COMMENTARY

Protein Particles: What We Know and What We Don't Know

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Abstract

All therapeutic protein products contain intrinsic particles formed by the aggregation of protein monomers. There is growing interest in understanding particles in biopharmaceutical products, fostered on one hand by significant advancements in particle analysis and on the other hand by concerns about potential impact of particles on product quality and safety. With currently available methods, particles in therapeutic proteins can be counted, sized, and characterized in a rudimentary way over a broad size range (from 10s of nanometers to 100s of micrometers). Here we review the known attributes of common protein particles, and then discuss the gaps in our current knowledge. The capabilities, limitations, and opportunities for improvement of common particle counting and characterization methods are listed. We conclude that further analytical progress is needed to better classify and characterize the diversity of particles encountered in therapeutic proteins, which may vary in the degree of protein unfolding, the inclusion of non-protein nucleation centers, and aggregate morphology. Very little is known about the potential correlation between specific particle attributes and increased immunogenicity. In this environment of uncertainty, a deeper understanding about specific particle attributes and potentially increased immunogenicity is greatly needed and will likely be an area of future intensive research.

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1. Introduction

Protein particles consist of reversibly or irreversibly associated protein molecules ranging in size from small oligomers to large, extended aggregates 100s of micrometers long. In well formulated therapeutic proteins, protein particles typically represent an extremely low fraction by mass of the total protein, [1] which decreases safety concerns. However, this low mass fraction also limits method development and characterization approaches, and thus our overall understanding about particles and their formation. Since particle formation is an inherent degradation pathway for proteins,[2] particles are ubiquitous and have been found, at some level, in all commercial therapeutic proteins.[1] Particle formation is often governed by slow or delayed-onset kinetics,[3] a phenomenon which poses substantial analytical characterization challenges (following changes over long periods) as well as formulation challenges (developing stable therapeutic proteins for intended use up to three years after manufacture).

With the present state of analytic instrumentation, basic attributes such as particle size, concentration, and morphology at sizes above 10 μm can be rapidly measured. Excellent reviews summarize the present state of the art for analytical methods as applied to the measurement of small, soluble aggregates[4] and of larger aggregates and particles.[5] Additional physicochemical attributes, including chemical composition or modification and protein secondary structure, can be measured with existing technology, but this process is slow and expensive, requiring the use of multiple, orthogonal analytical techniques. Improvement is needed in instruments that enable the rapid, simultaneous measurement of multiple particle attributes. Development of these tools would lead to a better understanding of the diversity and range of particle attributes, would promote the accurate measurement of rapidly changing particles, and would give better identification of particles of mixed chemical composition.

Improvements in particle characterization will complement other important research fields in coming years, such as developing methods for predicting particle formation as well as developing formulation tools for minimizing their formation.

Protein particles have been suggested as the cause of adverse events and of increased anti-drug antibodies in patients,[6-8] but the extent of the risk remains unclear. Mitigation of risk associated with immunogenicity of subvisible and visible particles is very important for the successful development of therapeutic proteins. While a clear correlation between the properties of protein particles and immunological response in patients has not been well established, manufacturers of therapeutic proteins are implementing various control strategies and continuous improvements to minimize particles such as

- Risk assessment of the role of manufacturing process, transportation, storage, clinical administration, etc. on their potential impact in fostering particle formation.
- Control of subvisible and visible particles over the shelf life of the product.
- Understanding the influence of the *in vivo* environment on promoting or mitigating particle formation.

These efforts are substantial and will be more focused and effective if we are able to identify and reduce those particles that have a higher likelihood of inducing adverse events or immunogenic responses. Thus evaluating the correlation between specific, measurable, particle attributes and potentially increased immunogenicity in patients will be a highly impactful and desirable area of intensive research in the coming years. These efforts necessitate the need to extensively characterize protein particles beyond our current capabilities, which will require enhancing analytic characterization of particles and overcoming the method limitations discussed in the last section of the commentary.

This commentary first summarizes what is known about protein particles, considers the limitations of our knowledge, and then discusses particular measurement technologies. We conclude with a discussion of opportunities for improving particle counting and analytical characterization methods.

2. What we know about particles

2.1 Variability of particle properties

Particles in therapeutic proteins have widely varying properties by count, size, morphology, optical density, chemical composition, and other physicochemical properties. By size, particles can be categorized into ranges: nanometer-sized aggregates, submicrometer particles up to 1 μm, 1 μm to 100 μm (commonly termed "subvisible"), and greater than 100 μm (commonly termed "visible").[9] Particles are dynamic and can easily change (count, size, morphology, etc) in response to minor changes in their environment.

Particles can be classified in two broad chemical-composition categories: homogeneous (one predominant chemical entity and source, e.g., protein) or heterogeneous (e.g., protein coating a non-protein core such as fiber, glass particle, silicone oil droplet, etc.).[10] Homogenous protein particles can also differ substantially in multiple aspects including size, protein structure, and morphology. In some cases protein structure is preserved and we classify the structure of these protein particles as native to be differentiated from denatured or partially denatured when loss of structure is detected. Examples of particles composed of native and denatured protein are presented in the next section of this commentary.

The morphology of protein particles is highly variable, ranging from nearly spherical aggregates to long, irregular fibers. Figure 1 presents a schematic of typical particles that may be found in therapeutic proteins. The morphology depends on both the type of stress and the choice of protein. The creation of fiber-like structures indicates a preferred orientation or axis in the aggregation process. Cryogenic electron microscopy reveals that even down to the molecular scale, typical aggregates are highly disordered and are not closely packed. Figure 2 illustrates possible particle structures at various length scales.

2.2 Mechanisms of formation and kinetics of protein particles

The formation of protein particles can be promoted by adsorption of proteins at interfaces (either air, solid, or liquid), certain chemical environments, elevated temperature, presence of nucleation sites; or mechanical stress.[2,10,11-13] Experiments on stressed protein solutions indicate that protein particles most commonly form as a result of a) partial or full unfolding of the protein followed by association with other proteins or protein particles, or b) adsorption of protein, possibly unfolded, to a non-protein particle or interface.

