HORIBA Scientific

Application Note

Accurate Protein Aggregation Analysis

AN221

Accurate Protein Aggregation Analysis by Multi-Laser Nanoparticle Tracking

Introduction

Protein aggregation is an important concern when developing and manufacturing biotherapeutics since these subvisible aggregate particles have been associated with the adverse drug reactions [1-2]. Protein aggregates may provoke adverse events through an unwanted immune response. And, regardless of mechanism of reaction, subvisible particle contamination has been a concern for manufacturers and regulators [3].

Therefore, it is important to quantify protein aggregation and particle formulation due to various stresses such as temperature, shear, high concentration and time. Values for particle concentration and size distribution allow direct comparison of samples and treatments. Such data is needed to guide formulation development, evaluate handling requirements, and monitor product quality.

To date, researchers have commonly analyzed this using optical microscopy, as described by United States Pharmacopeia (USP) <788> and European Phamacopeia 2.9.19. However, optical microscopy cannot be used for sizes down to 0.1 micron or smaller. Although electron and tunneling microscopy is an available imaging technique, scattering remains the method of choice for particles in this size range.

Nanoparticle tracking (NTA) holds significant promise as a method for monitoring aggregation. Unlike ensemble techniques, such as dynamic light scattering (DLS) and laser diffraction or static light scattering, scattering from each individual particle is monitored with a video camera. These tracks record particle motion, which can be converted to particle size with the Stokes-Einstein equation. Since data is collected on each particle, high resolution sizing information is extracted. Since the scattering volume is known along with the number of particles in the video, concentration is readily and accurately determined.

Conventional NTA is performed with a single laser and has proven successful with narrow size distribution samples. However, scattering is a very stiff function of particle size, and researchers cannot analyze particles that are outside of the range of the experimental setup (specifically, laser power and camera gain).

Particles that are too small will be missed since they do not scatter strongly enough to be detected by the camera. Particles that are too large cannot be tracked since the camera detects a large, saturated region of varying shape that defies analysis.

This issue was enshrined in ISO 19430: “Sample polydispersity affects the ability to track and therefore analyse different size fractions in the particle number-size distribution. … In a polydisperse sample, large particles scatter a lot more than small particles, making it difficult to detect or track small size particles.”

The issues with single-laser NTA described above can be resolved by implementing a multi-laser system. A multi-laser system uses multiple simultaneous lasers to illuminate the sample. Scattering from each laser is then collected with a video camera, and for each light source, a separate video is obtained. By collecting simultaneous videos, each particle will have a separate data set. Software can then determine the best track for each particle and use that track to ensure accurate size computation. Track analysis yields the size distribution and concentration of a wide range of particle sizes in the same sample.

As will be shown, many aggregated protein samples display a range of particle sizes. This range of particle sizes defeats analysis by conventional NTA, but can be readily analyzed by multi-laser NTA.

Methods

Multi-laser Nanoparticle Tracking

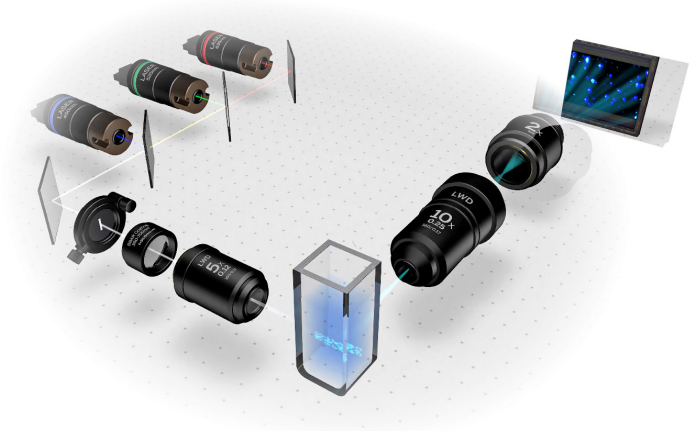


Diagram 1: Optical path of ViewSizer. The three lasers in the upper left are combined, focused and used to illuminate the sample in the cuvette in the lower right. A color video in the upper right records the scattering from each laser. This color video is used to track particle motion. Sophisticated algorithms automatically choose the best data (color) for each particle.

Analysis was performed with the HORIBA ViewSizer 3000, a multi-laser nanoparticle tracking analysis (NTA) system.

