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Analytical Gaps and Challenges for Particles in the Submicrometer Size Domain

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ABSTRACT This *Stimuli* article provides a technical discussion of the available technologies for submicrometer particle analysis, including consideration of the advantages, disadvantages, and technical gaps for each application. These methods can be used in the characterization of different protein aggregates as well as other types of particles in this size range. Changes are occurring rapidly in this field, so the *Stimuli* article and discussions focus on measurement principles and comparisons rather than specific instruments.

INTRODUCTION

Historically, for small molecule parenteral products, the primary concern regarding all particles in the micrometer size range has been the potential hazard of capillary occlusion, with the focus on particles in the ≥10 µm and ≥25 µm size ranges, as described in *Particulate Matter in Injections* ⟨788⟩. Recently, an additional emphasis on biologics has raised the possibility that protein aggregates <10 µm might be immunogenic (*1–2*). This has resulted in the development and application of techniques in addition to light obscuration (LO) for the determination of size, counts, and/or quantity and type of these aggregates. Furthermore, the new biologics-specific compendial chapter, *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, includes sample handling that is more appropriate for biologics, e.g., smaller sampling volume, and the recommendation to measure particles that are between 2 and 10 µm in size by LO. There is also a new informational chapter, *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩, which describes additional tools and instrumentation for analyzing these smaller subvisible particles (SbVP). These tools have been useful for characterizing proteinaceous particles during development and have been applied more broadly to understand the root cause of aggregate formation during process and formulation development, and to demonstrate process control (*3–4*).They have also been applied to SbVP to understand the relationship between particle attributes, such as size, chemical modifications, conformation, composition, and reversibility (*5*) and their potential for causing immunogenicity, as studied in both in vitro and in vivo model systems (*6–12*). A commentary co-authored by multiple biotherapeutics companies on strategy for analysis of these aggregates/particles has recently been published (*13*).

Protein aggregates span a continuum from dimer up to visible particles, with the concentration of particles present decreasing as the particle size increases. There is a gap in the analysis of species between those that can be analyzed by size-exclusion chromatography (SEC) and the SbVP covered by the analytical methods described in ⟨787⟩ and ⟨1787⟩. Proper assessment of the immunogenicity risk posed by proteinaceous aggregates (and particles) in the submicrometer size range requires information about both size and number, but preferably also about the structure and conformation of the protein. The analytical gap to obtain the size distribution of submicrometer particles and to otherwise characterize these species also complicates studies on the mechanism of protein aggregation and the development of mitigation strategies.

Analytical tools routinely and reliably applied to analyze submicrometer particles would greatly enhance studies of aggregation and root cause analysis. This approach also could potentially prove more sensitive as an early indicator of formation of protein aggregates before they become large enough to be detectable or measureable by SbVP techniques. Little has been published about the submicrometer particles, even for marketed products, with regard to their size distribution, counts/quantity, composition, morphology, stability, correlation to aggregates in the oligomer and SbVP and visible particle domains, and their biological consequences. This is being addressed in one example by the International Consortium on Innovation and Quality in Pharmaceutical Development (www.iqconsortium.org), which is conducting a cross-industry survey to determine the amount of particles between 0.1 and 1 µm in different types of biologics currently in the clinic or on the market.

This *Stimuli* article focuses on the analytical techniques that are being developed to address this submicrometer size range analytical gap, including the strengths and weaknesses of the tools currently available and areas that still need to be improved. It is hoped that this *Stimuli* article discussion will motivate work in this area and result in at least an informational chapter in the compendia.

GENERAL ISSUES

Currently there are a number of techniques that, in principle, are capable of analyzing submicrometer particles (see *Table 1*). These techniques are supported by the methods shown in *Table 2* that are applicable to analyzing SbVP. Ideally, one method would be able to size the particles, quantify/count them, identify the different particle species in the sample to be analyzed, and further characterize them, e.g., protein conformation and chemical modification (*5*). However, there is no single method available that is capable of covering all of these aspects in the wide size range of interest, i.e., from 20 nm to 100 µm. To achieve a comprehensive profile, it will likely remain necessary to compile results from multiple methods. It is therefore the scientist's responsibility to select the most suitable combination of methods for characterizing the samples and attributes of interest. The primary focus of this *Stimuli* article is the inherent particles present in the biologics drug product. These are particles arising from the protein itself (see ⟨1787⟩ for definitions of inherent, intrinsic, and extrinsic particles). For inherent particles, properties of interest include size, count, composition, association behavior, structure/conformation, chemical modification, and morphology (*5*). The second category of particles—intrinsic to the drug product but not to the active ingredient itself—is also discussed in this *Stimuli* article. It is important to be able to identify and quantify these particles, the most common of them being silicone oil droplets. Some of the techniques discussed in this article can be useful for this purpose, although they do not work perfectly. The third category, extrinsic particles, is comprised of all particles that are not a part of the formulation, process, or package.

