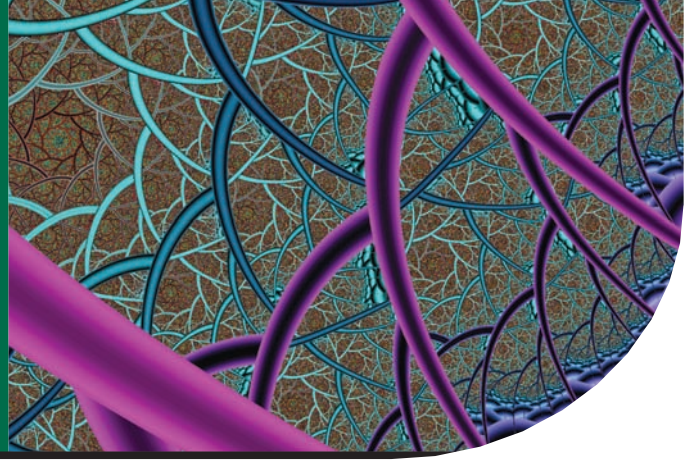

Nanoparticle Characterisation for Drug Delivery Systems

The use of nanoparticles within drug delivery is a growing area of research with wide ranging implications and is one of the major focuses of Professor Wim Jiskoot's group at Leiden University. To successfully use nanoparticles is a challenge in that their characterisation is not straightforward. If all the particles are of the same size, known as 'monodisperse', there are several techniques available. However, where there are mixes of particles sizes and ratios of different sized particles, it is more difficult to make measurements on these 'polydisperse' systems. Jiskoot's group has recently completed a review of a new technique called nanoparticle tracking analysis (NanoSight, Amesbury, UK) in comparison with other techniques.

> ***"The beam is caused to refract at the interface between the liquid sample and the optical element through which it is passed such that it describes a path close to parallel to the glass-sample interface."***

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The Centre for Drug Research, Leiden University is the home for Wim Jiskoot and his team of research scientists. Professor Jiskoot's research is focused on the formulation and delivery of biopharmaceuticals. Biopharmaceuticals are different from conventional drugs because they are based on large, complex molecules (mostly proteins), which are difficult to produce, stabilise, and administer to the patient. He has two lines of work.

The first is devoted to the study of unwanted immunogenicity of therapeutic proteins. Although highly pure and (nearly) identical to endogenous proteins, most therapeutic proteins elicit antibodies in patients. Improved fundamental insight into the causative factors of antibody formation will enable the design of better (for example, more effective and safer) protein drugs. The second research line is vaccine delivery, with the intent to make (for example, bacterial or viral) proteins as immunogenic as possible.

When formulated as a vaccine, these proteins should induce immunity, preferably life-long after a single administration. The aim is to identify the immunogenicity-limiting steps after a vaccine is administered to the patient and thus optimising the performance of the vaccine.

The vaccine delivery group aims to develop innovative delivery systems, such as polymeric nanoparticles and liposomes, for the delivery of different types of vaccines through the conventional (injection) or needle-free administration routes (such as transcutaneous or intranasal delivery). It is very important to know the size of the delivery systems as the size can influence the uptake by the cells of the immune system, the diffusion through the skin, the release of vaccine components, and thus the immune response. The protein characterisation group seeks to understand the causes of unwanted immunogenicity of therapeutic proteins and develop transgenic mouse models capable of predicting immunogenicity of human/humanised proteins in a preclinical setting.

For the protein group, a good size characterisation of protein aggregates is essential to better understand which size class is responsible for triggering unwanted immunogenicity of therapeutic proteins which is believed to be related to the presence of aggregates in the protein formulations. The group aims to stress and thoroughly characterise protein formulations to then test which ones are more immunogenic after their injection in the mouse models.

Prior to using NanoSight's LM-20 system, the Leiden group used a variety of established particle characterisation techniques such as Dynamic Light Scattering (DLS), Light Obscuration Particle Counting (LOPC) and Electron Microscopy (EM). However, each has deficiencies in terms of parameters such as sample preparation and speed of use. The work reported here evaluates the nanoparticle tracking analysis (NTA) technique, compares it with dynamic light scattering (DLS) and tests its performance in characterizing drug delivery nanoparticles and protein aggregates.

