



NANOPARTICLE TRACKING ANALYSIS

**A review of the first 1,000 reports of
applications and usage of NTA**

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Foreward

Since the commercialization of the technique in 2004, Nanoparticle tracking Analysis (NTA) has become increasingly prevalent in a wide variety of different research fields. Using data acquired from over 1000 papers that either include data produced using the technique or discuss the technology, this book is intended as a comprehensive review of NTA, the underlying technology behind it and the applications to which it has been applied.

Each chapter covers a basic explanation of the field to which NTA has been applied, a discussion of the relevance of the technique in the field and a review of the papers produced in that field of research. Applications covered in this publication include:

Exosomes and Microvesicle research

Protein Aggregation

Nanoparticle Toxicology and environmental impact

Drug Delivery and Targeting

Nanoparticle Production.

Thanks are due to all of the members of staff working on NTA and NanoSight instruments for their continued development of the technique and the researchers producing the papers without which this review would not be possible.

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Chapter I: Principles and Methodology

Due to the importance and relevance of nanoparticulates and their characterization, a variety of techniques have been developed allowing users to analyze particle size and size distribution. The most common of these techniques include Dynamic Light Scattering (DLS), Electron Microscopy (EM), Atomic Force Microscopy (AFM) and Analytical Ultracentrifugation (AUC). One of the most recent additions to the arsenal of apparatus available to those interested in particle characterization is Nanoparticle Tracking Analysis (NTA), a technique which not only allows the sizing and concentration measurement of nanoscale materials but also benefits from the ability to directly visualize materials within a sample. It is recognized, however, that when comparing any number of technologies in a field each of these methodologies comes with its own unique sets of benefits and limitations (Carr and Wright 2013).

EM and AFM both offer users images of the particles within a sample 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, though not an imaging technique, similarly provides high resolution information on the size distribution of particles in a sample but the technique requires a degree of previous knowledge with regards to the composition of the material, is time consuming and the initial investment for apparatus can be costly (Mächtle, 2006).

The most commonly utilized methodologies for nanoscale characterization are the ensemble techniques based on light scattering which interrogate a large number of particles in a suspension. While these techniques are ideally suited for the analysis of monodispersed systems, they are recognized as having a limited capability to analyze particles size distribution in polydisperse and/or heterogeneous systems. Also, being ensemble methodologies it is not possible to acquire accurate number concentrations of particulates within samples. The most widely used light scattering technique is DLS (alternatively known as Photon Correlation Spectroscopy (PCS) or Quasi Elastic Light Scattering (QELS)). This technique utilizes a digital correlator to analyze the timescales of fluctuations in intensity of light scattered by nanoparticles moving under Brownian motion in suspension. With over 40 years standing as an established technique it has been extensively reviewed (Pecora, 1985).

Through analysis of the resultant exponential autocorrelation function, average particle size can be calculated as well as a 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 can be significantly biased towards the larger, higher scattering particles within the sample. The resulting intensity weighted average particle size and particle size distribution information available can therefore be seriously misleading when analyzing polydisperse samples unless users have a very good understanding of the technique and the data produced.

Other optical techniques which normally measure particles smaller than one micron (e.g. static light scattering techniques based on diffractive Fraunhofer scattering or Multi-Angle Light Scattering (MALS) 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 0.3-0.5 μm diameter.

The addition of NTA to the range of techniques available to researchers offers the ability to directly visualize size and measure concentration of nanoparticles in liquid suspension. The ability of this technique to simultaneously analyze a population of nanoparticles on an individual basis means it is ideally suited for the real-time analysis of polydisperse systems ranging from 10-20 nm 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 the capability to visualize and analyze fluorescently labelled particles (NanoSight, 2012; Carr *et al.*, 2009).

Principles of operation

The properties of both light scattering and Brownian motion are utilized in order to obtain particle size distributions of samples in liquid suspension in NTA. A laser beam (of arbitrary wavelength but typically those available from commercially available laser diodes operating at 642 nm, 532 nm, 488 nm or 405 nm) is passed through a prism edged glass flat (or equivalent optical element) within the sample chamber. The angle of incidence and refractive index of the glass flat is designed to be such that when the laser reaches the interface between the glass and the liquid sample layer above it, the beam refracts to an intense low profile resulting in a compressed beam with a reduced profile and a high power density. The particles in the path of this beam scatter light in such a manner that they can be easily visualized at 90° via a long working distance, x20 magnification microscope objective fitted to an otherwise conventional optical microscope or equivalent optical train. The system then uses a CCD, EMCCD ((Electron Multiplied) Charged Coupled Device) or high-sensitivity CMOS camera, operating at typically 30 frames per second (fps) to capture a video file of particles moving under Brownian motion within a field of view of approximately 100 μm x 80 μm x 10 μm (Figure 1).

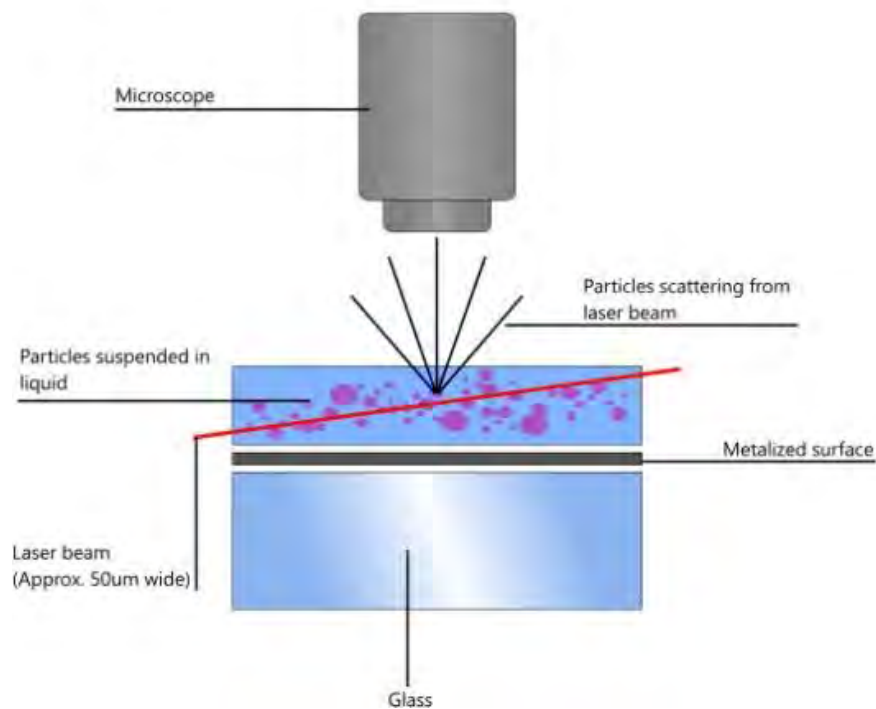


Figure 1 Schematic of the optical configuration used in NTA.

Sizing of Nanoparticles using NTA

Particles within the field of view are seen moving under Brownian motion, either directly by eye using the microscope oculars or via the image displayed on the computer screen recorded by the camera. The proprietary NTA software takes a video file (of typically 30-60 seconds duration) of the particles viewed and then simultaneously identifies and tracks the center of each particle seen on a frame-by-frame basis. The image analysis software then determines the average distance moved by each particle in the x and y planes. This value allows the particle diffusion coefficient (D_t) to be determined from which, if the sample temperature T and solvent viscosity η are known, the sphere-equivalent hydrodynamic diameter, d , of the particles can be identified using the Stokes-Einstein equation (Eq 1).

$$D_t = \frac{TK_B}{3\pi\eta d} \quad \text{Eq 1}$$

where K_B is Boltzmann's constant. See Fig 2 for a schematic of the basic principles of NTA analysis.

Obviously, Brownian motion occurs in three dimensions but NTA observes motion only in two dimensions. It is possible, however, to determine D_t from measuring the mean squared

displacement of a particle in one, two or three dimensions by using the following variations of the Stokes-Einstein equation (Eq 2a-c respectively);

$$\begin{array}{lll} \overline{(x^2)} = \frac{2TK_B t}{3\pi\eta d} & \overline{(x,y)^2} = \frac{4TK_B t}{3\pi\eta d} & \overline{(x,y,z)^2} = \frac{2TK_B t}{\pi\eta d} \\ \text{Equations} & 2a & 2b \qquad \qquad \qquad 2c \end{array}$$

Thus, in the case where measurement of movement in two dimensions is made the following equation can be used;

$$\frac{\overline{(x,y)^2}}{4} = Dt = \frac{TK_B}{3\pi\eta d} \quad \text{Eq 3}$$

Size range detectable

The lower limit of detection for instruments using NTA is determined by several factors, the most significant of which are the amount of light scattered by the particles and the capability of the optics used to detect this light.

The amount of light scattered by a particle in any given direction is a function of many variables including incident illumination power, wavelength, angle and polarization; particle size, refractive index (real and imaginary) and shape, as well as the refractive index of the suspending solvent. Similarly, the amount of light falling on a detector and strength of the resultant signal is dependent on a number of factors including the efficiency of the collection optics (e.g. Numerical Aperture) and the spectral response and sensitivity of the camera.

The theory of light scattering is well established (Bohren *et al.*, 1983; Kerker, 1969) and the formula for Rayleigh scattering of small particles of radius a , refractive index n_1 in a liquid of refractive index n_2 is given by (Eq 4);

$$\frac{I}{I_{in}} = \frac{16\pi^4 a^6}{r^2 \lambda^4} \left(\frac{n^2 - 1}{n^2 + 2} \right) \sin^2 \psi \quad \text{Eq 4}$$

where λ is the wavelength of the incident light beam, n relative refractive index (n_2/n_1), I_{in} is incident power per unit area, I_{scat} the scattered power per unit area a distance r from the scattering region and ψ is the angle between the input polarization and the scattering direction.

The total scattering (P_{scat}) into an aperture of collection angle θ (numerical aperture $NA = \sin \theta$) is then:

$$P_{scat} = \frac{64\pi^4 a^6}{\lambda^4} \left(\frac{n^2-1}{n^2+2} \right) \eta_o I_{in} \quad \text{Eq 5}$$

Where

$$\eta_o = \frac{(1-\cos\theta)}{4} + \frac{(1-\cos^3\theta)}{12}$$

For a detection system of fixed wavelength and incident laser power, NA and detection angle, the variables associated with the limit of sample NTA detection reduce to that of particle size and the refractive index difference between the material and the solvent in which the particles are suspended.

Thus, for materials of very high refractive index, such as colloidal gold or silver in water, it is possible to accurately determine size down to around 10-15 nm (depending on camera type used). For those particles of only moderate refractive index, such as metal oxides, some condensed polymers or refractile particles of a biological origin, the lower size limit may only be around 25 nm-35 nm. However, this minimum size limit will allow the analysis of most types of virus. For very weakly scattering materials (e.g. polymers, exosomes, liposomes), the smallest particle visible might only be 40 nm in diameter. The upper size limits of the system are approached when the rate of Brownian motion becomes so low that it approaches the same scale as exhibited by the small centering errors inherent in the tracking software and which therefore lead to sizing inaccuracies. This limit is typically found around 1-2 μm for particles in aqueous type systems.

NTA workflow:

- NTA captures a video of particles moving under Brownian motion
- NTA automatically locates and follows the centre of each and every particle and measures the average distance it moves per frame
- This is done simultaneously for all particles until hundreds or thousands of particles have been tracked
- NTA converts the distances moved into a particle size and plots accumulated results in real time as a particle size distribution profile.
- NTA analyzes the raw data, fits model distributions or displays different particle parameters (size vs relative intensity vs number) against each other. Concentration is also determined

Concentration ranges measureable

NTA is not an ensemble technique interrogating a very large number of particles, but rather each particle is sized individually, irrespective of the others. This means that in order to achieve statistically viable results it is important that a sufficient number of particles are analyzed within the sample time chosen. The optimal concentration to provide this number of particles within a 30-60 second analysis time typically lies somewhere between 10^7 to 10^{10} particles per mL.

Particles visualized by NTA move within a fixed field of view (approximately 100 μm by 80 μm) illuminated by a beam approximately 10 μm in depth. These figures allow a scattering volume of

the sample to be estimated and by measuring concentration of the particles within this field of view and extrapolating to a larger volume it is possible to achieve a concentration estimation in terms of particles per mL for any given size class or an overall total.

The effective scattering volume in which particles are detected and concentration measured varies as a function of several factors. These include both the particle size and difference in refractive index between the particles and the medium as well as the power, wavelength and dimensions of the illuminating laser (Eqs 4 and 5). Similarly, adjusting the camera sensitivity will affect the number of particles detected and therefore tracked and this will clearly impact on the concentration reading achieved. As a result of this, for more accurate determination of concentration of particles which scatter significantly differently from those for which the system is ideally optimized, calibration on particulate systems of known concentration is necessary.

Many samples of environmental or biological origin contain a wide range of particles sizes and types which, unless fractionated or partially purified, will frequently exhibit log-normal particle size distribution profiles. Similarly, many industrial nanoscale products are produced by grinding or milling of coarse starting material and even when a design size is reached may contain considerable numbers of fines whose presence is often unsuspected due to their being undetectable by conventional means. It will be appreciated, of course, that these lower number of larger particles (rare-event aggregates, contaminants, etc) may not be detected and concentration measured with the same accuracy as those of a smaller size and higher number.

It should be noted however that few, if any other, techniques are capable of determining nanoparticle concentration with such ease and fewer can be confirmed by the visual image of the sample afforded by the NTA technique.

Samples containing fewer than 10^7 particles per mL result in only a very limited number of particles being present within the field of view at any one time. Accordingly, extended analysis times (e.g. above 5 minutes) will be required in order to obtain statistically reproducible results. The NTA software will alert users to samples that contain fewer particles than required for optimal analysis and an automated facility is available which proposes suitable analysis times based on an initial estimate of sample concentration.

Samples containing a concentration of particles greater than 10^{10} particles per mL have a higher likelihood of particle trajectories crossing over one another before an adequate estimate of particle size can be made through tracking any given particle. This will degrade the quality of information obtained about the particles.

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

Given the above however, it is clear that accurate estimates of the number and concentration of any particular class of particles in a polydisperse and/or heterogeneous mixture of different particle

types will be subject to the different amounts of light they may scatter which will, in turn, determine their effective scattering volume thus any consequent estimate of concentration.

The typical error to be expected when determining the absolute concentration of even monodisperse sample can be as high as 20%. However, this figure can also be influenced by several parameters and will increase when;

- the polydispersity within the sample increases.
- concentration measurement is being made of small or weakly scattering particles on the limit of detection of the instrument.
- assessing particles on the larger end of the limit for sizing (800 nm+)
- concentration measurement is being made of highly asymmetrical particles.

Care should be taken if at all possible to reduce the effects that these parameters can have on the outcome of the analysis.

Absolute Accuracy and Resolution

Both NTA and DLS operate on similar basic principles; analyzing light patterns resulting from light scattered by particles moving under Brownian motion. Accordingly, if accurate information about the temperature and the viscosity of the solvent in which nanoparticles are present is given, then the absolute accuracy achieved by both techniques is effectively the same, around 2% under ideal conditions. As both techniques operate in the time domain they benefit from being uncommon examples of absolute methods of measurement in which (re-)calibration is unnecessary.

A simple comparison of the accuracy and reproducibility of NTA against a DLS instrument is shown in Table 1. Calibration polystyrene standards of 50, 100, 200 and 400 nm diameter were analyzed (averages of 5 repeat measurements are shown). As can be seen, NTA compares very favorably with DLS (Table 1).

Table 1	NTA (NanoSight)		DLS (Zetasizer)	
Nominal size (nm)	Average size (nm)	Standard deviation (nm)	Average size (nm)	Standard deviation (nm)
50	50.6	0.8	51.7	2.2
100	100.2	1.2	102.4	3.6
200	200.6	1.7	209.2	5.2
400	398.3	2.4	411.5	7.3

It should be recognized, however, that the Stokes-Einstein relationship between the measured D_t and the diameter reported assumes the particles are non-interacting, diffusing freely in the infinite dilution limit, spherical and measure the hydrodynamic diameter of the particle (which is that of the physical extent of the particle plus the hydrodynamic shell of structured solvent in close proximity to the particle surface). This hydrodynamic shell normally extends, dependent on solvent characteristics, some 1-2 nm from the surface for aqueous systems. For larger particles this contribution is relatively negligible, but for very small particles below 20 nm it becomes an increasingly significant percentage.

Unlike DLS, NTA measures the D_t of individual particles and, as such, does not suffer from the intensity weighting problems besetting DLS, nor is it an ensemble technique summing the motion of a large number of particles simultaneously. Accordingly, NTA is a technique of inherently higher resolution than DLS which, in practice, can rarely achieve resolutions < 3:1 or 4:1.

NTA estimation of accurate D_t relies, however, on being able to track any given particle's Brownian motion trajectory for a sufficient number of steps to generate an accurate average value of step-length with which to accurately determine size. Because of the very small depth of the scattering volume smaller particles, in particular, can often be present for a very limited period of time (<10 frames \equiv 0.3 seconds at 30 fps). The effect of this limitation manifests itself as an artifactual broadening of the distribution measured, though the mean of the estimated size remains accurate. The reduction in accuracy associated with these limited duration trajectories can, however, be mathematically modelled and compensated for (Saveyn *et al.*, 2010). Thus, for monodisperse samples, a 'finite track length adjustment' (FTLA) algorithm within NTA can be automatically applied which compensates for such effects and recovers the true distribution width (yellow) of narrow distributions of monodisperse, calibration quality nanoparticle suspensions (Figure 2).

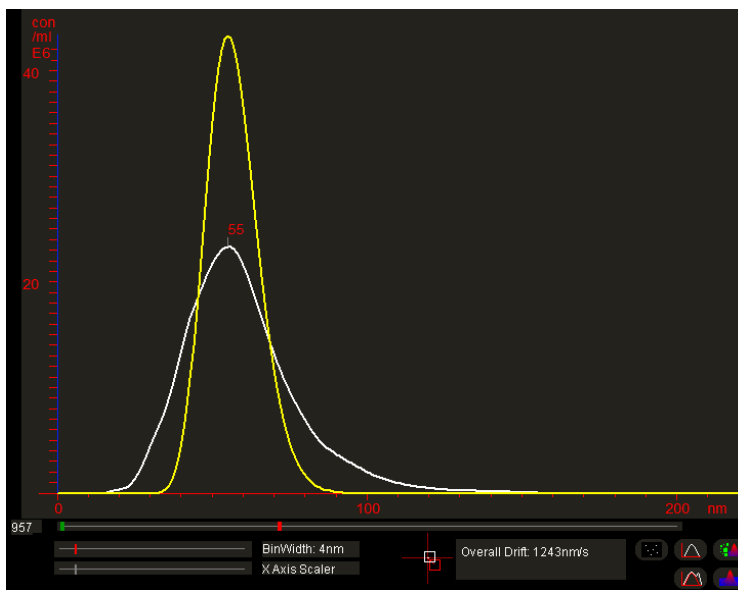


Figure 2. FTLA comparison for short track lengths.

Sample Preparation

In most circumstances sample preparation for use in NTA consists of little more than simple dilution so that particle concentration falls within the optimum ranges previously mentioned. A sample in which significant sedimentation is apparent implies the presence of particles which, by definition, are too large to be freely diffusing nanoparticles. In this case, an aliquot of the supernatant will contain any nanoparticles present and re-suspension of sediment should be avoided. The presence of particles sufficiently large to sediment in a short time period can interfere with NTA. If necessary, removal of particles $>1-2\ \mu\text{m}$ can be achieved by centrifugation, filtration or simple settling. As with any measurement, measuring a blank or control is strongly advised, so when diluting samples it is best to conduct a precautionary analysis of the diluting material in order to ensure the absence of contaminating nanoparticles.

It should be noted that efficient removal of such contaminants can sometimes be a non-trivial task but is clearly necessary to ensure accurate NTA analysis of the sample itself. It should further be recognized that the quoted pore size of common tortuous-pore membrane filters is a mean pore size only and that larger particles can initially pass through the filter and can be present in the early filtrate. Polycarbonate, track-etched membranes (e.g. Nucleopore®) exhibit very low distributions of pore sizes though suffer far lower flow rates.

Samples which, in concentrated form, appear colored rarely cause problems due to the fact that NTA requires significant sample dilution to work and any residual optical absorption of the nanoparticles at this dilution do not cause detectable heating effects. Furthermore, it should be recognized that NTA works in the time domain and the intensity of light scattered by the particles is not the property on which measurement of their size is based. However, significantly optically absorbing solvents must be avoided because they may suffer from thermal convection.

Many samples when analyzed using NTA require dilution by several orders of magnitude in order to reach the analysis optimum concentration of 10^7 To 10^9 particles per mL. It is recommended that this dilution is effected through a series of serial dilutions with no one step consisting of a dilution of $>200\times$ to reduce the risk of error from dilution technique.

Although it is required for many samples, dilution of materials is not without its problems. The first of these is the possible elimination of low numbers of particles in a population through over-dilution. Further to this is the possible change in sample stability on excessive dilution. Finally, the possible aggregation of unstable samples during dilution should be taken into consideration.

Repeat Measurements

Whenever a sample is characterized using NTA, a bulk sample is taken, often diluted, and sub-samples extracted from the bulk are analyzed. The measurement of sub-samples from a bulk can sometimes lead to sample bias with the result of the analysis only focusing on a certain population of materials. This is why it is recommended that conclusions about the size, distribution and concentration of particles within a sample are never drawn from a single analysis, but rather repeat measurements of different sub-samples should be taken. Owing to the large number of particles

within a sample in the NTA dilution regime (around 10^7 particles per mL) it would be impractical to measure all of them using this technique. However, correct sampling is important to ensure that the particles that are measured in the analyses are representative of the population as a whole. By analysis of only one field of view, the user could be biasing themselves towards a certain population within a sample and which might be unrepresentative of the whole sample.

Variation between repeat samples of the same preparation increases along with the degree of polydispersity and is indicative of an inadequate sampling regime or insufficient analysis time.

The reason for recommending repeat measurements rather than longer records is that repeat measurements ensure an entirely new field of view for each 60 second analysis therefore an entirely new sample population. If we record a longer video, then the majority of particles will stay within the field of view and a few new ones will enter from the sides. The longer the duration of the sample recording the more the rate of new particles entering the field of view reduces.

The number of repeat measurements that should be taken is related to both the sample size and the polydispersity of the sample. The larger the polydispersity the more repeat measurements should be taken in order to ensure the effects of sample bias are reduced.

There is however another way to reduce the effects of sample bias, through the use of precise, slow, constant flow of sample through the field of view. By connecting the sample chamber of the instrument to a digital stepper-motor syringe pump the user is able to apply a constant pressure to the sample causing it to flow in a uniform manner across the field of view. This means new particles are constantly being added to the field of view with a direct correlation between the length of the recording and the number of unique particles analyzed.

The syringe pump also improves the repeatability of concentration measurements, by continually introducing fresh sample volumes during analysis. This, in combination with batch analysis procedures, ensures the most precise and reproducible concentration measurements, especially for concentration measurement of larger contaminating particles and aggregates which are normally present in lower numbers.

The increased sampling population also allows for more accurate analysis of extremely dilute systems which would otherwise require extremely long capture durations to detect and track sufficient particles for statistically robust measurement.

A final advantage of the use of repeat measurements is the ability to use these readings to define the standard error of certain parameters (i.e. mean distribution, standard deviation of size and particle concentration). The standard error is calculated by taking the standard deviation between the repeats and dividing it by the square root of the number of samples (Eq 6).

$$Error \propto \frac{1}{\sqrt{n}}$$

Eq 6

Size vs. Intensity vs. Concentration 2D and 3D Plots

NTA uses particle Brownian motion rather than fluctuations in the intensity of light they scatter in order to measure the size of particles. However, one of the unique and beneficial features of NTA is the ability to simultaneously 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 and refractive index to be successfully discriminated.

When plotted on a graph of particle size against concentration (the regular output from an NTA reading) a mixture of 92 nm polystyrene and 90 nm gold nanoparticles would be seen as a single peak (Figure 3a). However, when the same plot is displayed as particle size against the relative intensity of light scattered it is possible to resolve the two similarly sized populations (Figure 3b).



Figure 3: a) particle size distribution profile of a mixture of 92 nm polystyrene and 90 nm gold nanoparticles showing a single peak when measured by size alone; b) a 2D plot of the same mixture when size of each particle is plotted as a function of the amount of light it scatters allowing the much higher refractive index gold to be discriminated from the same sized polystyrene nanoparticles.

A more complex demonstration of the extra resolving power afforded by this additional parameter can be seen in Figure 4. This plot shows a mixture of 30 nm Au, 60 nm Au and 100 nm polystyrene, all of which can be resolved in the 3D plot of Size v. Intensity v. Number.

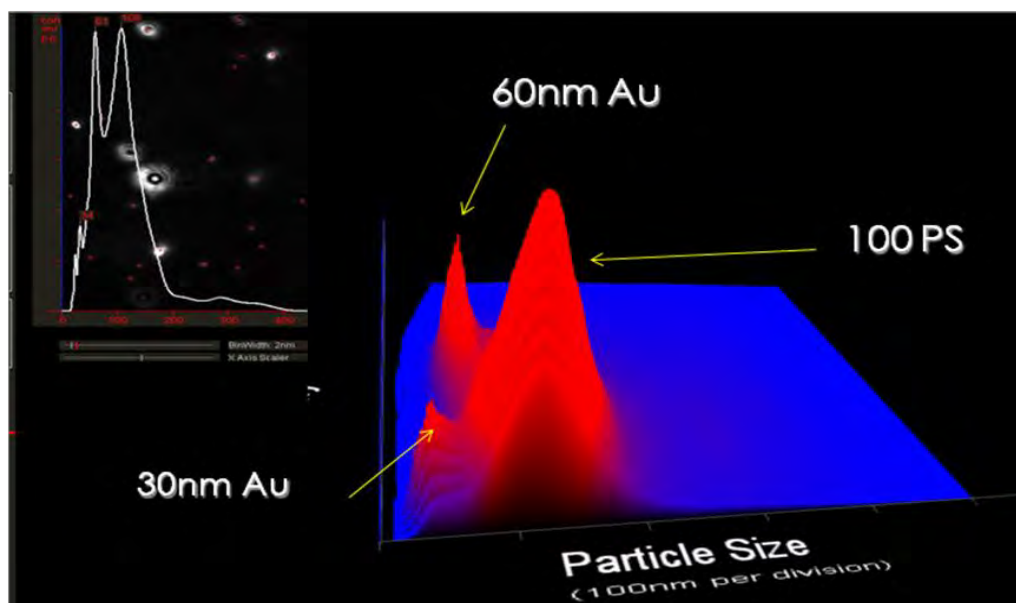


Figure 4. 3D representation of particle size against concentration against scatter

Fluorescence

While any wavelength of laser can be used to visualize and size, as well as measure concentration, nanoparticles under light scattering mode as previously described, shorter wavelength diodes such as blue 405 and 488 nm diodes and green 532 nm lasers can be used to allow fluorescent or fluorescently-labelled particles to be selectively identified and tracked through the use of appropriate optical filters.

It is therefore possible to selectively identify size and measure concentration of only certain sub-populations of particles in a mixture through fluorescent labelling. Such labelling may be effected through, perhaps, the use of antibody-mediated fluorophores allowing phenotyping of particular species of nanoparticles (Dragovic *et al.*, 2011). Alternatively, fluorescent stains and reagents, specific for certain sample constituents such as lipid, protein or nucleic acid can help differentiate nanoparticles from each other.

Of course, the degree to which fluorescence can be used successfully in this regard is dependent on the fluorescent signal being sufficiently intense for the particle to be seen and its dynamic behavior tracked accordingly. This strength of the fluorescent signal will depend on the choice of excitation laser wavelength which must be sufficiently well matched to the fluorophores excitation profile. The degree of absorption, or extinction coefficient, of the fluorophores, and the efficiency with which it subsequently fluoresces (its quantum yield) are also both important parameters determining the usefulness of a fluorophore in any given situation.

Obviously the optical filters used must be compatible with the excitation and emission profiles of the fluorophores used and the wavelength of the exciting source. Stable fluorophores are also required which do not decay within the time period required to allow their tracking by NTA (e.g. >0.5 seconds). There are methodologies for extending the life of fluorophores used in these

experiments. The first of these is to synchronize the camera shutter with a pulsed laser. Under normal conditions the laser is permanently on whilst the camera shutter is only open for a few fractions of a second per frame. This means that there is a significant proportion of each frame where the laser is exciting the fluorophore resulting in bleaching, but no signal is recorded by the camera. To circumvent this, on certain models of NTA instrument, the camera can be used to trigger the laser module. This means that the laser is then only illuminated when the shutter of the camera is open to collect data and as the fluorophore is only excited when the laser is active, the bleach rate of the material is significantly reduced.

Furthermore, for more rapidly bleaching fluorophores, it is possible to slowly flow the sample through the laser beam such that the population within the field of view is continuously refreshed. Rather than having material staying within the beam and being bleached by the laser throughout the entire analysis, this technique permits the continuous introduction of unbleached material thus maintaining analysis of larger numbers of particles over extended periods.

Finally, it is necessary for multiple fluorophores to be bound to the target nanoparticle because individual fluorescent molecules rarely generate a sufficient signal for detection by the CCD or even higher sensitivity EMCCD or sCMOS cameras as used by NTA. An interesting exception to this is the use of a class of fluorophores called 'quantum dots' which are 4-12 nm semiconductor nanocrystals (usually CdSe) whose optical characteristics (e.g. emission profiles) are related to their size. Quantum dots are extremely bright emitters and, usefully, sufficiently stable to be both detected and tracked by NTA on an individual basis. When functionalized with antibodies, these structures have been successfully used to phenotype biological exosomes by NTA (Dragovic, 2011).

The following example (Fig 5 a & b) shows the analysis of a mixture of polymeric microvesicles, some of which contained the fluorophore Rhodamine B, using a 405 nm laser-illuminated NTA system. From the 2D plot shown (Fig 5a), it can be seen that when analyzed under typical light scattering mode (shown by blue data points (Fig 5a) and curve (Fig 5b)) there is a far higher number of smaller non-labelled structures in the mixture than fluorescently labelled particles (green data points and curve in Fig 5a and Fig 5b respectively). The labelled particles, when measured under fluorescence mode (i.e. suitable optical filters are introduced into the light path), appear lower in number. Note that the larger particles are tracked and sized more successfully when seen under fluorescence labelling mode because when tracked under light scatter mode they frequently scatter so much light they saturate the detection systems and are not analyzed.

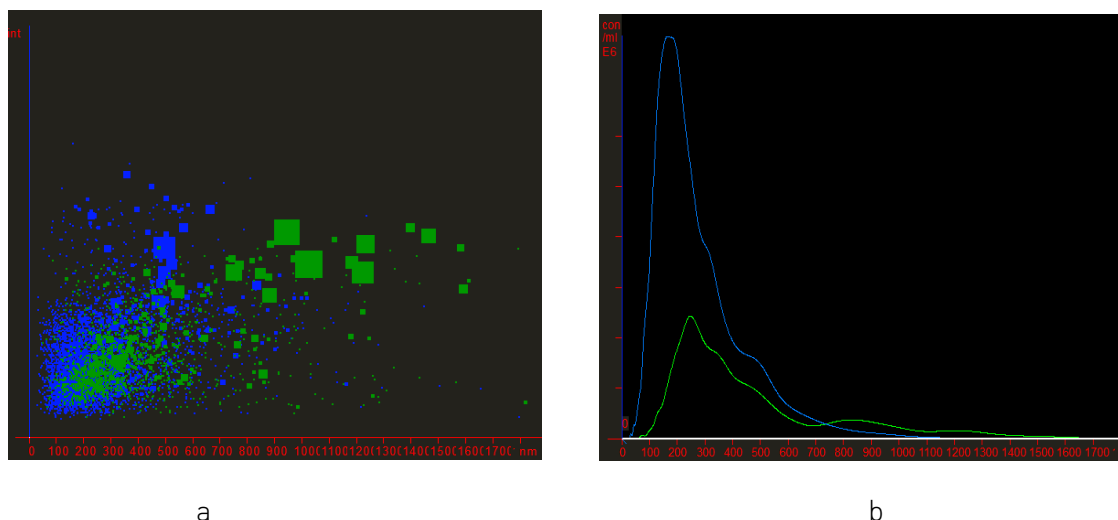


Figure 5 a) shows a 2D scattergram of size vs. light scattered (blue data points from all particles) or fluorescently emitted (green data points from Rhoda mine B labelled sub-population) of a mixture of polymeric microvesicles showing there is a far higher number of smaller non-labelled structures in the mixture than fluorescently labelled particles. Fig 5b confirms these data in a size vs. number plot of the mixture. Note that the larger particles are tracked and sized more successfully when seen under fluorescence labelling mode because under light scatter they frequently scatter so much light they can saturate the detection system.

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Chapter II: Assessments of NTA

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 FIFFF, (ii) the range of analytical tools that can be used to further characterize the nanoparticles after fractionation by FIFFF, (iii) how FIFFF results are compared to

other analytical techniques and (iv) the range of applications of FIFFF 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 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 D_t 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 "Tunable 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 offers 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 measurement 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 20×10^8 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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Chapter III: Nanoparticle Toxicity

At the same time as an increasing interest in, and rapid development of, a wide range of materials and products containing nanoscale structures and engineered nanoparticles, awareness has grown that the longer term potential toxic effects of such materials and their potential environmental impact are poorly understood. Existing methods have been assessed and new methods sought by which such materials could be analyzed on a routine basis during development and manufacture.

The use of Nanoparticle Tracking Analysis as a rapid and information-rich multi-parameter nanoparticle characterization technique which allows the user to obtain number frequency particle size distributions of polydisperse nanoparticulate systems has resulted in its rapid adoption as an interesting new technique in a wide range of sectors within environmental and toxicity studies. This Chapter addresses some of the latest work in the literature in which NTA has been proposed, used and assessed in the study of nanoparticle toxicity and environmental impact.

NTA has found use in a variety of investigations researching the toxicity and environmental impact of nanoparticles. As well as being used to determine the size of particles in investigations into the toxicity of carbon nanotubes and nanoparticulate metals, NTA has also been used in investigations on the interactions of nanoparticles with organisms at a cellular level and the development of methods for the testing of toxicity. NTA has proved to be a useful tool in determining both particle size and concentration of nanomaterials in wastewater analysis.