Table 1. Overview of Analytical Techniques for Analysis of Particles in the Submicrometer Size Range

Technique	Size[a] Range	Attributes	Typical/Ideal Applications for Protein Pharmaceuticals	Comments/Limitations
Analytical ultracentrifugation (AUC)	1–100 nm	Directly reported: Sedimentation coefficient distribution; Sedimentation velocity analytical ultracentrifugation; Molecular weight; Sedimentation equilibrium Derived: Concentration of monomer, oligomers, fragments; Molecular shape; Second virial coefficient	Quantification of monomer, oligomers, and fragments	Orthogonal method to SEC and FFF; Analysis in formulation buffer possible; Higher limit of quantitation (LOQ) than high pressure size exclusion chromatography (HP-SEC); Sample may need dilution
Field flow fractionation (FFF) including: Asymmetrical flow field flow fractionation (AF4); Hollow fiber flow field flow fractionation (HF5)	1 nm to several µm	Directly reported: Fractogram of monomer, oligomers, fragments separated by size and diffusion properties Derived: Concentration of monomer, oligomers, fragments; Size or molecular weight; Presence of submicrometer particles	Quantification of monomer, oligomers, and fragments; Qualitative analysis of SbVP in the submicrometer range when coupled with light scattering (LS)	Orthogonal method to HP-SEC and AUC; Requires more extensive method development compared to HP-SEC; Analysis in formulation buffer possible

Technique	Size[a] Range	Attributes	Typical/Ideal Applications for Protein Pharmaceuticals	Comments/Limitations
Atomic force microscopy (AFM)	1–500 nm	Directly reported: Size, shape of monomer, oligomer, and particles Derived: Conformation	3D images of particles	Requires separation of protein and aggregates from supernatant yet allows direct observation without further preparation; Need immobilization; Statistical robustness is a limitation
Electrical sensing zone (ESZ); Resistive pulse sensing (RPS)	100 nm to 1600 µm	Directly reported: Particle size and counts Derived: Particle shape	Quantification of subvisible particles	Orthogonal to light-based techniques; Not impacted by low optical contrast between particle and medium; Needs conductive medium; Compatibility with electrolyte needs to be assessed; Multiple apertures needed to cover wide size range; Smaller apertures are susceptible to clogging
Disc centrifugation, also differential centrifugal sedimentation (DCS)	Instrument-dependent. Lower size limit: 3–10 nm. Upper size limit: 30–100 µm. Depends on instrument and analyzed samples	Directly reported: Sedimentation coefficient distribution Derived: Particle size distribution (via Stokes' Law and estimated particle density)	Particle size distribution for SbVP	Not widely used
Flow cytometry	100 nm to 100 µm	Directly reported: Particle counts and distribution Derived: Particle types	Particle quantification and characterization; Particle sorting	Fluorescence channels are orthogonal to fixed-flow cell-based techniques; Some instruments are capable of classifying particle types based on imaging/fluorescence signals; Particle sorting, enrichment for further analysis
Light obscuration (LO); LS particle counter	300 nm to 200 µm	Directly reported: Particle size and counts Derived: Particle size distribution	Particle quantification via particle counting	Optical system, sizing relative to particle calibration standard used; Heavily impacted by refractive index difference
Dynamic light scattering (DLS)	1–1000 nm	Directly reported: Autocorrelation function of scattered light Derived: Diffusion coefficient, hydrodynamic radius, size distributions, polydispersity index	Detection of aggregates/particles, e.g., during formulation screenings	Optical system, limited to low protein concentration applications; No counts/concentration; Results skewed by presence of large particles

