

# **The Sensitive Method of Subvisible Particle Counting for the Detection and Quantification of Monoclonal Antibody Aggregation Caused by Freeze-Thawing: New Understanding of Particle Function in the Protein Aggregation Process**

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## **Abstract**

The purpose of this research was to measure the amount of subvisible particles formed throughout the freeze-thaw cycle of an IgG2 monoclonal antibody (mAb) using microflow imaging (MFI), a sensitive technique. Protein solutions in 20 mM histidine buffer (pH 5.5) were frozen and thawed three times before being examined using multiple-fraction isolation (MFI) and size-exclusion chromatography (SEC). While SEC could not identify aggregates, MFI demonstrated an increase in particle counts with each freeze-thaw cycle. Monitoring particle production enables the identification of protein aggregates containing just a tenth of a percent of the total protein mass, according to estimates of the total mass of particles generated. Even while SEC did not identify protein aggregation, variations in levels caused by various formulations or freeze-thaw

protocols were addressed. The purpose of the freeze-thaw process in phosphate-buffered saline was to determine whether the total aggregate mass estimates derived from SEC and MFI were quantitatively compatible. This procedure reduced the monomer peak area in the chromatogram, which allowed SEC to identify insoluble aggregates at a detectable level. The amount of monomer lost as measured by SEC and the total mass of subvisible particles as measured by MFI were in excellent agreement. The following is a copyright notice from Wiley-Liss, Inc. and the American Pharmacists Association: J Pharm Sci 100:492-503, 2011

Protein formulation, infrared spectroscopy, particle size, liquid chromatography, and protein aggregation are all relevant terms.

Introduction :

A decrease in product purity and quality and the possibility that aggregates may induce an immunogenic response in patients make therapeutic protein aggregation a big concern.<sup>1</sup> Proteins in solution may aggregate due to a variety of stressors, including heat, agitation, light, surface contact, and freeze-thaw cycles.<sup>2–8</sup> Most frequently, the resultant aggregation and loss of native protein may be recognized and quantified.

evaluated using size-exclusion chromatography (SEC). One drawback of utilizing SEC to detect aggregates is that it can only detect aggregates within a very small size range, around 5 to 1000 kDa, which is considered soluble.<sup>2</sup> One other drawback of SEC is that it takes around 0.1% to 0.5% of the total protein to be soluble aggregates and/or native protein lost before a change can be accurately detected in practice.<sup>9</sup>

Insoluble aggregates may also be indirectly quantified using size-exclusion chromatography. These aggregates are usually operationally characterized as those that can be extracted from solutions by means of filtering or short centrifugation at relatively low *g* values. The SEC quantification relies on the area loss for protein peaks in supernatant chromatograms or filter out.<sup>six, ten</sup> An indirect way to quantify the production of insoluble aggregates is by comparing the treated sample to the control sample and measuring the loss in area. However, before a change can be reliably detected and quantified, a significant amount of aggregates—approximately >1% of the total protein in the original solution—often needs to be formed. This is because the approach is an indirect measurement of aggregate

formation, and because of the limitations with SEC that were mentioned earlier.<sup>9</sup>

Turbidity has been measured using a variety of methods in other research to track the stress-induced formation of big (and perhaps intractable) aggregates.<sup>4, 11–13</sup> An rise in the sample's optical density can be noticed only when a large portion of the protein is in aggregate form, which is also required for turbidity. This method cannot provide quantitative information on the mass of aggregates in a specific sample as turbidity is dependent on both the concentrations and the sizes of the particles.

Multiple methods for counting subvisible particles have also proven successful in detecting and quantifying very big protein aggregates.<sup>3,14,11,13, and 6,7</sup> Previous research has shown that counting visible particles may be a more sensitive method for identifying and quantifying large protein aggregates than measuring turbidity or the loss of native protein. One research that looked at how stirring affected aggregate formation found that the saturation limit (around 18,000 particles/mL) was achieved or approached before a particle counting technique (light obscuration) showed a modest rise in turbidity.<sup>4</sup> The stress-induced aggregation methodology allowed for the reliable quantification of subvisible particles at much earlier time periods. Another experiment used a positive displacement pump to create hundreds of thousands of subvisible particles per milliliter by passing an IgG antibody through it. On the other hand, SEC did not reveal any soluble aggregates. The natural protein-based reduction in peak area in chromatograms from SEC was lost statistically insignificantly as a consequence of pumping.<sup>15</sup>

Based on these findings, we suggest that subvisible particle formation monitoring is a far more sensitive method than turbidity measurements or SEC for detecting and measuring levels of big protein aggregates. The first portion of the present investigation employed moderate freeze-thawing stress conditions to investigate the sensitivity of subvisible particle counting for the detection of large protein aggregates. We aimed to aggregate a small subset of the protein molecules in the sample. This was accomplished by creating an IgG2 mAb in a solution of 20 mM histidine (pH5.5), then freezing and thawing it three times. Following each cycle of freezing and thawing, the samples were examined using SEC, and microflow imaging (MFI) was used to count the subvisible particles.