Cytotoxic studies

At a cellular level, NTA has proved useful in studying the genotoxicity of cobalt NPs in human peripheral leukocytes (Colognato *et al.* 2008) and mouse fibroblasts (Ponti *et al.* 2009a). The ability of nanoparticles to cross the human placenta (Wick *et al.* 2009) including the transport of SiO₂ nanoparticles through human skin (Staroňová *et al.*, 2012). Similarly, Filton *et al.* (2012) reported on human skin penetration of cobalt nanoparticles through intact and damaged skin suggesting that Co applied as NPs is able to penetrate the human skin in an *in-vitro* diffusion cell system.

An increasing number of studies exploiting NTA address the potential hazards of different metal species in a variety of cellular and aqueous systems. These include the effect of gold (*et al.* 2010) silver (MacCuspie *et al.* 2011, Bouwmeester *et al.* 2011) and copper and chrome oxide nanoparticles (Studer *et al.* 2010, Khatoonet *al.* 2011).

An understanding of the dispersion a distribution of nanoparticle sizes prior to their introduction to cellular systems for cytotoxicological testing is crucial and NTA has proved useful in this regard compared to other nanoparticle characterization techniques such as DLS (Kendall *et al.* 2009, Patel *et al.* 2010, Munaro 2010, Karlsson 2010). The chemical interactions of nanoparticles of different types with various matrices of biological origin such as serum (Treuel *et al.* 2010) and organic pollutants (Ben-Moshe *et al.*, 2009) and dithiothreitol, (Sauvain *et al.*, 2008) have also been studied.

The toxicological effects of cobalt nano-particles (Co-NPs) aggregates were examined and compared to those of cobalt ions using six different cell lines representing lung, liver, kidney, intestine and the immune system. The overall findings were in line with the hypothesis that the toxic effects of aggregated cobalt NPs are mainly due to cobalt ion dissolution from the aggregated NPs. NTA was used to determine particle size distribution profiles (Limor *et al.*, 2011).

Christen and Fent (2012) showed that engineered silica nanoparticles and silver-doped silica nanoparticles induced endoplasmatic reticulum stress response and altered cytochrome P₄₅₀1A activity in human liver cells (Huh7) and *Pimephales promelas* (fathead minnow) fibroblast cells (FMH) with NTA being used to monitor stability of the particle in nanopure water.

Carbon black and related diesel exhaust nanoparticles have been studied in human epithelial cells (Frikke-Schmidt *et al.*, 2011) while Kadar looked at the enhancement of spermatotoxicity of stabilized nanoiron (Kadar *et al.*, 2011). Hemmingson *et al.* (2011) have used NTA in their studies of metabolic and genetic stress induced in a number of cell types exposed to conventional diesel and biodiesel nanoparticulate combustion products and showed biodiesel to be, on an equivalent mass basis, less toxic than conventional diesel. In other studies on diesel exhausts Jantzen *et al.* (2012) looked at oxidative damage to DNA by diesel exhaust particle (DEPs) exposure in co-cultures of human lung epithelial cells and macrophages concluding that exposure of mono-cultured cells to DEPs generated oxidative stress to DNA, whereas co-cultures with macrophages had lower levels of oxidatively damaged DNA than A₅₄₉ epithelial cells.

Suggesting that the toxicological effects of wood smoke particles are less investigated than traffic-related combustion particles, Forchhammer *et al.* (2011) compared the expression of adhesion molecules, monocyte interactions and oxidative stress in human endothelial cells exposed to wood smoke and diesel exhaust particulate matter using NTA to determine the particle size distribution of the wood smoke particles and those of standard reference material (SRM) 2975 diesel exhaust particles being used as benchmark particles. Similarly, Vesterdal *et al.* (2012a) looked at carbon black (CB) nanoparticles and vascular dysfunction in cultured endothelial cells and artery segments reporting that nanosized CB exposure activates endothelial cells and generates oxidative stress, which is associated with vasomotor dysfunction, using NTA to confirm nanoparticle stability during their experiments. Vesterdal *et al.* (2012) also used NTA to measure particle size in their study on pulmonary exposure to particles from diesel exhaust, urban dust or single-walled carbon nanotubes and oxidatively damaged DNA and vascular function in apoE^{-/-} mice. Vesterdal *et al.* (2013) have most recently looked at the accumulation of lipids and oxidatively damaged DNA in hepatocytes exposed to particles concluding that exposure to four different carbon-based particles (diesel exhaust particles, fullerenes C₆₀ or pristine single-walled carbon nanotubes) is associated with oxidative stress and steatosis in cultured human hepatocytes (HepG2). Zemanova *et al.* (2011) previously determined the: influence of C₆₀ fullerene derivative nanoparticle size on toxicity and radioprotectivity of water soluble fullerene derivative while Matthews has also investigated the transport of CNTs across pulmonary epithelium using an isolated perfused rat lung preparation using NTA (Matthews *et al.*, 2010 and 2009).

Using NTA to quantified microparticles in serum, Choudhary *et al.* (2013) evaluated the effect of cigarette smoke exposure on ventricular function and PA pressures. Progressive effects over

multiple weeks exposure, including an impaired RV systolic but without elevated PA pressure and an increase in circulating microparticles (in 108/ml) leading the researches to speculate that these effects may be due to exposure to circulating CS constituents or microparticles released in response to CS.

Mahmoudi *et al.* (2011) have discussed the opportunities and challenges surrounding the study of protein-nanoparticle interactions. Bulcão *et al.* (2012) investigated, for the first time, the toxicity of lipid-core nanocapsules (LNC) containing a polymer wall of poly(epsilon-caprolactone) (PCL) and a coating of polysorbate 80 (PS80) used as drug delivery devices (~245 nm as determined by NTA) in Wistar rats after single- and repeated-dose treatments. The findings were in agreement with earlier reports regarding no appreciable toxicity of biodegradable polymeric nanoparticles, indicating that LNC might be a safe candidate for drug delivery system.

More recently, Prina-Mello *et al.* (2013) have carried out a multiparametric toxicity evaluation of superparamagnetic iron oxide nanoparticles (SPIONs) by a high content screening technique in their identification of biocompatible multifunctional nanoparticles for nanomedicine. Mwilu *et al.* (2013) used a variety of methods including Absorbance Spectroscopy, High Resolution Transmission Electron and Scanning Electron Microscopy (TEM/SEM), Dynamic Light Scattering (DLS), and Nanoparticle Tracking Analysis (NTA) to follow changes in silver nanoparticles exposed to human synthetic stomach fluid; specifically the effects of particle size and surface chemistry. They found that generally, the smaller sized AgNPs (< 10 nm) showed higher rates of aggregation and physical transformation than larger particles (75 nm). Also working with silver, Kruszewski *et al.* (2013) found that oxidative DNA damage corresponds to the long term survival of human cells treated with silver nanoparticles. Silver nanoparticle aggregation was followed by NTA.

More recently, Dieni *et al.* (2013) have demonstrated that spherical gold nanoparticles impede the function of bovine serum albumin *in vitro*. In order to isolate strongly interacting BSA oligomers, irreversible BSA aggregates or strong BSA-nAu complexes induced by recruitment of BSA into the protein corona, BSA-nAu-cap suspensions were subjected to centrifugal filtration and native-PAGE. However, this methodology failed to detect any altered distribution of higher-molecular weight species of BSA compared to control (free of nAu), suggesting that any protein-protein or protein-nAu interactions that contribute to these altered properties of BSA are not irreversible and do not withstand high g-forces and/or electrophoresis.

Physicochemical properties of nanoparticles (NP) strongly affect their influence on cell behavior, but can be significantly distorted by interactions with the proteins present in biological solutions. In a recent study Bartczak *et al.* (2013) showed how different surface functionalities of zinc oxide (ZnO) NP led to changes in the size distribution as measured by NTA and dissolution of the NP in serum containing cell culture media and how this impacted on NP toxicity. NPs capped with weakly bound large proteins underwent substantial transformations due to the exchange of the original surface ligands to the components of the cell culture media. NTA was also used to determine size of particles in a study of the adsorption of nanoparticles and nanoparticle aggregates on membrane under gravity (Zhu *et al.*, 2013). Wiemann (2013) also showed NTA-derived data in his recent presentation on the agglomeration, uptake, biodistribution and *in vivo* toxicity of nanosized SiO₂-particles in the rat lung.

Mihaiescu *et al.* (2013) reported on Fe₃O₄/Salicylic acid nanoparticles behavior on chick CAM vasculature when using a modified ferrite co-precipitation synthesis to obtain core-shell Fe₃O₄/salicylic acid magnetic nanoparticles (Sa-MNP) with well-dispersed aqueous solution properties. They found a reversible and good controlled intravascular accumulation under static magnetic field, a low risk of embolization with nanoparticle aggregates detached from venous intravascular nanoblocked areas, a persistent blocking of the arterioles and dependent capillaries network and a good circulating life time and biocompatibility; all suggesting a possible biomedical application of these MNPs in targeted cancer therapy through magnetic controlled blood flow nanoblocking mechanism.

Given ecotoxic, non-degradable biocides with a broad protection range are now prohibited in Europe, the paint industry is considering engineered nanoparticles (ENPs) as an alternative biocide. However, there is concern that ENPs in paint might be released in run-off water and subsequently consumed by animals and/or humans, potentially coming into contact with cells of the gastrointestinal tract and affecting the immune system. Accordingly Kaiser *et al.* (2013) evaluated the cytotoxic effects of three ENPs (nanosilver, nanotitanium dioxide and nanosilicon dioxide) that have a realistic potential for use in paints in the near future. Using NTA to analyze changes in the size (i.e. agglomeration) of nanosilver and nanotitanium dioxide during incubation of gastrointestinal cells (CaCo-2) and immune system cells (Jurkat) in culture media, they showed that the results suggest that paints doped with ENPs do not pose an additional acute health hazard for humans

After passage through biological barriers, nanomaterials inevitably end up in contact with the vascular endothelium and physiological flow and can induce cardiovascular damage. In a recent study the toxicity and sublethal effects of six nanoparticles, including four of industrial and biomedical importance, on human endothelial cells was investigated using different *in vitro* assays (Ucciferri *et al.*, 2013), NTA being used to analyze the AgNPs used. Broggi *et al.* (2013) also showed that silver nanoparticles induce cytotoxicity, but not cell transformation or genotoxicity, on Balb3T3 mouse fibroblasts.

In recent years there has been an ongoing discussion whether traditional toxicological methods are sufficient to evaluate the risks associated with nanoparticle inhalation and which has led to the emergence of Air-Liquid interface toxicology. Svensson *et al.* (2013) thus describe the direct deposition of gas phase generated aerosol gold nanoparticles into biological fluids in which they analyze, using NTA as well as DLS and UV spectroscopy, corona formation and particle size shifts. They suggested that their results were important since the protein corona together with key particle properties (e.g. size, shape and surface reactivity) to a large extent may determine the nanoparticle effects and possible translocation to other organs.

Similar work on nanoparticle protein coronas recently under taken by Hayashi *et al.* (2013) who considered the proposal that, while cells recognize the biomolecular corona around a nanoparticle, the biological identity of the complex may be considerably different among various species. Using coelomocytes of the earthworm *Eisenia fetida* from which were extracted *E. fetida* coelomic proteins (EfCP) as a native repertoire and fetal bovine serum (FBS) as a non-native reference, they confirmed the determinant role of the recognizable biological identity during invertebrate *in vitro*

testing of nanoparticles. Their finding showed a case of species-specific formation of biomolecular coronas which suggested that the use of representative species may need careful consideration in assessing the risks associated with nanoparticles. Data from their presentation shows that NTA is less susceptible to the presence of aggregates than is DLS when the sample is measured by the two techniques.

Aquatic and Marine Toxicity

Methods such as NTA could be considered as one of a number of means by which the aquatic environmental impact and potential cellular toxicity of nanoparticles could be studied in the future (Hassellöv and Kaegi, 2009). Indeed, later studies showed that the complexity of interactions between NEPs and aquatic environmental matrices is extremely complex representing a significant challenge in both their quantification and modelling but in which NTA may play a role (Gornati *et al.*, 2009; Hartmann, 2011; Arvidsson *et al.*, 2011; Howard, 2010, Njuguna *et al.*, 2011; Tran *et al.*, 2009).

Large collaborative research projects began investigating the ecotoxicological effects of a variety of nanoparticles on the freshwater environment (Juhel 2009) including CeO₂ which is used increasingly being used as a catalyst in the automotive industry (Quik *et al.*, 2010; Van Hoecke *et al.*, 2009) and the effect of silver and gold nanoparticles on fish (Scown *et al.*, 2010) and rainbow trout hepatocytes and gill cells (Farkas *et al.*, 2010, Farkas *et al.*, 2011). Trumsina compared various methods for monitoring nanoparticle detachment from textiles during washing and discussed the relative benefits of DLS and NTA against a new method of gas discharge visualization (Trumsina *et al.*, 2011) and Piccapietra *et al.* (2011) considered the colloidal stability of carbonate coated silver nanoparticles in synthetic and natural freshwater.

Handy and his co-workers have carried out extensive studies on the effects, in terms of aquatic ecotoxicity, of various metal and metal oxide nanoparticles on fish. They looked at the effects of TiO₂ on the physiology and reproduction of zebrafish (Ramsden *et al.*, 2012) concluding there was limited evidence of toxicity but a discernible effect on reproduction. They also looked at the uptake of titanium from TiO₂ nanoparticle exposure in the isolated perfused intestine of rainbow trout, *Oncorhynchus mykiss*, (Al-Jubory and Handy, 2012; Shaw *et al.*, 2012 and Al-Bairuty *et al.*, 2012) and studied the histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of the same species. Assessing whether copper nanoparticles are more toxic than traditional forms of dissolved copper they studied the pathologies in gill, gut, liver, kidney, brain and muscle of juvenile specimens exposed in triplicate to either a control (no added Cu), 20 or 100 µg l⁻¹ of either being dissolved Cu (as CuSO₄) or Cu-NPs (mean primary particle size of 87 ± 27 nm) in a semi-static waterborne exposure regime. Overall the data showed that pathology from CuSO₄ and Cu-NPs were of similar types, but there were some material-type effects in the severity or incidence of injuries with Cu-NPs causing more injury in the intestine, liver and brain than the equivalent concentration of CuSO₄ by the end of the experiment, but in the gill and muscle CuSO₄ caused more pathology. In further work, they also showed that subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles were associated with gill rather than brain injury (Boyle *et al.*, 2012). In all of these studies, NTA was used to

determine the mean size and particle size distribution of the nanoparticles used. Recently, Windeatt and Handy (2012) have reported NTA work on the effect of nanomaterials on the compound action potential of the shore crab, *Carcinus maenas*.

In related research, the effects of particle size and coating on nanoscale Ag and TiO₂ exposure in zebrafish (*Danio rerio*) embryos was studied (Osborne *et al.*, 2012) the results of which showed titanium dioxide nanoparticles (nominally, 4nm, 10nm, 30nm and 134 nm) had little or no toxicity on the endpoints measured while Ag both in nano form (10nm and 35nm) and its larger counterpart (600-1600 nm) induced dose-dependent lethality and morphological defects, occurring predominantly during gastrula stage. Of the silver material tested, 10nm nanoparticles appeared to be the most toxic. More recent work on zebrafish has resulted in reports from Christen *et al.* (2013) who show that silver nanoparticles induce endoplasmatic reticulum stress response in zebrafish and compared their data to that obtained when tested on human hepatoma cells (Huh7).

Still working with zebrafish, Henry *et al.* (2013) showed that the association of Hg²⁺ with aqueous (C₆₀)_n aggregates facilitates increased bioavailability of Hg²⁺ in zebrafish (*Danio rerio*) using NTA to demonstrate an increase in aggregate size and settlement of nC₆₀ aggregates out of the water column over 24 h indicating that aqueous nC₆₀ can sorb Hg²⁺, transport Hg²⁺ to substrate surface, and increase concentrations of bioavailable Hg²⁺ in organisms located where settled nC₆₀ aggregates accumulate.

NTA has been central to studies on the influence of engineered Fe₂O₃ nanoparticles and soluble (FeCl₃) iron on the developmental toxicity caused by CO₂-induced seawater acidification (Kadar *et al.* 2010). Recently, Tatarkiewicz *et al.* (2012) have described the use of NTA in the concentration measurement and sizing of colloidal particles in the Arctic Ocean while Stuart *et al.* (2012) report proof-of-concept measurements relating to the impact of nanoparticles with an electrode potentiostatted at a value corresponding to the diffusion controlled oxidation of silver nanoparticles in authentic seawater media.

The increasing use of nanoparticles in a variety of textiles as antibacterial, antimicrobial, water resistant and protective agents has prompted the use of NTA in the study nanosilver mobilized in washing machine effluents (Farkas *et al.*, 2011). Wang *et al.* (2012) have also studied the aquatic toxicity of nanosilver colloids to different trophic organisms comparing the contributions of particles and free silver ion

Using a 15k oligonucleotide microarray for *Daphnia magna*, a freshwater crustacean and common indicator species for toxicity, to differentiate between particle specific and ionic silver toxicity and to develop exposure biomarkers for citrate-coated and PVP-coated AgNPs, Poynton *et al.* (2012) determined the degree of aggregation of AgNPs prior to studying their toxicity at the genomic level.

In further work studying the effect of nanoparticles on marine microfauna, Li *et al.* (2013) have reported on the accumulation of aqueous and nanoparticulate silver by the marine gastropod *Littorina littorea* concluding that Ag is most bioavailable to *L. littorina* when in true solution and that Ag measured in external tissues of the snail following exposure to nanoparticles arises from some physical association that does not result in significant transfer of the metal to internal organs.

Shaw *et al.* (2013) have proposed a simplified method for determining titanium from TiO₂ nanoparticles in fish tissue with a concomitant multi-element analysis claiming method precision and accuracy were good with coefficients of variation <7% with NTA data being used to confirm, where applicable, aggregate presence and size.

Batley *et al.* (2012) have recently reviewed the complexities associated with determining the fate and risks of nanomaterials in aquatic and terrestrial environments and Lambert *et al.* (2013a) have considered the effects of environmental conditions on latex degradation in aquatic systems as well as from products such as natural rubber latex condoms (Lambert *et al.*, 2013b). Samples were immersed in either demineralized water, artificial freshwater and marine water media and exposed for a period of 200–250 days with exposure starting at different times of the year. Effects of pH, agitation and the exclusion of light on degradation were also studied. At the end of the exposure period, recovery of polymer material $\geq 1.6 \mu\text{m}$ ranged from a low of 22.04% (± 16.35 , for the freshwater treatment at pH 5.5) to a high of 97.73% (± 0.38 , for the exclusion of light treatment). The disappearance of the bulk material corresponded to an increase in nanoparticles as measured by NTA and dissolved organic material in the test media. In the case of the condom study, the direct effects of the degradation mixture were investigated using two freshwater organisms with different life cycle traits, the water column crustacean *Daphnia magna* and the sediment-dwelling larval of *Chironomus riparius*. Ecotoxicity tests investigated both acute and chronic endpoints and were shown to exhibit no toxic effects. In another recent paper examining the effects of twelve carbon nanomaterials (CNMs) that differ in their core structure and surface chemistry to *Daphnia magna* over a 21-day chronic exposure Arndt *et al.* (2013) looked at the effect these materials have on daphnid mortality, reproduction, and growth: They concluded that 1) acute exposure assays do not accurately describe the impact of CNMs to biological systems, 2) chronic exposures provide valuable information that indicates the potential for different modes of action for nanomaterials of differing chemistries, and 3) core structure and surface chemistry both influence particle toxicity.

Recently, Hassellöv (2013) has comprehensively reviewed the occurrence, identification, fate and behavior of engineered nanoparticles and nanoscale pollutants in marine systems including, in addition to synthetic nanomaterials, many other types of micro- and nanoscale pollutants which have recently been identified as potential emerging pollutants, e.g. from road runoff, combustion, mining, waste and industrial processes.

Microbiota and Plants

The effect that nanoparticles have on microorganisms and their ecology has been studied using NTA to determine nanoparticulate properties and behavior.

In their investigation of the distribution and bioavailability of engineered nanoparticles (silver NP, cerium dioxide NP, titanium dioxide NP) in freshwater periphyton, Kroll and her co-workers used a variety of techniques, including NTA, to monitor material properties such as size, charge, and dissolution (Kroll *et al.*, 2011).

In work exploring the link between chemical composition and molar-mass distribution of the extracellular polymeric substances (EPS) released by the bacterium *Sinorhizobium meliloti* using

chemical, spectroscopic and fractionation techniques, NTA confirmed the size distributions and chemical heterogeneity of such materials as characterized by asymmetrical flow field-flow fractionation (Alasonati and Slaveykova, 2011).

Similarly, Turner *et al.* (2011) investigated the interactions of Ag nanoparticles with marine microalgae, *Ulva lactuca*, using NTA to characterize their Ag nanoparticle suspensions.

Finally, Chaudhari *et al.* (2012) used NTA, TEM and electron dispersive X-ray spectra to assess the effect of biosynthesized silver nanoparticles on *Staphylococcus aureus* biofilm quenching and prevention of biofilm formation.

Metal-containing nanomaterials have the potential to be used in dentistry for infection control, but little is known about their antibacterial properties. A recent study investigated the toxicity of silver, titanium dioxide and silica nanoparticles (NPs) against the oral pathogenic species of *Streptococcus mutans*, compared to the routine disinfectant, chlorhexidine. Thus, Besinis *et al.* (2012) investigated the antibacterial effects of Ag, TiO₂ and SiO₂ nanoparticles compared to the dental disinfectant chlorhexidine on *Streptococcus mutans* using a suite of bioassays using NTA for nanoparticle sizing while Bondea *et al.* (2012) similarly used NTA in their study of *Murraya koenigii*-mediated synthesis of silver nanoparticles and its activity against three human pathogenic bacteria claiming remarkable antibacterial activity against three human pathogenic bacteria when used in combination with commercially available antibiotics.

NTA was cited in data reported in other studies on bacteria. The antibacterial and water purification activities of self-assembled honeycomb structure of aerosol deposited titania film was studied by Park *et al.* (2012) and the determination of internalization of chromium oxide nano-particles in *Escherichia coli* by flow cytometry Khatoon *et al.* (2011). Carter *et al.* (2012) also showed that a bacteriophage cocktail significantly reduces *Escherichia coli* O157:H7 contamination of lettuce and beef, but did not protect against recontamination.

In a study of the effects of coating applied to zero-valent nano-iron (nZVI) on early life stage development of three key marine invertebrate species, Kadar *et al.* (2012) used NTA to study the dissolution of nZVI in sea water showing the coating help stabilize the nanometal suspension. Kadar has also studied the effect of NTA-analyzed industrially relevant engineered iron nanoparticles on growth and metabolic status of marine microalgae cultures in which he followed subsequent alterations in their growth rate, size distribution, lipid profiles and cellular ultrastructure (Kadar *et al.*, 2012).

NTA has also been used amongst other techniques to study nanoparticulates transport in fungi (Cunha-Azevedo *et al.*, 2011). Cunha-Azevedo also developed and tested an anti-fungal formulation of PLGA nanoparticles designed to release the active agent itraconazole in which size was considered an important feature and analyzed by NTA as an average of 174nm (Cunha-Azevedo 2011).

Hartmann *et al.* (2012) reviewed the challenges of testing metal and metal oxide nanoparticles in algal bioassays using titanium dioxide and gold nanoparticles as case studies. They showed that Au NP coating layers changed over time and TiO₂ nanoparticle aggregation/agglomeration increased as a function of concentration. While NTA was used to determine the hydrodynamic diameter and size

distributions of suspended particles, it was found that of three biomass surrogate measuring techniques evaluated (Coulter Counting, cell counting in haemocytometer and fluorescence of pigment extracts) fluorometric methods was found to be most suitable for quantifying biomass though complicated by algae-particle interactions and nanoparticle transformation. She concluded that optimization of the method is needed to reduce further particle interference on measurements.

The anti-microbial properties of nanosilver have been well established and as such much work has been carried out on determining the mechanism and effects of this material on microbial systems.

Piccapietra *et al.* (2011) used nanoparticle tracking analysis, dynamic light scattering, and ultraviolet–visible spectroscopy to measure changes in the physicochemical properties of silver nanoparticles (AgNP) in their investigation on the fate, mobility, and bioavailability of AgNP in aquatic systems including the influence of pH, ionic strength, and humic substances on the stability of carbonate-coated AgNP of average diameter 29 nm. He extended this work to include studies on the colloidal stability of silver nanoparticles and their interactions with the alga *Chlamydomonas reinhardtii* (Piccapietra, 2012).

Silver nanoparticles were also the subject of a recent study by Schacht *et al.* (2012) on microbial growth dynamics finding, to their surprise, that their data showed growth stimulation of *C. necator* at certain Ag(0) nanoparticle concentrations, as well as varying susceptibility to nanoparticles at different growth stages underscoring the need for time-resolved analyses of microbial growth inhibition by Ag(0) nanoparticles.

Using NTA to determine particle size distribution, Matzke *et al.* (2013a) recently discussed the effects of selected silver nanoparticles on freshwater microbial communities showing that differences in toxicity could be determined for the different particles with AgNO₃ being for almost all cases the most toxic compound with one exception. The same group subsequently described the toxicity of differently sized and coated commercially available silver nanoparticles to the bacterium *Pseudomonas putida*. The results indicated that the toxicity is driven by the Ag⁺ ions, implying that an environmental hazard assessment for microorganisms based on total silver concentration and the assumption that AgNPs dissolve is sufficiently protective (Matzke *et al.*, 2013).

In NTA-supported *in vitro* and soil experiments studying the impact of Ag and Al₂O₃ nanoparticles on the soil bacteria, *Bacillus cereus* and *Pseudomonas stutzeri*, Fajardo *et al.* (2013) showed that Al₂O₃ nanoparticles did not show significant toxicity at any dose or time assayed, whereas exposure to 5 mg L⁻¹ Ag nanoparticles for 48 h caused bactericidal effects. In a microcosm experiment, using two different natural soils, Al₂O₃ or Ag nanoparticles did not affect the *Caenorhabditis elegans* toxicity endpoints, growth, survival or reproduction. These changes were attributable to both the nanoparticles treatment and soil characteristics, highlighting the importance of considering the soil matrix on a case by case basis.

Tlili *et al.* (2012) also showed the short-term toxicity of silver nanoparticles on litter-associated fungi and bacteria from streams while Masurkar *et al.* (2012) showed that *Staphylococcus aureus* biofilm quenching and biofilm formation prevented activity of silver nanoparticles synthesized using *Saccharum officinarum* (sugarcane). Masurkar later extended this work to demonstrate biofilm

quenching activity of silver nanoparticles synthesized using *Bacillus subtilis* in his work promoting the green synthesis of silver nanoparticles as a basic need in the field of nanotechnology (Masurkar *et al.*, 2013). Similarly, Dhuldhaj *et al.* (2012) had previously described an eco-friendly approach via the *Tagetes erecta*-mediated phytosynthesis of silver nanoparticles. Gupta *et al.* (2013) also investigated the *Lawsonia inermis*-mediated synthesis of silver nanoparticles and its activity against human pathogenic fungi and bacteria with special reference to formulation of an antimicrobial nanogel using NTA to establish particle size distribution. Raheman *et al.* (2011) had previously proposed silver nanoparticles as a novel antimicrobial agent synthesized from an endophytic fungus *Pestalotia sp.* isolated from leaves of *Syzygium cumini*.

Zhdanov and Höök (2013) have reported on the nucleation in mesoscopic systems under transient conditions with respect to peptide-induced pore formation in vesicles given attachment of lytic peptides to the lipid membrane of virions or bacteria is often accompanied by their aggregation and pore formation, resulting eventually in membrane rupture and pathogen neutralization. The results obtained helped clarify the mechanism of the pore formation and membrane destabilization observed during interaction of highly active α -helical peptide with sub-100-nm lipid vesicles that mimic enveloped viruses with nanoscale membrane curvature.

Using NTA to determine particle size, it was found that alumina nanoparticles substantially increase biomass accumulation of the aquatic plant *Lemna minor* and that such a stimulatory effect of alumina nanoparticles on growth had not been reported previously (Juhel *et al.* 2011). NTA has also been used amongst other techniques to study nanoparticulates transport in seed germination (Vajpayee *et al.* 2011) and to study nanoparticulates transport in surface runoff through dense vegetation (Yu, 2011).

Finally, Schwabe *et al.* (2013) recently discussed the influence of two types of organic matter on interaction of CeO₂ nanoparticles with plants in hydroponic culture. They used hydroponic plant cultures to study nanoparticle–plant-root interaction and translocation and exposed wheat and pumpkin to suspensions of uncoated CeO₂–NP for 8 days (primary particle size 17–100 nm, 100 mg L⁻¹) in the absence and presence of fulvic acid (FA) and gum arabic (GA) as representatives of different types of natural organic matter. They showed that NP-dispersions were stable over 8 days in the presence of FA or GA, but with growing plants, changes in pH, particle agglomeration rate and hydrodynamic diameter were observed. None of the plants exhibited reduced growth or any toxic response during the experiment.

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Chapter IV: Environmental Impact of Nanoparticles

NTA in the Development of test methods for nanoparticle impact studies

Despite being a relatively new technique, NTA has been recognized as showing promise as an analytical method which could furnish not just information about nanoparticle size and, equally importantly, concentration but that it could do so in complex sample types of high polydispersity (Montes-Burgos *et al.*, 2010; Lynch 2008, Montes-Burgos *et al.*, 2007; Gornati *et al.*, 2009) and that methods such as NTA could be considered as one of a number of means by which the environmental impact and potential cellular toxicity of nanoparticles could be studied in the future (Borm *et al.*, 2006; Tenuta 2008, Tran and Anton 2009, Kuhlbusch *et al.*, 2010; Hassellöv and Kaegi 2009, Stolpe *et al.*, 2011) and methods to study their effects on other biological barriers have been addressed (Linn *et al.*, 2010). Tran *et al.* (2011) also developed a hypothetical model for predicting the toxicity of high aspect ratio nanoparticles while Karlsson (2010) compared the comet assay in nanotoxicology research to results obtained by NTA.

In another such study, NTA was considered to share many features in common with conventional flow cytometry but unique in the deeply sub-micron size range. NTA was considered “a direct and fast technique by which nanoparticles in their natural solvated state in a liquid can be rapidly detected, sized and concentration measured. The technique can be used to complement existing techniques for the sizing of nanoparticles (e.g., DLS, PCS) allowing data obtained from these methods to be validated by direct microscopical observation of the sample”. (Bendre *et al.*, 2011)

In an interesting and thought-provoking extension of assessing possible sources of nanoparticles Chen *et al.* (2012) undertook a TEM and NTA-assisted study in the characterization and preliminary toxicity assay of nano-titanium dioxide additive in sugar-coated chewing gum. They described a facile and highly reliable separation method of TiO₂ particles from food products (focusing on sugar-coated chewing gum) claiming their work to be the first comprehensive characterization study on food nanoparticles by multiple qualitative and quantitative methods. Surprisingly, their results showed that the number of food products containing nano-TiO₂ (<200 nm) is much larger than known and consumers have already often been exposed to engineered nanoparticles in daily life. Over 93% of TiO₂ in gum is nano-TiO₂, and it is unexpectedly easy to come out and be swallowed by a person who chews gum.

Noël also used NTA amongst other techniques for analysis of nanoparticle aggregates in his work on generating nano-aerosols from TiO₂ (5 nm) nanoparticles showing different agglomeration states as applied to toxicological studies (Noël *et al.*, 2012) and Cabot *et al.* (2012) used NTA to measure changes in tobacco smoke particle size over a series of different time points providing an input into residence time estimates, thus aiding dose calculations to the lower airways.

More recently Ling *et al.* (2013) have reviewed a range of detection and identification instruments and their calibration for the analysis of air/liquid/surface-borne nanoscale particles suggesting that microscopy analysis for particle morphology can be performed by depositing air-borne or liquid-borne nanoparticles on surfaces. Detection limit and measurement resolution of the liquid-borne

nanoparticles could be enhanced by aerosolizing them and taking advantage of the well-developed air-borne particle analyzers. NTA was tested on TiO₂ aggregate particles

Similarly, Asimakopoulou *et al.* (2013) discussed the development of a dose-controlled multiculture cell exposure chamber for efficient delivery of airborne and engineered nanoparticles. Their proposed technology was validated with various types of nanoparticles (Diesel engine soot aggregates, engineered nanoparticles for various applications) and with state-of-the-art nanoparticle measurement instrumentation to assess the local deposition of nanoparticles on the cell cultures. Final testing of the dose-controlled cell exposure system was performed by exposing A549 lung cell cultures to fluorescently labelled nanoparticles and delivery of aerosolized nanoparticles was demonstrated by NTA visualization of the nanoparticle fluorescence in the cell cultures following exposure.

Wang *et al.* (2013) addressed the need for suitable methods for high throughput screening of physicochemical properties of nanomaterials (NM) and their immediate environments to allow better understanding of NM bioactivities, prioritization of NMs for further testing, and the building of computational models to predict NM toxicity.

Recognizing that nanosilver, due to its small particle size and enormous specific surface area, facilitates more rapid dissolution of ions than the equivalent bulk material; potentially leading to increased toxicity of nanosilver, Reidy *et al.* (2013) critically reviewed current knowledge and recommendations relating to mechanisms of silver nanoparticle release, transformation and toxicity:

In anticipation of increasing regulatory measurements requirements for nanomaterials and their toxicity, a number of studies on metrology have been undertaken. Thus, Brown *et al.* (2013) have reviewed nano-object count metrology and a best practice framework. Emphasizing that harmonized methods for identifying nanomaterials by size and count for many real world samples do not currently exist and that, while particle size remains the sole discriminating factor for classifying a material as 'nano', inconsistencies in size metrology will continue to confound policy and decision-making they suggested that substantial scientific scrutiny is needed in the area of nanomaterial metrology to establish best practices and to develop suitable methods before implementation of definitions based solely on number percent nano-object content for regulatory purposes. Strong cooperation between industry, academia and research institutions will be required to fully develop and implement detailed frameworks for nanomaterial identification with respect to emerging concentration measurement-based metrics. When discussing NTA specifically, they pointed out that NTA may have difficulty with agglomeration since the agglomerate will appear as a single scattering centre. In these cases, the sample dispersion may have to be altered through further dilution or sonication. Dispersing aids may be used but should not index match the particles. Larger particles in a population may be removed through filtration, centrifugation or settling prior to analysis.

Similarly, Pettitt and Lead (2013) have outlined characterization requirements for nanomaterial regulation. They proposed some minimum physicochemical parameters required to adequately describe NMs for regulatory purposes and discussed the most appropriate mechanisms to obtain

those data in terms of the overarching delivery mechanism. Guiding principles for particle characterization during the hazard testing required to comply with regulations were examined.

Finally, Vincent (2013) has briefly reviewed NTA for the characterization of nanomaterials for toxicological assessment.

