# Nanoscale Material Characterization: a Review of the use of Nanoparticle Tracking Analysis (NTA)

PARTICLE SIZE

PARTICLE CONCENTRATION

FLUORESCENCE DETECTION - DELETED

## Summary

This white paper describes the central role of high resolution particle size and concentration measurement nanoparticle research. The technique of Nanoparticle Tracking Analysis (NTA) is described and compared to other characterization methodologies, and comparative papers are cited. A wide range of application studies is then summarized with specific reference to the use and value of NTA.

For those seeking a full listing of NTA experience to date by application type, the NanoSight publication "Nanoparticle Tracking Analysis - A review of applications and usage 2010 - 2012, (Carr B and Wright M (2013)) and its successors provides a detailed catalogue. This item is available from your local Malvern Instruments representative or email helpdesk@malvern.com.

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## Introduction

Nanoscale materials, in the form of nanoparticles, are playing an important and growing role across a range of different applications and industries which seek to exploit the unique properties exhibited by these materials, such as their very high surface area to volume ratio and high number. The overall properties and stability of many manufactured products often depends upon the ability to produce particle populations within fine tolerances, without contaminants or aggregates. The concentration of particles within a suspension is another factor that may have an effect upon the desired outcome of the product. It is clear then that there is a real need to characterize a variety of different properties when analyzing nanoparticles, in order to understand the relationship between the formulation and the overall bulk characteristics of the materials (Fedotov, 2011). Similarly, Paterson *et al.* (2011) have reviewed the requirement for quantified nanoparticle concentrations in environmental media in order to assess the risks to biological species due to potential nanoparticle exposure.

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 (EM), atomic force microscopy (AFM) and analytical ultracentrifugation (AUC). Each of these techniques comes with a unique set of benefits and limitations. EM and AFM both offer users images of the particles with high resolution information about both their size and morphology of the particles present, but both techniques also require time consuming preparation of samples, which requires the user to spend considerable time on analysis (Syvitski, 1991).

AUC 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 apparatus can be costly (Mächtle, 2006).

Ensemble methods based on light scattering, and which simultaneously interrogate a large number of particles in suspension, are ideally suited for the analysis of monodisperse systems but have a limited capability to analyze those that are polydisperse. Foremost of such techniques for the analysis of nanoparticles is DLS (alternatively known as Photon Correlation Spectroscopy (PCS) or Quasielastic Light Scattering (QELS)) which utilizes a digital correlator to analyze the timescales of fluctuations in the intensity of light scattered by a suspension of particles moving under Brownian motion and has been extensively reviewed (Pecora, 1985). Through analysis of the resultant exponential autocorrelation function, average particle size can be calculated as well as the polydispersity index. Furthermore, as the relationship between the size of particles and the amount of light that they scatter varies strongly as a function of

radius, the results are significantly biased towards the larger, higher scattering particles within the sample.

The recent development of the technique of NTA offers the ability to visualize size and count 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 polydispersed systems ranging from 10 nm - 30 nm up to 1 µm - 2 µm in size (depending on particle type). Additional parameters also allow users to acquire information on nanoparticle concentration, relative intensity of light scattered and also to visualize and analyze fluorescently labeled particles. (NanoSight, 2011, Carr *et al.* 2009).

## Nanoparticle tracking analysis: principles and methodology

NTA utilizes the properties of both light scattering and Brownian motion in order to obtain the particle size distribution of samples in liquid suspension. A laser beam is passed through the sample chamber, and the particles in suspension in the path of this beam scatter light in such a manner that they can easily be visualized via a 20x magnification microscope onto which is mounted a camera. The camera which operates at approximately 30 frames per second (fps) captures a video file of the particles moving under Brownian motion within the field of view of approximately 100 µm x 80 µm x 10 µm (Figure 1).
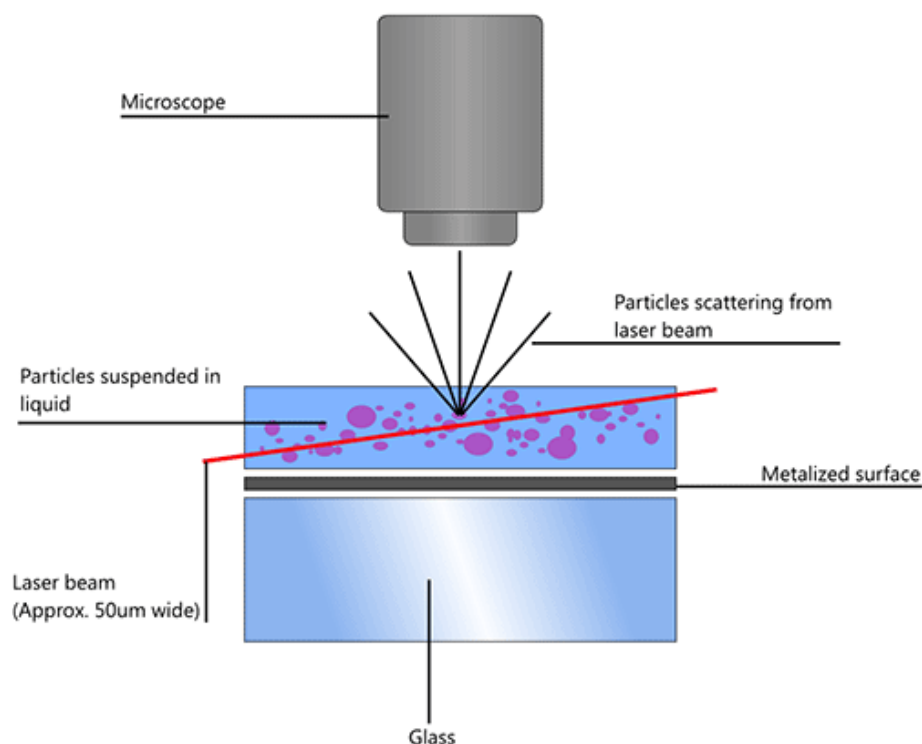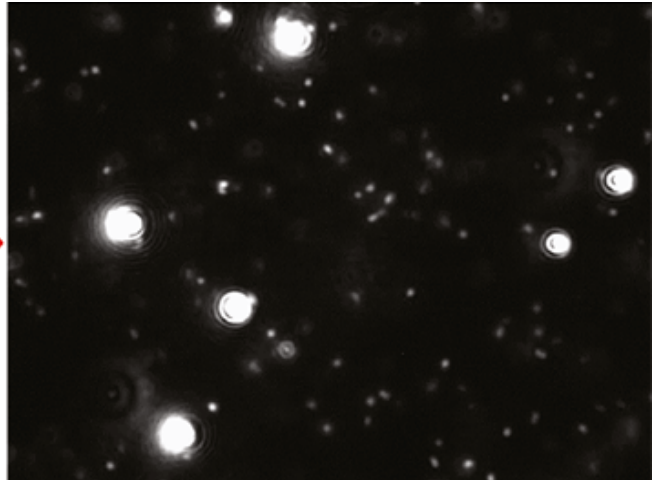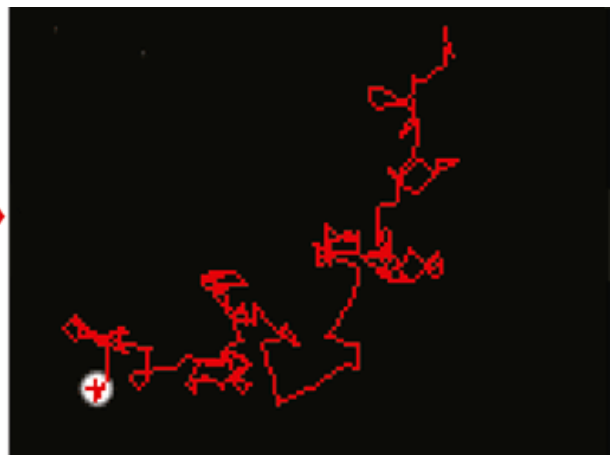
**Figure 1. Schematic of the optical configuration used in NTA.**

