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Mini-Review

Theme: Sterile Products: Advances and Challenges in Formulation, Manufacturing, Devices and Regulatory Aspects
Guest Editors: Lavinia Lewis, Jim Agalloco, Bill Lambert, Russell Madsen, and Mark Staples

Protein Particulate Detection Issues in Biotherapeutics Development—Current Status

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Abstract. Formation of aggregates and particulates in biopharmaceutical formulation continues to be one of the major quality concerns in biotherapeutics development. The presence of large quantities of aggregates is believed to be one of the causes of unwanted immunogenic responses. Protein particulates can form in a wide range of sizes and shapes. Therefore, a comprehensive characterization of particulates in biologics formulation continues to be challenging. The quantity of small size aggregates (*e.g.*, dimer) in a stable biologics formulation is well controlled using precision analytical techniques (*e.g.*, high-performance liquid chromatography). Particulate in clinical and commercial formulations is monitored using visual inspection and subvisible particulate counting assays. While visual inspection (by human eye or automated systems) is intended to detect particulates (intrinsic and extrinsic) of ~100 μm or larger, the subvisible counting methods cover smaller size ranges down to 10 μm. It is well recognized that research of particulates in the submicron (<1 μm) and low-micron (1–10 μm) ranges may provide important clues to understand the mechanism of particulate formation. The recent years have seen a significant increase in the development of newer technologies for more comprehensive characterization of particulates. This is attributed to increased awareness in this field of research over the past 5 years, stimulated by scholarly articles, commentaries, and robust discussions in various forums. This article provides an overview of emerging detection technologies that provide complementary characterization data encompassing a wider size range of particulates. It also discusses their advantages and limitations in the context of applications in biotherapeutics development.

KEY WORDS: biotherapeutics; formulation development; laser diffraction; particulate matter; protein aggregation.

INTRODUCTION

Biotherapeutic drug candidates continue to expand into new modalities and targets, resulting in a vast increase of biological molecules in clinical development (1–3). Indeed, the proportion of biotherapeutic medicines approved in the past decade for major unmet medical needs has increased significantly (4). With the increasing number of organizations and academic institutions involved in biotherapeutics research, understanding of biochemical and biophysical profile of many classes of biological molecules such as antibodies, fusion proteins, and drug conjugates has improved significantly over the past decade.

A biological molecule can undergo numerous degradations rendering its development as a stable, safe, and efficacious formulation highly challenging (5,6). Among all degradation routes, formation of aggregates and particulates in biopharmaceutical formulation continues to be one of the major quality concerns in biotherapeutic drug development (7–11). Aggregate/particulate formation poses challenges for quality, production yield, handling, and storage of biologics.[1] Three primary factors contribute to the concerns—(a) lack of adequate understanding of the mechanism of aggregate and particulate formation and consequently lack of predictability for formation of particulates; (b) unknown correlation of the quantity of particulates to the onset of adverse events; and (c) unlike other degradation routes, it is challenging to assay particulates by a single analytical technique because they can form in a very wide physical size range.

Aggregation involves biochemical and/or biophysical processes in which one or more drug molecules combine to form non-native oligomers that may remain soluble or become insoluble depending on size and other physical properties. The terms "soluble" and "insoluble" are qualitative descriptions that refer to visibility to the human eye. Non-native oligomers are formed by monomers that are structurally deformed, significantly unfolded, or fully denatured. Oligomer formation

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[1] The terms "biologics", "biopharmaceutical", and "biotherapeutics" are used interchangeably in this article; whereas "protein" or "protein pharmaceutical" refer to a sub-class of biologics that are protein-based. Also, "particle", "particulate matter" (PM), and "particulate" are used interchangeably.

can occur via covalent or noncovalent associations. Aggregation is the fundamental process that leads to the formation of small-size aggregates and large-size particulates. One can find a variety of nomenclature used by researchers in the literature to describe various sizes and types of aggregates. A recent article sheds light into the discrepancies of various qualitative nomenclatures (12).

Particulate (or, particulate matter (PM) or particle) historically refers to large-size species that are in tens of microns to sub-millimeter and millimeter size range and typically classified by visibility to human eye: visible (>~100 μm) or subvisible (>10 μm). However, exploration into the submicron and low-micron (1–10 μm) size areas may provide important data relevant to the mechanism of particulate formation, as noted by many researchers in the field (7–11). Particulates in biologics formulation can also originate from external sources such as contact with glass and rubber as well as environmental particles (13). Occurrence of extrinsic particulates is greatly minimized when a drug product is manufactured in tightly controlled environments for clinical and commercial uses (7).

In addition to non-native oligomers, aggregates may also form by oligomerization of native-like monomers where the monomers retain their secondary and tertiary structures (within the limits of detection) (14). It is important to understand if the oligomers formed by native-like monomers pose the same safety risk as their non-native counterpart. However, the size-based detection techniques can rarely explore the protein structural differences (see Table I). This article focuses primarily on size-based detection techniques, but it should be noted that a comprehensive *in vitro* characterization of aggregates and particulates should include biochemical/analytical (*e.g.*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), RP/IEX HPLC, *etc*., as applicable), protein secondary/tertiary structural (*e.g.*, circular dichroism, fluorescence, Fourier transform infrared spectroscopy (FTIR), Raman, *etc*., as applicable), and bioactivity assays.

Significant advances in protein chemistry, protein folding, and biologics formulation were made in the past two decades (15–21) to study the mechanism of many chemical and physical degradation pathways of protein-based biologics. In spite of these advances, the understanding of pathways leading to aggregate/particulate formation and prevention remains relatively poor. This article highlights the major issues with detection and quantitation of particulates in the subvisible and submicron size ranges (7–11). Significant technological advances in this field are also discussed for improved detection and characterization that can help in finding prevention strategies.

