The Next Frontier in Subvisible Particle Analysis: New Tools and Opportunities

Abstract

In the past decade, the biopharmaceutical industry has witnessed the arrival of a number of analytical technologies that are useful for characterizing subvisible particles in protein therapeutics. Even with the diverse tools that are available today, there are still important gaps that have not been filled but yet have a significant role in our ability to fully analyze particles for either product characterization or formulation development purpose. The goal of this article is to highlight some of these gaps and share the opportunities that may be captured by new tools that are on the horizon. The author will also use a case study to illustrate how simultaneous monitoring of sub-micron and micron-sized particles can assist biopharmaceutical formulation development and help fulfil current and future regulatory requirements.

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Introduction

Protein aggregation is a major obstacle to the successful development of stable, safe, and effective protein therapeutics[1]. As a well-recognized critical quality attribute (CQA), protein aggregation is usually monitored by size exclusion chromatography (SEC), an analytical method that is universally adopted for the detection and quantitation of soluble aggregates[2]. Protein aggregation can also lead to formation of subvisible particles (SVPs), which are insoluble aggregates that are excluded from the SEC column and yet too small to be visible to the naked eye (generally smaller than ~100 um in diameter). For the past few decades, the industry has exclusively relied on light obscuration (LO) and membrane microscopy (MM) with a focus on particle counts at 10 and 25 µm range to satisfy compendial testing requirements during product release. Even after the arrival of USP 787 and USP 1787, which were written specifically to address the needs of SVP analysis in biologics, LO and MM are still the most frequently implemented methods for particle quantification and identification, respectively[3, 4]. Following the 2009 commentary by Carpenter et al., SVPs, especially those below the 10 um size range, have become a greater concern to the biopharmaceutical industry[5, 6]. One key development following many studies and discussions is that now the regulators expect the industry to apply new orthogonal analytical methods to better characterize SVPs and provide quantitative data on particles in the 2 to 10 um range[7].

A key challenge for SVP characterization and its application as a quality control (QC) Tool is that aggregates and particles cover a size range of over 1 million fold (1x 10^6). Figure 1 shows why the existence broad size range of protein aggregates/ particles necessitates the use of orthogonal analytical methods in order to capture the full spectrum of particles in therapeutic protein products.

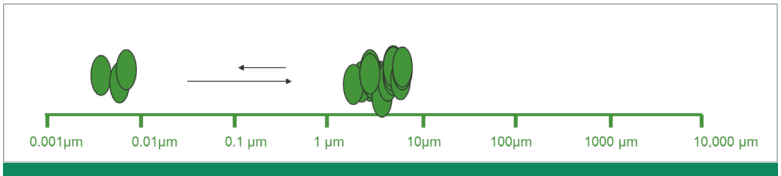


Figure 1

In terms of size classification, different nomenclatures are used to define a particle

Nomenclature	Size range
Oligomers	10 to 100nm
Sub-micrometer particles	0.1 - 1 µm
Micrometer particles	1 - 100 µm
Visible particles	> ~ 100 µm

Figure 2

Size Range	• Detects SVPs ranging from 0.1 - 100 µm.
Particle Count	• Ideal if it allows for validation and setting acceptable limits.
Particle Type	• Protein Aggregate vs Silicone Oil Droplet vs External Inclusions (Metal, rubber etc.)
Image of Particle	• Recording of particle image; Provision for visual identification and analysis.
No prior sample manipulation	• Stable Aggregates vs Dilution-dependent Transient aggregates.

Figure 3

according to its size range. Some examples are shown in Figure 2 (5 Narhi et al. 2012).

It is important to note that the potential safety risk posed by particles can be dependent on both their size and chemical composition[8]. Historically, the primary concern for SVPs in parenteral drugs is due to potential presence of extraneous particles (contaminates such as glass, rubber, or metal from the packaging container or manufacturing equipment), which may increase the risk of capillary clogging. For protein particles there is an additional cause for concern, which is a heightened potential for unwanted immunological response that can lead to patient morbidity or even mortality[9]. For these reasons, proper characterization of SVPs should include information well beyond size and number concentration data that are required by USP 788/787. This is why proper selection of new analytical tools is so critical today.

How does one develop a strategy for selection and evaluation of technology for SVP analysis? One way to simplify the process is to establish a "wish list" of strengths and capabilities and evaluate each technology according to its ability to fulfill the specific needs of the user. A sample of the capabilities that can serve as evaluation criteria for the "ideal analytical methodology" for SVP analysis is shown in Figure 3.