NTA captures a video of particles moving under Brownian motion.



NTA automatically locates and follows the center of each and every particle and measures the average distance moved per frame.



This is done simultaneously for all particles whilst NTA reports particle size vs concentration distributions.
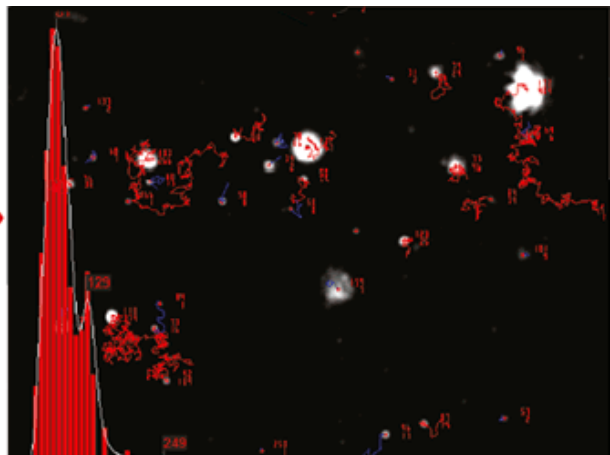
*NTA consolidates the particle-by-particle size measurements with 3D intensity vs. concentration vs. size plots.*
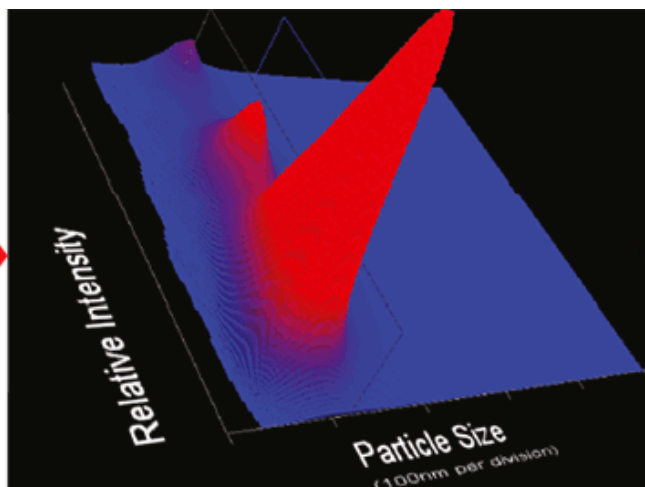
**Figure 2. The NTA particle sizing process.**

## Measuring concentration

While NTA particle sizing requires particles to be tracked for a number of consecutive frames to determine their size reliably, particle concentration can, in principle, be measured from scrutinizing one video frame alone. In practice, data from all the recorded frames contributes to a rolling average concentration measurement. This methodology eliminates concerns that NTA might count the same particle several times, had it been absent then reappeared - such a particle is indeed measured several times, but will ultimately give a contribution dependent on the total proportion of frames in which it was observed in the video (be it one long track or several short tracks). The concentration is calculated by taking this average count and dividing by the interrogated volume in which the number of particles is measured. The interrogated volume approximates to a cuboid 100 μm x 80 μm and with a depth of approximately 10 μm. The height and width of the cuboid are measured directly using a graticule in the microscope view. The depth of the cuboid is defined by both the depth of the laser beam, this being collimated and focused, and the depth of field gathered by the lens. Both of these approximate to 10 μm.

## Concentration ranges measurable

NTA is not an ensemble technique, which interrogates a very large number of particles to produce a single measurement, but rather each particle is sized individually, irrespective of the others. It is important that a sufficient number of particles are analyzed within the sample time to ensure that the data produced is statistically robust. A concentration in the region of $10^6$ to $10^9$ particles per mL provides the user with statistically sound and repeatable particle size distributions within a typical timescale of 30 - 60 seconds.
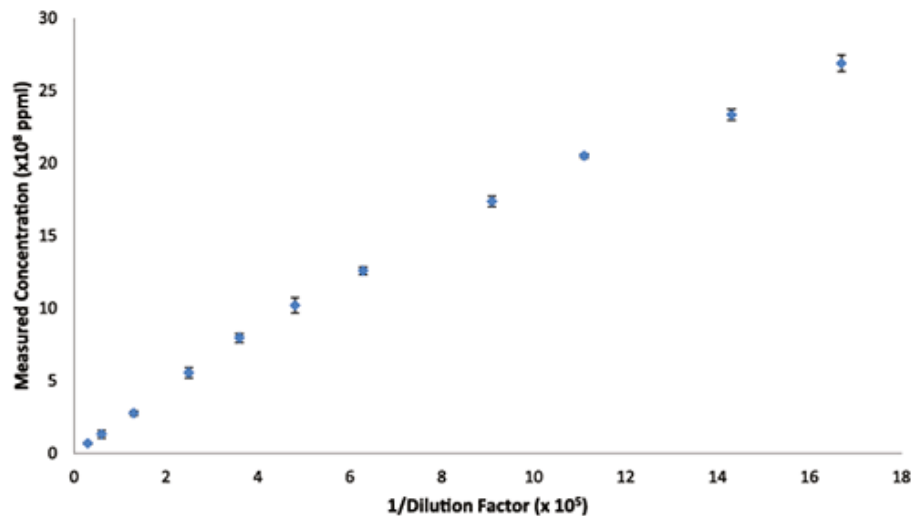
**Figure 3. NTA reported concentration vs. actual sample concentration for 100 nm latex particles.**

Under normal conditions, when analyzing optimal concentrations of nanoparticles exhibiting similar optical characteristics, such as a monodisperse polystyrene, concentration accuracies can be as good as ±5% - 10% if the sample is diluted to a suitable concentration range.

## Particle size determination combined with particle light scattering

While the size of nanoparticles is determined by NTA through measuring their Brownian motion, one of the unique and beneficial features of NTA is its ability to measure the amount of light it scatters ($I_{Scat}$) and plot the two measurements as a function of each other. This allows particles which may be of a similar size but different composition/refractive index to be successfully differentiated.