'CONTINUUM' OF SIZE AND QUANTITY

A majority of protein monomers have hydrodynamic diameters in the low-nanometers (~5–20 nm diameter). For example, bovine serum albumin is 7 nm in hydrodynamic diameter, and a human monoclonal antibody (IgG) is approximately 12–14 nm. A precision analytical assay such as size exclusion HPLC (SEC) can often resolve protein monomer from dimer and other small size aggregates and provides a dynamic range of few nanometers to ~20–30 nm depending on the type of column used. No other technique is able to achieve a similar degree of species resolution with matching robustness and reproducibility. Sensitivity of UV detectors play a critical role in quantifying the aggregates (22) because most often only a small amount (few percent) of aggregates can be measured in the presence of a majority of monomers. It is possible to measure as low as 0.1–0.5% aggregates (but a more reliable quantitation limit is 0.5%) in SEC using UV detectors. Additionally, it is also critical that each of the well-resolved populations (*i.e.*, sharp peak) is above certain threshold amount (approximately 20–100 ng) to be detected. Other detectors such as light scattering and fluorescence can increase the sensitivity of detection, but a reliable quantitation cannot be achieved. Considering the current state and gaps in both size range and level (quantity) of detection, one can ask if it is possible to develop an analytical SEC system that can resolve species in the size continuum of 1 nm to 100 μm as well as detect monomer and aggregates in picogram to microgram quantities.

Unfortunately, no such technique is available today that can cover a wide range of either size or quantity. Consequently, the current practices boil down to (a) risk assessment of a biologic candidate based on trends observed in the course of clinical development, (b) selection of appropriate orthogonal analytical and biophysical methods based on the risk assessment above, and (c) collection of data during the course of development to build a comprehensive molecular profile in the context of particulate formation under various bioprocessing, formulation, and storage conditions. For most biologic candidates, selection of orthogonal methods for characterization can be made from the ones described in Table I. Table I also includes the required tests such as visual inspection and subvisible particulate counting by light obscuration. Techniques such as light obscuration, light microscope, dynamic imaging, and related ones can detect particulates down to approximately 2 μm and thus, provide some characterization support to partly alleviate the so-called "subvisible gap." "Submicron gap" (for the size range of ~50 nm to ~1,000 nm, as noted in earlier sections), on the other hand, is much more challenging to address (see Fig. 1.) because very few solution/suspension techniques are available (laser diffraction, dynamic light scattering, and electron microscope) for this size range, and only limited characterization can be performed by these techniques.

ASSAYS FOR PARTICULATE COUNTING

Measurement of particulate size in pharmaceutical development has been discussed in several reviews recently (7–11). A number of recent studies (23–39) have addressed measurement and characterization of subvisible particulates in a variety of biological solutions. This section highlights the counting techniques that are most widely used (and required tests for certain stages in development) by biopharmaceutical organizations to monitor and characterize particulates and aggregates in biologics development. Advantages and limitations of these techniques are also discussed.

Visual Inspection Test and Characterization of Visible Particulates

Visual inspection for appearance is an important assay for biologics dosage forms, and it includes inspection for the presence of particulates (40–42) by the human eye or

Table I. Analytical and Biophysical Techniques to Detect and Characterize Particulates in Biopharmaceutical Development

Technique	Purpose/strength/use	Size range	Limitations
Assays for particulate counting			
Visual inspection (manual or automated)	Detection of visible particulates (protein and non-protein) Probabilistic detection Non-destructive, relatively simple Pharmacopeial method High throughput and improved reproducibility in automated systems Applications include release, stability, comparability, development	>~100–200 μm	Subjective (probabilistic detection); possible error due to fatigue (human); difficulty detecting transparent particulates
Light obscuration	Sub-visible particle count Industry standard Pharmacopeial method QC compatible Applications include release, stability, comparability, development	≥10 μm, ≥25 μm (and sizes between 2 and 10 μm)	Relatively large volume need (difficult for small volume dosage form, and in early development); difficulty with transparent particulates; no shape information; destructive test; issues for samples with high turbidity/viscosity/particle density
Membrane microscopy	Sub-visible particle count Industry standard Pharmacopeial method QC compatible Shape and morphology (actual microscopic image) Applications include release, stability, comparability, development	≥10 μm, ≥25 μm (and sizes between ~1 and 10 μm)	Difficulty with transparent particulates; destructive test; low throughput; potential alteration of fragile aggregate size/shape, and dehydration on membrane; issues for samples with high turbidity/viscosity/particle density
SE-HPLC	Not a particulate counting assay but critical test for aggregation monitoring in particulate study Detection/quantification of covalent and non-covalent aggregates, low molecular weight fragment High precision, robustness, throughput QC compatible Easy to use and industry standard Applications include release, stability, comparability, development	~1 to ~30 nm (or larger based on column choice)	Potential loss of aggregates in column/frit; potential matrix interactions, dilution of formulation; limited dynamic range (for size)
Particulate characterization assays			
Dynamic imaging analysis	Size distribution, count and shape of subvisible particulates based on actual image Richness of particulate morphology data—helpful in formulation development Superior detection of protein translucent particulates Applications include development and characterization	~1 to ~1,000 μm (cumulative size range from multiple measurements)	Relatively large volume need (difficult for small volume dosage form, and in early development); destructive test; low throughput (measurement and data analysis); technology still evolving; difficulty detecting transparent particulates (optical microscope limitation); issues for samples with high turbidity/viscosity/particle density

Table I. (continued)