Analysis of Environmental Samples

An early appreciation was published on the role that NTA could play in the analysis of nanoparticles contained within or extracted from complex environmental samples. Thus, Borm *et al.* (2006) and Tenuta (2008) cited NTA as a possible technology for the development of systematic research strategies concerning their analysis and specific examples were described relating to TiO₂ nanoparticles in natural aquatic media (Holmberg *et al.*, 2008) and the oxidation of organic pollutants in aqueous solutions by nanosized copper oxide catalysts (Ben-Moshe *et al.*, 2008)

More recent studies have shown that the complexity of interactions between NEPs and environmental matrices is extremely complex represents a significant challenge in both their quantification and modelling but in which NTA may play a role (Gornati *et al.*, 2009; Hartmann 2011, Njuguna *et al.*, 2011; Tran *et al.*, 2011; Kuhlbusch *et al.*, 2010; Sentein *et al.*, 2011)

Quik and his co-workers have also studied the role that natural colloids play in the sedimentation of CeO₂ nanoparticles using NTA to analyze natural river waters from two major European rivers in which they showed that heteroaggregation of the metal oxide with or deposition onto the solid fraction of natural colloids was the main mechanism causing sedimentation in relation to homoaggregation (Quik *et al.*, 2012). MacCuspie *et al.* (2011) had earlier studied the potential hazards of gold species in a variety of cellular and aqueous systems.

Schwyzer has studied both the colloidal stability (Schwyzer *et al.*, 2011 and 2012) and solubilization (Schwyzer *et al.*, 2010) of carbon nanotubes under natural conditions and Reed *et al.* (2012), in their study of the detection of single walled carbon nanotubes (CNT) by monitoring embedded metals (intercalated in the CNT structure), found that during analysis of split samples by both single particle inductively coupled plasma mass spectrometry (spICPMS) and NTA, the quantification of particle number concentration by spICPMS was several orders of magnitude worse than by NTA. They postulated that this was a consequence of metal content and/or size, caused by the presence of many CNTs that do not contain enough metal to be above the instrument detection limit, resulting in undercounting CNTs by spICPMS, though spICPMS is still a more sensitive technique for detecting the presence of CNTs in environmental, materials, or biological applications.

Domingos and his co-workers carried out a typical such study involving analysis of nanoparticle suspensions using several state-of-the-art analytical techniques (transmission electron microscopy; atomic force microscopy; dynamic light scattering; fluorescence correlation spectroscopy; nanoparticle tracking analysis; flow field flow fractionation). Theoretical and analytical considerations were evaluated, results were compared, and the advantages and limitations of the techniques were discussed. No “ideal” technique was found for characterizing manufactured nanoparticles in an environmental context as each technique had its own advantages and limitations (Domingos *et al.*, 2009).

NTA has also been used amongst other techniques to study and compare three Silica nanotracer nanoparticulates and their transport in soils (Vitorge *et al.*, 2010) and the nano-sized Fe₂O₃ waste powder adsorption with arsenite (As³⁺) in the steel industry has also been studied (Prasad *et al.*, 2011).

Given the increasing prevalence of nanoparticles in consumer products and processes, their release and appearance in wastewaters have attracted increasing attention. Thus the release of nanosized biocides from wood coatings have been studied with NTA (Künniger *et al.*, 2010) as have nanoscale components of toothpastes. (Peetsch and Epple 2011) and Farkas *et al.* (2011) have also reported the characterization of the effluent from a nanosilver producing washing machine using NTA to follow silver movement. Rezić (2011) had also described the determination of engineered nanoparticles on textiles and in textile wastewaters.

Similarly, the widespread use of TiO₂ as a major sunscreen component and the associated fate, behavior and environmental risks in the UK has been studied (Johnson *et al.*, 2011).

The use of NTA as a new tool in the study of nanoparticles in environmental samples and for toxicological studies has been reviewed recently. In a study of stability of CeO₂ in de-ionized water and electrolyte-containing fish medium the dispersions were monitored using various techniques, for a period of 3 days. NTA was found to provide useful data which was complementary to zeta potential, particle size via DLS, fluorescence and UV–Vis spectroscopy and SEM and specifically was shown to provide useful, quantitative information on the concentration of nanoparticles in suspension although limited in its ability to accurately track the motion of large agglomerates found in the fish medium (Tantra *et al.*, 2011).

Vähä-Nissi *et al.* (2011) have described the use of NTA in their study on the safe production and use of nanomaterials with special reference to aqueous dispersions from biodegradable and/or renewable polymers while Wilkinson *et al.* (2011) suggested solution-engineered palladium nanoparticles as a model for health effect studies of automotive particulate pollution.

In his assessment of the need for standardized methods and environmental monitoring programs for anthropogenic nanoparticles, Paterson reviewed the available techniques emphasizing the critical need for methods capable of qualitatively and quantitatively measuring such pollutants. He issued a challenge to national and international regulatory and research agencies to help develop standard methods, quality assurance tools, and implement environmental monitoring programs for this class of pollutants citing NTA as being one such technique that could supply important information. (Paterson *et al.*, 2011). von der Kammer *et al.* (2011) reiterated this point in their recent discussion on the general considerations associated with the isolation of engineered nanoparticles from highly complex environmental samples (von der Kammer *et al.*, 2011)

In other work related to the development of test methods for Health and Safety risk management, Dolez and her co-workers used NTA to measure the penetration of nanoparticles through protective gloves in conditions simulating occupational use. Involving nanoparticles applied as powder and colloidal solutions to different materials subject to various types of static and dynamic mechanical deformations simultaneously with nanoparticle exposure. In determining that the development of the test method also involved the identification of appropriate nanoparticle

detection techniques, Dolez concluded that while methanol-based sampling solutions could be centrifuged on grids or mica substrates for analysis by microscopy techniques, NTA and ICP-MS could also be used to directly detect nanoparticles in water-based sampling solutions (Dolez *et al.*, 2011).

In his assessment of new single particle methods for detection and characterization of nanoparticles in environmental samples, Tuoriniemi (2013) evaluated NTA for the measurement of number concentration and size distributions. The technique was considered suitable for monitoring and measuring exposure at “relatively high” ($> 10^6$ particles mL⁻¹) concentrations but NTA was considered relatively unspecific in the sense that it is difficult to distinguish particles of different materials. To increase sensitivity and specificity, single particle inductively coupled plasma mass spectrometry (spICPMS) was assessed for element specific characterization of particles in liquid samples. Also, recognizing that variable pressure or environmental scanning electron microscopes (ESEM) could be applied on a vast range of sample types with “no or very little sample preparation”, backscattered electron (BSE) imaging in such an instrument was chosen as a base for developing a method for quantification of particles in solid samples. The technique was applied for quantifying particles in toxicity tests involving soil biota and was considered to be sensitive enough to cover the concentration range that is typically of interest in such tests. It was concluded that due to the information obtained on a single particle basis, electron microscopy is a suitable complementing technique for spICPMS measurements, which otherwise give little information about the structure of the particles. It should be noted however, that this study did not apparently consider the high capital and running costs of these techniques nor the sample analysis time which might significantly curtail sample throughput which was a particular limitation.

Multiple complimentary techniques were used to characterize bare and polymer-coated nTiO₂ and nZnO particles under a range of environmentally relevant conditions: dynamic light scattering, nanoparticle tracking analysis, scanning electron microscopy and transmission electron microscopy. Percolation of suspensions of such materials through angular sand columns showed uncoated (bare) NPs demonstrated high retention within the water saturated granular matrix and both bare nTiO₂ and nZnO deposition onto sand was found to be time-dependent. In contrast to bare particles, polymer-coated NPs were highly stable in suspension and exhibited significant transport potential (Petosa *et al.*, 2011). Petosa *et al.* (2013) have more recently extended this work to the study of the mobility of nanosized cerium dioxide and polymeric capsules in quartz and loamy sands saturated with model and natural groundwaters. Laboratory-scale columns were used to examine the mobility of polyacrylic acid (PAA)-coated cerium dioxide nanoparticles (nCeO₂) and an analogous nanosized polymeric capsule (nCAP) in water saturated quartz sand or loamy sand. ENP suspensions were characterized using dynamic light scattering and NTA to establish aggregate size. Enhanced particle retention was also observed in loamy sand in comparison to the quartz sand, emphasizing the need to consider the nature of the aqueous matrix and granular medium in evaluating contamination risks associated with the release of ENPs in natural and engineered aquatic environments.

Raychoudhury *et al.* (2011) similarly investigated the straining of polyelectrolyte-stabilized (coated with carboxymethyl cellulose) nanoscale zero valent iron particles (CMC-NZVI) during transport

through granular porous media using NTA to demonstrate that CMC-NZVI particles, despite of their small size (NTA determined hydrodynamic diameters of 167–185 nm and transmission electron microscopy imaged diameters of approximately 85 nm), may be removed by straining during transport, especially through fine granular subsurface media. A tailing effect observed in the particle breakthrough curves, was attributed to detachment of deposited particles.

Mallampati *et al.* (2012) has used NTA to assess enhanced heavy metal immobilization in soil by grinding with addition of nanometallic Ca/CaO dispersion mixtures concluding that it might be due to adsorption and entrapment of heavy metals into newly formed aggregates, thereby prompting aggregation of soil particles and enclosure/binding with Ca/CaO-associated immobile salts. Shang *et al.* (2012) reported a study of transport and retention of engineered nanoporous silicate particles (ENSPs) that are designed for treatment and remediation of contaminants such as uranium in groundwater and sediments using NTA and DLS to periodically monitor the quality of the ENSP dispersion. NTA has also been used amongst other techniques to study nanoparticulates transport in soil organisms as diverse as earthworm (Hooper *et al.*, 2011) and the influence of humic acid on TiO₂ nanoparticles in test media (Mullinger *et al.*, 2011)

Tourinho *et al.* (2012) have recently reviewed the literature dealing with the fate and effects of metal-based NPs in soil. In the environment, the characteristics of NPs (e.g., size, shape, surface charge) and soil (e.g., pH, ionic strength, organic matter, and clay content) will affect physical and chemical processes, resulting in NP dissolution, agglomeration, and aggregation. They point to the lack of standards existing for toxicity tests with NPs and, more importantly, that the reporting of associated characterization data is sparse, or missing, making it impossible to interpret and explain observed differences in results among studies. NTA, with its ability to generate higher resolution particle size distribution information and number frequency distributions, is advantageous in this respect.

Ramirez-Garcia *et al.* (2011) reported on a highly successful and original protocol for the dispersion of titania nanoparticles in biocompatible fluids for *in vitro* and *in vivo* studies of the nanoparticle–biology interaction. Using stabilizers to obtain dispersions of 45 and 55nm diameters at concentrations up to 10mg/ml and the sizing techniques of Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA) and Differential Centrifuge Sedimentation (DCS) were used to characterize the different suspensions and the suitability of each was compared while Tenuta *et al.* (2011) have described the elution of labile fluorescent dye from nanoparticles during biological use.

In a comprehensive review of the use of flow field-flow fractionation (FIFFF) for the analysis and characterization of natural colloids and manufactured nanoparticles in environmental systems, Baalousha *et al.* (2011) compared numerous detection techniques applied to FIFFF (including inductively coupled plasma-mass spectroscopy, light scattering, NTA, UV-absorbance, fluorescence, transmission electron microscopy, and atomic force microscopy), demonstrating that FIFFF provides a wealth of information on particle properties including, size, shape, structural parameters, chemical composition and particle-contaminant association.

Exploiting the ability of NTA to more accurately measure the particle size distribution profile of polydisperse systems than other techniques, Raychoudhury *et al.* (2011) demonstrated that

aggregation resulted in a change in the particle size distribution (PSD) of carboxymethyl cellulose (CMC)-modified nanoparticles of zero-valent iron (NZVI) over time when they were investigated in laboratory-scale sand packed columns and that the change in PSD over time was influenced by the CMC-NZVI concentration in suspension. They showed that changes in particle sizes over time led to corresponding changes in single-collector contact efficiencies, resulting in altered particle deposition rates over time using a coupled aggregation-colloid transport model to demonstrate how changes in PSD can enhance or reduce the transport of CMC-NZVI in column experiments.

In studying the toxicity of ZnO nanoparticles to *Folsomia candida*, Waalewijn-Kool *et al.* (2012) showed that differences in methods of spiking exposure media to test dispersion size characteristics made little difference to the reproductive capacity of the organism, NTA and TEM both showing the toxicity of the ZnO was not related to particle size. Yu (2011) studied colloid transport in surface runoff through dense vegetation.

Hadioui *et al.* (2012) described a multimethod quantification of Ag⁺ release from nanosilver, suggesting part or all of the toxicity attributed to silver nanoparticles (nAg) may be due to the release of free silver (Ag⁺). Using NTA to determine nAg size and number prior to employing ion-exchange technique (IET) centrifugal ultrafiltration and single particle inductively coupled plasma mass spectrometry (SP ICP-MS) to determine very low concentrations of free or dissolved Ag in commercial suspensions of nAg.

Following earlier work in which Gallego-Urrea *et al.* (2011) considered the applications of NTA to the determination of size distributions and concentrations of nanoparticles in environmental, biological and food samples, Luo *et al.* (2013) have compared NTA to the use of atmospheric scanning electron microscopy (ASEM) in the visualization and characterization of engineered nanoparticles in complex environmental and food matrices such as supernatant of natural sediment, test medium used in ecotoxicology studies, bovine serum albumin and tomato soup, concluding that ASEM analysis was found to be a complementary technique to existing methods that is able to visualize ENPs in complex liquid matrices and to provide ENP size information without extensive sample preparation.

While Saleh (2013) showed aggregation behavior of nanomaterials under biological exposure conditions, Wilkinson (2013), in an attempt to gain a fuller insight into the health effects from PM, suggested it could only be achieved through practical investigation of the mode of toxicity from distinct types of particles and required techniques for their identification, monitoring, and the production of model fractions for health studies. Accordingly he undertook a comprehensive collection and chemical analysis of particulates at the origin of emission in order to provide clearer insight into the nature of the particulates at exposure and add detail to aid risk assessment. Taking the approach of *in vitro* cytotoxicity testing, nanoparticles of types typical to automotive emissions, were synthesized and extensively characterized using SEM-EDS, X-ray diffraction (XRD), transmission electron microscopy (TEM), dynamic light scattering (DLS), and nanoparticle tracking analysis (NTA). The produced model magnetite and palladium nanoparticles were found to induce toxicity in human pulmonary epithelial cells (A549 and PBEC) as well as impact severely on immunological and renal cells (221 B- and 293T-cells) in a dose-dependent manner.

Finally, Park *et al.* (2013) have posed the question of whether the results of regulatory ecotoxicity testing of engineered nanoparticles are the relevant to the natural environment. Proposing that many studies have explored the toxicity of ENPs to aquatic organisms but these studies have usually been performed with little understanding of ENPs behavior in the test media and the relationship between behavior in the media to behavior in natural waters, their study evaluated and compared the aggregation behavior of four model gold nanoparticle types (coated with neutral, negative, positive and amphoteric cappings) in standard ecotoxicity test media and natural waters. In standard media, positive and neutral nanoparticles (NPs) were stable whereas amphoteric and negative NPs generally showed substantial aggregation. In natural waters, amphoteric NPs were generally found to be stable, neutral and positive NPs showed substantial aggregation while negative NPs were stable in some waters and unstable in others. Humic acid addition stabilized the amphoteric NPs, destabilized the positive NPs and had no effect on stability of negative NPs. Given the dramatically different behaviors of ENPs in various standard media and natural waters, they suggest current regulatory testing may either under- or over-estimate the toxicity of nanomaterials to aquatic organisms and that, therefore, there is a pressing need to employ ecotoxicity media which better represent the behavior of ENPs in natural system. Prior to testing, all model study particles were characterized by TEM and NTA. Reed *et al.* (2013) and Reed (2013) used using single particle-inductively coupled plasma-mass spectrometry (spICPMS) to detect single walled carbon nanotubes by monitoring embedded metals using trace catalytic metals intercalated in the CNT structure as proxies for the nanotubes. Interestingly, analysis of split samples by both spICPMS and NTA showed the quantification of particle number concentration by spICPMS to be several orders of magnitude lower than by NTA. They postulated that this was a consequence of metal content and/or size, caused by the presence of many CNTs that do not contain enough metal to be above the instrument detection limit, resulting in undercounting CNTs by spICPMS. However, they claimed that since the detection of CNTs at low ng L⁻¹ concentrations is not possible by other techniques, spICPMS was still a more sensitive technique for detecting the presence of CNTs in environmental, materials, or biological applications.

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Chapter V: Applications in drug delivery and targeting

Nanoparticle Tracking Analysis represents a rapid and information-rich multi-parameter nanoparticle characterization technique allowing the user to obtain number frequency particle size distributions of polydisperse nanoparticulate systems. It has resulted in its rapid adoption as an interesting new technique in a wide range of sectors within the pharmaceutical sciences. This Chapter addresses some of the latest work reported in the literature in which NTA has been proposed, used and assessed in the study of nanoparticle-based drug delivery and targeting.

Nanomedicine

It is well established that the use of nanotechnology in medicine and more specifically drug delivery is spreading rapidly. Driven by the diminishing rate of discovery of new biologically active compounds that can be exploited therapeutically to treat disease and with fewer new drugs entering the market every year, interest in the use of nanoparticle's versatile and multifunctional structures for the delivery of existing drugs has grown rapidly. Nanoparticles offer better pharmacokinetic properties, controlled and sustained release, and targeting of specific cells, tissues or organs such (e.g. in new ways in which to cross the blood-brain barrier). All these features can improve the efficacy 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 in a large variety of compositions. Commonly defined nanoparticle vectors include: liposomes, micelles, dendrimers, solid lipid nanoparticles, metallic nanoparticles, semiconductor nanoparticles and polymeric nanoparticles. Therefore, nanoparticles have been extensively employed to deliver drugs, genes, vaccines and diagnostics into specific cells/tissues (Ram *et al.*, 2011).

However, while such nanoparticles are being increasingly used to reduce toxicity and side effects of drugs, it has been recognized that carrier systems themselves may impose risks to the patient. The kind of hazards that are introduced by using nanoparticles for drug delivery are beyond that posed by conventional hazards imposed by chemicals in classical delivery matrices. A multitude of substances are currently under investigation for the preparation of nanoparticles for drug delivery, varying from biological substances like albumin, gelatin and phospholipids for liposomes to substances of a more chemical nature like various polymers and solid metal containing nanoparticles. It has been previously recognized that the potential interaction with tissues and cells, and the potential toxicity, greatly depends on the actual composition of the nanoparticle formulation (De Jong and Borm 2008, Moquin and Winnik 2012).

Nanoparticles in Drug Delivery

Given the above, it is not surprising that the characterization of nanoparticles intended for drug delivery has been the subject of a recent review (McNeil, 2011a) in which the benefits of nanotechnology have been described but with warnings concerning the fact that the physical

nature of the nanoparticles can interfere with conventional and standardized biocompatibility and immunotoxicity testing protocols. In his further comprehensive review of the subject, McNeil (2011b) has also described many assays to determine physical and chemical properties of nanoparticles including batch-mode dynamic light scattering, MALDI-TOF, zeta potential measurement, AFM, TEM and SEM X-Ray microanalysis of nanoparticles present in tissue or cultured cell thin sections. Nanoparticle Tracking Analysis, being a recently developed technique was not considered in this review but is, however, gaining use in the characterization of nanoparticulate suspensions being developed for drug delivery usage, as is described below. An understanding of the dispersion a distribution of nanoparticle sizes prior to their introduction to cellular systems for cytotoxicological testing is crucial and NTA has proved useful in this regard compared to other nanoparticle characterization techniques such as DLS (Kendall *et al.*, 2010).

Following early work using NTA for the study of sodium caproate mediated promotion of oral drug absorption (Maher *et al.*, 2009), more recent work has used NTA to study holonium (Bult *et al.*, 2010)

Moddaresi *et al.* (2010) used NTA to show that semi-solid gel hyaluronic acid matrices used for topical application of drug delivery nanovesicles (tocopheryl acetate (TA) lipid nanoparticles) did, as expected, inhibit their mobility but deliberate manipulation of the particle mobility in the gel by varying the concentration of HA had little effect on TA delivery showing that drug release from the lipid nanoparticles was the rate limiting step in the delivery process and not the nanoparticle–vehicle–skin interaction. Bhuiyan (2010) showed that localized drug release from thermosensitive liposomes could be induced by hypothermia using NTA to characterize his liposome preparations.

More recently, Sunshine *et al.* (2012), in developing safe and effective delivery system based on poly(beta-amino ester)s (PBAEs) which show great potential as gene delivery reagents because they are easily synthesized and transfect a wide variety of cell types with high efficacy *in vitro*, have used NTA to determine particle size just prior to subretinal injection. The successful transfection of the RPE *in vivo* suggested that these nanoparticles could be used to study a number of genetic diseases in the laboratory with the potential to treat debilitating eye diseases.

Shirali *et al.* (2011) used NTA in the development of a poly(lactic-co-glycolic acid) (PLGA) nanoparticle formulation. PLGA nanoparticles are among the most studied polymer nanoformulations for several drugs against different kinds of malignant diseases, thanks to their *in vivo* stability and tumor localization exploiting the well-documented “enhanced permeation and retention” effect. Similarly, in treating the endemic disease Paracoccidioidomycosis, through a new formulation comprising the sustained release of encapsulated itraconazole in nanostructured PLGA, NTA was used to establish an average size of 174nm and which showed that the encapsulated delivery system exhibited improved performance and reduced cytotoxic effects (Cunha-Azevedo, 2011). PLGA nanoparticles loaded with curcumin have been shown to induce G2/M block in breast cancer cells. Using NTA to show full precipitation of the nanoparticle preparation, the PLGA nanoparticles proved to be completely safe, suggesting a potential utilization of this nanocomplex to improve the intrinsically poor bioavailability of curcumin for the treatment of severe malignant breast cancer (Verderio *et al.*, 2013).

Other examples of the importance of sizing and enumerating nanoparticulate drug delivery systems by NTA have been reported (Hsu *et al.* 2010, Park *et al.* 2010, Tagalakakis *et al.* 2010).

The successful transport of molecules across the cell membrane is a key point in biology and medicine. In most cases, molecules alone cannot penetrate the cell membrane, therefore an efficient carrier is needed. Sokolova *et al.* (2012 a and b) have investigated calcium phosphate nanoparticles (diameter: 100–250 nm, depending on the functionalization) as versatile carrier for small and large molecules across cell membranes using a number of techniques including NTA, DLS and EM.

In studying lipid exchange between membranes and the effects of membrane surface charge, composition, and curvature, Zhu *et al.* (2012) employed a quartz crystal microbalance with dissipation monitor method and showed that vesicle adsorption rate, membrane lateral pressure gradient, and lipid lateral diffusion coefficient are critical in deciding the lipid exchange kinetics between membranes and that NTA-determined vesicle size was inversely proportional to membrane contact area which directly affected the intermembrane lipid exchange rate.

The hemocompatibility of poly(beta-caprolactone) lipid-core nanocapsules stabilized with polysorbate 80-lecithin and uncoated or coated with chitosan as drug delivery systems has been studied by Bender *et al.* (2012) and NTA was also used to follow size changes in nanocapsules for intestinal delivery and enhanced oral bioavailability of tacrolimus, a P-gp substrate (Nyska and Benita 2009), as were other several other nanocarriers (Nasser *et al.*, 2009; Debotton *et al.*, 2010,).

Most recently, Abdel-Hafez *et al.* (2013) have utilized statistical designs and mathematical modelling to address questions about the different variables that influence the production of nanoparticles using the ionic gelation method between the biopolymer chitosan and tripolyphosphate ion. Nanoparticles were produced with diameters ranging from 52.21 nm to 400.30 nm, particle polydispersity from 0.06 to 0.40 and suitable morphology and NTA was performed to visualize the prepared particles and to ensure the absence of aggregates.

Using DLS and NTA to confirm formulation unimodal size distribution (with polydispersity value <0.1 from DLS) at the nanoemulsion as well as multi-unit pellet system (MUPS) stage Sangwai *et al.* (2012) reported a nanoemulsified poorly water-soluble anti-obesity drug Orlistat-embedded MUPS with improved dissolution and pancreatic lipase inhibition.

In a study to develop curcumin-loaded lipid-core nanocapsules (C-LNC) in an attempt to improve the antglioma activity of this polyphenol, visualization of the C-LNC was carried out by NTA (Zanotto-Filho *et al.*, 2012), the data obtained suggesting that the nanoencapsulation of curcumin in LNC is an important strategy to improve its pharmacological efficacy in the treatment of gliomas.

Cunha-Azevedo also developed and tested an anti-fungal formulation of PLGA nanoparticles designed to release the active agent itraconazole in which size was an important feature and analyzed by NTA as an average of 174nm (Cunha-Azevedo 2011)

For the development of novel thiolated dendrimers for mucoadhesive drug delivery, Yandrapu *et al.* (2012) used NTA characterized the dendrimer conjugates showing they exhibited sustained release of acyclovir and higher mucoadhesion.

Similarly, Kumru *et al.* (2012) have studied the compatibility, physical stability and characterization of an IgG4 monoclonal antibody after dilution into different intravenous administration bags using a combination of SE-HPLC, NTA, microflow-digital imaging (MFI), and turbidity measurements to follow the formation of soluble aggregates and particulates. He noted, however, that NTA quantification results were interfered with by the presence of polysorbate 20.

In the formulation design and characterization of different nanoparticle drug delivery systems, knowledge of the size, size distribution and number concentration remains central to understanding the behavior of the system. NTA has been shown to be very useful in this regard. Katzer *et al.* (2013) developed a castor oil and mineral oil nanoemulsion as a promising ocular drug delivery system. The formulations were developed by spontaneous emulsification and NTA used to show a mean particle size of 234 nm. Other ocular drug delivery system has been investigated using hyaluronic acid-based nanocomposite hydrogels, HA being a natural component of eye tissue with a significant role in wound healing. The size distribution of liposomes slowly released from the cross linked substrate was determined by NTA helping support the idea that these nanocomposite hydrogels, with controlled degradation properties and sustained release, could serve as potential drug delivery systems for many ocular diseases (Widjaja *et al.*, 2013)

NTA was used to determine the particle size distribution of hollow magnetic $\text{Fe}_3\text{O}_4\text{C}$ nanoparticles as drug carriers with high drug loading capability, pH-control drug release and MRI properties (Cheng *et al.*, 2012) and Morch *et al.* (2013) have developed a novel nanoparticle-microbubble platform in which NTA was the only technique suitable for its characterization.

Nanocapsule size of ~80 to ~100 nm was established by NTA in the work carried out by Piotrowsk *et al.* (2013) on the development of emulsion-core and polyelectrolyte-coated nanocapsules, designed as a water-insoluble neuroprotective drug delivery system. The results showed that nanoencapsulated form of MDL 28170 were biocompatible and protected SH-SY5Y cells against the H_2O_2 (0.5 mM/24 h)-induced damage in 20–40 times lower concentrations than those of the same drug added directly to the culture medium.

The field of nanoscale drug carriers to enhance effective oral drug delivery has recently been reviewed by Reis *et al.* (2013) and Howard and D Peer (2013) concluded that “Nanoparticle Tracking Analysis based on video capture of the trajectory of individual particles provides high-resolution particle size discrimination in polydispersed samples and is gaining popularity in their assessment of different techniques for standardizing nanoparticle-based drug delivery systems, including DLS, SEM and TEM though high-resolution cryo-TEM with environmental-SEM techniques can provide measurements in more natural states”. Another overview of advanced technologies potentially applicable in personalized treatment included discussion of techniques based on the measurement of particle’s Brownian motion (DLS and NTA) and on centrifugal sedimentation (Figueiredo, 2013). The overview was aimed at users who are not very acquainted with particle sizing issues but need to select the most adequate method to characterize their suspensions acknowledging that the techniques are quite different in their measuring principles and that may lead to rather different results, especially if the particles under analysis are far from spherical and exhibit broad size distributions.

As structures capable of crossing biological barriers, such as the membrane linings of various body tissues and the skin, Mbah *et al.* (2013) have discussed the importance of various vesicular carriers, namely liposomes, niosomes, transfersomes and ethosomes in drug delivery with greater emphasis on ethosomes. They concluded that vesicular carriers offer controlled and sustained drug release, improved permeability and protection of the encapsulated bioactives and that ethosomes in particular offer more efficient and enhanced bioavailability better than the older dosage forms owing to the high ethanol content. NTA was cited as a suitable technique for particle size analysis which did not suffer from the intensity weighting exhibited by more traditional methods such as DLS.

Drug release and liposome destruction were determined by photoinduced quenching and NTA respectively in a recent study by Garrier *et al.* (2013) in which they addressed the factors affecting the selectivity of nanoparticle-based photoinduced damage in free and xenografted chorioallantoic membrane models.

Mund (2013), in his study of titania nanoparticles for the intracellular delivery of paclitaxel in breast cancers, showed that NTA results determined more than 50% of particles aggregate in <100 nm dimension. He suggested that a concrete understanding of the particle size and concentration dependent dimension is able to predict drug loading and encapsulation efficiency and hence helpful in determining the effectiveness of drug-carrier conjugate in site dependent action and that therefore, simultaneous characterization through TEM and NTA is significantly valuable in drug delivery application.

Mendes *et al.* (2013) have recently described multicompartimental nanoparticles for co-encapsulation and multimodal drug (paclitaxel and genistein), delivery to tumor cells and neovasculature, in which NTA was used to help demonstrate that entrapment efficiency for both drugs in the nanoparticles was approximately 98%. Average particle diameter was 150 nm with a monomodal distribution. *In vitro* assays showed distinct temporal drug release profiles for each drug and that nanoparticles containing paclitaxel and genistein with a temporal pattern of drug release indicated that the combined effect of cytotoxic and antiangiogenic drugs present in the formulation contributed to the overall enhanced antitumor activity of the nanomedicine.

Finally, NTA has been cited in numerous recent patent applications in the field of nanoscale drug delivery development (Bloembergen *et al.*, 2013; Chen and Walsh, 2013 and Haag *et al.*, 2013).

Nanoparticles in Targeting

The targeting of drug delivery nanoparticles to specific sites frequently uses 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 presenting such biomarkers.

The addition of such capture molecules (frequently antibodies) to the surface of the drug delivery nanoparticle structure can, however, 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 enumerate any aggregates which may form during such modifications.

Accordingly, NTA has been used in a number of such studies including 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 drugs to the bone tumor (Segal *et al.*, 2009).

In the development of novel nanoscale immunization vector modules (Ag, adjuvant, and carrier) which were assembled into units that were optimized for stimulating immune responses to specific pathogens including the Dengue and West Nile (WN) flaviviruses, Demento *et al.* (2010) used NTA to help optimize immune responses in mouse.

Corradetti *et al.* (2012) also 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 Leukaemia 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.

Researchers investigating the dendritic cell maturation and T cell activation through the application of calcium phosphate nanoparticles encapsulating Toll-like receptor ligands and the antigen hemagglutinin used scanning electron microscopy, dynamic light scattering, NTA and ultracentrifugation to analyzing size, surface charge, and morphology of the nanoparticles (Sokolova *et al.*, 2010).

Dimitrova (2011) has recently described NTA in a discussion on the applications of sub-visible particle analysis in the development of protein therapeutics. Kolluru *et al.* (2012) also used NTA to develop the optimum formulation of albumin based theragnostic nanoparticles as a potential delivery system for tumor targeting showing that both NTA and DLS confirmed that the optimized nanoparticle formulation had a particle size of 125nm.

Geng *et al.* (2012) used NTA to establish that the development and characterizations of maleimide-functionalized biopolymer (Mal-PGA-Asp) as an effective targeted drug delivery carrier, synthesized from an amidation reaction between aspartylated PGA (PGA-Asp) and N-(maleimidohexanoyl)-ethylenediamine (NME), led to significantly enhanced cellular uptake of TP13-Mal-PGA-Asp3-Pt in the human hepatoma cell line SMMC-7721 as shown by fluorescence imaging and flow cytometry. NTA was used show the biopolymer had an average size 87 ± 28 nm. Satchi-fainaro *et al.* (2011) have recently patented a novel conjugate of a polymer having a therapeutically active agent and an angiogenesis targeting moiety attached, using NTA data in support of their claim.

Using a range of sophisticated techniques (including matrix assisted laser desorption ionization mass spectrometry (MALDI-TOF), NMR, and high performance liquid chromatography to characterize fullerene derivatives, stabilized Fc ϵ RI-mediated mast cells and peripheral blood

basophils, both DLS and NTA were used to demonstrate sizes of between 1 and 50 nm in aqueous solution (Dellinger *et al.*, 2013).

Rotan *et al.* (2013) used NTA to size the calcium phosphate nanoparticles they were using to transport supramolecular drugs across the cell membrane and Petersen *et al.* (2013) used NTA to size his bioresorbable polymersomes for targeted delivery of cisplatin.

DLS and NTA were used in conjunction to size transferrin(Tf)-containing gold nanoparticles to effect receptor-mediated transcytosis across the blood–brain barrier as a useful way to transport therapeutics into the brain. This transport is aided by tuning the nanoparticle avidity to Tf receptor (TfR), which is correlated with nanoparticle size (45 nm and 80 nm diameter being optimum) and the total amount of Tf decorating the nanoparticle surface (Wiley *et al.*, 2013).

Having previously demonstrated earlier that NTA-analyzed packaging of catalase into a polyion complex micelle ('nanozyme') with a synthetic polyelectrolyte block copolymer protected the enzyme against degradation in macrophages and improved therapeutic outcomes, Klyachko *et al.* (2013) have recently reported the manufacture of nanozymes with superior structure and therapeutic indices and shown that optimized cross-linked nanozyme loaded into macrophages reduced neuroinflammatory responses and increased neuronal survival in mice. Using NTA to measure particle size and concentration Look *et al.* (2013) described the nanomaterial-dependent modulation of dendritic cells and its potential influence on therapeutic immunosuppression in lupus, comparing the internalization of two nanoparticulate platforms: a vesicular "nanogel" platform with a lipid exterior, and the widely-used solid biodegradable poly(lactic-co-glycolic acid) (PLGA) system. Although both types of particles could mitigate the production of inflammatory cytokines and the up-regulation of stimulatory surface markers, nanogels yielded greater reductions. These *in vitro* measurements correlated with *in vivo* efficacy, where immunosuppressive therapy with nanogels extended the survival of lupus-prone NZB/W F1 mice whereas PLGA particles did not.