We used more demanding conditions and freeze-thawed the protein in phosphate-buffered saline (PBS) to generate greater quantities of subvisible particles. The SEC examination of the supernatant in centrifuged samples allowed for the easy quantification of monomer loss since this procedure transformed a significant proportion of protein to insoluble clumps. We compared the mass of aggregates found in subvisible particles calculated from particle counts with that found in SEC analysis. We were also able to track the growth of new particles and aggregate mass as a function of many freeze-thaw cycles by using the predicted protein mass population distribution in subvisible particles.

## **CONTENT AND APPROACH Materials and methods**

The antibody solution was supplied by Pfizer of Chesterfield, Missouri, USA, in

bulk, with a protein content of 20 mg/mL. A solution of 10 mM histidine, 222 mM trehalose dihydrate, and 0.02% polysorbate 80 (PS80) was prepared to make the bulk drug material. The pH of the solution was set at 5.5. The material was transported and kept at a temperature of 5° degrees Celsius. Mallinckrodt Baker of Phillipsburg, New Jersey, supplied the sodium phosphate monobasic, sodium phosphate dibasic, and trihydrate of trehalose. Chemicals such as histidine, histidine HCl, Tris, glycine, sodium chloride, and PS80 were acquired from Fisher Scientific in Fair Lawn, New Jersey. Millipore of Cork, Ireland, supplied the Amicon Ultra centrifugal filter devices.

## **Formulations**

The following solutions were used to create the protein:

First, 20 mM of histidine at pH 5.5 with 20 mg/mL of IgG2; second, 20 mM of histidine with 0.02% PS80 at pH 5.5 and 20 mg/mL of IgG2; third, 20 mM of histidine with 222 mM of trehalose dihydrate and 0.02% PS80 at pH 5.5 and 20 mg/mL of IgG2; and fourth, 10 mM of sodium phosphate and 140 mM of sodium chloride at pH 7.0 with 1 mg/mL of IgG2.

We used a Pharmacia FPLC machine and a Protein A Sepharose column from GE Healthcare in Piscataway, NJ, to remove the surfactant from the bulk IgG2 solution. This allowed us to make formulations without PS80. A mobile phase containing 20 mM Tris (pH 7.5) was used to trap the protein on the column. At least five column volumes (125 mL) of mobile phase were used to wash the captured protein. Then, 100 mM of glycine (pH 3.5) was used to elute the protein. Filters from Amicon 5000 MWCO Ultra 15 were used to transfer the protein

solution from the collection step into the correct formulation buffer.

### Methods for Freezing and Thawing

With a fill volume of 20 mL, 60 mL grade 316 stainless steel containers were filled with aliquots of the formed mAb. With each formulation that underwent the freeze-thaw condition, a minimum of three duplicate samples were used, with each sample being kept in its own tank.

The first freeze-thawing tests included putting the sample tanks in a freezer set to 20°C for the night. Thawing the samples was done by setting the tanks on the lab bench and letting them sit at room temperature for three hours. The freeze-thaw cycle was not applied to a control tank that was kept at 5°C. To guarantee a representative sample was collected, the protein formulation in the tank was gently swirled after thawing using a 1-mL pipette tip.

After the material had thawed, 1-mL portions were extracted and re-used for analysis. There were a total of three cycles of freezing and thawing the tanks, after which the procedure was repeated.

The samples were frozen in another series of studies by submerging the tanks in liquid nitrogen for around 10 minutes. The samples were defrosted by putting the containers in a fridge set at 5°C for about 48 hours. For three cycles, we followed this technique, removing 1-mL aliquots after each thawing cycle with moderate stirring.

