Precise Subvisible Particle Analysis with 10X Less Sample

Introduction

Monitoring protein aggregation in a biologic drug is crucial because aggregates can limit a product's shelf life[1–4] and is a leading indicator of a therapy's potential immunogenic threat. Measuring subvisible protein aggregates early in the development process is critical for ensuring formulation stability because by the time hundreds of microscopic protein aggregates can be measured, the formulation's stability may already be at serious risk. As a result, the FDA recommends that "strategies to minimize aggregate formation should be developed as early as feasible in product development"[5] and "an assessment should be made of the range and levels of subvisible particles (2–10 µm) present in therapeutic protein products initially and over the course of the shelf life."[5] Yet to this day, low volume subvisible protein aggregation analysis has remained elusive to biopharmaceutical researchers.

Both light obscuration (LO) and flow imagers (FI) can measure subvisible protein aggregation but require at least several hundreds of microliters of sample to do so. In biopharmaceuticals, however, this amount of sample may only be available in late stage development. Since the presence of microscopic protein aggregates point to an unstable formulation, the inability to measure this key parameter early in drug development can lead researchers to select inadequate formulation candidates, only to fail later in the development process after much time and resources have already been spent. As protein concentrations in formulations continue to increase, this already challenging problem only gets worse.

In this application note, we explore how the HORIZON® system, a novel subvisible particle analyzer, utilizes Backgrounded Membrane Imaging (BMI) to enable sensitive early stage subvisible particle analysis with less than 25 µL of sample, making it possible to take precise measurements early in the formulation process when sample is scarce. We describe three independent studies that test the HORIZON® system's ability to conduct rapid, low volume, and sensitive subvisible particle analysis of different biopharmaceutical formulations.

Sample preparation

Interfacially stressed IgG Aggregates (Int-mAb): Protein aggregates made of immunoglobulin G (IgG) antibody were generated using a periodic interfacial compression method.[6] Briefly, IgG protein was diluted into pH 7.4 phosphate buffered saline then filtered using 0.2 µm pore size sterile filters. Samples were rotated in a half-filled 50 mL conical tube at 15 rpm for 3 hrs (IgG) at room temperature.

Stir-agitated IgG aggregates (Stir mAb): Protein aggregates made of IgG were also generated using an a stir-agitation method.[7,8] Briefly, anti-streptavidin IgG1 was dialyzed using 10 kDa MWCO Spectra/Por 7 tubing

(Spectrum Laboratories). A concentration of 1 mg/mL was prepared with distilled, deionized water Milli-Q (Millipore) in 20 mM acetic acid (Fisher) adjusted to pH 5.0. The solution was stirred at 650 rpm for 24 hrs at room temperature.

Surrogate protein particle standard (ETFE): Surrogate protein particle standard ethylene-tetrafluoroethylene (ETFE) solution,[9,10] made to mimic the morphology and optical properties of protein-derived protein aggregates found in therapeutics, was acquired from NIST and used as received.

Study 1 — Precise low volume analysis of polydisperse biopharmaceutical samples using BMI

Subvisible particle measurements using 25 µL of highly disperse particle populations of agitated Int-mAb (N=20) aggregates, ETFE (N=20) and water control (N=4) were performed with BMI (Figure 1). The average of the 20 replicates of Int-mAb IgG samples was measured at 66,632 particles/mL with a CV of 6.2%, while the average of particles measured for ETFE was 13,580 with a CV of 9.7%. The water control had low counts, (an average of eight particles on the membrane) with particulates in the water control ranging from 1–11 particles per well. Total experiment time to analyze 44 samples was less than 60 minutes using the HORIZON® instrument — 30 seconds to background, 30 seconds to measure the sample, plus pipetting time and sample preparation.

Study 2 — Impact of volume on IgG aggregates and ETFE particle counts

Next, sample size impact on biopharmaceutical subvisible analysis on BMI was tested by measuring several volumes of an ETFE sample on the HORIZON® instrument (Figure 2). Samples measured at volumes of 10 µL, 25 µL, 50 µL and 100 µL, (N=6 wells for each volume) generated particle counts within 4% of each other for particles greater than 2 µm, proving equivalent counts were obtained when 10 µL and 100 µL of sample were analyzed. The average particle count was 11,121 particles/mL with CVs ranging from 5.0% to 8.1%, demonstrating system robustness. Thus, precise particle counts can be obtained using 10 µL of sample when sample limited, however we recommend using 25 µL for more quantitative work.