Gene, RNA and DNA Delivery

There is a requirement to bridge the gap caused by the diminishing rate of discovery of new biologically active compounds that can be exploited therapeutically to treat disease with clinical need. It is increasingly recognized that the use of nanotechnology in medicine, and more specifically drug and gene delivery, is set to spread rapidly. Interest is driven by the knowledge that nanoparticles represent versatile and multifunctional structures for the delivery of drugs allowing better pharmacokinetic properties, controlled and sustained release, and targeting of specific cells, tissues or organs (e.g. in new ways in which to cross the blood-brain barrier) (Malam *et al.*, 2011).

Of specific interest in this area is the use of nanoparticles for transporting and delivering cargoes of genetic material of a wide variety of types. Accordingly, non-viral gene delivery using polymeric nanoparticles has emerged as an attractive approach for gene therapy to treat genetic diseases as well as a technology for regenerative medicine. Unlike viruses, which have significant safety issues, polymeric nanoparticles can be designed to be non-toxic, non-immunogenic, non-mutagenic, easier

to synthesize, chemically versatile, capable of carrying larger nucleic acid cargo and biodegradable and/or environmentally responsive.

The delivery of siRNA to cell systems has been the subject of much recent work as a way to enhance human mesenchymal stem cell differentiation via RNA interference (RNAi) which could provide an effective way of controlling cell fate for tissue engineering, but a safe and effective delivery vehicle must first be developed. Tzeng *et al.* (2012) employed cystamine-terminated poly(beta-amino ester) to this end using NTA to follow size and concentration of different polymer formulations of nanoparticle production. Tzeng and Green (2012) then extended this work to explore subtle changes to the polymer structure and degradation mechanism to such structures for the highly effective short interfering RNA (siRNA) and DNA delivery to human brain cancer.

SiRNA delivery has also been studied through the use of cell-penetrating peptides (CPPs) which are short cationic peptides that have been extensively studied as drug delivery vehicles for proteins, nucleic acids and nanoparticles. They showed a newly developed CPP, PepFect 14 (PF14), forms non-covalent nanocomplexes with siRNA which are able to elicit efficient RNAi response in different cell-lines. NTA was used to demonstrate stability of the nanoparticles on drying and re-suspension (Ezzat *et al.*, 2012).

Similarly, Troiber *et al.* (2012) compared four different particle sizing methods for siRNA polyplex characterization given no standard technique for size measurements is available. Four different analytical methods were evaluated for their suitability to analyze the characteristics of homogeneous and heterogeneous siRNA polyplexes: DLS, AFM, "nanoparticle trafficking analysis" (NTA) and fluorescence correlation spectroscopy (FCS). While the smallest 40nm particles were of too low a refractive index to be tracked by NTA, larger particles of 120nm could be sized by all methods.

More recently, Sunshine *et al.* (2012) in developing safe and effective delivery system based on poly(beta-amino ester)s which show great potential as gene delivery reagents because they are easily synthesized and they transfect a wide variety of cell types with high efficacy *in vitro*, have used NTA to determine particle size just prior to subretinal injection. The successful transfection of the RPE *in vivo* suggested that these nanoparticles could be used to study a number of genetic diseases in the laboratory with the potential to treat debilitating eye diseases.

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 in their work 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, 2007b and 2007c; Ghonaim, 2008; Ghonaim *et al.*, 2009; 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 polymer in cell culture (Bhise *et al.*, 2010). Bhise recently further extended this work to develop an assay for quantifying the number of plasmids encapsulated by polymer nanoparticles using NTA to determine the number density of plasmids per 100nm nanoparticle (Bhise *et al.*, 2010).

Recent work has employed NTA to show that dendrimer structures being used as vehicles for siRNA delivery underwent changes in size and polydispersity at higher dendrimer concentrations which indicated that electrostatic complexation results in an equilibrium between differently sized complex aggregates (Jensen, 2011). This work allowed the optimum dendrimer structure to be identified for subsequent nucleic acid delivery. In another example, the self-aggregation of polyamidoamine dendrimers with hydroxyl surface groups was detected by NTA in Ciolkowski's study of the influence of PAMAM-OH dendrimers on the activity of human erythrocytes (Ciolkowski *et al.*, 2011).

Sander *et al.* (2013) and Stremersch (2013) have both reported on the encapsulation of siRNA into extracellular vesicles by electroporation. Sander suggested electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles while Stremersch found this phenomenon is biased by siRNA precipitation.

NTA was used to by Shimizu *et al.* (2013) for the determination of size and number of vesicles used in a study of the expression of miR-145 in Children with Kawasaki Disease. Such microRNAs are small non-coding RNAs that modulate gene expression at the post-transcriptional level and can be transported between cells in extracellular vesicles.

The synthesis and characterization of a novel, bioreducible linear poly(β -amino ester) designed to condense siRNA into nanoparticles and efficiently release it upon entering the cytoplasm was reported by Kozielski *et al.* (2013) and in which NTA was used alongside a ζ -potential-DLS method to characterize the nanoparticles. Delivery of siRNA using this polymer achieved near-complete knockdown of a fluorescent marker gene in primary human glioblastoma cells with no cytotoxicity.

Ballarín-González *et al.* (2013) used NTA to determine the hydrodynamic size of chitosan/siRNA complexes as used in their study by direct Northern and quantitative PCR (qPCR) detection, stability, gastrointestinal deposition, and translocation into peripheral tissue of nonmodified siRNA after oral lavage of chitosan/siRNA nanoparticles in mice. In contrast to naked siRNA, retained structural integrity and deposition in the stomach, proximal and distal small intestine and colon was observed at 1 and 5 hours for siRNA within nanoparticles, indicating an oral delivery platform that could have the potential to treat local and systemic disorders by siRNA.

Gene therapy utilizing lentiviral vectors constitutes a real therapeutic alternative for many inherited monogenic diseases. Therefore, the generation of functional vectors using fast, non-laborious and cost-effective strategies is imperative. Among the available concentration methods for VSV-G pseudotyped lentiviruses to achieve high therapeutic titres, ultracentrifugation represents the most common approach. However, the procedure requires special handling and access to special instrumentation, it is time-consuming, and most importantly, it is cost-ineffective due to the high maintenance expenses and consumables of the ultracentrifuge apparatus. Papanikolaou *et al.* (2013) have recently described an improved protocol in which vector stocks are prepared by transient transfection using standard cell culture media and are then concentrated by ultrafiltration, resulting in functional vector titres of up to 6×10^9 transducing units per milliliter (TU/mL) without the involvement of any purification step. They determined the viral functional titre by employing flow cytometry and evaluated the actual viral particle size and concentration in real time using NTA.

Similarly, adenoviral vectors hold immense potential for a wide variety of gene therapy based applications; however, their efficacy and toxicity is dictated by “off target” interactions that preclude cell-specific targeting to sites of disease. In order to overcome these limitations, Parker *et al.* (2013) have developed capsid modification strategies for detargeting Adenoviral vectors, using NTA to determine viral titre and stability.

NTA has also been used in the development of non-viral gene delivery systems based on a lipophilic plasmid DNA condensate (Do *et al.*, 2011), poly(β -amino ester)s (PBAEs) (Tzeng *et al.*, 2011) and in the screening of such structures *in vitro* (van Gaele *et al.*, 2011). More recently, in their evaluation of polymeric gene delivery nanoparticles by NTA and high-throughput flow cytometry, Shmueli *et al.* (2013) described a new protocol to characterize PBEA nanoparticles utilizing NTA. Such PBAEs are hydrolytically degradable and have been shown to be effective at gene delivery to hard-to-transfect cell types such as human retinal endothelial cells, mouse mammary epithelial cells, human brain cancer cells and macrovascular (human umbilical vein) endothelial cells. This NTA-based protocol was considered to be easily adapted to evaluate any polymeric nanoparticle and any cell type of interest in a 96-well or multi-well plate format for transfection assay for rapid screening of the transfection efficacy. Bhise *et al.* (2013) also evaluated, using DLS and NTA, the potential of poly(β -amino ester) nanoparticles for reprogramming human fibroblasts to become induced pluripotent stem cells. Evaluating the use of a biodegradable PBAE nanoparticle-based nonviral protocol and comparing it with an electroporation-based approach to deliver episomal plasmids encoding reprogramming factors for generation of human induced pluripotent stem cells from human fibroblasts, they screened a polymer library to identify the polymers most promising for gene delivery to human fibroblasts. They concluded, however, that certain nonviral reprogramming methods may not necessarily be safer than viral approaches and that maximizing exogenous gene expression of reprogramming factors is not sufficient to ensure successful reprogramming. The same group also evaluated the uptake mechanism of PBAE polyplexes and the dependence of cellular uptake on the end group and molecular weight of the polymer by synthesizing three different analogues of PBAEs with the same base polymer poly(1,4-butanediol diacrylate-co-4-amino-1-butanol) but with small changes in the end group or molecular weight. They showed that differential polymer structure tunes the mechanism of cellular uptake and transfection routes of poly(β -amino ester) polyplexes in human breast cancer cells (Kim *et al.*, 2013).

Troiber (2013) has described sequence-defined polycationic oligomers for nucleic acid delivery, in which NTA was considered ideal for the measurement of medium sized particles such as 332 polyplexes which displayed a mean diameter of 139 ± 47 nm and in which the resolution of NTA was therefore higher than for DLS.

Another method of separating nucleic acid polymer conjugates has recently been patented and in which NTA data was reported (Case, 2013).

Witwer *et al.* (2013) have suggested that real-time quantitative PCR (RT-qPCR) and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs in contrast to previous evidence that exogenous dietary miRNAs enter the bloodstream and tissues of ingesting animals and which has been accompanied by an indication that at least one plant miRNA, miR168, participates in “cross-kingdom” regulation of a mammalian transcript.

Employing RT-qPCR to measure plant and endogenous miRNAs from pigtailed macaques that received a miRNA-rich plant based substance, their results did not support general and consistent uptake of dietary plant miRNAs. NTA was used to show a shift in particle size and population following food intake.

In a sophisticated study using an X-ray free-electron laser, Demirci *et al.* (2013) carried out femtosecond X-ray diffraction of 30S ribosomal subunit microcrystals in liquid suspension at ambient temperatures, confirming crystal concentration was approximated as 10^{10} - 10^{11} /mL by NTA. Such high-resolution ribosome structures determined by X-ray crystallography have provided important insights into the mechanism of translation.

Mostaghaci *et al.* (2013) have developed a one-step synthesis of nano-sized and stable amino-functionalized calcium phosphate (low toxicity with simple and low cost synthesis) particles for DNA transfection, having used NTA to confirm that their refined wet-precipitation method yielded NPs with a narrow size distribution (~140 nm), a significant improvement on the previous results in which the transfection results varied because the precipitation lacked reproducibility and resulted in poly-dispersed, agglomerated particles.

Chitosan-based nanoparticles were also studied using NTA for gene-and siRNA-delivery (Malmo, 2012) and as permeating vectors for the blood–brain barrier when functionalized with alkylglyceryl (Lien *et al.*, 2012).

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Chapter VI: Drug nanocarrier design and Drug formulation

NTA in Nanomedicine

The role that nanoparticles play in the rapidly developing field of 'NanoMedicine' has been discussed previously in Chapter V. In this Chapter, we review the specific mechanisms by which such nanoparticles are designed and formulated and in which NTA has had a significant part to play.

Thus, Villaverde (2011) reviewed the emergence of nanoparticles in translational science and medicine and Banerjee *et al.* (2010) had focused on the role and biomedical applications that magnetic nanoparticles play in that nanomedicine. Similarly, the impact and biomedical applications of a different specific subset of nanoparticles, nanostructured carbiobeads, has also been reviewed (Stanishevsky *et al.*, 2011).

Wei *et al.* (2012), in exploring the challenges and opportunities in the advancement of nanomedicines, identified numerous needs including robust and general methods for the accurate characterization of nanoparticle size, shape, and composition as well as particle engineering for maintaining low levels of nonspecific cytotoxicity and sufficient stability during storage. They compared NTA and DLS when carrying out size analysis of nanocarriers composed of (a) trimethyl chitosan, (b) 50:50 poly(lactic-co-glycolic acid) (PLGA), and (c) commercial liposomes, showing that DLS rarely reported accurate data except in the more monodisperse sample types.

Bai *et al.* (2012) have recently presented evidence that homogeneous submicron particles can influence the growth rate of larger particles upon long-term storage in a temperature-dependent manner with implications for product stability and during which Interferon-beta-1a was thermally stressed at 50°C for 6h and characterized using NTA, microflow digital imaging (MFI), and circular dichroism (CD) spectroscopy.

Following an early assessment of NTA as an emergent technique (Filipe *et al.*, 2010), the role, impact and characterization of NTA has been more recently examined. Cho *et al.* (2013) discussed state-of-the-art challenges and emerging technologies associated with nanoparticle characterization given the importance they offer as promising tools to enhance target-specific drug delivery and diagnosis. The article provided a critical review of *in vitro* and *in vivo* techniques currently used for evaluation of nanoparticles and introduced emerging techniques and models that may be used complementarily and of which NTA was one. In an earlier review of the subject Poletto had discussed the used of polymeric nanocapsules in the development of nanocosmetics and nanomedicines (Poletto *et al.*, 2011).

Herring *et al.* (2013), focused on the role of cellular exocytic vesiculation in health, disease, and transfusion medicine as applied to the field of veterinary science assessing the advantages and disadvantages of various assays for the detection of microparticles.

Liposomes, microvesicles and micelles.

Following early work using NTA for the characterization of casein micelles (Thu *et al.*, 2007; Thu *et al.*, 2008) and in the study dispersion of poly(3,4-ethylenedioxythiophene) in organic liquids (Kim *et al.*, 2008), Sorrell and Lyon (2008) studied the deformation controlled assembly of binary microgel thin films.

Despite the frequently low light scattering properties of micellar systems and difficulty in their detection on an individual basis, NTA has been successfully used to characterize such structures (Vakurov *et al.*, 2009) and particularly in the development of drug delivery micellar formulations for controlled release of covalently entrapped doxorubicin (Talelli *et al.*, 2010) and the encapsulation of mithramycin (Capretto *et al.*, 2010; Capretto *et al.*, 2011). This latter study demonstrated that microfluidics was a powerful technology for microfluidic nanoprecipitation-based production of drug loaded polymeric micelles as compared to batch systems since it enabled better control, reproducibility, and homogeneity of the size characteristics of the produced micelles.

In attempting to improve the homogeneity of nano-sized lipid vesicles as drug delivery vehicles made by a constant pressure-controlled extrusion apparatus Morton *et al.* (2012) used NTA, DLS and EM to characterize their product's monodispersity as did Bhuiyan (2010) in his study of the application of hyperthermia for localized drug release from thermosensitive liposomes.

Wrenn *et al.* (2012) has used NTA to determine the number of liposomes and their diameter, under the application of ultrasound, in an initial attempt to distinguish mechanisms and quantify the relative contributions of liposome destruction versus diffusion through the bilayer. The overall number of liposomes decreased with ultrasound exposure time, with the most pronounced decrease (nearly 50%) occurring in the first four minutes of ultrasound exposure. This result strongly suggested that at least some vesicle destruction is occurring which was consistent with their prior studies.

Brinkhuis *et al.* (2012) used NTA to measure the zeta potential of polymersomes, self-assembled from the block copolymer polybutadiene-block-poly(ethylene glycol) in his investigation of the size dependent biodistribution and SPECT imaging of ¹¹¹In-labeled polymersomes to show that size, much more than for liposomes, will influence the pharmacokinetics, and therefore, long circulating preparations should be well below 100nm.

Ohlsson *et al.* (2012) reported on solute transport on the sub-100ms scale across the lipid bilayer membrane of individual proteoliposomes using NTA to check liposome stability and integrity.

Photoactive drug carriers were studied by Reshetov (2012), in which he used NTA to demonstrate that the inclusion of mTHPC into liposomes increases the structural stability of the carriers in serum compared to un-PEGylated liposomes which showed faster kinetics of degradation.

Knowing that membrane curvature and lipid composition regulates important biological processes within a cell, several proteins have been reported to sense and/or induce membrane curvatures, e.g. synaptotagmin-1 and amphiphysin and Morton *et al.* (2012) have identified a 25-mer peptide, MARCKS-ED, based on the effector domain sequence of the intracellular membrane protein myristoylated alanine-rich C-kinase substrate that can recognize PS with preferences for highly

curved vesicles in a sequence specific manner. These studies further contribute to the understanding of how proteins and peptides sense membrane curvature, as well as providing potential probes for membrane shape and lipid composition, NTA being used to monitor vesicle size. Zhu *et al.* (2012) investigated lipid exchange between membranes and the effects of membrane surface charge, composition, and curvature. Jing *et al.* (2013) have also studied phase transition-controlled flip-flop in asymmetric lipid membranes by taking advantage of distinct phase transitions in lipid membrane coatings where lipids exchange (flip-flop) between leaflets. The liposomes used in this study were characterized by NTA

Recently, Vader *et al.* (2013) showed that Taxol®-induced phosphatidylserine exposure and microvesicle formation in red blood cells is mediated by its vehicle Cremophor® EL.

Encapsulation

In a study to develop curcumin-loaded lipid-core nanocapsules (C-LNC) in an attempt to improve the antglioma activity of this polyphenol, visualization of the C-LNC was carried out by NTA (Zanotto-Filho *et al.*, 2012), the data obtained suggesting that the nanoencapsulation of curcumin in LNC is an important strategy to improve its pharmacological efficacy in the treatment of gliomas. More recently, Salehi *et al.* (2012) have also studied curcumin loaded NIPAAm/VP/PEG-A nanoparticles and studied their physicochemical and chemopreventive properties.

Following early studies in the formation of cholesteric and nematic emulsions (Tixier *et al.* 2006) NTA was used to follow size changes in nanocapsules for intestinal delivery and enhanced oral bioavailability of tacrolimus, a P-gp substrate (Nyska and Benita 2009) as were other several other nanocarriers (Nasser *et al.*, 2009; Debotton *et al.*, 2010; Sundar *et al.*, 2010; Smith *et al.*, 2010)

In a case study employing BaTiO₃, Pazik *et al.* (2011) explored the surface functionalization of the metal oxide nanoparticles with biologically active molecules containing phosphonate moieties. Using a battery of techniques including scanning electron microscopy/energy dispersive spectroscopy, pH-metric titration, NMR and IR spectroscopy, DLA, ζ potential, thermogravimetric analysis, radiometric measurements and NTA, they showed that the application of amino phosphonic acids as surface ligands provided nanoparticles with considerable solution stability in an aqueous medium at neutral pH and especially in the presence of electrolytes thus opening the broad prospect of applications for such nanoparticle dispersions in the domains of nano-optics and nanomagnetism.

Neville *et al.* (2010) described the fabrication and characterization of biosilicate nanoparticles formed by mimicking the peptides using polyethyleneimine and described, for the first time using TEM and NTA, the characterization of nanoparticles made with tetramethyl orthosilicate to entrap enzymes. This worked was explained further in a recent report on the fabrication and characterization of bioactive thiol-silicate nanoparticles (Neville and Millner, 2011). Zu *et al.* (2012) have also reported the preparation of ultrafine polyethylene-silica composite particles with a core-shell structure using scanning electronic microscope observation and nanoparticle tracking analysis to determine particle sphericity and a mean size of 160nm respectively.

Researchers investigating the dendritic cell maturation and T cell activation through the application of calcium phosphate nanoparticles encapsulating Toll-like receptor ligands and the antigen hemagglutinin used SEM, DLS, NTA and ultracentrifugation in analyzing size, surface charge, and morphology of the nanoparticles (Sokolova *et al.*, 2010). Similarly, recent developments of a nanoparticulate formulation of retinoic acid that suppresses Th17 cells and up-regulates regulatory T cells employed NTA to measure particle size (Capurso *et al.* 2010) and the stability of nanometer-sized prodrug (nanoprodugs) production by a spontaneous emulsification mechanism was confirmed by NTA to be constant at 120-140nm in diameter (Lee *et al.*, 2011).

Bhise *et al.* (2011) have described a novel assay for quantifying the number of plasmids encapsulated by polymer nanoparticles and Capretto *et al.* (2012) proposed mithramycin encapsulated in polymeric micelles by microfluidic technology as novel therapeutic protocol for beta-thalassemia.

Geng *et al.* (2012) used NTA to establish that the development and characterizations of maleimide-functionalized biopolymer (Mal-PGA-Asp) as an effective targeted drug delivery carrier synthesized from an amidation reaction between aspartylated PGA (PGA-Asp) and N-(maleimidohexanoyl)-ethylenediamine (NME) led to significantly enhanced cellular uptake of TP13-Mal-PGA-Asp3-Pt in the human hepatoma cell line SMMC-7721 as shown by fluorescence imaging and flow cytometry. NTA was used show the biopolymer had an average size 87 ± 28 nm.

Kolluru *et al.* (2012) also used NTA to develop the optimum formulation of albumin based theranostic nanoparticles as a potential delivery system for tumor targeting showing that both NTA and DLS confirmed that the optimized nanoparticle formulation had a particle size of 125nm.

The surface coatings of proteins on superparamagnetic iron oxide nanoparticles (SPIONs) that form immediately on contact with a biological milieu were assessed using a variety of techniques, including NTA, following stabilization of the SPION with citric acid, poly(acrylic acid), or double layer oleic acid (Jedlovsky-Hajdú *et al.*, 2012). SPION have also been exploited by Paquet *et al.* (2011) in their showing that particle architecture generating a synergistic enhancement of the t_2 relaxation in their study of clusters of superparamagnetic iron oxide nanoparticles encapsulated in a hydrogel.

In designing drug delivery vehicles capable of buccal delivery Mazzarino and her co-workers developed a chitosan-coated nanoparticles loaded with curcumin for mucoadhesive applications by the nanoprecipitation method using different molar masses and concentrations of chitosan and concentrations of triblock surfactant poloxamer (PEO-PPO-PEO) in order to optimize the preparation conditions. DLS studies at different scattering angles and concentrations had shown that the nanoparticles are monodisperse (polydispersity indices were lower than 0.3). The nanoparticle systems were also examined with NTA, and the results were in good agreement with those obtained by DLS. Colloidal systems showed mean drug content about 460 µg/mL and encapsulation efficiency higher than 99%. When coated with chitosan, these nanoparticles show a great ability to interact with mucin indicating also their suitability for mucoadhesive applications (Mazzarino *et al.*, 2012).

Clementi *et al.* (2011) determined the hydrodynamic diameter (at 200nm) and size distribution of PTX-PEG-ALN and of PTX-PEG conjugates by NTA in their work using dendritic poly(ethylene glycol) bearing paclitaxel and alendronate for targeting bone neoplasms.

Chitosan is a natural biodegradable cationic polymer with remarkable potency as a vehicle for drug or vaccine delivery. Zubareva *et al.* (2013) sought to produce stable nanosized range "Chi-gels" (nanogels, NGs) with different charge and to study the driving forces of complex formation between Chi NGs and proteins or peptides and showed that NGs preferentially formed complexes with oppositely charged molecules, especially peptides, as was demonstrated by gel-electrophoresis, confocal microscopy and HPLC. Complex formation was accompanied by a change in zeta-potential and decrease in size as measured by NTA.

Delivery and controlled release

The design and manufacture of micro- and nano-particles capable of releasing, or being triggered to release, a drug cargo in a specific location and at a specific time is one of the biggest opportunities and challenges in nanomedicine. Given knowledge of the size, size distribution profile and number concentration is central to the development and production of such systems, NTA has proved increasingly useful in furnishing this information at all stages through the manufacturing process. Thus, core particle size, the efficiency of addition of functionalized coatings (e.g. antibodies for targeting) and the behavior of such complex, multifunctional structures in biological environments has been the subject of intense study. Here, some examples of such use of NTA are given.

Following earlier work pointing to the potential of NTA in the investigation of complex, multifunctional nanoparticles (Lynch, 2007; Nyska and Benita, 2009), subsequent studies address a wide range of particle types and applications. Pagba and Lane (2010) reported the direct detection of aptamer-thrombin binding via surface-enhanced Raman spectroscopy (SERS) while Ciolkowski *et al.* (2011) discussed the influence of PAMAM-OH dendrimers on the activity of human erythrocytes ATPases.

The functionalization of nanoparticles was addressed by Park *et al.* (2011) in their work on the enhancement of surface ligand display on PLGA nanoparticles with amphiphilic ligand conjugates and Kusters *et al.* (2011) published their work on the functional immobilization of biological membranes in hydrogels. In all these cases, NTA was used to follow particle size during the development stages. Satchi-Fainaro *et al.* (2011) patented the use of a conjugate of a polymer, an anti-angiogenesis agent and a targeting moiety in the treatment of bone related angiogenesis conditions. ,

Simonsson *et al.* (2012) presented an amperometric study of content release from individual vesicles in an artificial secretory cell designed with the minimal components required to carry out exocytosis using NTA to measure catechol-filled LUVs at an average diameter of ~200nm. In fact, using NTA they observed that catechol filled vesicles are larger (mean diameter \approx 200nm) than vesicles typically obtained from extrusion through a 100nm pore sized polycarbonate filter. Hickerson *et al.* (2012) determined that siRNA-Invivofectamine 2.0 complexes were 100nm by NTA

in his intravital fluorescence imaging of small interfering RNA-mediated gene repression in a dual reporter melanoma xenograft model. Yandrapu *et al.* (2012) reported, as an acyclovir model formulation, the development and optimization of thiolated dendrimer as a viable mucoadhesive excipient for the controlled drug delivery while Jensen *et al.* (2011) elucidated the molecular mechanism of PAMAM-siRNA dendriplex self-assembly in terms of the effect of dendrimer charge density.

Narasimhan *et al.* (2012) have highlighted an industry perspective of the challenges and technical solutions associated with high-dose monoclonal antibodies via the subcutaneous route.

More recently, increasingly sophisticated structures and applications have been developed and NTA has been used to attempting to determine their structure and optimize the method of production. Chang *et al.* (2013) reported an aggregation-induced photodynamic therapy enhancement based on linear and nonlinear excited FRET of fluorescent organic nanoparticles, explaining that a binary molecule can self-assemble to form fluorescent organic nanoparticles because of the aggregation-induced emission enhancement property and which subsequently presents an efficient fluorescence resonance energy transfer to generate singlet oxygen under linear and nonlinear light sources. Nanoparticle sizes and sizing partition curves were directly measured using NTA.

Using confocal microscopy to identify the localization of carboxyfluorescein-labeled amylin in RIN-5F cells, Pillay *et al.* (2013) have developed a direct fluorescent-based technique for cellular localization of amylin. The size of the aggregates that formed on the cell membrane (size range of 130–800nm) were evaluated using NTA supporting previous findings that amylin was observed to interact with and remain associated to the cell membrane.

Targeted theranostics (combined therapeutic and diagnostic agents) are being developed by inducing clustered nanoconfinement of superparamagnetic iron oxide in biodegradable nanoparticles to enhance transverse relaxivity (Ragheb *et al.*, 2013). Poly(lactide-co-glycolide) nanoparticles were engineered to confine superparamagnetic iron oxide contrast for magnetic resonance imaging while enabling controlled drug delivery and targeting to specific cells. The work showed that clustering of superparamagnetic iron oxide in poly(lactide-co-glycolide), as measured by NTA, did not affect the controlled release of encapsulated drugs such as methotrexate or clodronate and their subsequent pharmacological activity, highlighting the full theranostic capability of the system

In her recent review of currently available drug carriers for oral delivery of peptides and proteins (discussing accomplishments and future perspectives) Reis *et al.* (2013) pointed out that while effective formulation for peptide and protein delivery through the oral route has always been the critical effort with the advent of biotechnology, stability, enzymatic degradation and ineffective absorption are common difficulties found for conventional dosage forms emphasizing the need for new drug-delivery approaches to circumvent these limitations and enhance effective oral drug delivery.

Design and Formulation

Using DLS and NTA to confirm formulation unimodal size distribution (with polydispersity value <0.1 from DLS) at the nanoemulsion as well as multi-unit pellet system (MUPS) stage, Sangwai *et al.* (2012) reported a nanoemulsified poorly water-soluble anti-obesity drug Orlistat-embedded MUPS with improved dissolution and pancreatic lipase inhibition. Inclusion of affinity tags greatly facilitated process development for protein antigens, primarily for their recovery from complex mixtures and although generally viewed as supportive of product development, affinity tags may have unintended consequences on protein solubility, susceptibility to aggregation, and immunogenicity.

Khan *et al.* (2011) employed NTA to establish particle sizes and, importantly, concentrations showing the influence of His-affinity tags on protein expression levels, solubility, secondary structure, thermal denaturation, aggregation and the impact on humoral and cellular immune responses in mice, the results of which suggested that the usefulness of protein tags may be outweighed by their potential impact on structure and function, stressing the need for caution in their use.

Recently, Heljo *et al.* (2012) explored the stability of rituximab in freeze-dried formulations containing trehalose or melibiose under different relative humidity atmospheres, using NTA to determine the diameter of the nanoparticles was between 50 and 1000nm. Kasper (2013) has recently comprehensively reviewed multiple aspects of the lyophilization of nucleic acid nanoparticles, including formulation development, stabilization mechanisms, and process monitoring emphasizing the importance of the freezing step. A brief overview on the basic concepts of pDNA and siRNA delivery in gene therapy was given and the need for lyophilized long-term stable formulations was accentuated.

Using NTA to confirm particle diameter, Chernousova *et al.* (2013) developed a novel genetically active nano-calcium phosphate paste for bone substitution. She showed that especially cationic nanoparticles showed a high transfection efficiency together with a low cytotoxicity. The nanoparticles could be either used in dispersion or added to a calcium phosphate paste for injection into bone defects. Rodrigues *et al.* (2012) had earlier synthesized and characterized nanocrystalline hydroxyapatite gel and studied its application as scaffold aggregation. Similarly, Stevens *et al.* (2012) had used NTA in their study of nanosponge formation from organocatalytically synthesized poly(carbonate) copolymers.

Do *et al.* (2011) undertook the characterization of a lipophilic plasmid DNA condensate formed with a cationic peptide fatty acid conjugate with NTA. Clementi *et al.* (2011) determined the hydrodynamic diameter (200nm) and size distribution of PTX-PEG-ALN and of PTX-PEG conjugates by nanoparticle tracking analysis technology in their work using dendritic poly(ethylene glycol) bearing paclitaxel and alendronate for targeting bone neoplasms.

Thermosensitive hydrogels were the subject of another NTA assisted study by de Graaf *et al.* (2012) in which they developed a micelle-shedding thermosensitive hydrogel based on poly(N-isopropylacrylamide)-poly(ethylene glycol)-poly(N-isopropylacrylamide) (pNIPAm-PEG-pNIPAm) as sustained release formulation for the delivery of the cytostatic agent paclitaxel (PTX). They showed

that, at the highest dose, PTX completely inhibited tumor growth for at least 3 weeks with a single hydrogel injection. This promising concept may find application as a depot formulation for sustained, metronomic dosing of chemotherapeutics.

Mun *et al.* (2013) have addressed the question of nanoparticle diffusion within non-Newtonian biological and synthetic fluids which though considered essential in designing novel formulations (e.g., nanomedicines for drug delivery, shampoos, lotions, coatings, paints, etc.) is presently poorly defined. Using NTA to visualize nanoparticle diffusion in various media, they reported the diffusion of thiolated and PEGylated silica nanoparticles, characterized by small-angle neutron scattering, in solutions of various water-soluble polymers such as poly(acrylic acid) (PAA), poly(N-vinylpyrrolidone) (PVP), poly(ethylene oxide) (PEO), and hydroxyethylcellulose (HEC) were probed using NTA. The water-soluble polymers retarded the diffusion of thiolated particles in the order PEO > PVP > PAA > HEC whereas for PEGylated silica particles retardation followed the order PAA > PVP = HEC > PEO. They concluded that in the absence of specific interactions with the medium, PEGylated nanoparticles exhibit enhanced mobility compared to their thiolated counterparts despite some increase in their dimensions.

In the development of new imaging agents in cancer therapy, plasmonic gold nanostars which exhibited tuneable plasmons in the near infrared tissue optic window generated intense two-photon photoluminescence capable of *in vitro* cell labelling and *in vivo* particle tracking, Yuan recently used the multiparameter analysis capability of a nanoparticle tracking analysis instrument fitted with a zeta potential module to determine the particles' hydrodynamic radius, ζ -potential and concentration. (Yuan *et al.* 2012) while Wand and Vo-Dinh (2011) had earlier studied plasmonic coupling interference nanoprobe for nucleic acid detection.

Using NTA to physically characterize their samples, Sunshine *et al.* (2012) showed that uptake and transfection with polymeric nanoparticles was dependent on polymer end-group structure, but largely independent of nanoparticle physical and chemical properties, while van Galen *et al.* (2012) demonstrated that the interaction of GPR-1 with lipid bilayers is regulated by alternative homodimerization. Liling (2008) had earlier employed NTA in his investigations of bio-responsive peptide-inorganic nanomaterials. Troiber *et al.* (2012) assessed NTA among three other sizing techniques in their comparison of four different particle sizing methods for siRNA polyplex characterization.

Jouffray (2012) described the use of an innovative cross-linked silicone coating in prefilled syringe technology to improve compatibility with biologics given that silicone oil is commonly used as a lubricant coating in prefilled syringes (PFS) and is becoming one of the most highly discussed topics in the PFS market, particularly for developers of highly sensitive biotech drugs. NTA was shown to reveal the presence and formation of sub-visible particles and he showed that the new oil formulation significantly reduced aggregation while retaining lubrication performance using NTA to show the reduction in numbers of 200-1000nm particles between the novel silicone formulation and baked silicone and conventionally lubricated syringes.

Banerjee *et al.* 2010 have studied magnetic nanoparticles for radio ablation and magnetic resonance contrast agent development while Smith *et al.* (2012) have used NTA to show that the

change in flux was not a result of a change in size due to aggregation of the haemoglobin at the different pHs tested when confirming that alginate hydrogel has a negative impact on *in vitro* collagen 1 deposition by fibroblasts.

Finally, Zhuang *et al.* (2013) have recently reviewed multi-stimuli responsive macromolecules and their assemblies using NTA to characterize micelles under both light scatter and fluorescent modes to explain the origin of employed mechanisms of stimuli responsiveness which may serve as a guideline to inspire future design of multi-stimuli responsive materials.

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Chapter VII: Protein Aggregation

The NTA as a rapid and information-rich multi-parameter nanoparticle characterization technique which allows the user to obtain number frequency particle size distributions of polydisperse nanoparticulate systems has resulted in its rapid adoption as an interesting new technique in a wide range of sectors within the pharmaceutical sciences. This Chapter addresses some of the latest work in the literature in which NTA has been proposed, used and assessed in the study of protein aggregation and in the characterization of virus preparations and viral vaccine products.

