Characterization of Nanoparticle Tracking Analysis for Quantification and Sizing of Submicron Particles of Therapeutic Proteins

CHEN ZHOU, AARON B. KRUEGER, JAMES G. BARNARD, WEI QI, JOHN F. CARPENTER

Department of Pharmaceutical Sciences, University of Colorado, Anschutz Medical Campus, Aurora, Colorado 80045

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ABSTRACT: Submicron particles may play important roles in therapeutic protein product quality, stability, and adverse effects in patients. However, quantitation of these particles has been challenging. Nanoparticle tracking analysis (NTA) is capable of both sizing and counting submicron particles. We investigated the effects of product and instrument parameters on NTA results for nanoparticle standards and therapeutic protein samples. To obtain proper particle size distributions, complete tracking numbers of at least 200 and 400 were required for latex nanobeads and protein nanoparticles, respectively. In addition, when set at suboptimal values, the minimum expected particle size parameter led to inaccurate sizing and counting for all particles types investigated. A syringe pump allowed for higher sampling volumes, and results were reproducible for nanoparticle sizing and counts at flow rates ≤7 μL/min. Finally, because therapeutic protein products are being formulated at relatively high protein concentrations, we investigated the effects of protein concentration on nanoparticle characterization. With high protein concentrations, nanoparticle sizing was not affected, whereas particle concentrations were significantly reduced. Linear relationships between particle count and dilution factor were obtained when a high protein concentration formulation was diluted into particle-free solutions at the same protein concentrations, but not when dilutions were made into buffer. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:2441–2450, 2015

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INTRODUCTION

Protein aggregation is ubiquitous during the development, production, and clinical use of therapeutic protein products.[1,2] Conditions during manufacturing, shipping, storage, and delivery to patients that can induce aggregation include exposures to light, elevated temperatures, freeze–thawing, agitation, particles of foreign materials, and various interfaces.[3,4] A major concern is that protein aggregates, including submicron and micron-sized particles, may lead to adverse and unintended immunogenicity in patients.[5,6] Accordingly, regulatory agencies are requesting that manufacturers provide more rigorous characterization and quantification of subvisible particles in their therapeutic protein products.[7,8]

One difficulty in meeting this regulatory expectation is that no single analytical method or instrument can cover the entire size range of particles from submicron to micron-sized to visible.[4,9,10] Also, each approach has key limitations and is critically affected by instrument parameters and sample characteristics, which must be evaluated rigorously in order to maximize utility of the method for analysis of therapeutic proteins. For micron-sized particles, light obscuration, electric sensing zone-based detection, and flow microscopy are available and have been evaluated in detail in earlier investigations.[1,4,9–13] Dynamic Light Scattering (DLS) and nanoparticle tracking analysis (NTA) are useful for submicron particles. DLS can provide size distributions based on scattering intensity fluctuations, but cannot determine particle concentrations. NTA provides both particle counts and size distributions, but there has been limited published research on the impact of operational and sample parameters on data acquisition, analysis, and robustness. In the current study, we use a model therapeutic protein and synthetic nanoparticles to critically evaluate such factors with the NanoSight LM20 NTA instrument.

An NTA instrument combines a high-resolution camera with a microscope to record the motions of individual nanoparticles in solution. Particles are visible in the system because of the light scattering that occurs when they are illuminated by the integrated laser. During sample analysis, individual particles are tracked spatially in the recorded videos. The diffusion coefficients of the particles are obtained and the hydrodynamic diameter of each particle is calculated according to the Stokes–Einstein equation.

Nanoparticle tracking analysis' utility and capabilities have recently been evaluated in a few research areas.[14–19] For example, NTA has been used to characterize nanoparticles arising in therapeutic protein products such as IgG and interferon-β-1a under various stresses[20,21]; and, along with other particle characterization techniques, to compare the particles in a series of commercial interferon-β products.[22] Also, the particle sizing capabilities of NTA have been found to be superior to DLS.[13,19] With polydispersed samples of protein aggregates and drug delivery system nanoparticles, NTA was better able to resolve different-sized populations of particles than DLS.

Although useful results have been obtained with NTA in such studies, the complexity of the method and the potential impacts of instrument settings and sample properties can lead to erroneous results. For example, a recent study by

Correspondence to: John F. Carpenter (Telephone: +303-724-6110; Fax: +303-724-7266; E-mail: John.Carpenter@ucdenver.edu)

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Weinbuch et al.[23] showed that the nanoparticulate impurities in excipients such as sugars could interfere with NTA measurements. In addition, the scattering intensity of particle species has been shown to have a profound effect on measuring particle concentration.[14,19] The particle concentrations of weak scatterers were undercounted by four orders of magnitude for DNA and around 20-fold for fibrillar protein aggregates when compared with their expected concentrations.[14] The goal of the current study was to systematically evaluate NTA instrument settings, operating parameters, sample handling methods, and sample properties (e.g., protein concentration) on the accuracy and reproducibility of nanoparticle sizing and counts with the NanoSight LM20 instrument. For this study, we used human serum albumin (HSA) as a model therapeutic protein.

