Published in final edited form as:

Methods Mol Biol. 2018 ; 1777: 145–158. doi:10.1007/978-1-4939-7811-3_7.

Nanoparticle Tracking for Protein Aggregation Research

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Abstract

Protein aggregates are the pathological agents in several neurodegenerative disorders such as Alzheimer's and Huntington's disease. In the pharmaceutical industry, protein aggregation poses significant challenges to the manufacture of biologics. Nanoparticle tracking is an emerging technology that allows particle-by-particle measurement of aggregate size and concentration. The technique is solution based, and requires no labeling. Here we describe protocols for using nanoparticle tracking in protein aggregation research, and provide a few examples for illustrative purposes.

Keywords

Nanoparticles; Protein aggregation; Amyloid; Light scattering; Diffusion

1 Introduction

Aggregation of proteins, particularly into amyloid fibrils, is of considerable interest from a clinical perspective because of its role in causing neurodegenerative disorders such as Alzheimer's and Parkinson's. On a different front, manufacture of protein biologics for pharmaceutical applications is a growing business, where unwanted protein aggregation raises concerns about low yield, reduced efficacy, and risk of immunogenicity. Researchers are motivated to investigate diverse aspects of protein aggregation including: solution conditions that cause aggregation, formulations that prevent aggregation, the role of mutations in aggregation propensity, the size and morphology of aggregates, and the kinetics and mechanisms of growth. Experimental investigation of protein aggregation is challenging. Aggregates are polydisperse in size, with a broad and sometimes multimodal distribution. They can range in morphology from fibrillar to amorphous. Preferably, techniques allow characterization of aggregates without labels, because labels can interfere with or redirect aggregation, and in solution, because surface interactions can affect aggregation. Most desirable are methods that can be carried out noninvasively and nondestructively, that can be used with a variety of solvents, and that allow measurements over time and over a range of concentrations. Experimental techniques for measuring aggregate concentration, size and morphology include size exclusion chromatography (SEC), dye-binding assays, electron and atomic force microscopy, and dynamic light scattering (DLS). Each technique provides distinctly different, and complementary, views of the "elephant," and each has advantages and limitations [1].

In this chapter we focus on nanoparticle tracking (NTA), a newer technique that holds much promise to fill a unique niche. In NTA, particles in the sample scatter light from an incident

laser, and the scattered light is tracked using a CCD camera (Fig. 1a). Submicron particles move in random directions due to Brownian motion. By measuring the two-dimensional trajectory of the diffusing particle, the diffusion coefficient D is obtained:

$$\overline{r^2} = 4Dt \quad (1)$$

where $\overline{r^2}$ is the mean squared displacement of the particle over a tracking time t . In NTA, a particle is detected because it scatters light, and its trajectory is tracked for a few seconds and video captured. The video is then evaluated frame by frame (typically at 30 frames per second), to measure the distance moved by the particle in each step, thereby building up a history of the particle motion. In the absence of any force fields or concentration gradients, the mean displacement $\overline{r} = 0$ (as long as sufficient statistics are built up) but the mean-squared displacement $\overline{r^2}$ is not (Fig. 1b). With a greater number of trajectories analyzed, the measured $\overline{r^2}$ approaches the true (statistical) value and therefore a more accurate measurement of D is obtained.

D is related to the hydrodynamic radius R_h of the particles by the Stokes-Einstein equation:

$$R_h = \frac{kT}{6\pi\eta D} \quad (2)$$

where k is Boltzmann's constant, T is the temperature, and η is the solvent viscosity. R_h of a spherical particle is simply the particle radius. For nonspherical particles, R_h is the radius of an equivalent spherical particle that has the same diffusivity as the particle of interest. In Eq. 2, it is assumed that particle motion is due to only translational diffusion. For high-aspect particles (such as needles or tubes, where the length-to-diameter ratio is large), rotational diffusion becomes a contributing factor. In NTA, rotational motion can cause "blinking" and distort the interpretation. Other factors that might complicate interpretation of the measured trajectory as simply R_h include flexing or bending motion of long flexible fibrils, or entanglement effects in highly concentrated solutions.

