PERSPECTIVES

An Industry Perspective on the Monitoring of Subvisible Particles as a Quality Attribute for Protein Therapeutics

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ABSTRACT: Concern around the lack of monitoring of proteinaceous subvisible particulates in the 0.1–10 μm range has been heightened (Carpenter et al., 2009, J Pharm Sci 98: 1202–1205), primarily due to uncertainty around the potential immunogenicity risk from these particles. This article, representing the opinions of a number of industry scientists, aims to further the discussion by developing a common understanding around the technical capabilities, limitations, as well as utility of monitoring this size range; reiterating that the link between aggregation and clinical immunogenicity has not been unequivocally established; and emphasizing that such particles are present in marketed products which remain safe and efficacious despite the lack of monitoring. Measurement of subvisible particulates in the <10 μm size range has value as an aid in product development and characterization. Limitations in measurement technologies, variability from container/closure, concentration, viscosity, history, and inherent batch heterogeneity, make these measurements unsuitable as specification for release and stability or for comparability, at the present time. Such particles constitute microgram levels of protein with currently monitored sizes ≥ 10 μm representing the largest fraction. These levels are well below what is detected or reported for other product quality attributes. Subvisible particles remain a product quality attribute that is also qualified in clinical trials. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:3302–3321, 2010

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INTRODUCTION

Therapeutic proteins have made an immense contribution to treatment of human diseases and

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represent an increasingly important part of the armamentarium available for this purpose. The life-saving benefits of products derived from recombinant protein technology, starting from the very first product insulin in 1982, have never been in dispute. However, concerns have always been present about the potential adverse consequences of aggregation of the protein in the product being dosed. Aggregation in biotherapeutic products is often discussed in conjunction with risk for immunogenicity,[1] although a clear connection between the two for protein therapeutics has not been demonstrated. Nevertheless, this concern has led the industry and regulatory authorities to use aggregation as a critical quality attribute for biologics.

Forming aggregates is to some extent an inherent property of a protein. While significant advances have been made in the understanding of the pathways of chemical degradation of proteins, the same does not completely hold for aggregation pathways or mechanisms. In light of this, judicious process and formulation development research is performed to control the development of aggregates during the production and storage of the product. The success of this effort is evident from the number of biotherapeutic products that have been commercialized and found to have favorable safety and efficacy profiles. It is quite reasonable to assume that these commercial products contain a range of aggregate levels and associated subvisible particulates, (see Tab. 1 for some examples), the latter being the focus of this article. Note, however, that the data in Table 1 do not distinguish between proteinaceous and extrinsic subvisible particles due to limitations of the technology, as discussed later.

The term aggregates covers a large variety of heterogeneous species from reversible to irreversible, native and nonnative, and from dimers to multimers that range in size from a few nanometers to visible particles in the hundreds of microns.[2–4] The technical ability to measure and quantify aggregates throughout this entire size range with one single method does not exist.[5,6] Carpenter et al.[7] have recently published a commentary highlighting concerns with protein aggregates in the subvisible size range between 0.1 and 10 μm. This article has been prepared in response to the commentary to provide an industry perspective around this topic.

Statement of Issue to be Discussed

The primary basis of the commentary by Carpenter et al.[7] lies in the assertion that the presence of protein in a product aggregates is a risk factor for immunogenicity. Under this umbrella concern, the commentary raises the following issues:

Table 1. Subvisible Particles (≥2, ≥5, ≥10, ≥25 μm) in some Marketed Biologics, Measured by Light Obscuration/HIAC Using Small-Volume Methods But Without Dilution

		Subvisible Particles (in Counts per mL) (Mean ± SD Where Available)				
	Product Info.	≥2 μm	≥5 μm	≥10 μm	≥25 μm	Estimated Protein Mass Represented by Subvisible Particles (g/mL)[a]
Product 1	Lyo/vial	2091 ± 1453	352 ± 225	29 ± 13	3 ± 0	2.95e−6
Product 2	Liquid/PFS	4477 ± 486	1220 ± 91	118 ± 26	2 ± 1	5.15e−6
Product 3	Lyo/vial	664 ± 191	59 ± 12	7 ± 7	0 ± 0	3.44e−7
Product 4	Lyo/vial	13081 ± 3035	1122 ± 581	99 ± 128	3 ± 5	1.56e−5
Product 5	Lyo/vial	4692 ± 725	385 ± 73	16 ± 4	0 ± 1	1.45e−6
Product 6						
Lot 1	Liquid/vial	5240 ± 117	1312 ± 33	227 ± 27	<10	8.46e−6
Lot 2	Liquid/vial	8826 ± 205	2016 ± 58	350 ± 17	<10	9.82e−6
Product 7 (CC = container/closure)						
CC2, Frmln 1, Lot 1	Liquid/PFS	2745 ± 46	823 ± 38	229 ± 22	21 ± 6	2.25e−5
CC2, Frmln 1, Lot 2	Liquid/PFS	6740 ± 57	1585 ± 53	343 ± 6	24 ± 7	2.66e−5
CC2, Frmln 1, Lot 3	Liquid/PFS	1191 ± 25	403 ± 19	163 ± 6	24 ± 8	2.57e−5
CC2, Frmln 1, Lot 4	Liquid/PFS	3679 ± 86	1108 ± 20	284 ± 12	22 ± 3	1.88e−5
CC3, Frmln 2, Lot 1	Liquid/vial	12 ± 3	<10	<10	<10	5.08e−6
CC3, Frmln 2, Lot 2	Liquid/vial	88 ± 12	<10	<10	<10	5.09e−6
Product 8	Liquid/vial	1054	150	13	1	3.05e−7
Product 9[b]	Liquid/vial	17145	4842	590	30	9.98e−6

[a]Protein mass in the form of subvisible particles has been estimated assuming spherical particles of density 1 g/cm^3. Mass estimation has been made using a worst-case size and number assumption. All particles in range ≥2 μm were assumed to be 5 μm; ≥5 μm were assumed to be 10 μm; ≥10 μm were assumed to be 25 μm; and ≥25 μm were taken to be 100 μm in size. Counts for each of the above sizes was taken as being equal to (mean + 3 × SD). Counts as given are cumulative but this was not corrected for in the calculations. Counts shown as "<10" were counted as 10. In all cases, it is the largest size range (≥25 μm) that constitutes the largest fraction of the total mass estimated.

[b]Product may contain proteinaceous visible particles.

(1) Proteinaceous subvisible particles (i.e., subvisible particles of protein-origin) have the potential to negatively impact clinical performance, since they could represent aggregates of thousands to millions of protein molecules.
(2) Currently, there is a gap in routinely measuring and controlling subvisible particles smaller than 10 μm in biotherapeutic products, with no pharmacopeial requirements for this size range.
(3) Recent studies have indicated the existence of proteinaceous particles in the subvisible size range below 10 μm (0.1–10 μm) in biotherapeutics.
(4) Uncertainty created by the lack of monitoring of (proteinaceous) particulates in this size range and the general risk for immunogenicity associated with aggregates implies that (a) further development of technologies that can measure particles in this size range is critical, and (b) more research is needed to investigate the relationship between aggregation and immunogenicity.

Objective

As with any new technology, collection of data across a broad set of samples is critical prior to interpreting the significance of the results. The industry has a demonstrated history of developing, adopting, and applying new technologies to characterizing products, and when appropriate, adding additional controls either in-process or at release. While we acknowledge that the published commentary will serve to accelerate this discussion, we feel it is important to thoroughly understand the methodologies prior to implementing any required controls. In addition, understanding the causes and sources of subvisible particles is critical to interpreting the data.

In this response, we want to:

(1) Examine the current state of knowledge in the connection between aggregation and immunogenicity, including the state of current therapeutics. We want to emphasize that available biotherapeutics have a strong record of safety, and there are no immunogenicity issues or other clinical findings that can be directly related to aggregates or to proteinaceous subvisible particles of any size, despite the gap in monitoring.
(2) Review currently available analytical tools and their capabilities for the measurements and identification of subvisible particles in the size range above 0.1 μm, and share current experience from the industry in the monitoring of subvisible particles of this size.
(3) Develop a common understanding between all interested parties, including academic laboratories, the industry and regulatory agencies, around the capabilities for monitoring particles in this size range using currently available technologies, and set realistic expectations around the value such measurements would provide.

There are no consistent or formalized definitions of subvisible versus visible particles. Visible particles are usually defined as having a lower size limit around 100 μm, based on an analysis of the size threshold at which a majority of inspectors consistently detected the particles under appropriate lighting and test conditions.[8] Light obscuration methods designed for subvisible size ranges can detect particles up to approximately 150 μm. Thus, although there is some overlap between the visible and subvisible size ranges, we will broadly refer to the subvisibles as between 0.1 and 150 μm, with the understanding that particles represent a continuum in size range, and the classification as subvisible versus visible is fluid and not very informative. Subvisible particles, for the purpose of this communication, therefore comprise the submicron and micron size ranges, spanning three orders of magnitude.

AGGREGATION, SUBVISIBLE PARTICULATES, AND IMMUNOGENICITY

Multiple publications have implied that protein aggregates may be one of the factors, if not the major factor, for tolerance reversal and induction of immune response against protein biotherapeutics.[1,9,10] This conclusion is based on theoretical considerations, circumstantial evidence obtained from investigations conducted on clinical products, as well as data from animal studies using artificially induced and stabilized aggregates.[11–14] Despite the fact that all biotherapeutics contain some level of aggregates (and subvisible particles as discussed later), there has been no direct evidence from clinical studies demonstrating aggregates as the true dominant risk factor contributing to the immunogenicity. The principal reason for this lack of a clear demonstrated connection is that clinical observations of immunogenicity are a consequence of combination of numerous (patient, therapy, product, dosing) factors.[15] This makes the task of deconvoluting the specific impact of aggregation very difficult. Furthermore, in many cases the incidence of immunogenicity is so low that to determine the true cause would be impossible (see, e.g., the survey by Hwang and Foote[16]). The broader concern with aggregates and the more specific

concern with proteinaceous subvisible particles, is therefore primarily due to the uncertainty around the risk they entail.

