

# THE METROLOGY OF COUNTING PROTEIN PARTICLES

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***Abstract - A common degradation pathway for protein-based drugs is the growth of protein aggregates or particles. Counting and characterization of these particles is needed to assure the quality, efficacy, and safety of this type of drug. The unusual physical attributes of protein particles lead to difficulties in obtaining accurate particle counts with traditional calibration standards and methods. We describe the development of instrument-response models, novel instrumentation, and novel reference materials to address this metrology issue.***

## Introduction

Protein-based drugs are a rapidly growing class of pharmaceuticals, driven by the ability of these drugs to treat conditions that do not respond to traditional small-molecule drugs and by the increased sophistication of protein design and manufacture. One degradation path for these drugs is the formation of particles, consisting of aggregated protein and possibly a non-protein nucleating core.[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 non-protein particles. There is some evidence that these particles, although constituting a very small volume fraction of the administered drug, may trigger an immune response.[3] In some cases, the immune response has no effect on the patient or on the drug efficacy, but in other cases, the response can lead to altered efficacy, or in very rare cases, development of patient antibodies that cause an auto-immune attack of the patient's own proteins. Figure 1 shows examples of bright-field microscopy images of protein particles.

Regulatory bodies and industry both desire to count and characterize the particles occurring in protein drugs. Particle characterization has important roles throughout the drug development process. Early in the development process, drug candidates are screened to see if they have acceptable resistance to aggregation. Prior to and during clinical trials, the product formulation is adjusted to give maximum stability, including resistance to aggregate formation. Characterization of the nature and count of intrinsic protein aggregates on exposure to various stresses is useful to assess the level of risk posed by particles. Once drug products are in production, simplified methods for particle counting are used as a release test to verify the quality of drug lots.

The size of protein aggregates varies from 10s of nanometers to 100s of micrometers. Protein particles have an irregular morphology and low optical contrast (equivalent to a small refractive index difference from the matrix fluid). Another complication is that protein particles are subject to dynamic changes in size.

The terminology for particle detection in the biopharmaceutical field reflects the evolution of particle counting efforts. Historically, the term 'aggregates' has been used primarily to refer to small protein oligomers with diameters below 100 nm, whereas 'particle' has been used to refer to larger objects that may or may not be composed of protein. In another category, silicone used to coat syringes pre-filled with drug product can leach into the drug product and form droplets. In cases where a particle counting instrument cannot differentiate between a protein aggregate, a silicone oil droplet, and foreign matter, or when only the total count of all objects is desired, the word 'particle' is used for all objects.

Methods used for particle and aggregate detection depend on the particle size. At the lower end of the range, for aggregates consisting of small protein oligomers, industry relies on size exclusion chromatography and analytic ultracentrifugation to monitor the level of aggregated protein. These methods are well established, and interferences to the methods are well understood.[4]

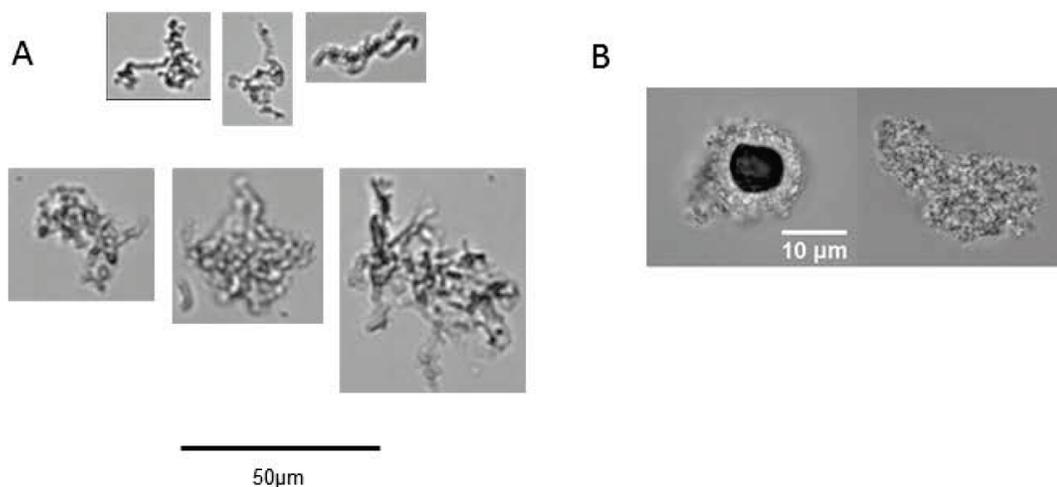


Fig. 1. Examples of protein particles: (A) particles formed from thermal treatment of the monoclonal antibody drug rituximab; (B) particles formed from aggregation of chemically destabilized polyclonal immunoglobulin (IgG). The heterogeneous particle (left) has aggregated around a precipitated-salt core.