A mixture of 30 nm Au, 60 nm Au and 100 nm polystyrene can be resolved in a 3D plot of size vs. intensity vs. number (Figure 4) and in which the smaller but high refractive index 60 nm Au particles can be seen to scatter more light than the larger 100 nm polystyrene (100PS).
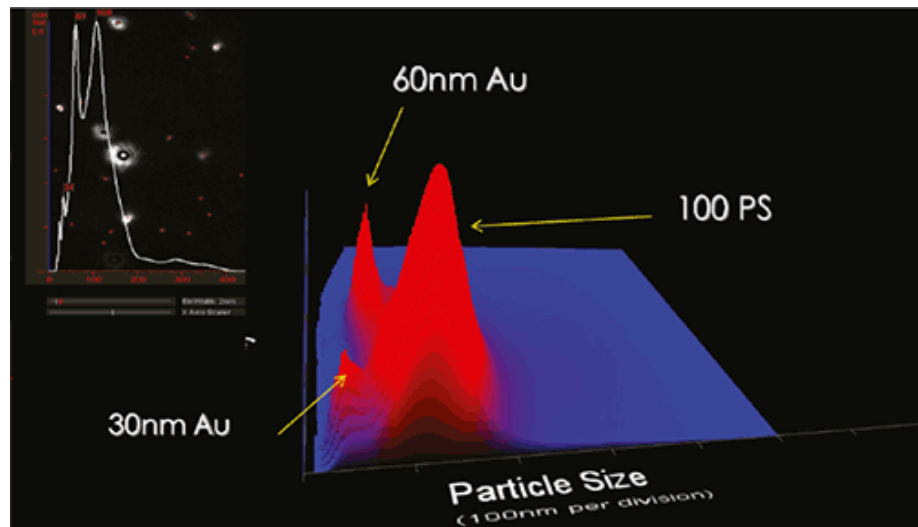
**Figure 4. A 3D plot of 30 nm and 60 nm gold, and 100 nm polystyrene particles in which the smaller but higher refractive index 60 nm gold particles can be seen to scatter more light than the larger 100 nm polystyrene.**

## Assessment of NTA

NTA has been assessed 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 two-dimensional 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. They showed that the simulation of a step length distribution allowed a much more reliable estimation of the particle size distribution to be determined, thereby reducing 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 modeling step is 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.

Filipe *et al.* (2010) undertook a critical evaluation of the NTA technique, compared to DLS, for the analysis of protein aggregates, mixtures of 60 nm to 1,000 nm polystyrene standard nanoparticles and drug delivery nanoparticles. In this comprehensive study, it was demonstrated that NTA could accurately analyze the size distribution of monodisperse and polydisperse samples by virtue of its ability to visualize and track individual particles. It was found that the presence of small amounts of large (1,000 nm) 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 the size of submicron aggregates. It was concluded that NTA is a powerful characterization technique that complements DLS and is particularly valuable for analyzing polydisperse nanosized particles and protein aggregates.

Sapsford *et al.* (2011) and Evtushenko *et al.* (2011) respectively reviewed techniques for the characterization of nanomaterials and assessed NTA for nanobiomaterials examination, protein aggregation studies and general nanoparticle characterization. Similarly, Njuguna *et al.* (2011) reported on progress in the development of techniques based on light scattering for single nanoparticle detection and, more recently, Boyd *et al.* (2011) have compared AFM, NTA and DLS for nanoparticle size measurements. They concluded that the different techniques gave different results, but all were consistent considering the exact nature of each measurand 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.

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

The American Society For Testing And Materials (ASTM) have published a standard guide for the measurement of particle size distribution of nanomaterials in suspension by NTA, in order to enable users of the technique to achieve standardization of results (ASTM E2834-12, 2012).

## Drug delivery and targeting

The use of nanoparticles in drug delivery continues to grow rapidly. Driven by the diminishing rate of discovery of new biologically active compounds that can be exploited therapeutically to treat disease, with fewer new drugs entering the market every year, interest in the use of nanoparticles' versatile and multifunctional structures for the delivery of drugs has grown quickly. Nanoparticles offer better pharmacokinetic properties, controlled and sustained release and targeting of specific cells, tissues or organs (e.g. blood-brain barrier transport). All these features may improve the efficacy, bioavailability and safety of existing drugs (Malam *et al.*, 2011).

Nanoparticles in this context have been defined as colloidal systems of submicron size that can be constructed from a large variety of materials across a large range of compositions. Commonly defined nanoparticle vectors include liposomes, micelles, dendrimers, solid lipid nanoparticles, metallic nanoparticles, semiconductor nanoparticles and polymeric nanoparticles. Nanoparticles have been extensively employed to deliver drugs, genes, vaccines and diagnostics into specific cells and tissues. (Ram *et al.*, 2011).

The targeting of drug delivery nanoparticles to specific sites frequently involves the addition of molecular structures with an affinity for specific cell surface biomarkers, which allows the drug-containing nanoparticle to be accumulated by the target cell types.

The addition of capture molecules (frequently antibodies) to the surface of a drug delivery nanoparticle structure can be problematic; retention of activity, sufficient loading and minimization of aggregation being necessary for optimum performance. Similarly, addition of other biochemical species designed to stabilize the functional structures added to the nanoparticles, or which act to reduce the immunogenicity of the nanoparticle, may result in similar deleterious effects. NTA is capable of detecting small changes in hydrodynamic diameter following the addition of layers of macromolecules to nanoparticles and can both detect and quantify any aggregates which may form during such modifications.

Accordingly, NTA has been used in a number of drug delivery studies including one which described the effect of conjugating polymer-alendronate-taxane complexes for targeting bone metastases (Miller *et al.*, 2009). The same group used NTA to show that successful conjugation for the targeting of angiogenesis-dependent calcified neoplasms using different polymers resulted in very much smaller sizes and narrower polydispersities and that, together with a cathepsin-K-cleavable system, they achieved a more specific drug release and therefore focused the toxicity of the free drug to the bone tumour (Segal *et al.*, 2009).

The successful transport of molecules across the cell membrane is a key point of research in biology and medicine. In many cases, molecules alone cannot penetrate the cell membrane; therefore an efficient carrier is needed. Sokolova *et al.* (2012) have investigated calcium phosphate nanoparticles (diameter: 100 nm - 250 nm, depending on the functionalization) as versatile carriers for small and large molecules across cell membranes using a number of techniques including NTA, DLS and EM. Ohlsson *et al.* (2012) reported on solute transport on the sub-100 milliseconds timescale across the lipid bilayer membrane of individual proteoliposomes, using NTA to check liposome stability and integrity.