NTA is not an imaging technique. Rather, it detects scattered light from each particle. The intensity of the scattered light depends on the scattering cross section of the particle as well as the intensity of the incident beam. The scattering cross section depends on the particle size and its refractive index increment—a measure of the extent to which the particle scatters light in excess of the solvent. Larger particles will scatter light more intensely, as will particles with greater refractive index increment. There is some threshold of scattered intensity under which particles will no longer be detected, which depends on the beam intensity, the sensitivity of the camera, the particle size, and the particle chemistry. In practice, for protein aggregates the minimum size detectable by current NTA technology is ~30 nm. The minimum size is smaller for gold or other strongly scattering materials. On the other side of the size spectrum, aggregates larger than 1000–2000 nm (1–2 μm) diffuse too

slowly for reliable measurement; these particles will scatter light strongly but one will not have an accurate measure of their size.

Since NTA is a particle-by-particle counting technique, one can directly determine the number (molar) concentration of particle aggregates. In contrast, SEC or dye-binding assays measure mass concentrations, and DLS provides no information about concentration. Thus, NTA measurements can be particularly advantageous for building kinetic models, because rates of reaction depend on molar and not mass concentration.

NTA and DLS are similar in that both are scattering techniques that measure particle diffusion. In DLS, one obtains an autocorrelation function that is the sum of contributions from all scattering particles in the sample volume. Obtaining size distributions from DLS measurements requires the use of constrained regularization or other statistical techniques, which are not always entirely reliable. Because individual particles are counted in NTA, the size distribution is directly obtained, as long as a sufficient number of particles are counted. The fact that larger particles scatter more strongly is a particular problem with DLS, where the signal from large particles can totally swamp out that from smaller particles. With NTA, this is less of a problem but is still not completely negligible. Because the laser used as the incident beam has a Gaussian distribution of intensity, smaller particles near the edge of the beam may miss detection whereas larger particles will be detected. Or, small particles may be hidden in the “halo” of strongly scattering particles. These factors can affect the accurate measurement of particle number concentrations, and are of particular concern when the sample size distribution is very broad.

One of the unique, but underutilized, advantages of NTA is that individual particles are measured for both size *and* intensity. One can thereby evaluate mixtures of particles with different material properties, as long as they have different refractive indices.

For more detailed information on the theory of nanoparticle tracking and analysis of data, readers are referred to [2] or [3]. Some additional useful references on applications of NTA include [4–10].

2 Materials

1. Buffers

Water and most aqueous buffers can be used. For example, we have used ultrapure water (distilled, deionized, and filtered water), Tris buffer: 200 mM Tris–HCl, pH 8.4, phosphate-buffered saline PBS: 100 mM NaCl, 10 mM phosphate, pH 7.4, CHES: 10 mM *N*-cyclohexyl-2-aminoethanesulfonic acid, 140 mM NaCl, pH adjusted to 9 with 1 M NaOH, or 8 M urea/0.01 M glycine–NaOH, pH 10. Buffers are filtered through a 0.02 μ m Whatman Anotop 10 filter (see **Note 1**).

2. Standards

Nanosphere size standard (NIST Traceable Mean Diameter) polystyrene latex beads in several different sizes (e.g., 60, 100, and 240 nm) are available from

Thermo Scientific (Fremont, CA) and can be used without further purification. Gold nanoparticles are available from BBI solutions (Cardiff, UK) and, because of their stronger scattering, are useful at smaller sizes (e.g., 20 nm).

3. Proteins

Purity should be established by SDS-PAGE or other appropriate techniques. Samples should appear clear and colorless by eye, with no turbidity or sediment. Samples should be filtered through an appropriate size filter to remove contaminants (*see Note 2*).

4. Nanosight

The Nanosight LM10 (Nanosight, Amesbury, UK) equipped with a 405 nm laser was used in all examples described here. Other laser wavelengths including 488 nm (blue), 532 nm (green), or 642 nm (red) are available, which are particularly useful for fluorescence-based methods (which are not discussed here). Two different cameras can be installed on the Nanosight: the standard CCD camera or a high-sensitivity CMOS camera. The higher sensitivity is better for very dilute solutions or smaller particles.

