Assessments of NTA

Part 2 of NTA 1000 publications review

4 PARTICLE CONCENTRATION

PARTICLE SIZE

Recent Developments in Methods for Nanoparticle Analysis

Nanoscale materials, in the form of nanoparticulates, play an important and growing role across a range of different applications and industries who seek to exploit the significantly enhanced properties exhibited by such materials when divided to ultra-fine dimensions (e.g. greatly increased surface area, number concentration, etc.). The overall properties and stability of many manufactured products often depends upon the ability to repeatedly produce particle populations with fine tolerances, without the presence of contaminants or aggregates. The concentration of particles within a suspension is another factor that may have an effect upon the desired outcome of a product. It is clear then that there is a real need to characterize a variety of different properties when analyzing nanoparticles in order to fully understand the relationship between the formulation and the overall bulk characteristics of the materials (Fedotov, 2011). Similarly, Paterson et al. (2011) have overviewed the requirement for quantified nanoparticle concentrations in environmental media in order to appropriately assess the risks to biological species due to potential nanoparticle exposure. In response to the recent European Commission definition of the term 'nanomaterial' in which a material is deemed to be such if it contains over 50% by number of particles with at least one dimension below 100nm, Linsinger et al. (2012) have reviewed the requirements on measurements for the implementation of the definition. Finally, Brown et al. (2013) have also recently highlighted current particle size metrology challenges faced by the chemical industry due to the move towards classifying industrial materials by their number content of sub–100-nm particles which could have broad implications for the development of sustainable nanotechnologies. They discuss advancing nano-object concentration measurement metrology as a path ahead to a best practice framework.

There are many techniques available for the analysis of particle size and size distribution, of which the most common include Dynamic Light Scattering (DLS), Electron Microscopy, Atomic Force Microscopy and Analytical Ultracentrifugation. However, each of these techniques comes with a unique set of benefits and limitations. Electron Microscopy (EM) and Atomic Force Microscopy (AFM) both offer users images of the particles themselves with high resolution information about both the size and morphology of the particles present, but both techniques also require time

consuming preparation of samples, which could be potentially damaging and require the user to spend considerable time on analysis (Syvitski, 1991).

Ultracentrifugation again provides high resolution information on the size of particles within a sample but the technique requires a degree of previous knowledge of the composition of the material, is time consuming and the initial investment for apparatus can be costly (Mächtle, 2006). Baalousha et al. (2011) have critically reviewed the technique of flow field-flow fractionation for the analysis and characterization of natural colloids and manufactured nanoparticles in environmental systems in which they help users understand (i) the theoretical principles and experimental consideration of the FlFFF, (ii) the range of analytical tools that can be used to further characterize the nanoparticles after fractionation by FlFFF, (iii) how FlFFF results are compared to other analytical techniques and (iv) the range of applications of FlFFF for natural and manufactured NPs.

Pace et al. (2012) have similarly undertaken a performance evaluation and method comparison in the determination of nanoparticle size by single particle inductively coupled plasma-mass spectrometry concluding that while further development of the technique is needed, spICPMS yields important advantages over other techniques when sizing nanoparticles in environmentally-relevant media. This view was supported by Laborda et al. (2013) who suggested the technique was a "a powerful tool for nanoanalysis" suggesting that although the number of applications reported to date is limited, the relatively simple instrumental requirements, the low number concentration detection levels attainable, and the possibility to detect both the presence of dissolved and particulate forms of an element make this methodology very promising in the nanoscience related areas. Fabricius et al. (2013) have discussed sample preparation and off-line fractionation strategies for the ICP-MS-based characterization of inorganic (Ag, TiO2, CeO2, ZnO, and Au) nanoparticles. Their general conclusions were that, after sample acidification and microwave-assisted digestion, acidified suspensions delivered better recoveries from 89.3 % (ZnO) to 99.3 % (Ag). For the quantification of dissolved fractions two filtration methods (ultrafiltration and tangential flow filtration), centrifugation and ion selective electrode were mainly appropriate with certain limitations, while dialysis and cloud point extraction could not be recommended. With respect to precision, time consumption, applicability, as well as to economic demands, ultrafiltration in combination with microwave digestion was identified as best practice. It was shown that a direct application of undissolved nanoparticle suspensions to an ICP-MS system does, applying steady state analyzes, "mostly not provide reliable data for total metal concentrations. In fact, without any further sample preparation, it is very likely that imprecise results and/or instabilities of the measurements occur".