Sub-micron particles in proteinaceous products

The subject of therapeutic protein aggregates 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 analytical centrifugation and dynamic light scattering (Arakawa *et al.*, 2007a) and FFF (Arakawa *et al.*, 2007b) in aggregation analysis while Krishnamurthy discussed emerging technologies for analysis of protein 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 now described as injectable solutions or injectables) are proscribed by national legislation (e.g. as laid out by US (USP), European (EP), and Japanese (JP) Pharmacopoeia standards). While the limits were based on the original counting efficiencies of available technology (e.g. USP22 test <788> as 10,000 counts per container at 10 μm and 1,000 counts per container at 25 μm) the recent revision of USP 23 <788> re-defines these limits as 6,000 counts per container at 10 μm and 600 counts per container at 25 μm (United States Pharmacopoeia, 2011).

The importance of detection and enumeration of sub-visible particles (down to 100nm in diameter) in therapeutic protein products has recently been the subject of much debate. Carpenter has suggested that the lack of understanding and the clinical significance of overlooking such particles may compromise product quality. He concluded that subvisible 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 particle concentration measuring instruments for particles as small as 0.1 μm and develop new instruments as needed (Carpenter *et al.* 2009). He more recently highlighted the potential inaccurate quantitation 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).

During a recent workshop on protein aggregation and immunogenicity, Barnard and Carpenter (2012) reviewed analytical methods for detecting aggregates showing that NTA optimally covered a range of significant interest in this area. Carpenter's group have most recently shown that recombinant murine growth hormone particles are more immunogenic with intravenous than subcutaneous administration, using NTA to measure, in their determination of the immune response in mice to injections of formulations of recombinant murine growth hormone (rmGH), the added controlled levels of protein particles (in addition to soluble, monomeric rmGH, the samples prepared contained either nanoparticles of rmGH or both nano- and microparticles of rmGH). No dependence of the immune response on particle size and distribution was observed but the immune response measured after the second injection was most pronounced when i.v. administration was used (Christie *et al.*, 2014).

However, in forwarding an industry perspective on the subject, Singh has reiterated that the link between aggregation and clinical immunogenicity has not been unequivocally established; and emphasized that such particles are present in marketed products which remain safe and efficacious despite the lack of monitoring. He concluded that while measurement of subvisible particulates in the <10 µm size range has value as an aid in product development and characterization, limitations in measurement technologies, variability from container/closure, concentration, viscosity, history, and inherent batch heterogeneity, make these measurements unsuitable as specification for release and stability or for comparability at the present time. (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 enumeration remains hampered by lack of instrumentation of adequate sensitivity. Zöls *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. Similarly, Fuh (2011) has reported on the challenges faced by industry in developing analytical tools for protein stability and ligand interactions, measurement of protein aggregates as small as 30nm and reducing production costs in which the need to eliminate protein aggregates early during bioprocessing was emphasized.

Hamrang *et al.* (2013) have highlighted a current need for evolution of analytical methodologies used in profiling biopharmaceutical aggregation and suggested some of the techniques discussed require validation and application to biopharmaceuticals. While the development of such technologies has enabled high-throughput assessment of compounds, the implementation of recombinant DNA technology and large-scale manufacture of monoclonal antibodies and which have resulted in the biopharmaceutical stronghold in the therapeutic market, aggregate prediction and profiling still remains a challenge in the formulation of biopharmaceuticals due to artefacts associated with each analytical method.

Similarly, Rad-Malekshahi *et al.* (2013) and Wiggnerhorn (2013) have also reviewed both the need and requirements of techniques capable of characterizing sub-micron particles in pharmaceutical products.

Finally, Zolls (2013) has comprehensively reviewed the subject and has identified and evaluated critical factors for protein particle analysis and applied this knowledge for the development of novel standardized protein-like particles, illustrating that it is crucial to not only comprehensively understand the techniques' principle and limitations, but to also evaluate data from different techniques carefully in order to draw reliable conclusions

NTA as a monitor of sub-micron particulates in pharmaceutical products.

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.

Thus Englesman, in his review of strategies for the assessment of protein aggregates in pharmaceutical products, concluded that NTA, as a single particle detection and characterization technique, was very useful for polydisperse samples though, compared to other techniques, it had a low sample throughput and, as an emerging technique, required trained operators (Engelsman, 2010). Similarly, Mire-Sluis *et al.* (2011) concluded that NTA is a useful method for the analysis of sub-micron aggregates though could be confounded by high concentration or opalescent background solutions and that while the technique could be considered promising had yet (at the time of writing) to be widely used in pharmaceutical applications while in a more 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 quantitation and characterization of aggregates of therapeutic proteins (Carpenter *et al.*, 2012; Zhao *et al.*, 2012; Printz and Friess, 2012). Singh and Toler (2012) have compared a wide range of techniques, including NTA, for the monitoring of subvisible particles in therapeutic proteins.

The subject of protein particles and their detection and analysis has been concisely reviewed in two recent publications (Ripple and Dimitrova, 2012 and Das, 2012) in which it was concluded that further analytical progress is needed to better classify and characterize the diversity of particles encountered in therapeutic proteins, which may vary in the degree of protein unfolding, the inclusion of nonprotein nucleation centres and aggregate morphology.

Similarly, Barnard *et al.* (2012), in their characterization and quantitation of aggregates and particles in interferon- β products to investigate potential links between product quality attributes and immunogenicity, used NTA (as well as microflow imaging and resonant mass measurement) to characterize particles while aggregates were characterized and/or quantified using size-exclusion chromatography (SEC), analytical ultracentrifugation, gel electrophoresis, and dot-blotting immunoassays the results of their study strongly suggesting that protein aggregate and particle contents are key product quality attributes in a given product's propensity to elicit the production of neutralizing Abs in patients.

Precipitation of alpha chymotrypsin in the simultaneous presence of ammonium sulphate and t-butanol (three phase partitioning) resulted in preparations which showed self aggregation of the enzyme molecules (Rather *et al.*, 2012). The presence of aggregates was confirmed by SEM and gel filtration on Sephadex G-200. While DLS reported aggregates in the range 242–1124 nm, NTA reported an aggregate range of 130–462 nm which probably reflected the sensitivity of DLS to being weighted incorrectly to the larger aggregates present.

van de Weert and Arvinte (2012) have recently discussed the use of protein aggregate-specific dyes such as Thioflavin T which is known to bind to amyloid.

Gruia (2011) described the characterization of submicron particle distributions in biological formulations and suggested that NTA was a novel technique which has the potential to enhance the current analytical capabilities for detecting, sizing and concentration measurement of particles in the sub-micron range.

In demonstrating that triethylenetetramine (TETA) prevented insulin aggregation and fragmentation during copper catalyzed oxidation, Torosantucci *et al.* (2013) used NTA to monitor the aggregation of insulin as part of this study concluding that TETA is a potential candidate excipient for inclusion in formulations of oxidation-sensitive proteins.

Ellison *et al.* (2013) developed a novel anodic particle coulometry method for agglomeration and aggregation studies based on sizing silver nanoparticles impacting a micro carbon electrode in a KCl/citrate solution. While this technique was shown to be in excellent agreement with NTA, they claimed that the electrochemical technique has the advantage of directly yielding the number of atoms per impacting nanoparticle irrespective of its shape, while they suggested NTA requires a correction for the non-spherical shape of agglomerated nanoparticles to derive reasonable information on the agglomeration state

Several other techniques have also recently been compared to NTA and were discussed in a recent book dedicated to biophysics for therapeutic protein development (Wei and Polozov, 2013; Wang *et al.*, 2013; Struble *et al.*, 2013).

Comparison of NTA to Dynamic Light Scattering (DLS)

The ensemble averaging technique of DLS, alternatively known as photon correlation spectroscopy (PCS), has historically been used for the detection of aggregates in proteins. As an ensemble technique it addresses very large numbers of particles but is limited in its ability to resolve polydisperse samples and suffers from being an intensity weighted technique which can be heavily biased to low numbers of larger particles. Furthermore, DLS cannot furnish information on particle concentration with any accuracy (Pecora, 1985).

Filipe *et al.* (2010) have critically evaluated the NTA technique for measurement of nanoparticles and protein aggregates stating that NTA was shown to accurately analyze the size distribution of monodisperse and polydisperse samples and that sample visualization and individual particle tracking were features that enabled a thorough size distribution analysis. They confirmed 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. These findings were subsequently further discussed in more general terms by Jiskoot *et al.* (2011).

In their comparison of DLS and NTA for the analysis of lysozymes, Li *et al.* (2011) used NTA to measure the size distribution of the 100nm dense liquid clusters that exist in lysozyme solutions and that DLS overestimates the mean size of the clusters because of the sixth power dependence of the scattered light intensity on the size of the scatterers. Furthermore, the factor of overestimation depends on the shape of the size distribution and was $\sim 1.6\times$ in the studied solution and the related underestimate of the cluster concentration is $\sim 10\times$. Similarly, the applicability of NTA, compared to DLS, to the monitoring of precipitation of a poorly water soluble drug was tested and found to give additional information not offered by DLS. Nanoparticle precipitation at the concentrations used was considered to be of relevance to high throughput screening in early drug discovery (Gillespie *et al.*, 2011).

Furthermore, Gillespie (2011), in discussing his comparison of NTA and DLS when monitoring drug precipitation, showed that, in the analysis of the poorly soluble anti-fungal compound Tolnaftate, NTA was capable of generating an image of the particle's scattering from which an estimate of particle concentration was available, while DLS could not generate concentration data nor detect changes in particle distributions or polydispersity over time.

In describing the success with which the development of poorly soluble and/or permeable drug molecules using nanocrystal formulations has proven to be highly successful, Wang *et al.* (2011) described not only the usual characterization techniques to determine physical properties such as DLS and SEM but also novel techniques such as NTA and dual polarization interferometry (DPI) as having recently emerged, pointing out that while NTA is based on DLS, it actually tracks the Brownian motion of nanoparticles quantitatively which enables the study of nanocrystal and stabilizer interactions

In comprehensively assessing the validity range of centrifuges for the regulation of nanomaterials: from classification to as-tested coronas, Wohlleben (2012), benchmarked analytical ultracentrifugation (AUC), DLS, hydrodynamic chromatography (HDC) and NTA against the known content of bimodal suspensions in the commercially relevant range between 20 nm and a few microns in an attempt to carefully validate methods for the quantification of dispersability and size distribution in relevant media, and for the classification according to the EC nanodefinition recommendation. He stated that "the results validate fractionating techniques, especially AUC, which successfully identifies any dispersed nanoparticle content from 14 to 99.9 nb% with less than 5 nb% deviation". He also claimed "In contrast, our screening casts severe doubt over the reliability of ensemble (scattering) techniques and highlights the potential of NTA to develop into a counting upgrade of DLS". He further concluded that the "recently introduced technique NTA" measured intrinsically number distributions, but was not standardized, especially not for the

determination of number% below a certain threshold. With NTA, they did adjust parameters for optimum conditions, knowing the expected results but would not have detected the bimodality with the same 'blind routine approach' that they took for DLS, HDC, and AUC. These findings were supported by a recent recommendation by the Environmental Protection Agency to use NTA for nanoparticle detection, but only when complemented by microscopic techniques. He finally pointed out that "the specific comment in the EC recommendation that the threshold is based on dividing the number of primary particles below 100 nm by the total number of primary particles. Hence, it is not sufficient to determine only the fraction below 100 nm, but the entire distribution is needed, which is a challenge for NTA".

Matayoshi and Wang (2013) have patented published a new method employing a novel imaging and data analysis method for the detection of particles in therapeutic products comparing results to those obtained by NTA and other techniques.

Applications in antibody preparations

NTA has been used specifically 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 at 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) given both techniques represented new methods worthy of assessment. In contrast to light obscuration, MFI was demonstrated to have the advantage of not underestimating proteinaceous particles. NTA, in contrast to DLS, was demonstrated to be a powerful technique for the determination of unbiased particle distributions of polydisperse samples. They found that all formulations became visibly turbid after several hours of agitation. It transpired that, for NTA-analysis, all samples had to be diluted prior to the measurement and a broad distribution of aggregated species was obtained with average values between 150nm – 400nm after stirring and slightly lower values after agitation. Standard deviations were generally rather high. With DLS it was possible to follow the loss of monomer but show that the particle distributions were also broad and partly biased to larger particles as compared to NTA. Reproducibility was better than with NTA and dilution was not necessary. Nevertheless, they concluded that the two novel methods presented powerful tools for the characterization of particles providing complimentary information to existing methods (Mickisch *et al.*, 2010).

Joubert *et al.* (2011) employed NTA for the classification and characterization of therapeutic antibody aggregates using multiple techniques capable of measuring percent aggregation, particle concentration measurements, size distribution, morphology, changes in secondary and tertiary structure, surface hydrophobicity, metal content, and reversibility. While they acknowledged no single technique was adequate for characterizing IgG aggregates, detection of particles in the nanometer range (20-1000 nm) for each stressed sample was achievable through NTA.

In a similarly broad study, Maddux *et al.* (2011) investigated multidimensional methods, including NTA, for the formulation of biopharmaceuticals and vaccines noting that determination and preservation of the higher order structural integrity and conformational stability of proteins, plasmid DNA, and macromolecular complexes such as viruses, virus-like particles, and adjuvanted

antigens were often a significant barrier to the successful stabilization and formulation of biopharmaceutical drugs and vaccines. In another study of PEGylated stress induced aggregation of insulin and mono-PEGylated insulin, Torosantucci *et al.* (2011) employed NTA to confirm that NTA characterization showed submicron aggregates in the size range between 50 and 500 nm, concluding that PEGylation does not protect insulin against forced aggregation.

On comparing NTA with Atomic Force Microscopy (AFM) for the analysis of monoclonal antibody aggregation intermediates, Lee *et al.* (2010) showed that, whereas DLS is an ensemble technique that tries to recover a particle size distribution from the combined signal of all particles present in the sample, NTA investigates the diffusion of individual particles. Thus, DLS calculates the average particle diameter by measuring fluctuation in scattering intensity and is therefore highly affected by the presence of a few large particles it subsequently tends to be weighted to the larger particles sizes.. Using DLS (Coulter N4-Plus Submicron Particle Sizer) and NTA for an identical FA-TEGALA nanoprodug, the average size calculated by DLS was 126 nm, which was larger than the size calculated by NTA (97 nm). The comparison of size distribution and average size from DLS and NTA indicate that a few larger nanoprodugs (>300 nm) have a significant influence on the size calculation in DLS (Lee *et al.*, 2010).

A variation of NTA has recently been described (Filipe *et al.*, 2011) in which fluorescently labelled IgG and aggregates thereof were tracked (as well as the presence of control 100nm fluorescently labelled beads in complex formulations). They also used fluorescence NTA to analyze fluorescently labelled IgG and human serum albumin subjected to heat stress. These initial studies were expanded in later work on the immunogenicity of different stressed IgG monoclonal antibody formulations in immune tolerant transgenic mice (Filipe *et al.*, 2012). The size, amount, morphology and type of intermolecular bonds of aggregates, as well as structural changes and epitope integrity were characterized and correlated with their immunogenic potential through analysis of anti-drug antibody (ADA) titres by bridging ELISA. Both unstressed IgG and freeze-thawed formulation did not induce measurable ADA levels.

Filipe *et al.* (2013a) have recently reviewed numerous analytical approaches to assess the degradation of therapeutic proteins such as murine IgG, including NTA, resonant mass measurement (RMM), electrical zone sensing (EVS) and fluorescence-activated microflow imaging. The review aimed to summarize the strengths and the pitfalls of current methods for assessing protein degradation, emphasizing the analytical challenges and discussing the most effective strategies during product development.

Additionally, Filipe *et al.* (2013b) have also described the *in vivo* fluorescence imaging of IgG1 aggregates after subcutaneous and intravenous injection in mice by fluorescently labelling a human mAb (IgG1), aggregated by agitation stress and injected in SKH1 mice through SC and IV routes. Their results showed differences in biodistribution and residence time between IgG1 aggregates and monomers. The long residence time of aggregates at the SC injection site, in conjunction with elevated cytokine levels, may have contributed to an enhanced immunogenicity risk of SC injected aggregates compared to that of monomers. NTA was used to determine the degree of aggregation of the material used.

Finally, Bell *et al.* (2013) have employed and compared DLS, NTA and DCS to quantify the degree of adsorption of IgG onto gold nanoparticles to better understand the behavior and fate of nanoparticles in biological systems. When the protein layer was formed completely, the results from all methods were consistent to within ~20% scatter and suggested that IgG adsorption on these 20 nm to 80 nm nanoparticles is rather similar to adsorption on flat gold surfaces with a water content of ~60% by volume. They reported that NTA and DLS provided, as expected, similar values that also correlated well with plasmon frequency shift. However, DCS analysis underestimated protein shell thicknesses in this regime and this may be explained through redistribution of the protein shell which reduces the frictional force during sedimentation.

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Chapter VIII: Exosomes and Microvesicles.

The study of exosomes and other cell-derived microvesicles is an area of rapidly growing importance and the subject of intense interest and research. The previous lack of suitable methods for their detection, analysis, enumeration and phenotyping is proving to be a significant limitation in these studies. This chapter shows the degree to which the technique of NTA is helping to address these problems.

Definitions and nomenclature

Extracellular vesicular bodies such as microvesicles and exosomes are currently under intense investigation due to their apparently ubiquitous presence in a broad range of prokaryotic and eukaryotic organisms and the wide role they appear to play, at a fundamental level, in many biological processes, both physiological and pathological. Their cellular origin, structure, function and characterization has been extensively reviewed, though still the subject of much debate.

In a recent and comprehensive review, Gyorgy (2011) discussed the technical pitfalls and potential artefacts in the rapidly emerging field, compared results from meta-analyses of published proteomic studies on membrane vesicles and summarized the clinical implications of membrane vesicles. Following an emerging consensus in terms of nomenclature, he subsequently described exosomes as being 50 -100 nm in diameter and microvesicles as 100 - 1000 nm, and listed those techniques which have been used most frequently in their isolation, purification, detection and analysis (Gyorgy *et al.*, 2011).

The definition and nomenclature of exosomes and microvesicles is, however, as yet variable. Simpson *et al.* (2009) define exosomes as 40 -100 nm diameter membrane vesicles of endocytic origin that are released by most cell types upon fusion of multivesicular bodies with the plasma membrane, presumably as a vehicle for cell-free intercellular communication. Because extracellular organelle terminology is often confounding, with many preparations reported in the literature being mixtures of extracellular vesicles, there is a growing need to clarify nomenclature and to improve purification strategies in order to discriminate the biochemical and functional activities of these moieties (Mathivanan *et al.*, 2010).

Similarly, Lee (2011) also confirmed that because microvesicles (MVs) are so heterogeneous this has led to the usage of multiple names for their designation under different experimental settings. Some of the most frequently encountered descriptors are MVs, microparticles, ectosomes, exosomes, exosome-like vesicles, shed vesicles and most recently oncosomes. Other names have also been used in various specific settings including argosomes, promininosomes, P4 particles, prostasomes, and several others. He stated that to some extent, this diversity reflects the culture of different fields in which MVs have been studied, but also the substantial biological diversity of the underlying biological process (Lee *et al.*, 2011).

In contrast, platelet-derived microparticles (PMP) are defined as heterogeneous populations of vesicles (<1 μm) generated from the plasma membrane upon platelet activation by various stimuli. They are a discrete population differing from the exosomes which originate from the intracellular

multivesicular bodies. PMP also differ from the microparticles derived from megakaryocytes despite the presence of several identical surface markers on the latter. The molecular properties and the functional roles of the PMP are beginning to be elucidated by the rapidly evolving research interest, but novel questions are simultaneously raised (Siljander, 2011).

In conclusion, it is clear that the diversity in nomenclature and definition of microvesicular bodies, be they microvesicles or exosomes, has arisen from the fact that they originate from a very wide range of cellular origins, through a multiplicity of causes and serve multiple functions, all of which are still to be clarified.

Similarly, Herring *et al.* (2013) focused on the role of cellular exocytic vesiculation in health, disease, and transfusion medicine, recognizing that microparticles (MPs), small membrane-derived vesicles which are derived from many cell types and released into the circulation under shear stress, complement activation, proapoptotic stimulation, cellular damage, or agonist interaction with cell surface receptors.

Origin, occurrence and role

MVs originate through at least three distinct mechanisms: (a) breakdown of dying cells into apoptotic bodies; (b) blebbing of the cellular plasma membrane (ectosomes); and (c) the endosomal processing and emission of plasma membrane material in the form of exosomes. Their generation may be triggered by pathways involved in oncogenic transformation, microenvironmental stimulation, cellular activation, stress, or death. Vesiculation events occur either at the plasma membrane (ectosomes, shed vesicles) or within endosomal structures (exosomes) (Gyorgy *et al.*, 2011; Lee *et al.*, 2011).

Exosomes are found in a wide range of bodily fluids such as urine, amniotic fluid, malignant ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk, saliva and blood (Simpson *et al.*, 2009) and multiple roles have been ascribed to exosomes given the number of different molecular structures associated with their construction. In the case of exosomes derived from breast milk, because exosomes carry immunorelevant structures, they are suggested to participate in directing the immune response and may be important for the development of the infant's immune system (Admyre *et al.*, 2007).

A recent patent filing (Chisholm *et al.*, 2013) concerns the extracellular release of vesicles by photo synthetic cyanobacteria, with NTA used to measure the size distribution of vesicles obtained from the cell-free *Prochlorococcus* supernatant.

Exosomes are thought to have a significant role in cell signaling and as such exhibit a strong relationship to disease progression. Lee *et al.* (2011) confirmed that MVs are increasingly recognized as mediators of intercellular communication due to their capacity to merge with, and transfer a repertoire of bioactive molecular content (cargo) to, recipient cells. Such processes may occur both locally and systemically, contributing to the formation of microenvironmental fields and niches. The bioactive cargo of MVs may include growth factors and their receptors, proteases, adhesion molecules, signaling molecules, as well as DNA, mRNA, and microRNA (miRNAs) sequences. As pointed out in numerous studies, the physiological function of exosomes is still a

matter of debate, but increasing results in various experimental systems suggest their involvement in multiple biological processes.

More recently, Cicero and Raposo (2012) have reviewed the cell biology of exosomes from an historical perspective and Yuana *et al.* (2012) have described the tools available to improve the detection of vesicles (including NTA), and the clinical applications being investigated using vesicles for diagnosis, prognosis, and therapy.

Preparation and detection protocol development

As previously discussed, there is an increasing recognition that methods of isolation and preparation of exosomes and microvesicles differ greatly and such differences can have a profound effect on any investigative results obtained. This lack of visibility regarding the true nanoparticulate nature of a sample under study (size, size distribution, number, etc.) has been considered in some detail by Yuana *et al.* (2011) in their assessment of pre-analytical and analytical issues in the analysis of blood microparticles. They concluded that while results of plasma microparticle (MPs) measurements reported in the literature vary widely, this is clearly not only related to the lack of well standardized MP assays, but also to variations in pre-analytical conditions. Emphasizing the desirability of obtaining fresh platelet-free plasma samples, they also cautioned against inadequate calibration of conventional flow cytometric analysis. When comparing DLS and NTA, they concluded that the sensitivity of DLS was lower in polydisperse sample types as exemplified by cell-derived MPs. NTA, on the other hand, can accurately size particles in a sample, however larger particles reduce the number of small particles detected by the software. The operation of NTA was not considered, as yet, to be as user friendly as that of DLS, and therefore required some skill in operation. Yuana *et al.* (2010) had previously found, however, that NTA confirmed the size and number concentration of MPs found by AFM.

The release of exosomes from Epstein-Barr virus transformed B cells has been studied, and NTA (as well as electron microscopy) used to confirm that the nanoparticulate structures observed during these studies were exosomes and not virions attaching to B cells in the samples (Johansson *et al.* (2010) and Vallhov *et al.* (2010)). In their study of the potential of exosomes for use in vaccine and immune therapeutic strategies, Vallhov used a number of sophisticated techniques (flow cytometry, confocal laser scanning microscopy, and multispectral imaging flow cytometry) to elucidate interactions with other cell types, but only EM and NTA were used to discriminate between exosomes and virions in the exosome preparation (Vallhov *et al.*, 2010).

Similarly, Ludwig and Giebel (2011) used both NTA and EM to size their exosome-enriched solutions, showing they mainly contained particles ranging from 80 to 160 nm, whereas the same sample, when prepared for and documented with EM-based technologies, appears significantly smaller. In a related study, Sokolova *et al.* (2011) characterized exosomes derived from three different human cell types (HEK 293T, ECFC, MSC) by NTA and SEM and investigated their stability during storage at -20 °C, 4 °C, and 37 °C. They showed the size of the exosomes decreased at 4 °C and 37 °C indicating a structural change or degradation. However, neither multiple freezing to -20

°C and thawing, nor multiple ultracentrifugation affected the exosome size. They concluded that NTA was well suited to study exosomes.

Taylor (2011) described the use of NTA for *in vivo* derived human extracellular vesicles to show sizes 30 to 300 nm. Vesicles at concentrations in the range of 10^{10} per mL were assessed following chromatographic and affinity isolation of circulating vesicles to identify specific populations of extracellular vesicles.

Gabriel and Giordano (2010) have discussed NTA under the title “Microparticle Sizing and Counting using New Light Scattering Methods” suggesting it offers many advantages to particle size distribution characterization. They suggested that in addition to its ease of operation, speed, and accuracy, the particle size, particle surface characteristics, interaction of the surface with specific ligands, and hydrodynamic volume of the particle are easily obtained. Extensions of these methods also permit the assessment of surface reactions in real time and without reporter group conjugation to the reactant. These methods offer the ability to examine binding constants and kinetics of binding without chemical modification and offer true advantages in product development and clinical diagnostics and therapeutic monitoring.

In describing the use of ultra-filtration (UF), a method which can potentially separate exosomes rapidly based on the characteristics of the physical size, Huang *et al.* (2012) compared it to more conventional ultra-centrifugation methods. They showed that NTA revealed the size distribution of the main population of particles were from 30 to 150 nm, fitting well to the definition of exosome, suggesting that the UF method is ideal for isolating tumor-associated exosomes from clinical samples. Similar results were showed in other lung cancer cell lines as well as cancer cells and immune cells derived from clinical malignant pleura effusion (MPE) samples. Similarly, Lässer *et al.* (2012) used NTA in their assessment of a 200 nm filtration before a final 120,000 x g ultracentrifugation as a valuable method of eliminating larger particles, and to evaluate the impact of the filtration step on the RNA profile of the isolated exosome fraction. They concluded that the method used for isolating exosomes affects the RNA profile of the exosome fraction.

Further studies on the use of myristoylated alanine-rich C-kinase substrate (MARCKS) peptide as a probe to target microvesicles (Morton *et al.*, 2012) employed NTA. It was also used to validate a method for the quantification and profiling of exosomes in human plasma using a protein microarray based on biotin labelled anti-tetraspanin antibodies, CD9, CD63 and CD81 (Jørgensen *et al.*, 2012), NTA being performed both as total quantification of all microvesicles and with fluorescence-labelling of the exosomes with the detection antibodies).

Soo *et al.* (2012) established that NTA permitted the determination of both the size distribution and relative concentration of microvesicles, including exosomes, in the supernatants of cultured cells and biological fluids during their study of the release of microvesicles from the human T lymphoblastoid cell lines Jurkat and CEM. They showed that, unstimulated, both cell lines release microvesicles in the size range 70 – 90 nm, which can be depleted from the supernatant by ultracentrifugation at 100,000 x g, and by anti-CD45 magnetic beads, and which (through

immunoblotting) also contain the exosome-associated proteins Alix and Tsg101. Incubation with known potentiators of exosome release, the ionophores monensin and A23187, resulted in a significant increase in microvesicle release that was both time and concentration dependent. They concluded that NTA can be effectively applied to monitor microvesicle release from cells of the immune system.

In a study aimed at the setup of a protocol for exosomes isolation from urine, and the quantification and analysis of surface markers and micro-RNA (miRNA) content, Dimuccio *et al.* (2012) compared and tested four protocols of exosome isolation, based on i) ultracentrifugation (100,000 x g at 4 °C for 1 hour); ii) nanomembrane concentrator Amicon (100k); iii) nanomembrane concentrator Vivaspin 500 (Sartorius); iv) denaturation of Tamm-Horsfall Protein (THP) with DTT (200 mg/mL) followed by ultracentrifugation. Exosome quantification was performed with Bradford assay for protein content, or with NTA concentration measurement. A total mRNA was extracted using mirVana kit (Ambion) and miRNA analysis was performed using quantitative RT-PCR. As exosomes were considered to be smaller than the lower limit of sensitivity of the cytofluorimetric analysis, it was performed after adsorption of isolated vesicles on 4 µm aldehyde-sulphate latex beads. They showed that the protein concentration tested with a Bradford assay only showed a very low exosome concentration for protocol number two, however but NTA analysis showed high concentration of exosomes in samples obtained using protocols one and two (4.7×10^8 and 3.5×10^8 exosomes/mL). Their study identified a protocol based on ultracentrifugation as the most suitable to obtain exosomes from urine, in which exosome concentration measurement using NTA was more reliable than protein quantification, possibly due to a contamination by urinary proteins, suggesting their findings could be a valid starting point for the further development of studies in a wide variety of renal pathologies.

Goda *et al.* (2012) have extended the development of methodologies for the detection of miRNA through the use of a label-free, microelectrode array exploiting the inherent miniaturization of the electrical biosensor meets requirements for massively parallel analysis of circulating microRNA as a non-invasive biomarker. Their study involved the isolation of exosomes from serum-free supernatant of cultured cells by centrifugation, filtration and ultracentrifugation. The isolated exosomes were characterized by NTA.

In their study of the impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles, Momen-Heravi *et al.* (2012) recognized that the different chemical and molecular compositions of biofluids have an effect on viscosity and this could affect movements of the particles inside the fluid. In addressing the issue of whether viscosity has an effect on sedimentation efficiency of microvesicles using ultracentrifugation they used different biofluids, spiked them with polystyrene beads, and assessed their recovery using NTA to demonstrate that MVs recovery inversely correlates with viscosity. They concluded that, as a result, sample dilutions should be considered prior to ultracentrifugation when processing any biofluids.

Of interest to researchers involved in the isolation, purification and, importantly, storage of exosome samples, Shiba *et al.* (2012) described their studies on the interaction between the

isolated exosomes (from cell culture) and solid materials (including SiO₂, Al₂O₃), and Fang *et al.* (2012) have highlighted NTA as a promising technique for exosome characterization and quantification in their recent assessments of analytical methods in renal research.

Tatischeff *et al.* (2012) described the fast characterization of cell-derived extracellular vesicles by NTA, cryo-EM and Raman tweezers microspectroscopy showing that NTA is valuable for studying the size distribution and concentration, Cryo-EM is outstanding for the morphological characterization, including observation of vesicle heterogeneity, while RTM provides the global chemical composition without using any exogenous label. Using cell-derived vesicles of *Dictyostelium discoideum*, a convenient general model for eukaryotic EVs, pointing out that the size distributions and concentrations of 2 different preparations of *D. discoideum* EVs obtained after 48 hours of cell growth as measured by NTA were different in terms of size distribution (if not number), meaning that different conditions for the 12,000 x g centrifugation can introduce a biased evaluation of the genuine size distribution of the vesicles in the extracellular medium.

Isolation and purification methodology

Because both cell-culture supernatants and biological fluids contain different types of lipid membranes, it is critical to perform high-quality exosome purification. Théry *et al.* (2006) described different approaches for exosome purification from various sources, and discussed methods to evaluate the purity and homogeneity of the purified exosome preparations.

Current isolation protocols for their isolation use a two-step differential centrifugation process. Due to their low density, exosomes are expected to remain in the low-speed (17,000 × g) supernatant and to sediment only when the sample is spun at high-speed (200,000 × g). However, other preparation methods have included sucrose gradient centrifugation, Annexin V-coated magnetic beads, immunoisolation, precipitation technologies (ExoQuick®) and filtration technologies (ExoMir®). A typical such isolation and analysis procedure may use a combination of techniques such as that described by Mathias *et al.* (2009) which employed size filtration followed by ultracentrifugation to isolate and purify exosomes from the colon carcinoma cell line LIM 1215. Morphological visualization and characterization was based on electron microscopy and western blotting, whilst protein identification was achieved using a combination of 1D SDS-PAGE and LC-MS/MS.

However, problems remain. Mathivanan *et al.* (2010) showed in their recent study on various strategies for purifying exosomes that the transport and propagation of infectious cargo, such as prions, and retroviruses, including HIV (suggesting a role in pathological situations), may be artefacts of exosome-purification strategies. Similarly, Quah and O'Neill (2007) described that exosome fractions of dendritic cells produced in long-term cultures were found to contain *Mycoplasma* contaminants. The study highlighted the close association between exosomes and infectious agents like *Mycoplasma* and cautioned about purification procedures for preparation of exosomes for studies on immunity. Furthermore, Bayer-Santos *et al.* (2012) have shown that the secretion of effector proteins into the extracellular environment by *Trypanosoma cruzi* is apparently complicated by the fact that *T. cruzi* releases proteins associated with vesicles that are formed by at

least two different mechanisms, as evidenced by proteomic analysis with NTA being used to discriminate different population sizes in parasite conditioned culture supernatant.

Of particular concern in this field is the problem of sample storage and transport, as well of those associated with methods of isolation and purification. Witwer *et al.* (2013) have investigated anticoagulant, freeze-thaw cycles and RNA isolation methods in EV research as applied to a model of HIV-1 disease. Blood was collected from healthy donors with anticoagulants, including sodium citrate, EDTA, ACD and lithium heparin. Blood was processed immediately to obtain “platelet-free” plasma, and NTA was performed with or without a lipid dye. Quantum dot-conjugated antibodies to surface proteins were also tested. They concluded that NTA indicated minimal effects of anticoagulant and freezing on particle size and number, but specific classes of EVs possibly responded significantly. As expected, some anticoagulants inhibited PCR assays. Newly available biofluids and RNA isolation protocols provided improvements over previous methods; however, RNA extraction should always be optimized carefully.

Sorokina *et al.* (2013) also undertook the qualitative and quantitative analysis of preservation techniques on extracellular microvesicles in order to facilitate the use of EV for clinical application, recognizing that it is crucial to develop efficient methods for their long-term storage without compromising their function. Quantitative analysis was performed on the EVs pre- and post-preservation (NTA and BCA protein assay) after EVs from lung were stored in PBS (1% DMSO) at 4 and -20 °C for up to 7 days but no differences were seen. Additionally, fresh and preserved EVs did not impact the viability of whole bone marrow cells in co-culture. Fast and efficient methods for isolation of exosomal-like vesicles from cell culture medium and body fluids (urine, saliva, plasma, serum) and exosome isolation, fluorescence labelling, analysis and characterization of cell uptake were also reported in a recent international conference on the subject in Boston USA by Kremenskoy *et al.* (2013) and Heusermann *et al.* (2013).