3 Methods

3.1 Instrument Setup, Sample Preparation and Data Collection

1. Assemble viewing unit. If necessary, switch off laser and remove power lead, and clean all parts (*see step 6*). Embed O-ring in its channel in the top plate. Lay the top plate onto the embedded optical flat gently. Tighten four metal screws alternately in opposite diagonal pairs. Do not overtighten. Attach the power lead to the viewing unit.
2. Check cleanliness. First check the cleanliness of the sample chamber and buffer preparation with ultrapure water and filtered buffer. Load water or buffer into the sample chamber using a syringe, allowing some excess to spill out of the outlet port. Advance sample slowly and avoid air bubbles (*see Note 3*). The observation of a completely blank background is required and represents the absence of scattering particles. If particles are detected in water or buffer, repeat cleaning of the instrument (*see step 6*) and filtering of the buffer until no particles are detected.
3. Load sample. Sample volume should be greater than 0.5 mL. Load the sample using the same technique as for water or buffer. Turn on power. The laser beam should be a thin line passing through the sample chamber if the sample is properly prepared and loaded with a proper concentration. Samples may need to be diluted to reach the target particle number concentration (*see Notes 4 and 5*).
4. Adjust the microscope and find the viewing field. Place viewing unit onto microscope stage. Adjust the position of microscope objective to find the optimum imaging position. This is illustrated in Fig. 2a. The laser beam path is indicated by the dashed lines. Adjust the *x*- and *y*-axis positions, and find the

fingerprint (flare spot). Adjust the x -axis position by moving to the left and find a line to the left of the flare spot. Continue moving to the left, and the optimum imaging position is on the left of the line. Then adjust the height of the microscope objective to obtain a clearly visible image of particles present within the beam, in focus. Particles should appear bright and sharp; avoid blurry or indistinct particles (Fig. 2b). It is easier to use the microscope to first find the approximate viewing field and then use the camera to find the optimal viewing field. You will need to adjust focus when switching from microscope to camera view.

5. Choose instrument settings and capture videos. Adjust the camera level so that all particles are visible but the background is dark (not milky). In SOP tab, use the standard measurement script function (five repeats of 60-s capture time, *see Note 6*), or load a script of your own (*see Note 7*). Run the script to initiate video capture. Be sure to keep the instrument steady and avoid any vibration or jarring. The instrument sits on vibration reduction pads and should be placed on a sturdy table, isolated from walls or any outside source of vibration such as traffic or trains. Check that the temperature in the cell is recorded automatically, or measured and recorded (depending on instrument model).
6. Switch off the laser and disconnect power. Empty the sample chamber of all residual fluid using a syringe via the Luer ports. Rinse the sample chamber with filtered buffer or water a few times and expel from chamber before disassembling. Unscrew the screws securing the top plate and take out the optical flat. Rinse top plate and optical flat twice with water and ethanol, and then dry with an air can, paying special attention to the input and output Luer ports. Dampen a lens paper with water and carefully wipe once over the surface of the optical flat in situ and top plate. Repeat with a lens paper dampened with ethanol (*see Note 8*).

3.2 Video Analysis

1. Start with the recommended default screen gain value at 10.0 and select an appropriate detection threshold. Evaluate the video and decide “by eye” what is a particle. Adjust the detection threshold so that every particle that you identify visually is highlighted with a red cross (+) located at the center of the particle (which appears as a white dot). Particles are selected for identification based on the scattered intensity as well as some other factors. The red cross indicates that the particle has been recognized and its trajectory will be analyzed. If the detection threshold is too low, multiple red crosses might be erroneously assigned to a single particle, whereas a high detection threshold might miss less intensely scattering particles (*see Note 9*).
2. Click OK in processing setting message and videos will be analyzed, displayed, and exported. Analysis includes size, size distribution, number concentration, and intensity.

3.3 System Performance Check

Although NTA does not require calibration, it is recommended that the performance be checked every 6 months, or with every new operator. For this purpose, standardized polystyrene latex spheres or gold nanoparticles are useful (see **Note 10**).