Ensemble methods based on light scattering and which simultaneously interrogate a large number of particles in a suspension are ideally suited for the analysis of monodispersed systems but have a limited capability to analyze polydisperse systems when trying to establish particle size distribution. Furthermore, being ensemble methods they are unable to provide users with quantitative results regarding the number concentration of their systems. Foremost of such techniques for the analysis of nanoparticles is DLS (alternatively known as Photon Correlation Spectroscopy (PCS) or Quasi Elastic Light Scattering (QELS)) which utilizes a digital correlator to analyze the timescales of fluctuations in intensity of light scattered by a suspension of nanoparticles moving under Brownian motion and, as a well established method for 40 years, has been extensively reviewed (Pecora, 1985). Through analysis of the resultant exponential autocorrelation function, average particle size can be calculated as well as polydispersity index. For multi-exponential autocorrelation functions arising from polydisperse samples, deconvolution can furnish only limited information about the particle size distribution profile (Harding et al., 1992). Furthermore, as the relationship between the size of particles and the amount of light that they scatter varies strongly as

a function of radius6, the results will be significantly biased towards the larger, higher scattering particles within the sample. The resulting intensity weighted average particle size and poor particle size distribution information available can therefore be seriously misleading when analyzing polydisperse samples.

Other optical techniques which normally measure particles >1micron (e.g. static light scattering techniques based on diffractive Fraunhofer scattering or Multi-Angle Laser Light Scattering (MALLS) or the widespread techniques of flow cytometry or Coulter Counting) can measure smaller particles but suffer, in practice, from a lower particle size analysis limit of between 0.3-0.5μm diameter.

Both Linn et al. (2010) and Bell et al. (2012) have discussed optical methods for the characterization of nanoparticles with the latter study being focused on silica.

The recent development of the technique of Nanoparticle Tracking Analysis (NTA) offers the ability to directly visualize, size and measure concentration of nanoparticles in liquid suspension. Due to the fact that this technique can simultaneously analyze a population of nanoparticles on an individual basis, it is ideally suited for the real-time analysis of polydisperse systems ranging from 10-20nm up to 1-2 micron in size (depending on particle type). Additional parameters and measurements also allow users to acquire information on nanoparticle concentration, zeta potential, relative intensity of light scattered and also to visualize\e and analyze fluorescently labelled particles. (Carr et al., 2009).

Nanoparticle Tracking Analysis

NTA is a relatively new technique through based on well understood principles of sizing by measuring the speed of Brownian motion of particles to give nanoparticle Dt and from which a spherical hydrodynamic diameter can be estimated. However, because the optical configuration employed in NTA allows nanoparticles to be simultaneously tracked and analyzed on an individual basis, the resulting data is not an intensity weighted mean as in DLS but a high resolution particle size distribution analysis in which different materials can be distinguished through their different refractive indices and, importantly, in which particle concentration can be recovered.

Furthermore, the ability to simultaneously measure additional parameters such as a nanoparticle's fluorescent properties or their dynamic behavior under an applied motive force (such as an electric or magnetic field) offers the user an unprecedentedly rich profile of nanoparticle properties. That the user also benefits from a direct visualization of the suspension is a further uniquely advantageous feature of NTA.