### Chromatography by Size Exclusion

Using a Tosoh G3000 SWXL 7.8 30-cm column with an in-line 0.22-µm filter, the Agilent 1100 or 1090 chromatography system from Santa Clara, CA was used. The

mobile phase used for elution consisted of 50 mM NaCl, 200 mM Sodium phosphate, and a pH of 7.0. The rate of flow was 0.7 mL/min during the 20-minute period. Elution was monitored by absorbance at 280 nm, and 20 µg of protein was put onto the column for each sample injection. The quantities of monomers and dimers were determined by dividing the total areas of the protein peaks by their respective totals. The following equation (Eq. 1) was used to determine the total protein recovery of each injected sample:

$$\frac{\text{Amount loaded}}{\text{Total relevant area} \times (\text{Flow rate} / 60)} = \frac{e \times \text{Path length}}{\text{mg}} \quad (1)$$

where flow rate = 0.7 mL/min; total relevant area = integrated area under the curve at 280 nm;  $e = 1.4 \text{ cm}^2/\text{mg}$ ; and path length = 1 cm.

### Particle Measurements

A Brightwell (Ottawa, ON, Canada) 4100 MFI system was used for particle counting and sizing measurements. For formulations 1 to 3, the instrument configuration was in “setpoint 3” mode, allowing for a minimum particle size detection of 1 µm. For formulation 4, low-magnification mode was used, allowing for a detection limit of 2 µm for particles. A higher volume flow cell is used in the low magnification mode. This mode was chosen to reduce clogging during analysis due to the large concentrations of particles generated by the formulation. The estimated mass of protein in particles was calculated in both setups using particles up to 50 µm in size, with bin increments of 0.125 µm. A Bel-Art degassing chamber from Pequann, NJ was used to degas the samples for a duration of 15 minutes. It was decided to keep the samples on ice

until processing. In order to determine the particle counts per milliliter, a 500- $\mu$ L sample was examined. In order to determine the particle count error, the independent duplicates had their mean and standard deviation for particle sizes ranging from 1.125 to 10  $\mu$ m and from 10 to 50  $\mu$ m determined. For every size category, we determined the %CV by dividing the standard deviation by the mean and then multiplying by 100.

In a separate experiment, three separate measurements were taken from the same sample using either a 500  $\mu$ L or 3 mL analytical volume to determine the variability of particle counts. As mentioned before, the calculation for the inaccuracy for these measurements was carried out.

### Protein Mass Estimation in Particles

We choose to represent particle size as an equivalent circular diameter in our data analysis using the Brightwell instrument software. This is the diameter of a circle that occupies the same pixel area as the actual pixel area of the particle observed. For each particle size bin, the volume of a sphere was calculated using the reported diameter. A density value of 1.43  $\pm$  0.03 g/mL has been recorded for over 30 distinct proteins.<sup>16</sup> Due to the presence of both protein and water in protein aggregates, their density is anticipated to fall between the range of 1.0 for water and the density of the monomeric protein. So, we guessed at random that there was 75% protein and 25% water in each particle. We estimated the mass of the protein by multiplying its density (1.43 g/mL) by its volume fraction estimate (0.75) and the volume of a sphere for each size bin. To get the mass per milliliter for each bin, we multiplied this mass by the particle detection rate in milliliters for each size bin

(Eq. 1). The sample's overall and integral masses were calculated by integrating these mass estimations throughout the whole particle size range.

$$\begin{aligned} \text{Estimated protein mass per size bin} \\ = (0.75) \times (\text{Volume}) \times (1.43 \text{ g/mL}) \\ \times (\text{Number of particles}) \end{aligned}$$

### Imaging using Infrared Light

A 1-mL portion of the PBS-based sample was transferred after the third freeze-thaw cycle and centrifuged at 14,000 g for 10 minutes. After removing the supernatant, 300  $\mu$ L of deionized water was used to wash the particle three times. For the next step, a pipette was used to spread the pellet over the infrared cell window of a Bio-Cell. Following the procedures outlined by Dong et al., we acquired and processed spectra.<sup>17</sup>

### Analytical Ultracentrifugation for Sedimentation Velocity

To make sure our SEC approach recognized soluble aggregate species properly, we employed sedimentation velocity analytical ultracentrifugation (SV-AUC). The experiment was carried out on protein samples that included 10 mM histidine, 222 mM trehalose dehydrate, and 0.01% PS80. For this analysis, we used a Beckman Optima XL-A analytical ultracentrifuge manufactured in Brea, California. The protein concentration was brought down to 0.5 mg/mL using a formulation buffer from both an unfrozen control sample and a sample that had been freeze-thawed three times. Using the SEC mobile phase previously described, the material that was freeze-thawed three times was further diluted to a protein concentration of 0.5 mg/mL. The sedimentation process was carried out at a rotor speed of 128,800 g. We

utilized SED-FIT, Version 1180, which can be obtained at NIH.gov, to model the raw absorbance data. Obtaining  $c(s)$  distributions is described in depth by Arthur et al.<sup>18</sup>, who employed model fitting parameters.