In the area of the development of nanoparticles as gene delivery vehicles, Ghonaim and his co-workers have reported extensively on the use of NTA on the effect of modifications to the chemistry of lipopolyamines and spermines in various non-viral plasmid DNA and siRNA delivery systems (Ghonaim *et al.*, 2007a;

Ghonaim *et al.*, 2009; Ghonaim, 2008; Ghonaim *et al.*, 2007b; Ghonaim *et al.*, 2007c; Soltan *et al.*, 2009; Ghonaim *et al.*, 2010). Similarly, Ofek *et al.* (2010) have employed NTA for the characterization of dendritic nanocarriers for siRNA delivery while Bhise measured particle size and size distribution by NTA in their study of gene delivery polymers in cell culture (Bhise *et al.*, 2010). Bhise recently further extended this work to develop an assay to quantify the number of plasmids encapsulated by polymer nanoparticles, in which he used NTA to determine the number density of plasmids per 100 nm nanoparticle (Bhise *et al.*, 2011).

Wei *et al.* (2012), in exploring the challenges and opportunities in the advancement of nanomedicines, identified numerous needs including robust methods for the accurate characterization of nanoparticle size, shape and composition, as well as particle engineering to maintain low levels of nonspecific cytotoxicity and to increase stability during storage.

Corradetti *et al.* (2012) used affinity-targeted biodegradable nanoparticles to mediate paracrine stimulation as an alternative approach to sustain the growth and pluripotency of mouse embryonic stem cells. They showed sustained release of Leukemia Inhibitory Factor (LIF) from nanoparticles composed of a solid poly(lactide-co-glycolic acid) polyester or a hydrogel-based liposomal system, which they termed Nanolipogel, replenished once after each cell passage. Other examples of the importance of sizing and enumerating nanoparticulate drug delivery systems by NTA have also been reported (Hsu *et al.*, 2010; Park *et al.*, 2010; Tagalakis *et al.*, 2010).

## Extracellular vesicles (exosomes and microvesicles)

Extracellular vesicles (EVs), comprising microvesicles and exosomes, are emerging as a significant class of sub-micron structures of potentially great importance in the development and diagnosis of a wide range of disease states. Generated by nearly all cells and in all organisms, they are believed to contain a wide range of signaling proteins, as well as genetic material of many different types.

Their detection has, to date, only been possible by electron microscopy or classical methods of analysis such as DLS. Flow cytometry has a lower limit of some 300 nm and therefore cannot "see" the majority of microvesicular material thought to be present.

NTA offers a means by which such structures can be seen and their concentration measured, and variations in the technique, such as fluorescence mode NTA, have allowed exosomes to be phenotyped. This multi-parameter capability, compatible with natural structures in their native environment, promises to be of significant value in the elucidation of the role these structures play in disease and the ways in which they may be exploited in a diagnostic or therapeutic application.

Vlassov and his co-workers have reviewed the subject of exosomes, providing an overview of current knowledge of their composition, biological functions, and diagnostic and therapeutic potential, and highlighted the following: i) exosomes are microvesicles containing nucleic acid and protein, secreted by all cells; ii) exosomes are found in abundance in all body fluids including blood, saliva, urine; and iii) exosomes' most intriguing role is intercellular communication. They described exosome composition, functions and pathways and discussed potential exosome use for diagnostic and therapeutic applications (Vlassov *et al.*, 2012). They also gave several examples of NTA analysis of exosomes in liquid samples, showing progressively lighter fractions through a sucrose, thus showing how easily NTA can be employed to furnish size and concentration information about such structures.

NTA was first assessed as a method for the analysis of exosomes and microvesicles by research groups working in the Departments of Haematology & Thrombosis and Reproductive Biology at the University of Oxford, England.

The first of these groups (Harrison, 2008 and 2009 and Harrison *et al.*, 2009a and 2009b) were primarily interested in identifying new methods by which the then current detection limit of >500 nm for the popular and widespread technique of flow cytometry could be improved on, given that the proportion of particles below this limit was then unknown. They assessed a conventional DLS instrument alongside NTA and showed that while both systems gave similar results on calibration quality beads over the size range 50 nm - 650 nm, in measurement of either purified microparticles (MP) or diluted normal Platelet Free Plasma (PFP), NTA reported a polydisperse MP distribution (up to 1000 nm) but with a predominant population from <50 nm to above 300 nm. Analysis of diluted PFP in PBS suggested that the concentration of particles was 200 - 260 × $10^9$/ mL which was 1000 fold greater than previous estimates. In further extensions of these studies, the latter group was interested in the use of exosomes as a potential biomarker for the condition of pre-eclampsia, a common disorder of pregnancy characterized by hypertension, proteinuria endothelial dysfunction and systemic inflammation (Sargent, 2010a and 2010b; Mincheva-Nilsson and Baranov, 2010). Circulating microvesicles shed by the placenta during pregnancy include syncytiotrophoblast microvesicles (STBM) and exosomes which have the potential to interact with maternal immune and endothelial cells and may have both proinflammatory and immunoregulatory effects, and it was suspected that increased shedding of STBM was associated with pre-eclampsia. NTA was used alongside flow cytometry and western blotting to confirm that excess shedding of syncytiotrophoblast vesicles in pre-eclampsia is a cause of the maternal syndrome. However, the number of STBM observed in the peripheral blood was much lower than predicted by the rate of shedding. Gardiner *et al.* (2012) hypothesized that this could be due to STBM binding to platelets and tested this using fluorescent NTA to show that there was no reduction in supernatant STBM following incubation in unstimulated platelet-rich plasma and <5% of platelets

demonstrated STBM binding. Concluding that STBM- dependent activation of the hemostatic system, and the subsequent binding of STBM to and internalization by platelets may account for the apparent scarcity of circulating STBM.

Dragovic *et al.* (2011a) have used both flow cytometry and NTA to rapidly size, quantify and phenotype cellular vesicles. Their interest was in the study of cellular microvesicles (100 nm - 1 μm) and nanovesicles (<100 nm; exosomes) isolated from the placenta, as they have major potential as novel biomarkers for the condition of pre-eclampsia. Such microvesicles have recently been shown to be implicated in a multitude of other pathological conditions. In common with all such studies, developments in this area were constrained by limitations in the technology available for measurement. Dragovic and her co-workers used a commercially available flow cytometer (BD LSRI) employing side-scatter thresholding and showed that they could analyze microvesicles ≥290 nm but nothing smaller. However, they showed that NTA could measure cellular vesicles down to approximately 50 nm.

Sheldon *et al.* (2010), in their study on notch signalling to endothelium at a distance by Delta-like 4 incorporation into exosomes, used NTA to confirm that their exosomes were only slightly larger than the suggested size (modal size of 114 nm for HUVECs and 120 nm for U87 cells, compared with published sizes of 50 nm - 100 nm). They stated that, while sizing of exosomes by electron microscopy was subjective and limited through underestimation of the size following fixing and dehydration, NTA allowed an objective and more accurate measurement of exosome size in a buffer such as PBS.