As an example of one pathway to particle formation, elevated temperature promotes local unfolding of proteins, exposing hydrophobic regions which can adhere to hydrophobic regions of nearby proteins. Temperatures above the protein melting point result in wide scale exposure of hydrophobic regions of therapeutic antibodies, which in many cases[14] results in rapid aggregation of nearly all protein in solution. Figure 3 illustrates the optical images and second derivative FTIR microscopy spectra of two particles from monoclonal antibodies. Although there is no discernible difference between the optical microscopy images, FTIR microscopy demonstrates that the native structure is preserved in (3a) while there is a substantial loss of a secondary structure in (3b). Thermal unfolding is only one mechanism of protein aggregation. Although shear rates characteristic of biopharmaceutical processing are only

marginally effective at unfolding monoclonal antibodies and smaller proteins,[11,15] agitation and other mechanical stresses likely promote the interaction of proteins with interfaces that do induce partial unfolding.[16] These examples illustrate the need to characterize protein structure and the chemical composition of particles to elucidate in depth the mechanisms of particle formation. Understanding of these mechanisms will in turn guide efforts to minimize and control particle formation.

In the last 5 years multiple publications have reviewed the emerging general trends for formulation strategies minimizing particle formation.[17] Addition of surfactants and sugars can often inhibit particle formation that may be triggered by incompatible interfaces, partial unfolding of proteins, and/or leachables in the solution.[18]

Protein particles in large number may be created by unit operations such as pump operations[19,20] or steps of the manufacturing processes.[10,11] Small particles generated from moving pump parts can act as nucleation sites for protein particle formation. It is also possible that the high shear stresses or the enhanced exposure of therapeutic protein to liquid-solid or liquid-air interfaces could induce the formation of chemically homogeneous protein particles.

For protein particles, both reversible and irreversible association can occur. While a therapeutic protein is subjected to a particle-inducing stress, the size distribution of the particles may vary considerably.[3] Following particle generation, while the particle-containing solutions were stored, Kiese et al.[21] observed dissolution of reversible particles over a time scale of several months. Interestingly, in this research spiking of monoclonal antibody with previously generated protein particles resulted in no accelerated particle growth, suggesting that protein particles themselves, in the absence of additional stresses, either were not efficient nucleation sites or that the kinetics of particle formation or dissociation were not limited by the availability of nucleation sites. However, in a different study[22] the concentration of large protein particles increased while stored at 2 to 8 °C over 12 to 18 months. This wide variability of behavior demonstrates that particle formation of therapeutic proteins must be investigated on a case-by-case basis.

2.3 Size distribution of particles

The resulting particles vary in size from small oligomers to particles as large as 100s of micrometers long. The particle size distribution is very broad and can often be modeled empirically with particle size distributions with one of two possible mathematical forms—either a power-law or an exponential distribution. For a power-law distribution, the particle concentration N is related to the equivalent particle diameter d:

$$N = N_0 d^{-\alpha}$$

where N_0 is a constant and α is approximately constant over a broad range of d values and in the approximate range 2 to 5.5.[13] The constant N_0 is proportional to the total concentration of protein particles. The parameter $-\alpha$ is the slope of the particle size distribution plotted on a logarithmic scale (i.e., the slope of log(N) plotted versus log(d)). For an exponential distribution, the relation between N and d is:

$$N = N_0 e^{-\beta d}$$

where N_0 is again proportional to total particle concentration and $-\beta$ is approximately a constant. Knowledge of which equation best models the data along with the numerical value of either α or β can give insight into the kinetics of particle formation and dissolution,[23] although modeling the non-equilibrium distributions typical of protein particles is mathematically challenging.

2.4 Particle morphology

Particles that form from thermally denatured protein have a morphology very similar to naturally occurring non-protein aggregates with known fractal characteristics, such as soot particles.[24, 25] On the other hand, fibrous protein aggregates formed from agitated protein solutions do not have an obvious fractal morphology. Fibrous aggregates often have a 'ribbon-like' appearance, with aggregate surfaces that appear smooth, relative to the surfaces of particles with highly irregular morphology. Analogies with particle formation of inorganic materials may provide useful guidance for protein particles. Highly branched, high-surface area structures are characteristic of diffusion-limited aggregation, in which the only limit for growth of a particle is the diffusion of an adherent monomer onto one surface of a growing particle. At the other extreme, growth of a crystal, with its smooth facets and perfect ordering, is limited by the proper orientation of a monomer onto a specific location where growth is favored.

3. What we don't know about particles

3.1 Absolute size and concentration of particles

Counting particles down to a few micrometers in size is now relatively routine. New methods enable counting down to diameters of a few tenths of a micrometer. In practice, measurements of particles using currently available methods can be very challenging. Examples include: when particles are continually growing over time, are weakly associated and therefore susceptible to being broken into many smaller particles during measurement and handling, or when particles are generated and/or introduced by the sample preparation and measurement procedures. Another challenge is that analytical instruments are typically calibrated with reference materials (e.g., spherical polystyrene latex beads) that differ substantially in physical characteristics from protein particles (e.g., see Section 4.3.1). To determine the absolute size and concentration of protein particles requires the development of analytical methods designed to accurately measure the size of protein particles; development of correction algorithms; or the development of new reference materials that mimic the properties of protein particles.[26]

3.2 Particle properties and formation kinetics

Beyond simple enumeration, there are several key areas where further knowledge is needed: the kinetics of protein particle formation, the internal structure of protein particles, and the relation of amount and physicochemical characteristics of protein particles to response by a patient's immune system. Industry also needs methods that can rapidly distinguish between subpopulations of particles. The literature suggests that there are multiple potential pathways for particle formation and particle formation will vary on a case-by-case basis for each individual system studied.[18]

Protein particle growth kinetics has been studied,[3] but a detailed understanding of particle growth is hampered by the lack of sensitivity of methods that measure particles in the size range from 0.1 μm to 1 μm. What is well known is that on an empirical basis, particles will initially be observed in a degrading protein solution at size scales of a few micrometers. However, this observation should not be construed as evidence that growth kinetics favor particles of a few micrometers in size—it may only be a result of the extraordinary sensitivity of commercial particle counting systems for particles of this size range.