Before a discussion of emerging SVP analytical technologies, it is appropriate to provide a summary of existing technologies based on the dynamic size range each can cover. Figure 4 shows the dynamic range for SVP analytical techniques that are widely adopted for either QC or characterization purposes.

Since 2008, Flow Imaging Microscopy (FIM) has been widely implemented by the industry for the analysis of particles in the 1 to 10 um size range. In more recent years, the need for better analytical methods for sub-micron particles was highlighted by the FDA, which underscored the importance of understanding how various stress conditions may alter the distribution and/or quantity of particles in the sub-micron range[10]. Current regulatory guidance suggests that the greatest unmet need is for analytical methods that can provide robust quantitative data on particles in the 0.2 - 2 micron size range. Along with this trend, the biopharmaceutical industry's interest in the detection, quantification, and characterization of sub-micron particles (0.1 – 1 um range) is also heightened by recent research, which suggests that protein aggregates in this size range may have greater potential for inducing unwanted immunological response[11]. In short, analytical characterization of sub-micron aggregates/particles and a better understanding of their role in the formation of larger sized particles is the next frontier in subvisible particle analysis.

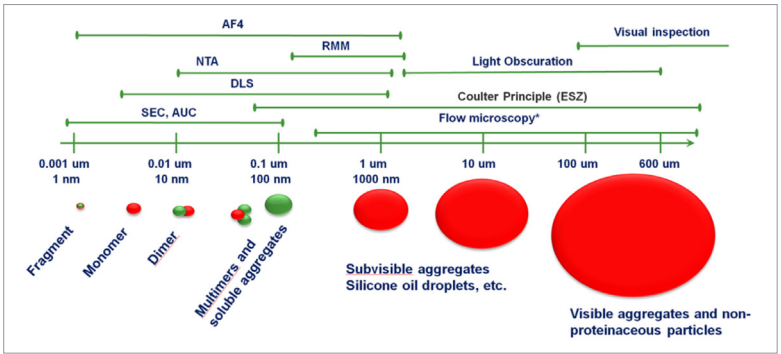


Figure 4

As mentioned earlier, the increasing acceptance of SVPs as a critical quality attributes in protein pharmaceuticals has resulted in an unprecedented interest in this field as well as the arrival of a large number of new technologies with potential utility for this application. A key goal in the quest to fully characterize particles in biopharmaceuticals is to have one or two analytical methods that can provide both quantitative and qualitative data on SVP from the nanometer size range up to 10 micron and beyond. At the present,

the number of technologies that can simultaneous monitor sub-micron and micron-sized particles is very limited and the new technology vendors often have little experience working with biologics. Therefore, it is incumbent on the scientist to evaluate each technology based on its true capability to bridge the sub-micron to micron gap in SVP data.

Looking forward, the big questions that remain are: 1. What is the relationship between submicron particles and larger SVPs? 2. What are the potential pitfalls of utilizing only submicron particulate data to choose formulations and how can we avoid them? 3. How relevant are submicron particles to biologic drug product development?

In this article, the author will share the insights gained from a study in which 4 different analytical technologies were evaluated for subvisible particle analysis: Mutltispectral Advanced Nanoparticle Tracking Analysis, Nano Flow Imaging/Oil immersion flow microscopy, Total Holographic Characterization (THC), and Single Particle Optical Sizing (SPOS). Rather than focusing on the details of any particular analytical technology, the author's goal is to provide a very brief introduction of each technology and demonstrate the importance of applying an analytical strategy that incorporates orthogonal methods that cover both sub-micron and micron-sized particles.

Specifically, the aims of this study were:

1. Compare data from 4 orthogonal SVP analytical methods that are generated using the same protocol and similar material.

2. Determine the utility of quantitative data in the sub-micron range and their potential relationship with micron-sized particles.

3. Show how orthogonal particle analytical technologies can be implemented to characterize SVPs in the 0.1 to 10 um size range and support formulation development.

Experimental method

Materials: The protein used was NIST reference material 8671 (NISTmAb), humanized IgG1κ monoclonal antibody. NISTmAb was chosen since it is a reference standard used for method development and validation, which can minimize potential variations in the samples tested by each method. The samples were prepared according to the NIST's instruction for use. After thawing, the stock solution of NISTmAb (10 mg/mL) was added to each well of a 96 well plate containing pre-mixed formulations to reach a final protein concentration of 1 mg/mL. iFormulate™ platform – a Design of Experiments (DOE) -based approach to formulation development was provided by HTD Biosystems. Each plate contains two sets of the same 25 buffer solutions. The rationale for development of this system was to provide a convenient predesigned formulation plate for rapid formulation of proteins. It is based on multivariable experimental response-surface design with 22 unique formulations plus 3 replicates that investigate effect of pH, ionic strength, buffer concentration, and stabilizer concentration[12].