1. To collect data on standardized latex beads: Prepare dilutions of 60, 100, or 240 nm Nanosphere size standard in deionized water. Adjust concentration if necessary. Inject into the sample chamber, capture videos, and analyze. The mean particle size should be very close to the standard. For example, in one experiment we obtained: 59 ± 3 nm for 60 nm beads, 99 ± 2 nm for 100 nm beads, and 239 ± 5 nm for 240 nm beads (mean $\pm$ SD from multiple repeat measurements).
2. Prepare a mixture of 60 and 100 nm latex beads at approximately 1:1 number ratio, and a mixture of 100 nm and 240 nm beads at 1:1 ratio. Inject into sample chamber, capture videos, and analyze. A bimodal distribution should be obtained. In one experiment, we used a 1.2:1 ratio of 60 and 100 nm particles, and observed a bimodal distribution, with the peak sizes shifted slightly toward the center (64 and 96 nm, respectively). The measured number concentration of 60 nm beads was about 15% lower than the number of 100 nm beads, even though the actual number of 60 nm beads was 20% higher (Fig. 3). Similar phenomena were observed for 100 nm and 240 nm mixture. This demonstrates that the detection threshold varies with particle size, and that some smaller particles may escape detection in the presence of larger, more strongly scattering, particles.
3. To collect data on gold nanoparticles: Prepare several dilutions of 20 nm gold nanoparticles (BBi solutions, Cardiff, UK). Inject into sample chamber, capture, and analyze. The number concentration should be close to expected, (within 5–10%) and there should be a linear response with dilution as long as the sample was within the target concentration range of 10^7 – 10^9 particles/mL. For example, in one experiment, we obtained a mean size of 20.3 ± 0.5 nm; plotting the measured versus calculated number concentration yielded a straight line with a slope of 1.04 (compared to ideal slope of 1.0) and R^2 of 0.997.

3.4 Examples

In the following section, we provide details of three different applications that illustrate use of NTA in protein/peptide aggregation.

1. Example 1: Aggregate size distribution. Transthyretin (TTR) is a homotetrameric transport protein (56 kDa, 7 nm hydrodynamic diameter,) that self-associates into fibrillar (amyloid) aggregates at acidic pH. Recombinant wt TTR was diluted to 0.35 mg/mL in acetate buffer (pH 4.4), quickly filtered, and immediately placed into the sample chamber. The size distribution is shown in Fig. 4a, and the result of binning the data is shown in Fig. 4b. The size distribution is unimodal and fairly broad. Virtually no particles of less than 40 nm diameter were detected. The lack of detection does not mean the lack of existence, rather it demonstrates

the lower size limit of detection and that small particles are more difficult to detect in a heterogeneous sample that also contains larger particles. Samples were also analyzed by dynamic light scattering (DLS), and the mean hydrodynamic diameter is shown on the figure (*see Note 11*).

2. Example 2: Aggregation kinetics (*see Note 12*). Peptides containing repeat runs of 20 or 24 asparagines (N20 or N24, respectively), along with flanking lysines for solubility, were synthesized using standard solid-phase peptide synthesis and purified by reverse-phase HPLC [11]. Peptides were disaggregated, filtered through a 0.22 μm filter, and diluted into Tris buffer to a final concentration of 30 μM . Samples were immediately filtered through a 0.02 μm filter, and loaded into the chamber. A custom script was written to collect data with 2-min intervals for the first 10 min and 5-min intervals for the last 50 min, for a total of 1 h (Fig. 5). For N20, almost no particles (one or none in the viewing field) were observed up to 35 min. At 35 min, weakly scattering, rapidly diffusing particles started to appear; these grew in both size and number concentration over the next half hour. With N24, a few small particles appeared at 2 min and the particle number concentration increased steadily over the next 15 min (*see Note 13*). After 25 min, very large, strongly scattering aggregates appeared suddenly; the diffraction rings attest to the micron (or greater) size of the aggregates.
3. Example 3: Intensity versus size analysis. Peptides containing a repeat run of 24 glutamines (Q24) or the Alzheimer-related peptide beta-amyloid ($\text{A}\beta$) were allowed to aggregate. Samples were loaded into the chamber and data was collected. By checking Particle Data in the export data window, information on every tracked particle is obtained, including its hydrodynamic diameter and its scattered intensity. The “True” particles were selected, and the normalized scattered intensity was plotted versus particle size for every counted particle (Fig. 6). We observed segregation into two separate clusters, with aggregates of Q24 scattering less light than $\text{A}\beta$ aggregates of equal size. This indicates that, for a given aggregate size, Q24 aggregates contain more water and are less “folded” than $\text{A}\beta$ aggregates. Considered another way, for a given scattered intensity, Q24 aggregates diffuse more slowly and are therefore less compact than $\text{A}\beta$ aggregates. Although both Q24 and $\text{A}\beta$ assemble into fibrillar aggregates, these data indicate that there are structural differences between these aggregates.