NTA has been recently assessed as a technique through a number of studies in a wide range of applications. In a study of the accurate particle size distribution determination by NTA based on 2-D Brownian dynamics simulation Saveyn et al. (2010) presented a physical model to simulate the average step length distribution during NTA experiments as a function of the particle size distribution and the distribution of the number of steps within the tracks. During this analysis, it was stated 'As compared to DLS, nanoparticle tracking analysis (NTA) has the advantage that it considers individual particles and hence may provide a higher resolution for multimodal samples. In addition, it provides direct visual information from which aggregation phenomena are visually observable.' They showed that simulation of a step length distribution allowed a much more reliable estimation of the particle size distribution to be determined thereby reducing the artificial broadening of the distribution, as is typically observed by direct conversion of step length to particle size data. As described above, a variation of this modelling step is now incorporated into the NTA algorithm as a 'finite track

length adjustment' which recovers the true distribution width of narrow distributions of monodisperse, calibration quality nanoparticle suspensions.

A further example of the visualization and sizing of particles as small as 87nm has been demonstrated recently. Haiden et al. (2013) used a microfluidic chip and manual 2-D tracking for size determination though the manual nature of the tracking led to extended (several seconds) analytical timescales and low numbers of particles detected.

Filipe et al. (2010) undertook a critical evaluation of the NTA technique, compared to DLS, for the analysis of mixtures of 60 to 1,000nm polystyrene standard nanoparticles and drug delivery nanoparticles as well as heat induced protein aggregates. In this comprehensive study they showed that NTA could accurately analyze the size distribution of monodisperse and polydisperse samples by virtue of its ability to visualize and track individual particles. They showed that the presence of small amounts of large (1,000nm) particles generally did not compromise the accuracy of NTA measurements, and a broad range of population ratios could easily be detected and accurately sized. NTA proved to be suitable to characterize drug delivery nanoparticles and protein aggregates, complementing DLS. Live monitoring of heat-induced protein aggregation provided information about aggregation kinetics and size of submicron aggregates. They concluded that NTA is a powerful characterization technique that complements DLS and is particularly valuable for analyzing polydisperse nanosized particles and protein aggregates. During the development of their 'Fluorescence Single Particle Tracking' (fSPT) technique for the characterization of submicron protein aggregates in human serum, plasma and formulations containing human serum albumin (HSA), Filipe et al. (2011) used NTA as a comparative technique to calibrate their findings. It was found 'the size distributions obtained by fSPT and NTA for the PEGylated beads in buffer are comparable, confirming the accuracy of fSPT to size nanoparticles.'

The developers of a variant of the electrozone sensing technique (i.e. Coulter counter) called "Tuneable Resistive Pulse Technology" have recently carried out a comparative study of the techniques of DLS, EM, NTA, Disc Centrifugation and their electrozone sensing method (Anderson et al., 2013). They claimed that only the Tunable Resistive Pulse Sensor and Disc Centrifuge provided the resolution required to detect all three particle populations present in the mixed 'multimodal' particle sample. In contrast, they reported that the light scattering based Particle Tracking Analysis and Dynamic Light Scattering techniques were only able to detect a single population of particles corresponding to either the largest (410 nm) or smallest (220 nm) particles in the sample, respectively. Some questions remain, however, as to the validity of the undescribed "peak detection algorithm" they employed in the generation of these results given that, in contrast to the results they obtained using NTA, correct operation of the NTA system employed and unadulterated analysis of an identical sample type (a selected mixture of 220, 330 and 410 nm polystyrene particles), showed NTA to be perfectly capable of resolving such a mixture with ease and indeed, was capable of resolving greater widths of particle size mixtures than could the electrozone sensing technique, without, furthermore, suffering from repeated blockage (a notorious problem with the electrozone technique) or changing pore diameter to accommodate this increased distribution.