Study design: NISTmAb in all 25 formulations was subjected to an accelerated stress study by isothermal incubation at 60°C. All formulations were stored at the same temperature for up to 48 hours and SVP concentration was monitored by each method over that time at fixed intervals. The study included 3 time points: T=0h, T=24h, and T=48h. Samples were analyzed by each of the 4 analytical methods at T=0 h and following storage. Data were processed in accordance with the requirements of each method to derive output of particle concentration per mL in each formulation for all 3 time points. Final results include other

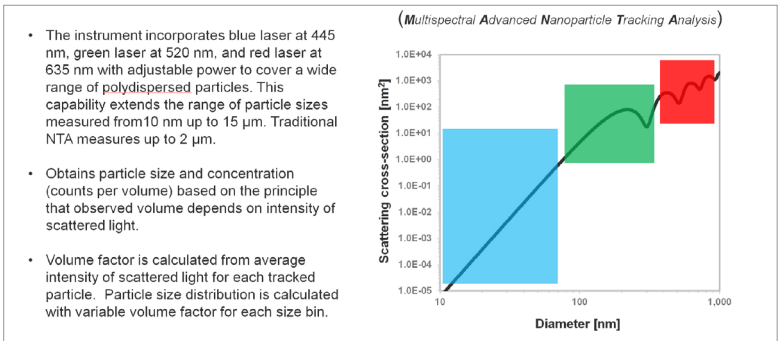
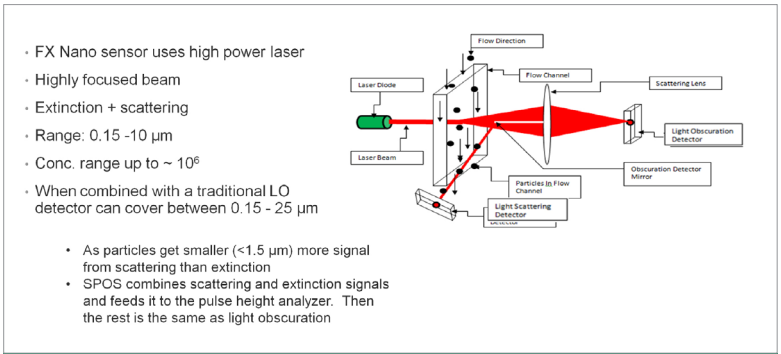


Figure 5



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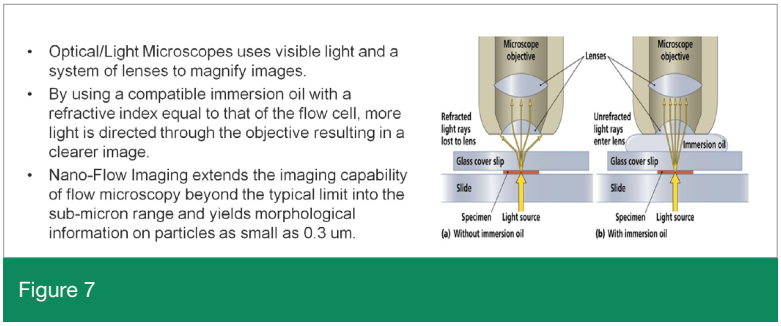

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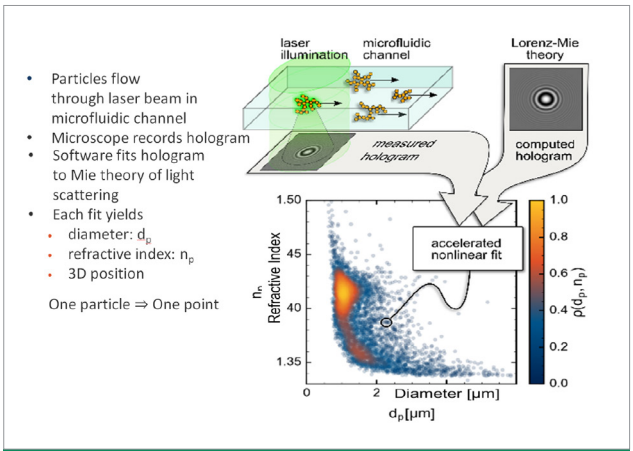


Figure 8

outputs (both quantitative and qualitative) that are unique to each method.