Drugs that are administered by other than oral or topical routes are visually inspected, which is effective at detecting particles greater than  $\approx 70 \mu\text{m}$  diameter. Detection of visible particles in biopharmaceuticals presents a number of challenges. First, traceability of any sort for visible detection requires training of inspectors using a challenge set of drug vials intentionally containing particles of known size and composition. At present, there are no existing standards for visible particles that mimic the optical properties of large protein aggregates. Another issue is that biological drugs are often opalescent, due to the very high protein concentrations ( $> 100 \text{ mg/mL}$ ), and this opalescence can obscure particles.

For diameters greater than  $10 \mu\text{m}$ , pharmacopeial methods for particle detection in therapeutic products were designed to identify manufacturing debris, such as glass, rubber or steel, that could cause blockage of patient capillaries.[5] For these types of particles, two effective counting techniques are filter microscopy and light obscuration. In filter microscopy, particles are collected on a membrane filter and the filter is then inspected under a microscope. In light obscuration, a stream of test sample passes through a flow cell traversed by a light beam; when a particle scatters light from the beam, the intensity of the beam drops. The magnitude of the intensity drop may be mapped to particle size. In each case, the particle size distribution of the particles is obtained.

When applied to biopharmaceutical products, traditional pharmacopeial methods give mixed results. Filter microscopy does not work at all for protein particles because the highly hydrated and flexible protein particles distort, flatten, and become invisible on the filter. Light obscuration can give highly repeatable measurements, but the reported particle counts can be in error because the physical properties of protein particles differ substantially from that of the polystyrene latex (PSL) beads commonly used to calibrate instruments.

Recently, a number of new technologies have been adopted by the biopharmaceutical industry to address the difficulty of counting and sizing protein particles.[6] The most common of these new techniques is the flow microscope, which consists of a microscope and camera, a flow cell for passage of the test sample through the focal plane of the microscope, and software that enables automated acquisition of images and identification of particles. Comparisons of the counts obtained from light obscuration particle counters and flow microscopes often give light obscuration counts that are a factor of ten or more lower than the particle counts obtained from flow microscopy.

Below the range of flow microscopy, but above the range of size exclusion chromatography, a number of novel methods are being developed, including field flow fractionation, nanoparticle tracking analysis, and resonance mass measurements.[6] Establishing traceability for each of these requires investigation into the principle of operation of each, and a review of the applicability of existing methods and reference materials.

## Calibration challenges

As defined by the International Vocabulary of Metrology, calibration consists of relating the indication of the instrument to the defined values of a measurement standard.[7] In the case of protein particles, the available standards consist of spherical beads of high optical contrast, and the relation of instrument indication to the values of the standards is not sufficient when the instruments respond to actual particles differently than to the standards. In other terms, the calibration process in no way guarantees that the indication of the instrument will be correct for a particle of properties far different than a PSL bead. In fact, accurate measurements are guaranteed only by:

- developing appropriate models for the instrument response,
- identifying and characterizing the physical properties of protein particles relevant to the counting method considered, and
- developing reference standards that mimic protein particles.

At NIST, we are conducting a program expanding on each of these themes. Our initial work focuses on providing methods and reference materials to enable traceability to the SI for equivalent diameters of 1  $\mu\text{m}$  and greater.

## Models of instrument response

Two examples of the development of instrument response models are a) the modeling of the light scattering process in light obscuration counters, and b) the simulation of electrical-sensing-zone (ESZ) counters for the detection of porous and irregularly shaped particles.

To model the light scattering process, we treat the protein particles as randomly oriented spheroids with an effective refractive index, and determine the scattering amplitude from an eikonal approximation.[8] This optical approximation is appropriate for particles with small refractive index difference from the surrounding matrix, which is the case for protein particles. The extinction efficiency factor  $Q$  (the ratio of the scattering cross section to the geometric cross section of the particle) is then calculated by integrating the scattering amplitude over the appropriate range of angles for the particle detector considered. These calculations reveal that the finite detection aperture of the light obscuration counter must be accounted for, and that the most important particle attribute is the refractive index difference between the particle and the matrix liquid, as shown in Fig. 2. Modeling of the particle scattering process demonstrates why light obscuration tends to undercount the number of particles above a given threshold: the light obscuration counter interprets the reduction in transmitted light as the diameter of a PSL bead that would create the same reduction in transmitted light. Since the  $Q$  of protein particles is substantially less than that of PSL beads, across the whole size range of the instrument, the equivalent PSL diameter is less than the actual protein particle size. In contrast, a flow microscope has better abilities at correctly identifying the size of low-contrast particles.

