An Interlaboratory Comparison of Sizing and Counting of Subvisible Particles Mimicking Protein Aggregates

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ABSTRACT: Accurate counting and sizing of protein particles has been limited by discrepancies of counts obtained by different methods. To understand the bias and repeatability of techniques in common use in the biopharmaceutical community, the National Institute of Standards and Technology has conducted an interlaboratory comparison for sizing and counting subvisible particles from 1 to 25 μm. Twenty-three laboratories from industry, government, and academic institutions participated. The circulated samples consisted of a polydisperse suspension of abraded ethylene tetrafluoroethylene particles, which closely mimic the optical contrast and morphology of protein particles. For restricted data sets, agreement between data sets was reasonably good: relative standard deviations (RSDs) of approximately 25% for light obscuration counts with lower diameter limits from 1 to 5 μm, and approximately 30% for flow imaging with specified manufacturer and instrument setting. RSDs of the reported counts for unrestricted data sets were approximately 50% for both light obscuration and flow imaging. Differences between instrument manufacturers were not statistically significant for light obscuration but were significant for flow imaging. We also report a method for accounting for differences in the reported diameter for flow imaging and electrical sensing zone techniques; the method worked well for diameters greater than 15 μm. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:666–677, 2015

Keywords: image analysis; imaging methods; light scattering; microparticles; particle size; physical characterization

INTRODUCTION

Protein particles, consisting of aggregated protein and possibly a nonprotein nucleating core, can form in biopharmaceutical drugs.[1,2] Stresses that can lead to the formation of protein particles include changes in chemical environment, exposure to interfaces, agitation, elevation of temperature, or the introduction of nonprotein particles.[3–7] Counting and characterizing these particles is necessary to assure the quality of these drugs. Although the size of aggregated proteins may vary from 10s of nanometers to 100s of micrometers, the most sensitive analytical techniques cover the approximate range from 1 to 100 μm.[3,8]

In contrast to possible nonprotein impurities (e.g., glass chips, stainless steel particles, and fibers) protein particles have low optical contrast (equivalent to a small refractive index difference from the matrix fluid) and are subject to dynamic changes in size and concentration as particles are formed or dissolve back into solution.[3,9,10] Industry has made great strides in adopting new technologies to count protein particles routinely down to sizes of approximately 2 μm, but particle counts obtained with different types of instruments often differ by as much as a factor of 10.[11,12] Particle counting instruments are commonly calibrated using polystyrene latex (PSL) beads, which have high optical contrast and spherical shape; the observed count discrepancies indicate that instrument calibrations with PSL beads do not suffice to standardize instrument response to protein particles.

Comparison of analytical measurements of particle size and count has been hampered by the instability of the protein particles themselves, which can aggregate further on shipping or revert back to smaller aggregates or monomer protein molecules. As an alternate path to producing a suitable reference material, the National Institute of Standards and Technology (NIST) is developing a reference material comprising irregular particles of a low-refractive index fluoropolymer, ethylene tetrafluoroethylene (ETFE). The morphology and optical contrast of this material closely resembles that of typical protein particles.

As an initial step in implementing this reference material and to assess the level of agreement among different laboratories, NIST has conducted an interlaboratory comparison for sizing and counting subvisible particles from 1 to 25 μm, using a polydisperse polymer suspension that closely mimics actual protein particles. As listed in Table 1, a total of 23 laboratories participated, including 15 from biopharmaceutical manufacturers, one from biomedical device manufacturers, two from instrumentation manufacturers, three from government laboratories, and two from academic laboratories.

This paper describes the design, production, and characterization of the particles (section *Materials and Methods*); gives an overview of the bias between different counting methods (section *Results and Discussion*); and then discusses results for the four methods[8,13] used by participants: flow imaging,[12] light obscuration, electrical sensing zone (ESZ),[14,15] and resonant mass measurement (RMM)[16] (sections *Overview and Identification of Outliers to Resonant Mass Measurement*). Sections

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Table 1. List of Laboratories That Participated in the Study

