PARTICLESENTRY AI™

Using Deep Machine Learning in Formulation Development and Early Process Design of Biologic Drug Substances and Products

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Introduction

Analytical method choices made during drug formulation and early process design and development follow the drug through review, regulatory approval, and beyond. As such, these decisions have significant implications as a product moves through its lifecycle. Detailed characterization of subvisible particles, including proteinaceous aggregates, inform defining the Target Product Profile (TPP), Chemistry, Manufacturing, and Controls (CMC) strategy, and development of product Critical Quality Attributes (CQAs). Ultimately, these contribute to a robust particulate control strategy.

Analytical methods employing subvisible particle imaging techniques deliver critical data on the changes a drug substance may undergo during the manufacturing process. For instance, critical steps like viral clearance, pumping, recirculation, or filtration impose stress on the drug substance that can result in the creation of subvisible proteinaceous particles that may be harmful to a patient or could decrease drug efficacy. Classically, the value of subvisible particle imaging techniques has been limited by the restrictions imposed through the availability of only subvisible size and count data. A Subject Matter Expert (SME) can study an image set and make qualitative observations, but no way has existed to validate SME observation as an analytical method. This situation left the rich morphological data embedded within the images unexploited.

Recently, significant advances in computing and graphics processing have spawned the age of artificial intelligence (AI). Machine Learning (ML) and Deep Machine Learning (DML) have revealed the potential value contained within the collected particle images. When combined with DML and computational statistics behind ParticleSentry^{AI} software, these images can unlock new quantitative information. Using robust Design of Experiments (DoE), the software delivers a fingerprint of the morphological features of a population of particles, including differentiations by stress. This statistical representation of the heterogeneous particle population at given process delivers quantitative, actionable data and particle outlier detection to improve process yield, ensure patient safety and drug efficacy – throughout the product lifecycle.

This article discusses current areas where subvisible particle image analysis using ParticleSentry^{AI} software can add new insight to existing processes and assist in providing a solid analytical platform for new drug products.

Particle Analysis in Biotherapeutics

Particulate matter in injectable drugs is categorized into three general groups: (a) extrinsic, or materials foreign to the substance and its production, (b) intrinsic, materials resulting from instability of the components used the manufacturing process (e.g., component wear over time from manufacturing equipment like metals from tubing, gasket material, filter membranes, etc.), and (c) inherent, particles resulting directly from the substance itself. In biologic drug products, these are typically proteinaceous aggregates. Aggregates range in size (0.1 – 100 µm) and have long been associated with possible immunogenic response and lowered therapeutic benefit when administered to patients. Aggregates can, and usually are, produced at any point in the life cycle of a protein therapeutic.

While this article focuses on inherent particles, and specifically, proteinaceous aggregates found in biologic drugs, protein particles can and do attach to the surface of intrinsic particle materials during the manufacturing process. Strictly speaking, these particles are considered intrinsic, but can provide a surface for protein aggregation to occur. Throughout this text, references to these kinds of particles are commonly referred to as subvisible particles – whether they contain proteinaceous material or not.

Protein Aggregation

Protein aggregation is a major challenge during the development and commercialization of biologic drug products. Protein molecules are highly impacted by the stresses caused during mixing, pumping and filtration of the drug substance, as depicted in Figure 1.