Technique	Purpose/strength/use	Size range	Limitations
Laser diffraction particle analyzers	Size distribution for submicron, subvisible and visible particles Widest dynamic range of particle size Applications include development and characterization	~10 nm to 2,000 μm	Dilution needed—potential for alteration in aggregate size; relatively large quantity of sample needed; interference from dust
DLS	Submicron particulates along with small aggregates and monomers In original formulation (no dilution or change in formulation needed) Very wide dynamic range of size Back scattering allows high concentration measurements High sensitivity to detect larger size Low sample volume (~10–100 uL) Easy and quick measurement Applications include development and characterization	~0.3 nm to ~10 μm (depends on equipment model)	Semi quantitative (relative quantity); poor resolution of species close in size; dust can skew results; high sensitivity towards larger species can mask scattered intensity from small species (*e.g.*, monomer)
FTIR imaging	Spectroscopic study of particulate and precipitate possible Protein structural information (secondary structure) Spectra collection in membrane microscopy or via ATR (isolated particulates) Fingerprinting of foreign particulates Wide compatibility with formulations Applications include development, characterization, and comparability (as needed)	>~5 μm	Low resolution of secondary structural components; poor sensitivity to low level aggregates/degradants; interference from water band and water vapor spectrum; difficulty with overlapping particulates
Raman imaging	Spectroscopic study of particulate and precipitate possible Protein structural information (secondary structure) Spectra collection in specialized membrane microscopy or automated mode (rapID) Fingerprinting of foreign particulates Wide compatibility with formulations Applications include development and characterization	>~5 μm	Fluorescence background from denatured protein may obscure Raman signal; difficulty with overlapping particulates; low resolution of secondary structural components
Fluorescence microscopy	Sensitive detection of ultra-low level of aggregates (labeling by fluorescent dye needed) Characterization of certain types of aggregates (fibril) Applications include characterization	>~1 μm (lower size range possible with microscope capability)	Low throughput; not quantitative; labeling needed (sample manipulation required); interference from non-specific labeling
Coulter counter	New uses for counting subvisible and visible particulates Counting not dependent on color, shape, transparency, *etc.*	~0.4 μm to ~1,600 μm	Adequate conductivity of media required; may need to change formulation to saline for measurement; no shape information; applications in subvisible particulate counting not fully tested
Flow cytometry (FACS)	Established technique for cell biology applications Fluorescence labeling can distinguish proteinaceous and foreign particulates Sensitive detection of ultra-low level of aggregates (labeling by fluorescent dye needed) High throughput possible (plate-based)	~1 μm to ~100 μm	Labeling needed (sample manipulation required); interference from non-specific labeling; applications in subvisible particulate counting not fully tested

Table I. (continued)

Technique	Purpose/strength/use	Size range	Limitations
SMR	Particulate "count" based on mass detection Femto-gram level of mass detection	~0.1 to 5 μm (not fully tested)	New technology—applications in particulates research not tested; conversion of mass to count with assumptions of density, shape, *etc*.
NTA	Sizing of particulates (analogous to DLS) Superior size resolution of closely lying size groups (than DLS)	~30 to ~1,000 nm	Can not cover small size aggregate and protein monomers (dynamic range less than in DLS); new technology—applications in particulates research not fully tested
TEM, SEM, AFM	Morphology of particulates with nanometer resolution Applications are as-needed basis	~1 nm to submicron and higher (dynamic range depends on choice of resolution)	Low throughput; high cost of equipment; extensive sample manipulation needed that can breakdown fragile particulates
Nephelometry	Qualitative information on the presence of submicron/subvisible particulates High throughput (plate-based) Low sample need Can establish trends of (assumed) particulate formation in screening studies	Cannot be measured	Indirect and qualitative information on presence of particulates; interference from scattering/opalescent medium
SDS-PAGE	Solubilized particulates can be tested for covalent *vs.* non-covalent aggregates, and fragments Low cost, easy to use	~1 to <~15 nm	Semi-quantitative, low-precision, extensive manipulation of sample

QC quality control, *SE-HPLC* size-exclusion high-performance liquid chromatography, *DLS* dynamic light scattering, *FTIR* Fourier transform infrared, *ATR* attenuated total reflectance, *FACS* fluorescence-activated cell sorting, *SMR* suspended microchannel resonator, *NTA* nanoparticle tracking analysis, *TEM* transmission electron microscopy, *SEM* scanning electron microscopy, *AFM* atomic force microscopy, *SDS-PAGE* sodium dodecyl sulfate-polyacrylamide gel electrophoresis

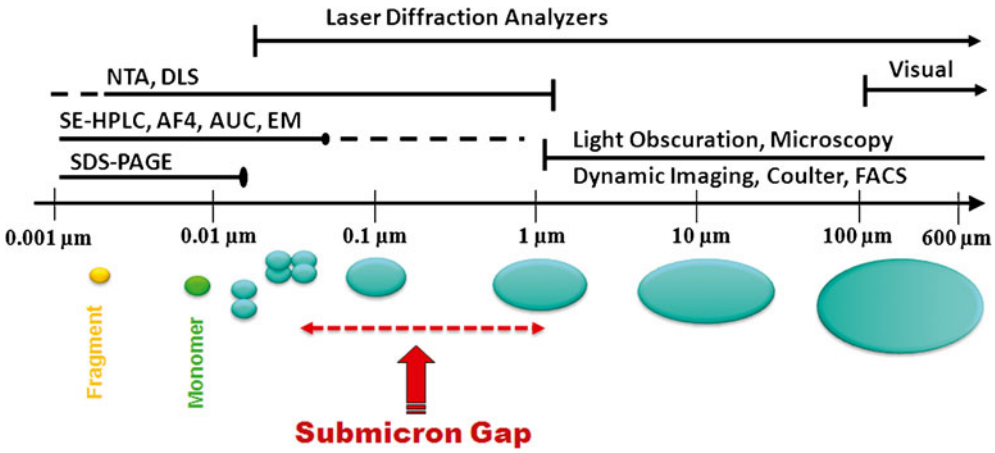

Fig. 1. Schematic representation of 'submicron gap' (not drawn to scale)

automated systems (*e.g.*, transmitted light (static division) Automated Inspection System, Eisai Machinery USA, Inc., NJ, USA; Inspection Machine, Seidenader Maschinenbau GmbH, Germany; automated inspection machines, Brevetti CEA S.p.A., Italy). Visual inspection is a required test in establishing the quality profile of a clinical or commercial dosage form. Hence, it is important to discuss the advantages and limitations of this assay. The human eye is incredibly powerful in detecting the presence of particulates in injectable solutions. However, the visual inspection assay can be subjective and may produce variability in data for some types/sizes of particulates. Visualization of particulates not only depends on an inspector's visual acuity but also on the nature of particulates (*e.g.*, transparency) and test conditions used. Although no strict size limit can be assigned when particulates become visible to human eye, it is generally recognized that particulates of >100 µm size are detected with relatively high probability when appropriate testing conditions (*e.g.*, light intensity and background) are used (41–43). The process of detection is probabilistic, with the probability of detection increasing with increasing particulate size. The human eye can detect particulates once the size reaches approximately 50 µm for a spherical particulate, albeit with very low probability of detection. This probability increases to ~40% for a 100 µm particulate and further improves to >95% for particulates greater than or equal to 200 µm (41–44).

Some biopharmaceutical manufacturers employ automated inspection system designed and calibrated to yield visual inspection data. Sometimes, the automated system can be more sensitive and reproducible than inspection by the human eye.