### **The outcomes Mass Calculations in Theory**

To investigate the possibility of using the estimated mass of protein in particles as a sensitivity measure for aggregated protein, we computed the mass of the particles (Eq. 2). For protein particles in the size range of 1 to 2  $\mu\text{m}$ , it takes hundreds of thousands to millions of particles to equal a mass of 1 g, in contrast to the about 15 particles of 50  $\mu\text{m}$  size that represent around 1 g total mass. As a result, particle counting offers a very sensitive way to track the process of protein aggregation formation.

### **Histidine-Buffered Formulations for Freezing mAb Samples**

From 20°C to room temperature, freezing and thawing the mAb solutions produced thousands of particles per milliliter after the first cycle, and the number of particles increased with the two subsequent cycles, even in formulations that provided significant stabilization to the mAb (Fig. 1). Also, throughout the freeze-thaw process, the particles generated in all three formulations were mostly smaller than 5  $\mu\text{m}$ . Imaging using Infrared Radiation

Centrifuged at 14,000 g for 10 minutes, a 1-mL aliquot of the PBS-based sample was transferred after the third freeze-thaw cycle. Following the removal of the supernatant, the particle was washed three times using 300 : L of deionized water. The next step included spreading the pellet across the Bio-infrared Cell's cell window using a pipette.

We collected and analyzed spectra in accordance with the methods described by Dong et al. Analysis of Sedimentation Velocity via Analytical Ultracentrifugation (17)

We used sedimentation velocity analytical ultracentrifugation (SV-AUC) to verify that our SEC method correctly identified soluble aggregate species. Samples of proteins with 10 mM histidine, 222 mM trehalose dehydrate, and 0.01% PS80 were used in the procedure. Our analytical ultracentrifuge for this study was a Brea, California-made Beckman Optima XL-A. The protein content was reduced to 0.5 mg/mL by using a formulation buffer derived from a control sample that had not been frozen and another sample that had undergone three cycles of freezing and thawing. The sample that had been freeze-thawed three times was further diluted to a protein concentration of 0.5 mg/mL using the SEC mobile phase that had been previously described. With a rotor speed of 128,800 g, the sedimentation process was executed. For this study, we modeled the raw absorbance data using SED-FIT, Version 1180, which is available at NIH.gov. Arthur et al.<sup>18</sup> provides a detailed description of the process of obtaining  $c(s)$  distributions using model fitting parameters.

### **The results Theoretical Mass Calculations**

By calculating the mass of the particles (Eq. 2), we aimed to examine the feasibility of use this mass as a sensitivity measure for aggregated protein. In contrast to the about 15 particles of 50  $\mu\text{m}$  size that constitute roughly 1 g total mass, hundreds of thousands to millions of smaller protein particles are needed to achieve a mass of 1 g. Therefore, particle counting provides a

very sensitive method for monitoring the aggregation forming process of proteins.

### **Anti-Freeze mAb Samples in Histidine-Buffered Formulations**

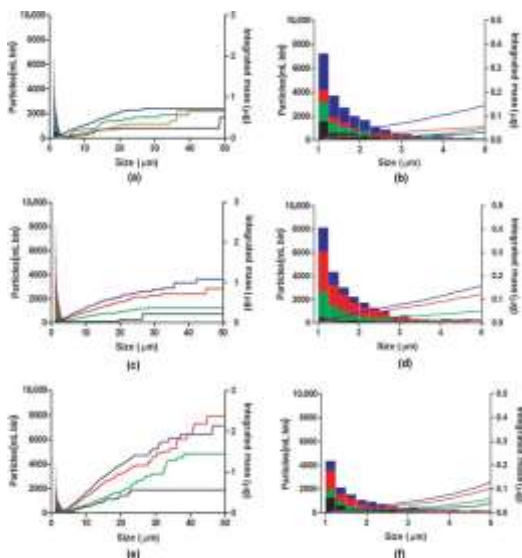
The number of particles per milliliter was thousands after the first cycle of freezing and thawing the mAb solutions from 20°C to room temperature. This number continued to rise with the two cycles that followed, even in formulations that significantly stabilized the mAb (Fig. 1). Particles produced by the freeze-thaw cycle in all three formulations were mostly less than 5 : m in size.