To model the electrical sensing zone counters, we have used finite element modeling to understand the perturbation in ionic conductance as a particle passes through the orifice. The amplitude and shape of the electrical resistance variation depend on the path of the particle through the orifice, the particle geometry, and the particle orientation

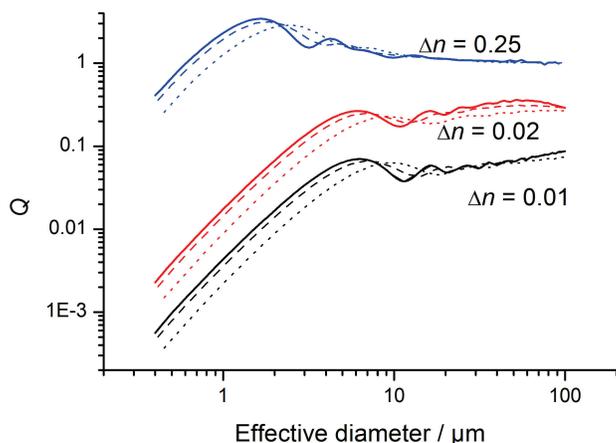


Fig. 2. Extinction efficiency factor  $Q$  as a function of particle diameter, for prolate spheroids of refractive index difference from the matrix fluid,  $\Delta n$ , and for different aspect ratios (1 for solid lines, 2 for dashed lines, and 4 for dotted lines). For PSL beads in water,  $\Delta n \approx 0.25$ ; for protein particles,  $\Delta n$  may range from  $\approx 0.005$  to  $0.07$ .

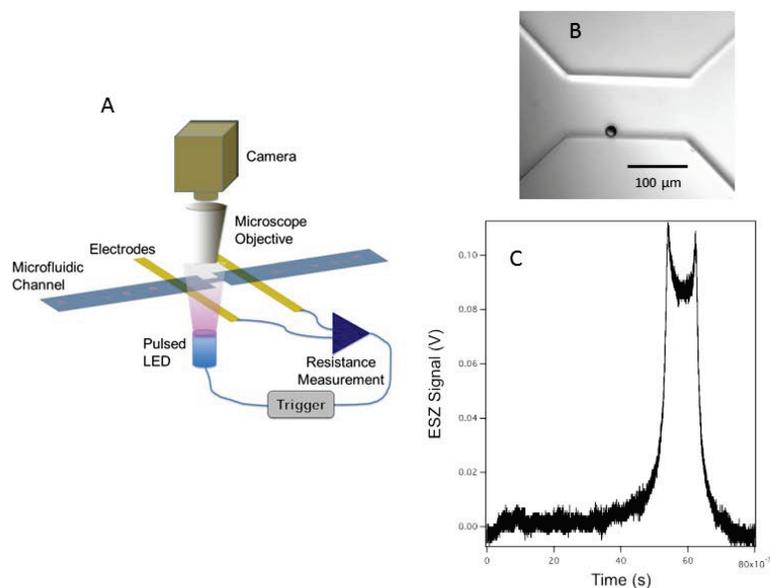


Fig. 3. (A) Schematic of a microfluidic, electrical-sensing zone particle counter combined with a flow microscope, (B) a particle traversing the orifice of a microfluidic electrical-sensing-zone counter, and (C) the corresponding electrical signal.

inside the orifice. Predictions of the model can be confirmed by measuring non-spherical particles of known geometry. To further investigate the differences between optical imaging and the ESZ technique, we have developed a device that combines optical imaging and a microfluidic electrical sensing zone counter, as shown in the schematic of Fig. 3A. The electrical sensing zone technique measures the increase in ionic resistance across an orifice when a particle passes through this orifice. To make an ESZ device that is compatible with optical microscopy, we have fabricated the orifice and flow cell using microfluidic techniques. As the sample flows through the orifice, electrical detection of a particle triggers illumination and capture of the particle image by the camera. For simple solid shapes, such as cells, the resistance increase can be related to the volume of the particle. For more complex shapes, such as irregular aggregates of protein particles, it is not clear if the particle volume can be inferred from the resistance increase. Figure 3B shows the optical image (by bright field microscopy) of a particle passing through the microfluidic orifice along with the corresponding increase in an electrical signal proportional to resistance change. Image analysis of the microscopy results will give a measure of typical morphological parameters, such as the apparent particle area and aspect ratio. Comparison of these parameter values with the electrical-sensing-zone signal for individual particles will give us insight into the differences in response of the two counting methodologies. We are improving the device further by adding a high-speed fluorescence camera to the microscope, which will enable us to take real-time pictures of fluorescently dyed particles passing through the orifice.

