Particle analysis of therapeutic protein formulations with ImageStreamX® Imaging Flow Cytometry

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Abstract

The formation of protein aggregates (PA) and other subvisible particles within protein-based drugs is a major concern for the pharmaceutical industry because they can impact drug activity or cause unwanted immunogenicity in patients (1). It is critical to monitor the presence of these particles to ensure the safety and efficacy of these drugs, especially since their formation can be highly sensitive to production and storage conditions. Currently US and European pharmacological regulations require testing of subvisible (>10 μm) and visible (>25 μm) particles of therapeutic agents, and recent literature has highlighted the importance of also measuring particles in the 1-10 μm size range (2).

Another emerging requirement is the need to classify particle type, as multiple contaminants including silicone oil droplets and bacteria may be present in this size range (3). No currently accepted industry method meets the need to simultaneously size, enumerate, and classify particles within this critical 1-100 μm size range, thus illustrating the need to develop new approaches for this application (4). In this study we evaluate the potential for the Amnis ImageStreamX® MKII® (IXS) imaging flow cytometer to address these criteria and provide a comprehensive particle characterization for therapeutic protein formulations. The major advantage of the IXS is the coupling of flow cytometry with microscopy, which allows particle type to be classified using specific fluorescence stains and quantitative measurement of morphological properties such as size from high resolution imagery. Though traditional flow cytometry has been previously applied for this application (4), it is limited compared to imaging flow cytometry because it cannot directly size particles, is less sensitive for detection of small and translucent particles, and does not measure other potentially important morphological parameters such as shape and contrast. In this study we used Enzo Life Sciences ProteoStat® Protein Aggregation Standards as a model PA system. ProteoStat® Protein Aggregation Standards are the only commercially available PA standards, and provide a useful tool for measuring the linearity and sensitivity of the IXS for PA analysis.

Additionally, the ProteoStat® Protein Aggregation assay was used to fluorescently label PAIs with high specificty. This simple mix-and-read assay offers high selectivity for PAIs using a proprietary fluorescent (FL) probe that displays 20-99 fold brightness enhancement upon binding to the cross-beta spinnaceous domain of aggregated proteins. As silicone oil droplets are a common contaminant for PA applications, we further evaluated the ability of IXS to differentiate this contaminant using Bodipy FL (BF) oil droplets. We have determined that the IXS offers multiple advantages for PA analysis compared to currently accepted approaches, including (i) direct measurement of particle size and other morphological characteristics through quantitative image analysis (i.i) classification of subvisible particles types, (ii) incorporation of additional fluorescent stains, (iii) collection of statistically robust data sets via rapid image acquisition, (iv) measurement of absolute particle concentrations using a volumetric syringe pump, and (v) small minimum sample volume requirement of 20 μL.

Materials and Methods:

Sample preparation:

IgG only samples

ProteoStat® Protein Aggregation Standards (Enzo-SI-0109) were reconstituted in filtered deionized water to final concentrations of 12.5%, 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.20%, and 0.1% aggregated IgG (total protein concentration fixed at 1 mg/mL). Samples were fluorescently labeled using the ProteoStat® Detection Assay® (Enzo -SI-0123) following the protocol provided by the manufacturer.

Mixed IgG and silicone oil samples

A solution containing silicone oil microspheres (SIOI) was prepared by mixing 100 μL silicone oil with 50 mL of water and vortexing vigorously for one minute. 100 μL SIOI mix samples were prepared by mixing 100 μL of SIOI solution with 900 μL of deionized water. The concentration of SIOI was 30% by volume. Samples were incubated with ProteoStat® Detection Reagent and Bodipy dye (final concentration 1 μM) for 15 minutes to fluorescently label PAIs and SIOI respectively.

Data acquisition and analysis:

The ImageStreamX® MKII® was used to collect imaging flow cytometry data for both analytes. Imaging was set at 60x (0.23 um pixel resolution). A 488 nm excitation laser (200 mW) was used to excite Bodipy and ProteoStat fluorescence. brightfield (BF) imagery was collected using an LED-based BF illumination. A dedicated 785 nm laser (20 mW) was used to collect side scatter (SSC) measurements. The number of counts per sample was 50,000 for IgG only experiment and 2,000 for mixed IgG and SIOI experiment. Image analysis was completed using quantitative image algorithms available in the IXS, an image analysis software package. SpeedBead® Calibration reagent events were removed from analysis using forward-side scatter discrimination, and clamped objects were eliminated using the Raw Centroid filter. Volumetric percent was calculated by multiplying particle concentration by mean BF particle diameter using the appropriate unit conversion. A fluorescence plate reader (Infinite 200 Pro-Tecan) was used to measure absolute sample fluorescence.

References