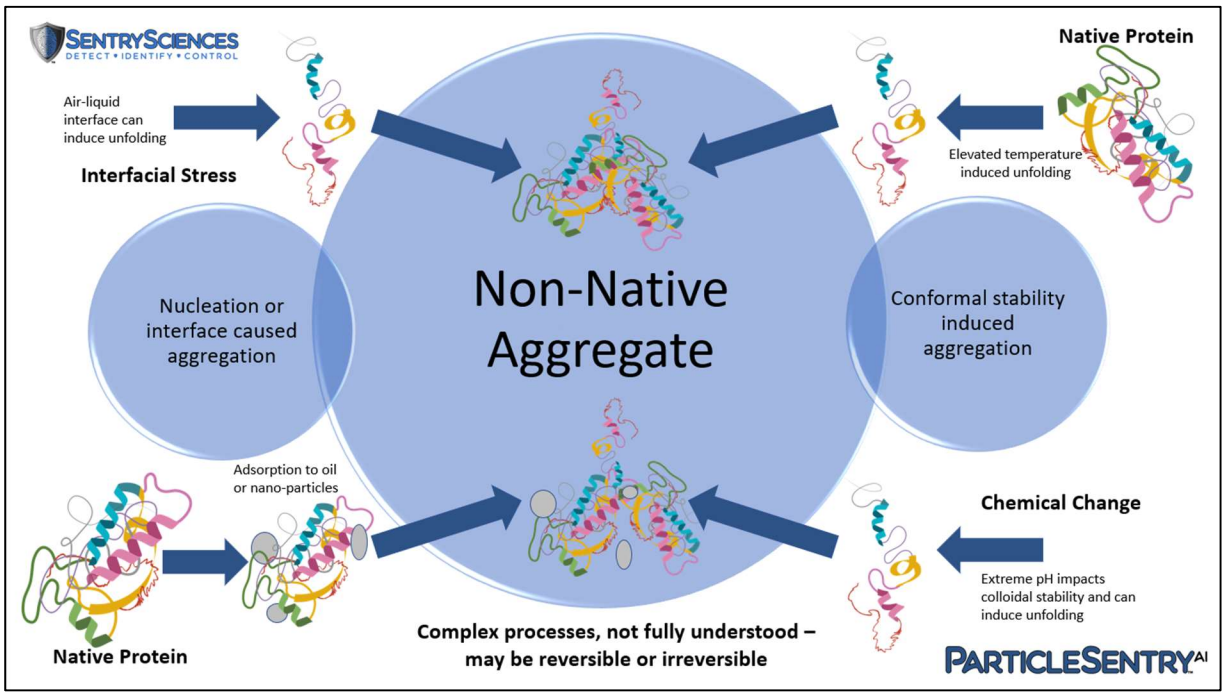

Figure 1: An illustration of some of the various pathways of protein instability that lead to formation of aggregates and particles.

Figure 1 illustrates some of the pathways for protein degradation and aggregation. Philo further breaks down these pathways into mechanisms that can occur at many places throughout the production process [1]. These mechanisms continue through shipping to the clinic, and even patient administration. The protein monomer surface is described as containing "sticky or complimentary patches" that attract other monomers to form higher order complexes as aggregate mass increases. This aggregation mechanism is often irreversible and can modify bioactivity at higher concentrations. Other mechanisms can cause conformational changes that are significantly increased by stressors, such as temperature and shear, during manufacturing. Additionally, nucleation can occur when protein monomers attach to an intrinsic particle, like a silica particle from glass, steel particles shed by piston pumps, silicone particles from tubing or lubricants used throughout the manufacturing process and in prefilled syringes. Finally, protein conformational changes brought about by hydrophobic or electrostatic interactions can result in binding to a surface (air-liquid interface, ice crystals formed during freeze/thaw, etc.). Following conformational change, the monomer can become prone to aggregation and the presence of these non-native monomers may impact drug potency and immunogenicity [1].

Aggregates are heterogeneous in both size and morphology relative to other subvisible particles contained within a proteinaceous drug product. Process variables related to the sequence, stage, and solution conditions for a drug substance, and more importantly, the co-existence of multiple methods of aggregation, make development of a single model to predict and control protein aggregation unrealistic [1]. As such, development and understanding of the degradation pathways, and temperature and pH changes the protein experiences during the manufacturing process are key to monitoring and controlling protein aggregation. Drug formulation and production processes must balance critical process steps that contribute to aggregation with analytical solutions to monitor the impact on the quality of the drug product [4].

Analytical Techniques

Biologic drug formulations consist of multiple constituents, all of which contain particulates. Regulatory acceptance criteria for the presence of particles are governed by US Pharmacopeia (USP) Chapters <787> Subvisible Particulate Matter in Therapeutic Protein Injections [5] and <788> Particulate Matter in Injections [6]. These chapters are harmonized with both the European Pharmacopoeia and the Japanese Pharmacopoeia and the acceptable particle limits are based upon the number of particles per container or per mL of drug product. Accepted test methods are the Light Obscuration Particle Count Test (Method 1; LO), or the Microscopic Particle Count Test (Method 2; MM not discussed) which is primarily used only for samples with reduced fluid clarity, increased viscosity, or products containing significant air or gas bubbles.

LO has been widely used for particulate counting in parenteral drugs since 1985. When enumerating particles in proteinaceous drugs, LO encounters significant technical challenges. LO measures the presence of a particle, as well as its size, based on the time a single particle blocks a laser beam at a fixed fluid flow rate. Proteinaceous aggregates, which have an index of refraction close to that of the formulation buffer they are suspended in, are not consistently detected due to the low difference in refractive indices. Furthermore, LO robustly counts air bubbles and silicone oil droplets that may be present in samples, resulting in inflated counts from the non-relevant materials. While LO is currently the preferred compendial method for quantifying particle size and count in formulated biologics, other techniques are identified in USP <1787> Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections [7] and USP <1788> Methods for the determination of Particulate Matter [8]. These techniques include subvisible particle imaging techniques that expand the focus from strict particle size and count, encouraging both qualitative and quantitative methods for analyzing particulate images to extract valuable morphological detail.

Testing for and understanding the mechanisms of aggregation during formulation and process design is necessary for development of a robust aggregate control strategy. Use of orthogonal methods to quantify and characterize aggregates throughout the development process is essential, because no single method provides comprehensive detail for understanding the mechanisms of aggregate formation and subsequent mitigation. The following analysis of techniques does not include all possible methods for subvisible particle analysis; rather, it focuses on those most widely deployed in biologics development today.