size, with over 95% of the total particles found to be less than 10 : m. Particles in this size range contributed a rather little amount to the overall mass, even though they were quite numerous (Fig. 1). All three formulations had their x-axis cut at 5 : m for better data visualization and to highlight these spots (Figs. 1b, 1d, and 1f). Figures 1b, 1d, and 1f show that particles ranging in size from 1.25 to 5 m still had an integrated mass below 0.2 g even after three freeze-thaw cycles. Particles with a diameter larger than 5 : m were responsible for the majority of the mass contributions, despite the fact that their quantities were very small.

Figure 1 shows that after three freeze-thaw cycles, the particle distributions and total mass of the histidine and histidine + PS80 formulations were quite comparable. The freeze-thaw cycle significantly reduced particle counts with the histidine plus trehalose and PS80 formulation (Figs. 1e and 1f). Figures 1e and 1f show that more particles in the larger size range were formed, resulting in a higher overall mass of particles compared to the other formulations.

Figure 2 shows that compared to freezing at 20°C and thawing at ambient temperature, freezing in liquid nitrogen and thawing at 5°C produced much more particles with a larger mass. Take note of the size disparity since in the two Image 1 and image 2. Similar to the results shown with the 20°C to room temperature freeze-thawing, the particle count distributions were such that over 95% of the particles were less than 10 : m in diameter. The total number of particles produced was significantly higher in the presence of PS80 compared to histidine alone (Figs. 2a-2d). On the other hand, the histidine plus PS80 production inhibited the creation of bigger particles (Figs. 2a-2d). Hence, the histidine-only formulation had a higher total protein mass after three freeze-thaw cycles, even if the particles had a higher number of them. Just like the findings from 20°C to room temperature, adding trehalose caused a decrease in the total number of particles but an increase in the amount of protein in those particles (Figs. 2e and 2f). The histidine plus trehalose mixture resulted in a higher production of the bigger particles, which is the reason for this.

It was unexpected to see that the total mass of particles generated after the first freeze-thaw cycle was much higher than the total mass of particles during the third freeze-thaw cycle in the histidine plus PS80 simulation (Figs. 2c and 2d). Compared to the other two sample replicates, one showed significantly higher particle counts during the first freeze-thaw cycle (Fig. 3). Possible causes of this phenomenon include ice nucleation temperature, directed solidification, and other undetectable variations in key parameters throughout the freeze-thaw process.



**Figure 1.** Particle distribution (left y-axis) and integrated mass data (right y-axis) for IgG<sub>2</sub> samples frozen and thawed from 20°C to room temperature. Formulations tested were: histidine formulation (a); histidine formulation results with truncated x-axis (b); histidine, polysorbate 80 (PS80) formulation (c); histidine PS80 formulation results with truncated x-axis (d); histidine, trehalose, PS80 formulation (e); and histidine, trehalose, PS80 results with truncated

x-axis (f). In all panels, particle counts are for before freeze-thawing (black), one freeze-thaw cycle (green), two freeze-thaw cycles (red), and three freeze-thaw cycles (blue). For all reference to color in the legends, the reader can obtain the web version of the article.

concentrate, and gradients of concentration, and freeze. The particle counts and total mass of protein in particles might be significantly impacted by a little variation in the mass percentage that was converted into particles during the freeze-thaw process, as only a small fraction of the protein

molecules, about less than 0.1%, were transformed into particles. Whatever the reason may be for the greater particle count in one sample, these findings highlight the need of doing tests on duplicate samples. We computed %CV for all formulations for each to get a feel for how repeatable the findings were. Fig. 4a shows the freeze-thaw cycle. For particle counts ranging from 1 to 10 m and from 10 to 50 m, the %CV was typically around 20% and 50%, respectively. However, during the first freeze-thaw cycle using the histidine plus PS80 formulation, the percentage of chlorine gas was 40% in the 1- to 10-meter area and 60% in the 10- to 50-meter range.