The density of amorphous protein is established from evaluation of the adsorption of proteins onto surfaces.[27] What is not well established is the density of aggregated proteins, which may have a much more open structure than a dense, amorphous sphere of protein.[26] Density is a key particle parameter in

relating optical scattering to a physical particle size, as applicable in light obscuration or the scattering channels of a flow cytometer. Conversely, for methods that directly measure size, lack of knowledge of particle density prevents accurate assessment of the mass of protein bound as aggregates.

3.3 Immunogenicity of particles

How physicochemical particle characteristics relate to immunogenicity is unknown. For instance, we currently do not know how particle size, morphology or molecular structure affects immunogenicity. Multivalent binding of antigens has been demonstrated to activate B cells, yet this activation is quite complex, depending on molecular recognition of the antigens, the spacing of antigens, and the ratio of B-cell receptors to available antigens.[28] In the field of vaccine research, Mant et al.[29] demonstrated that the size and degree of aggregation of protein-coated polystyrene particles strongly influenced the surface presentation of antigens by dendritic cells. The vaccine adjuvant alum, which is a high-surface area particle,[30] has long been known to cause an immune response, although the exact role of alum in enhancing immune response remains surprisingly elusive.[31] Non-protein materials incorporated in heterogeneous protein particles may have a similar role.[8] These examples demonstrate that multiple particle attributes may influence the immune response. Much research remains to be done before the detailed interaction of protein particles with the immune system[7] and how the physical and chemical structures of particles correlate with immunogenicity will be understood.

Given the complexity of antigen recognition, identification of aggregate structures with high immunogenic potential may require development of sensitive molecular probes. It is possible, though, that readily identified physical attributes may correlate with potential immunogenicity. Smooth surfaced particles with oriented proteins may represent a repetitive antigen pattern that can efficiently stimulate B-cell recognition. Conversely, particles with a high surface-area-to-volume ratio present a greater area of protein, which provides a possible alternative path to high immunogenicity. For compact geometrical shapes, such as a sphere, the surface area scales as the particle dimension squared. For fractal objects, the surface area will scale as a non-integer power that may be substantially different than two. If electron or optical microscopy images of sufficient resolution are available, analysis of particle morphology is possible using existing algorithms developed in other fields.[25] Such analysis has not yet been applied to protein particles but may be of great use in understanding the biologically relevant surface area of protein particles.

In the immune response dependent on T-helper cells, dendritic cells (DCs) recognize, take up, and digest antigens. These steps, and the subsequent cell-surface presentation of protein-particle derived peptides may also plausibly depend on a number of physicochemical particle attributes. There is an upper limit to the size of particles that can be taken up by DCs; therefore the size of the particle may matter. Additionally, particle density and morphology may alter the efficiency of proteolytic digestion of the particle and subsequent antigen presentation.

4. Particle counting and characterization methods

4.1 General principles

To understand the nature of particles in a protein solution and assess potential patient risk, the particles must be both counted and characterized. This process can be broken down into three steps:

- Separation: distinguishing a particle from background or other particles,
- Analytical characterization: measuring the physicochemical properties of particles, and
- Classification: assigning particles to subpopulations

Separation can be an intrinsic part of the particle detection process, such as distinguishing the outline of a particle suspended in a homogenous solution of protein monomer on a microscopic image. Alternatively, the separation and detection stages may be performed sequentially, as in the use of field-flow fractionation to physically separate particles on the basis of size followed by the use of light scattering to characterize the separated particles.

Particle detection instruments can be broadly classified as those that count individual particles (e.g., microscopes, light obscuration counters) and those that characterize an ensemble of particles (e.g., dynamic and static light scattering). When the separation of particles is high and when the attainable signal-to-noise ratio is sufficient to permit single particle detection at a high level of confidence, extremely good sensitivity may be achieved. In a dynamic imaging system that images fluid flowing through a cell and automatically identifies particles through software analysis of the acquired images, a single particle of 10 µm diameter, corresponding to a volume of only 500 μm^3, could potentially be measured unambiguously out of a sampled fluid volume of 500 mm^3, representing a volume fraction of only 10^{-9}. Comparison of this sensitivity with other common measures of aggregate or particle concentration is informative. Pharmacoepial limits[32] on the total number of particles exceeding 10 µm and 25 µm in diameter are equivalent to particle volume fractions of approximately 10^{-6} to 10^{-5}, orders of magnitude higher than the sensitivity attainable with dynamic imaging.

For detection of very small particles, single particle detection may not be achievable. In this case, ensemble methods provide acceptable sensitivity. However, ensemble methods have two drawbacks. First, if particle counts are inferred by comparison of a monomer peak area with areas of secondary peaks corresponding to higher molecular weight or size (as with dynamic light scattering, for example), sensitivity is limited by the dynamic range of the detector, which must be able to measure both the monomer and secondary peak areas. Second, ensemble methods have poor selectivity when applied to the measurement of mixed populations of particles, unless paired with a separation stage (e.g., light scattering detectors following field-flow fractionation separation) that adequately separates the expected particle subpopulations. As an example, size exclusion chromatography can detect soluble, nanometer-sized aggregates with a sensitivity of approximately 0.1 % of the total protein concentration. For a protein therapeutic at a concentration of 100 mg/mL, this sensitivity is equivalent to a volume fraction of approximately 10^{-4}, orders of magnitude higher than either pharmacoepial limits or the detection sensitivity by single-particle methods for larger particles.