Technique	Size[a] Range	Attributes	Typical/Ideal Applications for Protein Pharmaceuticals	Comments/Limitations
Static light scattering (SLS) (ensemble)	1–1000 nm	Directly reported: Intensity of scattered light at single angle or multiple angles Derived: Average size, shape, molecular weight	As detector for fractionation techniques, e.g., SEC/FFF; Stand alone in batch mode for molecular weight[b] and size	Optical system, commonly used as detection for dilute solutions
Nanoparticle tracking analysis (NTA)	200–1000 nm	Directly reported: Diffusion of individual particles tracked via imaging of light scattered from particles Derived: Particle counts (concentration); Hydrodynamic radius; Size distribution	Analysis of protein aggregates, or particulate active pharmaceutical ingredients (API), e.g., liposomes	Orthogonal to DLS; Optical system, heavily impacted by refractive index difference and light scattering by monomeric protein/species <50 nm, dilution may be necessary for high-concentration samples; Presence of a few large particles easily skews results; Representative sampling critical
Suspended microchannel resonator (SMR); Resonant mass measurement (RMM)	200 nm to a few μm	Directly reported: Buoyant mass of individual particles, counts Derived: Particle size and distribution; Particle classification (positively or negatively buoyant)	Quantification of oil droplets and differentiation of oil droplets from non-oil particles	Particle density is sample dependent; Particle size calculated from the measurement depends on the density value used; Representative sampling critical
Transmission electron microscopy (TEM)	5–1000 nm	Directly reported: Particle image Derived: Size, shape	High definition images of particles	Representative sampling critical; Labor and skill intensive
Turbidity/nephelometry	Not applicable	Directly reported: Light scattering/blockage Derived: Turbidity	Ensemble assessment of particle presence in liquid samples	Results are relative and nonspecific
Hyperspectral imaging	200–1000 nm	Directly reported: Images and electromagnetic spectra of selected objects Derived: Particle classification	Optical observation and quantitative spectral analysis of nanoscale species	Particle classification based on spectral library

[a] Size is a generic term here, referring to the length dimension (particle diameter) determined by a technique. Commonly used particle size terms include hydrodynamic radius or diameter (r_h, d_h) by DLS and NTA; radius or gyration (r_g) by SLS; equivalent circular diameter (ECD) by LO or digital image analysis technique (DIA); equivalent spherical diameter (ESD) by ESZ; maximum Ferret diameter by DIA; and longest dimension by microscopy.

[b] Molecular weight = weight-average molecular weight.

Table 2. Overview of Analytical Techniques for Analysis of Particles in the Micrometer Size Range

Technique	Size Range	Attributes	Typical/Ideal Applications for Protein Pharmaceuticals	Comments/Limitations

Electrical sensing zone (ESZ); Resistive pulse sensing (RPS)	100 nm to 1600 µm	Directly reported: Particle size and counts Derived: Particle shape	Quantification of subvisible particles	Orthogonal method to LO, DIA, and other light-based methods; Compatibility in electrolyte needs to be assessed; Multiple apertures needed to cover wide size range
Dynamic imaging analysis (DIA); Flow imaging microscopy	1–400 µm	Directly reported: Particle size, counts, shape, and morphology Derived: Particle type	Product development, stability, comparability, and compatibility; Particle classification and differentiation between silicone oil and non-silicone oil particles	Orthogonal to LO; Optical system, impacted by refractive index difference; Particle differentiation inference from images; Differentiation possible for particles ≥5 µm
Light obscuration (LO)	1–200 µm	Directly reported: Particle size and counts	Pharmacopoeia method (⟨787⟩ and ⟨788⟩); Routine use for conformance	Well established method; Optical system, heavily impacted by refractive index difference; No differentiation of particles
Fourier Transform Infrared (FTIR) microscopy	>10–20 µm	Directly reported: FTIR spectrum, particle image Derived: Particle identification	Investigational forensic tool for particle identification	Particle needs to be isolated (e.g., on filter membrane); Reference spectra may be needed; Low throughput
Raman microscopy	>1 µm	Directly reported: Raman spectrum, particle image Derived: Particle identification	Investigational forensic tool for particle identification	Particles can be analyzed in situ or after isolation (e.g., on filter membrane); Reference spectra may be needed; Low throughput
Fluorescence microscopy (with dye staining)	>1–2 µm	Directly reported: Particle image/fluorescence signal Derived: Particle type	Visualization of protein particles by dye (better contrast); Direct analysis in the formulation	Particles need to be stained by dye; Interference by polysorbate; Low throughput
Scanning electron microscopy with energy dispersive X-ray spectroscopy	>1 µm	Directly reported: Particle image and EDX spectrum Derived: Particle identification	Investigational forensic tool for particle identification	Particles need to be isolated and dried; Low throughput