The primary risk with aggregates evolves from the concept of pathogen associated molecular patterning wherein repetitive arrays of proteins are found to be potent modulators of immune response. This raises the possibility that aggregates of human biotherapeutics, including those in the micron range (subvisible proteinaceous particles) could be immunogenic in patients through the same mechanism.[1] To the best of our knowledge, there are no published reports examining the structure of naturally occurring protein aggregates which either prove or discount the presence of such repetitive motifs in therapeutic proteins. Moreover, aggregates produced by different methodologies can have very different biochemical and biophysical characteristics and morphologies,[17] and could produce completely different immunological effects when injected into the same animal model.[13,14,18]

Due to the difficulty in clearly delineating the impact of aggregates in clinical studies, animal models, including transgenic mice, are being developed and employed.[12–14,18] The use of animal studies to assess immunogenicity of aggregates and/or particulates comes with caveats. As already noted above, aggregates produced by different methods can lead to different immune response in the same animal model. Immunogenicity observed in animal studies of human protein drugs may not be predictive of what could happen in humans.[19–21] Jahn and Schneider[20] report that a range of scenarios have been observed during dossier evaluation, ranging from an almost absent immune response in animals but high immunogenicity in humans, to (the more usually observed) high immunogenicity in animals but low immunogenicity in humans. The utility of animal models would therefore lie in assessing the relative immunogenicity risk of aggregates or particles, although the translation of an observation of "increased" or "decreased" immunogenicity in the animal model, to the human clinical response would still be difficult. This relative ranking of risk would likely be dependent on the biotherapeutic class and on the construct of the animal model.

In summary, the immunogenic potential of aggregates in general, and of proteinaceous subvisible particles in particular, is complicated to assess. Animal models have limited utility and clinical studies specifically designed to test such product-related impurities would not be ethically justifiable. Ultimately, the assessment of subvisible particles may have to be similar to the approach currently applied to other product-related impurities: an acceptable product safety and immunogenicity profile is demonstrated in clinical studies with drug product lots containing a certain historical range of product-related impurities (in this case subvisible particles). However, the ability to do this from a QC or specifications perspective has some practical and technical limitations as discussed in the subsequent sections of this article.

STATE OF CURRENT PROTEIN PRODUCTS

The specification for protein oligomers (generally measured by SEC) in the therapeutic proteins on the market are set prior to clinical studies and qualified in these studies. Subvisible particles larger than 10 μm are routinely measured for these products, while the range below 10 μm is not. Despite the lack of a complete picture about subvisible particles/aggregates, it is likely that such particulates/aggregates (including those below 10 μm) existed during clinical studies and are present in the marketed product. There are no reports to suggest that these currently marketed products are in anyway unsafe as a consequence of these subvisible particles.

An example showing subvisible particle counts in nine marketed, efficacious and safe biotherapeutic products is given in Table 1. It should be noted that for particles in the $\geq$2 and $\geq$5 μm ranges, the standard deviation in a number of cases are quite high, and thus precision is low. Variability between different lots of the same product and between different presentations of the same protein is significant, and is likely a consequence of product, including the device and presentation of the drug, as well as method of measurement (see discussion on Product-Related Factors Leading to Variability in Subvisible Particulates Data Section; all measurements were carried out with small-volume methods, see discussion on Sample Requirements Section). Although limited, the data show that a wide range of particle counts exists in marketed products in the size range below 10 μm, even within the same product. However, a larger survey would be useful, as discussed later in the Risk Assessment Section.

Using the particle count data in Table 1, an estimate of protein mass contained in the subvisible particles has also been made under some worst-case assumptions about size and number, and is likely an overestimation by at least an order of magnitude. The results demonstrate that under this worst-case estimation, and assuming that all these particles were proteinaceous, they comprise less than 30 μg of mass, and in most cases less than 5 μg. The proteinaceous particles thus constitute a negligible fraction of the total dose of most products. Furthermore, in the above calculations, >90% of the particle mass actually arises from the $\geq$25 μm particles (which were all assumed to be of 100 μm size for

the calculation), with the next highest contribution arising from the ≥10 μm particles (assumed to be 25 μm). Thus, the size ranges that are currently already monitored represent the greatest mass of proteinaceous subvisible particles. Although it is not clear if it is the mass of antigens or the number of antigens that is critical to an immune response, White et al.[22] suggested that micron-sized particles of ovalbumin were more immunogenic in mice than submicron-size particles at the same total mass dose, as measured by an *in vivo* CTL activity assay. These aggregated particles were also found to generate a better anti-ovalbumin IgG response in the mice compared to solubilized aggregates, suggesting that size is more important than number. Whether this size versus number translates to a human clinical situation with a protein that is not inherently immunogenic (unlike ovalbumin to mice) is however not known.

Package inserts for a number of marketed biotherapeutic products also mention the possibility of the presence of visible proteinaceous particles (see, e.g., asparaginase, laronidase, imiglucerase, alglucosidase, cetuximab, infliximab, etanercept, orthoclone OKT3, ofatumumab, panitumumab). These solutions are also likely to contain subvisible particles in a wide size range. However, such products continue to be safe and efficacious.

SUBVISIBLE PARTICLES AND THE MEASUREMENT GAP

The current standard limits or specifications for subvisible particles and information about methods in the compendia are summarized in Table 2. The current standards arose to mitigate the risk associated with the presence of extraneous particles in intravenous injection solutions. The particle sizes monitored (≥10 and ≥25 μm) were set based on the risk for blood vessel occlusion by intravenous administration of small particles. Modern aseptic production technologies have reduced the occurrence of high extraneous particle counts such that there are moves to tighten the standards.[23]

The general consensus is that IM or SC routes of administration are more immunogenic than IV. The current USP 32 <1> (as well as the forthcoming USP 33 <1>) exempts SC/IM products explicitly from the existing subvisible particulate requirement of USP <788>; the Ph.Eur.6.0 and JP 14th Ed. do not (see Tab. 2). From a protein therapeutic perspective, this was an unfortunate gap as pointed out in the commentary.[7] However, these exemptions in USP<1> are proposed to be removed per USP PF35.3. Interestingly, USP PF35.3 also states that "parenteral products for which the labeling specifies use of a final filter prior to administration are exempted from the requirements of USP <788>, provided that scientific data are available to justify this exemption." This harmonizes the USP with Ph.Eur. The Ph.Eur. also states that "in the case of products for SC and IM injection, higher limits may be appropriate."

Current Measurement Techniques: Performance and Limitations

Aggregates and particulates can be looked upon as a continuum in size, ranging from dimers to visible multimers. In this context, subvisible proteinaceous particles refer to aggregates that have grown in size to fall in the subvisible size range. The ability to detect and quantitate these aggregates/particles in protein solutions is a function of their size, amount/number, and the capability of the technique being employed. Figure 1 illustrates some analytical techniques and the approximate size ranges over which they are viable. Although there may be differences in the ranges proposed by different authors, it is generally accepted that no single technique can cover the whole range of interest.[2–6] The capture, detection, and quantitation of particulates is a stochastic process and the accuracy of the results are impacted by their concentration/number and size. The particle measurement landscape as a function of number and size of aggregates/particles is illustrated in Figure 2. The straight lines in this figure represent constant mass of particles (under the assumption of spherical particles of density 1 g/cm^3). The detection and quantitation ability of some methods is mapped on this chart. Sizes above ~1 or 2 μm can be detected and quantified by counting methods while those below ~0.1 μm can be detected and quantified by concentration-based methods. Intermediate sizes can be detected by light scattering methods but obtaining an accurate quantification is not feasible, as discussed later. The ability to both detect and accurately quantify the number of particles in solution in the 0.1–1 μm size range represents the true measurement gap in currently utilized techniques, and is discussed in more detail below.

The various techniques summarized in Figure 1 also report results in different ways, depending on the principles of measurement. The counting techniques (e.g., light obscuration) quantify numbers in a size range, while SEC or AUC quantify by mass fractions of the various species. The results reflect differences in the sensitivity of the techniques/detection technology used. Conversion between various modes of reporting data requires making several assumptions about the properties of the particles. This makes studies exploring the conversion from one species

Table 2. Summary of Compendial Requirements for Subvisible and Visible Particulates in Parenterals

