Application Note

Direct Visualisation, Sizing and Counting of Aggregation in Proteins

Introduction

Characterising the state of aggregation in proteins is of paramount importance when trying to understand biopharmaceutical product stability and efficacy. Product quality, both in terms of biological activity and immunogenicity can be highly influenced by the state of protein aggregation.

A wide variety of aggregates are encountered in biopharmaceutical samples ranging in size and characteristics (e.g., soluble or insoluble, covalent or noncovalent, reversible or irreversible). Protein aggregates span a broad size range, from small oligomers (nanometers) to insoluble micron-sized aggregates that can contain millions of monomer units.

Protein aggregation can occur at all steps in the manufacturing process (cell culture, purification and formulation), storage, distribution and handling of products. It results from various kinds of stress such as agitation and exposure to extremes of pH, temperature, ionic strength, or various interfaces (e.g., air–liquid interface). High protein concentrations (as in the case of some monoclonal antibody formulations) can further increase the likelihood of aggregation.

Therefore, aggregation needs to be carefully characterised and controlled during development, manufacture, and subsequent storage of a drug substance and formulated product. Similarly, by monitoring the state of aggregation, modification or optimisation of the production process can be achieved.

NanoSight now offer a new laser-based Nanoparticle Tracking Analysis (NTA) system which allows nanoscale particles, such as protein aggregates, to be directly and individually visualised and counted in liquid in real-time, from which high-resolution particle size distribution profiles can be obtained.

The technique is fast, robust, accurate and low cost, representing an attractive alternative or complement to existing methods of nanoparticle analysis such as Dynamic Light Scattering, DLS (also known as Photon Correlation Spectroscopy, PCS) or Electron Microscopy.

Imaging Protein Aggregates

The NanoSight instrument offers a unique insight into protein aggregation in the range of 30nm - 1000nm.

Having visually inspected the sample for the presence of aggregated material (Figure 1), the user can rapidly generate a particle size distribution profile and a count (in terms of aggregate number concentration) of the aggregates seen (Figure 2).

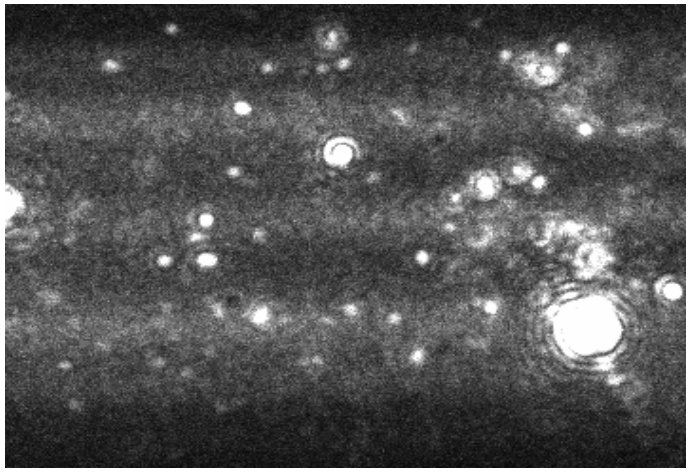

Figure 1. A typical image produced by the NanoSight technique. The image allows the users to instantly recognise certain features about their sample and the presence of aggregates.

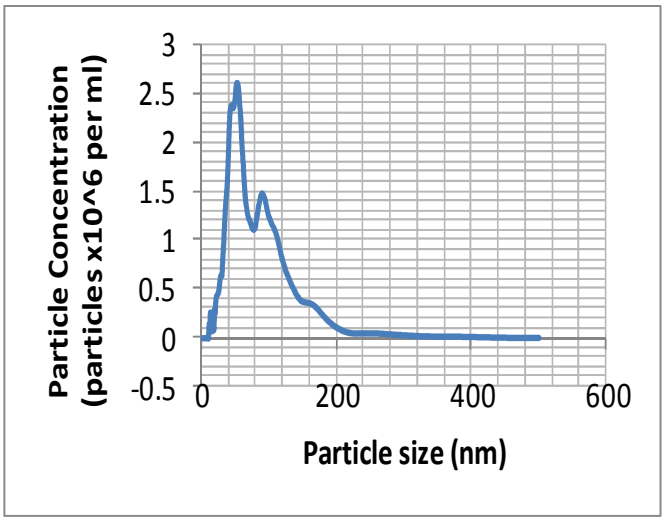

Figure 2. Particle size distribution (number distribution) produced from the sample shown in Figure 1.