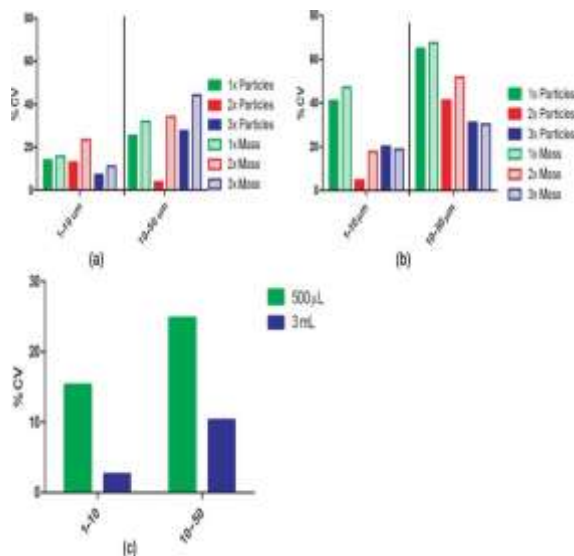
Important cautions must be considered when interpreting the total mass estimations due to the higher %CV values seen for particle counts in the 10- to 50- m range. As previously stated, almost 95% of the

There was no discernible decrease in soluble protein as measured by SEC, accounting for 0% of the total protein mass in solution.

Selected samples underwent sedimentation velocity analytical ultracentrifuge to guarantee reliable detection of aggregated species by SEC. After being freeze-thawed three times, the protein was diluted to 0.5 mg/mL in either the formulation buffer or the SEC mobile phase after being formed in histidine, trehalose, and PS80. A control sample that was not frozen was likewise diluted to a concentration of 0.5 mg/mL using the formulation buffer. The findings from SV-AUC analysis did not reveal the presence of higher order oligomers in any of the samples, which is in line with what was found in SEC (data not shown). The dimer level (about 0.4%) in these samples is below the detection limit of this approach, hence it

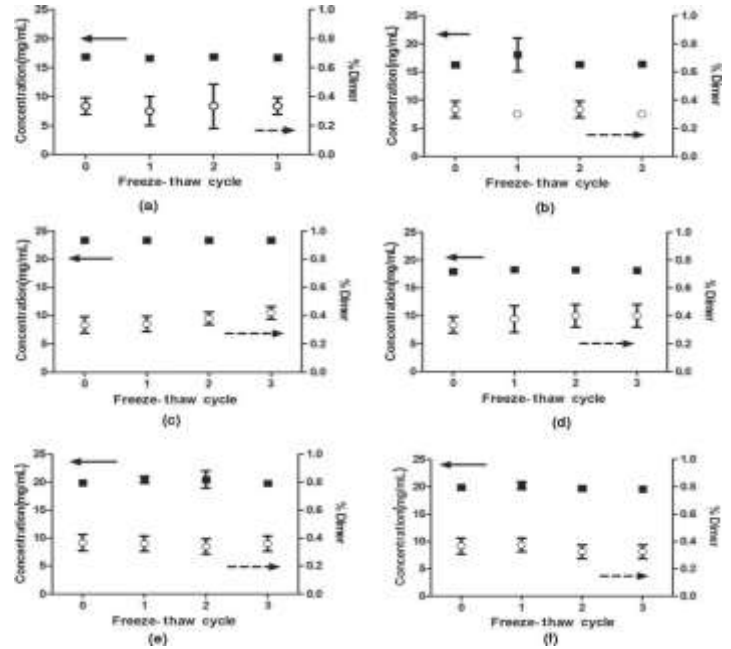
is not unexpected that SV-AUC could not detect dimer either.<sup>18</sup>

The classic aggregation pathway, in which monomeric protein combines to form dimers and progressively higher order species, has been described in more detail by others<sup>2,19,20</sup>. It may seem at odds with this theory that no increase in soluble aggregates was detected under experimental conditions where increases in particle counts were observed. But the particles found only make up a tenth of a percent of the protein in the solution, as stated before. Thus, in the event that



**Figure 2** Error of particle counts and mass estimates reported as %CV. Panel (a) shows the error observed between replicates of the histidine, polysorbate 80 (PS80), trehalose formulation frozen and thawed from 20°C to room temperature and is representative of the typical amount of error observed. Panel (b) shows the error observed between replicates of the histidine, PS80 formulation frozen and

thawed from 196°C to 5°C. Panel (c) shows the error of three replicates from the same sample using sampling volumes of 500:Lo or



**Figure 3** Size-exclusion chromatography results for histidine formulations, 20°C to room temperature (a); histidine formulation, 196°C to 5°C (a); histidine, polysorbate 80 (PS80) formulation, 20°C to room temperature (c); histidine, PS80 formulation, 196°C to 5°C (d); histidine, trehalose, PS80 formulation, 20°C to room temperature (e); and histidine, trehalose, PS80 formulation, 196°C to 5°C (f). Closed square symbols are the soluble protein detected (left-axis) and the open circles are % dimer detected (right-axis). Error bars represent the standard deviation of three independent replicates.