Visibility of biologics particulates may also be negatively impacted by their degree of transparency in aqueous solution, as noted above. Additionally, the assessment of shape/morphology of biologic particulates is highly challenging because of their extremely irregular shape, resulting in qualitative size distribution data of particulates.

Because visual inspection is a required test for injectable biotherapeutics and it is applicable to thousands of clinical candidates and commercial products worldwide, it is worthwhile to briefly discuss the guidelines and regulatory expectations. The United States Pharmacopeia (USP 35–NF 30) *Chapter <1> Injections (Foreign and Particulate Matter* section) (45) [also see exceptions for certain types of biological products (45)], states that "Each final container of all parenteral preparations shall be inspected to the extent possible for the presence of observable foreign and particulate matter ("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." Similarly, *EP Parenteral Preparations* (46,47), states "solutions for injection, examined under suitable conditions of visibility, are clear and practically free from particles." It is also recognized that the terms "essentially free" and "practically free" have been difficult to define because particle detectability is influenced by their number and size (see discussion above), among other factors (48).

It is obvious that some upstream development work and testing are necessary to increase the confidence in the stability of formulations and to minimize the risk of failure in clinical drug product manufacturing. It is beneficial to apply additional testing for characterization of visible particulates during formulation development, for example, to identify if the particulate is intrinsic (*e.g.*, protein aggregate) or extrinsic (foreign matter) or a mix. Visible particulates require specialized protocols for enumeration (*e.g.*, modifications of standard use protocols of light obscuration, light scattering, and light microscope) and characterization (imaging-based techniques for size/shape, spectroscopic methods such as FTIR or Raman, and elemental analysis such as scanning electron microscopy (SEM)-EDX for metal contamination) (7,10,49). Additional difficulties in characterizing visible particulates include (a) small quantity and (b) handling/isolation. It may be necessary to centrifuge the sample containing particulate to make a pellet followed by solubilization in dispersing/denaturing solvents and further examine by spectroscopic or biochemical (*e.g.*, SDS-PAGE) techniques. Finally, finding the root cause of visible particulate formation continues to be challenging, and prevention strategies may include significant trial and error procedures in the absence of any reliable predictive tools. Some of the root cause possibilities include (a) interaction with silicone oil (23,24,31,35,50–54), (b) denaturation due to frothing (stress at air–water interface) (27,55–60), (c) cryo-concentration (61), and (d) incompatibility with preservatives (62–69).

Light Obscuration Test and Sources of Subvisible Particulates

Light obscuration particle count is another test required for clinical and commercial injectable drug products. This test is based on the principle of light blockage and allows for an

automatic determination of particulate size and the number of particulates according to size. Equipment from multiple manufacturers are available (such as Hach Company, CO, USA; Particle Measuring Systems, CO, USA) to conduct this test following pharmacopeial methods. It is an important assay to establish limits of subvisible particulates and thereby ensuring quality and safety of injectable products such as parenteral biotherapeutic formulations. Commercial biotherapeutic products used worldwide over the decades have established the benefit of this test, and no adverse events (to the best of knowledge of this author) are linked to particulate counts within approved limits. However, emerging research data indicate some limitations of the technique discussed below. Additionally, new ideas to enhance the benefits of this test as well as updates in the compendial guidelines are also discussed in this section.

USP chapter <788> [(70), effective from Aug 2011] defines PM for injectable drug products as consisting "….extraneous mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions." The current definition includes both intrinsic and extrinsic sources of particulates. This general chapter is now harmonized with the corresponding texts of the *European Pharmacopoeia* (71) and/or the *Japanese Pharmacopoeia*. The acceptable count limits of ≥10 and ≥25 μm particulates are described in the references above (70,71). Another important point as a reminder to readers is that the current *USP <788>* guidance (70) does include intramuscular and subcutaneous injections in its scope. These products must meet requirements described in *USP <788>*. A guideline for particulates in ophthalmic products is also available in *USP <789>* (72). Ophthalmic products covered in this pharmacopeial guide are products intended for topical application to the eye but are also applied for subtenon, intravitreal, and conjunctival injections to the eye. It is challenging to apply *USP <789>* for small fill volume (*e.g.*, 50 μL) ophthalmic dosage forms. It is necessary to discuss with the appropriate regulatory agencies in advance to determine a suitable course of action.

Particulates can form intrinsically (*i.e.*, made of protein) in a protein formulation by a multitude of factors including purification, handling, shear, temperature, and other storage conditions. It should be noted, however, that any subvisible or visible particulates carried over or formed during manufacturing of protein drug product are removed using ~0.2 μm filters. Therefore, establishing the stability of a biologics formulation is key to prevent formation of new particulates in the finished drug product. When subvisible particulates are observed in a biologics formulation, it is important to distinguish between the extrinsic (*e.g.*, silicone oil) and intrinsic types in relation to development of a stable formulation. One of the goals in developing a stable and clinically usable formulation is to understand and control the intrinsic particulate. In early stages of biologics development, the occurrence of extrinsic particulates may be more common especially in laboratory environments. Most often, such extrinsic particulates are eliminated when the process is transferred to a GMP environment for clinical manufacturing (7,9). The presence of extrinsic particulates, such as pieces of glass/rubber, and silicone oil droplets may sometimes act as so-called "nucleating sites" in promoting aggregation of biologics. As occurrence of extrinsic particulates decreases in a formulation manufactured in a GMP environment, the probability of nucleation-induced aggregate formation is reduced.

Formation of intrinsic particulates in biologics formulation is a complex phenomenon and proceeds via kinetic control of association of monomers through either sequential steps or cooperative growth (5,73). It should be noted that protein formulations of therapeutic use are almost never aggregate-free and that the presence of a small amount of aggregate/particulates does not always lead to formation of additional amount. Rather, it depends on aggregation propensity of a protein in a particular formulation medium.

The light obscuration test is challenging to apply to certain types of formulations. Formulations with high turbidity or viscosity or particle density may not work in this test. Pretreatment (*e.g.*, dilution) of such samples may be needed to get accurate counting. Formulations that tend to generate bubbles are likely to produce erroneous (exaggerated) counting. Finally, certain types of protein particulates may appear semi-transparent in the light path resulting in inaccurate particulate counts. For such samples, additional assays including fluorescence microscopy and imaging techniques might be needed to establish any trend of increasing proteinaceous particulate formation.