Up until recently, analytical characterization of particles was limited to assessing the number of particles of equivalent diameter above certain limits, as called out by pharmacoepial standards for particle counts in parenteral or opthamological drugs. For small-molecule drugs, the primary risk of particles is capillary blockage, and an emphasis on particle diameter is sensible. However, with the advent of therapeutic proteins, measured particles must be characterized by more than just size to assess patient risk adequately. In addition to manufacturing impurities or contaminants, common particles found in therapeutic proteins include silicone oil droplets (from the lubricant in pre-filled syringes and other combination devices), the inherent protein particles, and heterogeneous particles (e.g., stainless steel pump debris coated with protein).

The classification process has several levels. At the coarsest level, particles can be categorized as belonging to subpopulations of: protein particles, silicone oil droplets, foreign particles (fibers, glass

particles), etc. At the next finer level, heterogeneous particles can be differentiated in the appropriate subpopulations. At the finest level, the chemical identity, protein conformation, and molecular structure of a particle can also be defined.

A high degree of precision and sensitivity alone do not suffice to categorize particles adequately. Figure 4 illustrates this point. For all particle types likely to be encountered in therapeutic proteins, the particle size distributions are quite broad and these distributions likely overlap. Increased precision or accuracy of the particle size cannot improve the differentiation of the subpopulations in Fig. 4A. Instead, the particle must be categorized by an additional property other than particle diameter to allow successful classification.

The goal of measuring multiple attributes of a particle leads to a dilemma. Although there are multiple analytical methods for characterization of particles, successive measurement of protein solutions on multiple instruments will not give as clear a characterization of subpopulations as simultaneous measurements on single particles. Furthermore, application of advanced analytical methods to characterize the chemical composition or protein conformation is expensive and slow, and the methods are relatively insensitive.

4.2 Interpretation of what is measured

Although many analytical instruments report the concentration versus size of measured particles, what is reported as the effective particle diameter will depend on the type of instrument. Each particle-analysis instrument measures a particular physical property of the tested protein particles (e.g., optical image, optical scattering, particle diffusion, excluded volume) and then converts the measured value of that property into an observed effective diameter. Biases or uncertainty of the reported diameter can arise either through inaccurate assumptions used in the conversion algorithm (see Section 4.3.1) or through the difficulty of comparing results from different types of measurements. The relationships between various size measures used with many particle counting methods is known for prolate (elongated) or oblate (compressed) spheroids.[33] For irregular particles, though, determining the relationships between different measures of effective diameter will require both an understanding of the structure of protein particles and mathematical modeling of the measurement process.

4.3 Measurement methods

4.3.1 Light scattering

Particles in solution will scatter light, which can be measured either by a drop in the intensity of the transmitted beam or by detection of the scattered light. A particle passing in a flow cell through a collimated light beam will scatter this light. The amount of scatter can be detected in two separate ways. If the particle passes through the beam, the amount of transmitted light will drop by an amount equal to the light scattered or absorbed by the particle. This is the operational principle of the light obscuration particle counter. Or, the scattered light can be detected directly. This is the operational principle of static light scattering methods.

The primary difficulty with light scattering methods is that the relation between the amount of light scattered and the particle size depends somewhat on particle morphology and very much on the refractive index of the particle, relative to the matrix fluid. As discussed in Section 3, the density of protein particles is not well known, and since the refractive index of the particle relative to the solution

matrix will vary linearly with the protein particle density, the refractive index is likewise not well known.

Figure 5 illustrates how the scattering efficiency (i.e., the ratio of the optical scattering cross section to the geometrical cross section of a particle) of a spherical particle varies with the difference Δn between the refractive indices of the particle and matrix fluid. Without knowledge of the true scattering efficiency, the actual physical diameter of the particle cannot be reported—what is instead reported is the diameter of a polystyrene latex bead that gives the same scattering signal as the measured particle. As the scattering efficiency drops with low Δn, this reported diameter can be as small as 1/5 of the actual effective diameter. Optical modeling can provide the basis for correction of this bias. Alternatively, if the scattering strength of a particle could be measured simultaneously with an independent measure of particle size, that combination may provide an excellent means of categorizing particles.

Multi-angle light scattering (MALS) is an ensemble technique that sacrifices accurate particle-by-particle counting for detailed knowledge of the angular distribution of the scattered light. For particles below approximately 0.5 μm, the angular distribution can be used to unambiguously ascertain the particle size without a priori knowledge of the scattering efficiency. MALS has been used successfully paired with size-exclusion chromatography[4] and asymmetrical field-flow fractionation[34] for characterization of oligomer-size aggregates.

Dynamic light scattering (DLS) is an ensemble technique that measures the hydrodynamic radius of particles by measurement of the temporal fluctuations of scattered light. Because large particles scatter light much more efficiently than small particles, the presence of a few large particles can obscure the signals from smaller particles. Thus, for polydisperse protein particles, DLS is only useful when paired with a size-selective separation technique.[35]

4.3.2 Flow microscopy

Although microscopy is fully capable of imaging protein particles down to effective diameters of approximately 1 μm, a standard microscope samples a very small volume of fluid and is not optimized for automated counting of particles. In a flow microscope, protein solution is passed through a thin flow cell, the cell is stroboscopically illuminated, and techniques of dynamic image acquisition are used to record sequential images and automatically identify and analyze particles.[3,36] Strengths of the method are its very high selectivity and the simultaneous acquisition of effective diameter and particle morphology.

Although commercial instruments report particle counts to effective diameters as small as 1 μm, there are fundamental optical limits that reduce the available morphology information and diameter accuracy. The optical configurations necessary to obtain in-focus images for particles throughout the depth of the flow cell, limit the spatial resolution relative to what can be achieved with standard, stationary object microscopy.

Flow microscopy has proven to be a very effective tool for characterizing particles because of its high sensitivity and selectivity. Further improvements are possible. Statistical image analysis will be useful in identifying extended, nearly transparent particles and reducing measurement artifacts. Improvements in fluorescence techniques for tagging proteins or non-protein particles may also be adopted in flow microscopes.

