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Variability in Flow-Imaging Microscopy Measurements and Considerations for Biopharmaceutical Development

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ABSTRACT

Flow-imaging microscopy is widely used in the biopharmaceutical industry to characterize populations of subvisible (1-100 μm) particles due to high sensitivity and the ability to discriminate different particle morphologies. The present work provides a comprehensive assessment of the capabilities of flow-imaging microscopy by exploring the impacts of a variety of factors on the observed variability of these measurements. A novel graphical presentation is proposed to facilitate both determination of expected levels and detection of potential atypical results. Data collected across different products and container-closure systems illustrate that a substantial amount of historical experience is typically required to adequately define the expected levels of subvisible particles for any specific system. It is also shown, however, that an appropriate level of control can be demonstrated without the need to pool large numbers of containers or perform replicate measurements.

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Introduction

The development and manufacture of biopharmaceuticals have made significant strides since the introduction of recombinant insulin, yet aggregate formation remains an inherent property of these protein drug formulations.[1-3] In addition, there is potential for formation of proteinaceous particles through nucleation from intrinsic particles.[4-7] Given the risk for heterogeneous nucleation, there is concern that even without changes to the formulation or container and closure system, the number and type of protein particles present in a product can vary during the filling process.[8,9] It is therefore recommended that aggregates of all sizes be appropriately monitored and characterized during development as a potential component of the overall control strategy.

Particles of primary concern are in the size range from approximately 0.1 to 100 μm. Although visual inspection is probabilistic,[10] 100 μm is often cited as the size at which visual inspection can identify particles with reasonable probability[11]; below 0.1 μm, size exclusion chromatography provides accurate, precise measurements of aggregate levels.[12,13] Light obscuration (LO) is routinely employed for measuring particles greater than 10 μm.[14-16] However, this methodology may underestimate populations of smaller transparent particles,[13,17-19] can be dependent on the matrix,[13,20-22] and cannot distinguish particle morphology.[13,23,24] Flow-imaging microscropy is an orthogonal technology to LO to analyze particles less than 10 μm in size. It provides high sensitivity in detecting and imaging transparent particles and has a unique capability to differentiate subpopulations of particles with different morphologies, making it possible to distinguish between potentially proteinaceous particles and other types of particles such as silicone oil microdroplets and air bubbles.[17]

There are many examples highlighting the use of flow-imaging microscopy for characterization of sub 10-μm particles in parenteral bioproducts.[18,21,23-26] However, a systematic and comprehensive evaluation of the impact of product, container and closure types, sampling strategy, and number of measurement replicates required for flow-imaging microscopy analysis has not been completed. The current work explores these factors in detail, presents a multivariate framework for characterizing expected levels of subvisible particles, and provides specific recommendations for presentation and interpretation of flow-imaging microscopy data. Attempting to fully elucidate the underlying root causes of the observed differences between different products and container-closure systems is left to future work.

Materials and Methods

A variety of materials in different container-closure systems were investigated as part of this study. The materials include

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several classes of therapeutic biopharmaceuticals and placebo (Table 1). The concentration of the drug products ranged from 3 mg/mL to 120 mg/mL and covered 3 container types; prefilled syringes, vials, and cartridges. All drug products were liquid solutions in aqueous buffer systems. Two fill volumes in the prefilled syringes were evaluated; 0.5 and 1 mL. In addition to the materials summarized in Table 1, a National Institute of Standards and Technology traceable 5-μm polystyrene bead standard (Thermo Scientific, Waltham, MA) was evaluated to help assess method variability.

Experimental Design

A full-factorial experimental design was used to assess the impact of multiple analysts, instruments, sample pooling strategies, and replication strategies across multiple sample and container types on particle level and variability. In general, each sample type in its corresponding container was analyzed on 6 separate occasions by 2 analysts using 3 instruments. For each analysis, the sample was prepared using 3 pooling strategies to determine the minimum amount of sample required for analysis without adversely impacting the variability of the particle data. To assess the triplicate injection variability and to allow for an initial injection to flush the system with the material of interest, a minimum of 5-mL volume was needed. This constraint was the basis for the minimum number of units pooled for analysis. The requirement for number of units/volume needed for United States Pharmacopeia <788> by LO was used to define the highest number of units pooled for analysis. See Table 1 for additional details. The 3 levels of pooling were 5-mL, 10-mL, and 25-mL pools for the syringe products; 3, 5, and 10 vials (i.e., a range of 30-200 mL) for the vial products; and 3, 5, and 10 cartridges (i.e., a range of 9-30 mL) for the cartridge product. Each prepared sample was then analyzed 4 times to determine whether the first replicate needed to be used as a flush and to evaluate the impact of the replication strategy on the variability of the reported data.