Analytical methods:

The following is a brief overview of the analytical technologies that was provided by courtesy of each manufacturer.

MANTA – According to the manufacturer, Multispectral Advanced Nanoparticle Tracking Analysis covers particles ranging from 10 nm to 15 µm (Figure 5).

Single Particle Optical Sizing (SPOS) – this instrument uses two sensors; a light scattering detector to measure from 0.15-0.6 µm and a Light Obscuration (LO) sensor to achieve a combined range of 0.15 to 25 µm (Figure 6).

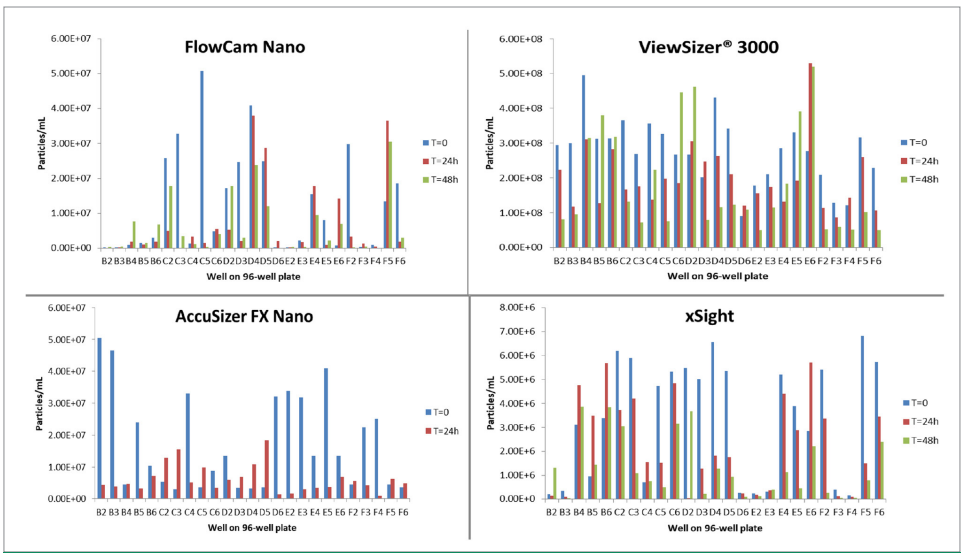
Nano-Flow Imaging (also known as oil immersion flow microscopy) - uses oil immersion technology to extend dynamic range of conventional flow imaging microscopy, which enables it to capture and analyze images of particles ranging from 0.3 to 10µm (Figure 7).

Total Holographic Characterization (also known as Holographic Video Microscopy) - measures size and refractive index of particles on a single-particle basis. It is effective for detecting and counting particles ranging from 0.5 to 10µm (Figure 8).

Data analysis: Data generated from the 4 instruments were analyzed using DOE software. The results are presented by three dimensional response surface diagrams, which provide an intuitive description of formulation design space for the NISTmAb.

Results

In this study, the rate of particle formation in NISTmAb under 25 solution conditions was monitored for 48 hours using 4 orthogonal analytical technologies. Each well in the iFormulate™'s 96-well plate contains a unique formulation, which enables a direct comparison of the data from different technologies under a more diverse number of solution conditions. Normally, when a protein is exposed to thermal stress its three dimensional conformation becomes perturbed along with increased exposure of the hydrophobic amino acids in its interior, which increases its propensity for aggregation. Depending on the protein's inherent conformational and colloidal stability as well as solution condition (formulation), aggregation



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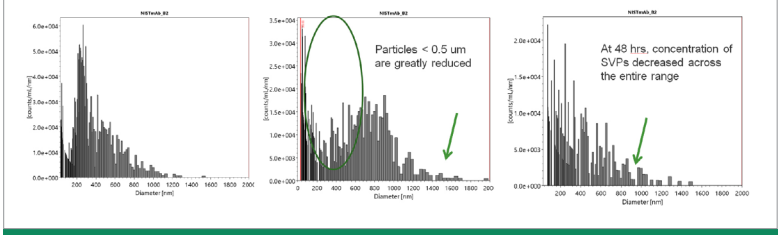

Figure 10

will occur at different rates[13]. The prevailing thought is that when the soluble aggregates reaches a certain size and exceeds its equilibrium solubility, phase separation occurs and insoluble particles are formed[14]. SVP analytical technologies that monitor micron-sized particles have been immensely useful for quantifying these particles; and in most cases one will see a progressive or rapid increase in SVP concentration corresponding with the length of exposure to stress. The results from this study, however, were not as expected. Figure 9 shows the composite data gathered by the four technologies. Surprisingly, all four instruments showed that, when NISTmAb was exposed to stress by isothermal incubation, the total concentration of SVPs actually decreased in the vast majority of formulations (Figure 9).