In reviewing the subject of standardization of collection, handling and detection of extracellular vesicles at the same conference, Yuana *et al.* (2013) pointed out that while the most commonly used method to detect EV is flow cytometry (FCM), it detects only 1-2% of all EV present and accordingly, results from EV research are difficult to compare between laboratories. Thus, they aimed to develop standard collection and handling protocols, and to perform sensitive detection of EV using suitable techniques such as resistive pulse sensing (RPS) and NTA. They found that, in comparison to flow cytometry, RPS and NTA detected 1,000 - 10,000-fold more particles in all EV preparations. In contrast to the above, however, generally the concentration and particle size of EV were more affected by the single freeze/thaw cycle than by centrifugation conditions. Their conclusion was that the type of EV, reconstitution solution and detection limit of techniques used to measure EV are important factors to standardize protocols. The subject of standardization of sample collection, isolation and analysis methods in extracellular vesicle research has also been recently comprehensively reviewed by Witwer *et al.* (2013). Having been the subject of a recent series of international meetings on the isolation and analysis of EV, purification and analysis of associated RNA molecules, and molecular engineering of EV for therapeutic intervention, it was recognized that there was a clear need for standardization of specimen handling, appropriate normative controls, as well as isolation and analysis techniques to facilitate comparison of results,

but also that continual development and evaluation of techniques will be necessary as new knowledge is amassed.

Methods for the isolation and analysis of exosomes and microvesicles has been the subject of much recent patent activity, in which NTA is used as proof of the exosomal nature of the isolates (Vlassov *et al.*, 2013; Antes and Kwei, 2013; Jones and Knox, 2013).

Given the importance and potential role of the RNA cargo carried by microvesicles and exosomes, the development of methods for the extraction and RNA profiling of exosomes has received much attention. Confirming the identity and purity of exosomes by NTA and EM (with Western blots for CD63 marker), Zeringer *et al.* (2013) aimed to develop protocols for isolation of exosomes from HeLa cell culture media and human blood serum and characterization of their RNA content. Through the use of a "Total exosome RNA and protein isolation kit", they claimed their isolation procedure was completed in a fraction of the time, compared to the current standard protocols utilizing ultracentrifugation and allowed the recovery of fully intact exosomes in higher yields. Zeringer *et al.* (2013) then extended this work in a subsequent report in which they used NTA to show their isolation protocol represented a set of reagents and a workflow allowing fast and efficient extraction of exosomes, followed by isolation of RNA and its analysis by qRT-PCR and other techniques.

In an attempt to find out whether spin filtration with size exclusion chromatography (SEC) fractioning might represent a more scalable and reliable method than conventional ultracentrifugation (UC), Nordin *et al.* (2013) compared UC, spin filtration and spin filtration with sequential LC fractioning for isolation of exosomes from cell culture media. Through RNA and protein content analysis, Western blotting (WB), NTA and electron microscopy, they showed that by simple spin-filtration and sequential LC fractionation, high yields of exosomes can be purified from large media volumes but needed further development to become the gold standard for exosome purification.

An alternative method, involving a novel peptide with affinity for canonical heat shock proteins (HSPs) as a tool for capture and enrichment of extracellular microvesicles (eMV), was proposed by Chute *et al.* (2013). They showed that eMVs can be purified and evaluated by protein content, and NTA proved comparable to other established methods of eMV isolation. Accordingly, the efficiency of HSP affinity peptide (Vn96)-mediated capture of eMVs matches or exceeds currently accepted methods of eMV isolation while also providing greater specificity in capturing eMVs of particular clinical interest.

Finally, Stensballe *et al.* (2013) have reported the proteomic analysis of exosomes enriched using exosome microarray.

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Chapter IX: Exosomes and Microvesicles: Characterization

While the principles underpinning the Nanoparticle Tracking Analysis (NTA) technique have been described in Chapter 1, it must be reiterated that the use of high intensity laser beams combined with a low-background optical configuration allows particles of deeply sub-micron dimensions to be visualized, the lower range of particle sizes measureable depending on particle refractive index. While for very high refractive index particles, such as colloidal gold, accurate determination of size can be achieved down to 15 nm diameter, for lower refractive index particles, such as those of biological origin such as exosomes, the smallest detectable size might only be 30-40 nm. This minimum size limit allows, however, the analysis of microvesicles and exosomes of a size which would normally be far below the detection threshold of 300 nm for most commercially available flow cytometers. The upper size limits are approached when the Brownian motion of a particle becomes too limited to track accurately, typically 1-2 μm diameter.

The laser with which the nanoparticles are illuminated can be exchanged for one with which fluorescence could be excited, allowing nanoparticles labelled with fluorescent molecules to be visualized, tracked and thus sized and concentration measured specifically through the use of appropriate optical filters. Accordingly, instead of the usual 638 nm red laser, a 532 nm green laser diode can be used to excite a range of organic fluorophores, while a deep blue/violet 405 nm laser diode allows semiconductor CdSe nanocrystals (also known as quantum dots) to be detected on an individual basis. A 488 nm laser diode can similarly be used to excite more conventional dyes as used historically in flow cytometry.

Through the use of antibody-mediated fluorophore labelling of specific sub-populations of exosomes, phenotyping within complex mixtures can therefore be achieved. Of specific importance in this regard is the ability to speciate a particular exosome type by means of Antibody(Ab)-labelling, while simultaneously measuring the size of the exosome by analyzing its Brownian motion, the two measurements being independent of each other. Note also that the concentrations of such labelled exosomes can still be recovered and compared to the total number of similar sized structures whether labelled or not.

Comparison of NTA to Flow Cytometry and Electron Microscopy

NTA is an absolute technique in which the size of the nanoparticles is obtained through measurement of their dynamic Brownian motion behavior and which is independent of the amount of light scattered by the particle (as well as being independent of particle mass or density). This is, of course, not true of flow cytometry, in which size estimates are based purely on the intensity of light scattered by a particle (usually at low angle) and which thus requires, for accurate measurements, pre-calibration with particles of very similar refractive index to that of the sample nanoparticles or which requires significant *a priori* knowledge of the sample nanoparticles themselves in terms of their light scattering properties. Thus, while Nolte-'t Hoen *et al.* (2011) described the development of a fluorescence-based quantitative and qualitative flow cytometric analysis of nano-sized cell-derived membrane vesicles, NTA was used to calibrate the system to the calcein-labelled liposome preparations and CFSE-labelled mouse hepatitis virions with which the

system capabilities were demonstrated. However, wide angle flow cytometric forward scattering could be used for larger and higher refractive index 100 nm and 200 nm fluorescently labelled calibration beads. This group then expanded this work to study CD4+ T cell activation promotion of the differential release of distinct populations of nanosized vesicles (van der Vlist *et al.*, 2012).

The question of the validity of flow cytometers calibration with polystyrene beads when the application is the study of microparticles and exosomes has been addressed by van der Pol *et al.* (2012). Recognizing that polystyrene beads have different optical properties to biological vesicles, and because the mechanisms causing the detection signal are incompletely understood, there are contradictions between expected and observed results. In an attempt to overcome these limitations, this group attempted to model this using Mie theory of light scattering. However, they found that irrespective of the applied gating, multiple vesicles smaller than 220 nm or multiple 89 nm silica beads were counted as a single event signal at sufficiently high concentrations. They concluded that vesicle detection by flow cytometry is attributed to large single vesicles and swarm detection of smaller vesicles, i.e. multiple vesicles are simultaneously illuminated by the laser beam and counted as a single event signal. Swarm detection allows the collective detection of smaller vesicles than previously thought possible and explains the finding that flow cytometry underestimates the concentration of vesicles. This finding was supported by comments by Harrison and Gardiner (2012).

Gyorgy *et al.* (2012a) analyzed synovial fluid (SF) derived MVs, plasma and SF samples of patients with osteoarthritis (OA), rheumatoid arthritis (RA) and juvenile idiopathic arthritis, using electron microscopy and NTA to determine the particle size distributions in SF samples as well as using flow cytometry 'differential detergent lysis' method. They showed that while the different techniques gave concordant results regarding the size distribution of MVs in SF samples (80–400 nm), NTA analysis and Mass Spectrometry (MS) revealed that most of the events were related to protein aggregates rather than cell-derived vesicles.

More specifically, György *et al.* (2012b) compared an improved flow cytometric (FC) methodology to reveal distinct microvesicle (cell-derived microparticle) signatures in joint diseases. In acknowledging that the analysis of MVs in body fluids has not been fully standardized yet, and there are numerous pitfalls that hinder the correct assessment of these structures, they showed that EM and NTA showed that substantial amounts of particles other than MVs were present in synovial fluid (SF) samples of patients with osteoarthritis (OA), rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). Interestingly, total particle concentration, as measured by NTA, were two orders of magnitude higher than the total counts detected by FC. This supports the 'iceberg' theory which assumes that FC only detects particles above 200–300 nm (although the detection threshold is also dependent on the refractive index of the particles) and most of the particles in SFs fall below this range. On the other hand, NTA detects any particles, whereas by FC they enumerated only the true (AX-positive, Triton sensitive) vesicle-related events. They pointed out that using the fluorescence capability of the NTA system and specific labelling, individual populations may also be analyzed.

Dragovic *et al.* (2011b) made the first attempts to develop a combined method involving flow cytometry and fluorescence NTA to characterize cellular microvesicles and nanovesicles. Following earlier work using a human placental vesicle preparation in combination with a fluorophore labelled anti-placental alkaline phosphatase antibody (NDOG2-Qdot605), flow cytometry showed that 93.5% of the vesicles labelled positive for NDOG2 with over 90% of the vesicles being below 1000 nm in diameter, the main population being between 300-400 nm in diameter (Dragovic *et al.* 2011a). However, when the same sample was studied by fluorescence NTA, the results showed a size distribution of NDOG2-labelled vesicles ranging from 50-600 nm, with peaks at 100 nm and 180 nm. Analysis of total cellular vesicles in ultracentrifuge pellets of platelet free plasma (n=10) revealed that ~200 fold more vesicles were detectable using NTA (mean vesicle size 251 ± 35 nm) vs. flow cytometry. They concluded that these results demonstrate that NTA is far more sensitive than conventional flow cytometry and greatly extended their capabilities for the analysis of microvesicles and nanovesicles (Dragovic *et al.* 2011b).

Despite the fact that flow cytometry is widely recognized as being unable to routinely measure exosome preparations, Robert *et al.* (2012) have reported that a high-sensitivity flow cytometry provides access to standardized measurement of small-size microparticles and the use of flow cytometry for the study of microparticles and exosomes has recently been comprehensively reviewed by Baj-Krzyworzeka *et al.* (2012a).

Current detection and analysis methodologies

One of the major problems associated with the isolation and purification of exosomes from complex matrices like body fluids is the paucity of techniques by which fractions can be assessed for exosomal content and concentration measurement.

Van der Pol *et al.* (2010) suggested that despite increasing scientific and clinical interest, no standard procedures are available for isolation, detection and characterization of microparticles and exosomes, because their size is below the reach of conventional detection methods such as flow cytometry. They compared the theoretical performance of a variety of currently available and potentially applicable methods for optical and non-optical determination of size, concentration, morphology, biochemical composition, and cellular origin of microparticles and exosomes. He concluded that several (combinations of) methods could detect clinically relevant properties of microparticles and exosomes, though, because of the biological complexity of body fluids, isolation of microvesicles has proven to be extremely difficult. As a consequence, recovery and contamination cannot be reliably quantified and isolation protocols have not been standardized. In a comprehensive comparison of different techniques he thought the light scattering techniques of DLS and NTA were potentially capable of measuring relative and absolute size distributions of microvesicles within minutes. While Raman spectroscopy, on the other hand, could potentially detect the size, concentration, and biochemical composition of single microvesicles without labelling, the measurement time is in the order of hours. From the optical methods based on fluorescence, fluorescence NTA (fNTA) and Fluorescence Correlation Spectroscopy (FCS) were potentially capable of measuring the absolute size distribution and obtaining biochemical information by applying fluorescent antibody labelling, but it was recognized that this was not easy

to perform and involved several practical and optical problems. fNTA was considered to be the most suitable method to detect size, concentration, biochemical composition, and cellular origin of microvesicles at high speed, especially since the method can determine the relevant characteristics of microvesicles directly in body fluids.

Müller (2012) has recently discussed the emergence of novel tools for the study of cell type-specific exosomes and microvesicles (EMVs) citing numerous suitable technologies for analysis of the size, density and molecular composition of EMVs together with methods for their improved isolation and purification out of heterogeneous vesicle populations. In addition, he thought the recent revolution in mass-spectroscopy, (micro-) flow cytometry, atomic force microscopy, nanoparticle tracking and biosensing will considerably facilitate the quantitative and qualitative analysis of all the constituents assembled in EMVs. Technologies will be preferred that provide signatures specific for EMV subsets rather than a single or a few parameter(s) averaged for the total EMV population. Accordingly, “many of the problems and disadvantages associated with current single-parameter technologies could be overcome by the recently introduced method of NTA which enables the direct and real-time visualization as well as quantitative evaluation of nanoparticles (NPs) in fluidic samples”.

In a similar assessment of NTA, Zheng *et al.* (2012) monitored the Rab27 associated exosome pathway using NTA, showing that it could be used to monitor the inhibition of exosome secretion from MDA-MB-231 breast cancer cells expressing inhibitory RNA targeted for Rab27a, a known component of the exosome pathway. They concluded that their data showed that “nanoparticle tracking analysis can be used effectively and rapidly to monitor the disruption of exosome secretion”.

New commercial tests

- Such is the speed with which interest is building in this area, numerous new reagents and technologies for the isolation, purification and, sometimes, analysis of exosomes or their content have been recently developed and made commercially available; some of which are outlined here:
- Exomir™ uses an alternative approach in which samples are passed over syringe filters to capture exosomes and larger membrane-bound particles, which are then flushed with an RNA extraction reagent to lyse the captured particles for subsequent analysis by qPCR.
- Exotest™ is a proprietary sandwich ELISA kit to capture and quantify exosomes in plasma based on expression of housekeeping proteins (CD63 and Rab-5b) and a tumor-associated marker, caveolin-1 (Logozzi, 2009) for the detection of exosomes in plasma of melanoma patients as a potential tool for cancer screening and follow-up.
- Based on studies by Balaj *et al.* (2011), Exosome Diagnostics Inc. is developing a number of molecular diagnostics employing libraries of binding reagents specific for tumor-specific biomarkers to isolate exosomes from cancer patients for subsequent analysis by more conventional sandwich immunoassay techniques.
- Using technology developed by Delcayre *et al.* (2005), Anosys Inc. employ a novel methodology called Exosome Display enabling the manipulation of exosome composition and tailoring of exosomes with new desirable properties.
- ExoQuick™ is a polymer-based proprietary exosome precipitation reagent that facilitates one-step microRNA and protein biomarker extraction from exosomes in plasma and other bodily fluids for

subsequent profiling by qPCR. Interestingly, NTA was used to confirm the precipitation of exosomes by this technology (Systembio Technical Manual 2011).

- A blood-based diagnostic technology, called Carisome™, which captures and characterizes circulating microvesicles, including exosomes, is also being developed by Caris Life Sciences and is based on work originally carried out by Skog *et al.* (2008).
- Exosome Sciences (2011), Inc. have developed a 96-well assay that allows researchers to isolate exosomes in blood and other fluids using their Enzyme Linked Lectin Specific Assay (ELLSA) which is specific for exosomes, analysis thereof being possible through detection molecules such as antibodies linked to a specific biomarker on the exosome.
- Life Technologies, Inc. has recently described a new reagent for the isolation of exosomes from complex media and biological fluids for use with their RNA marker identification system Ion Torrent (Magdeleno, 2012). This reagent has been recently promoted as a “complete exosome workflow solution: from isolation to identification of the RNA markers using the Ion Torrent Personal Genome Machine” by Vlassov (2012a), using NTA as proof that their reagent is as effective as ultracentrifugation at the isolation of exosomes. Similarly, Zeringer (2012) has described the use of this reagent for the concentration of exosomes from different sample types for downstream analysis.
- More recently, a PureExo® Exosome Isolation Kit (2013) has been produced by 101Bio Inc. which claims 95% isolation efficiency of intact exosomes in <2hours from serum or plasma without requiring ultra-centrifugation.
- Another kit, Exo-spin™, is advertised as suitable for the preparation of pure, functional exosomes from a variety of biological fluids including blood plasma/sera, cell culture media, urine and saliva. It is also claimed to be faster than ultracentrifugation and more efficient than competitor kits (Exo-Spin, 2013). Again, they used NTA to confirm the quality of their product.
- Norgen’s Urine Exosome RNA Isolation Kit is also advertised as constituting an all-in-one system for the concentration and isolation of exosomal RNA from urine and tissue culture media. Separation and purification from urine is based on spin column chromatography using Norgen’s proprietary resin as the separation matrix, following which the exosomes are lysed to release the RNA, which is then bound to Norgen’s resin (BIND) for subsequent analysis
- Finally, HansaBioMed (2013) provides products for exosome research including immunobeads. They also sell NTA-analyzed exosomes standards claiming their “purified lyophilized exosomes were obtained from different biological sources that includes exosomes from cell culture supernatant, human plasma and urine samples...” and that “....isolation is obtained through a combination of ultracentrifugation and microfiltration procedures.....Exosomes are subsequently quantified and validated for overall protein content and particle number by NTA with NanoSight. Effects of lyophilization on stability of exosomal proteins were comparable to other storage methods such as storing fresh exosomes at -20 °C and confirming their stability over 12 months at 4 °C”.

All of these products are alternatives for exosome isolation but might present a lack of specificity because of the precipitation step which may precipitate exosomes among a lot of impurities.

It should be further recognized, moreover, that all of the above tests focus on the isolation of exosomal structures from complex biological fluids (e.g. blood, urine, etc.) for subsequent analysis by more conventional mechanisms (ELISA, qPCR, etc.). As such, they could be considered as bulk purification/separation protocols which offer no opportunity to individually characterize, phenotype and enumerate the exosomes themselves. As is shown below, such a capability would offer significant advantages in the exploitation of exosomes in diagnostics and is offered by the technique of NTA.

The emergence and assessment of NTA as a method for MV characterization

Following early work on the application of Dynamic Light Scattering (DLS) to measuring microparticles (Harrison, 2008; Harrison *et al.*, 2009), Gardiner *et al.* (2009 and 2010) started using NTA for the visualization, sizing and concentration measurement of cellular microparticles and exosomes. Other research groups began to assess NTA in their discussion of pre-analytical and analytical issues in the analysis of microparticles in blood (Yuana *et al.*, 2011) and of microparticle sizing and concentration measuring using light scattering methods (Gabriel and Giordano, 2010).

Subsequently, Dragovic *et al.* (2011) extended their work to both the sizing and phenotyping of cellular vesicles using NTA, while Sokolova *et al.* (2011) described the characterization of exosomes derived from human cells by NTA and SEM. Further studies followed specifically on the use of NTA for the analysis and concentration measurement of (circulating) microparticles (Gardiner, 2011); the analysis of cell exosome and nanovesicle secretion (Powis *et al.*, 2011); the analyzes of *in vivo* derived human extracellular vesicles (Taylor, 2011) and the monitoring of microvesicle and exosome secretion from immune cells (Soo *et al.*, 2012). Cicek Gercel-Taylor *et al.* (2012) later used NTA in the analysis of circulating cell-derived vesicles in ovarian cancer patients.

In studying other methodologies, NTA was also compared in the quantification and profiling of exosomes in human plasma using protein microarray (Jørgensen *et al.*, 2012) and in the isolation, concentration measurement and characterization of exosomes from normal urine (Dimuccio *et al.*, 2012).

Vlassov and his co-workers have reviewed the subject of exosomes, overviewing current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials 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 also describe exosomes composition, functions, and pathways and discuss exosomes used for potential diagnostic and therapeutic applications (Vlassov *et al.*, 2012b). They gave several examples of NTA analysis of exosomes in liquid samples, showing progressively lighter fractions through a sucrose gradient as shown by the more defined size of the particles in these preparations, thus proving how easily NTA can be employed to rapidly furnish size and concentration information about such structures compared to the more conventional industry standard methods of EM and DLS.

The fast characterization of cell-derived extracellular vesicles by NTA, cryo-electron microscopy and Raman tweezers microspectroscopy was reported by Tatischeff (2012) while Arigi *et al.* (2012) used NTA in her proteomic profiling and characterization of human endometrial cancer cell-derived extracellular microvesicles. Huang *et al.* (2012) have described the isolation of tumor associated exosomes from clinical samples using the ultra-filtration method.

Increasingly, NTA is being used routinely for the analysis of microparticles and exosomes in a wide range of studies. The following highlights some of the studies in which NTA has proved central to identifying the physicochemical nature of the microvesicular structures under study.

Cantaluppi *et al.* (2013) reported NTA data (as well as FACS, western blot, bioanalyzer and RT-PCR) in their presentation on the isolation, characterization and pro-angiogenic activity of microvesicles (MVs) derived from human pancreatic islets while Katsuda *et al.* (2013) showed that human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes in their study on the accumulation of β -amyloid peptide (A β) in the brain affected by Alzheimer's disease (AD). On the premise that Neprilysin (NEP) is the most important A β -degrading enzyme, they explored virus-mediated NEP gene delivery using NTA and TEM to confirm the size of purified ADSC #4-derived exosomes at 175 nm. Protein amounts and particle numbers of harvested exosomes were determined by the Bradford method and NTA, respectively.

Hajj *et al.* (2013) reported the unconventional secretion from certain cells of co-chaperone stress-inducible protein 1 (STI1) via a heterogeneous population of extracellular vesicles. STI1 lacks a signal peptide and pharmacological approaches pointed that it does not follow a classical secretion mechanism. Using NTA specifically to measure concentration of the number of EVs during their studies, they showed that astrocytes secrete a diverse population of EVs derived from MVBs that contain STI1 and suggest that the interaction between EVs and neuronal surface components enhances STI1–PrPC signaling.

Having previously demonstrated that macrophage-derived matrix vesicles (MVs) are correlated with the formation of microcalcifications within the fibrous cap of atherosclerotic plaques, Hutcheson *et al.* (2013) identified a role for formation of microcalcifications in vulnerable plaques during regulated release of macrophage-derived matrix vesicles from lipid raft domains. NTA was used to characterize the release of MVs from the RAW264.7 macrophage cell line, following treatment with the proinflammatory cytokine TNF- α (@ 20 ng/mL). Lipid raft domains were identified by confocal microscopy using cholera toxin-based staining of GM1 gangliosides. Kinetic studies using NTA indicated that untreated RAW cells released MVs at a constant rate (average R²=0.95) over 24 h. Treating RAW264.7 cells with TNF- α led to a 1.8-fold increase in MV secretion rate for 6 h. After this initial rate increased, further MV release was completely suppressed up to 24 h.

NTA was also used to determine the contribution of fetal calf serum exosomal RNA in *in-vitro* experiments (Shelke *et al.* (2013)) and assist in the study on microRNA content of extracellular vesicles from rat's urine for distinguishing between healthy vs. polycystic kidney disease (Moggio *et al.*, 2013). Antone *et al.* (2013) showed that cigarette smoking induces and increase in neutrophil/monocyte microvesicles in susceptible subjects.

In searching for a novel source for non-invasive disease biomarkers and showing that extracellular vesicles released by hepatocytes also carry RNA, Royo *et al.* (2013) have most recently demonstrated that these vesicles, likely to be involved in the activation of stellate cells, might become a new source for non-invasive identification of the liver toxicity markers. NTA was used to characterize extracellular vesicles released in two non-tumoral hepatic models: primary culture of rat hepatocytes and a progenitor cell line obtained from a mouse fetal liver.

Raposo and Stoorvogel (2013) have recently produced an excellent and comprehensive review on the subject of extracellular vesicles, exosomes, microvesicles and related structures, focusing specifically on the characterization of EVs and on currently proposed mechanisms for their

formation, targeting, and function recognizing that deficiencies in our knowledge of the molecular mechanisms for EV formation and lack of methods to interfere with the packaging of cargo or with vesicle release, however, still hamper identification of their physiological relevance *in vivo*.

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Chapter X: Exosomes and Microvesicles: Cancer studies

Potential of Exosomes as biomarkers in Cancer

The exacerbated release of exosomes in tumor cells, as evidenced by their increased levels in blood during the late stage of a disease and their over expression of certain tumor cell biomarkers, suggests an important role of exosomes in diagnosis and biomarker studies (Simpson *et al.*, 2009). Furthermore, recent findings that exosomes contain inactive forms of both mRNA and microRNA that can be transferred to another cell and be functional in that new environment, have initiated many microRNA profiling studies of exosomes circulating in blood. These studies highlight the potential of exosomal microRNA profiles for use as diagnostic biomarkers of disease through a non-invasive blood test (Simpson *et al.*, 2009).

Similarly, tumor cells emit large quantities of MVs containing procoagulant, growth regulatory and oncogenic cargo (oncosomes), which can be transferred throughout the cancer cell population and to nontransformed stromal cells, endothelial cells and possibly to the inflammatory infiltrates (oncogenic field effect). These events likely impact tumor invasion, angiogenesis, metastasis, drug resistance, and cancer stem cell hierarchy. Ongoing studies explore the molecular mechanisms and mediators of MV-based intercellular communication (cancer vesiculome), with the hope of using this information as a possible source of therapeutic targets and disease biomarkers in cancer (Camussi *et al.*, 2011). For a list of exosome protein markers that are most often identified in exosomes, see ExoCarta, an exosomal protein and RNA database (<http://exocarta.ludwig.edu.au>).

Exosomes have also been studied as biomarkers for Prostate Cancer (PCa). While the biomarker protein, prostate-specific antigen (PSA), has been considered the gold standard for the detection of PCa and has acceptable sensitivity, it lacks the specificity for discriminating benign prostate diseases (e.g. benign prostatic hyperplasia and infection), indolent PCa and aggressive PCa. Accordingly, screening for PSA is also associated with a high risk of over-diagnosis and over-treatment based on findings on complementary diagnostic prostate biopsies. In a recent paper, Duijvesz *et al.* (2010) focused on the potential role of exosomes as novel biomarkers for PCa. They showed that exosomes, being small vesicles (50–100nm) secreted by almost all tissues, represent their tissue origin. Purification of prostate- and PCa- derived exosomes allow the profiling of exosomes as a promising source of protein and RNA biomarkers for PCa.

In a further interesting development, dendritic cell (DC)-derived exosomes have been shown to allow targeted RNAi delivery to the brain after systemic injection, demonstrating the first proof-of-concept for the potential of these naturally occurring vesicles as vehicles of drug delivery with the added advantages of *in vivo* safety and low immunogenicity. Ultimately, exosome-mediated drug delivery promises to overcome important challenges in the field of therapeutics, for example as delivery of drugs across otherwise impermeable biological barriers, such as the blood brain barrier, and using patient-derived tissue as a source of individualized and biocompatible therapeutic drug delivery vehicles (Lakhal and Wood 2011). Indeed, NTA has already been used in such work (Montecalvo *et al.*, 2011).

Ezrin *et al.* (2012) have characterized blood derived exosomes from glioblastoma patients following exogenous loading with Gliolan to determine if tumors loaded with Gliolan could shed circulating microparticles containing 5-ALA-derived fluorophores as a novel tool to endogenously label, track, and quantify tumor-derived microparticles. Microparticles were isolated by gel filtration and characterized using NTA and bicinchonic acid assay (BCA) for microparticle size/number and protein content, respectively. Endogenous fluorescence from the microparticles was also assessed using NTA in the fluorescence detection mode ($\lambda_{\text{ex}} = 405 \text{ nm}$ and $\lambda_{\text{em}} > 430 \text{ nm}$). Preliminary results suggested that microparticles (mode diameter of 50-100 nm) were present at a concentration of about 10^{11} particles/mL of serum (protein content = $283.5 \pm 47 \text{ ug/ml}$ of sera). They claimed that this was the first evidence that a small molecule drug following oral dosage can be absorbed by tumor cells, enzymatically modified, and shed back into circulating microparticles within hours of dosing and that this direct measure of tumor function affords multiple therapeutic and drug development implications for this novel “liquid biopsy” procedure. Ezrin (2013) has also, using NTA data, patented a pharmaceutical composition comprising 5-aminolevulinic acid (5-ALA) to detect the level of conversion of 5-ALA to protoporphyrin IX (PPIX) associated with brain-derived microparticles in a biological sample from the subject, thereby detecting WHO grade III or grade IV brain tumors.

The subject of microparticles and exosomes as biomarkers has been recently reviewed by Burger *et al.* (2013) in which they summarize approaches for the detection of microparticles and examine novel concepts relating to the formation of microparticles and their biological effects and well as the evidence for microparticles as both biomarkers of, and contributors to, the progression of disease.

Morton *et al.* (2012a) have described microvesicles as indicators of cancer progression using biomarkers in a further methodology belying their more familiar role in proteomics and genomics. Balaj (2012) has carried out BEAMing qRT-PCR analysis of mutant IDH1 mRNA in tumor microvesicles in a diagnostics context and has carried out a direct comparison of glioblastoma large oncosomes and exosomes/ microvesicles reporting that two EVs populations from glioblastoma U87 and HUVEC cells (separated by differential centrifugation and sucrose gradient and compared by NTA, cryoEM, immunofluorescence (IF), qRT-PCR, western blotting and mNMR), both produce more EMs than large oncosomes suggesting that large oncosomes may better represent the content of tumor cells.

Because exosomes carry a range of membrane and cytosolic proteins comprising endosomal compartment and transport/fusion proteins, and because specific proteins indicative of cell type and functional state and are ubiquitously found on exosomes from different biological samples and referred to as common identification markers, their expression can vary across exosomes from different sources. Following capture on an ExoTEST (2013) plate by incubation with precleared plasma samples, different capture/detection antibodies as well as NTA analysis were used to define the correspondence between expression profiles and number of exosomes in each sample (Guazzi *et al.*, 2013). They concluded in this study that, when used in a quantitative immunoassay, some commonly acknowledged exosomal proteins can act as specific markers of tumor type and stage due to variations in overall exosome number or altered protein levels.

Lunaavat *et al.* (2013) used NTA in their comparison of RNA profiles between microvesicles and exosomes derived from melanoma cells, while Polanco *et al.* (2013) undertook a proteomic study of prostate cancer cell-derived microvesicles for identification of therapeutic targets.

In order to characterize exosomes from the saliva of oral cancer (OC) patients isolated by different methods and to compare them to exosomes from the saliva of healthy individuals (HI), Zlotogorski *et al.* (2013) showed that exosomes isolated from saliva of cancer patients differ from those of healthy individuals. Exosomes were isolated by two methods: chemical – Exoquick® (EQ, System Biosciences, CA, USA), and physical – ultracentrifugation (UC, 120,000 g for 3 hours) and isolated exosomes were characterized by ELISA, NTA, EM and AFM. ELISA performed on saliva of OC patients using the exosomal marker CD63 presented higher concentrations compared to HI saliva by both methods, EQ and UC. These results were confirmed in the examination of OC saliva with NTA, which revealed a higher concentration of exosomes that were of a larger size compared to HI saliva. They concluded that exosomes isolated from the saliva of OC patients seem to differ from those of HI saliva in their concentration, distribution and size and that these differences should be further explored for diagnostic and therapeutic purposes. Similarly, Yoshioka *et al.* (2013) undertook a comparative marker analysis of extracellular vesicles in different human cancer types. To confirm the presence of EVs in the preparations, they pointed out that researchers have utilized so-called EV marker proteins, including the tetraspanin family proteins and such cytosolic proteins as heat shock 70 kDa protein 4 (HSP70) and tumor susceptibility gene 101 (TSG101). However, studies have shown that some EV markers are not always present in all EVs, which not only complicated the identification of EVs but also precluded the quantitative evaluation of EV proteins. Thus, it was strongly required to explore well-conserved EV marker proteins that were present at similar levels, regardless of their tissue or cellular origin. In their study, they compared the presence of 11 well-known EV marker proteins by immunoblotting using EVs isolated from 4 human prostate cell lines and 5 human breast cell lines, including cancer cells with different phenotypes and found that all the tested EVs were positive for CD9 and CD81, with similar abundance that was irrespective of the EV origin. In contrast, other EV marker proteins, such as TSG101, Rab-5b and CD63, were detected in an inconsistent manner, depending on the origin of the EVs. Thus, they proposed that the detection of CD9 and/or CD81 should ensure the presence of EVs.

NTA was used to determine exosome size distribution and concentration in an examination of quantitative proteomics of fractionated membrane and lumen exosome proteins from isogenic metastatic and nonmetastatic bladder cancer cells which reveal differential expression of EMT factors (Jeppesen *et al.*, 2013). Similarly, NTA-identified urinary exosomal microRNAs in incipient diabetic nephropathy was studied by Barutta *et al.* (2013) in which they showed that urinary exosomal miRNA content is altered in type 1 diabetic patients with incipient diabetic nephropathy and miR-145 may represent a novel candidate biomarker/player in the complication.

Having previously demonstrated that the scaffolding protein plectin is a robust biomarker for pancreatic ductal adenocarcinoma (PDAC), one of the most aggressive malignancies, Shin *et al.* (2013) reported an unexpected gain of function for plectin due to mislocalization in pancreatic cancer, DLS analysis revealing that the mean size of PDAC particles was 63.53 ± 4.46 nm in diameter and that the mode size was 50.75 nm. NanoSight analysis showed similar results ($57.67 \pm$

20.00 nm). They proposed that it is now clear that this PDAC biomarker plays a role in PDAC, and further understanding of plectin's contribution to PDAC could enable improved therapies.

Cancer Studies in Exosomal Intracellular Communication involving NTA

Given it is now accepted that a) microvesicles (MVs) and exosomes play a pivotal role in cell-to-cell communication and that b) tumor cells have specifically been demonstrated to release such membranous structures, described as microvesicles or exosomes depending on specific characteristics, including size and composition, and that c) these cell-derived vesicles can exhibit an array of proteins, lipids, and nucleic acids derived from the originating tumor, it is now recognized that these vesicular components are critical conveyers of intercellular communication and mediate many of the pathological conditions associated with cancer development, progression, and therapeutic failures. Accordingly, the role that exosomes and microvesicles play in cancer is currently one of the most important subjects of study and most frequently reported use of NTA in the analysis of exosomes. Following earlier disclosures that tumor microvesicles contain retrotransposon elements and amplified oncogene sequences (Balaj *et al.*, 2011) and reviews on brain tumor microvesicles and their role in intercellular communication in the nervous system (van der Vos, 2011) and a review on historical and perspectives of the cell biology of exosomes (Cicero and Raposo, 2012), a significant amount of research has been carried out recently in the role that exosomes play in cellular communication and the potential impact this has on tumor genesis and progression. NTA has, as one of the few techniques by which these small structures can be detected, sized and concentration measured, found itself at the centre of a wide range of such studies.