Microflow Imaging

Microflow imaging (MFI) measurements were performed using 3 equivalent DPA 4200 systems (Protein Simple, Santa Clara, CA) located in 3 different laboratories. All systems were equipped with 100 μm, 1.6 mm, silane-coated flow cells (Protein Simple). Polystyrene bead standard was introduced using a 10-mL glass syringe. All other samples were gently pooled into particle-free glassware (Thermo Scientific, Waltham, MA) in a laminar flow hood to minimize potential interference from air bubbles or extrinsic particles from the environment. Pooled samples were allowed to equilibrate at room temperature for at least 1 h before injection.

Table 1
Summary of Materials and Pooling Levels

Product	Concentration (mg/mL)	Container Type	Fill Volume (mL)	Pooling Levels	
				Units (#)	Total Volume (mL)
P265	3	PFS 1	0.5	10, 20, 50	5, 10, 25
M426	120	PFS 2	1	5, 10, 25	5, 10, 25
	80	PFS 2	1	5, 10, 25	5, 10, 25
M294	120	PFS 1	1	5, 10, 25	5, 10, 25
M300	20	Vial	20	3, 5, 10	60, 100, 200
P541	20	Vial	10	3, 5, 10	30, 50, 100
	20	Cartridge	3	3, 5, 10	9, 15, 30
Placebo	N/A	PFS 2	1	5, 10, 25	5, 10, 25

PFS 1, 1-mL long 29 G TW BD Hypak for Biotech syringe with FN siliconization; PFS 2, 1-mL long 27 G STW BD Hypak for Biotech syringe with DN siliconization.

Table 2
Definitions of Additional Reportable Parameters of Potential Interest in Microflow Imaging Measurements

Parameter	Definition
Circular particles ≥5 μm	ECD ≥5 μm and AR ≥0.85
Noncircular particles ≥5 μm	ECD ≥5 μm and AR <0.85
Circular fraction ≥5 μm	(ECD ≥5 μm and AR ≥0.85)/ECD ≥5 μm
Noncircular fraction ≥5 μm	(ECD ≥5 μm and AR <0.85)/ECD ≥5 μm

Each injection used a purge volume of 0.2 mL, an analyzed volume of 0.6 mL, and a flow rate of 0.17 mL/min. Illumination optimization was performed at the start of each injection using the sample itself. Data were collected and analyzed using MVSS software version 2 (Protein Simple), automatically converting the raw 10-bit grayscale images of the particles to binary (black/white) representations to extract information on size and morphology. Equivalent circular diameter was used as the measure of particle size throughout, defined as the diameter of a circle having the same total area as the binary representation. Nominal performance of each instrument was verified at the start of each measurement day using a criterion of ≤200 counts/mL (particles ≥1 μm) measured in 0.22-μm filtered water.

The first of 4 readings was treated as a system flush, and injections 2 through 4 treated as triplicate readings. Evaluation of the first injection (see Supplementary Fig. S1) verified that the common practice of flushing the system with sample material is an important and effective step.[18,20,21,27] Even after the system flush, however, there were instances where some results appeared atypical based on visual inspection of the data. These replicates were investigated further and found to contain a far greater number of false counts—images falsely attributed as particles—than the next closest replicate for the same sample run and excluded from further analysis (additional detail is provided in Results).

Several approaches have been described for creating image filters with the intent of discriminating between silicon oil microdroplets and air bubbles and potentially proteinaceous particles,[17,24,28] which is important to deliver a robust characterization package and ensure product quality. Sharma et al.[29] demonstrated that reliable results could be obtained using a simple, single parameter filter based on particle aspect ratio (AR), where less circular particles have an AR approaching zero. Therefore, in addition to total particle counts ≥2 and ≥5 μm, 4 related reportable parameters of potential interest are defined (Table 2).

MFI data were statistically analyzed using JMP® software 11.1.1.

Results

Defining Expected Levels and Detecting Atypical Results

Counts for particles ≥5 μm for each of the triplicate readings are shown in Figure 1. Because particle counts ≥2 μm were highly correlated with counts ≥5 μm (see Supplementary Fig. S2), and no morphological information can be obtained for this size, the current work focuses on the larger particle counts. Correlation between particle counts at different size ranges has been observed elsewhere.[30] Although the variability between triplicate particle counts is relatively low, occasionally an injection differed greatly from the other two. Points that stood out through visual inspection were subsequently investigated and found to contain falsely high particle counts are depicted as solid circles. Using the MFI View Analysis Suite software (version 2), it was determined that the atypical results stemmed from either background noise getting

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