Because only a minute sample volume is recorded and analyzed with this instrument (the dimension of the sample viewed is approximately $100 \times 80 \times 5\ \mu m^3$ or 4×10^{-8} mL), a multiplication factor of approximately 15 million is used to convert the raw particle numbers measured to particles per milliliter. To increase sample volume/tracking numbers measured during an experiment, a syringe pump connected to the sample chamber may be used. We therefore investigated the effect of sample flow rate through the chamber on particle counting and sizing. We also evaluated the effect of the raw number of particles detected during each measurement (referred to as "complete tracking number" in the instrument software) on the robustness of the particle data. During image analysis with the NanoSight LM20 instrument (software NTA 2.2), the minimum expected particle size (MES) must be set by the operator. Because this parameter can greatly affect results for a given sample, we also tested the effect of MES settings on particle concentration and on the accuracy of particle size determination.

Finally, it is becoming common to formulate therapeutic proteins such as monoclonal antibodies at concentrations of 50–100 mg/mL or even higher.[24,25] Samples at such high protein concentrations can cause difficulties during particle characterization with methods such as microscopic imaging and DLS.[10,26] In the current study, we investigated the capabilities of NanoSight for counting and sizing particles in samples with high protein concentrations by studying different particle species (latex beads, gold nanoparticles, and protein aggregates) spiked into particle-free HSA solutions at various protein concentrations.

MATERIALS AND METHODS

Materials

Human serum albumin (fraction V fatty acid free) was purchased from MP Biomedical (Carlsbad, California) and formulated with phosphate buffer (0.1 M sodium phosphate, pH 7.09). Latex beads size standard (100 nm) was purchased from Thermo Scientific (Waltham, Massachusetts). Gold particle size standard (50 nm) was purchased from Nanocs (New York City, New York). All chemicals were purchased from Fisher Scientific (Hampton, New Hampshire) and were of reagent grade or higher quality.

Methods

Instrument Configuration

The NanoSight (Model LM20; NanoSight Ltd., Amesbury, United Kingdom) instrument was equipped with a 405-nm laser light source (Model #LM12B). NTA 2.2 software was used to collect and analyze videos of particle tracks. For each experiment, unless otherwise noted, 500 μL samples were drawn into 1 mL silicone oil-free plastic syringes (National Scientific Company, Rockwood, Tennessee), which were used for sample injection into the instrument sample chamber.

In our studies with the NanoSight Model LM20 instrument, data were acquired using the video capture mode, which records videos of the sample for post hoc analysis. In this mode, the user defines the length of video acquisition time from 10 to 215 s, depending on the observed particle concentration.

The current study was performed on the older LM20 system, which is no longer marketed but still used in many laboratories. Also, results obtained with the LM20 system should be directly applicable to any of the current NTA systems available, including the NanoSight LM10, NS300, and NS500 systems.

Evaluation of Complete Tracking Number

The raw number of particle detected during analysis is referred to as complete tracking number. To initially investigate the importance of this parameter, a suspension was prepared by diluting the 100-nm latex beads size standard with MilliQ water to achieve a desired number of particles per video frame (>10). For complete tracking number evaluation, the sample was measured with video record mode, and analysis was stopped when desired complete tracking numbers were obtained: 50, 100, 200, 400, and 800. During analysis, the detection threshold was set at 8, and MES was set at 100 nm.

Also, blur and minimum track length were set at auto. Blur is a smoothing function used to remove scattering noise around and within a particle. A large value may reduce the noise but also cause smaller particles to be overlooked. Minimum track length determines the minimum number of steps that a particle must take before its size value can be included in the final result. A large minimum track length value will omit small fast-moving particles and cause bias toward a larger than expected size distribution.[27] In our evaluations, we used the auto setting for those two parameters because we found it to be sufficient for the samples we studied.

Next, the effect of complete tracking number was studied with HSA particles. To generate protein particles, a HSA solution (30 mg/mL) was agitated in a 15-mL polypropylene conical tube at 300 rpm with an orbital shaker for 24 h at room temperature. The agitated HSA solution was then diluted to a protein concentration of 1 mg/mL with phosphate buffer. The sample was evaluated with complete tracking numbers of 50, 100, 200, 400, 800, and 1200. During analysis, the detection threshold was set at 6, MES was set at 50 nm, and blur size and minimum track length were set at auto.

Evaluation of MES

The effect of choice of MES was investigated with gold nanoparticles, latex beads, and HSA particles. An aliquot (3 μL) of the original stock solution of 50 nm gold particles was diluted with 1 mL MilliQ water. The 100-nm latex bead stock solution was diluted 10^5-fold with MilliQ water. To prepare HSA samples with particles, 0.1 mg/mL HSA was agitated in a 15-mL polypropylene conical tube at 300 rpm with an orbital shaker for 24 h at room temperature. Videos were recorded and analyzed with MES settings of 30, 50, 80, 100, 150, 200, 250, 300, and 400 nm. For evaluation of all samples in the study of effects