Silicone oil, specifically when added to aid in the processing of the product (e.g., stopper flow in the bowl) and to ensure the functionality of primary packaging (e.g., prefilled syringes), requires special consideration because it is a source of subvisible and submicrometer particles. These silicone oil droplets are seen as particles by most instrumental techniques. Droplets arising from silicone oil are considered intrinsic particles and frequently account for a significant fraction of the total particle count in a product, in all size ranges, especially for prefilled syringes. The contribution of silicone oil droplets to total particle counts also tends to change over time, and syringe-to-syringe variability can be substantial with respect to silicone oil content. The possibility that silicone oil droplets could interact with protein, in the absence of surfactant, and cause denaturation/aggregation (*17*), and thus carry risk for potentiating immunogenicity (*14*), highlights the importance of silicone oil quantitation and control. The ability to identify and subsequently count and size silicone oil particles is therefore quite important, as discussed in ⟨1787⟩. However, recent comprehensive reviews of typical particulate matter and the medical risk factors do not include silicone oil alone as a toxic agent (*15–16*). According to Felsovalyi et al. (*17*), silicone has extensive long-term stability data and a long history of use with no safety issues. Regardless of any safety concerns, it is important to differentiate between silicone oil and proteinaceous particles in the product.

For setting up a proper particle characterization strategy, the knowledge of the measurement principle of the analytical technique — including sample requirements, method limitations, and how to evaluate the results — is essential for generating relevant, robust data. The following general aspects typically play a role in this context.

Ensemble vs. Single-Particle Techniques

As the detection of single, submicrometer particles can be difficult, a number of techniques infer the particle size distribution (PSD) from the properties of a large number of simultaneously measured particles. For example, the PSD may be inferred from the angular dependence of the light scattered from a particle suspension. These techniques, e.g., dynamic light scattering (DLS), static light scattering (SLS), and laser diffraction, as discussed in *Table 1*, are termed ensemble measurements. The ensemble measurements have the advantages that the signal-to-noise ratio of the measurement is increased, the size range may be broad, and some instruments allow low sample volumes. However, there are significant disadvantages to ensemble methods as well, including the following:

- Deconvolution of the measured data to obtain the PSD may be inaccurate or subject to large errors for highly polydisperse suspensions (such as those with typical aggregated proteinaceous particles).
- The protein monomer population may dominate the ensemble signal, particularly for high protein concentration solutions, masking out the particle population of interest.
- The presence of a few large particles can skew the PSD profile.
- There is often no capability to distinguish between particles of different types (e.g., silicone oil droplets versus aggregated protein particles).
- There is no capability to provide the absolute amounts of particles or aggregates.
- Determination of a quantitative PSD requires a reliable model that relates the measured signal to the particle concentration.
- Sizing accuracy is based on the correctness of assumptions for the solution viscosity (DLS, SLS) and particle refractive properties (laser diffraction using Mie theory).

Imaging techniques (optical microscopy, electron microscopy, and atomic force microscopy) inherently measure single particles, provided that the particles are well dispersed during sample preparation. With these techniques, however, it is difficult or impossible to characterize sufficient numbers of particles in their native, suspended state to generate a profile that accurately represents the entire particle population present in the sample. Optical microscopy may be useful for suspended particles down to the diffraction limit, at about 0.3 μm.

A number of relatively new analytical techniques do achieve measurements of single particles in suspension and can measure sufficient numbers of particles. These techniques have limitations that are summarized in *Table 1*. Examples include electrical sensing zone (ESZ, e.g., Coulter counters), suspended microchannel resonator (SMR), resonant mass measurements (RMM), and nanoparticle tracking analysis (NTA).

Concentration

An important parameter that determines applicability of a method is sample concentration, both that of the protein and that of the particles within the drug product solution. Light-based methods, such as DLS, NTA, laser diffraction, and LO, may be significantly impacted by high protein concentration due to 1) background light scattering of the monomer/dimers, 2) reduced optical contrast or signal due to increased refractive index of the bulk liquid at high protein or excipient concentration, or 3) secondary effects of high concentration such as non-ideality or increased viscosity.

All instruments have an optimum range of particle size, size distribution, and concentration. Particle counting methods where single particles are detected in a sensing zone, such as ESZ, SMR, or LO counters, may give incorrect sizes and counts when there is significant probability of coincidence of particles within the sensing zone or measurement cell at high particle concentrations. Similar interferences occur in imaging or tracking instruments as a result of overlap of particle tracks or images. Very low particle concentrations can result in poor statistical sampling, especially if the volume of sample analyzed is a small fraction of the total sample of interest. Overall, the user should be aware of the concentration ranges of bulk protein and of particles that are required for the method of choice.