General methods used for particle analysis and characterization:

Common Particulate Techniques:

Chromatographic methods, while critical to downstream processing, deliver limited detail on protein aggregation. They are discussed here for informational purposes as they relate to protein aggregation analysis: High-performance liquid chromatography (HPLC), which includes size exclusion chromatography (SEC), gel permeation chromatography (GPC) and gel filtration chromatography (GFC), and similarly liquid chromatography mass spectrometry (LC-MS), are often used for analytical characterization of aggregates in biologic formulation development and manufacture. Variations of these technologies are used to understand aggregation reversibility/dissociation [9]. These processes are ensemble methods that do not examine the individual particles in the population [22]. They illuminate the method of aggregation by identifying the presence of specific chemical modifications, however isolation of particles and sub-populations is challenging.

Analytical ultracentrifugation (AUC) is often used for measuring the size distribution of sub-micron particles in biologic drugs. However, AUC throughput is low, it requires substantial investment, necessitates an experienced SME for data analysis and interpretation, and is difficult to transfer to manufacturing from development environments [10].

Fourier-Transform Infrared microscopy (FTIR), a vibrational spectroscopy, is commonly used in biologics for Higher Order Structural (HOS) analysis by examination of the secondary structural elements of a bulk protein, expressed by the Amide I and Amide II bands. FTIR has also seen widespread deployment as a robust tool for raw material analysis, as well as for forensic identification of particulates. However, modern

FTIR analysis of particulates requires contact with an ATR probe, is low throughput, and is constrained to particulates larger than 10 µm. It is also exquisitely sensitive to water, so is not suitable for investigation of formulated biologics without particulate isolation. FTIR has been utilized with molecular labels (dyes) to investigate the hydrophobicity of the protein surface that indicate unfolding, and exposure of residues normally buried in the folded structure of a protein [3,9,11].

Raman spectroscopy, a second vibrational spectroscopic technique, has seen increased use in HOS determination of secondary and tertiary protein structural elements. Raman has also been used for raw materials analysis and forensic particulate identification in biologics laboratories. Using a focused excitation source (laser) Raman has successfully interrogated particulates smaller than 1 µm and is not sensitive to water making it useful for both suspended and isolated particulate identification. Raman spectroscopy is sensitive to both organic and most inorganic matter and can be used not only for extrinsic particulate identification, proteinaceous aggregate identification and structural analysis, but for excipient (polysorbate) degradation studies (as discussed below). Throughput is low, and care must be used to avoid laser-induced damage of the sample [11,15].

These methods provide valuable characterization details to assist in understanding the chemical nature of the primary protein as well as conformation, aggregation, and self-associated species. Except for chromatographic methods, each of these tools are lab-based and typically are not carried through to in-process measurements.

Particulate Imaging Techniques:

Flow-Imaging Microscopy and Microfluidic Imaging technologies (collectively referenced as FIM) collect digital images (grayscale or color) of suspended particulates greater than 2 µm. These techniques are considered orthogonal to LO by regulatory agencies and are widely accepted as offering superior proteinaceous aggregate detection. FIM, through particulate images, provides particle count and accurate particle size, in addition to morphological features. Historically, these analytical methods have been used to correlate particle size and count data delivered by LO instrumentation, but without exploiting the morphological data encoded in the digital images.

Backgrounded Membrane Imaging (BMI) uses a modern image processing algorithm to analyze particles on a multi-sample filter plate. A background image of the filter plate is taken, the sample is aliquoted to individual filters, vacuum is applied isolating any particulates, and the plate is then re-imaged. The background image is then subtracted from the sample image, eliminating the background membrane features leaving only the particulate images. This technique resolves limitations posed by refractive index matching between inherent particles and the sample fluid, delivering images of particles and their morphologic and textural detail. BMI requires low sample volume and eliminates interference from air and gas bubbles, though suspended fluid droplets such as silicone oil will traverse the membrane. BMI is attractive for work in formulation development where buffer and excipient screens can be compared [12,24]. The technique can be extended with fluorescent labels specific for protein and other common material types, providing chemically sensitive differentiation of particulate species.

Imaging flow cytometry (IFC) is a technique typically used in cellular imaging. This method expands on the brightfield-based analysis mechanisms employed in MFI/FIM by adding additional imaging options: side scattering and fluorescence channels. Like BMI, the fluorescence channel employs dyes. IFC data analysis can be complex and requires mask definition, which can be aided to some extent by manufacturer software [21, 42].

Total Holographic Characterization (THC) is a single-particle light scattering technique based upon Lorenz-Mie Theory. In addition to particle size and count data for particulates in the 0.5 to 10 µm size range [13], the technique provides the refractive index of individual particles, a property that is linked to particle chemistry. THC is considered complementary to FIM and covers the subvisible gap. While this technique is relatively new to the analytical laboratory, it offers promise to provide valuable information for particle size, count, and chemistry in the submicron range.