Furthermore, using human placental vesicle preparation in combination with a fluorophore-labeled anti-placental alkaline phosphatase antibody (NDOG2-Qdot605), flow cytometry showed that 93.5% of the vesicles labeled positive for monoclonal antibody NDOG2 with over 90% of the vesicles measuring below 1000 nm in diameter, the main population being between 300 nm - 400 nm in diameter (Dragovic *et al.*, 2011b). However, when the same sample was studied by fluorescence NTA, the results showed a size distribution of NDOG2-labeled vesicles ranging from 50 nm-600 nm, with peaks at 100 nm and 180 nm. Analysis of total cellular vesicles in ultracentrifuge pellets of platelet free plasma revealed that ~200 fold more vesicles were detectable using NTA (mean vesicle size 251 nm ±35 nm) vs. flow cytometry. The group concluded that these results demonstrate that NTA is more sensitive than conventional flow cytometry and greatly extends capabilities for the analysis of microvesicles and nanovesicles (Dragovic *et al.*, 2011b).

Results generated by these groups on the use of NTA for the detection of exosomes and other circulating microvesicles have been the subject of numerous presentations (Gardiner *et al.*, 2009, 2010, 2011 and Gardiner, 2011). Redman and his co-workers have established that there is a large 'hidden' population of microvesicles and nanovesicles (including exosomes) which are hard to detect

because of their size, despite being of significant importance in signalling in the maternal syndrome of pre-eclampsia. Using NTA to measure the size and concentration of syncytiotrophoblast vesicles prepared by placental perfusion, they found that the vesicles range in size from 50 nm to 1 µm with the majority being <500 nm (including both exosomes and microvesicles). They speculated whether changes not only in the numbers but also in the size of beneficial syncytiotrophoblast exosomes and harmful microvesicles might be important in pre-eclampsia (Redman *et al.*, 2011).

In an attempt to standardize the characterization and enumeration of exosomes, El-Andaloussi *et al.* (2012) published a standardized (3 week) protocol for the exosome-mediated delivery of siRNA in-vitro and in-vivo. Their protocol covers i) the generation of targeted exosomes through transfection of an expression vector (comprising an exosomal protein fused with a peptide ligand), ii) how to purify and characterize exosomes from transfected cell supernatant, iii) crucial steps for loading siRNA into exosomes and finally, iv) how to use exosomes to deliver siRNA in-vitro and in-vivo in mouse brain. As part of the crucial characterization step, they describe a 30 minute protocol for NTA analysis of exosome preparations including verification using NIST-traceable polystyrene microspheres, dilution to appropriate concentrations, repeat measurements for adequate statistical reproducibility and, finally, data analysis.

## Protein aggregation

The subject of therapeutic protein aggregation has been studied in depth for many years, and Arakawa has comprehensively reviewed the area in a series of papers covering general aspects of the mechanisms of aggregate formation and analysis (Arakawa *et al.*, 2006), the use of AUC, DLS (Arakawa *et al.*, 2007a) and field flow fractionation (Arakawa *et al.*, 2007b) in aggregation analysis, while Krishnamurthy discussed emerging technologies for the analysis of proteins during production (Krishnamurthy *et al.*, 2008).

The detection of microcontamination, specifically non-soluble particulates such as aggregates in liquid formulations (historically known as parenteral solutions but which are often now described as injectable solutions or injectables) is prescribed by national legislation as laid out by US, European and Japanese Pharmacopoeia standards, (USP, EP and JP respectively).

The importance of detection and quantification of sub-visible particles (down to 100 nm in diameter), in therapeutic protein products is the subject of much debate. Carpenter *et al.* (2009) suggested that the lack of understanding and the clinical significance of overlooking such particles may compromise product quality. He concluded that sub-visible protein particles have the potential to negatively impact clinical performance to a similar or greater degree than other degradation products, such as soluble aggregates and chemically modified

species that are evaluated and quantified as part of product characterization and quality assurance programs, and that current USP particulate testing is not designed to control the potential risk of large protein aggregates to impact protein immunogenicity. Analytical methods that can assess particulate characteristics (including composition, amount and reversibility of the protein aggregate) are critical for developing scientifically sound approaches for evaluating and mitigating risk to product quality caused by large protein aggregates. Furthermore, he advocated that pharmaceutical and academic researchers and instrument manufacturers should work together to help define the quantitative capabilities of current instrumentation for concentration measurement for particles as small as 0.1 µm and develop new instruments as needed (Carpenter *et al.*, 2009). More recently he highlighted the potential inaccurate quantification and sizing of protein aggregates by size exclusion chromatography suggesting the use of orthogonal methods to assure the quality of therapeutic protein products was essential (Carpenter *et al.*, 2010; Barnard *et al.*, 2012).

However, in providing an industry perspective on the subject, Singh and his colleagues have reiterated that the link between aggregation and clinical immunogenicity has not been unequivocally established, and they emphasized that such particles are present in marketed products which appear safe and efficacious despite the lack of monitoring. (Singh *et al.*, 2010).

It is clear, however, that elucidation of the potential problems associated with sub-micron contaminants and aggregates in proteinaceous products, and the ability to legislate for their detection and quantification remains hampered by lack of instrumentation with adequate sensitivity. Zölls *et al.* (2012) have reviewed the available analytical methods for the analysis of visible and sub-visible particles in therapeutic protein formulations and describe the underlying theory, benefits, shortcomings, and illustrative examples for quantification techniques, as well as characterization techniques for particle shape, morphology, structure, and identity.

The ability of NTA to visualize, size and measure concentration of sub-micron particles has attracted the attention of numerous workers in this field and the technique has been assessed and applied to the real-time study of proteinaceous aggregates and their formation in several applications. In a recently published book on the analysis of aggregates and particles in protein pharmaceuticals (Mahler and Jiskoot, 2012), a number of chapters discuss the role that NTA can play in the quantification and characterization of aggregates of therapeutic proteins (Carpenter *et al.*, 2012; Zhao *et al.*, 2012; Printz and Friess, 2012), and Singh and Toler (2012) have compared a wide range of techniques, including NTA, for the monitoring of sub-visible particles in biotherapeutics.

Similarly, Barnard *et al.* (2012) in their characterization and quantification of aggregates and particles in interferon-*β* products to investigate potential links

between product quality attributes and immunogenicity, used NTA alongside techniques such as microflow imaging, resonant mass measurement, size-exclusion chromatography, analytical ultracentrifugation, gel electrophoresis and dot-blotting immunoassays, the results of their study strongly suggesting that protein aggregate and particle content are key product quality attributes in a given product's propensity to elicit the production of neutralizing antibodies (Nabs) in patients.

NTA has been used for monitoring and analyzing aggregation antibody preparations. Mickisch *et al.* (2010) used both NTA and microflow imaging (MFI) for the analysis of sub-visible particles in a monoclonal antibody formulation (IgG @ 1mg/ml) formulated in phosphate buffer (pH 7.2) exposed to agitation stress (stirring for 48 h and agitation in vials for up to 1 week). In contrast to light obscuration, MFI was demonstrated to have the advantage of not underestimating proteinaceous particles. NTA was demonstrated to be a powerful technique for the determination of unbiased particle distributions of polydisperse samples. This study found that all formulations became visibly turbid after several hours of agitation. NTA analysis detected a broad distribution of aggregated species was detected with average sizes between 150 nm - 400 nm after stirring and slightly lower values after agitation. It was concluded that the two novel methods presented powerful tools for the characterization of particles providing complementary information to existing methods (Mickisch *et al.*, 2010).