Principle of Measurement

Methods for characterizing submicrometer particles can be categorized by the basic physical principle of detection (see *Table 1*):

- Quantitative measurement of scattered light
- Size-dependent particle diffusion, observed by time-dependent light scattering or optical particle tracking

- Perturbation of a measured property resulting from displacement of fluid volume or fluid mass and mass/density
- Direct imaging of particles, by profiles of intermolecular forces (atomic force microscopy) or electron interactions (transmission electron microscopy)

Some of these detection methods may be combined with separation methods that fractionate particles by mass or volume. Field flow fractionation (FFF) methods rely on hydrodynamic effects to separate particles by size, and appropriate detectors are coupled to the FFF system to provide PSD and relative amounts of the eluted particles. In contrast, analytical ultracentrifugation (AUC) separates particles by sedimentation, and quantitative analysis of the resulting particle concentration profiles in the centrifugation cell provides PSD and amounts.

Mathematical Correlation Across Techniques

The basic principles of the measurement determine how the reported diameter is defined. For example, diffusion-based measurements give the hydrodynamic radius of the particle, whereas static, angle-dependent light scattering gives the radius of gyration. These fundamental differences in reported sizes can lead to difficulties in comparing PSDs obtained with different instruments. There are two complementary approaches to this problem. In the first approach, the geometry of the particles can be either assumed or independently measured, and from the geometry, conversion factors for use between different diameters may be obtained. Unfortunately, little information is available on the properties of aggregated proteins in the submicrometer size range; this type of information is necessary for determining conversion factors. As an example, SMR measures particle mass directly, and reporting protein particle concentration as a function of particle mass is straightforward. Converting these measurements to a report of particle concentration versus diameter, however, requires knowledge of the aggregate packing fraction (the volume fraction of the aggregate that is dry protein). Similarly, light scattering results for protein particles cannot be interpreted correctly unless the average refractive index of the particles (which can be inferred from the packing fraction) is known. The same lack of knowledge of the attributes of submicrometer aggregates also limits the development of reference materials that closely resemble protein aggregates in density, optical contrast, and/or morphology.

In the second approach to comparing PSDs, the ratio of different types of diameters may itself give valuable information on the morphology of particles that are difficult to analyze by direct microscopic methods. For example, the ratio of the hydrodynamic radius to the radius of gyration gives information on the packing density of the particle as a function of distance from the particle center.

Correlating the results of size, counts, and PSD obtained using different techniques can be complicated by measurement biases of the individual techniques. Many techniques show a drop in sensitivity near the upper and/or lower size limits of the technique, resulting in an artificial drop in particle concentrations at these detection limits. Control experiments with particles of known size and other properties relevant for detection should be conducted to understand the practical limits of the techniques for the particle types being investigated. The results of such experiments can define the boundaries of reliable data for each instrument. The PSDs obtained from ensemble techniques, in the absence of prior size separation steps, are especially prone to errors, and these methods should be carefully assessed with poly-disperse particle suspensions of a known PSD.

Sizing and counting results obtained using (some) optical techniques often are dependent on the calibration procedure and standards used. One approach is to apply correction factors to the measured particle diameter and/or the particle concentration (*18*). However, algorithms for application of corrections have not been developed fully for instruments that measure submicrometer particles (*19*), and application of correction factors will be difficult without knowledge of the type and properties of the measured particles. Commercial concentration standards, based on PSL beads, are available in the submicrometer range. For optical methods, measurements on particles with reduced refractive index relative to the matrix liquid are recommended as a complement to PSL measurements (*20*).

Sample Preparation

Sample preparation can be a source of artifacts for most analytical methods. To avoid contamination by foreign particles, sample preparation may need to be conducted in a controlled environment using items such as a laminar flow hood, particle-free containers, and/or particle-free buffers for dilutions. Suitable controls should be used to show that the sample preparation itself does not lead to the uptake or formation of foreign particles or loss of inherent particles. Entrapment of air bubbles is also a common source of artifacts, especially for highly viscous samples and may require degassing. Degassing procedures should be standardized, and it should be confirmed that they do not cause further aggregation or generation of particles.

Careful pipetting and sample handling are crucial because air bubbles can be difficult to remove once they have been introduced into the sample. Also, attention to homogeneous and representative sampling is important. Particle stability as a function of time, temperature, and handling needs to be understood and taken into consideration. Sonication should not be used as a means to homogenize protein samples.

Other non-apparent differences in sample handling and preparation or differences in equipment can result in confounding data as exemplified in intra-laboratory sample analysis.