4 Notes

1. Some liquids must not be used in NTA, including (a) solvents with significant optical absorption, (b) any glass etching solutions (hydrogen fluoride, potassium fluoride, etc.), or (c) O-ring incompatible solvents (DMSO, DMF, acetone, acetic acid, etc.). For aqueous buffers that contain high concentrations of solutes, or for nonaqueous solvents, the viscosity must be adjusted in SOP-Advance. Also, viscosity is a strong function of temperature and the operator should ensure that the correct viscosity is used. This is a critical step, because the relationship

between diffusion coefficient and hydrodynamic radius is a function of solvent viscosity (Eq. 2).

2. Membrane filter sizes are given as a mean pore size, and there is a size distribution. Larger particles can initially pass through the filter, so the early few drops of filtrate should be discarded. Sedimenting particles will interfere with NTA measurements and must be avoided. These large particles can be removed by centrifugation or filtration.
3. After pulling the sample into the syringe, any air bubbles should be removed by inverting and tapping the syringe lightly, to force bubbles to float up, and then pushing gently till the liquid reaches the tip of the syringe. Having the sample chamber completely dry prior to sample loading helps to avoid air bubbles. The cell unit can be tilted (with the syringe injecting vertically upward) to avoid trapping air bubbles. The sample chamber should be filled slowly until the liquid reaches the tip of the nozzle. It is important that sample solutions do not leak under the O-ring and around the optical flat as it could cause irreparable damage to the laser.
4. The optimum concentration range is 10^7 – 10^9 particles/mL. Samples containing fewer than 10^7 particles/mL have an insufficient number of particles in the field of view for statistically valid sampling. Samples containing more than 10^{10} particles/mL are likely to have particle trajectories that “cross over” one another. In a typical sample of an aggregation-prone protein, the protein mass concentration is determined by absorbance. However, neither the aggregate size nor the fraction of protein that is aggregated is known prior to NTA measurement, so one generally cannot a priori prepare a sample at the desired particle concentration range. The optimum concentration usually needs to be found by serial dilution, with no step greater than 100-fold dilution. A simple way to determine if a sample is too concentrated is to load the sample in the sample chamber and turn on the power. If the beam appears very bright and fuzzy, and “blooms” within the sample (looking at the sample cell chamber), or if the background appears like static on a television and particles are not distinguishable from the background (looking at the camera capture screen), then the sample is much too concentrated and needs further dilution. One word of caution: dilution may in some cases interfere with accurate characterization of the sample, for example, if aggregation is concentration-dependent.
5. A syringe pump increases the number of sample particles captured and tracked during a run. This provides a more representative sample population, thus yielding a more reproducible size distribution and more accurate concentration measurement. A syringe pump is particularly useful for very dilute samples where insufficient particles are tracked to provide a statistically robust measurement. A syringe pump can also be extremely useful to prevent settling of the sample during data collection. However, using a syringe pump requires higher sample volumes. It should also be noted that the shear stress produced by

the syringe pump might induce aggregation of samples sensitive to these types of stresses.