Membrane Microscopy

Light obscuration is the preferred method for subvisible particulate counting described above. Microscopic particulate count tests are conducted when light obscuration tests either fail or cannot be applied (70,71). This test involves filtration of a solution using an appropriate membrane filter of ≤1 μm nominal pore size to retain the particulates, followed by counting of particulates using a suitable microscope with the recommended magnification. The details of the method as well as acceptable limits of ≥10 and ≥25 μm particulates by membrane microscopy are described in the pharmacopeial guides (70,71). The limits are different from those for light obscuration.

Disadvantages in membrane microscopy counting include (a) potential detectability issues with certain types of protein particulates that are semi-transparent and (b) particulates too fragile to retain their original size upon filtration and subsequent sample handling (*e.g.*, dehydrated on filter). Finally, membrane microscopy (as well as other types of light microscopy) provides a "static" image of particulate that may not be identical to its true shape in solution. "Dynamic imaging" techniques, discussed in a later section, can provide valuable data on particulate morphology in solution conditions.

Pharmacopeial Tests in Early Stages of Development

Although the pharmacopeial guides are adequate for ensuring the quality of final product concerning particulate matter, the formulation development and formulation characterization of biologics may need to employ additional techniques especially when a product candidate is suspected to form aggregates or particulates during storage stability. At the early stages of development, the propensity of particulate formation in a biologics formulation may not be known. Therefore, it is desirable to conduct suitable stress tests during formulation development and use appropriate detection and characterization assays for particulates that are amenable to a small quantity of sample (*e.g.*, nephelometry, dynamic light scattering

(DLS)). Light obscuration and other particle counting tests require relatively high sample volumes. Therefore, it may not be feasible to apply these assays in early stages of formulation development, especially for high concentration subcutaneous dosage forms (*e.g.*, 100 mg/mL active) that are likely to be packaged in small volumes. Adaptation of the light obscuration particulate counting test to work with smaller sample volume sample might be possible when such modified methods are appropriately qualified. Once a formulation is nominated for clinical manufacturing, it is worthwhile to start collecting particulate count data (and particle imaging data, as needed) to de-risk future clinical manufacturing and release. Furthermore, it may also be useful to collect data of additional size groups at under 10 μm (*e.g.*, 2, 5, and 8 μm) from the light obscuration test (9). Commercially available equipment can collect data under 10 μm in the same assay when larger-size buckets are measured, *i.e.*, no additional sample is needed for testing under 10 μm.

Size Exclusion Chromatography

SEC is generally out of scope to analyze protein particulates as discussed in the earlier sections. However, it is a critical assay to monitor small-size aggregates in protein formulations and a required test for release and stability of clinical dosage forms (22). SEC data of release and stability studies also provide a snapshot of the "initial state" and potential correlation (or lack of) to kinetic events leading to particulate formation.

PARTICULATE CHARACTERIZATION ASSAYS

In addition to the tests described above, this section highlights selected techniques for detection and characterization of particulates covering the subvisible and submicron (under 1 μm) ranges.

It is impractical to try to apply all of these techniques in formulation characterization. The user can select some of these techniques based on specific needs and any particulate formation trends seen in the course of development. Advantages and limitations of these techniques are also discussed to provide guidance on which techniques would be more appropriate to use for a given need.

Dynamic Imaging Analysis

Most analytical measurements for particulate sizing (small or large) use either spherical approximation for shape, or indirect methods (*e.g.*, mathematical algorithm) to extract shape information. Microscopy methods, however, provide direct visualization of morphology in addition to size information. Microscopy is an established technique for studying protein particulates (74,75) for shape as well as enumeration (pharmacopeia method) as described in an earlier section. Microscopy methods use static imaging, *i.e.*, the particulates under study are held still (*e.g.*, on a membrane filter) when recording images. More recently, advanced capabilities have been introduced by manufacturers to record particulate imaging in "dynamic" mode (38,39,76). The dynamic imaging equipments allow measurement of size and shape of particulates in solution or suspension. Dynamic imaging refers to the group of imaging techniques that record digital images of particulates suspended in fluid (*i.e.*, stirred solution/suspension in a cell, or in a predetermined volume flowing through a capillary/channel) (38,76). Flow-based image recording is especially beneficial to count particulates in a certain volume and to generate size distribution as well. The optical microscopes used in the dynamic imaging typically have a lower size limit of approximately 1.0 μm. Therefore, dynamic imaging techniques allow complementary size analysis of subvisible as well as visible particulates (up to approximately 0.3 mm or larger depending on equipment model). Although the total size range of the imaging systems is ~1 to >1,000 μm, the user should realize that a single measurement does not cover the whole range. When the size limit is pushed to cover the low end, the high end is sacrificed, and vice versa.

Examples of flow-based systems are FlowCAM imaging particle analysis (Fluid Imaging Technologies, ME, USA), Micro-Flow Imaging particle analysis (ProteinSimple, ON, Canada), and flow particle image analyzer (Malvern Instruments Limited, UK). A cuvette-based measurement system is also available (EyeTech particle size and shape analyzer, Ankersmid Lab, The Netherlands) for size distribution analysis in solution/suspension. Although various flow-based systems produce similar information on size/shape of particulates, they use different methods for sample flow and analysis. Additional differences include (a) quality of digital image, (b) ability to handle range of particulate concentrations (and need for dilution), and (c) percentage of particulates actually analyzed. The user is advised to review the specification details of each of the models.

Digital images of particulates are recorded and analyzed to extract parameters such as Feret diameter, aspect ratio, circularity, and intensity. Building a "particulate profile" of a protein candidate under normal and stress conditions in various formulations can be helpful in mitigating future particulate events. Dynamic imaging offers the benefit of measuring images in real time and under conditions where particulates remain suspended—an advantage over static imaging. This may allow superior imaging of highly irregular shaped particulates and monitoring the dynamic behavior of particulates if the size distribution is changing over time. Such information is valuable during formulation development of biologics to characterize particulates and finding potential preventative measures. Disadvantages of dynamic imaging systems include (a) the inherent complexity in determining a true size distribution of biological particulates from imaging data because of extreme irregularity in shape and size and (b) the size distribution and particulate count from dynamic imaging cannot be directly compared with such information obtained from light obscuration or laser-diffraction analyses.