Attributes	USP33	Ph.Eur.6.0	JP 15th Ed
	Unit Product Volume Definition ≤ 100 mL = Small Volume Parenteral (SVP) >100 mL = Large Volume Parenteral (LVP)		Unit Product Volume Definition <100 mL = Small Volume Parenteral (SVP) ≥ 100 mL = Large Volume Parenteral (LVP)
Specifications: light obscuration (LO) (preferred method)			
SVP	$\geq 10\ \mu m$: ≤ 6000 counts/container $\geq 25\ \mu m$: ≤ 600 counts/container	$\geq 10\ \mu m$: ≤ 6000 counts/container $\geq 25\ \mu m$: ≤ 600 counts/container	$\geq 10\ \mu m$: ≤ 6000 counts/container $\geq 25\ \mu m$: ≤ 600 counts/container
LVP	$\geq 10\ \mu m$: ≤ 25 counts/mL $\geq 25\ \mu m$: ≤ 3 counts/mL	$\geq 10\ \mu m$: ≤ 25 counts/mL $\geq 25\ \mu m$: ≤ 3 counts/mL	$\geq 10\ \mu m$: ≤ 25 counts/mL $\geq 25\ \mu m$: ≤ 3 counts/mL
Specifications: microscopy (M) (second stage to LO or in case LO cannot be used)			
SVP	$\geq 10\ \mu m$: ≤ 3000 counts/container $\geq 25\ \mu m$: ≤ 300 counts/container	$\geq 10\ \mu m$: ≤ 3000 counts/container $\geq 25\ \mu m$: ≤ 300 counts/container	$\geq 10\ \mu m$: ≤ 3000 counts/container $\geq 25\ \mu m$: ≤ 300 counts/container
LVP	$\geq 10\ \mu m$: ≤ 12 counts/mL $\geq 25\ \mu m$: ≤ 2 counts/mL	$\geq 10\ \mu m$: ≤ 12 counts/mL $\geq 25\ \mu m$: ≤ 2 counts/mL	$\geq 10\ \mu m$: ≤ 12 counts/mL $\geq 25\ \mu m$: ≤ 2 counts/mL
Intraveneous injections	Limits apply	Limits apply	Limits apply
Injections solely for intramuscular (IM) or subcutaneous (SC) dosing	Limits do not apply per USP33<1>; USP PF35.3 proposal: Limits to apply for IM and SC products also	Limits apply. Higher limits may be appropriate	Not mentioned separately
Non IM/SC dispersed systems	M (no special procedure described)	M (no special procedure described)	M (no special procedure described) Suspension particle $\leq 150\ \mu m$ Emulsion drop $\leq 7\ \mu m$
Non IM/SC solutions or powders for injection			
With final filter before injection	Excluded from requirements per USP33<1>; USP PF35.3 proposal: parenteral products for which the labeling specifies the use of a final filter prior to administration are exempt from the requirements provided that scientific data are available to justify the exemption	Exempt from requirements, providing it has been demonstrated that the filter delivers a solution that complies	Not mentioned
High viscosity	Dilution followed by LO	Dilution followed by LO	Dilution followed by LO
Other solutions	LO alone or followed by M	LO alone or followed by M	LO alone or followed by M
Test protocol			
Statistically sound sampling plan	Required for <25 mL/unit For ≥ 25 mL/unit, 10 units acceptable	Required for <25 mL/unit For ≥ 25 mL/unit, 10 units acceptable	Required for <25 mL/unit For ≥ 25 mL/unit, 10 units acceptable
<25 mL/unit	Pool ≥ 10 units to obtain >25 mL, test $4 \times$ NLT 5 mL aliquots, discard first result	Pool ≥ 10 units to obtain >25 mL, test $4 \times$ NLT 5 mL aliquots, discard first result	Pool ≥ 10 units to obtain >25 mL, test $4 \times$ NLT 5 mL aliquots, discard first result
≥ 25 mL/unit	No pooling, tested individually, test $4 \times$ NLT 5 mL aliquots, discard first result	No pooling, tested individually, test $4 \times$ NLT 5 mL aliquots, discard first result	No pooling, tested individually, test $4 \times$ NLT 5 mL aliquots, discard first result

Visual or machine inspection	Each final container of all parenteral preparations shall be inspected to the extent possible for the presence of observable foreign and particulate matter (hereafter termed "visible particulates") in its contents. The inspection process shall be designed and qualified to ensure that every lot of all parenteral preparations is essentially free from visible particulates. Qualification of the inspection process shall be performed with reference to particulates in the visible range of a type that might emanate from the manufacturing or filling process USP <1>	Light box observation for 5 s. Solutions for injection examined under suitable conditions are clear and practically free from particles (EP 2.9.20) Monoclonal antibodies: free of visible particulates (EP6.6 2031E)	JP6.06: Inspect with the unaided eyes at a position of light intensity of approx. 1000 lux under an incandescent lamp Acceptance criteria: Injections either in solutions, or in solution constituted from sterile drug solids (Method 1): Clear and free from readily detectable foreign insoluble matters Injections with constituted solution (Method 2) Clear and free from foreign insoluble matters that is clearly detectable
Clarity			
Turbidity compared to reference suspension	Not mentioned	Turbidity is the same as that of water *R* or of the solvent used, or not more than that of reference suspension 1	Not mentioned
Visual examination compared to reference suspension	Not mentioned	Turbidity is the same as that of water *R* or of the solvent used, or not more than that of reference suspension 1	Not mentioned

(size) to another over time, or comparing products/samples, difficult to interpret.

Detection and Measurement of Submicron Particles (0.1–1 μm)

As discussed earlier and shown in Figure 1, dynamic light scattering (DLS) techniques can in principle cover a wide size range (0.1 nm to ~3 μm; Philo[3]) but have poor size resolution as well as report a hydrodynamic size instead of a direct size value. Geometry or shape therefore plays a big role in the results obtained. Small particles can be masked if some large particles are present.[5] DLS response is highly dependent on the solution conditions and protein concentration, and also cannot distinguish between particle types. Furthermore, DLS cannot give absolute quantitative counts of particles. Static light scattering techniques also suffer from the limitation of lack of absolute counting/quantifying ability common to all light scattering systems.

Field flow fractionation (FFF)-based separation and analytical ultracentrifugation (AUC) are currently used as alternative (to SEC) techniques to quantitate oligomers. AUC can be applied in the ~0.01–0.1 μm range, while FFF offers a broader dynamic range from 0.01 μm to several μm dependent on the separation mode and detection.[4,24] The sensitivity of these techniques is limited by the sensitivity of the detectors used. A limitation of these methods is the difficulty of use of instrumentation as well as complexity of data analysis. These techniques, especially AUC, are therefore very useful for development and characterization, but not for batch release or stability. There are no commercially available techniques or instruments that can be used for quantitation of particles in this size range on a routine basis for QC applications.

Detection and Measurement of Micron-Range Particles (>1 μm)

The primary methods for "subvisible particles" analysis in the micron range include (i) light obscuration and (ii) microscopy-based methods.

Theoretically, light obscuration can quantify particles in the size range of approximately 1–150 μm or larger, however, with varying degrees of precision and reproducibility.[25–27] The HIAC instrument is the industry standard and can be applied to sizes between 2 and 150 μm. This method has been used to quantify particles in biotech formulations in the size range of 2 μm and larger.[17] Particle concentrations between ~10 and 18,000 counts/mL can be quantified. The instrument is commonly available and is robust and simple to use. There are certain limitations when it is used for analyzing protein-based products. A major limitation is that the instrument cannot differentiate

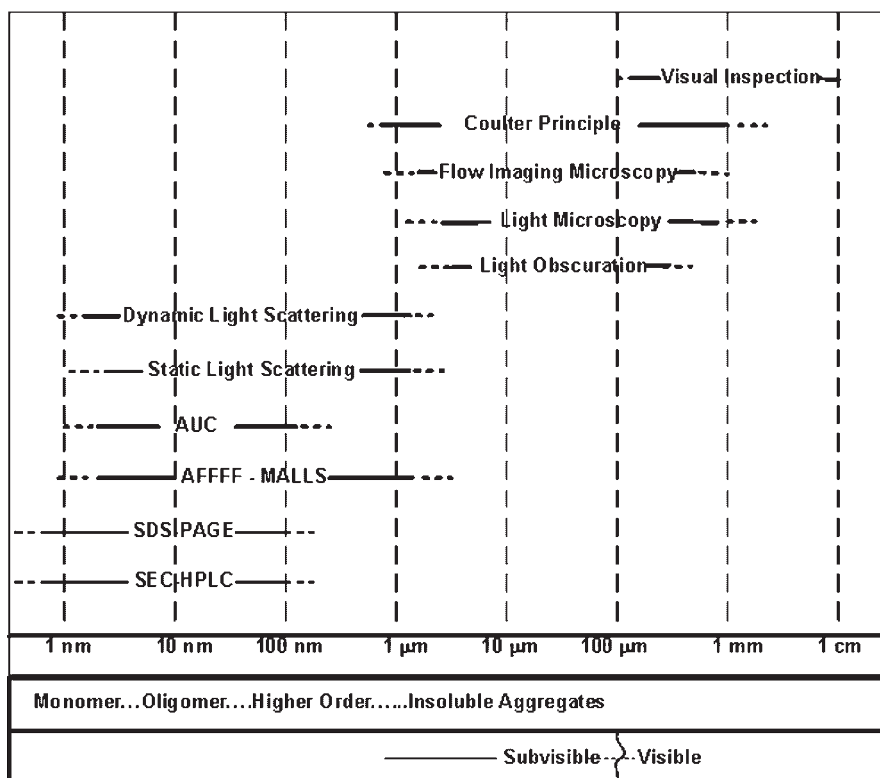

Figure 1. Schematic representation of particulate size detection ranges for various techniques (adapted from Mahler et al.[2]).

between particles from protein aggregation, particles from extraneous sources, silicone oil and air bubbles. This can potentially lead to false high counts. False low counts may result if the transparency of the particle is high, that is, some very transparent particles might not obscure enough light and therefore might not be detected. Artifacts in the results may also be seen at high particle concentrations if the channel becomes physically blocked or if the limit of detection is reached. Dilution of samples can reduce the probability of simultaneous blockage of the incident laser beam, but could also cause dissociation of protein aggregates thereby changing the particle distribution. Most importantly, the sample preparation and handling procedure is critical to obtaining good quality results by light obscuration (as well as other techniques). For example, reconstitution of lyophilized protein samples can result in the generation of microscopic air bubbles as a consequence of the diluent distribution and cake dissolution process (see, e.g., Tab. 3). Removal of air bubbles can be difficult, especially in high concentration protein formulations, and can have a significant impact on results obtained. Vacuum degassing has been found to be extremely useful in this respect (in contrast to sonication as recommended in USP <788>). In general, analyzing high-concentration protein formulations with light obscuration is a challenge, due to their higher turbidity and viscosity. Dilutions can be used but if the results are not linear, impact on particulates by dilution must be suspected, as mentioned above. The bottom-line however is that this technique, when properly used, can be relied upon to give consistent relative values between different samples, and has helped to assure the quality of products on the market. One aspect that hinders improved reliability of this (and other such techniques) in quantitating proteinaceous particles is the lack of an appropriate calibration standard mimicking the small differences in refractive index between protein particles and the medium.