DYNAMIC LIGHT SCATTERING

While Dynamic Light Scattering (DLS) methods (also known as Photon Correlation Spectroscopy - PCS) is an industry standard technique that is used routinely and very successfully for the analysis of monodisperse and homogenous sample types. It is however well recognised that DLS can become unreliable when presented with heterogeneous samples which contain a wide range of particle sizes, and that the mean particle size (z-average) will be intensity weighted towards the larger brighter particles within the sample.

Furthermore, successful analysis of the correlation function by classical deconvolution algorithms to extract, for instance, multimodal distributions are realistically limited to sample types containing only two (or exceptionally three) monodisperse particle sizes, each needing to differ from each other by a size factor of, in practice, >3:1. DLS is also limited in its ability to allow the user to recognise when the sample is unsuitable for analysis by that method and that the data (for example, the particle size distribution profile) obtained should accordingly be treated with some suspicion.

NANOPARTICLE TRACKING ANALYSIS

An alternative light scattering method for nanoparticle analysis is Nanoparticle Tracking Analysis (NTA). It is being increasingly used for determining nanoparticle size through simultaneously but individually tracking and analysing the trajectories described nanoparticles undergoing Brownian motion in a fluid.

HOW DOES NTA WORK?

The technique is centred on a sample analysis module *(Figure 1)*, which comprises a small metal housing containing a solid-state, single-mode laser diode (<35mW, 638nm) configured to launch a finely focused beam through the sample of liquid containing a dilute suspension of nanoparticles placed directly above a specially designed optical flat. The sample chamber is approximately 250µl in volume and 500µm deep. Samples are introduced by syringe via a Luer port and allowed to thermally equilibrate for 20 seconds prior to analysis.

The beam is caused to refract at the interface between the liquid sample and the optical element through which it is passed such that it describes a path close to parallel to the glass-sample interface.

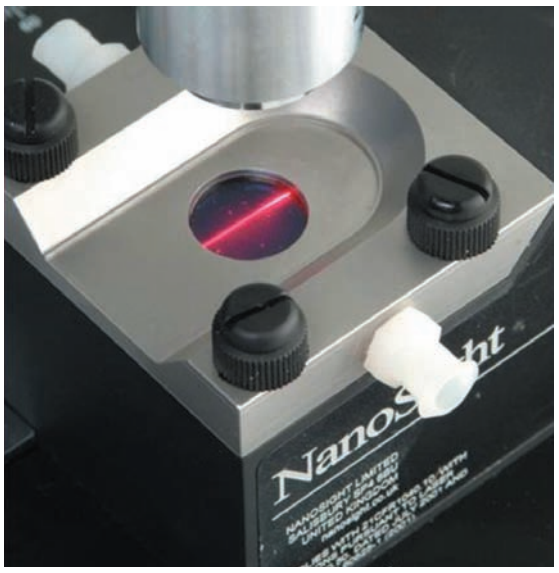

Figure 1. Picture of laser module showing beam passing through sample and viewed from above via microscope objective.

Particles in the beam (which is approximately 100µm wide by 15µm deep) are visualised by a conventional optical microscope aligned normally to the beam axis to collect light scattered from each and every particle in the field of view.

Given NTA is not an imaging technique per se; the total magnification of the system is quite modest (x100 via a x20 0.4 NA long working distance microscope objective). The particles are seen down the microscope as small points of light moving rapidly under Brownian motion *(Figure 2a)*.