Designing for Analytical Characterization

In 2004, the US FDA began emphasizing the importance of science- and engineering-based activity for pharmaceutical development and manufacturing. A cornerstone of this work is the guiding principle ensuring that "science-based policies and standards form the foundation upon which product quality regulation is based." [14]. The result was a shift from their previous review system to a risk-based system, placing new importance on in-depth process understanding and control of change. This shift has emphasized investigation and use of new technologies, including Process Analytical Technologies (PAT) to support risk mitigation and drive quality improvements.

To ensure the highest quality products, appropriate analytical methods must be used to assess the risk related to each element of the manufacturing process for both small- and large-scale production. Analytical techniques that deliver images provide visualization of the particulates to aid in the interpretation of test results. Particle image clarity, contrast, and shape may indicate possible sources of particle types, but often require SME interpretation for robust qualitative categorization. Extending these analyses with basic ML approaches to classify subvisible particles based upon their morphological features [17,37,41,42] has become common. However, basic ML categorization doesn't aid in identification of new conditions, such as the presence of a foreign body the model is not trained on, or the presence of proteinaceous particles with new morphological features – the outlier is simply placed into the nearest trained category – leaving possibly critical information buried within trained categories [17]. The requirement of basic ML, forcing particles into known classifications, fails to capture novel morphological entities, thereby missing statistically significant changes.

Increasing Data Value through DML

Imaging and video techniques are evolving rapidly. Pictures and video are worth a thousand words – especially when the morphological characteristics of subvisible particles and aggregates often defy human description because of their small size. SMEs may be able to "learn" key features contained in the amorphous grayscale images, but the size of the datasets and human interpretation of the images make such analysis qualitative at best.

Previous work with imaging data sets from analytical techniques such as FIM have demonstrated the ability to visually differentiate images of aggregates based upon their formulation stressor [12,17-20]. These images can be extracted, and based upon their mechanism of creation, provide valuable insight into their detection and control [2,12,17-21,31].

Leveraging images captured through robust DoE, ParticleSentry^{AI} software employs a convolutional neural network, principal component analysis and dimension reduction to the image collection and returns fingerprints that enhance process understanding (Figure 2). When deployed during formulation research, early process design, and CMC development, this approach provides the foundation for analytical and statistical tools needed to rationalize CQAs and fine-tune processes. These fingerprints follow the drug through formulation R&D and into process design, providing a framework that delivers the statistical data needed for characterization of the inherent subvisible proteinaceous aggregates in a biologic drug product. Unlike simple ML classifiers, fingerprints capture statistical shifts in the population of subvisible particles, indicating changes in the heterogeneous mix of subvisible particles in the sample. Further, the fingerprint can follow the drug substance throughout its lifecycle, providing critical data to support future root-cause analysis when a process excursion occurs.