Even though this trend was observed for all the technologies, it was still tempting to attribute these surprising results to the experimental design, analyst error, or even the possibility that NISTmAb is not the appropriate protein for this experiment. If protein aggregation is expected to result in the formation of more SVPs, why was there a decrease in total particle concentration? The answer to this question can be found in the detailed quantitative and qualitative information that are provided by each technology.

First, with a quick glance of the T=0 size distribution data gathered by Multispectral Advanced Nanoparticle Tracking Analysis (MANTA), one can see that the concentration of sub-micron particles was exponentially higher than micron-sized particles at T=0 (Figure 10). After 24 hours of incubation, there was a small increase in particles that are in the 1 to 2 um size range. At the same time, however, the concentration of particles smaller than 0.5 um showed a dramatic decrease. Based on the finding from this method, it appears that thermal stress-induced aggregation of the mAb led to assembly of the sub-0.5 um particles into larger sub-micron and micron-sized particles. Since the concentration of sub-0.5 um particles was much higher at T=0, agglomeration of these small particles resulted in an increase in particles that are relatively larger (~ 0.8-1.6 um) but actually a decrease in the total number of subvisible particles in the solution. This effect is even more apparent after 48 hours as the concentration of particles is further reduced across the entire range. One can hypothesize that this is due to extensive aggregation of the protein, which