Sample dilution may also cause particles to aggregate, break apart, or dissolve, thereby resulting in either higher or lower particle concentrations and altered PSDs (after correction for the dilution factor) relative to the non-diluted sample.

Controls

Use of appropriate controls is important to assess and ensure the quality of the obtained results. The measurement of negative controls (blanks) and positive controls should be integrated into the measurement routine.

CONTROLS FOR SYSTEM SUITABILITY

For particle sizing/counting methods, regularly scheduled calibration and maintenance is often done with multiple PSL standards of different sizes covering a wide size range, whereas system suitability is often checked with just one or two standards prior to analysis. However, because protein particles have different properties compared with polystyrene spheres (e.g., refractive index, shape, and size distribution) additional controls may be beneficial for judging the suitability of a method to quantify and characterize proteinaceous particles. The difference in the refractive index between the solution and the particle being analyzed is an important parameter. In general, a lower image contrast or smaller difference in the refractive indices between the particles and solution downgrades sensitivity of optical methods.

ADDITIONAL CONTROLS

Controls to assess the impact of sample preparation are also important. The procedure should involve conducting the same sample preparation for a placebo as that used when measuring actual product samples. Analysis of the particle concentration of water or a particle-free buffer when it comes in contact with the equipment and/or disposables should also be used as a control.

Use of controls to assess the impact of sample dilution should include experiments to test for the influence of dilution on not only particle concentrations but other sample properties, e.g., by analyzing the samples with additional/orthogonal methods.

Controls for background levels of sample matrix/excipients should include analysis of the active pharmaceutical ingredient (API)-free formulation in parallel with the drug product.

Artifacts, Matrix Effects, and Points to Consider

- Entrapment of air bubbles during sample handling can lead to artificially high counts (*21*).
- Foreign matter (e.g., dust, sample-container or measurement-cell debris) may skew the measurement or lower the signal-to-noise ratio.
- Schlieren lines (optical artifacts resulting from inhomogeneous refractive index of the matrix liquid) can cause erroneous results for the optical-based method. Schlieren lines can occur, for instance, if pre-run volumes are too low, resulting in retention in the flow cell of some liquid with a different refractive index, which can mix with the incoming sample. Care must be taken to verify proper flow-cell priming when the sample, pre-run, or rinse fluids have very different viscosities.
- High particle counts may arise from excipients, for example, the presence of surfactant micelles, nanoparticle impurities within sugars (*22*), and other species that appear as particles for the counting method being used.
- For light-scattering and light-imaging methods, a low refractive index difference between proteinaceous particles and formulation can lead to the underestimation of particle sizes and counts. For DLS and NTA, the measured size of individual particles is not greatly affected, but a low refractive index difference can result in particles not being detected, leading to reduced particle counts and/or a shift in PSD. A low refractive index difference will cause large size errors for SLS and LO measurements throughout the full size range of the instruments. Dynamic imaging analysis (DIA) measurements can be in error as a result of reduced image

contrast, especially for particles that are less than approximately 5 µm, which might not be detected at all, or as a result of the detection of large particles as multiple smaller fragments.

- Light-based particle characterization methods are not suitable for measuring turbid or opalescent samples, unless the purpose is to measure sample turbidity. If light-based methods must be used for such samples, dilution is recommended to minimize the interference caused by turbidity or opalescence.
- The presence of highly-scattering particles can interfere with measurements based on light scattering. The significance of DLS data as an ensemble technique can be impeded by a few highly-scattering particles, masking out smaller particles. NTA may be disturbed as well, which can result in undercounting or can render it impossible to perform a reasonable measurement.

REQUIREMENTS FOR ROBUST ANALYSIS

One of the main challenges for submicrometer particle analysis is the robustness of the methods. Important factors to consider include statistical relevance of results, appropriate application of methods, reduction and elimination of artifacts, and correct interpretation of the data.

Current experience with measurements of particles in the submicrometer size range indicates that particle concentration increases substantially as the size of the particles decreases. However, measurements in the submicrometer size range have significant issues with data reliability (both size and count), and therefore the validity of the magnitude of particles reported should be ascertained with benchmarking data or with historical trending.

Robust analysis of these large counts requires the following, purely from a statistical standpoint:

- The sample aliquot used for measurement must be representative of the whole sample and of the population of particles present. This is especially important in techniques that count particles singly, such as NTA and SMR. Multiple measurements on independent aliquots may be needed.
- An adequate number of particles should be counted to obtain results that are statistically sound. Counting a larger number of particles will reduce the standard error of the mean, as the general practice is to analyze three aliquots from each test article. If counting fewer particles per aliquot, a larger number of aliquots would be required to reduce the standard error of mean. Analysis should also be on a large enough fraction of the total sample to avoid magnification of errors due to large correction factors when reporting particles for the entire sample volume (*23*).