The microscopic method for determination of subvisible particles is also featured in USP<788> and Ph.Eur.2.9.19, based on a manual or automatic count of particles with the use of a binocular microscope. The sample is vacuum filtered onto a grid-lined filter and, once dried, is placed under the microscope for counting. For this to reflect the actual numbers of particles present in the sample, the particles on the filter should not arise from the preparation environment, equipment or from the personnel, and should not be altered by the sample preparation procedure. For soft proteinaceous particles, these requirements may be difficult to meet. The wash step can alter the particle distribution. For protein particles below 10 µm, isolation is very

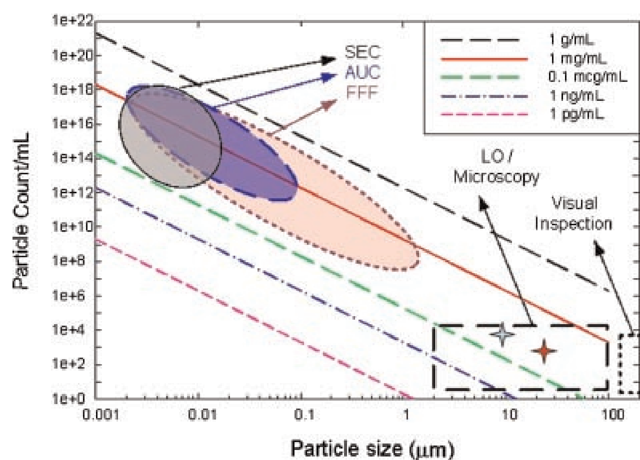

Figure 2. The particulate detection and measurement landscape. The straight lines represent boundaries for constant mass of protein particles (under the assumption of spherical particles of density 1 g/cm^3). The 0.1 μg/mL line has been taken as a detection limit for UV/RI detectors. Approximate operating regions for SEC, AUC, and FFF are shown. Note that the upper size limit for SEC is a consequence of "filtering" or entrapment of larger aggregates/particles on the column. The standard use of AUC is for aggregates from dimer to 20 mers representing an upper range of around 0.01–0.02 μm. Gravitational sweep sedimentation can be used to work in the 0.01–0.1 μm range. FFF is currently used to detect aggregates or particles up to 1 μm. The USP and Ph.Eur. limits for ≥10 and ≥25 μm sizes are also shown (figure adapted courtesy Gabrielson and Kendrick, unpublished).

difficult. It is possible for the amorphous protein particles to pass through the filter, or they can also spread out on the filter surface such that they become very difficult to visualize. Calibration is performed; however this can be difficult and tedious, and when performed with particle standards, is not representative of the results obtained for protein particles. Similar to light obscuration, counting is better when the particle contrast/translucency is high compared to background. The specification limits in the pharmacopeias by the microscopic method are much tighter compared to light obscuration (Tab. 2).

The manual optical microscopy technique has been enhanced with the development of *flow-imaging*. Four instruments are available that take images of particles as they flow through the microscope. The instruments [Micro-Flow Imaging (MFI) by Brightwell Technologies Inc. Ottowa, Canada; Flow Particle Imaging Analyzer (FPIA) from Malvern Instruments Ltd., Malvern, UK; FlowCam from Fluid Imaging Technologies, Yarmouth, ME; ParticleInsight from Micromeritics Inc., Norcorss, GA] employ a combination of microscopy in a flow-through mode coupled to an image processing system to enable automated analysis and counting of particles in liquid formulations. In this case the manipulations involved in particle filtration and isolation are avoided, and recent developments in digital imaging are leveraged. Besides number and size, the flow microscopy techniques also claim to assess parameters such as transparency and circularity/shape, thus potentially helping to differentiate between, for example, silicone droplets or air bubbles and extraneous particles.[28,29] However, assessing some of these parameters require particles to be of a certain minimum size so as to image an adequate numbers of pixel for the analysis to be carried out. Figure 3 shows an example of the images obtained with two of these instruments (MFI and FPIA). The images of a particle counting standard used to calibrate the instruments, of a silicone oil droplet, and of a protein particle are quite different. Some protein particles look like agglomerates of smaller particles, with uneven irregular density and borders. The quality of the optics and the fraction of total volume analyzed vary in these two instruments (based on magnification used), but both are destructive, low throughput instruments useful for research and development but not suitable for routine quality testing. The picture also illustrates the wide range of particle morphologies and optical properties possible in a sample. This makes it difficult to make a proper estimation of the size of these particles, with each instrument using a different algorithm to measure the same object. Results are therefore dependent on the algorithm used to select, classify, and "size" particles, with the same (sample) image potentially yielding different results if reprocessed.

The *Coulter principle* is another method that can quantify subvisible particles. The technique provides absolute particle counts in dilute conducting liquids. Recent instruments utilizing this principle (COULTER COUNTER® Multisizer 4, Beckman Coulter, Fullerton, CA, and the Micromeritics Elzone II 5390®), have good sensitivity and can measure particles from 0.4 to about 50 μm, though different apertures and instrument settings are needed for in order to span the entire range. Depending on the formulation buffer properties and experimental conditions used, modification of the ionic strength of the sample (either by dilution or addition of salt) may be necessary, which in turn could either create or break aggregates/particulates. It is likely that the actual impact of this manipulation is dependent on the protein and the nature of the aggregate, thus creating some uncertainty about the results reported. Loose aggregates carrying a large fraction of enclosed solution, and/or particulate geometries differing significantly from spherical, can also lead to difficulties in sizing by the Coulter principle. Table 3 shows a comparison of the experimental characteristics of light obscuration, flow imaging and the Coulter

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Table 3. Comparison of Current Particle Counting Technologies

Attributes	Light Obscuration	Flow Microscopy (MFI)	Flow Microscopy (FPIA)	Flow Microscopy (Flow CAM)	Coulter Principle (Multisizer 4)
Measurement principle	Light obscuration	Microscopic imaging	Microscopic imaging	Microscopic imaging	Coulter principle (may require modification of ionic strength)
Measurement outcome	Particle concentration (counts/mL)	Particle concentration (counts/mL) and shape/ morphology[a] (image quality medium)	Particle concentration (counts/mL) and shape/ morphology[a] (image quality high)	Particle concentration (counts/mL) and shape/ morphology[a] (image quality medium)	Particle concentration (counts/mL)
Particle sizes limits for[b]					
Detection vs. identification/ classification		5X: detect 1 μm, id 4 μm		4X: detect 4 μm, id 15 μm; 10X: detect 1.5 μm, id 5 μm; 20X: detect 1 μm, id: 4 μm	
Minimum particle sizes detected	≥2 μm	5X: ≥1 μm	20X: ≥1.5 μm	20X: ≥1 μm	≥0.4 μm
Sample handling	Degas if required by allowing to stand or apply vacuum	To be defined by user	To be defined by user	To be defined by user	To be defined by user
Minimum sample volume required	≥25 mL (USP/Ph.Eur.) ~3–5 mL (scaled down)	5X: 0.5 mL	≥1 mL	4X: >1 mL; 10X: >0.25 mL; 20X: >0.25 mL	Total cell volume 10 mL (after dilution into conducting buffer)
% Total sample volume analyzed	≤60%	5X: ~65%	≤1%	4X: ~65%; 10X: 20–40%; 20X: 5–10%	Set by user
Particle concentration range	≤18,000 counts/mL	5X: ~850,000 counts/mL at 2.5 μm size	20X: ~500,000 counts/mL at 1.5 μm size	10X: 1E6/mL at 2.5 μm size	~1E6–2E6 counts/mL
Detection limited by[b]	Particle transparency	Particle contrast and pixels available	Particle contrast and pixels available	Particle contrast and pixels available	Solution conductivity

[a]Shape/morphology information may help to differentiate particle types (e.g., silicone oil), for particles above 4 μm.
[ab]Detection and classification in imaging systems is dependent on pixels available at sample plane.

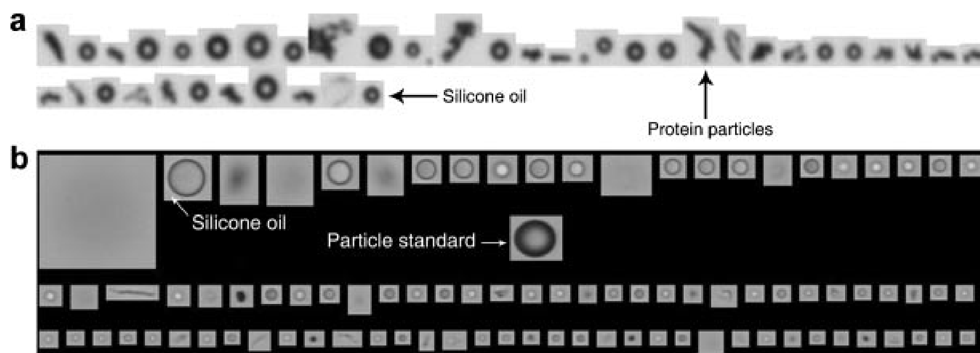

Figure 3. Subvisible particle images obtained with two different flow microscopy instruments. Difference in clarity of images reflects differences in the optical capabilities of the two instruments. (a) Representative particle images by MFI; (b) representative particle images by FPIA.

principle techniques. Some illustrative data generated by these methods is presented and discussed below to allow a comparison of the performance of the methods. It is clear that absolute number of counts varies between techniques but are consistent within a technique across test samples.

