	-
	-
Type of Glass and dimensions	USP/Ph. Eur. Type I 1-mL long syringe	USP/Ph. Eur. Type I 1-mL long syringe	Same
Adhesive	No	Yes	Different
Siliconization process	“baked” on siliconization	Spray siliconization (free silicone)	Different
Type of Silicone	Dimethicone emulsion	Dimethicone oil	Similar

Table 7. Subvisible particle levels measured by light obscuration in SIN-PFS in comparison to Luer-type PFS (product X)

Subvisible Particle Levels	Pre-Change: Luer-Type Syringes (Batches A and B) (particles/container)	Post-Change: SIN-PFS (Batches A and B) (particles/container)
$\geq 10\text{ }\mu\text{m}$		
Mean	29	200
Max	93	303
Min	6	88
$\geq 25\text{ }\mu\text{m}$		
Mean	0	3
Max	2	5
Min	0	1

Table 8. Subvisible particle levels measured by light obscuration in SIN-PFS in comparison to Luer-type PFS (product X) – stability data (at intended storage conditions)

Subvisible Particle Levels	Storage time/ temperature	Pre-Change: Luer-Type Syringes (Batches A and B) (particles/container)	Post-Change: SIN-PFS (Batches C, D, E) (particles/container)
$\geq 2 \text{ um}$	Initial	1201 – 1534	907 – 4788
	1 month/5 °C	565 – 1355	1439 – 4542
	3 months/5 °C	1118 – 1602	4734 – 8380
	6 months/5 °C	873 – 1292	2389 – 8363
$\geq 5 \text{ um}$	Initial	265 – 336	272 – 1931
	1 month/5 °C	110 – 283	363 – 1384
	3 months/5 °C	234 – 339	861 – 2056
	6 months/5 °C	165 – 268	680 – 1996
$\geq 10 \text{ um}$	Initial	46 – 53	88 – 303
	1 month/5 °C	14 – 42	67 – 235
	3 months/5 °C	37 – 38	106 – 323
	6 months/5 °C	22 – 29	120 – 333
$\geq 25 \text{ um}$	Initial	0 – 2	1 – 5
	1 month/5 °C	0 – 2	3 – 7
	3 months/5 °C	0	2 – 8
	6 months/5 °C	0	3– 9

Table 9. Characterization of subvisible particles in luer-type and SIN-PFS by flow imaging

Representative images of subvisible particles $\geq 10\text{ }\mu\text{m}$	
Pre-Change: Luer-Type Syringes (Batch A, stored 5 months/5 °C)
Post-Change: SIN-PFS (Batch D, stored 9 months/ 5 °C)

Table 10. Characterization of subvisible particles in luer-type and SIN-PFS by light microscopy

Representative images of subvisible particles

Pre-Change:
Luer-Type Syringes
(Batch A, stored 5
months/5 °C)

Post-Change:
SIN-PFS
(Batch D, stored 9
months/ 5 °C)