In the area of reference materials, we are developing a reference material consisting of a polydisperse distribution of abraded ethylene-tetrafluoroethylene (ETFE) particles.[9] ETFE has several desirable properties: it is chemically inert and very rugged, and has a refractive index (1.40) very close to that of amorphous protein adsorbed on surfaces.[10] When abraded from a solid block, the morphology of the resulting particles closely mimics that of typical protein particles, as seen in Fig. 4, even though the mechanism for protein particle formation is completely different than the abrasion process. Oscillatory motion of a piece of ETFE tubing against a diamond abrasive creates a dilute slurry of highly contorted ETFE particles. The resulting particles are suspended in a pH 7 to pH 8 buffer with an anionic surfactant to promote particle dispersion. The particle size distribution is very broad, depending on the size of the abrasive grains used to produce the particles. We are focusing our present efforts on creating a reference material with effective diameters certified over a range from 0.5 μm to 30 μm.

Once generated, the particle suspension must be characterized in a manner traceable to the SI. We intend to spike into the ETFE suspension a known concentration of PSL spheres, collect samples of the spiked suspension on alumina filters, and then count the collected particles using scanning electron microscopy (SEM). Existing NIST reference materials for PSL beads are certified for diameter, but not concentration. We plan to calibrate the PSL bead concentration by modifying a light obscuration apparatus that will measure in a single pass an initial prime volume of

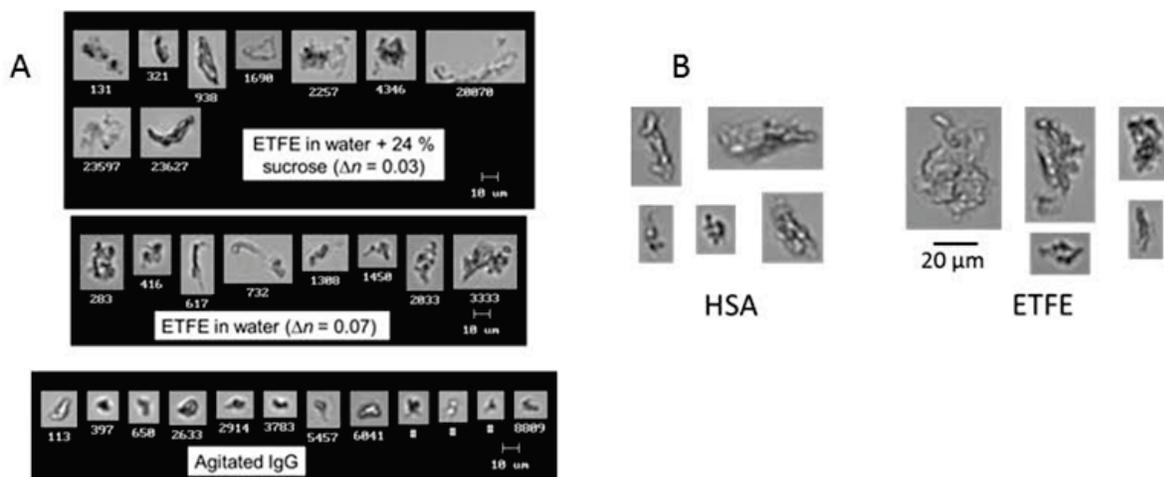


Fig. 4. A: Comparison of a candidate reference material (abraded ETFE) with protein particles formed from agitated polyclonal immunoglobulin (IgG); B: Comparison of a candidate reference material (abraded ETFE) with protein particles formed from agitated human serum albumin (HSA).

particle-free water, a known volume of PSL bead suspension, and finally a rinse of particle-free water. Bracketing the PSL beads with water volumes will make the measurement independent of start/stop and will also reduce the loss of PSL beads by diffusion into dead volumes or by adsorption on the walls. By spiking the ETFE suspension, all of the counts obtained by SEM are relative to the number of observed spheres, which is significantly simpler than performing SEM in an absolute counting mode. Success in using PSL beads as a reference for concentration will require understanding the filtration process in detail and ensuring that both PSL beads and ETFE particles are captured equally effectively. Use of the ETFE standard will, we hope, provide a means of quantifying the instrument response for particles of both low optical contrast and irregular morphology.