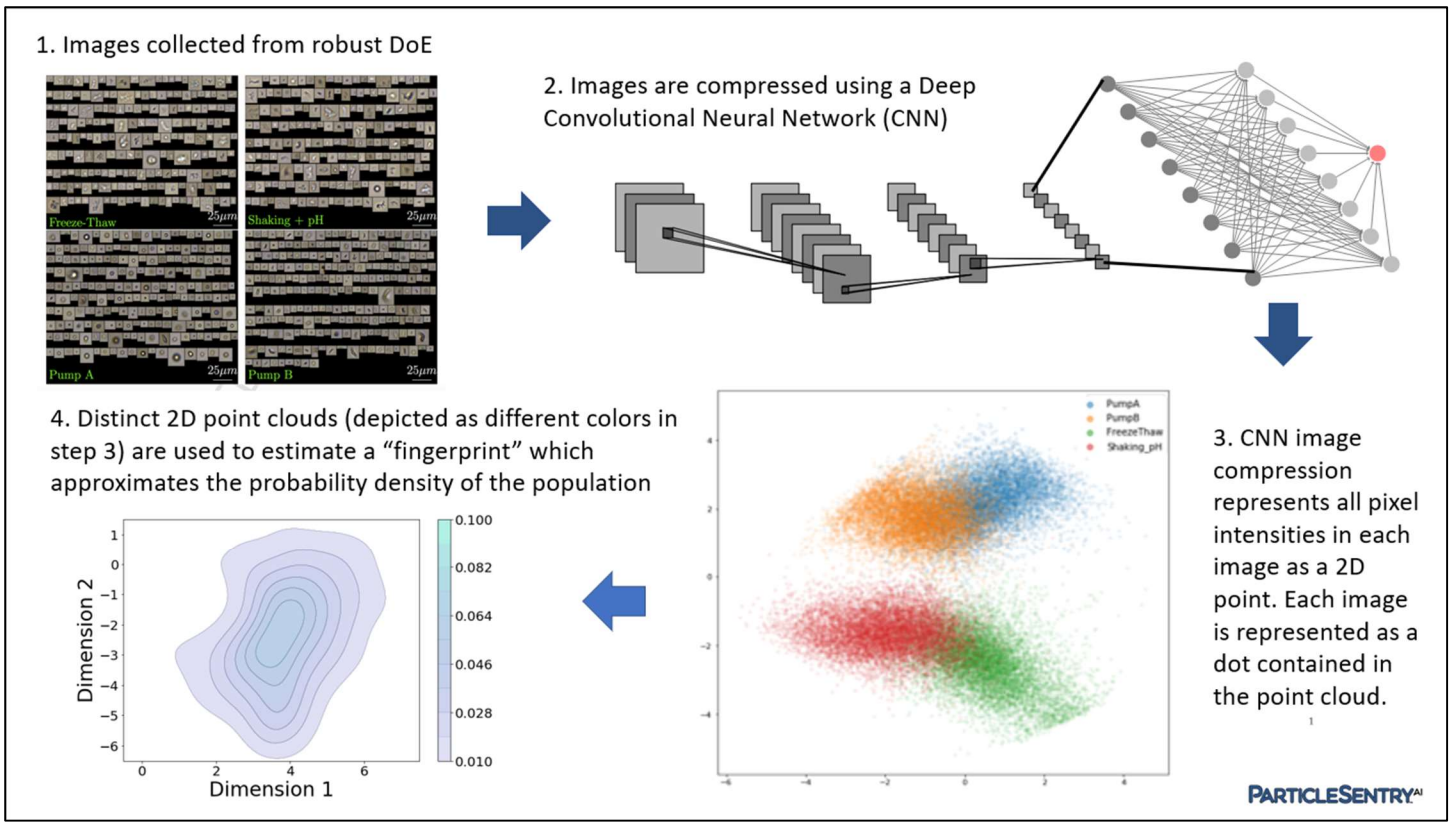

Figure 2: Image analysis using DML, principal component analysis, and dimension reduction delivers a fingerprint.

In 2022, Calderon et al demonstrated that DML and the technique employed in ParticleSentry^{AI} software, can be employed for FIM diagnostics as well as valuable analysis of pharmaceutically relevant particles. Testing was conducted using a NIST protein surrogate made from abraded ethylene tetrafluoroethylene (ETFE). They were able to characterize the effects of imaging instrument variability that can be systematically quantified and accounted for in hypothesis testing. Further, employing the morphological characteristics contained in particle images can be useful in precisely monitoring biologic drugs and can identify process-upset conditions, making it useful both in production and as a quality assurance tool. Finally, they also noted that results are not limited to FIM only and the methods are complimentary to BMI, flow imaging cytometry, etc. [21]

Formulation development

While analytical requirements in formulation R&D may vary from those of CMC process design, Morar-Mitrica indicated that harmonization of analytical methods, where possible, across these groups is critical to understanding and solving problems that arise during GMP manufacturing. Further, they note the potential benefit of "expanding the particle knowledge space through adding particle characterization to established knowledge pillars such as developability assessment and forced degradation studies." The premise for this statement lies in the fact that desired 'stability' of a drug substance extends through the manufacturing process to drug product transport and administration, and that protein aggregation and particle formation is not a random process, but a result of "interaction between the formulation and specific stress condition(s)."[20] Image analysis, coupled with ParticleSentry^{AI} software provides valuable fingerprint data on stress conditions and quantitative data for future process design, enabling root-cause analysis when an out of specification condition occurs.

Developing the target product profile (TPP) is a typical output of the formulation development process. The TPP is used to aid in finalizing drug substances and, ultimately, drug product CQA's. The TPP can benefit from DML image analysis in different areas, including:

- Thermodynamic stability is linked to the kinetics of protein aggregation and provides an early basis for optimizing stability of a biologic formulation [22].

- Freeze-thaw studies induce protein degradation, and thus aggregation, due to water and excipient crystallization, cold denaturation of protein molecules, pressure, and mechanical stressors related to volume expansion during transformation of water to ice. Since most biologic drug substances are stored in a frozen state and thawed prior to buffer exchange and fill-finish into the final drug product, it is critical that the impact of freeze-thaw and storage conditions are understood to enable both small- and large-scale production, and importantly, how choices about container size/material and storage temperature, impact protein aggregation and subvisible particle formation [23,24].