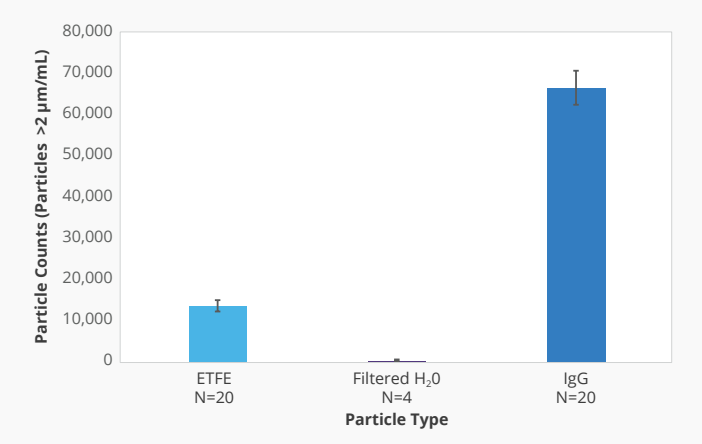

FIGURE 1: Low volume analysis of polydisperse samples in BMI. 25 µL samples of ETFE and IgG were measured with 20 replicates each. CVs for ETFE and IgG samples were 9.7% and 6.2%, respectively.

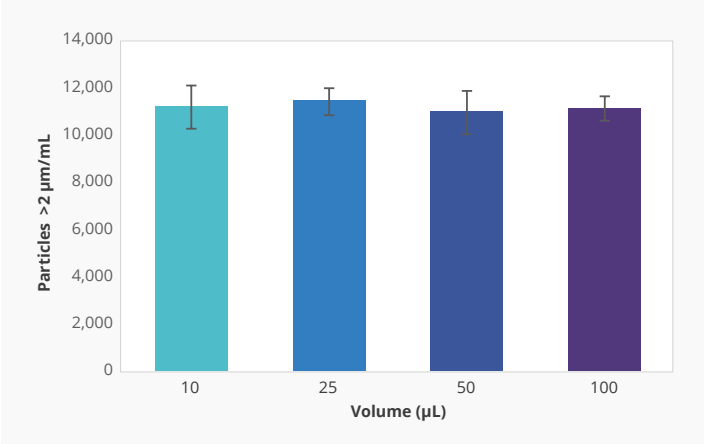

FIGURE 2: Impact of volume on polydisperse samples in BMI. Varying volumes of ETFE samples were measured. Particle counts were all within 4% of each other, demonstrating sample volume did not impact measurement precision.

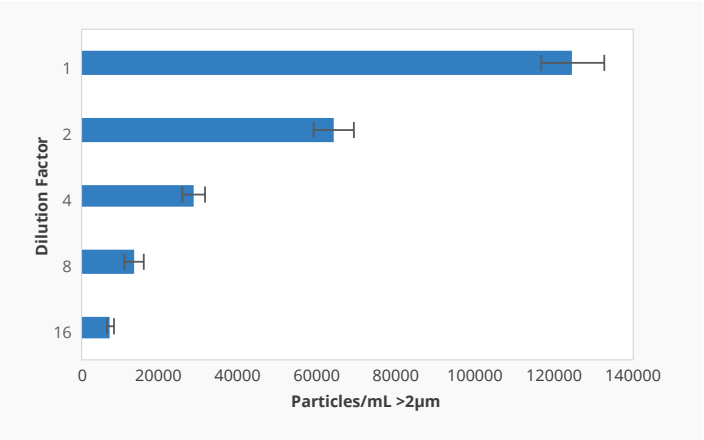

FIGURE 3: BMI linearity. Dilution series of Int-mAb aggregate samples. R^2 = 0.9979 demonstrates count linearity.