Mahl et al. (2011) investigated the possibilities and limitations of different analytical methods (including SEM, DLS, NTA and analytical disc centrifugation) for the size determination of a bimodal dispersion of metallic nanoparticles both as pure populations and mixtures of 1:1 by weight While the detection of 15nm gold particles (which approach the detection limit of NTA) was precluded by the presence of 70nm Ag nanoparticles in both NTA and DLS, electron microscopy was poor at the accurate

detection of aggregates. He stated that "The inspection of a large number of particles also permits the analysis of mixtures of small and large particles. For both pure silver or gold nanoparticle dispersions, the analysis gave very satisfactory results. However, the particle size distribution was broader than obtained by other methods, and a clear differentiation between silver and gold nanoparticles was not possible".

Sapsford et al. (2011) and Evtushenko et al. (2011) have respectively reviewed techniques for the characterization of nanomaterials and assessed NTA for nanobiomaterials examination, protein aggregation studies and general nanoparticle characterization. Similarly, Zhu et al. (2011) reported on progress in the development of techniques based on light scattering for single nanoparticle detection, such as NTA, in which he emphasized the difficulties associated with the sixth power dependence of Rayleigh scattering on particle size which makes it very challenging to detect individual nanoparticles of small sizes. Despite these limitations, single particle detection remains attractive as it offer a simple and efficient approach for the size, size distribution, and concentration analysis of nanoparticles in which intrinsic heterogeneity or rare events are masked by ensemble averaging techniques, as exemplified by dynamic light scattering (DLS), can be revealed.

Boyd et al. (2011) have compared atomic force microscopy, NTA and dynamic light scattering for nanoparticle size measurements. They concluded that the different techniques gave different results but these are all consistent considering the exact nature of each measureand and their physical conditions. They showed that while AFM analyzed individual particles with agglomerates not being detected, NTA detected both and combining the two techniques allowed the effect of agglomerates on DLS to be quantified. Recently, Carter et al. (2013) have carried out an extensive review of advances in the analysis of metals, chemicals and materials, specifically with regard to the technique of Atomic Spectroscopy. NTA was discussed among many other comparative techniques.

Recently, Walker (2012) and his colleagues (Walker.et al., 2012) have proposed improvements to the methods by which nanoparticles can be tracked using NTA which account for the finite number of steps in each particle track and consequently for the measurement uncertainty in the step-length data and in which computer simulation and experimental results were presented to demonstrate the performance of the new approach compared with the current method. He also described an alternative approach involving processing multiple images of a sample of particles suspended in a liquid undergoing Brownian motion. From each image, the centres of the particle positions were measured, and then a histogram of the vectors connecting the centres in each image with all the centres in the next image was formed. This vector histogram contained information about the particle size distribution. A maximum-likelihood data inversion procedure to invert the data then yielded a particle size distribution (Walker et al., 2012).

Van der Meeren et al. (2012) have discussed the relevance of two-dimensional Brownian motion dynamics in applying nanoparticle tracking analysis emphasizing that an understanding of the basic principles underlying the technique helps avoid incorrect analyzes. Wagner et al. (2013) suggested that a further improvement to measurements of well-defined particle populations and Monte-Carlo simulations showed that the analysis of polydisperse particle dispersion could be improved with mathematical methods. Logarithmic transform of measured hydrodynamic diameters led to improved comparability between different modal values of multimodal size distributions. Furthermore, an automatic cluster analysis of transformed particle diameters could uncover otherwise hidden particle populations both of which markedly improved the interpretability of multimodal particle size distributions as delivered by particle tracking measurements.

NTA is unique amongst optical methods of deeply submicron particle analysis in its ability to furnish information about particle number concentration. Röding et al. (2013) have presented a method that they claim enables for the first time highly accurate size and absolute concentration measurements of polydisperse nanoparticles in solution, based on fluorescence single particle tracking, that are self-calibrated in the sense that the detection region volume is estimated based on the tracking data. They proved their method on polystyrene nanospheres in water/sucrose solution and extended their results to show quantify aggregation and clearance of different types of liposomes after intravenous injection in rats, where additional and more accurate information can be obtained that was previously unavailable. It should be noted, however, that their technique was only demonstrated on relatively monodisperse and homogenous particle types, the sensitivity of the technique to polydisperse and complex systems being a consequence of the requirement for assumptions based on assumed and fixed light scattering properties for any given particle type.