- Surfactant screening studies. Previous work [12] has demonstrated the value of image analysis in subvisible particle and protein aggregate analysis. As discussed in a recent Morar-Mitrica paper, "the impact assessment of variability (and concentration optimization) in the levels of critical formulation components (such as surfactants) on particle formation under specific process stress before a formulation decision is made" [20]. Calderon et al demonstrated both high classification accuracy and particle image resolution by combining BMI and ParticleSentry^{AI} software to distinguish between protein and fatty acid particles, while still maintaining the ability to identify new morphological features as a shift in a heterogeneous fingerprint of particle images. Further, they showed how the approach delivers reproducible results when analyzing stability data over time [24].
  - Polysorbates, such as PS20 and PS80, are considered inactive ingredients by regulatory authorities and are widely used to stabilize proteins in biologic drug substances. Surfactants can protect proteins against physical degradation from interfacial stress and help to minimize formation of protein aggregation [25]. They are typically deployed at low concentration (typically 0.1 to 0.5 mg/mL); however, their stabilization power is not without complication.
    - PS manufacturers produce material using one of two synthetic development routes. Depending on the supplier, manufacturing method and purity, the raw material will have differing levels of impurities. As a result, they are inherently heterogeneous and include various fatty acid esters and polyunsaturated and long-chain fatty acids. These esters and fatty acids may be susceptible to enzymatic host cell protein (HCP) induced polysorbate degradation, or free fatty acid (FFA) agglomeration [29]. These issues can exist even with high-purity grade PS, resulting in necessary care protocols for handling and storage (long and short-term), as well as analytical characterization of PS materials on arrival and before use in a drug substance.
  - Oxidation can occur due to improper closure of PS storage containers between uses and exposure to air/light and transition metals. This degradation/destabilization pathway is complex and occurs both in the polyoxyethylene (POE) and the fatty acid chain moieties. This results in POE esters and other short chain degradants that further increase complexity when coupled with buffer components like histidine and citrate [25,26].
  - Hydrolytic degradation is typically caused by enzyme-mediated hydrolysis and is believed to be the primary root cause of PS hydrolytic degradation in biopharmaceutical formulations. It is caused by trace amounts of HCPs that have been co-purified with the therapeutic protein. While this degradation pathway may be less complex than oxidation, its impact on the final drug product can be more severe by releasing free fatty acids (FFAs) that can eventually agglomerate and form subvisible and even visible particles. [26].

- Forced degradation, stress, and stability testing challenge drug formulations, helping to identify the leading to protein aggregation [30]. These tests create conditions allowing the impact of degradation of a formulation to be studied. Challenging formulation candidates to identify the degradation pathways for temperature shift, photolytic exposure, mixing, filtration, and pumping are all critical to understanding the strength of a formulation to resist aggregation from these potential sources. Morar-Mitrica describes the importance of stability studies of a candidate through mechanically stressed samples. They assert these tests are "especially important as particles may be revealed during stability following stress and not immediately after stress." [20] The use of analytical imaging techniques such as BMI and FIM, combined with DML and statistical analyses delivered by ParticleSentry^{AI}, enables the distillation of quantitative and actionable data embedded in the morphological characteristics of the particulates formed by specific stress conditions. The compression of this data into fingerprints by the software provides the statistical precision needed to guide process design and monitoring by capturing patterns of impact on the heterogeneous aggregate populations before and after a stressor.

Assessment of subvisible particles and protein aggregation is critical to formulation R&D, providing the benefit of robust process development and manufacturing strategy. As discussed by Morar-Mitrica et al "The observation that particles often occurred during GMP (manufacture and not during development activities) led us to conclude that analytical method harmonization is a critical key action. Harmonization of the analytical assessment of visible and subvisible particles includes standardization of equipment, procedures, extent of testing as well as instrument and analyst qualification in development and up to commercial testing." [20]. They further conclude, "Overall, the early-phase particle mitigation strategy encourages an early engagement and investment, aims at assessing formulation *against* process stress (not in parallel or sequentially), embraces high-throughput tools, and points to the interface between technical and clinical development to explore the safety profile of inherent particles." [20] It is essential that data gathered during these early stages carries forward to the CMC and process development phases of a drug product's lifecycle because of their potential to inform the documented protein aggregate control strategy.

Early process development

Effective control strategies require analytical techniques and complimentary orthogonal methods to correlate data across platforms and inform robust process design. For example, techniques that can identify chemical and structural changes in proteinaceous aggregates may illuminate their cause but may not support a clear understanding of where and how they were created during the production process. An effective control strategy is dependent upon correlated data across techniques to drive thorough process understanding. Techniques that deliver imaging, when deployed pre- and post-process deliver before/after data that can be valuable when designing operational steps for small- and large-scale drug substance production. Coupling imaging techniques with DML and ParticleSentry^{AI} enables fingerprints that can be used to study and predict manufacturing component life (tubing, filters, etc.), and aid in root-cause analysis when a change is detected during routine operations. When used during process development, these data are a major enabler to design process control and monitoring throughout the production process.