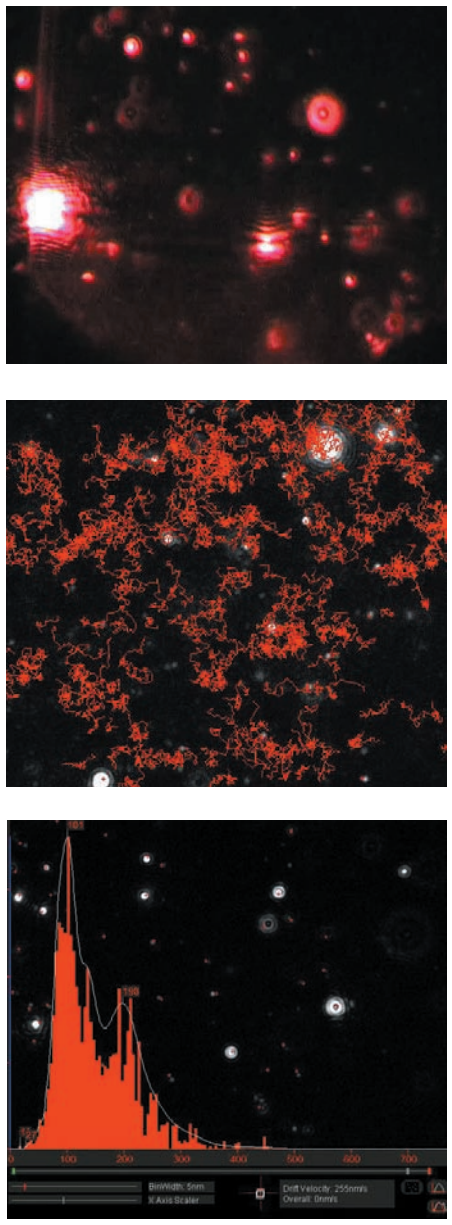

Figure 2a. Still image of nanoparticle suspension as seen by microscope in the path of the laser beam; b. trajectories of individual particle Brownian motion as plotted by the tracking analysis program and c. particle size distribution profile as generated by analysis of particle trajectories.

A video of 20-60 seconds is taken of the moving particles at 30 frames per second. The video is analysed by a proprietary analysis program on a frame-by-frame basis, each particle being identified and located automatically and its movement tracked *(Figure 2b)*. The results are finally displayed as a particle size distribution plot *(Figure 2c)*.

A DEMONSTRATION OF THE RESOLUTION OF NTA: COMPARISON OF DATA FROM MIXTURES OF MONODISPERSE POLYSTYRENE BEADS HAVING A FIXED NUMBER RATIO

One of the pitfalls of DLS is its low peak resolution, i.e. it can only resolve particle populations that differ in size at least by a factor of three. Thus, to demonstrate the resolution of NTA, monodisperse polystyrene standard beads were mixed at a fixed number ratio (60nm and 100nm; 100nm and 200nm; 200nm and 400nm; 400nm and 1,000nm) and analysed with both techniques.

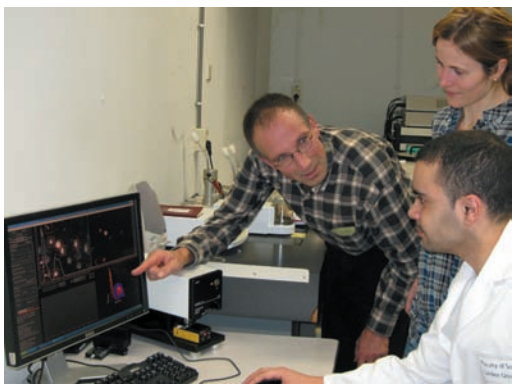

Figure 3. Professor Wim Jiskoot with Andrea Hawe and Vasco Filipe at Leiden University discuss results from the NanoSight LM-20 system.

The two-dimensional (2D) size distributions of DLS and NTA, with the corresponding NTA video frames and three-dimensional (3D) graphs (size vs. intensity vs. concentration) are shown in *Figure 4*. From these results, the difficulty of DLS in resolving peaks of polydisperse samples becomes apparent, as it was not possible to separate the two bead sizes of any of the mixtures. On the other hand, NTA was able to resolve and distinguish the two size populations in all mixtures and yielded accurate size estimations of the beads in the mixtures. The 2D size distributions show that DLS only gives a single peak for the mixtures shifted towards the larger particle size present, which is again related to its bias to larger particles. The error bars of the DLS results of the two mixtures with the larger bead size *(Figure 4c and d)* are larger than the ones of the NTA results. This is related to the difficulty that the DLS software has to fit the data of an autocorrelation curve of a sample that has two populations with size differences smaller than the peak resolution limit of this technique. As a result, the single peak as calculated by the DLS software is prone to changes in shape and position from measurement to measurement, giving rise to relatively large error bars in the average result.