Laser Diffraction Particle Analyzers

When a laser beam is incident on particulates of various sizes, light is scattered, and the direction and intensity of scattered light are related to particulate size. The diffraction of light can be described mathematically by Fraunhofer or Mie theory. The traditional laser light diffraction analyzers typically use an array of detectors to cover a size range of approximately 0.5–2,000 μm. The detectors when placed at low angle can size large particulates with good accuracy. Performance of

these detectors is inadequate for smaller size particulates. Advancements such as combinations of different wavelengths, polarization ratio, and white light scattering expand the size range of the analyzers to <0.1 μm to ~1 cm. Polarization intensity differential scattering (PIDS™) (laser diffraction particle size analyzer, Beckman Coulter Inc, FL, USA) is one of the techniques that can push detectability down to approximately 17 nm (17 nm to 2,000 μm). With PIDS technology, a particulate is sequentially illuminated with vertically and horizontally polarized light source, and the differential of scattered light is measured over a range of angles and with multiple wavelengths of incident light to enhance the richness of information. The most significant benefit is achieving a wide coverage (~20 nm to 2,000 μm) of particulate size from a single measurement and therefore alleviating some of the concerns of "submicron gap." The size distribution (and relative amount) of particulates in this wide range can provide vital clues to understand the mechanism and kinetics of particulate formation. Other laser diffraction analyzers (100 nm to 8,750 μm in particle size analysis with laser diffraction, Sympatec, Germany; 10 nm to 3,500 μm in Mastersizer particle size analyzer, Malvern, UK) are also able to provide a significant coverage of the submicron size range. The particle size analyzers typically are built to the specifications of ISO 13320 "Particle size analysis–laser diffraction methods."

One of the most significant disadvantages of laser diffraction particle analyzers is the requirement of larger sample volumes. However, the sample requirement is highly dependent on particulate concentration in the sample, *i.e.*, the sample need decreases (*e.g.*, to 1–2 mL) with increasing particulate concentration. Sample analysis using laser diffraction analyzers often requires large dilution of samples. Therefore, if aggregates and particulates are unstable (*i.e.*, size changes upon dilution), special attention should be paid to understand the dilution effects. Finally, size is determined by calculating the equivalent spherical diameter in most light scattering measurements, irrespective of actual shape of the particulates.

Dynamic Light Scattering (DLS)

DLS measures fluctuations (microsecond and longer time scale) of scattered light caused by Brownian motion of molecules in solution and therefore relates to the diffusion coefficient. Hydrodynamic radius (Rh, not radius of gyration) can be extracted from diffusion coefficient values by Stokes–Einstein equation with spherical approximation (77). DLS is sometimes referred to as photon correlation spectroscopy or quasi-elastic light scattering. DLS provides a relatively easy and fast measurement of size (Rh) and covers a large dynamic range (<1 nm to ~1–10 μm) in one single measurement. The measurement can be done with liquid/suspension of a formulated drug substance or drug product without any alteration/dilution (*e.g.*, a 50 mg/mL monomeric antibody formulation can be studied without dilution) using a very low sample volume (~10–100 uL). DLS is the only technique that is able to carry out solution measurements in a wide size range covering ~0.3 to >1,000 nm, thereby partly mitigating the submicron analysis gap. DLS is most frequently used in batch mode with unfractionated samples placed either in sample cells or in well plates. Batch mode DLS is able to detect large aggregates/particulates (up to a few microns depending on detection angle) along with the protein monomers. Therefore, DLS is a very useful "one-stop shop" tool in formulation development and bioprocessing covering protein monomer (~5–20 nm), aggregates, as well as submicron particulates, and it can be applied in early and late stages of development due to low sample volume need.

Disadvantages of DLS include limitation in size resolution, lack of shape information, artifacts due to dust particles, and masking of scattered intensity of smaller particulates in the presence of significant quantity of larger particulates. It can resolve two size groups only if they differ by more than 2–5× in hydrodynamic diameter (78,79). For example, a monomer and its dimer cannot be separated by DLS; instead, an average value of size is measured.

Raman/FTIR Imaging

The microscopic imaging systems (described in earlier sections) provide additional benefits when they are coupled with spectroscopic characterization tools such as FTIR or Raman. A traditional characterization method involves isolation of particulate in membrane microscopy followed by recording of individual FTIR or Raman spectrum of the isolated particulates. Raman and FTIR (39,80) are powerful methods to distinguish extrinsic (foreign) and proteinaceous particulates by spectral fingerprinting because both techniques reveal characteristic protein spectra (amide bands). Some of the excipients used in biologics formulations (in relatively large quantity, *e.g.*, mannitol or sucrose) can also be identified, as they possess unique vibrational bands. Additionally, material of composition of a foreign particulate is likely to have a unique FTIR or Raman spectrum. Therefore, it is possible to identify the source of foreign particulate contamination. This feature is extremely valuable in finding root causes of particulates seen in development and manufacturing.

An additional advantage of Raman (over FTIR) is noninterference of water in measurements with aqueous formulations. Newer Raman spectrometers provide adequate sensitivity to collect good-quality spectrum in a short period allowing higher throughput. One application of such throughput method is rapid characterization of particulates (>~2 μm) by optical imaging and Raman identification (Particle Explorer, rap.ID Particle Systems GmbH, Germany). The formulation is filtered on a gold-plated filter to collect the particulates (foreign matter as well as intrinsic particulates) followed by Raman identification of a statistically relevant number of particulates (or all) to understand the nature and distribution of particulates. Most foreign matter (types of plastic, rubber, fiber, polymers, glass, organics, *etc.*) have unique Raman spectral fingerprint, while the protein-based particulates display characteristic backbone amide bands (80). Fluorescence is often a problem (high background) with Raman measurements of biologics formulations, especially if the particulates are formed by denatured protein emitting high intensity of intrinsic fluorescence. High fluorescence background can be significantly reduced by selecting high-wavelength lasers far away from the near-UV range.

Fluorescence Microscopy

Fluorescence microscopy can be used where micron-size (or larger) aggregates can be stained with appropriate

fluorescence dyes (81,82) such as thioflavine T or SYPRO orange. Due to high sensitivity of dye fluorescence, a small number of aggregates can be detected along with their size and shape. Confocal microscopy can provide additional enhancement in image quality and resolution (83). Various binding properties of commercially available dyes offer some degree of specificity (hydrophobic binding, charge-based binding, affinity to amyloid structures) that can be helpful in the characterization of particulates.