Covering the Size Range

Historically, a number of techniques have been used to characterise proteins and protein aggregation. Often separation techniques are used to discriminate proteins and protein aggregates, with further analysis performed on a separated sample.

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Analytical Techniques:

- Dynamic Light Scattering (DLS)
- Multi-Angle Light Laser Scattering (MALLS)
- UV Spectroscopy
- Light Obscuration
- Micro-Flow Imaging (MFI)
- Nanoparticle Tracking Analysis (NTA)

Separation techniques:

- Size Exclusion Chromatography (SEC)
- Field Flow Fractionation (FFF)
- Capillary Electrophoresis. (CE)
- Analytical Ultracentrifugation (AUC)

Sub 30nm

It is common to find SEC paired with DLS, MALLS or UV spectroscopy. Size Exclusion Chromatography can be used to separate protein monomers from aggregates. Subsequent analysis using DLS for example, can produce accurate size or molecular weight analysis for purified fractions. Above the exclusion limit of the SEC column there is no separation and hence bulk analysis systems such as DLS become less well suited. MALLS analysis can help reduce the effect of larger aggregates in non-fractionated samples but the technique requires interpretation.

30 - 1000nm Range

The NanoSight technique allows protein aggregates within the size range of 30 - 1000nm to be **individually** imaged and sized by tracking their Brownian motion on a **particle-by-particle** basis. Particle-by-particle analysis allows high-resolution number distributions to be generated. This region is often poorly served by DLS with high concentration of protein monomer and low number of large, bright aggregates often dominating the signal.

Whilst fractionation can be performed such as with FFF to aid DLS analysis, the dilution that is often required for FFF can make this route undesirable due to the potential for further aggregation. Furthermore, dilution of these 'mid-sized' aggregates often takes them below the concentration sensitivity limit for DLS. The NanoSight technique frequently requires no dilution as the 30 - 1000nm protein aggregates often fall within the optimum concentration range for this technique.

The cut off limit of the NanoSight technique (approx. 30 nm for protein aggregates) means that it is well suited to complement SEC/DLS or SEC/UV above the exclusion limit of SEC. The upper limit of the NanoSight technique represents the point at which conventional single particle imaging/obscuration techniques become applicable. With no prior separation of aggregates, DLS would typically produce a bimodal result for the aggregated sample shown in Figure 2.

The primary peak would be formed from the large number of monomeric particles, while the secondary peak would be formed by very large aggregates which scatter significant intensities of light. A poorly resolved DLS analysis would show no particles between these points despite their existence as the primary monomeric particles and the few larger aggregates would dominate the signal.

The NanoSight technique would be unable to measure the primary monomeric size as the particles would fall below the detection limit of the technique. Above 30nm, the technique provides particle-by-particle analysis of protein aggregates, uniquely forming a high-resolution number distribution of aggregated particles.

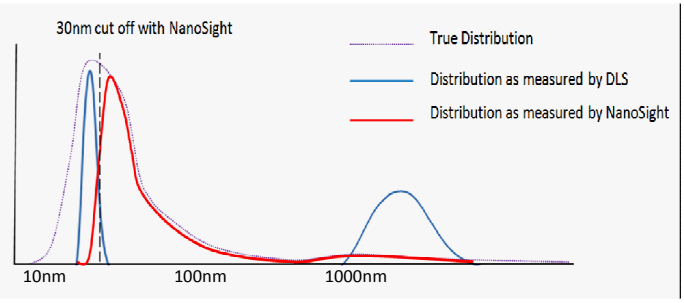

Figure 3. A representation of the distribution of particle sizes which may be contained within an aggregated protein sample.