## Viral vaccines and VLPs (virus like particles)

Virus sizes, typically 15 nm - 300 nm, match well with NTA's optimal size range. As NTA allows suspensions of nanoparticles to be visualized, sized and enumerated on a particle-by-particle basis, its ability to determine the concentration and direct number frequency based particle size distribution profile means that virus preparations in are ideal candidates to be studied in higher detail. It is frequently the case in vaccine production and manufacture that the size of any particular virus or bacteriophage particle is of secondary importance to the estimation of virus particle concentration and degree of aggregation. In this regard, the ability of NTA to determine virus concentrtion through direct visualization, irrespective of virus infectivity, is of significant value.

Normally, the titer (concentration) of 'phage (bacteriophage) and other virus particles is established by plaque assay, where virus particles are grown in confluent cell layers to produce plaques (zones of destroyed cells) which can be counted. While providing a direct count of individual infective virus particles, non-infective virus particles do not produce plaques and possible aggregates containing many virus particles will produce only single plaques. Often, the manufacturer needs to know the number of virus particles in the preparation, whether infective or not, and the degree, if any, to which the preparation is undergoing aggregation as an early indicator of limited product shelf life. The

applicability of NTA within the growing subsector of virus-like particles (VLPs) is greater still, as these moieties lack the genetic material to be grown in plaque assay.

Kramberger *et al.* (2012) have evaluated NTA for total virus particle determination by testing its ability to quantify latex particles, adenovirus 5, and influenza virus over several consecutive days NTA was also used to quantify chromatographic fractions of adenovirus and influenza virus after purification on a CIM monolithic column. NTA results were compared and evaluated against hemagglutination assay and end point dilution assay, determining total and infective virus particle number, respectively. The results demonstrated that nanoparticle tracking analysis is a method for fast estimation of virus concentration in different samples. In addition, it can provide insight into the level of virus aggregation.

NTA was compared to conventional plaque assay (PA) and quantitative polymerase chain reaction (qPCR) for the detection and analysis of 3 'phage types in a comprehensive study (Anderson *et al.*, 2011) in which it was concluded that, while NTA operated best only in a relatively clear medium over an optimum concentration range and compared to conventional PA methods is more capital expensive, the technique generated results within an impressive ≤5 min timeframe, which was significantly faster than PA and the qPCR method (18-24 hrs and 3-4 hrs, respectively) and that its performance does not require any additional reagents. The authors suggest that, once optimized for phage, it is likely that the NTA-based method will be reproducible amongst laboratories, with accuracy comparable to PA but significantly faster, and may be very useful for future basic and applied research with bacteriophages.

Cowpea Mosaic Virus (Aljabali *et al.*, 2011) and Tobacco Mosaic Virus (Bromley *et al.*, 2008) have both been used for the production of nanoparticles using a variety of metals including cobalt, nickel, iron, platinum, cobalt-platinum and nickel-iron and, in the case of Tobacco Mosaic Virus, gold nanowires in which successful deposition of highly refractive metal layers to the surface of the viruses were seen as an increase in scattered intensity without a significant change in particle size which would have indicated aggregation. Other studies have prepared virus templated nanoparticles of silica (Steinmetz *et al.*, 2009) and iron- platinum (Shah *et al.*, 2009).

## NTA in nanomaterials regulation

Given the recognized importance of nanoparticles and their analysis, and the fact that nanoparticles are already used in several consumer products including food, food packaging and cosmetics, and their detection and measurement in food represents a particularly difficult challenge, the European Commission published in October 2011 its recommendation on the definition of a 'nanomaterial'. This will have an impact in many different areas of legislation, such as the European Cosmetic Products Regulation, where the current nanomaterial definitions will come under discussion. The new definition calls for the measurement of the number-based particle size distribution in one or more dimensions of all the primary particles present in the sample, which measure between 1 nm and 100 nm in diameter, regardless of whether they are in a free, unbound state or as part of an aggregate/agglomerate. Recently, Linsinger *et al.* (2012) analyzed the requirements on measurements for the implementation of the European Commission definition of the term 'nanomaterial'.

Calzolai *et al.* (2012) subsequently reviewed methods for measuring nanoparticle size distribution in food and consumer products. They gave an overview of the current state of the art, focusing particularly on the suitability of the most commonly used techniques for size measurement of nanoparticles when addressing this new definition of nanomaterials and illustrated the problems to be overcome in measuring nanoparticles in food and consumer products with some practical examples. In assessing NTA in comparison, the other such techniques, they acknowledged that NTA was effective when confronted with mixtures of relatively similarly sized particles.

## Nanoparticle design and production

Kendall (2011) discussed problems of particle aggregation in ceramics presenting three types of scenario to illustrate the theory that small interatomic forces between ceramic particles have a major influence on the aggregates formed during processing and on the final ceramic product's microstructure and strength. The first is a theoretical problem of ceramic particle aggregation to define the weak interatomic forces between spheres. The second concerns the better processing that can be applied to dispersed particles, delivering improved ceramic properties by adding polymer to ceramic dispersions, redusing particle attractions which lead to aggregation. The last is the application of polymer extrusion to make improved ceramic fuel cells which can start up in a short time to provide auxiliary power to new applications.

Reduction in the formation of aggregates by the use of surfactants has been investigated using NTA and other techniques. Accordingly, Polleto *et al.* (2011) used ionic and non-ionic surfactants for the control of platinum nanoparticle aggregation in proton exchange membrane fuel cells. Platinum nanoparticles

were prepared in aqueous dispersion using tetradecyltrimethylammonium bromide (C14TAB), cetyltrimethylammonium bromide (C16TAB) and nonylphenolethoxylate (NP9). The aggregation behavior of the nanoparticles was studied using Transmission Electron Microscopy (TEM), NTA and DLS. NTA was used specifically to characterize the aggregate's particle size distribution profile. In further work, the same group used NTA to study the aggregation behavior of these materials which helped them conclude that the surfactant molecule selection is vital in obtaining effective fuel cell catalyst (Newton *et al.* (2011).

Herrington *et al.* (2010) studied the effect of the size and size distribution of $BaTiO_3$ nanoparticles on the electro-optic properties of nematic liquid crystals, and Jawor-Baczynska *et al.* (2012) have shown 250 nm glycine-rich nanodroplets are formed on dissolution of glycine crystals but are too small to provide productive nucleation sites, both studies using NTA amongst other techniques for determining nanoparticle size and number.