Given the production of microvesicles (MVs) appears to be closely linked to activation of the cell-death programme, apoptosis, but the functional attributes of MVs released from apoptotic cells have not been defined in detail, Willems *et al.* (2012) hypothesized that MVs produced by apoptotic tumor cells are involved in conditioning of the tumor microenvironment, a critical aspect of tumor evolution and progression using NTA to measure concentration of MVs.

Cicero and Raposo (2012) have reviewed the general area of the cell biology of exosomes from a historical perspective and Taylor and Cicek (2012) have discussed how circulating cell-derived vesicles mediate tumor progressions. In the latter report it was suggested that through the expression of components responsible for angiogenesis promotion, stromal remodelling, signaling pathway activation through growth factor/receptor transfer, chemoresistance, and genetic intercellular exchange, tumor exosomes/microvesicles could represent a central mediator of the tumor microenvironment.

Attempting to define the mechanisms by which fetuin-A mediates the adhesion of tumor cells, Watson *et al.* (2012) used the concentration measuring capability of NTA to demonstrate that the secretion of exosomes increases as a function of intracellular calcium ion concentration. Graner (2012) has ebulliently reviewed the role that extracellular vesicles play in cancer and emv-target cell

interactions and Arigi *et al.* (2012) described the proteomic profiling and characterization of human endometrial cancer cell-derived extracellular microvesicles.

The secretion, composition and biological activity of tumor derived exosomes were shown to be regulated by heparinase (Thompson *et al.*, 2012) and King *et al.* (2012) have demonstrated the hypoxic enhancement of exosome release by breast cancer cells. In this study, proposing that hypoxia is an important feature of solid tumors which promotes tumor progression, angiogenesis and metastasis, potentially through exosome-mediated signaling, King and his co-workers showed that exposure of three different breast cancer cell lines to moderate (1 % O₂) and severe (0.1 % O₂) hypoxia resulted in significant increases in the number of exosomes present in the conditioned media as determined by NTA and CD63 immunoblotting.

As outlined earlier (Lee *et al.* 2011), exosomes are thought to have a significant role in cell signaling and as such exhibit a strong relationship to disease progression. Because extracellular organelle terminology is often confounding, with many preparations reported in the literature being mixtures of extracellular vesicles, there is a growing need to clarify nomenclature and to improve purification strategies in order to discriminate the biochemical and functional activities of these moieties and that NTA is a potentially useful method for exosome detection and enumeration (Mathivanan *et al.*, 2010).

A number of studies have begun to utilize NTA for the detection and concentration measurement of exosomal sized microvesicular structures to investigate their role in intracellular communication, specifically in the study of prostasomes, which are exosome related structures released by prostate acinar epithelial cells (Ronquist *et al.*, 2012); transcriptomics profiling of hepatic extracellular microvesicles (Falcon-Perez *et al.*, 2012); exosomal transfer of RNA based signals between the hematopoietic system and the brain in response to inflammation (Oesterwind *et al.*, 2012); Syndecan–syntenin–ALIX regulation of the biogenesis of exosomes (Baietti *et al.*, 2012); and the induction of phosphatidylserine exposure and microvesicle formation in erythrocytes by an excipient in the conventional clinical formulation of paclitaxel (Vader *et al.*, 2012). Most recently, van Balkom (2012) has described recent developments in exosome signaling in endothelial function and angiogenesis.

Shao *et al.* (2012) used protein typing of circulating microvesicles to allow real-time monitoring of glioblastoma therapy and employed NTA to obtain size, size distribution (log normal) and number of MVs to develop a dedicated microfluidic chip, labeled with target-specific magnetic nanoparticles and detected by a miniaturized nuclear magnetic resonance system which exhibited a much higher detection sensitivity and which could differentiate glioblastoma multiforme (GBM) microvesicles from nontumor host cell-derived microvesicles.

It is known that one component of the adaptive stress response is that innate immunity is primed by circulating endogenous danger-associated molecular patterns (DAMPs). Extracellular heat shock protein 72 (eHsp72) is a DAMP that is upregulated intracellularly after acute stress, but its mechanism of release is unknown. In a study on the role that exosome-associated eHsp72 plays following exposure to acute stress. Beninson *et al.* (2012) used NTA and EM to confirm successful

exosome isolation and reported that exposure to an acute stressor increased exosome expression of eHsp72, but not other stress-inducible proteins (IL-1 β and IL-6). Additionally, exosomes from stressed, but not control, rats facilitated *in vivo* bactericidal inflammatory response ($p < 0.05$) and an *in vitro* LPS-evoked inflammatory response ($p < 0.05$). These data suggested that exposure to stress can alter the proteomic composition of circulating exosomes, thereby enhancing the innate immune response. Wallner (2012) has analyzed extracellular vesicle (EV) mediated signaling in an *in vitro* model of atherosclerotic lesions using NTA to calculate that low density lipoprotein-induced granulocyte microparticles are produced equally over the size range 100-400nm though the LDL particles might have exhibited, in part at least, a common size range.

The role played by exosomes in prostate cancer and their analysis by NTA has been the subject of many recent research projects (Kharaziha and Panaretakis, 2012). Deep *et al.* (2013) have shown that exosomes secreted under hypoxia enhance invasiveness in prostate cancer cells. Human PCA LNCaP cells were exposed to hypoxic (1% O₂) or normoxic (20% O₂) conditions. Media was collected and exosomes, secreted under hypoxic and normoxic conditions, were isolated by ultracentrifugation and precipitation (ExoQuick) methods. Hypoxic and normoxic exosomes were characterized by NTA and EM to confirm the size/structure of the exosomes. Ramteke *et al.* (2013) from the same Group, showed also that exosomes secreted under hypoxia enhance invasiveness and stemness of prostate cancer cells by targeting adherens junction molecules. NTA revealed that ExoHypoxic have smaller average size as compared to ExoNormoxic. This supported other data which suggested that hypoxic exosomes are loaded with unique proteins that could enhance invasiveness, stemness, and induce microenvironment changes, thereby promoting prostate cancer aggressiveness. Chowdhury *et al.* (2013) reported that prostate cancer exosomes alter the fate of mesenchymal stem cell differentiation. Their exosomes were purified using a sucrose cushion and characterized by Western blot, NTA and flow cytometry of exosome-coated beads (flow cytometry being unable to detect the exosomes directly). Hosseini-Beheshti *et al.* (2013) showed prostate cancer derived exosomes could promote prostate cancer progression via activation of the ERK cell signaling pathway and Kim *et al.* (2013) reported that microvesicles shed from DIAPH3-silenced, amoeboid prostate cancer cells enhanced growth of other tumor cells and suppressed proliferation of immune cells. NTA was used to assess the degree of MV shedding from DIAPH3 knockdown DU145 cells (DIAPH3 KD) and controls. They showed that DIAPH3 KD cells secreted about 2-fold more MVs than control DU145 cells suggesting that MVs produced in mCRPC by the loss of DIAPH3 expression may condition the tumor microenvironment, by activation of cancer cells and suppression of tumor-infiltrating immune cells. Ogorevc *et al.* (2013) discussed the role of extracellular vesicles derived from bladder cancer cells in intercellular communication using NTA to help further investigate the mechanisms of intercellular transfer of bladder cancer cell-derived EVs to non-cancerous and cancerous cells. Finally, Karmate *et al.* (2013) have for the first time shown the expression of membrane receptors such as EGFR in exosomes derived from prostate cancer cell lines, LNCap xenograft serum and patient plasma/serum. Exosomes isolation was validated by TEM, expression of exosomal markers and NTA and their study also revealed that a sucrose-assisted centrifugation method was superior for exosomes isolation as compared to ExoQuick.

Melanoma cell lines have been shown to release the stress inducible protein 1 via extracellular vesicles (Dias *et al.*, 2013) in which size and concentration of EVs were evaluated using NTA and TEM while Burdek *et al.* (2013) have studied the effect of human melanoma exosomes on the function of antigen-specific CD8+ T cells. In a related study, Wang *et al.* (2013) demonstrated a positive role for bone marrow stromal cell (BMSC)-derived exosomes in the facilitation of multiple myeloma (MM) cell survival through inhibition of the JNK pathway, using NTA to determine the size of exosomes derived from naïve BMSCs, 5T33 BMSCs and 5T33MMvt cells. Similarly, Boswell *et al.* (2013) have undertaken miRNA expression profiling and proteomic analysis of circulating exosomes from multiple myeloma patients using NTA for the detection and sizing of their exosomes.

Exosomes have also been implicated in lung cancer though their contribution is still largely unknown. Huang *et al.* (2013) evaluated the roles of nanometer sized extracellular vesicles on lung cancer progression investigating the roles of EVs in lung cancer using a malignant pleural effusion (MPE) model, in which soluble components play important roles. EVs were isolated using both ultra-centrifugation (UC) and ultra-filtration (UF) methods, and evaluated by TEM, NTA and Western blotting, as three techniques which are now commonly used in exosomal research. TEM and NTA revealed that EVs isolated using both methods were closed vesicles of nanometer size. Their results demonstrated that the UF method is ideal for isolating tumor-associated EVs from both cell culture and clinical samples and that lung cancer-associated EVs may contribute to cancer progression by triggering oncogenic signals with the IL-6 and VEGF cargos. Wong *et al.* (2013) showed extracellular vesicles (EVS) from activated fibroblasts promoted lung fibrotic remodelling, the EVs being characterized by EM and NTA.

Given the central role that exosomes and EV appear to play in a wide variety of disease conditions and the fact that NTA is proving to be an extremely useful tool in their characterization and enumeration, these have been many reports of such work over the last year. NTA has been used routinely in studies on their mediation of oligodendrocyte–neuron communication by neurotransmitter-triggered transfer of exosomes (Frühbeis *et al.*, 2013); possible defects of communication between neuron and Schwann cells (Zhu *et al.*, 2013); the over expression of a single oncogene altering the proteomic landscape of microparticles (Amorim *et al.*, 2013); the increased abundance of active lysyl oxidase-like-2 on the surface of exosomes by endothelial cells following stimulation with collagen-I or hypoxia (de Jong *et al.*, 2013); the potential of B-cell derived exosomes to activate naïve B Cells (Gutzeit *et al.*, 2013).

The release of humoral factors between cancer cells and the microenvironmental cells is critical for metastasis. However, the roles of secreted miRNAs in non-cell autonomous cancer progression against microenvironmental cells remain largely unknown. Kosaka *et al.* (2013) have recently demonstrated that the neutral sphingomyelinase 2 regulates exosomal miRNA secretion and promotes angiogenesis within the tumor microenvironment as well as metastasis using NTA to measure their exosomes. NTA has also been used to measure EV emission by NB4 cells derived from an acute promyelocytic leukaemia (APL) patient with t_(15;17), the reciprocal translocation between chromosomes 15 and 17 which is a major causative agent in APL (Fang *et al.*, 2013)

Gastrointestinal Stromal Tumors (GIST) are the most common mesenchymal tumors of the digestive tract and several studies have shown that tumor cells produce and utilize exosomes, transporting various cargo reflective of the cells of origin, to communicate with and alter the surrounding microenvironment. In a recent study, Atay *et al.* (2013) demonstrated for the first time that GIST-derived exosomes, detected by NTA and flow cytometry, could induce a GIST-like phenotype in human smooth muscle cells via the transfer of mutant KIT.

Iglesias *et al.* (2012) have shown that human mesenchymal stem cells, from amniotic fluid or bone marrow, reduce pathologic cystine accumulation in co-cultured mutant fibroblasts or proximal tubular cells from cystinosis patients and that paracrine effect is associated with release into the culture medium of stem cell microvesicles (100–400 nm diameter) containing wildtype cystinosis protein and CTNS mRNA as identified and confirmed by NTA following ultracentrifugation. In work reflective of the studies carried out by the Oxford researchers described above, Alam *et al.* (2012) have reported that immunomodulatory molecules are secreted from the first trimester and term placenta via microvesicles. Wang *et al.* (2013) have subsequently shown that mesenchymal stem cell-derived exosomes interact with monocytes and mesenchymal stem cells.

Aggressive epithelial cancer cells frequently adopt mesenchymal characteristics and exhibit aberrant interactions with their surroundings, including the vasculature. Whether the release/uptake of extracellular vesicles (EVs) plays a role during these processes had not been studied. Garnier *et al.* (2012) have now shown that cancer cells can indeed be induced to express mesenchymal phenotype release exosome-like extracellular vesicles carrying tissue factor using NTA to measure the number of size and size distribution of these EVs. That exosome uptake depended on ERK1/2-heat shock protein 27 signaling and that lipid raft-mediated endocytosis was negatively regulated by caveolin-1 was described by Svensson *et al.* (2013), the presence and purity of isolated exosomes again being confirmed by TEM with size measurement being carried out by NTA. Exosome secretion from multivesicular endosomes, as quantitated by NTA, was enhanced by specialized invasive actin structures called invadopodia and has been shown to drive invasive behavior of cancer cells (Hoshino *et al.*, 2013).

Davila *et al.* (2013) used NTA and DLS to analyze the size distribution of particles in conditioned medium (CM) or plasma fractions in their research into microparticle association and heterogeneity of tumor-derived tissue factor (TF) in plasma. In attempting to find out whether it was important for coagulation activation, they found that particles $<0.1\mu\text{m}$ and the supernatants of both CM and plasma gained TF activity after addition of exogenous phospholipids. While TF was found in MP-free CM supernatants, it was also present in CM and plasma pellets. They concluded that tumor-derived particles $<0.1\mu\text{m}$ and non-sedimentable TF are, or can, become procoagulant in the presence of phospholipids and may contribute to the procoagulant potential of circulating TF.

Arguing that the bioactivity of exosomes resides not only in their protein and RNA contents but also in their lipidic molecules, Record *et al.* (2013) have proposed exosomes as representing new vesicular lipid transporters involved in cell-cell communication and various pathophysiology. Because exosomes can vectorize lipids such as eicosanoids, fatty acids, and cholesterol, and their

lipid composition can be modified by *in-vitro* manipulation. Because they also contain lipid related enzymes such that they can constitute an autonomous unit of production of various bioactive lipids, the lipid content of circulating exosomes could be useful biomarkers of lipid related diseases.

Finally, Tadokoro *et al.* (2013) have shown that exosomes derived from hypoxic K562 leukaemia cells cultured under normoxic (20%) or hypoxic (1%) conditions for 24 hours and quantitated by NTA enhanced tube formation in endothelial cells while Haga *et al.* (2013) demonstrated that PDGF-BB induces apoptotic priming of cancer-associated fibroblasts in cholangiocarcinoma. EVs were isolated by differential centrifugation, verified using EM and quantitated using NTA. Their results allowed them to conclude that EV transfer from KMBC increases fibroblast-like activity and selectively alters mRNA expression and secretion of IL6 and other cytokines/chemokines by mesenchymal stem cells that can, in turn, alter KMBC proliferation. Thus, tumor cells can "educate" MSC to modulate the microenvironment and thereby facilitate tumor growth. This was claimed to be a previously undescribed and unique mechanism by which tumor cells can modulate the microenvironment and facilitate tumor growth and offered new opportunities for therapeutic intervention in cholangiocarcinoma and possibly other cancers.

Exosomal Cancer Therapeutic Potential involving NTA

Most recently, Beckler *et al.* (2012) have carried out a proteomic analysis of exosomes from mutant KRAS colon cancer cells to identify intercellular transfer of mutant KRAS which occur in 30-40% of colorectal cancers and NTA allowed them to enumerate the number of exosomes per μg protein.

Tumor-derived exosomes are emerging mediators of tumorigenesis and Peinado *et al.* (2012) showed, by using NTA to analyze exosomes isolated from fresh plasma derived from healthy controls and melanoma subjects, that exosome production and transfer and education of bone marrow cells supports tumor growth and metastasis, has prognostic value and offers promise for new therapeutic directions in the metastatic process. Itoh *et al.* (2012) demonstrated that prostate cancer cells *in vitro* released microvesicles into the culture medium, which was shown by electron microscopic study and NTA for the first time, and Huang *et al.* (2013) recently reviewed extracellular miRNAs embedded inside circulating microvesicles as biomarkers for diagnosis and prognosis of disease, or even as therapeutic agents for targeted treatment. They summarized recent publications involving extracellular miRNA profiling studies in three representative urologic cancers, including: prostate cancer, bladder cancer, and renal cell carcinoma, focussing on the diagnostic, prognostic, and therapeutic potential of these miRNAs in biological fluids such as serum, plasma, and urine which they had concluded were present at concentrations of 0.8×10^8 to 13.4×10^8 /mL of stocked serum or plasma.

Baj-Krzyworzeka *et al.* (2012b) have focussed on the interactions of tumor-derived microvesicles (TMV) with human monocytes, which are precursors of tumor associated macrophages. Their work has shown that monocytes pre-exposed to TMV and restimulated with tumor cells show M2-like cytokine secretion and that TMV significantly modulate biological activity of monocytes and may affect their function during tumor progression, thus suggesting TMV mimicks the effect of tumor

cells on monocytes. They postulate that TMV should be considered as a modulator of monocyte/macrophage functions in the tumor bed and in peripheral blood.

Mizrak and his co-workers reported the first use of a therapeutic mRNA/protein via microvesicles (MVs), analyzed by NTA, for treatment of cancer (Mizrak *et al.*, 2012). They first generated genetically engineered MVs by expressing high levels of the suicide gene mRNA and protein–cytosine deaminase (CD) fused to uracil phosphoribosyltransferase (UPRT) in MV donor cells. MVs were isolated from these cells and used to treat pre-established nerve sheath tumors (Schwannomas) in an orthotopic mouse model. They subsequently demonstrated that MV-mediated delivery of CD-UPRT mRNA/protein by direct injection into Schwannomas led to regression of these tumors upon systemic treatment with the prodrug 5-fluorocytosine, which is converted within tumor cells to 5-fluorouracil – an anticancer agent. Excitingly, these studies suggest that MVs can serve as novel cell-derived “liposomes” to effectively deliver therapeutic mRNA/proteins to treatment of diseases.

In their work on determining the quantitative proteomics of extracellular vesicles derived from human primary and metastatic colorectal cancer cells, Choi *et al.* (2012) used NTA to measure the diameters of 500ng/ml extracellular microvesicles in PBS while Fonsato *et al.* (2012) showed that the delivery of selected miRNAs by MVs (confirmed by NTA to have been successfully isolated from stem from human liver cells) may inhibit hepatoma tumor growth in SCID mice and stimulate apoptosis. Hepatocellular carcinoma metastasis and the role export mechanisms played by exosomal microRNAs in this disease were addressed by Janas *et al.* (2013) while Takahashi *et al.* (2013) showed that chemoresistance in hepatocellular cancer, a common problem, was mediated by an increase in long non-coding RNA-ROR in tumor cell exosomes. Exosomal content was verified by density gradient centrifugation, NTA and EM in malignant (HepG2, Hep3B, HepG2ST, Huh7 and PLC) or non-malignant hepatocytes.

Bruno *et al.* (2012) have shown that microvesicles derived from human bone marrow mesenchymal stem cells inhibit tumor growth. The 145nm (NTA-measured) microvesicles, when administered intra-tumor into established tumors generated by subcutaneous injection of these cell lines in SCID mice, significantly inhibited tumor growth. Furthermore, MVs from human mesenchymal stem cells inhibited *in vitro* cell growth and survival of different tumor cell lines and *in vivo* progression of established tumors, suggesting a future role in tumor treatment. Penfornis *et al.* (2013) similarly described exosome-mediated tumor stromal support of mesenchymal stem cells.

Huan *et al.* (2013) reported that hypoxia alters exosome release and RNA incorporation by acute myeloid leukaemia cells while Katsuda *et al.* (2013) worked on the potential role of osteosarcoma-derived exosomes in pre-metastatic niche formation in the lung. Wong *et al.* (2013) showed that therapeutic treatment of glioblastoma modulated extracellular vesicles dynamics. NTA was used in all these cases to determine exosome size and concentration.

T-cell tolerance of allergic cutaneous contact sensitivity induced in mice by high doses of reactive hapten is mediated by suppressor cells that release antigen-specific suppressive nanovesicles. In

order to determine the mechanism or mechanisms of immune suppression mediated by the nanovesicles, Bryniarski *et al.* (2013) induced T-cell tolerance by means of intravenous injection of hapten conjugated to self-antigens of syngeneic erythrocytes and subsequent contact immunization with the same hapten. Using NTA, tolerance was shown due to exosome-like nanovesicles in the supernatants of CD8+ suppressor T cells that were not regulatory T cells. Nonsuppressive nanovesicles could be made suppressive by adding antigen-specific antibody light chains or miRNA-150 thus showing, for the first time, that T-cell regulation through systemic transit of exosome-like nanovesicles could deliver a chosen inhibitory miRNA to target effector T cells in an antigen-specific manner by a surface coating of antibody light chains. In a related study, Bryniarski *et al.* (2013) showed antigen-specific, antibody-coated, exosome-like nanovesicles could deliver suppressor T-cell microRNA-150 to effector T cells to inhibit contact sensitivity thereby highlighting a possible link to inflammatory skin diseases.

TNF-related apoptosis-inducing ligand (TRAIL) is a protein functioning as a ligand that induces the process of cell death and has been shown to kill *in vitro* a wide variety of tumor cells with minimal effects on normal cells but has so far shown limited efficacy *in vivo*. In contrast, recent reports have shown that significant apoptosis can be observed both *in vitro* and *in vivo* when TRAIL is expressed on the cell membrane (mTRAIL). By innovatively delivering the bioactive proapoptotic TRAIL through its expression by extracellular vesicles (EVs), Buttiglieri *et al.* (2013) have demonstrated potent *in vivo* anti-tumor activity of extracellular vesicles isolated from genetically engineered primary mesenchymal stromal cells, thus paving the way to the use of EVs for therapeutic purposes. NTA revealed that EVs had a variable size, up to approximately 400 nm in diameter, with a predominant peak at 273 nm. Similarly, Huber *et al.* (2013) employed mTRAIL-armed exosomes as a novel and effective anti-tumor therapy which represented a more efficient tool for delivering death signals to the tumor, as compared to soluble TRAIL. The poorly immunogenic erythroblastoid cell line K562 was stably transduced with a lentiviral vector containing membrane-bound TRAIL. Subsequently, TRAIL-transduced K562 cells secreted significant amounts of highly mTRAIL-positive exosomes which induced relevant apoptosis in SUDHL4 (80%) and KMS11 (40%) haematological cancers and melanoma cell lines. Exosomes were isolated by differential centrifugations. TRAIL expression and exosomal nature were assessed by flow cytometry, ELISA, electron microscopy, and NTA.

Tian *et al.* (2013) described another example of a targeted drug delivery vehicle with low immunogenicity and toxicity for cancer therapy, namely a doxorubicin delivery platform using engineered natural membrane vesicle exosomes. Purified exosomes from mouse immature dendritic cells were loaded with doxorubicin via electroporation, with an encapsulation efficiency of up to 20%. The α_v integrin-specific iRGD peptide targeted exosomes showed highly efficient targeting and doxorubicin delivery to α_v integrin-positive breast cancer cells *in vitro* as demonstrated by NTA, confocal imaging and flow cytometry. The intravenously injected targeted exosomes delivered doxorubicin specifically to tumor tissues, leading to inhibition of tumor growth without overt toxicity.

Bretz *et al.* (2013), in showing that body fluid exosomes promote secretion of inflammatory cytokines in monocytic cells via TLR signaling, suggested that exosomes (confirmed by NTA as 100-

300nm in diameter) triggered TLR-dependent signaling pathways in monocytic precursor cells but possibly also in other immune cells. They concluded that this process could be important for the induction of immunosuppressive mechanisms during cancer progression and inflammatory diseases.

Finally, the role that exosomes may play in the fight against infection has become of interest recently and NTA has been used routinely in visualizing and quantifying these structures. Szabo *et al.* (2013) have shown that Exoquick-purified and NTA-analyzed exosomes in *Hepatitis C virus* (HCV) infection mediate CD81-independent transmission and are rich in Ago2-miR122-HSP90 complexes and, as such, can significantly suppress exosomal transmission of HCV infection, suggesting their use when treatment failure occurs with anti-HCV immune therapies. Similarly working with HCV and with HIV, Gupta *et al.* (2013) demonstrated that activated monocyte-derived exosomes mediate miRNA transfer to neural cells with implications for neurodysfunction in HIV/HCV coinfection. Hu *et al.* (2013) reported that release of luminal exosomes from the biliary and intestinal epithelium is increased following infection by the protozoan parasite *Cryptosporidium parvum* and contributes to TLR4-mediated epithelial shuttling of antimicrobial peptides (cathelicidin-37 and beta-defensin 2) in antimicrobial defence, thereby revealing a new arm of mucosal immunity relevant to antimicrobial defence. A time-dependent apical exosome release was detected in infected H69 monolayers by NTA and other techniques. Finally, Twu *et al.* (2013) showed that *Trichomonas vaginalis*, a common sexually transmitted parasite that colonizes the human urogenital tract, produces exosomes (sized by NTA) that deliver cargo to host cells and mediate host:parasite interactions. They suggested that exosomes from highly adherent parasite strains increase the adherence of poorly adherent parasites to vaginal and prostate epithelial cells and that these studies are the first to reveal a potential role for exosomes in promoting parasite:parasite communication and host cell colonization. The *T.vaginalis* microvesicles had physical and biochemical properties similar to mammalian exosomes.

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Chapter XI: Exosomes and Microvesicles: Blood/Platelets; Pregnancy and Diagnostics/Therapeutics Potential

Extra-cellular 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. Found to be 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 by classical methods of analysis such as DLS. Flow cytometry has a lower limit in practice of some 300nm and therefore cannot see the majority of microvesicular material thought to be present.

NTA offers a means by which not only can such structures be seen and concentration measured, but 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.

Platelet-derived microparticles (PMV)

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 group (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 >500nm for the popular and widespread technique of flow cytometry could be improved on, given the proportion of microparticles below this limit was then unknown. They assessed a conventional DLS instrument and NTA and showed that while both systems gave similar results on calibration quality beads over the size range 50–650nm, measurement of either purified microparticles (MPs) or diluted normal Platelet Free Plasma (PFP) NTA gave a polydisperse MP distribution (up to 1000nm) but with a predominant population from < 50nm to above 300nm. Analysis of diluted PFP in PBS (1:40–1:160) suggested that the concentration of particles was 200–260×10⁹/L which was 1000-fold greater than previous estimates. They concluded that while both techniques were rapid and capable of measuring over the entire size range of MP sizes to be expected in biological fluids, NTA exhibited superior resolving power in broad distributions. Gardiner *et al.* (2011) subsequently reported on the use of NTA in the analysis of cell-derived microvesicles and nanovesicles in plasma while Yuana *et al.* (2010) had compared NTA to atomic force microscopy in the detection of nanosized blood microparticles.

In further extensions of these studies, Aleman *et al.* (2011) investigated differential contributions of monocyte- and platelet-derived microparticles towards thrombin generation, fibrin formation and stability using a variety of techniques including transmission electron microscopy, NTA, flow

cytometry, tissue factor (TF) activity, prothrombinase activity, thrombin generation, and clot formation, density and stability, concluding that microparticles from platelets and monocytes differentially modulate clot formation, structure and stability. Fibrinolysis was also studied by Lacroix *et al.* (2012) in which report they suggested leukocyte- and endothelial-derived microparticles represented a circulating source for fibrinolysis.

Siljander (2011) reviewed the subject of platelet-derived microparticles (PMV), pointing out that while the molecular properties and the functional roles of the PMV are beginning to be elucidated by the rapidly evolving research interest, novel questions are simultaneously raised regarding the methodological problems and the paradoxical role of the PMV in health and disease. Aatonen *et al.* (2012a) analyzed the distribution of PMV sizes by NTA and EM, confirming that size distributions by the two techniques correlated well showing that over 90% of PMVs were <500nm and over 70% were <250nm irrespective of the method of activation by various physiological stimuli in comparison to Ca^{2+} -ionophore. These findings showed that the majority of PMVs were much smaller than previously defined by flow cytometry and that the data suggest qualitative agonist-dependent differences in the PMV-specific cargo which respectively influenced their function. They concluded that novel detection methods, such as NTA, and a broader understanding of microvesicle physiology were changing the understanding of MP/exosome sizes and properties. Aatonen subsequently reviewed the role of platelet-derived microvesicles as multitasked participants in intercellular communication (Aatonen *et al.* 2012b) and discussed the methodological issues of PMV detection and analysis in the light of recent advances within the field, as exemplified by NTA. Most recently, Aatonen has shown that the properties of PMV depend on the activation pathway. PMV number and size distribution were analyzed by NTA and correlated with total protein, lipids and EM, respectively. They systematically compared PMVs generated by different platelet-activating pathways and found that 64-85% of MVs were <250 nm depending on activation and 95-99% were <500 nm in diameter. Only 0.5-5% of PMVs was in the 0.5-1 μm range. Their data showed that the activation pathway significantly modulated the quantity and the molecular composition (protein/lipid) of the subsequently formed PMVs, but not so much their size distribution. They concluded that the apparent scarcity of PMVs in the 0.5-1 μm range strongly necessitated the re-evaluation of data obtained with 1st generation flow cytometers, NTA having shown that the bulk of PMVs were below the detection level of such instrumentation (Aatonen *et al.*, 2013).

The development of standardized methods for the analysis of platelet-derived extracellular vesicles (PL-EVs) in human platelet hemapheresis products was described by Ors  *et al.* (2012) in which resistive pulse sensing, FFF, NTA and flow cytometry were compared and found to produce varying results though NTA showed consistency of size of exosomal preparations in different media. Schmitz *et al.* (2012) have discussed the differential composition of subpopulations of platelet derived extracellular vesicles(PL-Evs) related to platelet senescence.

Using differential centrifugation followed by NTA analysis, Pienimaeki-Roemer *et al.* (2012) have shown, for the first time, that stored platelets alter glycerophospholipid and sphingolipid species in stored platelet concentrates and which are differentially transferred to newly released extracellular

vesicles with implications for the effect that storage has on the activity and viability of platelet-derived extracellular vesicles.

Coagulation has been repeatedly linked to the generation and presence of circulating microvesicles in recent years. Owens *et al.* (2011) reported using NTA in demonstrating that monocyte tissue factor-dependent activation of coagulation in hypercholesterolemic mice and monkeys is inhibited by simvastatin. de Vooght *et al.* (2013) have recently shown that cardiopulmonary bypass procedure induces extracellular vesicle formation but these vesicles are rapidly cleared in patients. Barteneva *et al.* (2013) reviewed current knowledge about microparticles (MPs) and provided a systematic overview of last 20 years of research on circulating MPs, with particular focus on their clinical relevance. They suggested that MPs have large diagnostic potential as biomarkers; however, due to current technological limitations in purification of MPs and an absence of standardized methods of MP detection, challenges remain in validating the potential of MPs as a non-invasive and early diagnostic platform. They concluded that improvements in the effective deciphering of MP molecular signatures will be critical not only for diagnostics but also for the evaluation of treatment regimens and predicting disease outcomes. Cantaluppi *et al.* (2013) showed that anticoagulation and enhanced permeability hemodialyzers limits sepsis-associated acute kidney injury through the increased clearance of inflammatory cytokines (IL-6) and microvesicles. Using FACS, Nanosight and RNA profiling, they showed that during extracorporeal blood purification for sepsis, regional citrate anticoagulation inhibits inflammation and decreases mortality. Enhanced permeability hemodialyzers reduce plasma levels of inflammatory mediators including IL-6. Moreover, Zecher *et al.* (2013) showed that erythrocyte-derived microvesicles amplify systemic inflammation by thrombin-dependent activation of complement. Given that transfusion of aged blood has been associated with increased morbidity and mortality in critically ill patients and that during storage, erythrocytes release increasing numbers of microvesicles (red blood cell-derived microvesicles [RBC-MVs]), they hypothesized that RBC-MVs mediate some of the deleterious effects of aged blood transfusions. Using NTA, their results point toward a thrombin-dependent mechanism of complement activation by RBC-MVs independent of the classical, lectin, or alternative pathway. Besides identifying RBC-MVs as potential mediators of transfusion-related morbidity, they suggested their findings may be relevant for other inflammatory disorders involving intravascular microvesicle release, for example, sickle cell disease or thrombotic microangiopathy.

Similarly, Tissot *et al.* (2013) discussed various detection and analytical techniques, including NTA, in their assessment of blood microvesicles and their various roles and properties from proteomics to physiology. Mullier *et al.* (2013) have also addressed pre-analytical issues in the measurement of circulating microparticle regarding current recommendations and pending questions while Rauova and Cines (2013) have again tried to clarify the nomenclature and definitions of microparticles in blood, emphasizing that NTA and AFM, which can resolve particle sizes 1 to 3 orders of magnitude lower than the 200-nm threshold for flow cytometry, indicate that 90% of MPs are below this detection limit.

Pregnancy

As mentioned above, NTA was also assessed as a method for the analysis of exosomes and microvesicles by research groups working in the Department of Reproductive Biology at the University of Oxford, England.

This group was interested in the use of exosomes as potential diagnostics 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. Alam *et al.* (2012) have reported that immunomodulatory molecules are secreted from the first trimester and term placenta via microvesicles.

Historically it has been believed that in pre-eclampsia the maternal symptoms are thought to be caused by the increased shedding of STBM into the maternal circulation. However, the number of STBM observed in the peripheral blood is 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 PRP and <5% of platelets demonstrated STBM binding and that STBM-dependent activation of the haemostatic 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 most recently used both flow cytometry and NTA to rapidly size, quantitate and phenotype cellular vesicles. Their interest was in the study of cellular microvesicles (100nm-1µm) and nanovesicles (< 100nm; exosomes) isolated from the placenta as they have major potential as novel biomarkers for pre-eclampsia, such microvesicles having been previously shown to be implicated in a multitude of other pathological conditions. In common with all such studies however, developments in this area were constrained by limitations in the technology available for their measurement. Dragovic and her co-workers used a commercially available flow cytometer (BD LSRI) employing side-scatter threshold and showed that they could analyze microvesicles ≥ 290nm but nothing smaller. However, they showed that NTA could measure cellular vesicles down to approximately 50nm.

Sheldon *et al.* (2010), in their study on notch signaling 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 of exosomes (modal size of 114nm for HUVECs and 120nm for U87 cells, compared with published sizes of 50-100nm). They stated that, while sizing of exosomes by EM was subjective and limited though underestimation of size following fixing and dehydration, NTA allowed an objective and more accurate estimation of size of exosomes in a buffer such as PBS.