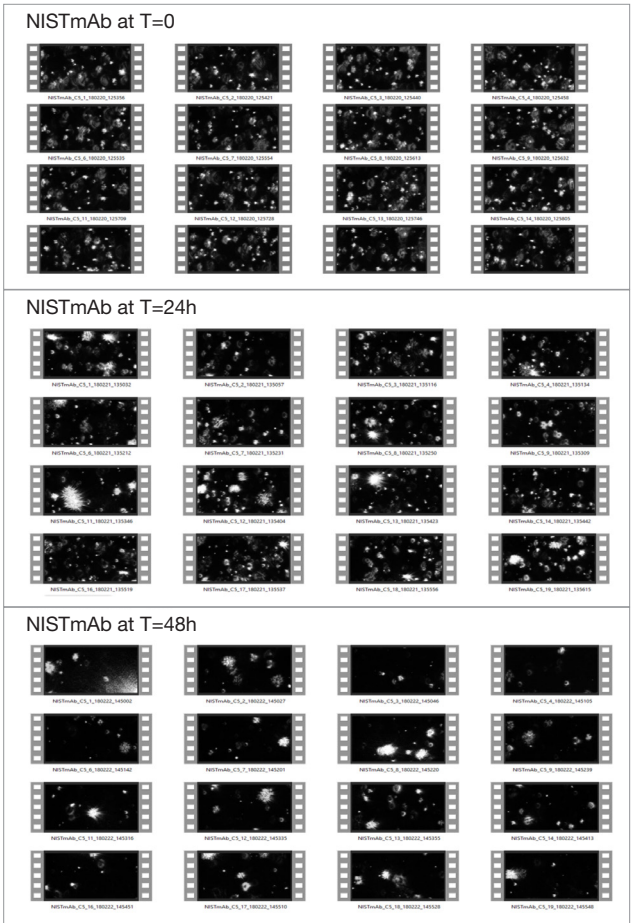

Figure 11

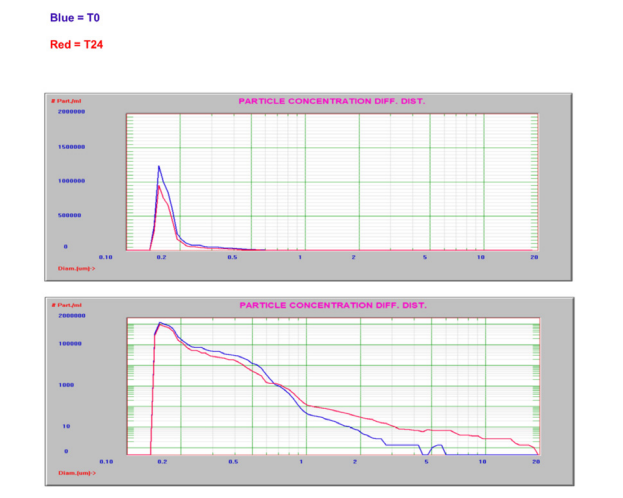

Figure 12

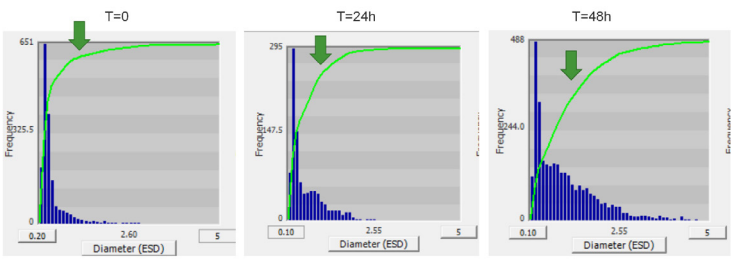

Figure 13

leads to generation of particles that are simply too large to be detected by MANTA.

One piece of evidence that supports this hypothesis can be found in the snapshots from the video recordings obtained by MANTA (Figure 11).

One can see that as time progresses the number of small particles captured by the instrument becomes much lower; but at the same time a few larger particles have formed. In order for a particle to be detected by MANTA it must exhibit Brownian motion[15]. It appears that in the case with NISTmAb, as the protein aggregates, the particles formed become progressively larger and larger until they disappeared from view. One possible reason for these findings is that, once a particle reaches a certain size (approximately 1.5 - 2 um as shown in this experiment) it longer moves under Brownian motion; and there is an increased tendency for it sediment on the bottom of the sample cell and become undetectable by the instrument. Based on the video images from MANTA, this is likely the reason why the number of particles detected was significantly lower after 48 hours of stress exposure.

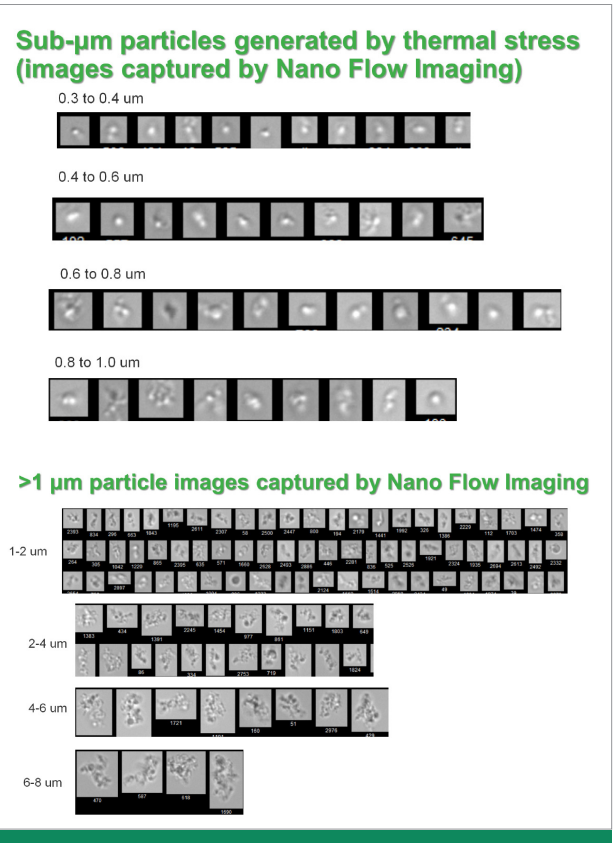

Figure 14

After being surprised by the initial data from one analytical technology, we proceeded to examine the results obtained by Single Particle Optical Sizing (SPOS). As one can see in Figure 12, raw size distribution data (top graph) showed that T=24 hour sample has fewer particles than the sample at T=0. Again, this was inconsistent with our original expectation; however, due to the fact that orthogonal analytical techniques were available, we were able to investigate further. In this case, simply by showing the particle concentration (y-axis) of the size distribution graph in log scale (lower graph), we can see that there was indeed a population redistribution from the smallest particles (<0.7 um) to larger particles (from >0.7 um up to 20 um) when NISTmAb was exposed to thermal stress. These results corroborate well with the conclusions drawn from the data obtained by MANTA. In fact, SPOS data proved that the stressed sample did contain significant number of particles that are larger than 2 um, which were not detected by MANTA.

Next, we reviewed the Nano-Flow Imaging size distribution data for one of the formulations across the 3 storage time points. As can be seen in Figure 13, consistent with the results from MANTA and SPOS, we saw a reduction in the concentration of submicron particles, along with a gradual increase in the concentration of particles above 1 um in size. Instead of causing particle numbers to increase, isothermal incubation actually resulted in a decrease in total particle concentration. The same trend was observed for many of the formulations tested. Nano-Flow Imaging appears to be suitable for monitoring submicron particles and micron-sized particles simultaneously; its distinguishing feature is that the images that are captured can provide additional dimensions of information based on morphological features of the particles that are detected (Figure 14). Due to recent advancements in this technology, its imaging capability has now been extended to size ranges that were unobtainable in the past.

