Counting and Sizing of Virus and Protein Aggregates by Nanoparticle Tracking Analysis (NTA)

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ABSTRACT

A new analytical system is described which provides total particle count and high-resolution size measurements for biological structures such as virus and protein aggregates. This system builds on the Nanoparticle Tracking and Analysis (NTA) technique for the simultaneous visualization and individual sizing of nanoparticles based on their Brownian motion.

Virus production and purification processes rely on overall measurements of concentration such as optical density and infectivity assays which may take days to obtain results. The NTA method provides a total particle concentration result in minutes, providing a clearer picture of how the sample has been attenuated or killed by the purification process. Fluorescence measurements can be used to isolate a sub-population of interest.

NTA provides the only alternative for concentration and sizing of polydisperse protein aggregates formed in the sub-micron range.

Keywords: virus, protein, aggregate, nanoparticle, sizing

1 INTRODUCTION

The analysis of nanoparticle properties is an increasingly important requirement in a wide range of applications areas and size analysis is usually carried out by either electron microscopy or dynamic light scattering (DLS). Both techniques suffer from disadvantages; the former requiring significant cost and sample preparation, the latter frequently generating only a population average size, which itself can be heavily weighted towards larger particles within the population.

A new method of microscopically visualizing individual nanoparticles in a suspension, called Nanoparticle Tracking Analysis (NTA), allows their Brownian motion to be analyzed and from which the particle size distribution profile (and changes therein in time) can be obtained on a particle-by-particle basis [1-3]. The technique offers significant advantages over traditional light scattering techniques (such as DLS- and SLS-based systems) for the characterization of polydispersed populations of nano-scale particles. Independent of particle density or refractive index, NTA dynamically tracks individual particles within the range of 10 - 1,000nm and provides size distributions along with a real-time view of the nanoparticles being measured.

This technique also provides a measurement of particle count within the measured volume. By knowing the interrogated volume, this particle count can be converted to a total concentration measurement.

2 MEASUREMENT METHODOLOGY

A small (250µl) sample of liquid containing particles at a concentration in the range 10^6-10^{10} particles/ml is introduced into the scattering cell through which a finely focused laser beam (approx. 40mW at λ=635nm) is passed. Particles within the path of the beam are observed via a microscope-based system (NanoSight LM10 or NS500) onto which is fitted a CCD camera.

The motion of the particles in the field of view (approx. 100 x100 µm) is recorded (at 30 frames per second) and the subsequent video analyzed. Each and every particle visible in the image is individually but simultaneously tracked from frame to frame and the average mean square displacement determined by the analytical program. From this can be obtained the particle's diffusion coefficient. Results are displayed as a sphere-equivalent, hydrodynamic diameter particle distribution profile. The only information required to be input is the temperature of the liquid under analysis and the viscosity (at that temperature) of the solvent in which the nanoparticles are suspended. Otherwise the technique is one of the few analytical techniques which is absolute and therefore requires no calibration. Results can be obtained in typically 30-60 seconds and displayed in a variety of formats.

The minimum particle size detectable depends on the particle refractive index but for highly efficient scatterers, such as colloidal silver, 10nm particles can be detected and analyzed. For weakly scattering (e.g. biological) particles, the minimum detectable size may only be 30-50nm. The upper size limit to this technique is defined by the point at which a particle becomes so large (>1000nm) that Brownian motion becomes too limited to be able to track accurately. This will vary with particle type and solvent viscosity but in normal (e.g. aqueous) applications is approximately 800-1000nm. See www.nanosight.com for details.

3 SIZE DETERMINATION BY NANOPARTICLE TRACKING ANALYSIS

Brownian motion in a Newtonian fluid is governed by the Stokes-Einstein equation. Whilst the motion clearly occurs in three dimensions, NTA observes motion only in two dimensions. It is possible to determine the diffusion coefficient from measuring the mean squared displacement of a particle in the two observed dimensions;

$$\overline{(x,y)}^2 = \frac{4TK_B t}{3\pi\eta d}$$

where the first term is the mean squared displacement, T, is temperature, K_B is Boltzmann's constant, t is the time period (here given by 1/*framerate*), η is viscosity and d is the hydrodynamic diameter.

By tracking the centers of the particles the mean squared displacement for each and every particle is calculated. This process is depicted below in figure 1. By recording a video of particles (fig 1a), tracking them (fig 1b) and compiling the resulting sizes a particle size distribution is established (fig 1c).

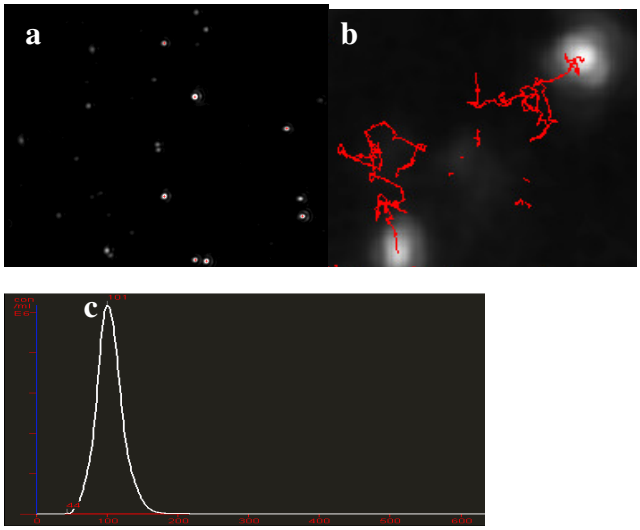

Fig 1. a) A still from a video of 100nm polystyrene calibration particles, b) showing only some (for clarity) of the Brownian motion trajectories analysed and c) subsequent particle size distribution.