Amgen, Inc., Formulation and Analytical Sciences, Thousand Oaks, California
BD Medical, Pharmaceutical Systems, Pharmaceutical technology/R&D, Pont de Claix, France
Biogen Idec, QC Analytical Technology, Research Triangle Park, North Carolina
Bristol Myers Squibb, Biologics Analytical Development and Testing, Pennington, New Jersey
Boehringer Ingelheim Pharma GmbH and Company KG, Biopharmaceuticals, Biberach an der Riss, Germany
Coriolis Pharma, Martinsried, Germany
Eli Lilly and Company, Biopharmaceutical Research and Development, Indianapolis, Indiana
F. Hoffmann-La Roche Ltd, Pharma Technical Development Europe (Biologics), Basel, Switzerland
Food and Drug Administration,[a] Laboratory of Plasma Derivatives, Center for Biologics Evaluation and Research, Bethesda, Maryland
Fluid Imaging Technologies, Yarmouth, Maine
Genentech, Inc., Roche Group, Late Stage Pharmaceutical and Processing Development, South San Francisco, California
Hach Company, Grants Pass, Oregon
GlaxoSmithKline R&D, Biopharm Product Sciences (BPS), King of Prussia, Pennsylvania
GlaxoSmithKline (formerly Human Genome Sciences), Gaithersburg, Maryland
Health Canada, Centre for Biologics Evaluation, Biologics and Genetic Therapies Directorate, Ottawa, Canada
Janssen R&D, Schaffhausen, Switzerland
MedImmune, Formulation Sciences Department, Gaithersburg, Maryland
National Institute of Standards and Technology, Bioprocess Measurements Group, Gaithersburg, Maryland
Novartis Pharma AG, Biologics Process R&D, Basel, Switzerland
Pfizer, Inc., Biotherapeutics Pharmaceutical Sciences, Chesterfield, Missouri
Sandoz Biopharmaceuticals, Pharmaceutical and Device Development, Drug Product Analytics, Sandoz GmbH, Langkampfen, Austria
University of Kansas, Department of Pharmaceutical Chemistry, Macromolecule and Vaccine Stabilization Center, Lawrence, Kansas
University of Leiden, Leiden/Amsterdam Center for Drug Research, Department of Drug Delivery Technology, Gorlaeus Laboratories, Leiden, The Netherlands

[a] Although US FDA laboratory participated in the scientific study and/ or discussion, please note that FDA does not recommend, endorse, or recognize this standard development and further, the content of this communication represents the authors' views and does not bind or obligate FDA.

Particle Morphology and *Resonant Mass Measurement* also describe an initial attempt to adjust the reported diameter of ESZ instruments to be equivalent to the diameter reported by flow imaging instruments.

The results give a snapshot of the level of agreement between different laboratories for the particle counting methods in common use today in the biopharmaceutical industry. As expected from published results on protein particles, particle counts differed significantly depending on the counting method. For each specific method, statistically significant deviations were observed primarily because of differences in instrument response. There were also several outliers (~10% of the reported data) likely related to insufficient resuspension of the ETFE particles and contamination of the ETFE particles by debris from vial-thread abrasion. Surprisingly, data obtained by light obscuration agreed well for small diameter particles [relative standard deviation (RSD) of <26% for lower diameter limits from 1 to 5 μm], but the level of agreement was significantly worse for large particles. For flow imaging, there were statistically significant differences between data sets acquired on different instrument models, resulting in a large variability of counts (RSD values of 33%–61% for all flow imaging data). For specified instrument settings and models, the variability was reduced, with RSD values of 13%–49% over the full size range of the comparison. ESZ instruments gave anomalously high counts for the lowest diameter limits.

MATERIALS AND METHODS

Preparation of the Particle Suspension

The samples circulated for testing consisted of a polydisperse suspension of particles created from the polymer ETFE. ETFE is attractive because it has low refractive index[17] (≈1.40, similar to that of protein films adsorbed on surfaces[18]) and is chemically inert and mechanically strong.[19]

The particles were produced by abrading a solid polymer sample of ETFE against a diamond lapping disc. Although the process of producing the ETFE particles in no way corresponds to the aggregation mechanism of actual protein particles, the morphology of the ETFE particles is remarkably similar to protein particles. Thus, the ETFE particles can serve as a surrogate to actual protein particles, with similar morphology and optical contrast. Like actual protein particle suspensions, but unlike PSL standards, the ETFE suspensions are polydisperse, with particles ranging in approximate sizes from greater than 50 μm down to less than 0.5 μm.

We produced polydisperse ETFE particles by first abrading ETFE against a diamond abrasive (45 μm nominal grit size, nickel bonded to a compliant backing) while submersed in an aqueous solution of 0.03 mol/L 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and 0.1% mass concentration sodium dodecyl sulfate (SDS) buffered to pH 6 (we intended to use pH 7.5, but inadvertently used a pH 6 buffer for the particle fabrication). At approximately 1 h intervals, the particle suspension was withdrawn by pipette from the well holding the abrasive disc. To prevent clogging of analytical instruments, large particles were filtered out by passing the suspension through a nylon screen with nominal 53 μm square openings. The nylon screen did not shed an appreciable number of particles if it was securely mounted, not folded or manipulated during the filtering process, and thoroughly rinsed with particle-free water prior to use. As harvested, the particle count was too high for direct measurement in some instruments. The suspension was diluted to the desired particle count with additional HEPES/SDS solution buffered to a pH of 7.5. Prior to use, the HEPES/SDS solution was filtered through a 0.45-μm PVDF syringe filter (Millex-HV; EMD Millipore, Billerica,