As an alternative method, we are also developing particles using lithographic techniques. An epoxy-based photoresist (SU-8) was deposited over a release layer on a silicon wafer; the SU-8 was exposed to ultraviolet light through a mask and baked to selectively polymerize the particles, and then the particles were released from the wafer by dissolving the release layer. Examples of these particles are shown in Fig. 5. We have produced particles as large as 300 μm and as small as 50 μm long rods with a 2.5 μm x 2.5 μm square cross section. As one example of the utility of this type of particle, the elongated particles in the upper right corner of Fig. 5 were measured using a calibrated, standard microscope and by two automated flow microscopes, all using bright field microscopy. The uncertainties of the NIST measurements are dominated by the ambiguity of the physical edge of the particles.[11] Table 1 presents the results, demonstrating that both instruments achieve acceptable performance for the measurement of perimeter and area of an irregular particle. Even larger particles may be useful as a standard for the counting of visible particles. When released from the silicon wafer, large planar SU-8 particles if sufficiently thin are flexible and take on three-dimensional shapes, as seen in Fig. 5. PSL beads scatter light evenly regardless of orientation. The lithographic particles, like protein particles, have a ‘twinkling’ appearance as they rotate relative to the light source and observer. One disadvantage of the lithographic particles is their relatively large density. PSL, with a density of 1050 kg/m<sup>3</sup>, is nearly neutrally buoyant in water. SU-8 has a density of 1200 kg/m<sup>3</sup>, although the rate of fall of flexible sheets (see Fig. 5) is significantly smaller than if the SU-8 were fabricated as spheres.

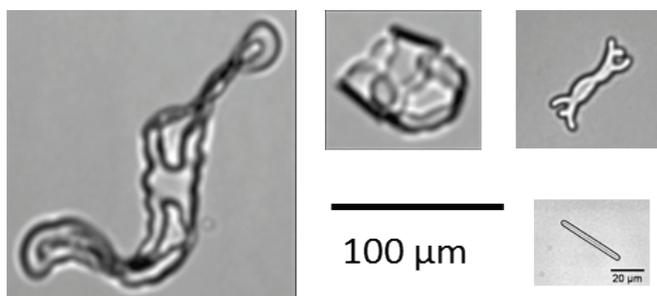


Fig. 5. Examples of polymer particles produced by lithographic techniques over a range of sizes.

Table 1. Measurement of a monodisperse, lithographically fabricated particle by three microscope instruments. The maximum feret is the maximum dimension of the particle;  $S_r$  denotes the relative standard deviation;  $U_r$  denotes the relative expanded uncertainty at a coverage factor of two. Data for instrument A from Clark Merchant, ProteinSimple; data for instrument B from Dave Palmund, Fluid Imaging Technologies. [12]

|                                | NIST |       |              | % dev. from NIST |          |
|--------------------------------|------|-------|--------------|------------------|----------|
|                                | Mean | $S_r$ | $U_r(k = 2)$ | Instr. A         | Instr. B |
| Max. feret, $\mu\text{m}$      | 56.0 | 1%    | 3 %          | -9 %             | 2 %      |
| Equiv. diameter, $\mu\text{m}$ | 29.1 | 2 %   | 6 %          | 1 %              | 15 %     |
| Aspect ratio                   | 0.39 | 3 %   | 5 %          | -11 %            | 7 %      |
| Area, $\mu\text{m}^2$          | 666  | 4 %   | 12 %         | 2 %              | 33 %     |
| Perimeter, $\mu\text{m}$       | 189  | 3 %   | 3 %          | -24 %            | -7 %     |

Smaller size particles are useful to study the response of instruments to non-spherical particles. We have fabricated rod-like particles to study the orientation of particles in flow cells and to understand the response of electrical-sensing zone counters to non-spherical particles. The lithographic particles are highly reproducible; however, the refractive index of SU-8 (1.59) is too high to truly mimic the low optical contrast of typical protein particles. We are engaged in identifying other polymer types that will enable the development of rugged particles with reduced optical contrast.

## Future challenges

Measurement of well-defined reference materials and studies of the operational principles of each type of instrument enable identification of measurement biases and uncertainties. We anticipate this effort to continue as new types of instruments come into use. However, any corrections that require extensive knowledge of the properties of the measured particles may prove to be impractical. One of the key challenges is to discern a path to traceable, reproducible measurements, while not burdening biopharmaceutical laboratories with overly complex procedures.

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