4.3.3 Flow cytometry

Flow cytometry has traditionally been used to identify subpopulations of cells by measuring the scattering of light off of the cells and the simultaneous fluorescence of labels that are specific for certain cell properties (such as the abundance of particular receptors). A number of common dyes have affinity for hydrophobic chemical environments and preferentially adsorb to protein particles. Recently a highly selective, molecular-rotor dye has been introduced that is fluorescent only when intercalated between the crossed-beta strand structures that are often found in aggregated proteins.[37]

With the combination of two light-scattering and multiple fluorescence channels, flow cytometry shows excellent promise as a means of rapidly characterizing and categorizing particles.[38,39] As usually practiced, flow cytometry provides relative fluorescence counts of measured cells with high repeatability and precision, but poor accuracy in terms of absolute fluorescence. Recent work on quantitative flow cytometry demonstrates that these instruments can be calibrated to give absolute fluorescence measurements.[40] These techniques for instrument standardization may be applicable to the counting of protein particles.

Challenges for achieving reliable particle characterization with flow cytometry include the development and validation of dyes specific to the types of particles likely to be encountered, the understanding of possible interferences and cross-sensitivities associated with dyeing mixed-particle populations, and the development of absolute standardization methods and reference materials for both the scattering and fluorescence channels.

4.3.4 Field-flow fractionation plus optical detectors

Field-flow fractionation is primarily a separation method based on the size dependent response of particles to hydrodynamic flow. It is typically used in conjunction with a MALS or another type of optical detector. In the most common variant, asymmetric-flow field flow fractionation (AF4), a cross flow transverse to the main channel will sweep particles to one side of the channel.[34] Although AF4 has an extraordinarily broad range of size separation, application of AF4 to protein particles has been problematic due to adsorption of protein particles on the transverse-flow membrane. This adsorption phenomenon calls into question whether the total number of harvested particles is representative of the original sample and whether the particles have been distorted in size or conformation in the separation process. A possible alternative approach is centrifugal flow fractionation, in which the suspension flows through a channel that is subjected to a strong centripetal acceleration. Centrifugal flow fractionation has potential advantages over AF4: separation is based on a combination of particle density and size, and there are no membranes. Centrifugal flow fractionation instruments are commercially available, but application of these instruments to protein particles has been minimal to date.

4.3.5 Electrical sensing zone particle counters

Cells have been reliably counted for many years with Coulter counters, and the measurement technique has recently been applied to protein particles.[41,42] In this technique, an ionic current passes through an orifice. As protein solution is pumped through the orifice, passage of a particle or cell through the orifice will result in a transient drop in electrical conductance. The magnitude of the drop is a quantitative measure of the particle volume, from which an equivalent effective particle diameter can be inferred. Because of the electrical detection mechanism, the method is alternatively named the electrical

sensing zone (ESZ) technique. For particles with simple, enclosed shapes (such as cells), the particle size is easy to infer. Protein particles, though, are known to be highly hydrated and not tightly packed, and ions may be able to pass through the particles, leading to an under-reporting of particle size. Conversely, ESZ counters have the potential of identifying protein aggregates that are too small or optically transparent to be detected by imaging methods. More work needs to be done to understand the relation between protein particle structure and the ESZ signal, and to correlate the ESZ observations with microscopy results. Additionally, ESZ is difficult to use in systems with low ionic strength, and addition of salts may perturb the particle structure.

4.3.6 Novel methods

Recently, several commercial instruments have become available with unique capabilities in either the size range of the detected particles or the mechanism of detection.

The Brownian motion of small particles, below the optical resolution of a microscope, can be tracked by scattering laser light from the particles[35] or by inducing fluorescence of a particle.[43] This technology is the basis of nanoparticle tracking analysis. From the pattern of this motion, the hydrodynamic radius can be inferred, as in DLS. A distinct advantage of single-particle tracking is that accurate measurements may be made of polydisperse samples. The method does have a relatively limited range of particle density, dictated by the seconds-long times needed for accurate characterization of the Brownian motion and by the upper limit on particle density so that individual tracks may be distinguished. A challenge of the method is to differentiate particle translation motion from the internal motion of flexible protein particles. The method may allow categorization of different subpopulations by examination of the scattering intensity.

Another novel method (resonant mass measurements) detects particles on the basis of their buoyant mass, Δm, which is the mass of the particle minus the mass of the displaced matrix fluid.[44] Sample fluid passes through a micro-machined channel on a vibrating cantilever arm. When the buoyant mass is positive, the cantilever mass increases, and the resonance frequency drops. This method provides very clear differentiation of silicone oil droplets (negative Δm) versus protein particles or manufacturing impurities (positive Δm). Further differentiation of particles would be possible if the method could incorporate independent sizing of the particles by optical or electrical sensing.

4.3.7 Advanced characterization

Analytical and biophysical methods for characterizing protein particles have advanced over the past five years.[45] There is an increased focus on improving methods for particle characterization, not just in extending measurements of particle size distribution and morphology to sizes below 1 μm, but also in expanding capabilities for chemical and structural characterization. Protein particles have been successfully isolated and then analyzed by a variety of analytical methods. With such particles, the primary concern is whether the isolation process itself has altered the particle structure, the particle size distribution, or the protein conformation. High resolution images of particles can be achieved utilizing electron microscopy and atomic force microscopy. Cryogenic electron microscopy, although tedious, can distinguish individual protein monomers, is capable of resolving patterns in the molecular structure of protein particles, and likely does not distort the aggregate structure during sample preparation. SEM-EDX, or scanning electron microscopy with energy dispersive spectroscopy detector and FTIR microscopy are most commonly applied for compositional analysis of particles,[22] which is of value in determining the source of unexpected particles introduced in the manufacturing process. Simple protein

conformation measurements, such as determining the proportion of alpha helix and beta strand secondary structure, can also be successfully carried out on isolated protein particles (Fig. 3). FTIR and Raman microscopy are often used to analyze the protein structure of subvisible and visible particles, capable of differentiating native from denatured protein particles.[46] Hydrogen-deuterium exchange mass spectrometry identifies the exposure of specific peptide regions to the surrounding water. This technique has recently been applied to protein particles induced by freeze/thaw cycles to identify both the degree of native protein structure and the protein domains involved in protein aggregation.[47]