By incorporating three variable power light sources (blue, green and red) the instrument collects useful data for a wide range of particle sizes at once. If one light source is too bright, data from a dimmer source is available for analysis. This approach overcomes the limitations of conventional NTA when analyzing polydisperse samples and enabling a much larger range of particle sizes to be visualized. The optical arrangement of the instrument is shown in Diagram 1.

The ViewSizer 3000 employs a simple, easy-to-use and easy-to-clean cuvette assembly. This is particularly important for typically sticky protein solutions which introduce cleaning issues. The cuvette assembly can be completely dissembled to enable mechanical cleaning or more effective ultrasonic cleaning. All cuvette components can be cleaned with soap or bleach.

Lysozyme

Lysozyme from chicken egg white (Sigma Bioultra) was dissolved in pH 7.4 phosphate buffered saline (PBS) to a concentration of 1 mg/mL. After initial measurement, the lysozyme solution was warmed in a bath to 65 °C for 12 minutes to induce aggregation, allowed to cool to room temperature and then measured again. The solution was then heated to induce aggregation and analyzed by multi-laser NTA. In addition, the solvent system (PBS) was also heated and analyzed for comparison.

BSA

Bovine serum albumin (Sigma Aldrich) was allowed to dissolve overnight under refrigeration in PBS to a concentration of 50 mg/mL.

NIST mAb

A sample of National Institute of Standards and Technology monoclonal antibody reference material (NIST mAb) and diluted to 1 mg/mL in proprietary formulation buffer. Aggregate particle concentration and particle size distribution before and after shear induced by stirring. Shear was applied with a stir rate of 1400 rpm for five minutes.

Drug Formulation

A proprietary candidate protein drug formulation with, among other components, 0.2% polysorbate 80 was prepared. Samples were stressed at 25 °C for various periods and particle concentration was analyzed with the HORIBA ViewSizer 3000.

Results

Thermal Aggregation: Lysozyme

Figure 1 is a representative screen capture of the video for pure PBS buffer that has been heated to 65 °C for 15 minutes. As expected, the blank images and corresponding size distribution show a negligible background particle count. Buffers encountered in many pharmaceutical settings do not have significant quantities of particles, presumably due to sterile filtration. However, particles that arrive either through the air or from crystallization of buffer components will lead to significant quantities of background particles. Therefore, buffers should be checked for particulate contamination with the same analytical technique before solutions are prepared.

Figure 1: Screen capture of video from PBS buffer only. Note the lack of scattering from any particles, that is a black image, indicating a clean buffer.

Figure 2 below shows color images from NTA of the pure protein solution. Note the absence of spots or scattering from large particles. Under these solution conditions (low concentration, good solvent), lysozyme is not expected to aggregate and therefore, the only "particles" are individual protein molecules in solution. These can be analyzed by static or dynamic light scattering, but not by NTA.

Figure 2: Screen capture of video from lysozyme solution before heating. Note the lack of scattering from any particles, that is, a black image, indicating that there are few, if any, aggregates in this dilute protein solution.

After heating, the protein solution is qualitatively different. Figure 3 shows a color image from NTA of the same solution after heating to promote aggregation. Here, the scattering from multiple aggregates is readily identified. The motion of the aggregates in the video can be used to infer particle size. Since these are images of scattering from the particles, particles smaller than the Rayleigh limit can be analyzed.

Figure 4 shows the observed number-based size distribution and cumulative particle concentration. Note that the number distribution data is more reliable than that obtained by ensemble techniques such as dynamic light scattering (DLS) and the wide range of sizes observed in the sample that would defeat analysis by single laser NTA.

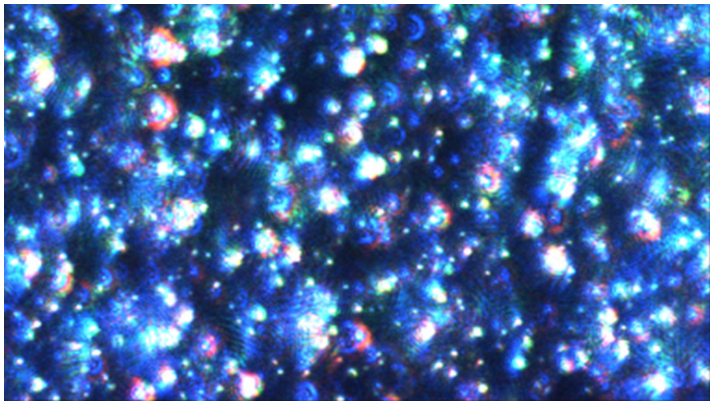


Figure 3: Screen capture of video from lysozyme solution after heating to promote aggregation. In contrast to figure 2, there are numerous aggregate particles which can be individually sized and counted with the ViewSizer.