Coulter Counter

A Coulter counter (for example, Multisizer, Beckman Coulter Inc, FL, USA) uses an electrical sensing zone method to detect particulates based on the principle that particulates placed in an electric field will modify the current flow (37). The method for size and count determination by a Coulter counter is described in ISO 13319 "Determination of particle size distributions—electrical sensing zone methods." It is able to cover a wide size range (approx. 0.4–1,600 μm) cumulatively by conducting several measurements. A significant advantage of the Coulter counter is that the response is unaffected by color, shape, composition, or refractive index of a particle. Additionally, this method provides high resolution between the size groups. Some studies using Coulter counter demonstrate the utility of this technique in studying particulates in protein formulations and biological systems (11,25,84,85). However, the formulations containing particulates need to be suspended in an electrolyte (saline or high-conductivity formulation buffer) that may cause artifacts by changing the composition and count of particulates.

Flow Cytometry (Fluorescence-Activated Cell Sorting)

Flow cytometry is a well-known technique traditionally applied in cell biology applications such as cell sorting and platelet aggregation (86). Newer fluorescence-activated cell sorting (FACS) systems have several fluorescence detectors to collect wavelength-selective (using emission filters) fluorescence emission data and light scattering detectors to measure the approximate size (87). Recent reports (33,34) suggest utility of FACS in studying protein particulates in the ~1–100 μm size range. Obviously, the target study material needs to be labeled using a suitable fluorescence dye such as Alexa Fluor 647 or SYPRO Orange (33,34). The requirement of fluorescence labeling of samples for FACS analysis puts it at a slight disadvantage compared with optical-imaging- and light-scattering-based methods that require little or no manipulation of sample. In addition to protein particulates, it has been shown that FACS is able to analyze silicone oil droplets (stained with suitable dye) to study protein adsorption onto silicone oil droplets (33). Size determination using FACS has similar limitations (approximation of size, lack of shape information) as in any light-scattering-based size analyzer. FACS systems are available from several manufacturers including Beckman Coulter Inc (FL, USA), BD Biosciences (USA), and Amnis Corporation (WA, USA).

Suspended Microchannel Resonator

The resonance frequency of a suspended microfluidic channel with micrometer-thin walls and a thin fluid layer is highly sensitive to the presence of microparticles whose mass density differs from that of the solution (88). Suspended microchannel resonator (SMR) is a relatively new technology applied successfully to various biological applications to detect extremely small differences (femto-gram) in buoyant mass of cells and micro-particulates (88–91). Buoyant mass is the mass of a particle over that of the fluid it displaces. Concentration and size (approximately 0.1 to 5 μm) of such particulates can be calculated by applying density values. A new commercially available benchtop machine (Archimedes Particle Metrology System, Affinity Biosensors, CA, USA) uses SMR technology, intended to measure the mass of individual aggregates and foreign particulates in protein formulations. The volume of sample needed is few hundred microliters depending on particulate concentration in a formulation. When protein particulates flow in their native formulation through the microfluidic channel, shifts in the sensor resonant frequency are measured to determine particulate mass in femto-gram resolution. The corresponding size distributions are derived by translating the mass of each particulate to equivalent-sphere diameter. SMR is an emerging technology that merits further evaluation for protein particulate research.

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) (Nanosight, UK) is a relatively new technology for sizing particulates in the range of approximately 30–1,000 nm (92). The lower detection limit depends on the refractive index of the particulates. For protein particulates of low refractive index, the lower limit is approximately 40–50 nm. NTA combines laser light scattering microscopy with a CCD camera to enable visualization and recording of particulates in solution. Movement of individual particulates under Brownian motion relates to particle size derived from the Stokes–Einstein equation. A comparative analysis (92) with DLS shows advantage of superior size resolution with NTA, but it requires larger sample volume (300 uL) than DLS (~10–100 uL). Additionally, NTA is not able to size the monomeric form and small aggregates of proteins that are typically less than 30 nm in size.

TEM, SEM, and AFM

Advanced microscopic methods such as transmission electron microscopy (TEM), SEM, and atomic force microscopy (AFM) can offer fine-quality imaging of particulates with nanometer resolution. Both TEM and SEM require extensive sample processing (81,93). AFM can capture the surface topology (image) of particulates with nanometer resolution (94,95). These microscopic methods are generally suited for only specialized applications due to high equipment cost, low throughput, and concern of altered sample conditions.

MECHANISM OF PARTICULATE FORMATION

Formation of aggregates and particulates can be caused by several stress factors (5,63,96–100) including temperature, oxidative agents, agitation, shear, freeze–thaw (cryo-concentration), organic solvents, preservatives, high protein concentration, and extremes of pH and ionic strength. Aggregates and particulates discussed in the preceding sections are

primarily classified in terms of size (dimension, molecular weight). Understanding of the structural basis of aggregates and particulates is also important when it comes to elucidation of mechanism. Structures can be either covalent or non-covalent. Covalent structure is generally irreversible. An example of a covalent structure is disulphide-scrambled species, which is often dissociable by a reducing agent. Non-covalent structure is typically held together by either strong association (*i.e.*, not dissociated by simple dilution or mild treatments) or weak association (*i.e.*, may be reverted to monomer by dilution).

The chemical and physical events implicated in initiating protein aggregation include protein denaturation, misfolding, partial unfolding (*e.g.*, molten globule), self-association, surface adsorption, and covalent bond formation. Exposure of hydrophobic regions of proteins is believed to initiate aggregation. Additionally, the presence of trace quantity of non-native protein structure may also provide nucleation. However, detection of such non-native species in an ensemble of native state populations is very challenging by using conventional analytical techniques (19). Detection of nucleating species becomes further challenging because the initial state in a protein formulation is already highly heterogeneous. Although it is tempting to assume that pathways leading to small size aggregate and particulate share some common intermediate states, it is very challenging to delineate the sequential events of particulate formation.