## Monitoring and treatment of waste and contamination

Sachse *et al.* (2012) have studied the effect of nanoclay on dust generation during drilling of polymer nanocomposites, using NTA to follow particle size distribution and quantity. Whilst there is currently a lack of information available in the literature on the nano and ultrafine particle emission rates from these, it was shown that the influence of nanoclay on mechanical drilling of PA6 composites, is to generate more particles in the size range 175 nm to 350 nm diameter over a shorter period of time. In a similar type of application, Njuguna *et al.* (2011) investigated the nanoparticles generated from nanofiller-reinforced polymer nanocomposites during structural testing.

Rezić (2011) reviewed analytical techniques for the characterization of engineered nanoparticles on textiles. In this context, the increasing number of nanomaterial-based consumer products raises concerns about their possible impact on the environment. In assessing of the effluent from a commercially available silver nanowashing machine Farkas *et al.* (2011) used inductively coupled plasma mass spectrometry (ICP-MS) and TEM to confirm the presence of an average of 10nm silver nanoparticles, but employed NTA to determine that 60 nm - 100 nm particles were also present. The effluent was shown to have negative effects on the natural bacterial community, and it was suggested that if washing machines capable of producing AgNPs become a common feature of households in the future, wastewater will contain significant loadings of AgNPs which may cause damage to the environment.

Nanoparticle-containing matrices are increasingly being investigated for the ability to remove environmental pollutants from industrial process wastewaters. NTA was employed by Prasad *et al.* (2011) in their study of the adsorption of

arsenite (As3+) on nano-sized $Fe_2O_3$ waste powder from the steel industry, while Savu *et al.* (2010) earlier assessed the generation of airborne nanoparticulates during pulsed laser welding processes and considered methods for their removal.

Cheng *et al.* (2012) have recently described the synthesis of carbon-coated, porous and water-dispersive $Fe_3O_4$ nanocapsules with a diameter of about 120 nm as determined by NTA and their excellent performance for heavy metal removal applications. The heavy metals removal test they employed demonstrated the excellent affinity of nanocapsules for different metals (>90%), 79 mg/mL adsorption capacity for Pb2+ and ultrafast removal process (Pb2+, 99.57% within 1 minute).

## Paper, inks and coatings

Lamminmäki and her co-workers described studies using NTA on the reported short timescale inkjet ink component diffusion as an active part of the absorption mechanism into inkjet coatings (Lamminmäki *et al.*, 2011) and the limitations of current formulations when decreasing the coating layer thickness of papers for inkjet coating. The rate of uptake of inks is strongly related to the number of fine diameter pores in the substrate and a critical parameter in industrial scale printing processes both in terms of speed and coating density. The results showed that, under the external pressure caused by the surface tension and impact of the ink droplets themselves, the permeability of the coating layer dominates after at least 4 milliseconds from the time of ink application on a high-speed inkjet printing press.

Kosmala *et al.* (2011) also reported the development of highly concentrated aqueous silver nanofluid and inkjet printing on ceramic substrates in which the effect of substrates, printing temperature and dot spacing on the size and morphology of printed silver features was investigated. NTA was used in the analysis of silver nanoparticles and zeta potential (dependent on pH) for the nanosilver powders treated with IPA and acetone. The use of high solid loading inks reduces the number of printed layers required for thick, dense and conductive film, thus leading to a cost reduction, and increased efficiency of the printing process (Kosmala *et al.*, 2011).

Nanocelluloses can be used to fabricate and reinforce hemp fibers. Thus, Dai, Fan and Collins (2012), developed a novel fabrication which was employed to produce nanocelluloses from natural fibers (hemp) and the developed nanocellulose was then used as "coupling agent" to modify hemp fibers themselves. The size distribution of nanoparticles (nanocellulose) was measured by NTA and results showed that the oxidation–sonication-developed nanocellulose had a wider size range (29 nm - 281 nm) than the average size (100 nm - 112 nm) before modification. Mechanical testing showed that this nanocellulose modification could improve the mechanical properties of natural fibers significantly.

## Filtration

Co-workers Boulestreau and Schulz carried out extensive studies of filtration using NTA as the primary method for testing filter efficiency and performance. Thus, in describing the online analysis of nanoparticles to prevent membrane fouling by a secondary effluent, Boulestreau *et al.* (2011a and 2011b) tested NTA in terms of reliability and reproducibility of the filterdevice as well as the impact of prefiltration on the measurements made. They showed that NTA was able to measure the particle size distribution and the absolute particle concentration of particles sized between 100 nm and 1000 nm in secondary effluent. Results clearly showed a relationship between the amount of nanoparticles below 200 nm and filtration behavior.

More recently, Boulestreau has described the on-line use of NTA in which it was used to optimize the ozonation and coagulation conditions in a filter system. They stated that the fact that the absolute size and concentration of the nanoparticles can be observed within a few minutes allows the user to detect the effect of ozonation and coagulation and concluded that the NTA instrument is "a highly capable device to analyze the nanoparticles" (Boulestreau *et al.*, 2012).

## Nanobubbles

Seddon comprehensively reviewed the area of nanobubbles at surfaces and in bulk, and has considered the current understanding of their formation, stability, physicochemical properties and current and future applications (Seddon *et al.*, 2012). In principle, a nanobubble in the bulk should be less stable than one of the same volume at an interface. The bulk nanobubble has a larger gas/liquid interface to allow diffusion of gas out of the bubble.

Also, the curvature of the surface of the bulk bubble is greater, thus leading to a greater pressure differential across the interface for a bulk bubble of the same volume. Nonetheless, several groups have presented evidence for their existence. The most startling evidence for bulk nanobubbles is the recent work which reports small nitrogen, methane and argon bulk nanobubbles of radius 50 nm that are stable for up to 2 weeks. The bulk nanobubbles, which were produced by mechanical means that led to extreme supersaturation, were imaged from freeze-fracture replicas by scanning electron microscopy (SEM) and were produced in such large quantities that the bulk density of the solution was substantially reduced.

Takaya *et al.* (2011) and Kikuchi *et al.* (2011) described the formation of nanobubbles by water electrolysis and their analysis with NTA while Ioka at el (2011) investigated their stability and weight having determined their size distribution with NTA.

Uchida *et al.* (2011) used TEM observations of nanobubbles and their capture of impurities in wastewater. They generated a nanobubble solution by introducing pure $O_2$ gas into ultra-high purity water with an MNB generator and used NTA to provide the resulting number concentration, estimated to be on the order of $10^7$ particles per mL of solution under the same sample preparation conditions. Ushida also investigated the efficiency with which nanobubbles could replace detergents in the washing of laundry, as it has been estimated that mechanical work has been found to account for 50% of the washing effect and nanobubbles can achieve the same mechanical action. A combination of nanobubbles and reduced detergency resulted in a 10% increase in washing efficiency (Ushida *et al.*, 2011).

Ushida *et al.* (2012) have recently investigated the drag reduction effect of nanobubble mixture flow through micro-orifices and capillaries in which the nanobubble-containing mixture was shown to contain 1.0% volume nanobubbles by NTA. Results of studies using nanobubble mixtures for water and glycerol which were passed through several sizes of micro-orifices and capillaries suggested that the addition of nanobubbles to a liquid results in excellent drag reduction.