Beyond these general requirements, there are points to consider based on which instruments and techniques are being used:

1. The sample preparation procedure must not require significant manipulation, as this can result in changes to the particles. If dilution is required, the dilution procedure must be checked to ensure the lack of impact on the results [(see ⟨1787⟩)] (*21*), including selection of the dilution medium. The minimum dilution factor that is suitable should be used, ascertained by performing a dilution series. Other sample manipulations must be controlled and verified similarly.
2. Blanks and appropriate placebo controls should be included as part of the analysis to understand the background counts and any contributions from non-protein components of the sample.
3. Risk of blockage of the measurement channel or cell by any large particles present in the samples should be considered and monitored when using small-bore cells to measure small particles.
4. Preferably, measurements should be performed in the middle of the dynamic range (for size and especially count) of the instrument to obtain accurate and reproducible results. Diluting or concentrating samples to minimize instrumental artifacts may have undesirable effect(s) on the sample.
5. Instruments in the submicrometer range usually measure a small aliquot of the sample. Adsorptive losses to tubing and other contact surfaces should be considered, although with high particle counts in the submicrometer size range, these losses may not introduce significant error.
6. Stressed samples can be used to identify the threshold for detection of a change between samples for the different techniques. Orthogonal methods may be used to supplement the analysis.
7. The protein monomer in solution creates a background that will depend on the protein concentration of the sample and will change the detection threshold or characteristics. The other excipients [especially those present in larger amounts, e.g., sucrose or submicrometer particle impurities within sucrose (*22*) will also have an impact. For light-based techniques (DLS, NTA) the optical properties will change, whereas for SMR, the background density will be affected.

8. When considering the differentiation of particles, it should be recognized that the size and classification output is derived from a combination of measured and assumed parameters, for example, SMR estimates size based on assumed densities for silicone oil and protein particles (*24*). The parameters may have to be revised for different samples.

Finally, data interpretation is an integral part of obtaining robust results. This includes having a good understanding of the measurement principles involved, recognizing the limitations of each technique and therefore avoiding over-interpretation, and discarding results that are not trustworthy. For example, a single peak from a DLS measurement should not be interpreted as a monodisperse distribution of protein or particles in the tested solution, and a multi-modal distribution profile is often an indication of poor data. One needs to evaluate the goodness of the correlation function to assess the quality of the data instead of directly reporting the values and profiles produced by the software.

APPLICATION OF TECHNIQUES: CONSIDERATION OF STRENGTHS AND WEAKNESSES

As alluded to in the introduction, measurements of particles in the size range of 20 nm to 1 µm are both more difficult and less common in practice than measurements below 20 nm (e.g., protein oligomers measured by SEC) or above 1 µm (e.g., SbVP by LO or DIA).

There are reasons, however, for extending particle measurements into the submicrometer range. The gap between maximum size for SEC and the lower limits of DIA and LO is greater than one order of magnitude. Not knowing amounts, size distribution, and the characteristics of the particles in the size range from approximately 20 nm to 1 µm increases the risk of incomplete knowledge of drug product characteristics; for instance, it is not possible to determine whether there is a relationship between protein aggregates across the size continuum without the ability to measure the protein particle population across the entire range. Studies on the mechanism of protein aggregation and subvisible and visible particle formation, and on the root causes of these particles, are confounded by the difficulty of counting and characterizing particles in the submicrometer range. We cannot determine if there is a mathematical correlation between the particles of different sizes until we have a way of reliably measuring every population.

Similarly, it is not possible to properly correlate immunogenicity, efficacy, and other biological consequences of these submicrometer particles without, at a minimum, the ability to count and size them. An overarching question when studying immunogenic potential of particles is whether the total immune response to a set of particle species (if seen) correlates best to particle concentration, mass, volume, or other characteristics, in addition to particle size. Typical PSD profiles for aggregated protein show rapidly increasing particle concentrations as the diameter decreases. Thus, if the response is proportional to particle concentration independent of size, measurements in the 20 nm to 1 µm range may be critical for accurately assessing the immunogenicity risk of a therapeutic protein formulation. The particle mass or volume distribution, however, may peak at a size greater than 1 µm, and acquiring data below 1 µm may not add significant value to the risk assessment; this can only be understood with reliable measurements of the population across the entire range.