- Host-cell proteins (HCPs) are endogenous proteins from the host cells used for bioproduction and they form a major class of process-related impurities that can impact the safety and efficacy of biopharmaceuticals. Detection and testing of HCPs is typically conducted with immunobead assay, though testing is imperfect and doesn't identify individual HCPs.
    - Ultrafiltration and diafiltration (UFDF) with high-performance tangential-flow filtration (HPTFF) are unique membrane separation processes used in downstream processing. They allow purification, concentration, and buffer exchange in one step. HPTFF differs from regular tangential-flow filtration (TFF) in that it exploits both size and charge differences of biomolecules and can separate protein solutes that are

similar in size [29]. One of the challenges with this method is recirculation and agitation of the drug substance through pumping, which is known to cause aggregation due to mechanical stresses the protein monomers experience when they are compressed against the tubing wall by the rollers on the pump head.

- Viral clearance is typically accomplished in biomanufacturing via heat, chromatographic separations, low-pH treatments, solvent/detergent (S/D) viral inactivation and nanofiltration. The most common method for viral clearance is chromatography, but filtration is also widely used. Regulatory guidelines require biomanufacturers to incorporate separate individual methods of viral clearance, rather than repeat the same mechanism more than once [27].
    - Viral filtration is a size-based removal technique that poses challenges like those posed by other filtration methods – filter clogging. Though these issues can be managed with judicious control of start/stop of the flow and regular examination of the filter materials. [28]. One of the challenges with this method is recirculation and agitation of the drug substance through pumping.
- Non-traditional mechanisms of aggregation
    - Interfacial stress has been extensively studied and indicates the importance of mixing and shear forces experienced during small and large-scale production. Thite, et. al. summarized that "particle formation pathways may not be mutually exclusive, and multiple pathways (e.g., particle formation at air-water interfaces and air-water-container interfaces) may operate simultaneously" [31]. As a result, interfacial stress is often overlooked as a contributing factor in protein aggregation.
    - Nucleation of particles can occur when an intrinsic particle is shed (e.g., a shard from a glass vial, a stainless-steel particle from a tank weld, or immiscible liquid droplets from component and device lubricants) and proteins aggregate on the shed particle [1].
- Drug Substance/Product Stability and chemical & microbiological stability
    - Chemical degradation leads to a conformal change in the protein molecules and can change the concentration of the drug substance. When anti-microbial preservatives are added to multi-dose formulations, they have been shown to drive further conformal changes that, in-turn, increase the potential for aggregates in those preparations [1,2].
    - Microbiological stability. Sterile filtration is used to remove microorganisms from the drug substance and can occur in bulk or in-line in advance of drug product fill-finish. This process captures microorganisms and larger proteinaceous aggregates. During filtration it is possible for proteins and excipients to adsorb to the filter membrane, causing particulate formation. It is also possible for the filter to shed intrinsic particles of filter material, creating new nucleation sites for protein aggregates.
- Movement of materials during manufacture introduces new aggregation sources during both small- and large-scale production.
    - Pumping of drug substances occurs at several stages throughout the manufacturing process, including the final fill-finish. The mechanical energy imparted by pumping of protein drug substance/product is known to produce particulates and proteinaceous aggregates [33]. Pump types (rotary piston, rolling diaphragm, time pressure, and peristaltic) used throughout the production process need to be extensively tested for unintended consequences. These challenges make pump selections, and

design of substance movement, critical to process design and development.
    - Mixing and compounding occurs during multiple steps in the production process for both drug substances and drug products. Mixer design (top- or bottom-mounted, magnetically coupled impeller/drive, magnetic stirring bar), speed, time, solution temperature and potential for grinding can have a direct impact on shear forces experienced by the drug product, on subvisible particle creation, protein aggregation, and as a result, drug substance potency. In high concentration formulations these design choices are even more critical to understand [32].
    - Tubing qualification of the silicone tubing (polydimethylsiloxane, PDMS) used almost exclusively in pharmaceutical production provides hydrophobic and hydrophilic surfaces that promote aggregation based upon the coatings used inside the tubing [34].
- Container, closure, compatibility, and packaging considerations. Container compatibility and packaging has historically been heavily influenced by analysis of the particle count and size distributions captured during accelerated stability studies. As discussed above, this analysis may not effectively characterize the influence of aggregation caused by interfacial phenomena within the container (e.g., storage containers, vials, storage bags and large- and small-sized IV bags) and can result in an underestimation of the resulting aggregation from container interfaces [31]. Container selection (vials, prefilled syringes, etc.) is critical to safe, efficacious drug delivery. These decisions have far-reaching impact because some mechanisms of failure may not be captured without intentional long-term stability and analysis.
    - Glass delamination can create the opportunity for intrinsic particles (glass/silica flakes) creation (as discussed above). These problems often occur after the liquid drug substance has been stored in the container for some period [35]. Testing for compatibility of the substance and container selection prior to clinical trials is essential to eliminate delamination and nucleation as a potential source of aggregation.
    - Headspace has been long considered a contributing factor to particle creation and adds another interface (air-water-container) to the drug solution [31].
    - Dosage presentation and formulation differences. Daniels, et. al. analyzed the FIM data generated from an FDA study on peginesatide and applied a Kullback-Leibler Divergence analysis with the images. Their analysis focused on the morphological differences between the particles and identified significant morphological differences in the particle populations contained in the Single-Use Vial (SUV) and Multi-Use Vial (MUV) presentations. Both formulations contained identical concentrations of peginesatide and sorbitol, but the SUV included a phosphate buffer and Tween 20, and the MUV contained a methionine buffer and phenol preservative. While the morphological differences were difficult to detect (because of the size of the particles), they found distinct differences in morphological characteristics between the SUV and MUV presentations [2].
    - Silicone oil and pre-filled syringes (PFS). PFS has become an important method of drug product delivery, due to their ease of use, accurate administration of dosage and elimination of some contamination pathways. Most glass PFS use silicone-oil as a lubricant and a small amount of the oil can migrate into the DP as droplets [36]. These droplets can become nucleation sites and can induce protein particle aggregation [40].
- Shipping of biologics subjects drug products to low g-force mechanical stresses and nearly