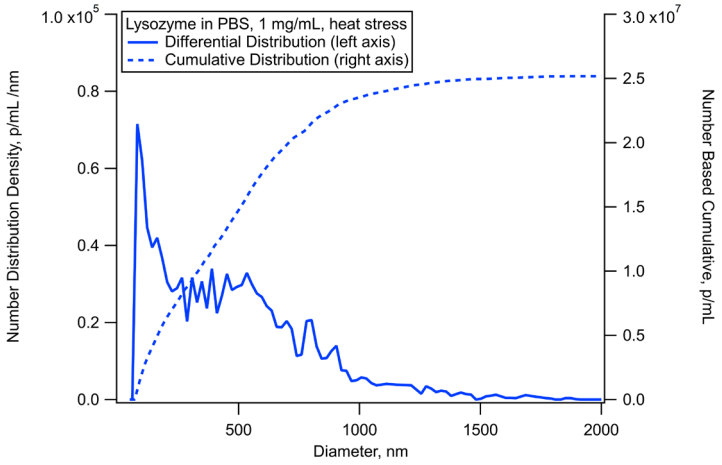
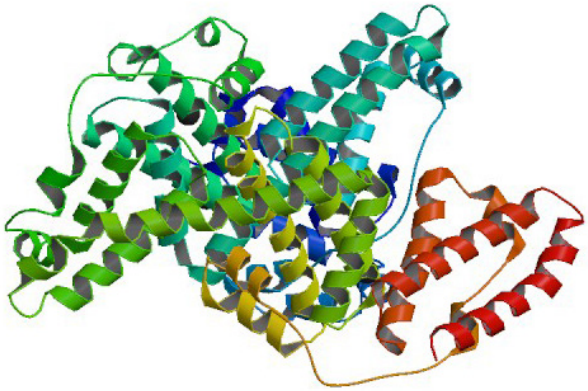


Figure 4: Observed number based size distribution of lysozyme aggregates created by briefly heating to 60 °C. Note that absolute concentration data is also obtained.

It is useful to note here that nanoparticle tracking analysis cannot be used to analyze well dissolved proteins (e.g., monomer). This extends to protein dimers and tetramers, which are better analyzed by dynamic light scattering (DLS) with the HORIBA SZ-100. However, the ViewSizer 3000, with multi-laser nanoparticle tracking can be used to determine the size distribution and concentration of the larger aggregates that can provoke an immune response. This information is important for evaluating formulation and manufacturing performance for protein therapeutics.

Concentration Induced Aggregation: BSA

Thermal stress is not the only source of protein aggregation. A high concentration solution of BSA was prepared to demonstrate concentration induced aggregation. In this case, the high protein concentration leads to aggregation. In order to accurately determine size distribution, the correct value of solution viscosity is required. Therefore, solution viscosity was measured by using tracer particles. Figure 5 shows the size distribution of particles obtained for BSA at a concentration of 50 mg/mL. Here, the size distribution is broader than that obtained for the lysozyme sample above.



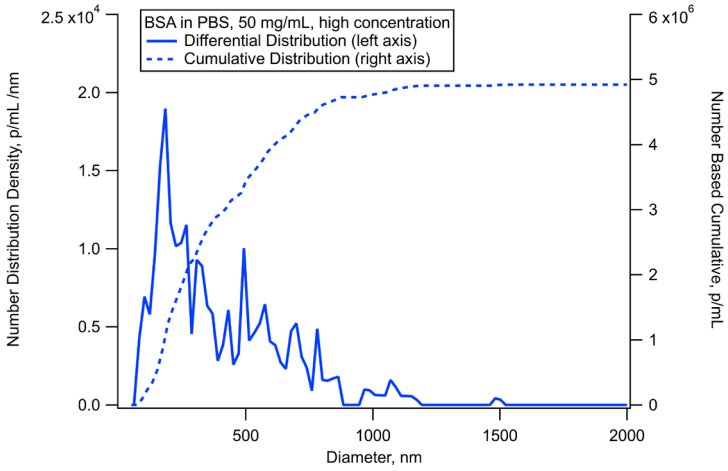


Figure 5: Observed number based size distribution of BSA aggregates at 50 mg/mL in PBS.