Table 4 shows particle count results obtained for two lyophilized recombinant drug product presentations (rP1, Lots 1 and 2) and (rP2, Lot 1) by three different techniques. For HIAC measurements, each sample was analyzed on 3 days by two analysts on two different instruments essentially following the current USP <788> and Ph.Eur.2.9.19 to obtain an estimate of intermediate precision. Ten individual containers were pooled for each analysis performed by each analyst. The mean values (Day 1, Day 2, and Day 3) are in agreement between analysts with similar distribution ranges. However, the largest variability in the particle count and the largest variability range of the results are observed for the ≥2 and ≥5 μm sizes. In this study, sample preparation (reconstitution) technique and waiting time prior to analysis had a significant impact on the results. Higher counts were seen if analysis was performed immediately after reconstitution (data not

Table 4. Intermediate Precision Results of Two Lyophilized Drug Product Presentations Obtained by Two Analysts Using Light Obscuration Measurement (HIAC), Membrane Microscopy and Flow Imaging (MFI)

		Subvisible Particles (Counts per mL) Mean $_{\text{Day 1 to Day 3}}$ + Deviation From the Mean to the Maximum		
Analyst and Instrument	Particle Size (μm)	rP 1, Lot 1	rP 1, Lot 2	rP 2, Lot 1
Light obscuration (HIAC)				
1	≥2	350 + 156	4338 +2735	966 + 785
	≥5	73 + 44	838 + 587	257 + 219
	≥10	14 + 9	84 + 47	46 + 28
	≥25	1 + 1	3 + 4	3 + 2
2	≥2	430 + 205	4247 + 3962	1179 + 955
	≥5	100 + 65	710 + 854	359 + 322
	≥10	18 + 8	67 + 50	57 + 27
	≥25	1 + 1	1 + 1	3 + 1
Membrane microscopy				
1	≥2	Not measurable	Not measurable	Not measurable
	≥5	54 + 6	45 + 9	72 + 3
	≥10	36 + 6	29 + 5	46 + 3
	≥25	14 + 2	11 + 4	17 + 2
2	≥2	Not measurable	Not measurable	Not measurable
	≥5	54 + 8	48 + 9	77 + 2
	≥10	36 + 5	29 + 4	51 + 4
	≥25	15 + 3	12 + 4	21 + 2
Flow imaging (MFI)				
1	≥2	3424 + 1047	3741 + 2128	2862 + 817
	≥5	489 + 291	597 + 713	593 + 117
	≥10	88 + 17	88 + 45	206 + 58
	≥25	15 + 5	15 + 1	40 + 18

shown). Submicroscopic bubbles formed after reconstitution that persisted in solution, were the main cause of the variability and could not be controlled even though an established preparation procedure was followed. Note that while the absolute numbers of subvisible particles are different, the relative numbers or trends between samples, are the same. This also demonstrates the inadequacy of the compendial sample handling procedure for many products.

The reconstituted samples, when assessed by membrane microscopy, show significantly lower counts in the size range $\geq 5\,\mu m$ (Tab. 4). The data were obtained from 10 different individual containers. The microscopy was performed using a gray filter to enable the analysts to accurately observe and count different types of particles.

MFI data for the same product were obtained from 10 different individual containers in three replicates similar to HIAC analysis (Tab. 4). Subvisible particle counts are generally higher with the MFI compared to the HIAC results, although not always. The deviation from the mean to the maximum is also higher, suggesting that the MFI data are subject to a similar range of variability and errors as the HIAC.

The significantly lower counts by the membrane microscopy method suggests that the HIAC and MFI results are either (1) impacted by sample preparation procedure and are likely to be counting the microscopic bubbles resulting from the reconstitution procedure, or (2) the optical microscopic method is missing a large number of true particles due to inherent difficulties in their isolation, or (3) a combination of both these effects.

Other comparisons of results by light obscuration and flow-imaging have been published. Huang et al.[28] noted much larger differences in particle counts when comparing measurements by light obscuration and MFI. Measurements in a number of participating authors' laboratories also confirms the observation that MFI reports significantly higher counts than HIAC, particularly in the smaller size ranges. One such set of observations is shown in Figure 4 below. Although particles below $10\,\mu m$ were not measured on the HIAC in this case, the trend for all sizes is similar in both instruments, leading to the conclusion that the subvisible particulate results correlate well with the applied stress conditions. Similar observations have been made in other laboratories (data not presented).

Huang et al.[28] attribute the differences between light obscuration and flow microscopy results primarily to the refractive index of the smaller particles, although the morphology of the particles is also expected to play a role. The hypothesis proposed is that the smaller the particles, the closer their refractive indices are to that of the formulation buffer. In this situation, the MFI detector, which

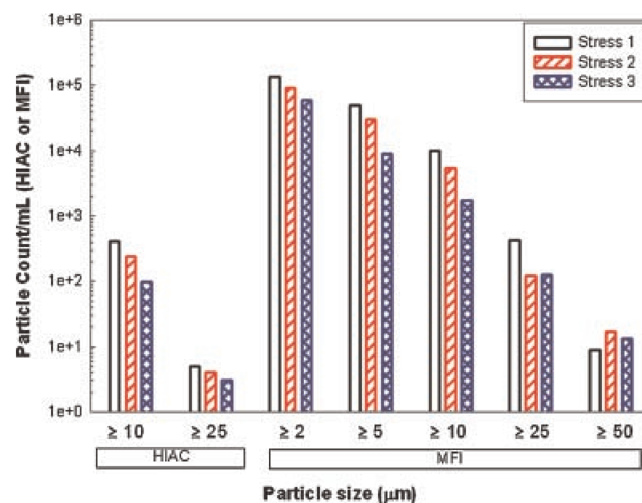

Figure 4. Comparison of particle counts for a MAb formulation by HIAC and MFI. The samples were exposed to three different stress conditions.

captures an "image" as opposed to the HIAC detector which captures a "shadow," is purported to give a more accurate count. Furthermore, it is proposed that particles with noncircular morphologies are not accurately counted by light obscuration. Differences could also arise due to the different algorithms used to calculate size in these different techniques. The flow microscopy instruments all use algorithms that include assuming the protein particle is a sphere, or at best a cylinder. At present, it is not possible to say which technique (including isolation and microscopy, or the newer flow imaging techniques) is giving the most accurate quantitation of these particles.

Finally, some illustrative data are given in Table 5 for a test system with bovine serum albumin (BSA), where the results by Coulter principle (Multisizer 4) and flow imaging (MFI) are compared in the range 1–$10\,\mu m$. The results are mostly comparable, except in the high count sample. Here, the MFI gives almost 10 times as many counts for the $\geq 2\,\mu m$ range, and even greater factors for larger sizes. The difference could arise because either the aggregates are reversible when diluted in saline for Multisizer 4 (conductive media) or the result is simply a reflection of the difference in measurement principles. However, measurements in other laboratories have found the reverse trend between flow imaging and Coulter principle measurements of the same sample, suggesting that the results are product specific.

The difference in numbers of subvisible particles obtained by the various techniques shown above, highlights that a single value for subvisible particulates is difficult to interpret, especially when dealing with nonspherical, ill-defined amorphous proteinaceous particles. None of the results are incorrect,

Table 5. Data from a Coulter Counter® (Multisizer 4) Compared to Flow Microscopy (MFI)

Multisizer 4	≥1 μm	≥2 μm	≥3 μm	≥4 μm	≥5 μm	≥6 μm	≥7 μm	≥8 μm	≥9 μm	≥10 μm
BSA in PETG bottle	1399	466	166	133	33	33	33	33	0	0
BSA with glass beads unshaken	1834	1434	434	134	67	67	33	33	0	0
PBS shaken with glass beads	2433	1266	533	233	133	33	33	0	0	0
BSA shaken with glass beads	333149	12265	1332	399	99	66	66	33	33	0
BSA shaken and filtered	332	299	166	133	66	33	0	0	0	0

MFI	≥1 μm	≥2 μm	≥5 μm	≥10 μm
BSA in PETG bottle	322	187	57	18
BSA with glass beads unshaken	1267	363	84	11
PBS shaken with glass beads	2351	572	118	18
BSA shaken with glass beads	605769	128816	10684	678
BSA shaken and filtered	127	53	15	3

but accurately determining the absolute number of proteinaceous particles and distinguishing them from extraneous particles, is currently not possible. Results therefore need to be placed in context of the method used for the measurement, and should not be compared across techniques. Differences in reported results for the same sample, between the techniques discussed above (and other techniques) will arise due to the different measurement principles, the wide range of optical and morphological properties of the particles being counted, and also the individual algorithms to estimate size and count. The various techniques detect particles differently, measure different characteristics and therefore provide differing counts. Furthermore, sample preparation and handling to adapt the sample to the instrument, is a critical step that adds to the variability in reported value.

A limitation when comparing results from various techniques is the lack of appropriate counting reference standards for proteins, as mentioned earlier. The polystyrene particle counting standards differ considerably from the proteinaceous particles in all their characteristics (morphology, refractive index, shape, texture, deformability, etc.). Interestingly, the absolute numbers obtained on polystyrene standards by the different techniques agree well with each other, in contrast to the data obtained on protein samples above. Figure 3 also illustrates the difficulty in defining appropriate standards for proteinaceous particles given their morphological heterogeneity. It also illustrates the inappropriateness of the current polystyrene standards which differ significantly from the protein particles in both optical properties and morphology. Hence, creation of such a standard or of a standardized procedure to create a laboratory standard, would be of great value, allowing comparisons across techniques/laboratories/products.

Product-Related Factors Leading to Variability in Subvisible Particulates Data

The data (in Tabs. 1, 4, and 5) show that variability in levels of subvisible particles can be quite high, especially in the smaller size ranges. We now examine some of the product-related factors that impact these counts.