The fourth and final technology that was evaluated in this study is Total Holographic Characterization (THC). With THC, particles pass through a laser beam in a microfluidic channel and the hologram produced by the particle is recorded by a microscope and fitted according to Mie theory of light scattering. The result is that for the

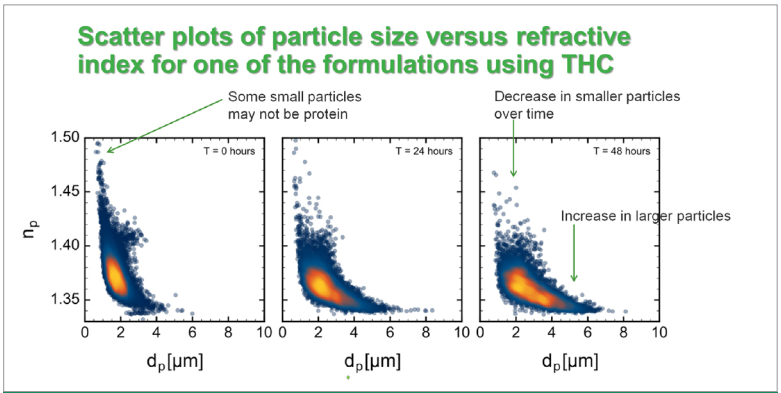

Figure 15

particle detected, information on size and refractive index can derived on an individual basis[16]. Since protein drug products often contain impurities that are not proteinaceous and have refractive index within a range that is different from protein particles, the added information on refractive index means that there is also the potential to use THC to differentiate non-proteinaceous particles (e.g., silicone oil, etc.) from protein particles[17].

Looking at the particle concentration data gathered by THC from T=0 to 48 hours, it is apparent that the changes follow the same pattern that was shown by the other 3 technologies (Figure 9). There is no question that the total particle concentration decreased in the majority of formulations over the course of 48 hours. A review of the scattered plots from THC for each time point provides better insights into this dynamic (Figure 15):

By following the changes that were recorded at each time point, we can see that aggregation of NISTmAb results in the population of SVPs shifting from smaller particles to larger particles that have a lower refractive index (n_p). There is a decrease in particles <2 um with a concomitant increase in particles in the 2 to 8 um size range. Unless one follows the protein aggregation process by implementing analytical methods that can monitor this particle population redistribution across a sufficiently wide size range, it is easy to be misled by simply counting particles. Together, these data illustrate that, by carefully examining all the information that can be extracted by each analytical technology, one can avoid making an erroneous conclusion by relying on particle concentration data alone. Sub-micron particle is clearly a highly sensitive and early indicator of protein aggregation. The actual change in particle number concentration (increase or decrease), however, can be dependent on the protein, the specific stress condition applied, as well as the time of sample analysis.