Figure 2 shows a schematic of pathways that might be involved in particulate formation. Kinetics and stability of the intermediate states dictate what are actually detected in the analytical assays. The starting point in this scheme is a folded monomeric state. Heterogeneity of species present in the initial state is an important factor because they may have potential to alter the kinetics of subsequent events. Although the number of different species (*e.g.*, glycan variants, charge variants, deamidated at different locations, various oxidized species, *etc.*) present in the initial state can be overwhelming, the model assumes folded monomeric state for these species. This is a valid assumption because most often these species are not directly linked to aggregate formation. On the other hand, a small population of any aggregated or truncated species is likely to have altered structure. Some degree of unfolding or altered structure in the monomeric state can initiate reversible association of partially folded monomers (or association between folded and partially unfolded monomers). Subsequent steps lead to an increase in size of the aggregates (5,63,101). The various steps in the pathway (Fig. 2) may include (a) structural or conformational rearrangement of oligomers forming key stabilizing inter-protein contacts that make the resulting aggregate nucleus net irreversible; (b) growth of nuclei or other pre-existing aggregates via monomer addition; (c) growth of aggregates via aggregate–aggregate coalescence to form larger soluble (molecularly dispersed) aggregates; and (d) growth via phase-separation to form insoluble particulates (5).

It is important to understand the limitations of analytical or biophysical assays when such data, especially from limited utility assays, are used to delineate aggregation pathways (102,103). The assays should be designed to address detection of various intermediates that might be present. In addition to the most commonly used assays such as SEC, orthogonal methods are increasingly being used to explore protein aggregation and particulate formation (79,104–107). When it comes to visually detectable particulates, an additional challenge is to understand the mass balance. The presence of a very small quantity of protein (*e.g.*, microgram quantity) can be visually detectable, and formation of such visible particulates may or may not affect (within limits of detection) the population of other intermediates starting from the initial monomer.

Surface adsorbed species either in contact or in equilibrium with bulk population can also contribute to the particulate formation pathway. Emerging techniques such as quartz crystal microbalance with dissipation might be able to detect structural changes in the adsorbed protein layer (108–111).

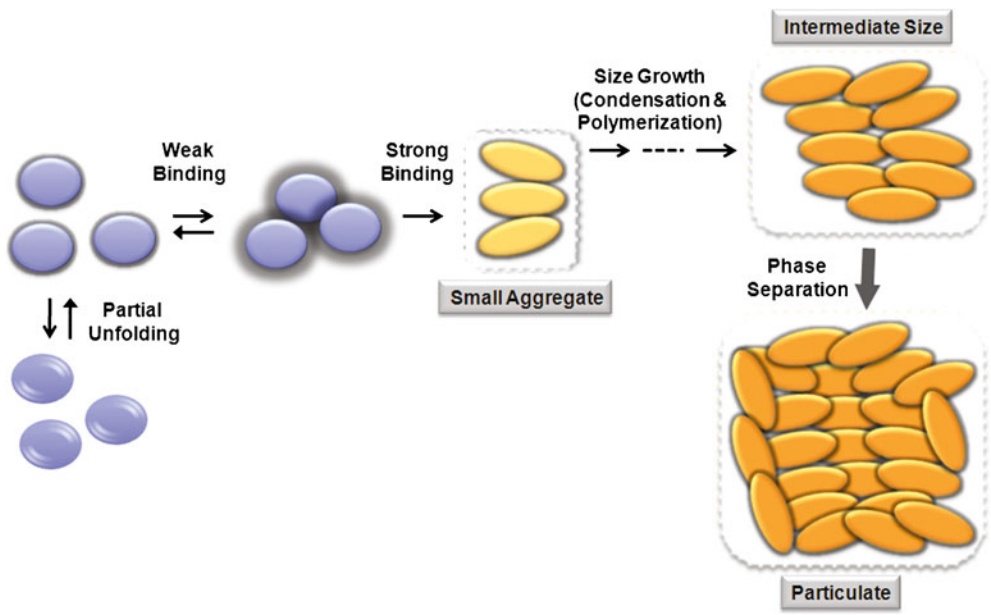

Fig. 2. A schematic representation of mechanism of particulate formation [adapted from (5)]

Finally, interactions with leachables from primary packaging (*e.g.*, silicone oil, tungsten) can initiate aggregation events (27,33,51,53,54,112).

CONCERN OF ADVERSE EVENTS

The presence of aggregates in biopharmaceutical formulations continues to be one of the major quality and safety concerns in biotherapeutics development (113,114). Aggregate formation poses challenges for quality, production yield, handling, and storage during biologics development. Recent discussions include both aggregates and particulates in the context of potential immunogenicity (8,9). However, the mechanism of how aggregates and particulates lead to potential adverse events may be fundamentally different, primarily because of their size difference. Some of the safety concerns, especially about particulates, remain theoretical due to lack of any clinical data (9). Additionally, clinical observations of immunogenicity are a consequence of a combination of many complex factors including patient, therapy, product, and dosing (115) that make it impossible to connect particulates to immunogenicity.

Therefore, much of the discussions often center on the quantity of aggregates or particulates that could be considered safe. Attention should be paid also to the importance of types (physical/chemical characteristics) of aggregates or particulates (including extrinsic or proteinaceous), considering "not all aggregates (or particulates) are made equal." Preclinical studies often use non-representative amount or type of aggregate/particulate to induce immune response (96,116–118). Preclinical studies are not currently capable of predicting clinically relevant immunogenicity potential (119,120) of particulates that constitute a very small fraction (*e.g.*, microgram quantity) of active in a biopharmaceutical product (9).

CONCLUSIONS

In the past several years, there has been a lot of focus on emerging techniques and novel methods to study particulates in biotherapeutics. A stage-appropriate strategy of comprehensive characterization is key to understanding the many facets of physical and chemical degradation events leading to particulate formation. Detection of potential intermediates in the particulate pathway is expected to provide vital clues to decipher the mechanism as well as finding prevention. Emerging techniques are expected to produce valuable characterization data to bridge the current gap in subvisible and submicron size ranges which is important to build the history of a given product candidate. A good understanding of the common factors (including relevant forced degradation conditions) causing particulate formation is important to develop a stable, safe, and efficacious formulation. Pharmacopeial particulate counting methods serve very well in defining methods and safe limits of particulate load in clinical and commercial products. However, it is recognized that additional methods are necessary to enhance our understanding of protein particulates.

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