Real Examples 1 —Shear Stress

In the following example a virus was correctly measured by NTA at 45nm diameter (Figure 4a). However, following agitation of the same sample by simple shaking for a few seconds, shear stress was seen to have induced aggregation in the virus sample (Figure 4b).

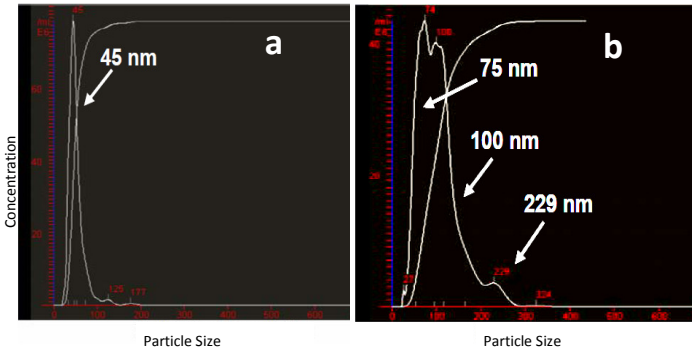

Figure 4. Particle size distribution profile of a virus sample a) before and b) after shear stress induced aggregation. Note the change in scale of the normalised vertical axis shows a drop in the concentration of particles on aggregation (from approximately 80x10^6 particles/ml to approximately 50x10^6 particles/ml). Such information is unavailable to other ensemble light scattering techniques such as DLS.

From; Moser M., (2008) Emerging analytical techniques to characterize vaccines, Proc. Intl. Conf. Vaccines Europe, Brussels, December 2008.

Real Examples 2 — Heat Stress

In this example, a sample of IgG was heat stressed at 50°C for 35 minutes in the NanoSight sample chamber and the aggregation followed in real-time using the batch capture facility in the NTA programme. In Figure 5. the size distribution (middle panels) with the corresponding NTA video frame (left panels) and 3D graph (size vs. intensity vs. concentration; right panels) are shown.