continuous low-intensity vibrations, coupled with sporadic, high g-force mechanical shocks that may induce cavitation. Witeof, et. al. using FIM analysis coupled with the technology behind ParticleSentry^{AI} demonstrated that the morphology of aggregates produced during cross-country shipping were like those created during lab shaking analysis, but distinctly different than those generated from a rotary high repetition drop instrument. Their analysis suggested that stresses imposed by shaking rather than occasional g-force events encountered during shipping were the primary drivers of protein aggregation during shipment [31, 37].

Developing an Aggregate Control Strategy

An effective aggregate control strategy begins with a target product profile (TPP) in formulation development. The TPP follows through drug development and into production process design, where the target product quality profile (TPQP) is defined. The TPQP includes the physical, chemical, and biological properties of the drug, and culminates in final development of the CQAs and ultimately the process parameters to achieve the CQAs reliably [38].

- A robust aggregate control plan requires a complete understanding of how aggregate size and type can influence product quality [40]. The control strategy must consider the differences encountered between small-scale production and scale-up. Changes in the equipment used, process and hold times and storage conditions, among other factors, can all impact subvisible particle formation. Small scale models may drive unjustified confidence in process robustness but can be leveraged to assess and define the "edge-of-failure" of a product by exaggerating relevant stress conditions. They can also be used to assess the impact on CQAs [20].
- Statistical understanding of data and control of processes is a critical function in developing an aggregate control strategy. Use of control charts in conjunction with process capability analysis form the matrix of data required to demonstrate process control for regulatory authorities [15].
- ICH Q11 Guideline on development and manufacture of drug substances indicates that a "control strategy can be developed through a combination of approaches, utilizing the traditional approach for some CQAs, steps, or unit operations, and a more enhanced approach for others." In short, this means the traditional focus on narrow operating ranges and CQAs can be improved upon by using an iterative approach as knowledge about variability and of the process and product increase [38].

Morar-Mitrica indicated clearly "The particle mitigation strategy informs innovation efforts." [20] Finished drug products are tested for quality by assessing whether they meet manufacturer and FDA approved specifications and requirements. If they don't meet these requirements, they are discarded, and failure root causes are not often well understood. Failure to understand aggregation during development, technology transfer and manufacturing can cause submission delays and loss of batches, which in turn leads to revenue loss and increasing costs.

DML analysis of particle images delivers actionable and quantitative insight that can be used to inform and improve the manufacturing process at small and large scale. ParticleSentry^{AI} Software for image analysis throughout formulation development and process design captures the morphological characteristics of degradation pathways and lays the foundation for improved root-cause-analysis and diagnosis when an excursion occurs – and it will. Further, ParticleSentry^{AI} software provides the statistical basis for comparison of the heterogeneous mix of particles contained in the drug substance at lot release. The fingerprint comparison doesn't require a subject matter expert and can be done near- or at-line.

Conclusion

There are numerous causes of protein aggregation and subvisible particulate formation extant in biologic drug substance manufacturing processes. Aggregation sources can change and become more pronounced as drug substance moves from small batch production through scale-up and ultimately to large-batch production. It is widely understood by the biopharmaceutical industry that LO is unable to correctly size and count these particulates, yet LO remains the primary method for lot release by regulatory agencies. Modern analytical techniques deliver enhanced sensitivity through the recording of particle images, providing much more than just orthogonal particle size and count verification. ParticleSentry^{AI} software expands the utility of these particle imaging modalities by enabling quantitative and statistical analysis of the morphological features of subvisible particles and protein aggregates that are linked to particulate genesis. The fingerprint of particulate population produced by ParticleSentry^{AI} can be incorporated at any point in the drug development process, but is especially valuable for formulation development, process development and scale-up, and during commercial manufacturing as an aid to root cause analysis.

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