4 VIRUS AGGREGATE AND CONCENTRATION MEASUREMENTS

The development of viral vaccines requires viruses to be cultured in live cells, harvested and then purified. Vaccine manufacturers are interested in monitoring the purity of the viral preparation at various key stages of the purification process and understanding the concentration of virus material present. The particle-by-particle approach to sizing and counting viruses can easily distinguish viruses (of known size) from larger non-viral cell debris from the culture process or aggregates of virus particles containing many individual virions. In either case, such aggregates or contaminants represent a possible problem to the manufacturer, and the high-resolution number-based size distributions can be used to calculate the number of viruses vs. the number of virus aggregates.

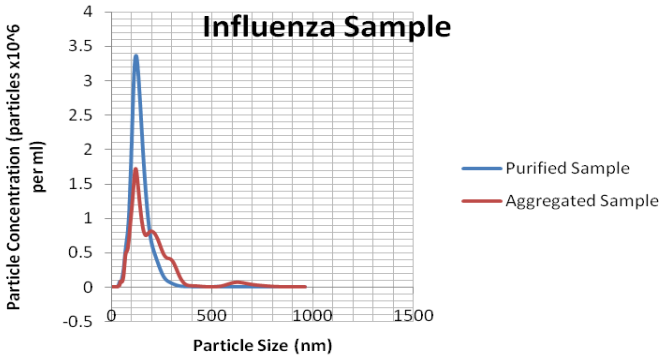

Fig. 2. Measurement of purified influenza preparation, before and after aggregation.

Estimating the concentration of viruses present is essential in understanding the loss of product at each step of the purification process (and hence can be used to optimize the process in terms of product yield). Also virus concentration is essential when trying to understand dosage in the final product.

The ability of NTA to size and count a virus whether it is live or inactive allows the users to obtain an idea of the relative concentrations of infective particles vs. total particles, when used in conjunction with conventional TCid50 or plaque infectivity assays. This ability is of particular value for measurement of inactivated virus or virus-like particles (VLP), which are not infective and therefore not applicable to these techniques.

As the technique is equally able to analyze fluorescent particles, a sub-population of interest (generally the virus), may be fluorescently tagged and isolated from the remainder of the material in the total sample. The fact that the light being measured at the detector is fluorescent light has no effect on the measurement, so all of the measurement capabilities are the same whatever mode is being utilized. The analytical system differs from the standard arrangement only in the selection of an appropriate fluorophore, incident laser wavelength, and optical filter.

4 CHARACTERIZAING PROTEIN AGGREGATION

Characterizing 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 characterized and controlled during development, manufacture, and subsequent storage of a drug substance and formulated product. Similarly, by monitoring the state of aggregation, modification or optimization of the production process can be achieved.

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 [4]. 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.

Fresh preparations may show few, if any, particles within the measureable range. Over time, or when subject to stress such as shear or heat, the early onset of aggregation is seen at smaller sizes and lower concentration, following by a gradually increasing size and concentration profile. Characterizing this early-stage aggregation, compared to traditional counting techniques which are restricted to measurements larger than approximately one micron can provide a useful early warning and greater understanding for this critical subject.

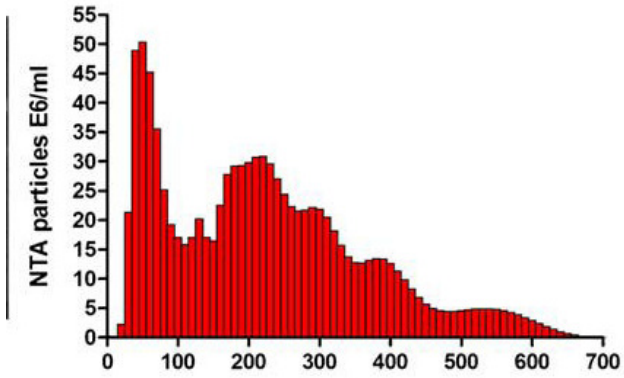

Fig. 3. A sample of IgG was heat stressed at 50°C for 35 minutes in the sample chamber and the aggregation followed in real-time

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5 CONCLUSION

The NTA technique is a robust and direct method for characterizing particle size distributions and concentrations for a wide range of biological particulate materials. It represents an attractive alternative or complement to higher cost and more complex methods of nanoparticle analysis such as light scattering or electron microscopy that are currently employed. The technique uniquely allows the user a simple and direct qualitative view of the sample under analysis (perhaps to validate data obtained from other techniques) and from which an independent quantitative estimation of sample size, size distribution and concentration can be immediately obtained. [5-7].

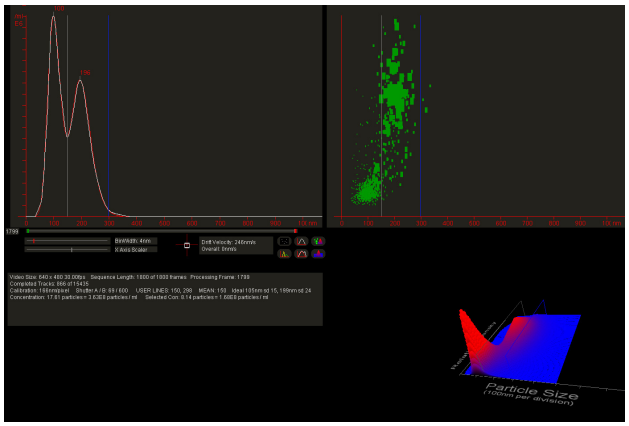

Figure 5: Particle size distribution, intensity scatter plot, and intensity map for a 100 and 200nm polystyrene mix.

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