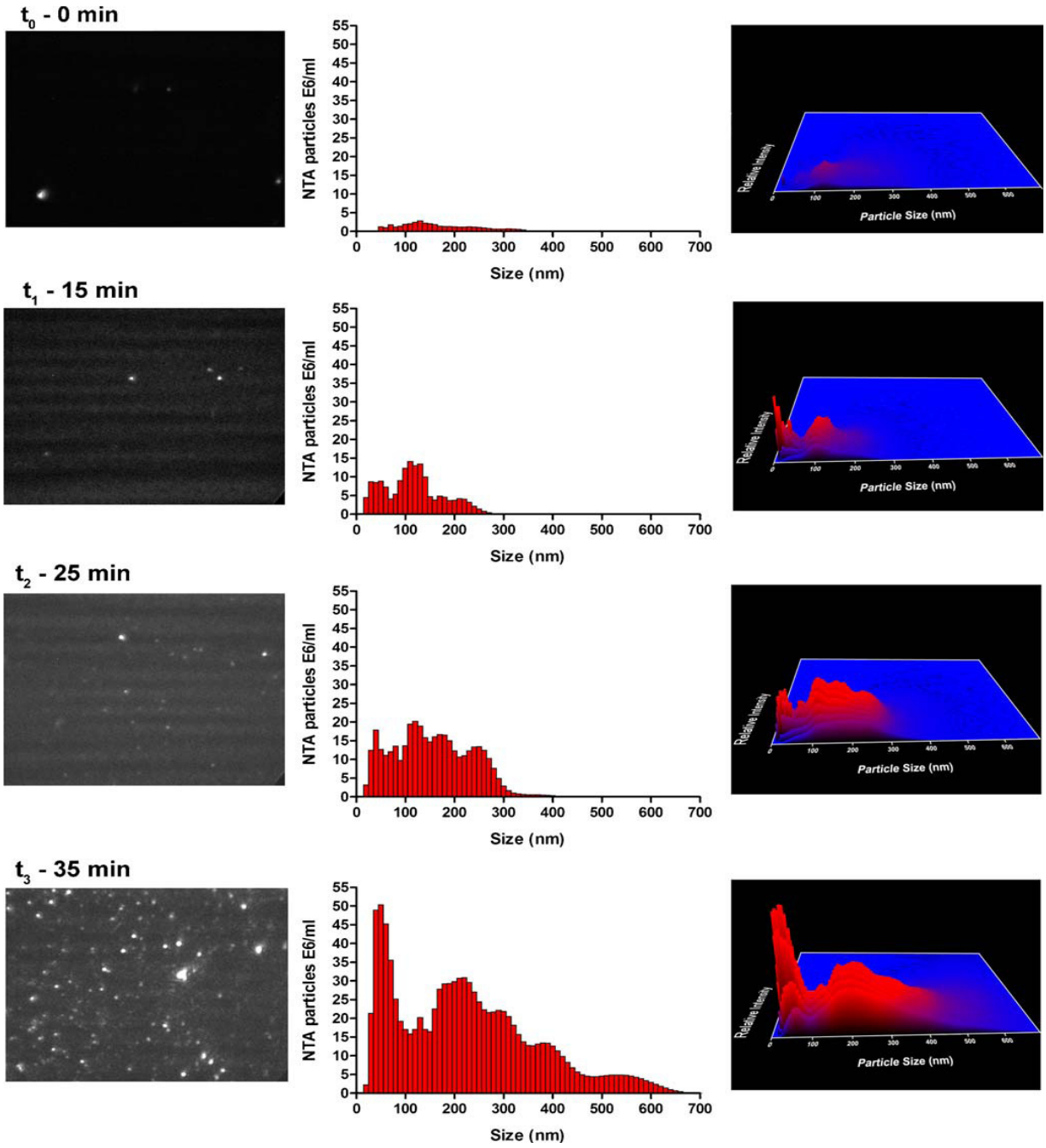

Figure 5. Time course of heat induced aggregation of IgG protein at 50°C.

Data reproduced from Filipe, Hawe and Jiskoot (2010) "Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates", *Pharmaceutical Research, DOI: 10.1007/s11095-010-0073-2*

Real Examples 3 — Aggregation Following Dilution in Different Quality Waters

In this example, two nanoparticle sample types (chitosan nanoparticles and gold calibration nanoparticles) were diluted in waters of varying quality.

1. Tap-water (from hard-water area)
2. Deionised water (for use in batteries)
3. High purity, reagent grade (18MΩ) water

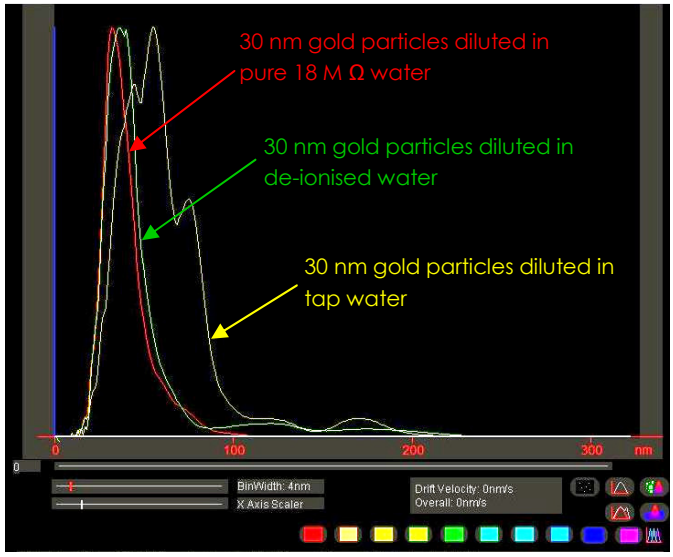

Figure 7: Comparing particle size histogram obtained by NanoSight from a 60 second video of 30nm NIST gold particles diluted in various types of water.