6. The duration of each capture depends on the number of particles in the field of view; typically 60 s are sufficient if 20–60 particles are in the field of view in any single frame. (An individual particle is tracked for only a few seconds; during a 60-s capture particles will move in and out of the field of view.) A minimum of 500 total particle tracks per measurement should be collected. Longer measurement time is needed for samples containing fewer particles, with the maximum measurement time about 3–4 min. At least three repeat measurements should be taken. Samples with high polydispersity and/or low particle concentrations require more repeats to reduce sampling biases. For kinetic studies where smaller time intervals are needed, shorter capture time (30-s) can be used.
7. As an example, the following script will collect five repeats of 60-s captures.
CAMERASETTINGMSG
REPEATSTART
MESSAGE Please advance sample, then press OK
CAPTURE 60
DELAY 1
REPEAT 4
PROCESSINGLESETTING
EXPORTRESULTS
8. To remove any adsorbed protein in the sample chamber, fill the chamber with 100 mM NaOH solution and hold for 30 min. Remove NaOH solution with syringe and follow the normal cleaning procedure.
9. It is a good idea to vary the detection threshold and compare distributions and number concentrations to see if there are any biases or other unwanted effects. Additional security against unwanted artifacts is provided by the software, as it will evaluate each identified particle and decide if particles are “true” or “false” particles. In general, it is better to go too low on detection threshold (false positives) than too high (false negatives), because the software will delete marginal (“false”) particles. On the other hand, choosing a very low detection threshold means that a lot of particles are analyzed and determined to be false, which increases analysis time.
10. For materials with high refractive index (e.g., gold or silver colloidal), the lower size detection limit is 10–15 nm diameter. For particles with moderate refractive index (e.g., condensed polymers, protein aggregates), the lower size limit is around 25–35 nm. For weakly scattering materials (e.g., polymers, liposomes), detection limit is approximately 40 nm.

11. There are two important differences between NTA and DLS. First, the mean size determined by DLS is *z*-(intensity) averaged, which weights larger particles much more heavily. On the other hand, unaggregated protein and small (<40 nm) aggregates contribute to the DLS signal, but are too small to contribute to the distribution as measured by NTA.
12. Starting from time zero is important in kinetics study. The instrument setting should be adjusted well before sample injection. After loading the sample in chamber, adjust microscope quickly to get optimum view and start to capture immediately.
13. The particle number concentration (million particles per mL) is calculated by NTA. In our experience, the reported number is reasonably accurate for polystyrene spheres and gold nanoparticles, but large protein aggregates are under-counted [10].

Acknowledgements

The authors acknowledge helpful discussions with Dr. Dennis T. Yang. Financial support was provided by NSF CBET-1262729 and NIH R01AG033493.

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Fig. 1.

(a) A schematic illustrating the principle of NTA operation. Light from the incident laser beam is scattered by the particles in solution, and the scattered light is captured by a camera perpendicular to the fluid plane. (b) Snapshots of a particle undergoing Brownian motion create a trajectory; the displacement r can be measured at each time interval t . The theoretical distribution of displacements follows a bell-shaped curve, which broadens with longer time intervals. The mean distance is zero, but the root-mean squared distance is not. For a 100 nm diameter particle diffusing in two dimensions in water at 298 K, with 1/30 s time intervals, $\sqrt{r^2} = 750$ nm

Fig. 2. Illustration of NTA data collection method. **(a)** Find the viewing field. Adjust the position of the microscope objective to find the flare spot (fingerprint) first, then move the objective toward left. The optimum imaging position is the left side of the vertical line. **(b)** Adjust the height of the microscope objective to obtain a clearly visible image of particles present in focus within the beam, as shown in the left panel. Avoid blurry particles shown in the right panel

Fig. 3. Measured size distribution of a mixture of 60 and 100 nm particles at a 1.2:1 ratio. Arrows show true size of latex particles

Fig. 4. Size distribution of TTR sample. The overall distribution is shown, as well as the option for binning

Fig. 5.

NTA analysis of N20 and N24 aggregation kinetics. Peptides were prepared at 30 μ M in Tris buffer from monomeric stocks. (a–d) Still images taken from videos collected for (a) N20 at 35 min, (b) N24 at 2 min, (c) N20 at 40 min, and (d) N24 at 40 min. (e) Particle number concentration as a function of time for N20 or N24, and the script for 1 h measurement with 2-min intervals for the first 10 min and 5-min intervals for the last 50 min

Fig. 6. Relationship between hydrodynamic radius and scattering intensity of individual particles. Comparison of Q24 (cross mark symbol) with A β (open circle)