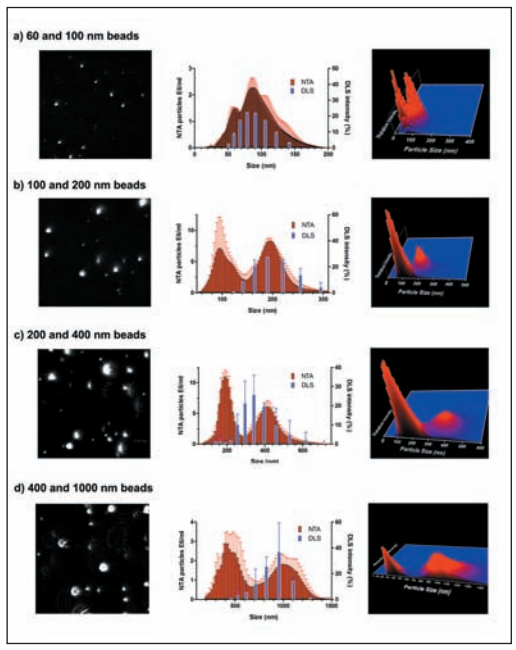

Figure 4. Size distribution from NTA and DLS measurements of mixtures of monodisperse polystyrene beads (middle panels) with the corresponding NTA video frame (left panels) and 3D graph (size vs. intensity vs. concentration; right panels). a) 60-nm/100-nm beads at a 4:1 number ratio; b) 100-nm/200-nm beads at a 1:1 number ratio; c) 200-nm/400-nm beads at a 2:1 number ratio; d) 400-nm/1,000-nm beads at a 1:1 number ratio.

The two different bead sizes with different scattering intensities can be observed in the NTA video frames and 2D size distribution graphs and can be clearly distinguished in the 3D graphs.

COMPARISON OF RESULTS FROM DIFFERENT DRUG DELIVERY NANOPARTICLES

In order to evaluate the analytical performance of NTA for nanoparticles commonly used in the pharmaceutical field, PLGA (polylactic-co-glycolic acid) particles, TMC (N-trimethyl chitosan) particles and liposomes were analysed with NTA and the results compared to DLS (*Figure 5*).

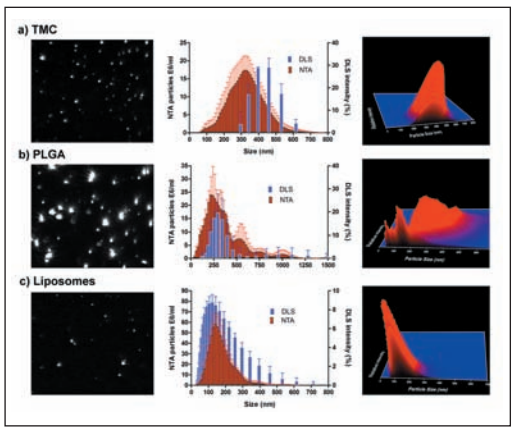

Figure 5. Drug delivery nanoparticles measured with NTA and DLS. The size distribution (middle panels) with the corresponding NTA video frame (left panels) and 3D graph (size vs. intensity vs. concentration; right panels) are shown.

The TMC result shows a mean particle size of 320nm by NTA whereas the DLS result is about 410nm. This shift may be explained by the fact that size distributions obtained by DLS are intensity distributions. Because NTA counts each individual particle, it is providing a number distribution.

The PLGA results show a system which is much more polydisperse than the TMC. This is very clearly seen in the visualisation of the sample in the video of the NTA measurement. It is shown that the main population of particles by DLS is shifted to larger sizes than those reported by NTA, which also clearly shows the polydisperse nature of the sample.

In the final liposome example, the DLS result is slightly lower than that obtained using NTA. This may be a function of detection limits of the two techniques and perhaps further analysis by other techniques is required to clarify these observations.

Further examples illustrating the preferred use of NTA to study heat induced protein aggregation have also been published by the Jiskoot group [1] .

CONCLUSION

The team concluded that NTA has been shown to accurately analyse the size distribution of monodisperse and polydisperse samples. Sample visualisation and individual particle tracking are features that enable a thorough size distribution analysis. The presence of small amounts of large (1,000nm) particles generally does not compromise the accuracy of NTA measurements, and a broad range of population ratios can easily be detected and accurately sized. NTA proved to be suitable to characterise drug delivery nanoparticles and protein aggregates, complementing DLS.

Commenting on the NTA method, principle user Vasco Filipe, said: "We are able to visualise the sample which gives us confidence in our results. Individual particle tracking enables a much better peak resolution than DLS so making it better suited to study polydisperse samples."

REFERENCE

[1] NanoSight wishes to acknowledge that the applications shown here along with other examples comparing NTA and DLS were first published in a paper by Vasco Filipe, Andrea Hawe and Wim Jiskoot entitled 'Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates'. Pharmaceutical Research (2010) DOI: 10.1007/s11095-010-0073-2 (open access at Springerlink.com).