## Silica

Monodisperse spherical silica particles may be available for various applications as building blocks for photonic crystals, chromatography stationary phase, and drug support for controlled release. Immobilization of a molecular-recognizable unit to the surface of the spherical particles is important in such applications. Okada *et al.* (2012) used NTA in their study of swellable microspheres. The spheres consisted of a layered silicate produced by using monodisperse silica particles. The study showed that silica spheres of sub-micron size were covered with a swellable layered silicate, which played a role in accommodating cationic species.

Yang *et al.* (2010) obtained relevant particle size distributions to estimate the effects of particle size-matching filling of spherical silica on the flowability of epoxy molding compounds for large-scale integrated circuits packaging.

Zu *et al.* (2012) described the preparation of ultrafine polyethylene-silica composite particles with a core-shell structure, using scanning electronic microscopic observation and NTA to determine that the composite particles possess a spherical morphology and the mean size is about 160 nm.

## Nanoparticulate silver

Khaydarov *et al.* (2010) used NTA to test the aggregation characteristics of silver nanoparticles in the development of a novel method of continuous fabrication of aqueous dispersions of silver nanoparticles using cellulose fibers showing that the synthesised colloidal dispersions had a pronounced antibacterial effect, as evidenced by low minimum inhibitory concentration values obtained for Escherichia coli, Staphylococcus aureus and Bacillus subtilis cultures. Hodges (2011) made anti-microbial self-assembling click monolayers utilizing silver nanoparticles for indwelling medical devices, testing the dispersions with NTA.

Ranville *et al.* (2012) analyzed metal-containing nanoparticles using single particle ICP-MS (SP ICP-MS) in environmental matrices. Their aim was to develop SP ICP-MS using spherical monodisperse metal NP "standards" (Au, Ag) and extend this capability to other metal-containing NPs; $TiO_2$, $CeO_2$, ZnO, Ag nanowires, and carbon nanotubes. NTA revealed a broader size distribution than was detected by the other techniques.

Silver nanoparticles, synthesized using Saccharum officinarum (sugarcane), have been shown to quench and inhibit biofilm formation in Staphylococcus aureus by Masurkar *et al.* (2012). NTA measurements revealed that the mean size of synthesized silver nanoparticles was 32 nm with a concentration of $1.7 \times 10^{11}$ particles per ml. No aggregates or debris were detected by NTA. Similarly, Dhuldhaj *et al.* (2012) demonstrated Tagetes erecta mediated phytosynthesis of silver nanoparticles as an eco- friendly approach for nanomaterials synthesis using NTA and TEM to confirm the synthesis of the polydisperse spherical silver nanoparticles of size 20 nm - 50 nm, with an average size of 30 nm.

## Iron oxide

Cheng *et al.* (2012) described the synthesis of carbon-coated, porous and water-dispersive Fe3O4 nanocapsules of about 120 nm (about 50 nm cavity) as measured by NTA and claimed excellent performance for heavy metal removal applications. They showed that when protected by a porous carbon layer, the nanocapsules displayed excellent acidic resistance and adsorption properties even at pH 3.

The synthesis, solution stability and 64Cu2+ labelling of magnetite nanoparticles (NPs) coated using different macro cycles has been reported by Barreto *et al.* (2011) using NTA to demonstrate that the NPs formed a stable colloidal suspension in 0.05M aqueous 2-(N-morpholino)ethanesulfonic acid (MES) buffer, consisting of larger aggregates with a mean hydrodynamic size of about 200 nm.

In a systematic examination of the effect of four common polymers on the size, surface chemistry, colloidal stability, and sedimentation behaviour of

nanoparticles of non zero valent iron (NZVI), Cirtiu *et al.* (2011) measured the size, surface characteristics and colloidal stability of NZVI NPs iron nanoparticles post- and pre-treatment. TEM images and NTA revealed that iron nanoparticles synthesised in the presence of the polymers were larger in diameter, with TEM mean diameters ranging from 84.5 nm to 189 nm, compared to a mean diameter of 59.1 nm for bare NZVI NPs, when synthesized with the same initial Fe2+ concentration.

When developing efficient water oxidation catalysts based on readily available iron coordination complexes, Fillol *et al.* (2011) carried out different analyses to investigate the possible formation of nanoparticles in solution. Experiments used DLS for determination of particle size distribution (from 10 nm to 1000 nm) and by real- time visualization and tracking analysis of nanoparticles in a liquid (NTA). Catalytic reactions produced a very low concentration of nanoparticles in solution (< 0.1 ppm), that was below the limit of detection for DLS and it was not possible to get a reliable size distribution measurement. NTA provided measured values of particles per mL in the same magnitude order ($7.6 \times 10^7$ particles per mL) as the blank experiments.

## Metallic gold

Vogel *et al.* (2011) have reported a new route for mass production of uniform metal nanoparticles in water by means of laser light induced processes in which NTA showed that pulsed laser ablation from a gold plate in water results in a large amount of nanoparticles with radii in the range of R=75nm with a relatively broad size distribution of sigma=31%. They also found that this broad size distribution was subsequently narrowed in a single irradiation step to sigma=20% without a significant change of the mean nanoparticle radius, utilizing selective laser tailoring.

## Carbon and carbon nanotubes

To assess the removal efficiency of formaldehyde using nanosized carbon colloid (NCC), which was produced by a comparatively easy and cheap method, Kim *et al.* (2012) produced nanosized carbon colloid based on water by an electro- chemical method. This was then used as a gaseous formaldehyde pollutant. NTA was used to monitor carbon particle size in production. Lv *et al.* (2011) used NTA to determine the size of graphene oxide nanoparticles in the design and production of graphene oxide membranes for possible use in new optical devices.

In the case of carbon nanotubes (CNTs), despite their highly asymmetric shape, NTA has been used to determine the sphere equivalent diameter as an indicator of sample monodispersity and behaviour in different matrices. Thus Schwyzer *et al.* (2012) have studied the influence of the initial state of carbon nanotubes on their colloidal stability under natural conditions over a period of many days.

They showed that the initial state of the CNTs (dry vs. suspended) and the medium composition are critical determinants for the partitioning of CNTs between sediment and the water column. This work was subsequently extended into a study on the long-term colloidal stability of 10 carbon nanotube types in the absence/presence of humic acid and calcium.

## Conclusion

NTA is a relatively new technique though based on well-understood principles of sizing by measuring the speed of Brownian motion of particles to give nanoparticle diffusion constant, 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 but a high resolution particle size distribution analysis in which different materials can be distinguished through their different refractive indices and, importantly, from which particle concentration can be recovered.

Furthermore, the ability to simultaneously measure additional parameters such as a nanoparticle's fluorescent properties offers the user an unprecedentedly rich profile of nanoparticle properties. The user also benefits from a direct visualization of the suspension, a unique feature of NTA.

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