In more general terms, Gayatri et al. (2012) and Liu (2012) have assessed the preparation and characterization of nanoparticles and, more specifically Du et al. (2012) have used NTA and DLS to measure aggregation and adhesion of gold nanoparticles in phosphate buffered saline. Troiber et al. (2012) have recently undertaken a comparison of four different particle sizing methods (dynamic light scattering (DLS), atomic force microscopy (AFM), nanoparticle trafficking analysis (NTA) and fluorescence correlation spectroscopy (FCS)) for siRNA polyplex characterization pointing out that while NTA was unable to measure the smaller 40nm primary particles, it alone could analyze the larger polydisperse 120nm aggregates. It was concluded that a comprehensive analysis by more than one method is of particular importance.

Gallego-Urrea et al. (2011) critically discussed the advantages and limitations of NTA for the analysis and to characterization of NPs in low concentrations in complex matrixes such as environmental, biological and food samples. Dean (2012) discussed the requirement to produce stable reference materials and prevent agglomeration by modifying the surface of the particles citing NTA as a suitable method for visualizing and analyzing particle size and size distribution by relating the rate of Brownian motion to particle size advocating combining NTA with a label free, real-time, cell-electronic sensing system was used to measure changes in cell number following nanoparticle exposure.

In a critical assessment of NTA in which he compared NTA against other particle tracking methods, Gallego-Urrea concluded that NTA had the benefit of being a minimum perturbation method that gave high sensitivity in terms of particle concentration, and provision of number-based size distributions of high resolution for aquatic samples. It was also rapid, easy to use and low cost. While NTA gave linear calibration curves in terms of number concentration and accurately reproduced size measurements of certified reference material nanoparticles, the accuracy of the size distributions obtained with this method exhibited a high dependence on set-up parameters and the concentrations were shown to be strongly correlated with the refractive index of the material under examination. The size distributions for the contrasting environmental samples were fairly reasonable compared with other studies and were less sensitive to the presence of large particles or aggregates but an underestimation of small sizes was observed, which can be explained by a material-dependent lower detection limit in terms of size. The number concentrations obtained for the natural nanoparticles ranged from 0.5 to 20 × 108 particles mL–1 and correlated well with conventional turbidity measurements. (Gallego-Urrea et al. 2011, Gallego-Urrea 2010) and Fedotov has recently reviewed methods of fractionation and characterization of nano- and microparticles in liquid media (Fedotov et al. 2011).

Nikitin et al. (2013) have recently reviewed the examination of biologically active nanocomplexes by NTA concentrating specifically on an immunogenic complex (a candidate nanovaccine) comprised of spherical particles (SPs) generated by thermal remodelling of the tobacco mosaic virus and Rubella virus tetraepitopes exposed on the surface of SP.

The American Society For Testing And Materials (ASTM) has recently published a standard guide for the measurement of particle size distribution of nanomaterials in suspension by NTA, through adoption of which users of the technique can achieve standardization of results (ASTM E2834 – 12, 2012).

Given that one of the key challenges in the field of nanoparticle (NP) analysis is in producing reliable and reproducible characterization data for nanomaterials, in a most recent study, Hole et al., 2013 have reported an interlaboratory comparison (ILC) of size measurements on nanoparticles using NTA. They described the protocol development and presented both the data and analysis of results obtained from 12 laboratories, mostly based in Europe, who are primarily QualityNano members. QualityNano is an EU FP7 funded Research Infrastructure that integrates 28 European analytical and experimental facilities in nanotechnology, medicine and natural sciences with the goal of developing and implementing best practice and quality in all aspects of nanosafety assessment.

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