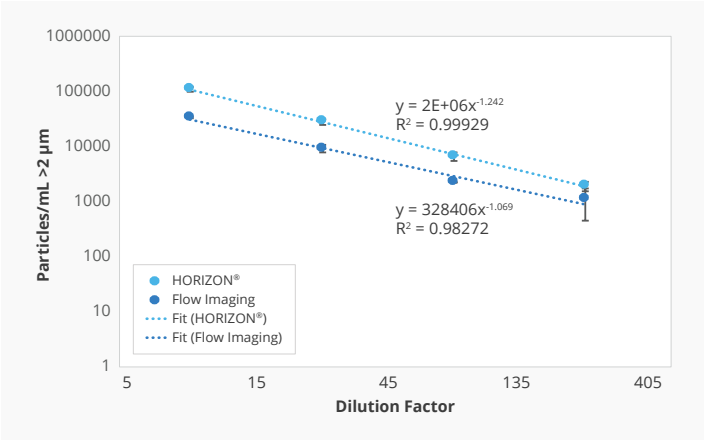

FIGURE 4: Impact of volume on polydisperse samples in BMI and FI. Varying amounts of ETFE samples were measured. Both systems demonstrated strong linearity.

Study 3 — Low volume dilution linearity with BMI and FI

Measurement linearity on the HORIZON® instrument was tested (Figure 3) by serially diluting concentrated Int-mAb IgG protein aggregates by two-fold into filtered phosphate buffered saline (PBS). 50 µL of sample was processed for each dilution condition (N=16 each). A least-squares fit on the counts for this dilution series resulted in R^2 of 0.9979 indicating that dilutions are not affecting the count accuracy.

Dilution linearity between the HORIZON® system and FI was then compared (Figure 4). Stir mAb IgG protein aggregates were serially diluted by three-fold into filtered phosphate buffered saline (PBS). Each sample was run on both a HORIZON® instrument (N=4 wells at 50 µL per well) and a flow imager (N=3 at 100 µL measured per sample). For each 100 µL measured in FI, an additional 200 µL was needed to purge the flow cell. Total particle counts in the HORIZON® system were roughly 3.26X more than total counts in FI over the entire concentration range. BMI produced average CVs of 14.4% while FI produced average CVs of 20.1%. A least-squares fit of 0.9995 between both methods was obtained, demonstrating strong linearity between the two systems.

Conclusion

BMI analysis of ETFE and IgG aggregates using as little as 25 µL of sample precisely measured both highly polydisperse and low refractive index contrast biopharmaceutical samples. Also, particle counts obtained with as little as 10 µL of solution were equivalent to counts obtained with 100 µL, as demonstrated for the ETFE solution measured at various volumes. Thus, precise subvisible particle measurements can be generated with as little as 10–25 µL of material using BMI, though 25 µL should be used for quantitative work.

The HORIZON® system exhibited linear behavior during serial dilution experiments of Int-mAb. For a serial dilution of two, one would expect an outcome where every dilution factor increase of 2X would result in ½ the measured particle counts. This equates to exponentially decreasing particle counts of the formula $y = A^{x-1}$ where A is the number of particles at dilution of 1. Fitting an exponentially decreasing line to this data produces a fit of $125668^{x-1.058}$ with an R^2 value of 0.9979. This shows that there is less than 6% sampling, inter-instrument, and pipetting error (1.058 vs 1.000) for this formulation.

Both FI and BMI were highly linear with respect to each other (R^2 fit =0.9995). However, the FI system required

roughly 300 µL per sample with >60% of this volume used for purging the flow cell, while the HORIZON® BMI system required 6X less sample. The fact that BMI produced lower CVs, even at lower volumes, can be probably attributed to sensitivity of the BMI technique. The sensitivity is attained because BMI has a greater refractive index contrast between the nearly translucent particles and the surrounding media (see Halo Labs *Application Note 1*).

The HORIZON® system is a fluidics-free subvisible analyzer that is free of the issues that have historically impeded low volume measurements. Powered by Backgrounded Membrane Imaging, a volume invariant method, the HORIZON® instrument delivers early and robust insights on a formulation's subvisible particle content by analyzing volumes as low as 25 µL quantitatively and as low as 10 µL for quick, qualitative screens. Ultra-low volume analysis means researchers can now quickly measure if they have an unstable formulation, or get to the right formulation conditions, with just a fraction of the volume to access this key stability parameter as early as candidate selection.

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