Efforts to determine whether there is a specific size of protein aggregate that has the greatest potential to be immunogenic have yielded a variety of conclusions, depending on whether the aggregate studied was in the presence of adjuvant, such as virus-like particles in vaccines (*25–26*), or was generated by stressing a protein solution (*7,27*). Protein aggregates in the absence of adjuvant in the submicrometer size range appear to have substantially less immunogenic potential than the SbVP that are between 2 and 10 µm (*27–28*). Studies like these are very important, but to date they have not included sufficient characterization and quantification of submicrometer particles. This further demonstrates the need for development of analytical methods for these species.

Until recently, the number of techniques that could be applied to the submicrometer size range was limited. However, an increasing number of applicable technologies is being developed or adapted for this purpose. The established techniques of flow cytometry and FFF have been applied successfully to the measurement of protein particles (*29*). The newer methods of SMR, NTA, resistive pulse sensing (RPS, a variation of ESZ) and SLS are now available as commercial instruments. In principle, this instrumentation diversity provides a set of orthogonal measurements sufficient to distinguish different particle types. FFF and AUC in combination with appropriate detectors can provide both particle size and mass distributions (over a wider size range than other methods), and NTA, RPS, and SMR are single-particle methods that give particle size, concentration, and buoyant mass, respectively.

However, challenges remain in the routine study of submicrometer particles. Instruments covering the submicrometer size range require care in operation, environmental considerations, and a high level of operator understanding and skill; discrepancies between different methods can be quite large; and there are no established methods for comparing or standardizing measurements from different instrument types. Working with suspensions of protein particles presents challenges that are not present when measuring monodisperse and nonadherent samples. For example, polydisperse and morphologically irregular protein particles readily clog small channels used for SMR and RPS, and proteins can adsorb onto membranes used in FFF, leading to poor recovery and repeatability. The sensitivity of detectors and small sample mass typically used for FFF can limit the ability to detect and quantitate the data generated. Diluting samples to match optimum concentration ranges can result in formation or dissolution of particles. As a result of these factors, confidence in data from the submicrometer size range is not high at present.

To promote reliable, routine measurements in the submicrometer region, the greatest needs are improved reproducibility, robustness and simplicity of instruments, and ability to measure the sample directly.

Standardized methods and ways of harmonizing the different instrument outputs would also improve measurement reproducibility and utility. This in turn would improve the likelihood of making submicrometer particle measurements routine. The availability of abundant submicrometer-particle data will facilitate evaluation of the safety impact of these particles, their value as a product quality indicator, and their criticality to the aggregation pathway. To clarify the relationship of submicrometer particle concentrations to product quality risk, further research is needed:

1. Further measurements on the immunogenicity of size-selected particles would improve our understanding of which particle properties and size ranges correlate best with immune response and biological properties (such as potency).
2. Data sets for protein particle size distributions, over a size range from submicrometer to 100 µm, are needed to understand whether particle concentrations in the micrometer size range may be extrapolated to smaller sizes. These experiments should be conducted using formulations and particle-generating stresses that yield particles that are highly comparable to those generated in actual drug production and storage.
3. Better methods are needed to differentiate particle types and also to determine protein conformation (native, unfolded, partially unfolded, and secondary and tertiary structure) in the submicrometer size range.
4. Appropriate methods and reference materials need to be developed to ensure accurate counting and sizing of particles in the submicrometer range.

CONCLUSION

It is apparent that technological advances over the last few years have begun to close the so-called "subvisible gap" in measurements of particles in therapeutic protein products (*30–31*). To some extent, particles in the submicrometer size domain that are present in these products can now be detected, sized, counted, and also classified, in increasing order of difficulty and decreasing order of robustness. The techniques used when analyzing the same samples will report results that differ from each other due to the variety of detection and characterization principles involved. The robustness of the analysis is also currently limited due to lack of appreciation of factors that can lead to artifacts and incorrect interpretation of results, as well as technical limits of the instruments. It is expected that increased experience with the techniques, as well as the innovation and development of technology and instrumentation, will help to increase our understanding of their advantages and limitations, as well as bridge the gap between techniques.

We hope that this *Stimuli* article will encourage scientists to explore the utility and applicability of the techniques on a diverse range of samples, develop best practices for their use, and share the experience with the community through publications and presentations. At the same time we hope that the instrumentation community can also engage with development scientists to further improve the technologies and techniques as well as their applicability to diverse range of product types.

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