Shear Induced Aggregation: mAb

Shear can increase aggregation in concentrated protein solutions. Figure 6 shows the increase in aggregation when a monoclonal antibody formulation is subjected to shear with the stirrer in the VewSizer sample cell. Confounding effects of shear during sample transfer are avoided by applying shear *in situ* with the stir bar in the ViewSizer.

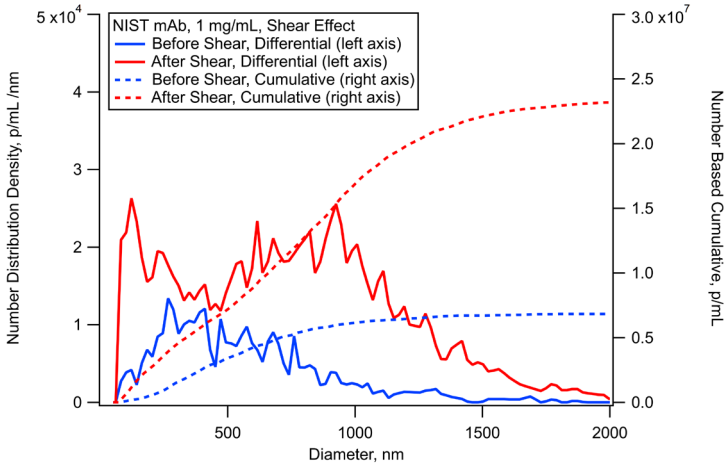


Figure 6: Aggregate size distribution and concentration before and after applying shear. Note the increased particle concentration with applied shear.

Note the average particle size and the concentration of particles increases with shear. While the applied shear field is complicated, it can be tightly reproduced in order to compare the effect of shear on different formulations with the Viewsizer.

Time Study: Drug Candidate Formulation

The more slowly a formulation changes over time, the longer the shelf life. Aggregation can continue, sometimes slowly, with time. In the example below, a candidate drug formulation was prepared, stored, and analyzed after two, three, and six months. Figure 7 shows the change in size distribution and concentration. Here it is clear that the particle concentration is increasing with time, but more slowly as time goes on.

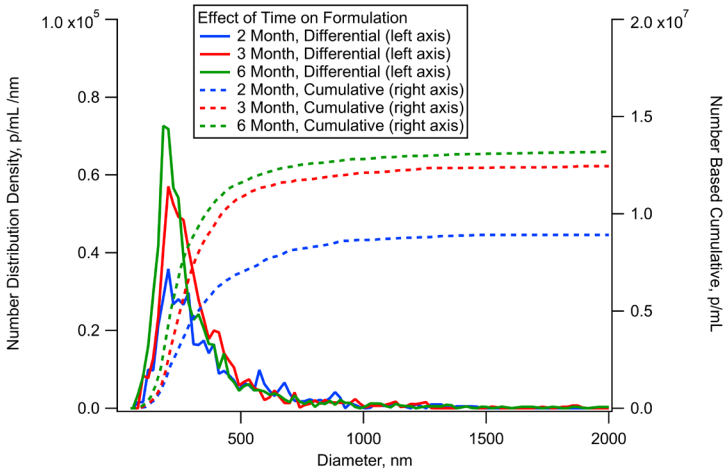


Figure 7: Particle size distribution measured after different storage durations.

Conclusions

Researchers can use the ViewSizer 3000 to monitor the concentration and size distribution of protein aggregates under a large variety of conditions. Measurements of unaggregated (monomeric) protein are not possible. But the three laser design of the ViewSizer 3000 allows accurate analysis of the broad size distributions typical of many aggregated protein systems.

References

[1] Kotarek J, Stuart C, De Paoli SH, Simak J. Subvisible Particle Content, Formulation, and Dose of an Erythropoietin Peptide Mimetic Product Are Associated With Severe Adverse Postmarketing Events. *J. Pharm. Sci.* 2016; 105(3):1023-7. doi: 10.1016/S0022-3549(15)00180-X

[2] Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJ. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. *J Pharm Sci.* 2009;98(4):1201-5. doi: 10.1002/jps.21530.

[4] A presentation on subvisible particles at PEGS 2019 suggests that manufacturers generally exceed USP requirements by a significant margin, indicating manufacturing skill is well ahead of regulatory requirements.