Some level of particulates is unavoidable in parenteral products. For a given product, actual levels of particles can conceivably be impacted by processing and postproduction handling including freeze–thaw, transportation, shock-drop and the overall time-temperature history. Container/closure systems can contribute significantly to the background level of (extraneous) particulates in products making it difficult to monitor proteinaceous particles. For products in prefilled syringes (PFS) or cartridges, monitoring can be even more problematic due to silicone-oil-based particles potentially making up a large part of the total population of subvisible particles. Furthermore, the content, distribution and release of silicone-oil from the individual PFS varies widely. Use of surfactant in the formulation may enhance the dispersion of silicone-oil, while shock/vibration history, storage orientation, stopper movement and head-space volume can impact the distribution into the product solution. The variability introduced by container/closure is demonstrated in Figure 5a and b which compare the subvisible particles in two lots of the same protein in vials and PFS. Particle counts in the PFS are almost an order of magnitude higher. Inability to distinguish between silicone-oil and proteinaceous particles is a limitation of current techniques (discussed later). Indeed, it was found in one of the participating author's laboratories that the particle counts in placebo from PFS were often indistinguishable from that of product, especially below 10 μm. Experience suggests that as a consequence, a greater degree of

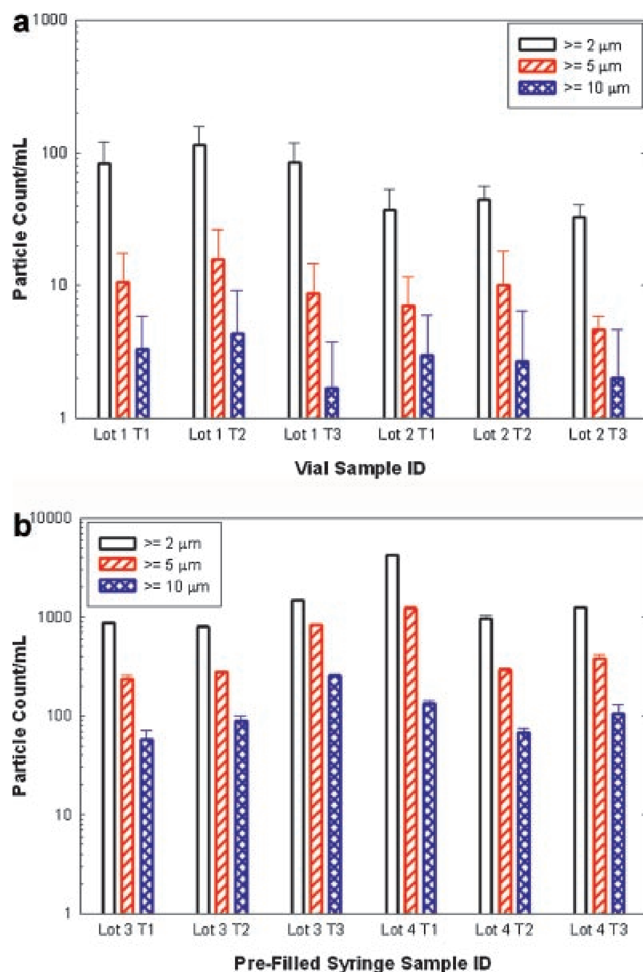

Figure 5. Subvisible particles measured by HIAC on two lots of the same protein filled into (a) vials or (b) prefilled syringes.

variation will be seen in the particle counts below 10 μm.

MFI and FPIA claim to be able to distinguish between silicone oil and proteinaceous particles based on the geometry/morphology as illustrated in Figure 3. However, attempts to develop mathematical models/algorithms to differentiate between the populations can result in significant over/under counting of the protein particles.

The variability due to microscopic bubbles created during reconstitution of lyophilized products has already been illustrated in Table 3. Proper removal of microbubbles becomes more difficult for higher concentration products due to the increased viscosity. Surfactants, a very common ingredient in the formulations, add to this difficulty of sample preparation.

Another factor for consideration when analyzing reconstituted products is the contribution to particulates from the diluent itself. Saline, WFI, or other diluents including those packaged in infusion bags or vials are a possible contributor to thousands of particles in the lower ($\leq$5 μm) size ranges, as measured in laboratories of a number of the contributing authors (see, e.g., Mahler et al.[30]).

In summary, subvisible particulate measurement results can be impacted by a number of product and process factors. A consequence of the variability introduced by such factors is that it might not be possible to generate reliable baseline data for all product/formulation/container combinations. The variability also makes the setting of specifications, or establishing comparability criteria very problematic. For example, changing from a frozen solution in a vial to a refrigerated liquid in a PFS will affect the number of subvisible particles, making comparability difficult to establish between materials made at different stages of a project. Furthermore, it may lead to the situation that the same biologic in different packaging presentations and/or different concentrations has different subvisible particle limits (even on a normalized basis). An example is shown by the data for Product 7 in Table 1 where the same product in two different formulations and two different container closures has dramatically different particle counts. A single specification based on a comparison of these numbers would not be justified. Any specification would need to be context (formulation, strength, package, volume) dependent and not simply molecule dependent. This is unlike other product attributes. Oxidation, for example, is an attribute that can also be potentially impacted by the above mentioned factors, but the mechanism is well understood, it is readily measurable, it is not impacted by transportation, it can generally be preserved unchanged by freezing, and it does not vary significantly within a batch. Molecule specific attributes such as glycosylation do not change once a molecule has been defined. In contrast, proteinaceous subvisible particulates vary as a consequence of all the above factors and are also impacted by product history while a mechanistic understanding of the cause of their formation is seldom very clear.

Sample Requirements

In order to have the possibility of understanding the impact of subvisible particles on safety, their counts and identity must be known. With this objective and in light of the large differences seen in results reported by the techniques (e.g., HIAC and MFI), it would be important that the same method is used during development, for release, and throughout the commercial lifecycle of a product.

The standard USP <788>/Ph.Eur.2.9.19 methodology places a heavy demand on sample required for testing (summarized in Tab. 2), although smaller volume tests can be qualified as discussed below. However, once the HIAC is calibrated from 2 to

10 μm, no extra sample volume is required beyond what is needed for standard pharmacopeia measurements. Thus, in formal stability programs, this data can be gathered. However, if high numbers of particles in the 2–10 μm range are present, the resultant saturation of the instrument and error message can lead to assay failure. Dilution into formulation buffer might be required if the compendial method is used, which would entail method development and qualification to ensure that the results are not impacted.

The use of HIAC in development studies has some limitations. Generally, the full sample volumes as suggested in USP/Ph.Eur. are not available. Smaller measurement volumes down to 0.5 mL can be qualified in the HIAC. However, smaller measurement volumes are also inherently more prone to counting errors, further increasing the variability in the data. MFI requires approximately 0.8 mL; however, replicates are necessary for reliable counting, which raises the volume requirements to about 3 mL after allowing for accurate sample removal. Studies in which such measurements are to be made therefore have to be selected and designed judiciously.

Development samples also have another weakness—these are generally not manufactured in a Class A environment. Environmental contamination can confound the results, causing high variability between vials and artificially inflated counts that can be a distraction from development. The question remains as to how to interpret the data generated during development. Current regulatory opinion based on informal discussions is that the data should be evaluated for "trends." The accuracy and precision of measurements in this size range, as well as consistency of measurement techniques used over the course of product development, should be kept in mind when evaluating the data.

In summary, the appropriate number of samples (sample size) to test depends on the number of subvisible particles present, the sample-to-sample variability associated with the particular product, and the level of acceptable risk, among other factors. Confidence in the reliability of the results obtained from testing a limited number of samples to represent the entire lot is significantly affected by the sample size tested. Subvisible particles measurements during development and characterization (as illustrated later) will therefore emphasize trending and relative differences. In these applications, a smaller sample size with the greater inherent variability in results may be acceptable.

Identification of Particulates—Under What Circumstances and How?

The ability to identify the source/nature of particles is important in order to be able to eliminate them. Identification of particulates in the lower subvisible size ranges is especially difficult. Thus, if an aberrant high count is seen, the question becomes "what should we do with this." Isolation of these particulates is not simple. The particles can go through the filter, the handling itself can break them apart, or generate fibrils, and it can be very difficult to detect a few particles of small size and uncertain edges on a neutral background under the microscope. Some new techniques have been developed that propose to help address this issue, but require further validation. Most identification technologies also focus on a few particulates, having been designed to look at foreign matter, leaving open the question whether the subset is representative of the sample.

Brightwell's MFI technology suggests that the morphology is indicative of whether a particulate is proteinaceous or extraneous. However, morphological characterization of particles is limited by current camera technology and is useful only on particles of size greater than 4 μm.[31] Various algorithmic filtering techniques can be created to distinguish bubbles or silicone oil droplets,[29] although validation of these algorithms for quality control purposes is considered problematic, and can also result in mis-assignment of a large fraction of the protein particles as silicone oil (up to 20%, data not shown). More experience will be required to confirm these capabilities, and whether it can distinguish between a protofibril and a cellulose fiber or other material such as rubber/plastic (considering the size limitation given above). The technology will be useful as an investigative tool in case of a "deviation" and as a developmental tool.

Rap.ID (Berlin, Germany) using Raman spectroscopy may also provide some clues. However, in the authors' experience, it is not always unequivocal in identification of particles as proteinaceous or not. The quality of the database and statistics of scanned particles as well as quality of spectral overlays impacts the analytical output significantly. Particles in protein solutions can be heterogeneous, and this also confounds the spectral identification.

If the difficulty of isolation is overcome, attempts can be made to further characterize and analyze aggregates on a structural level. Techniques used for the structural analysis such as circular dichroism (CD), Fourier-transformed infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (NMR) or intrinsic fluorescence might be considered. Other techniques that can be used to identify particulates, that is, to differentiate whether they are proteinaceous or from an external source, are Raman or FT-IR (micro)spectroscopy, Electron microscopy with elementary analysis may also be utilized.

Data analysis (e.g., spectral comparison and overlays) should be considered with care. For example spectra overlays may be difficult to interpret due to

differences in signal intensities and signal broadening of aggregated and isolated species compared to the signal of the species in solution, different contributions from buffers, etc. Additionally, the sensitivity of structural analysis methods such as FT-IR to small conformational changes (<5% change in signal) is low. Furthermore, even extraneous particles may be of proteinaceous nature (e.g., human dander). Dissecting and identifying individual components in heterogeneous particles containing more than two species is very difficult. Therefore, an unambiguous composition of particles cannot always be given by any available method to date. Due to the limitations of the methods used, any resultant data should be interpreted with care and on a case-by-case basis.