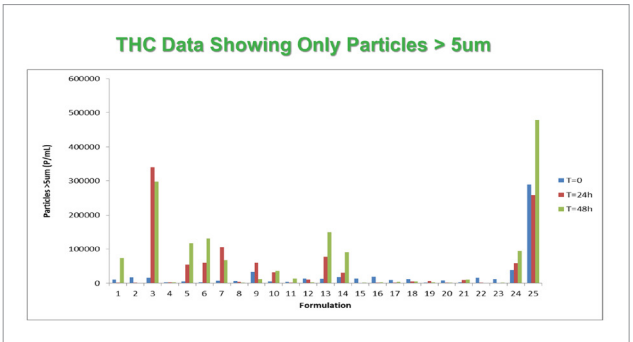

Figure 16

The final goal is this study was to determine whether the particle data obtained from the instruments can be helpful in selecting a suitable formulation for NISTmAb. To achieve this objective, we tested the stability of NISTmAb in 25 solution conditions using Design of Experiments (DOE), a statistical method that uses pre-chosen solution conditions based on multivariable experimental response-surface design that investigates effect of pH, ionic strength, buffer concentration, and stabilizer concentration, with the goal of obtaining a formulation design space for the protein in one experiment[18]. In this case, the ability of a formulation to control the growth (in number and/or size) of subvisible particles was the main criterion for selection. Since all 4 analytical methods indicate that micron-sized particles were increasing over time despite the apparent decrease in total SVP concentration, we reanalyzed the data to only show the changes in the concentration of particles in the micron range. Figure 16 shows THC data from all 25 formulations when we only consider the changes in the concentration of SVPs that are equal or greater than 5 um. As expected, some of the formulations saw a large increase in SVP concentration during the 48 hour incubation period; these formulations would be classified as sub-optimal formulations. Conversely, there were formulations (e.g., formulations 15 to 23) that saw essentially no increase in particles larger than 5 um (Figure 16). When these data were inputted into the DOE software, the result is a 3 dimensional response surface diagram that shows how different formulation variables (e.g., pH, ionic strength, etc.) influence particle concentration, the rate of particle formation as well as any interaction that may exist between the solution variables (Figure 17).

In Figure 17, we can see that when particle concentration (for 5 um or larger particles) is the only performance criterion for formulation selection, the optimal solution pH for controlling particle formation in NISTmAb is around pH 5. Also, at this pH buffer concentration is not critical since it has minimal impact on stability. To take into account the observed dynamic relationship between sub-micron particles and micron-sized particles, we also performed

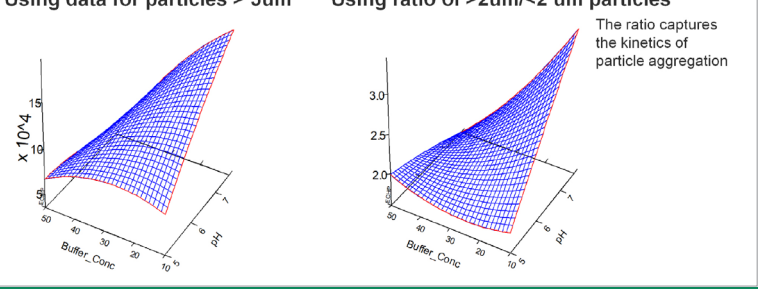

Figure 17

DOE analysis using the ratio between particles greater than 2 um and particles smaller than 2 um (>2um/<2um). This ratio captures the population shift from sub-micron particles to micron particles across all the time points; at the same time it can also serve as an indicator of protein stability in each formulation. Remarkably, the results of DOE analysis show that the best formulation for reducing growth of > 5 um particles is highly similar to the one that minimizes the >2um/<2um ratio (Figure 17). With these results we have now demonstrated how novel analytical technologies, when combined with proper experimental design, data interpretation, and implementation of DOE, can be highly useful in protein formulation development.

Conclusions

In this article, we described the findings from an investigation that was undertaken to compare data from 4 SVP analytical methods using the same protocol and similar material. Contrary to our original expectation, many samples in the study showed a decrease in particle concentration after exposure to thermal stress. Based on both quantitative and qualitative data gathered by the different instruments, it was proven that, while isothermal incubation led to an decrease in the total number of particles in many samples, this phenomenon can be attributed to a population redistribution when the smallest particles, which were far more abundant in the beginning, consolidated into fewer larger particles over the course of the study. In light of this knowledge, we have gained significant insights on the utility of particulate data in the sub-micron range and their potential relationship with micron-sized particles. Oftentimes, investigators gather data in the sub-micron and micron size ranges using separate instruments that are based on different principles of detection, which can lead to difficulties in understanding the dynamic relationship between the two populations. The potential pitfall of relying on only submicron particulate data is that one may make an incorrect assessment about the stability of a sample due to missing information (i.e., whether larger particles are actually increasing). The results from this study underscore the importance of simultaneously monitoring both sub-micron and micron-sized SVPs, ideally with an instrument that can cover both size ranges. We have also shown how each of the 4 orthogonal methods evaluated in this study contributed unique and important insights that extended our ability to understand the underlying phenomenon behind our findings. Using data from analytical technologies that have different principles of detection and a wider dynamic range, we have successfully demonstrated how an orthogonal approach to SVP characterization can enable a better understanding of the various potential mechanisms of subvisible particle formation. By taking advantage of these new insights, we were able to quickly identify the most suitable formulation for NISTmAb. Therefore, this study provides further evidence to support the use of orthogonal methods for SVP characterization and protein formulation development.

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Acknowledgments

The author would like to thank the following individuals and companies for their contribution this study: Camilo Denbrok and Kathryn H Roache-Johnson (Yokogawa Fluid Imaging Technology), Julie Chen Nguyen (Horiba Instruments), Mark Bumiller (Entegris Inc.), Rajiv Nayar (HTD Biosystems), and Laura Phillips (Spheryx Inc.).