The formation of the subvisible particles was caused by soluble aggregates, the quantities of which would be far lower than the detection limit of SEC.

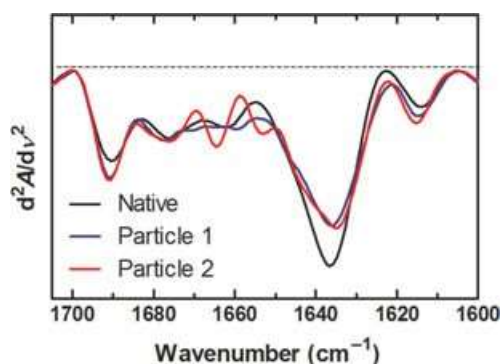
Sample Freezing and Thawing in Phosphate-Buffered Saline

The purpose of the freezing and thawing process in PBS, which has a pH of 7.0, was to intentionally produce a high concentration of protein particles. It is possible to eliminate these particles.

centrifugation removed the monomer from the solution, and SEC measured the amount of monomer reduced in the supernatant. Thus, these particles might be categorized as "insoluble aggregates" according to the operational definition. Particle counting was used to determine the amount of protein in the particles, and this mass was compared with the mass of monomer reduction in the supernatant. At a relatively low protein content (1 mg/mL), the protein formulation was frozen and thawed in PBS from 20°C to room temperature. The

### Talking about

In contrast to SEC, our findings with histidine-based formulations show that subvisible particle counts and estimates of protein mass in particles offers a sensitive way to identify protein aggregation. Screening formulations and stress settings that are more pharmaceutically relevant than those generally utilized for accelerated



degradation tests becomes possible with the possibility for greater sensitivity in identifying and measuring the conversion of protein into aggregates. As an example,

particle counting might be a way to settle discrepancies in real-time storage stability investigations, such as those using aqueous solution formulations kept at 4-8°C. of various formulations' stabilizing properties.

The choice to add a surfactant to a formulation and at what concentration usually involves putting the protein formulation through stresses of multiple freeze-thaw cycles and agitation during the final stages of formulation development. Due to the fact that the optimal pH, buffer salt, and stabilizing excipients would have been determined by the time development was well underway, aggregation caused by freezing and thawing might not be detectable by SEC or turbidity measurements until a large number of freeze-thawing cycles have been employed. The same holds true for aggregation caused by agitation; conventional SEC analysis can miss it unless the agitation tests use speeds and durations that are irrelevant to the actual world. Nevertheless, by keeping an eye on the development of subvisible particles, it may be possible to identify very minute levels of aggregates—microgram quantities—long before other approaches can detect or quantify aggregation. One case study in the literature employed this method to design formulations; it included freezing and thawing hemoglobin solutions with varying concentrations of surfactant and sugar to detect subvisible particles.<sup>8</sup> Particle formation was modestly decreased by sucrose and substantially reduced by PS80. It was not possible to directly compare the sensitivity of monitoring subvisible with other approaches since SEC and light scattering were not used. To find the optimal PS80 dosage to prevent agitation-induced aggregation of deoxyhemoglobin,

researchers in another piece of published work used particle measurements using light obscuration methods.<sup>7</sup> The authors determined the lowest polysorbate concentration necessary to prevent agitation-induced partial gel formation using this method. For instance, a PS80 concentration of just 0.019% was enough to inhibit

### IN THE END

Microgram amounts of invisible protein particles were identified against a background protein concentration of 20 mg/mL by using the whole distribution data to estimate the mass of the particles. This method is far more sensitive than previous aggregation detection approaches, since it can identify aggregated protein forms that constitute a fraction of the total protein in solution. While more research with other proteins and stress situations is required, preliminary results indicate that particle counting might be the most sensitive method currently available for detecting aggregated proteins. More research that uses numerous methods to assess aggregation at the same time is also required.

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#### Conflict of Interest

The Author declares that there is no conflict of interest.

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