5. Forward looking opportunities

From the standpoint of measurement technology, the past few years have seen the growth of several new techniques to rapidly measure and characterize particle populations. Unambiguous and rapid categorization of mixed populations of particles, though, will require simultaneous measurement of multiple parameters. Improved analytical tools capable of rapidly measuring multiple particle attributes, at particle concentrations of therapeutic protein formulations, are a high priority for the field. Rapid characterization methods can be categorized by the measured property of the particle. The list of properties utilized by commercially available particle counters is relatively short: size, light scattering intensity or angular dependence, morphology, density, and affinity for fluorescent dye. A primary goal for future work is to take this short list and to develop instruments that will provide a sufficient combination of attribute values to enable categorization of each particle. An additional goal is to extend this list to additional particle properties, such as particle heterogeneity or protein conformation.

For particles larger than 10 μm in effective diameter, flow microscopy gives simultaneous measures of size and morphology of single particles. Flow cytometry, using dyes validated to bind to known chemical targets, shows great promise for providing enhanced characterization at sizes below 10 μm. Like the selectivity of fluorescent dyes, particle density is an attractive parameter to use as a basis for particle categorization, since the range of densities varies so greatly between silicone oil, protein, residual impurities, and others. Combined detection of particle size and mass would provide an excellent basis for particle categorization.

As analytical instrumentation advances, identification and counting of protein particles down to diameters below 1 μm will become routine. An important area for future research is the identification of specific particle attributes and characteristics that correlate well with product safety and immunogenicity. An exact understanding of the most relevant particle properties to measure awaits further progress in the field of protein particles and immunogenicity. Nonetheless, the physical structure of an aggregated protein could plausibly modify the immune response in several ways.

In Section 3, we described some particle attributes that could plausibly affect the immune response to a particle. These considerations imply that particle characterization in the future will need to ascertain, in addition to particle size and composition, protein packing density and the particle structure at length scales below the resolution of current rapid imaging techniques. To do this routinely will be a great challenge.

Careful work will also be needed to understand how the *in vivo* chemical environment affects particle properties, and also how biological degradation pathways for aggregates depend on the particle morphology or protein conformation.

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Fig. 1. Schematic of typical particle types found in therapeutic proteins.

Fig. 2. Typical structures seen in protein particles. Micrographs of various protein particles illustrating structures on a wide range of length scales. (A) cryogenic TEM image of protein particles induced by freeze-thawing of immunoglobulin IgG2. (B) homogeneous protein particle of agitated and chemically denatured polyclonal immunoglobulin G. (C) heterogeneous protein particle of agitated polyclonal immunoglobulin G surrounding a core of unknown composition. (D) extended, fibrous particle of bovine serum albumin.

Fig. 3. Optical images and second derivative FTIR microscopy spectra of two particles from monoclonal antibodies. Although there is no discernible difference between the optical images, the FTIR microscopy demonstrates that the native structure is preserved in (3a) while there is a substantial loss of secondary structure in (3b).

Fig. 4. Separation of particles by size and density. Size-based separation (A) is ineffective when there are multiple subpopulations with overlapping size ranges. Density-based separation (B) may be an effective alternative.

Fig. 5. Scattering efficiency for light scattering detection of spherical particles with a refractive index Δn larger than the matrix fluid, and a detector acceptance angle of 0.05 rad.