Similar to particulate counting, the primary limitation in all identification methods is sample preparation and handling, and must be kept in mind when interpreting data. Well-trained operators must preferably be used. The use of such techniques cannot be left to every development scientist. A highly skilled forensic laboratory would be required to isolate and identify particles of 20 μm and larger, and this would not be a routine task. Identification of particles below 20 μm may sometimes be possible but with significant challenges in unequivocal identity assignment.

From a risk management perspective, given the lack of ability to identify the particle population involved, the assumption may have to be made that all particles in the size range below ~10–20 μm are proteinaceous, adding to the error and variability of this parameter. The only situation where an alternative assignment could be made is if a placebo manufactured in parallel shows similar amounts of subvisible particles, in which case they could be assessed to be "most likely not protein." However, such a possibility is not always available and also assumes similarity in the amounts of contaminating extrinsic particulates, including the silicone oil droplets that are present in any product from PFS.

VALUE OF MONITORING SUBVISIBLE PARTICLES OVER A BROAD RANGE

Available observations, albeit limited, suggest that particle counts within the various size ranges are positively correlated. The data presented below (Fig. 6) on a lyophilized product stored under stressed conditions shows that subvisible particles in all size ranges increased concurrently. The number of particles in size range ≥10 μm correlated with the counts for the smaller sizes.

A similar correlation between the subvisible particle counts in various size ranges was detected for another antibody when a stressed and an unstressed sample were compared (see Fig. 7). The numbers of subvisible particles increased due to light and heat stress, but the size distribution remained constant as indicated by the parallel lines, especially above the LOD/LOQ of the techniques (10 particles per mL).

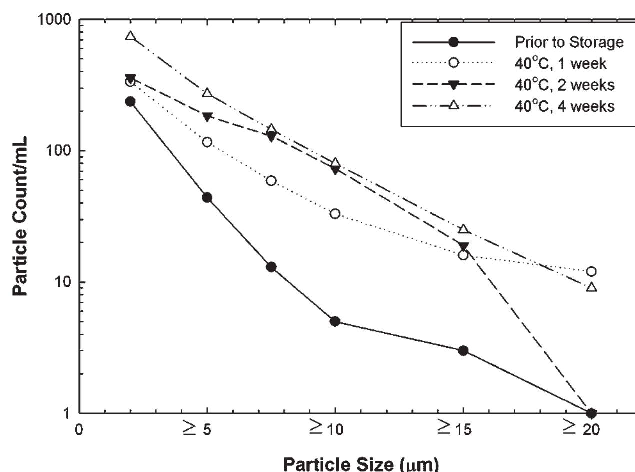

Figure 6. Subvisible particles measured after reconstitution for a lyophilized product stored under stressed storage conditions, using a small volume HIAC method.

Although the positive correlation between particle sizes shown in the above two examples may not always hold, it is apparent from these and the previous examples that the numbers of particles in the ≥2 and ≥5 μm ranges represent a good tool for product development. These numbers tend to be high and are therefore a sensitive measure to assess impact of variables of interest during developmental studies. However, as discussed above, the high sensitivity is also associated with higher variability

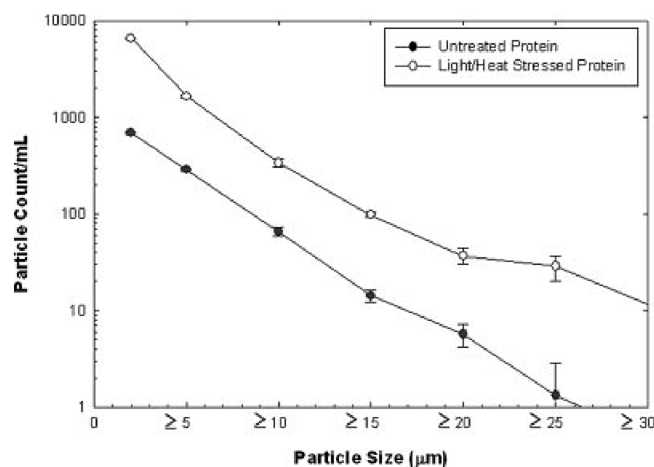

Figure 7. Subvisible particles measured by (a small volume) HIAC method on untreated and stressed monoclonal antibody.

Table 6. Impact of Filtration on Subvisible Particle Populations Measured by Light Obscuration (Narhi et al.[6])

Sample Type	Particles/mL, $\geq$2 μm	Particles/mL, $\geq$5 μm	Particles/mL, $\geq$10 μm	Particles/mL, $\geq$25 μm
Unfiltered, Lot 1	339 ± 78	29 ± 2	<10	<10
After 1× filtration Lot 1	29 ± 6	11 ± 5	<10	<10
After 2× filtration Lot 1	19 ± 9	14 ± 9	<10	<10
After 5× filtration Lot 1	50 ± 14	12 ± 9	<10	<10
Unfiltered, Lot 2	680 ± 51	36 ± 4	<10	<10
After 1× filtration Lot 2	20 ± 3	5 ± 2	<10	<10

or noise. Data interpretation must be made with care to prevent time and effort being expended on false trails. Keeping these caveats in mind, this type of data can be useful in designing optimal formulations and in troubleshooting.

The utility of measuring particles under 10 μm sizes for examining a process step is illustrated by the data in Table 6 where the efficacy of the filtration step in removing these particle is shown.[6] The number of particles in the $\geq$10 μm size range was below LOQ (about 10 counts/mL) and therefore not useful for process development. Results for the $\geq$2 μm size show that a single filtration step is adequate and subsequent filtration steps do not provide added benefit in particle clearance.[6]

To be useful, these types of comparative analyses must be performed under similar conditions (concentration, container/closure) and by the same methodology (sample preparation procedure, technology/instrument).

Setting Specifications for Sizes <10 μm?

When performed properly, measurements of subvisible particles below the 10 μm range, can have utility in guiding product development and in establishing clinical experience with the product. However, the ability and utility of setting specifications for particles in this size range requires much more critical thought. The variability of the methods for measuring particles under 10 μm and the other difficulties in analysis discussed earlier (silicone oil in PFS/cartridges, bubbles, high concentration/viscosity solutions, environmental contaminants, impact of container/closure, handling, time/temperature history, etc.) need to be considered for the specific product before it can be determined whether meaningful specifications can be established. Heterogeneity within a batch is quite likely, increasing the sampling requirements to get a statistically relevant number. This is quite unlike other quality attributes. In order to accommodate the variability, product history will need to be acquired over multiple drug substance and drug product batches. In the case of PFS or cartridge products, this need will be magnified to cover various silicone loads. If such a path is taken, it is likely that specifications for each size range will be driven by the product/container configuration and not by any characteristic of the biologic itself.

Subvisible particles assessment as part of comparability exercises may present several logistical challenges. For most quality attributes, the preferred approach to establishing comparability is to perform a side-by-side analysis of the two processes/formulations/configurations to be compared, to minimize discrepancies contributed by day-to-day assay variability. Material (samples) retained from various critical (toxicology, clinical) batches would need to be available in sufficient number and volume to be part of this study. This would require significantly more retained samples than is current practice as these samples would be required to support any change in process, container/closure, strength, volume, etc. The above-mentioned heterogeneity within a batch will also significantly increase the number of samples required to get a statistically relevant comparison. Furthermore, it is very possible that the subvisible particle profile would change during storage of these samples, making the true assessment of comparability difficult. This is all the more likely if the retained samples are stored frozen, as is the practice so that other quality attributes do not change. These limitations may require that comparability be assessed by evaluating the particulate profile in relation to historical information available for the product, thus necessitating a good understanding of the variability of the method used for measuring the subvisible particulates as well as the normal variability observed for the product. Modifications in container/closure and/or formulation during the course of development (e.g., going from vial to syringe or from lyophilized to liquid) could result in differences that have no clinical impact, such as an increase in reported particulates due to silicone oil droplets. With these constraints, arriving at a meaningful conclusion would be challenging, especially given the generally limited history and experience with this attribute during development.

Risk Assessment

The mass represented by proteinaceous subvisible particles over a wide size range is shown in Figure 2. Particle sizes that are currently already specified represent the greatest mass of protein in particulate

form. On the other hand, particles between 1 and 10 μm, even if present at 10,000 counts/mL, represent about 0.1 μg of material, while the protein aggregates at sizes below 1 μm comprise material in the ng to pg levels (see Fig. 2). The risk of an immunologic reaction with this amount of material is low considering that even vaccine doses lie in the μg–mg range, and further require the inclusion of adjuvants. The authors realize that the comparison with vaccines is not perfect since unlike vaccines, certain protein therapeutics are dosed chronically, while vaccines are purposely dosed at levels high enough to create an immune response. However, given the lack of clarity around the impact of aggregates on immunogenicity, transferring learnings from related fields may help to put the risk from aggregates in perspective.

Another aspect to assess is the level of the proteinaceous subvisible particles relative to other product-related impurities, variants or degradants. Subvisible particles comprising 0.1 μg of protein represent about 0.0002% of a 50 mg/mL monoclonal antibody product and about 0.01% of a 1 mg/mL therapeutic protein product. For most other product quality attributes (e.g., oxidation, deamidation, charge-variants, glycosylation variants, etc.), these levels are neither detected nor reported, and are considered to be well below the safety concern threshold.

More experience with the measurement and monitoring of subvisible particles, similar to other product quality attributes is therefore required to develop thresholds for control and monitoring of proteinaceous subvisible particulates. A step in this direction would be to assess the current state of affairs in more detail. A survey of subvisible particulate levels in current marketed products (along the lines in Tab. 1) could help set possible benchmarks to judge individual products during development, especially prior to accumulating clinical experience. For instance, questions like “is 10,000 counts/mL of size 2–10 μm high, low or about average?” would be very helpful in decision making. Given the lack of experience as well lack of clarity around the clinical impact of aggregates, this may provide an avenue to approach risk assessment around this question. Such an assessment can put the subvisible particle data in perspective, especially if the risk is broken down by therapeutic area, functional modality, biologic type (monoclonal antibody or therapeutic protein), duration of therapy (acute or chronic), dosing site (IV, IM, SC), disease, and other patient-related factors.