A) Chitosan Nanoparticles

Firstly, samples of chitosan nanoparticles (a bioadhesive polysaccharide) developed for use in drug delivery applications (supplied by IPATIMUP - Instituto de Patologia e Imunologia Molecular da Universidade do Porto) was diluted in the three water types shown above and the size of population measured immediately on dilution and after 5 - 10 minutes.

The effect of reduction of ion and mineral content in water on aggregation can be clearly seen in Figure 6 below. The left hand plot shows significant aggregation in tap water (red line t=0, white line t=5 - 10 minutes), the middle plot shows reduced aggregation in deionised water and the right-hand plot shows no aggregation when diluted with ultra-pure water containing no ions.

B) Gold Nanoparticles (NIST Standards)

Calibrated 30nm gold particles (NIST) were diluted into the same three types of water: tap, de-ionised and 18MΩ water (all free from nanoparticles) then analysed with the same concentration using the NanoSight system. Figure 7 shows that the degree of aggregation depends on water purity with only the pure 18MΩ water causing no aggregation.

Contact Details

For further information, contact NanoSight or your local distributor, listed at **www.nanosight.com**

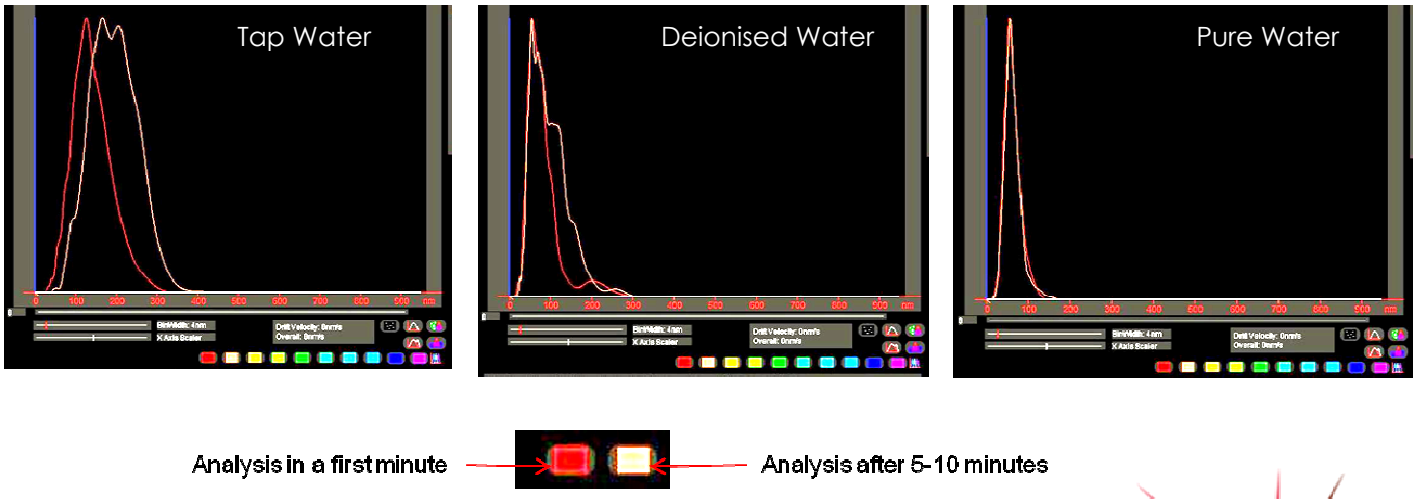

Figure 6. Comparing Chitosan aggregation process in various types of water with time (in first minute of analysis and after 5 - 10 minutes).