Fig. 1

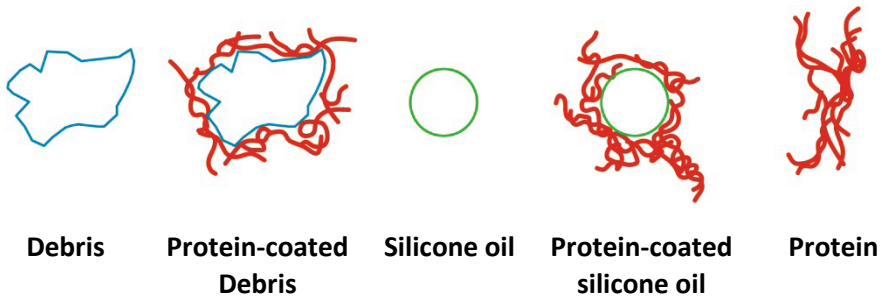

Debris
Protein-coated Debris
Silicone oil
Protein-coated silicone oil
Protein

Fig. 2

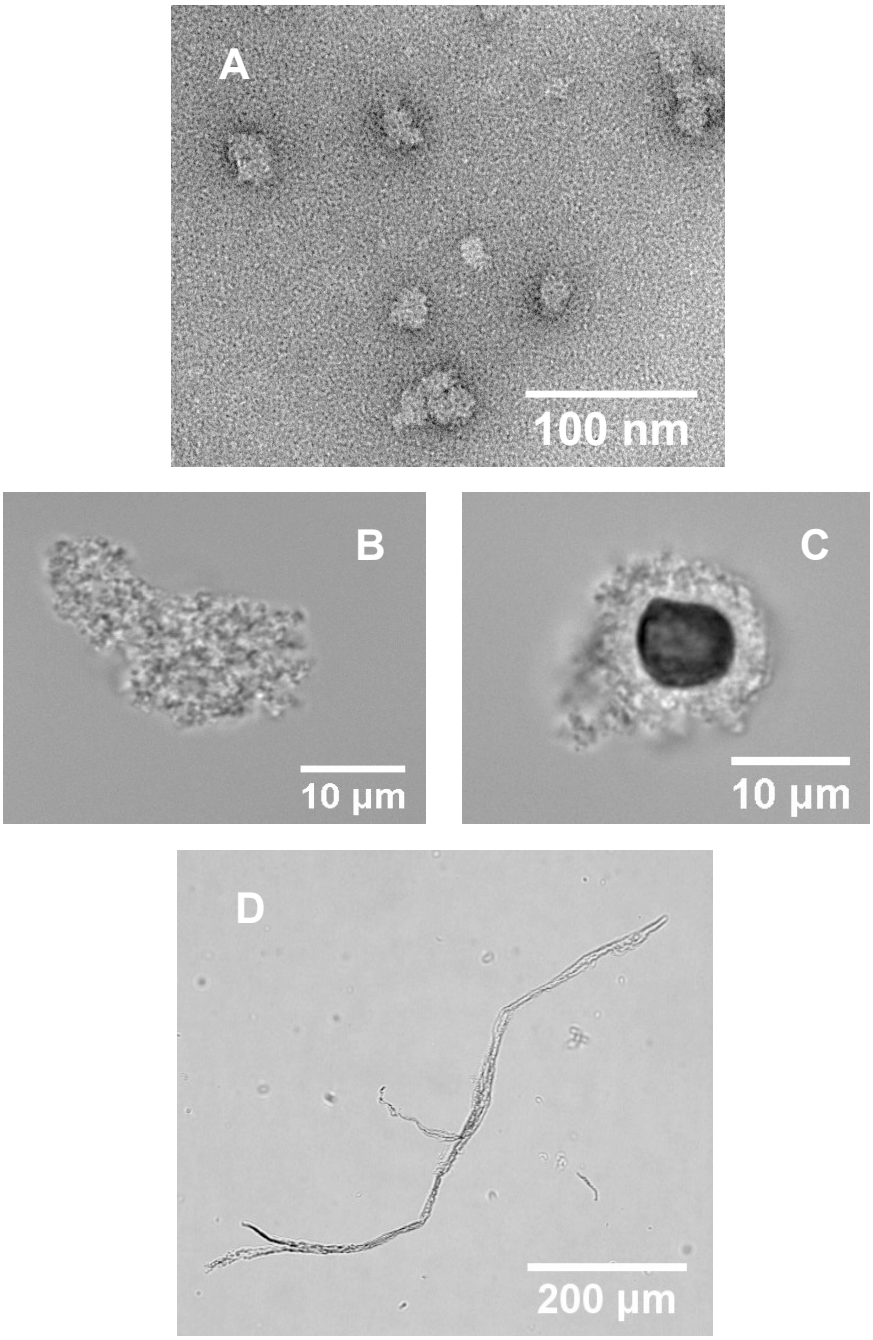


Fig. 3

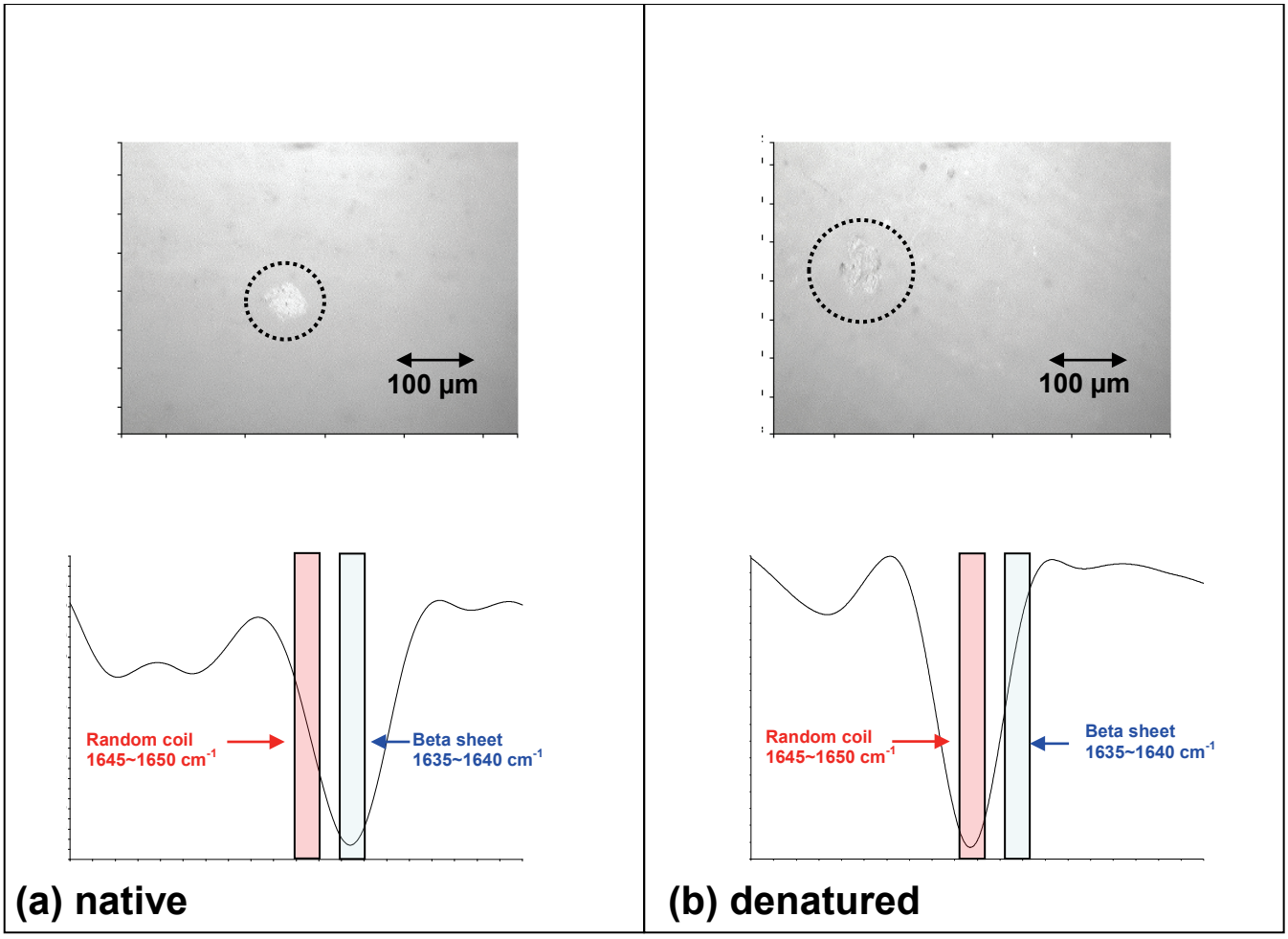

100 µm
Random coil
1645~1650 cm-1
Beta sheet
1635~1640 cm-1
(a) native
100 µm
Random coil
1645~1650 cm-1
Beta sheet
1635~1640 cm-1
(b) denatured

Figure 4.