CONCLUSIONS AND RECOMMENDATIONS

(1) Concerns about aggregation as a potential cause of immunogenicity in human biotherapeutics have been around for a long time. The multiplicity of determinants of immunogenicity in a clinical setting makes the deconvolution of factors very difficult, and no direct evidence from clinical studies has been forthcoming to demonstrate that aggregates are a true dominant risk factor. It is also difficult to determine the immunogenicity potential of naturally occurring aggregates of a particular human protein in human patients, based on the results of animal studies using artificially created aggregates due to differences in the morphology and structure of the aggregates. However, we concur with Carpenter et al.[7] in their suggestion that this remains an important area for continued research.

(2) The authors agree that research is needed and new technology must be developed for accurate particulate (and aggregate) characterization and quantitation. Development of proteinaceous subvisible particle standards would be of immense value in improving the results from the current techniques. Simultaneously, research into the clinical immunogenic potential of aggregates in general and proteinaceous (subvisible) particles in particular must be progressed, as stated above. However, it is also likely that such a link may never be unequivocally demonstrated. An effort to assess of the current state of affairs among marketed products, broken down by therapeutic area and class of biologic among other factors, may be an approach to understand the true risk potential.

(3) Proteinaceous subvisible particles represent large assemblies of protein molecules, and therefore are subject to the same concerns as aggregates in general. Measurements on a limited set of marketed products suggest that these (and presumably most if not all marketed products) contain large but varying numbers of subvisible particles in the size range below 10 μm. These small particles represent protein content in fractions of μg or lower, with the largest portion of this mass represented by the size ranges above 10 μm, the population that is currently monitored. To place this amount in perspective, other product quality attributes such as oxidation, deamidation, charge-variants or glycosylation-variants, are neither detected nor reported at these levels, and are considered well below the safety concern threshold. Human biotherapeutics currently on the market continue to be safe and efficacious from an immunologic perspective, despite the gap in the monitoring identified in the commentary and further detailed in this article.

(4) Measurement of subvisible particulates smaller than 10 μm size range has value as an aid in product development and characterization. Data can be generated as a "for information only" test, assisting in the understanding of the product behavior. However, lack of ability to differentiate proteinaceous from extrinsic particles between 0.1 and 10 μm particles, and variability introduced as a consequence of factors such as container/closure, product concentration, viscosity, product history, and inherent heterogeneity within a batch, imply that the results would differ considerably between phases of the development cycle. Furthermore, considering the limitations of the methodologies (namely low precision and reproducibility) demonstrated here, as well as limited experience and the lack of understanding of the impact of this attribute, it is not appropriate to add specifications for subvisible particles in the size range $<10\,\mu m$ at release and stability, or for comparability at the present time.

(5) Characterization of product quality attributes including subvisible particulates, is performed on all products in R & D during clinical development. Preclinical studies are not currently capable of assessing the clinically relevant immunogenicity potential of product attributes such as aggregates and/or particles smaller than 10 μm. Preclinical studies are also not currently capable of assessing the clinically relevant immunogenicity potential of any type of product variants or degradants present at fraction of μg levels or less. Thus, the safety and efficacy profile of biotech products is established during research and development by appropriately designed nonclinical and clinical studies. Immunogenicity is monitored and evaluated during clinical studies using the clinical study material, and thereby product quality attributes which could be potentially relevant for safety and/or efficacy are qualified. Subvisible particles, of which the currently monitored range $\geq 10\,\mu m$ population would represent the largest fraction of protein aggregate mass (if proteinaceous), remain a product quality attribute that is also qualified in this fashion.

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REFERENCES

1. Rosenberg AS. 2006. Effect of protein aggregates: An immunologic perspective. AAPS J 8:E501–E507.
2. Mahler H-C, Freiss W, Grauschopf U, Keise S. 2009. Protein aggregation: Pathways, induction factors and analysis. J Pharm Sci 98:2909–2934.
3. Philo JS. 2006. Is any measurement method optimal for all aggregate sizes and types? AAPS J 8:E564–E571.
4. Philo JS. 2009. A critical review of methods for size characterization of non-particulate protein aggregates. Curr Pharm Biotechnol 10:359–372.
5. Das T, Nema S. 2008. Protein particulate issues in biologics development. Am Pharm Rev 11(4):52–57.
6. Narhi LO, Jiang Y, Cao S, Benedek K, Shnek D. 2009. A critical review of analytical methods for subvisible and visible particles. Curr Pharm Biotechnol 10:378–381.
7. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJA, Middaugh CR, Winter G, Fan YX, Kirshner S, Verthelyi D, Kozlowski S, Clouse KA, Swann PG, Rosenberg A, Cherney B. 2009. Overlooking subvisible particles in therapeutic protein products: Gaps that may compromise product quality. J Pharm Sci 98:1202–1205.
8. Knapp JZ. 1999. The scientific basis for visible particle inspection. PDA J Pharm Sci Tech 53:291–302.
9. Rosenberg AS, Worobec A. 2004. A risk-based approach to immunogenicity concerns of therapeutic protein products. Part II: Considering host-specific and product-specific factors impacting immunogenicity. BioPharm Int 17(12):34–42.
10. Schellekens H. 2005. Factors influencing the immunogenicity of therapeutic proteins. Nephrol Dial Transplant 20:vi3–vi9.
11. Braun A, Kwee L, Labow MA, Alsenz J. 1997. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of Interferon alpha (IFN-a) in normal and transgenic mice. Pharm Res 14:1472–1478.
12. Hermeling S, Jiskoot W, Crommelin DJA, Bornaes C, Schellekens H. 2005. Development of a transgenic mouse model immune tolerant for human Interferon beta. Pharm Res 22: 847–851.
13. Hermeling S, Schellekens H, Maas C, Gebbink MFBG, Crommelin DJA, Jiskoot W. 2006. Antibody response to aggregated human Interferon alpha2b in wild-type and transgenic immune tolerant mice depends on type and level of aggregation. J Pharm Sci 95:1084–1096.
14. Purohit VS, Middaugh CR, Balasubramanian SV. 2006. Influence of aggregation on immunogenicity of recombinant human Factor VIII in hemophilia A mice. J Pharm Sci 95:358–371.
15. Schellekens H. 2008. How to predict and prevent the immunogenicity of therapeutic proteins. Biotechnol Annu Rev 14: 191–202.
16. Hwang WYK, Foote J. 2005. Immunogenicity of engineered antibodies. Methods 36:3–10.
17. Kiese S, Pappenberger A, Friess W, Mahler H-C. 2008. Shaken, not stirred—Mechanical stress testing of an IgG1 antibody. J Pharm Sci 97:4347–4366.

18. Fradkin AH, Carpenter JF, Randolph TW. 2009. Immunogenicity of aggregates of recombinant human growth hormone in mouse models. J Pharm Sci 98:3247–3264.
19. Bugelski PJ, Treacy G. 2004. Predictive power of preclinical studies in animals for the immunogenicity of recombinant therapeutic proteins in humans. Curr Opin Mol Ther 6:10–16.
20. Jahn E-M, Schneider C. 2009. How to systematically evaluate immunogenicity of therapeutic proteins—Regulatory considerations. New Biotechnol 25:280–286. 10.1016/j.nbt.2009.03.012.
21. Rosenberg AS, Worobec A. 2005. A risk-based approach to immunogenicity concerns of therapeutic protein products. Part III: Effects of manufacturing changes on immunogenicity and the utility of animal immunogenicity studies. BioPharm Int 18(1):32–36.
22. White PJ, Anastasopoulos F, Church JE, Kuo C-Y, Boyd BJ, Hickey PLC, Sze Tu L, Burns P, Lew AM, Heath WR, Davey GM, Pouton CW. 2008. Generic construction of single component particles that elicit humoral and cellular responses without the need for adjuvants. Vaccine 26:6824–6831.
23. Nath N, McNeal E, Obenhuber D, Pillari B, Shelton L, Stevens-Riley M, Sweeney N. 2004. Particulate contaminants of intraveneous medication and the limits set by USP General Chapter <788>. Pharmacopeial Forum 30:2272–2280.
24. Cao S, Pollastrini J, Jiang Y. 2009. Separation and characterization of protein aggregates and particles by field flow fractionation. Curr Pharm Biotechnol 10:382–390.
25. Chrai S, Clayton R, Mestrandrea L, Myers T, Raskin R, Sokol M, Willis C. 1987. Limitations in the use of HIAC for product particle counting. PDA J Parenteral Sci Technol 41:209–214.
26. Etzler FM, Sanderson MS. 1997. Particle size analysis. A comparative study of various methods I. Part Part Syst Charact 12:217–224.
27. Etzler FM, Sanderson MS. 1997. Particle size analysis. A comparative study of various methods II. Part Part Syst Charact 12:278–282.
28. Huang CT, Sharma D, Oma P, Krishnamurthy R. 2008. Quantitation of protein particles in parenteral solutions using Micro-Flow Imaging. J Pharm Sci 98:3058–3071.
29. Sharma D, King D, Moore P, Oma P, Thomas D. 2007. Flow microscopy for particulate analysis in parenteral and pharmaceutical fluids. Eur J Parenteral Pharm Sci 12:97–101.
30. Mahler H-C, Fischer S, Randolph TW, Carpenter JF. 2010 (in press). Protein aggregation and particle formation: Effects of formulation, interfaces and drug product manufacturing operations. In: Wang W, Roberts C, editors. Formulation and process development strategies for manufacturing of a biopharmaceutical. New York: John Wiley.
31. Brown L. 2009. Imaging particle analysis: Resolution and sampling considerations. http://www.fluidimaging.com/imaging-particle-analysis-white-papers.aspx (accessed June 2009).