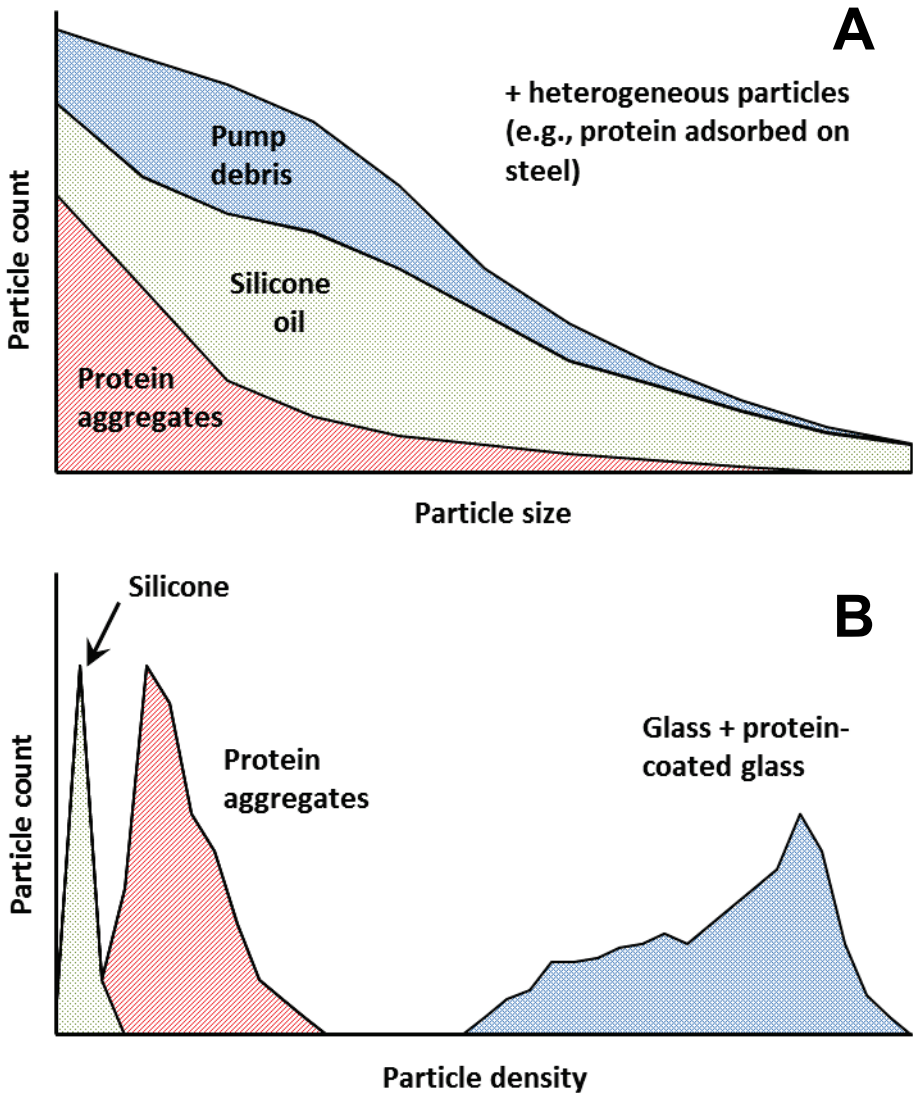
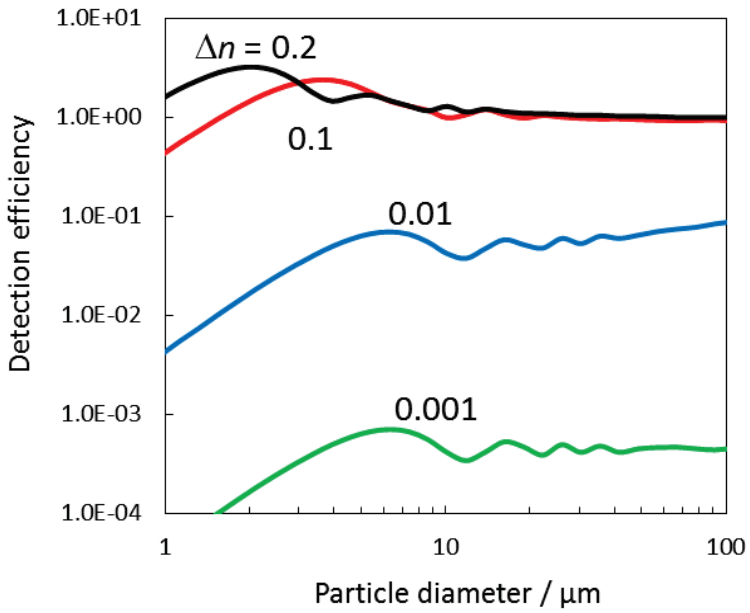

A
+ heterogeneous particles
(e.g., protein adsorbed on
steel)
Pump
debris
Silicone
oil
Protein
aggregates
Particle count
Particle size
B
Silicone
Protein
aggregates
Glass + protein-
coated glass
Particle count
Particle density

Figure 5.


1.0E+01
1.0E+00
1.0E-01
1.0E-02
1.0E-03
1.0E-04
Detection efficiency
Δn = 0.2
0.1
0.01
0.001
1
10
100
Particle diameter / µm