Furthermore, using a human placental vesicle preparation in combination with a fluorophore labelled anti-placental alkaline phosphatase antibody (NDOG2-Qdot₆₀₅), flow cytometry showed that 93.5% of the vesicles labelled positive for NDOG2 with over 90% of the vesicles being below 1000nm in diameter, the main population being between 300-400nm in diameter (Dragovic *et al.*, 2011b). However, when the same sample was studied by fluorescence NTA, the results showed a size distribution of NDOG2-labelled vesicles ranging from 50-600nm, with peaks at 100nm and 180nm. Analysis of total cellular vesicles in ultracentrifuge pellets of platelet free plasma (n=10) revealed that ~200 fold more vesicles were detectable using NTA (mean vesicle size 251±35nm) vs. flow cytometry. They concluded that these results demonstrate that NTA is more sensitive than conventional flow cytometry and greatly extended their capabilities for the analysis of microvesicles and nanovesicles (Dragovic *et al.*, 2011b).

In a further extension to their work, the Oxford group (Alvarez-Erviti *et al.*, 2011) used NTA to show that exosomes played a role in the transmission of alpha-synuclein, aggregation of which is known to be important in Parkinson's disease pathology. These mechanisms they elucidated were considered to potentially provide a suitable target for therapeutic intervention.

Results generated by these groups on the use of NTA for the detection of exosomes and other circulating microvesicles has been the subject of numerous presentations in different applications (Gardiner *et al.*, 2009, 2010, 2011 and Gardiner 2011).

Knowing that flow cytometry detects only a fraction of cell-derived microvesicles and nanovesicles in plasma (PMV), Gardiner *et al.* (2011) recently exploited the sensitivity of NTA and showed NTA sizing is not dependent on the refractive index of the exosomes, whereas sizing of exosomes by flow cytometry requires suitable calibration. Furthermore, fluorescence NTA of PMV, achieved by labelling with a quantum dot-conjugated cell-tracker peptide, produced vesicle concentration measurements of 1.49x10⁷/μL for PFP and 1.20x10⁷/μL for the reconstituted pellet with >95% of all pelleted particles being labelled with the cell tracker, compared to <0.1x10⁷/μL (<0.02%) of the vesicles in the supernatant. The latter were stained with a lipophilic dye, indicating that these were probably lipoprotein vesicles which have a similar size profile to PMV and low density. This suggested that PFP comprises a large population of low density vesicles that are not cellular derived. The presence of lipoproteins will become problematical for flow-cytometry as particle size detection limits continue to fall. The mean PMV (pelleted) concentration measurement was 1.82x10⁷/μL (SD 0.78), with a mean modal size of 92.7nm (SD 6.9nm) and a mean median size of 107.3nm (SD 9.8). The size distribution showed that the 75% of PMV were <150nm, while <2% were greater than 300nm; the minimum size detection limit of conventional flow-cytometers. Pointing out that even the new ultra-sensitive flow-cytometers only detect between 10,000 and 40,000 PMV/μL, Gardiner concluded that NTA detects approximately 100 times more PMV than the most sensitive flow-cytometers.

More recently, Redman and his co-workers have established that there is a large 'hidden' population of microvesicles and nanovesicles (including exosomes) which are hard to investigate because of their size, despite being of significant importance in signaling 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 50nm to 1μm with

the majority being <500nm (which includes both exosomes and microvesicles). They speculated whether changes not only in the numbers, but also in the size (beneficial syncytiotrophoblast exosomes and harmful microvesicles) might be important in pre-eclampsia (Redman *et al.*, 2011).

To enable the identification of the cellular origin of plasma microvesicles and exosomes, specific markers are required and *in vitro* derived vesicles provide the ideal platform to determine whether surface antigens specific for a particular cell type are also present on vesicles derived from them. Dragovic *et al.* (2012) used flow cytometry and NTA in parallel to rapidly size, quantitate and phenotype *in vitro* derived vesicles from platelets, red blood cells (RBCs), endothelial cells, lymphocytes, monocytes and granulocytes. They found that, while using a side-scatter threshold to determine that their standard BD LSRII flow cytometer could analyze vesicles ≥ 290 nm but nothing smaller, NTA could measure cellular vesicles down to approximately 50nm in size and that NTA of platelets, RBC and endothelial-derived vesicles revealed that their size distribution differed, ranging from 50-900nm, 50-400nm and 50-650nm respectively. They showed that vesicle concentration measurements, as determined by NTA vs. flow cytometry were elevated by 75-fold for platelet vesicles, 2855-fold for RBC vesicles and >10,000 fold for endothelial vesicles. From differences in the expression of cell surface antigens on these populations (as determined by NTA vs. flow cytometry) they concluded that vesicles do not necessarily have the same antigenic repertoire as their parent cells and brings into question the use of several standard cellular markers for quantifying plasma vesicles. Dragovic *et al.* (2013) have most recently employed multicolor flow cytometry and NTA of extracellular vesicles in the plasma of normal pregnant and pre-eclamptic women. They developed a multi-color flow cytometry antibody panel to enumerate and phenotype STBM in relation to and other EVs in plasma from non-pregnant (NonP), normal pregnant (NormP) and women with late-onset PE using NTA to determine EV size and concentration. NTA showed that the total number of EVs in plasma was significantly elevated in NormP and late-onset PE women compared to NonP controls, and that EVs were smaller. In general EVs were elevated in pregnancy plasma apart from platelet EVs which were reduced. They also suggested there is scope to develop NTA using a fluorescence capability and could therefore be used to phenotype EVs using fluorescent antibodies.

In an attempt to standardize the characterization and enumeration of exosomes, El-Andaloussi *et al.* (2012) have 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 (crucial steps for loading siRNA into exosomes) and finally, iii) how to use exosomes to efficiently deliver siRNA *in vitro* and *in vivo* in mouse brain. As part of the crucial characterization step, they describe a 30minute protocol for NTA analysis of exosome preparations comprising verification using NIST-traceable polystyrene microspheres, dilution to appropriate concentrations, repeat measurements for adequate statistical reproducibility and, finally, data analysis.

Hypothesizing that exosomes or the slightly larger microvesicles (100–300 nm) are released from the endometrial epithelium into the uterine cavity, and that these contain specific micro (mi)RNA that could be transferred to either the trophoctodermal cells of the blastocyst or to endometrial

epithelial cells, to promote implantation, Ng *et al.* (2013) have shown that exosomes/mv containing specific miRNA are present in the microenvironment in which embryo implantation occurs and may contribute to the endometrial-embryo cross talk essential for this process.

Using STBM prepared by placental lobe dual perfusion and mechanical disruption and analyzing them by four color flow cytometry, NTA and Western blotting to determine vesicle size, purity and Flt-1 and endoglin (Eng) expression, Tannetta *et al.* (2013) have recently found differences in physical and antigenic characteristics of normal and PE placenta STBM preparations produced by placental perfusion or mechanical disruption.

Ferreira *et al.* (2013) have shown that human embryos release extracellular vesicles (EV) which may act as indicators of embryo quality. Using NTA to provide real-time visualization and characterization of EV size and concentration, they confirmed that EV characterization may predict recovery of slow developing embryos, which might be associated with miscarriages though the relationship between EV, embryo quality and oocyte quality requires further investigation. Nevertheless, EV may be a key way in which the embryo signals to the endometrium which may have important implications for both IVF protocols and determining the implantation potential of an embryo. Ouyang *et al.* (2013) have recently reviewed placenta-specific microRNA in exosomes centering on extracellular miRNAs that originate in trophoblasts and that could mediate crosstalk between the feto-placental unit and the mother during pregnancy. They specifically detailed the function of miRNAs from the primate-specific chromosome 19 miRNA clusters which are highly expressed in human placentas and in the serum of pregnant women. They also showed, using NTA, that such miRNAs are also packaged into extracellular vesicles of diverse sizes, including exosomes, and endowed non-trophoblastic cells with resistance to a variety of viruses.

Kilpinen *et al.* (2013) have recently shown extracellular membrane vesicles (MV) from umbilical cord blood-derived MSC protect against ischemic acute kidney injury, a feature that is lost after inflammatory conditioning. NTA-analyzed MVs resuspended with PBS following ultracentrifugation, they demonstrated by both *in vitro* and *in vivo* models accompanied with a detailed analysis of molecular characteristics that inflammatory conditioning of MSCs influence on the protein content and functional properties of MVs revealing the complexity of the MSC paracrine regulation.

Sargent (2013a and 2013b) has recently reviewed the role of MVs in pregnancy and the use of NTA in their detection.

Diagnostics and Therapeutic Potential

Diagnostic potential

The potential of microvesicles and exosomes as diagnostic agents, based in the presence of multiple biomarkers on and in such structures acting as early diagnostics for the onset of a wide range of disease conditions, has been described extensively.

As well as the work carried out by the University of Oxford described previously, several other groups have been studying the use of exosomes in diagnostics. Schorey (2012) proposed that exosomes can be used as diagnostic and prognostic markers in detection and treatment of prostate cancer. Thamilarasan *et al.* (2012) investigated the presence and differential expression of

microRNA (miRNA) located in peripheral blood microvesicles of multiple sclerosis patients under treatment of interferon-beta-1b, in which they confirmed the presence of microvesicles in their preparation using two laser-based detection systems: Fluorescence-activated cell sorting (FACS) analysis and NTA.

While Gercel-Taylor *et al.* (2012) confirmed that cell-derived vesicles are recognized as essential components of intercellular communication, and that many disease processes are associated with their aberrant composition and release and, as such, circulating tumor-derived vesicles have major potential as biomarkers, they pointed out that the diagnostic use of exosomes is limited by the technology available for their objective characterization and measurement. In their study, they compared NTA with submicron particle analysis (SPA), DLS and EM to objectively define size distribution, number and phenotype of circulating cell-derived vesicles from ovarian cancer patients. Using vesicles isolated from ovarian cancer patients, they demonstrated that NTA could measure the size distributions of cell-derived vesicles, comparable with other analysis instrumentation. Size determinations by NTA, SPA, and DLS were more objective and complete than that obtained with the commonly used electron microscopic approach. They confirmed that NTA could also define the total vesicle concentration. Further, the use of fluorescently-labelled antibodies against specific markers with NTA allowed the determination of the phenotype of the cell-derived vesicles. Recently, using NTA to determine particle size distribution profile and concentration estimation, Marcus and Leonard (2012) have modified exosomes to interrogate cargo incorporation and Witwer (2012) has studied the influence of food intake on circulating extracellular vesicles and microRNA profiles based on the fact that circulating miRNAs have provoked intense interest as potential diagnostic or prognostic biomarkers for a wide variety of diseases, from cancers to sepsis. Dietary influence on circulating miRNA profiles - including the potential direct contribution of dietary miRNAs - has received comparatively less attention but could profoundly influence our understanding of proposed biomarkers, since qualitative and quantitative diet alterations have been reported in association with, for instance, cancers and infectious disease. The influence of food intake and fasting on circulating biological nanoparticle carriers of miRNAs was assessed by NTA), which was used to quantitate and characterize small (<500 nm) particles in serial pre- and post-prandial (1, 4, and 12 hour) plasma samples from an animal model. MicroRNAs were isolated from the same samples and profiled using low-density qPCR arrays.

The fact that exosomes and related microvesicular structures frequently contain proteins on the surface that can act as biomarkers for a wide range of disease conditions has attracted the attention of many researchers and it is widely recognized that NTA, in combination with other techniques, can be used to detect these structures at unprecedentedly early stages in disease progression. Burger *et al.* (2012) undertook one of the earlier reviews of this subject and in which NTA was explicitly proposed as a suitable detection methodology and Marcus and Leonard (2012) also discussed the concept of engineering exosomes to interrogate cargo incorporation. As discussed earlier, Morton *et al.* (2012) have discussed the potential of biomarkers on microvesicles as indicators of cancer progression.

As a platform for glioblastoma biomarker development, Akers *et al.* (2013) investigated the use of miR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF). While NTA was used to determine EV number and size, reference transcripts commonly used for quantitative PCR (including GAPDH, 18S rRNA, and hsa-miR-103) were unreliable for assessing EV miRNA. Accordingly, in a panel of glioblastoma cell lines, the cellular levels of miR-21 correlated with EV miR-21 levels ($p < 0.05$), suggesting that glioblastoma cells actively secrete EVs containing miR-21 and that CSF EV miRNA analysis of miR-21 may serve as a platform for glioblastoma biomarker development. The differential expression of miR-145 in children with Kawasaki disease was similarly studied by Shimizu *et al.* (2013) again using NTA for exosome quantification and sequencing of small RNA species allowed the discovery of microRNAs that may participate in Kawasaki disease pathogenesis. miR-145 may participate, along with other differentially expressed microRNAs, in regulating expression of genes in the TGF- β pathway during the acute illness suggesting a model of Kawasaki disease pathogenesis whereby miR-145 modulates TGF- β signaling in the arterial wall. Hajj *et al.* (2013) have also shown that astrocytes secrete a heterogeneous population of microvesicles that share many exosomal markers and contain the co-chaperone STI1 while Kowal *et al.* (2013) have re-assessed the distribution of exosome markers within subpopulations of extracellular vesicles.

In an extension of his earlier work, Cantaluppi and his colleagues have discussed the potential protective role of blood purification techniques in their work on the use of plasma extracellular vesicles as biomarkers and mediators of sepsis associated acute kidney injury (AKI). EV analysis was carried out by NTA, FACS, proteomic and RNA profiling during different blood purification techniques. They were able to conclude that in sepsis, plasma EVs are potential biomarkers of outcome and are involved in the pathogenetic mechanisms of AKI through the triggering of apoptosis in kidney cells. Blood purification using citrate may limit the release of EVs from activated leukocytes and platelets protecting from AKI and multiple organ failures (Cantaluppi *et al.*, 2013).

Nieuwland (2013) also comprehensively discussed the metrological characterization of microvesicles from body fluids as non-invasive diagnostic biomarkers given the recognition that while measurement results need to be quantitative and traceable, the detection of EV is a challenge due to their small size (average diameter less than 100 nm) and heterogeneity and the complexity of body fluids. He included NTA among the state-of-the-art EV detection techniques considered. As part of a major European study (Euramet, 2011) into a multidisciplinary approach to improve the traceable characterization of MV in body fluids, he discussed methods to (a) explore, compare and develop methodologies for dimensional characterization and to measure concentration, morphology and (bio) chemical composition using techniques such as small-angle X-ray scattering, anomalous SAXS and AFM, (b) develop methods for standardized collection and handling of human body fluids and (c) select and test synthetic and biological reference materials to allow traceable calibrations of EV measurements.

Zarovni *et al.* (2013) have described improved immunoassays for detection and quantitative analysis of selected protein biomarkers in human plasma exosomes in order to overcome the problem of unspecific immunoreactivity that interferes with assays outcome and results in false positives or

blockade of a specific signal. NTA and IR spectrometry were used to validate FACS and ELISA analysis of plasma exosomes which revealed unspecific binding of secondary Ab that hampered detection and comparison of specific target proteins in different samples. This issue was complicated by interindividual variability in immunoreactivity and exosome amount in healthy individuals and affected by exosome purification method, while unaltered by sample storage.

Therapeutic potential

The potential for exosomes to be employed as drug and gene delivery vehicles has been discussed earlier (Lakhal and Wood, 2011; Maguire *et al.*, 2012; Morton *et al.*, 2012 and 2013; Fonsato *et al.*, 2012; Biancone *et al.*, 2012 and O'Loughlin *et al.*, 2012; Yuana *et al.*, 2012).

van Dommelen has reviewed the potential for microvesicles and exosomes to be used in drug delivery given they would appear to be capable of delivering lipids, proteins, mRNA and microRNA to change the phenotype of the receiving cells. He concludes that although a number of limiting factors in the clinical translation of the exciting research findings so far exist, it is promising for the development of a potentially novel generation of drug carriers (van Dommelen *et al.*, 2011).

Tumor microvesicles isolated from a variety of cell lines were analyzed for exoRNA content as a function of exosome particle size distribution profile as determined by NTA (Balaj *et al.*, 2011), from which they proposed that tumor microvesicles also carry DNA in addition to a selected set of proteins and RNAs, thus expanding the nucleic acid content of tumor microvesicles to include: elevated levels of specific coding and non-coding RNA and DNA; mutated and amplified oncogene sequences; and transposable elements. Thus, tumor microvesicles contain a repertoire of genetic information available for horizontal gene transfer and potential use as blood biomarkers for cancer. In a related paper, van der Vos *et al.* (2011) used NTA to identify microvesicles shed by brain tumor cells in their study of the novel intercellular communication route they represent the potential physiological role of microvesicles in brain tumorigenesis.

Powis *et al.* (2011) suggested the capabilities of NTA may represent a significant step forward in the characterization of exosomes, allowing them to monitor the release of exosomes in the range 30-150nm after activation with a variety of immune stimuli, relevant to both normal and aberrant immune responses in a way not previously visible with flow cytometry. Most recently, Montecalvo *et al.* (2011) have used NTA to size exosomes during their investigation into the mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Similarly, Weisshaar *et al.* (2012) used TEM to visualize the exosomes isolated from autologous conditioned cell free serum (ACS) and NTA to quantify them but both techniques matched size and concentration of $5.2 \times 10^8/\text{ml}$ with a mean size of 180nm which proved to be mainly aggregates. In their study of cellular stress conditions being reflected in the protein and RNA content of endothelial cell-derived exosomes, de Jong *et al.* (2012) used NTA to help quantify exosome concentration, from which they could show that several proteins and mRNAs displayed altered abundances after exposure of their producing cells to cellular stress, which were confirmed by immunoblot or qPCR analysis.

Cantaluppi and his co-workers have shown that microvesicles derived from endothelial progenitor cells protect the kidney from ischemia–reperfusion injury by microRNA-dependent reprogramming

of resident renal cells, indicating the potential of microvesicles to reverse acute kidney injury by paracrine mechanisms and that microvesicles released from these progenitor cells activate an angiogenic program in endothelial cells by horizontal mRNA transfer. The mean size and particle concentration values were calculated by NTA (Cantaluppi *et al.*, 2012).

In an interestingly orthogonal study, Maguire *et al.* (2012) have recently shown that Adeno-associated virus (AAV) vectors, known to exhibit remarkable efficiency for gene delivery to cultured cells and in animal models of human disease, show limitations after intravenous transfer, including off-target gene delivery (e.g. liver) and low transduction of target tissue. They have, however, shown that during production, a fraction of AAV vectors are associated with microvesicles/exosomes, termed vexosomes (vector-exosomes). These were visualized by EM and their size and concentration routinely determined by NTA allowing their purification for future use as a unique entity which offers a promising strategy to improve gene delivery.

In describing a systematic approach to exosome-based translational nanomedicine, Hood and Wickline (2012) compared DLS with NTA, concluding that NTA has an advantage over DLS in that it is multimodal. Furthermore, they confirmed that if fluorescent antibody labelling of exosomes is combined with NTA, the result is a highly effective means to identify exosome subpopulations and pursue exosome biomarker studies, but the use of fluorescent antibody-based NTA is not appropriate for the production of exosome-based semi-synthetic nanovesicles (EBSSNs) because of its inability to discern single vesicles from vesicle clumps, whose formation is exacerbated by antibody-mediated vesicle cross linking. They suggested it is important to size exosomes prior to pelleting as described above or develop new methods to carefully disaggregate exosomes prior to sizing.

Recently, Vojtech *et al.* (2012) have studied the effect of exosomes in semen on mucosal immunity to viral pathogens in which they used NTA to measure concentration of seminal exosomes and found them to number between 4.7×10^{11} and 1.2×10^{12} /ml (equivalent to 2-34 trillion per ejaculate). Weisshaar (2012) has also studied the anti-inflammatory and anti-microbial activity of exosomes isolated from autologous conditioned cell free serum.

Yeo *et al.* (2013) have recently reviewed the development of mesenchymal stem cell (MSC) exosomes as a potential first-in-class therapeutic given the rationale for MSC therapeutic efficacy remains tenuous and is increasingly rationalized on a secretion rather than differentiation mechanism and in which recent studies have identified exosomes as the secreted agent.

According to Biancone *et al.* (2012), several studies have demonstrated that mesenchymal stem cells have the capacity to reverse acute and chronic kidney injury in different experimental models by paracrine mechanisms. They discussed whether MVs released from mesenchymal stem cells have the potential to be exploited in novel therapeutic approaches in regenerative medicine to repair damaged tissues, as an alternative to stem cell-based therapy. Biancone *et al.* (2012) subsequently pointed out that this paracrine action may be accounted for, at least in part, by microvesicles (MV) released from mesenchymal stem cells, resulting in a horizontal transfer of mRNA, microRNA and

proteins. MVs, released as exosomes from the endosomal compartment, or as shedding vesicles from the cell surface, are now recognized as being an integral component of the intercellular microenvironment. By acting as vehicles for information transfer, MVs play a pivotal role in cell-to-cell communication. Both these studies have used NTA as the means by which MVs can be detected and enumerated in support of such studies. Iglesias *et al.* (2012) have also reported, using NTA-based supported data, that stem cell microvesicles transfer cystinosin to human cystinotic cells and reduce cystine accumulation *in vitro*.

Glutamate transport through astrocytic excitatory amino-acid transporters (EAAT)-1 and EAAT-2 is paramount for neural homeostasis. EAAT-1 has been reported in secreted extracellular microvesicles (eMV, such as exosomes) and, because the Protein Kinase C (PKC) family controls the sub-cellular distribution of EAATs, Gosselin *et al.* (2013) have explored whether PKCs drive EAATs into eMV. While Western-blots show that EAAT-1 is present in eMV from astrocyte conditioned medium, together with NaK ATPase and glutamine synthetase all being further increased after PMA treatment, NTA revealed that PKC activation did not change particle concentration which raises the possibility that microvesicular EAAT-1 may exert extracellular function.

Tetraspanin molecules are commonly used as protein markers of extracellular vesicles, although their role in the unexplored mechanisms of cargo selection into exosomes has not been addressed. For that purpose, Perez-Hernandez *et al.* (2013) have characterized the intracellular tetraspanin-enriched microdomains (TEM) interactome by high-throughput mass-spectrometry, in both human lymphoblasts and their derived exosomes, revealing a clear pattern of interaction networks. Using NTA to measure concentration and size of their extracellular vesicles, their data provided evidence that insertion into TEMs may be necessary for protein inclusion into the exosome structure.

The loading of siRNA into extracellular vesicles by electroporation has been studied by Kooijmans *et al.* (2013). Using NTA to determine aggregate formation (while loading efficiency was determined by fluorescence spectroscopy (FS) and quantitative reverse transcription PCR), they showed that electroporation of EVs with siRNA is accompanied with extensive siRNA aggregate formation, which may cause severe overestimation of the amount of siRNA actually loaded into EVs. The low loading efficiency under precipitate-reducing conditions highlights the necessity for more efficient loading methods.

Verhage *et al.* (2013) have looked at cardiomyocyte progenitor cells (CMPCs) type stem cells for the regeneration of myocardium and the role that secretion of paracrine signals by CMPCs may play in enhancing endogenous repair. Vesicles derived from CMPCs showed presence of several exosomal characteristics, including an average NTA-determined size of 85 nm and expression of exosomal markers flotillin-1 and CD9 around a density of 1.12 to 1.16 g/ml. They concluded that the constitutive secretion of exosomes by CMPCs was shown to be a distinct component of the paracrine signaling pathway by inducing angiogenesis both *in vitro* as *in vivo*, and implicates the use of exosomes as potential therapeutic for cardiac regeneration. Yellon *et al.* (2013) have also reported that rat plasma exosomes are cardioprotective.

In order to better understand the relationship between exosomes and infection, Mantel *et al.* (2013) have studied the production of microvesicles derived from the red blood cells (RBCs) from humans and mice infected with different *Plasmodium* strains. NTA ensured correct identification of microvesicles in their study which allowed them to show that malaria-infected erythrocyte-derived microvesicles mediated cellular communication within the parasite population and with the host immune system. NTA sizing of extracellular vesicles (EV) also allowed Kang *et al.* (2013) to show that EV derived from gut microbiota, especially *Akkermansia muciniphila*, protect the progression of dextran sulphate sodium-induced colitis.

Given that a small subset of unconventional human T cells, gdT cells, play complex roles in host immunity against pathogens and cancers and play a key function in antigen presentation, comparable to that of dendritic cells, Welton *et al.* (2013) used cryo-EM and NTA to reveal the majority of extracellular vesicles secreted by these cells to be <100 nm, with very small electron dense (30 nm) vesicles being the most prevalent. They used flow cytometry to show the expression of gdT cell receptor, MHC Class I and II and tetraspanins while multivesicular body and lysosomal markers, TSG101 and LAMP2, were observed by Western blot. They accordingly were able to describe a previously unstudied exosome source, showing that gdT cells produce a heterogeneous population of mainly small and dense vesicles phenotypically similar to exosomes. These vesicles exhibited peptide-presenting function, activating chemokine secretion by antigen-specific abCD8 T cells with a potential to be therapeutically valuable.

Finally, NTA was discussed in a recent review of the current knowledge of exosome composition, biological functions and diagnostic and therapeutic potential (Vlassov *et al.*, 2013).

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Chapter XII: Viruses and Viral Vaccines

Viruses and viral vaccines

As NTA allows suspensions of nanoparticles to be visualized, sized and enumerated based 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 particular can be studied in higher detail.

It is frequently the case in vaccine production and manufacture that the size of any particular virus or phage particle is known and of secondary importance to the estimation of virus particle concentration measurement and degree of aggregation. In this regard, the ability of NTA to determine virus concentration measurement through direct visualization, irrespective of virus infectivity, is of significant value.

Similarly, NTA's ability to detect, size and, most importantly, measure concentration of all but the smallest viruses has led to numerous studies and assessment in this area, where it is considered to compare favorably with existing techniques.

Virus Characterization, Sizing and Concentration Measurement

Normally, the titre of phage and virus particles is established by plaque assay or, in the case of animal cell viruses, by cell based bioassay. In these assay systems, infective virus particles are grown in confluent cell layers to produce plaques (zones of destroyed cells) which can be detected. While a direct count of individual infective virus particles can be obtained, non-infective virus particles do not produce plaques and therefore cannot be detected. In addition, 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 (an early indicator of limited product shelf life).

Accordingly, NTA has been assessed for this purpose. Early work established the technique's potential for fast and low cost analysis of small volume samples of high polydispersity but recognized the then early stage of development and limited data on which to base estimates of reproducibility and accuracy (Moser, 2008).

Subsequent studies have furnished much more detailed information. In testing the ability of NTA to determine number concentration of both artificial and natural nanoparticles (such as adenoviruses), Du and his colleagues concluded that NTA had improved accuracy compared to mathematical calculation and spectrophotometer methods (Du *et al.*, 2010), although the concentration range over which NTA was applicable was a potential limitation. Nevertheless, it was shown that NTA could have significant applications in a number of fields ranging from nanoparticle synthesis, aggregation, and drug delivery to vaccine production. The same group also used NTA to study the adhesion of polystyrene and virus particles (Kendall *et al.*, 2010).

More recently, 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 by using known concentrations of the subject particles. NTA analysis 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 (HA) and end point dilution assay, determining total and infection 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 a better insight into the sample status, regarding the level of virus aggregation.

NTA has also been 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). Here, it was concluded that, while NTA only operates best 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 hours and 3-4 hours, respectively) and that its performance does not require any additional reagents. The authors suggested that, once optimized for phage, it would be likely that the NTA-based method will be reproducible among various laboratories, with accuracy comparable to PA performed by various investigators. However, it would be significantly faster and may be very useful for future basic and applied research with bacteriophage.

Similarly, Filipe *et al.* (2011) compared NTA to qPCR as a means by which a greater understanding of viral preparations can be gained in support of traditional and frequently limited techniques (Filipe *et al.*, 2011). They emphasized that while the qPCR technique is advantageous, in that it is highly specific and can be used to quantify DNA containing viruses in a harvest material, it cannot distinguish the amount of aggregation within a sample preparation. In addition, the technique measures only viruses that actually contain DNA or RNA (filled capsids) while many viruses formed in vaccine or gene therapy production contain no RNA or DNA (empty capsids) and thus cannot be measured using qPCR. The fact that NTA visualizes, sizes and measures concentration of all virus particles and, importantly, aggregates thereof allows the user to calculate the ratio of filled and empty capsids (despite varying levels of aggregates in a given sample). They concluded that the greatest value of NTA in this field is in making it possible to quantify, visualize and size not only viruses, but also aggregates. Finally they recognized the potential of NTA in its ability to measure fluorescently labelled virus particles thus extending the usefulness of this technique into harvest materials, for which it is essential to discriminate viruses from background host-cell debris and proteins.

In his study of the use of viral quantitative capillary electrophoresis (Viral qCE) for measuring concentration of intact viruses, Mironov used NTA to verify the quantitation of intact oncolytic *Vaccinia* virus particles by Viral qCE after calibrating the instrument with 100, 200 and 400 μm latex beads (Mironov *et al.*, 2011). In another study involving the detection of oncolytic viruses using small molecule single stranded RNA/DNA oligonucleotide aptamers, Chechik and Berezovski (2011) used NTA to evaluate the binding of DNA aptamers to *Vaccinia* virus and vesicular stomatitis (VSV). They showed that aptamers conjugated with beads caused the aggregation of virus particles and the intensity of light scattered by the aggregates was directly proportional to the affinity of

aptamer pools to viruses. They concluded that NTA brings a valuable tool for all further developments of biomolecular binding analyzes and aptamer selection.

Vesina *et al.* (2012) also obtained particle size distribution on their plant-derived virus-like particles in their development of methodology for their preparation. Trifonova *et al.* (2012a and 2012b), on the other hand, used structurally modified plant viruses as nanovaccines using NTA to assist in their characterization, while Azizi *et al.* (2012) have most recently used NTA to discriminate between variations in binding of the fluorescent label YOYO-1 to viral and ribosomal RNA differential binding in their development of a quantitative capillary electrophoresis technique for concentration measurement and quality control of RNA virus.

The use of lentiviral vectors for gene therapy necessitates the generation of functional vectors using fast, non-laborious and cost-effective strategies. Eleni *et al.* (2013) have described an improved protocol in which vector stocks are prepared by transient transfection using standard cell culture media and are then concentrated by ultrafiltration, resulting in functional vector titres of up to 6×10^9 transducing units per milliliter (TU/mL) without the involvement of any purification step. They determined the viral functional titre by employing flow cytometry and evaluated the actual viral particle size and concentration in real time by using NTA. Searing *et al.* (2013) have also recently reported characterizing lentiviral vector particles by NTA-determined size and composition.

The potato virus X (PVX) virion can be reconstituted *in vitro* from the virus coat protein (CP) and RNA with heterologous RNAs capable of being used as well. In a recent study, Petrova *et al.* (2013) showed the structure and properties of cognate and heterologous viral ribonucleoproteins (vRNPs) to be similar to those of native virions, using NTA to analyze an incubation mixture which was diluted 10,000 times in 10 mM Tris-HCl buffer, pH 7.5, to a protein concentration of 0.5 ng/mL.

As one of the vectors of choice for gene delivery and expression of foreign proteins in gene therapy and vaccination purposes, recombinant adenoviruses are highly efficient at gene transfer for a broad spectrum of cell types and species. Silva *et al.* (2013) have used NTA in the development of new scalable and reproducible production processes of adenoviral vectors (human and canine) using stirred tank bioreactors.

Vaccines and virus-like particles

The use of NTA in vaccine research has been reviewed recently by several research groups. In a study aimed at the development of large scale production protocols of virus-like particles (VLPs) which offer great promise as candidates for new vaccine strategies, Cervera *et al.* (2013a) described the generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium, as compared to the more conventional use of the baculovirus expression system. They used NTA to show that the maximum cell density attained using the optimized Freestyle culture medium was 5.4×10^6 cells/mL in batch mode, almost double of that observed using the unsupplemented medium (2.9×10^6 cells/mL). Cervera *et al.* (2013b) subsequently extended this work to demonstrate that the human embryonic

kidney 293 (HEK 293) is the preferred host system due to the many industrially relevant features this cell line offers including high transfectability, ability to grow in suspension, ability to grow to high cellular densities and adaptation to serum-free culture conditions. The great majority of Gag-GFP recovered from cell culture supernatants was shown to be correctly assembled into VLPs of the expected size and morphology as demonstrated by NTA. The scalability of this strategy was demonstrated using a 1L WAVE bioreactor and the quantity and quality of Gag VLPs generated using the described platform was considered suitable for pre-clinical studies in mice. The same group has shown that, as an alternative to time-consuming ELISA based assays, the development and validation of a new fluorescence-based quantitation assay for Gag VLP titres were in good agreement with those obtained using TEM and NTA (Gutiérrez-Granados *et al.*, 2013a). They concluded that this simple, rapid and cost-effective quantitation assay should facilitate development and optimization of bioprocessing strategies for Gag-based VLPs. Gutiérrez-Granados *et al.* (2013b) have most recently reported on the characterization and quantitation of fluorescent Gag virus-like particles. Using a Gag-GFP fusion construct, the viral particle titres estimated were compared with those obtained by p24 ELISA (Innotest®, Innogenetics, Belgium), densitometry, TEM and NTA. Upon transfection, Gag-GFP was expressed in the cytoplasm of the producer cells and accumulated in the vicinity of the plasma membrane where the budding process takes place.

Mather (2013) has described the work of the Lomonosov Moscow State University (MSU; Moscow, Russia) who used NTA technology for vaccine characterization and standardization, as the technology enabled real-time measurement of virus particles in liquid without contamination or particle aggregation (clustering). The results enabled the research team to analyze and control the size, state of aggregation, and concentration of artificial plant virus particles and small spherical plant and animal viruses. It also allowed them to see the formation of immunogenic complexes (candidate vaccines) by using the indirect immunofluorescence or immunogold staining methods

Similarly, Patois *et al.* (2013) recently reviewed the applications of NTA for the characterization of biopharmaceuticals and procedures were proposed for the determination of size distributions. Size distributions of two subunit seasonal influenza vaccines (a) Influvac (Abbott) and (b) Agrippal (Novartis) were measured within 60 seconds using NTA. The review also discussed techniques such as fluorescence microscopy with Nile Red staining, micro-flow imaging, 90° light scattering, and flow field-flow fractionation (FFF) and concluded that NTA is a suitable technique for a wide range of applications in the field of biopharmaceuticals.

In the development of virus-like particles for the study of virus-host interaction of hepatitis E virus (HEV) Kapur *et al.* (2011) used TEM and NTA to show near uniform particles of approximately 30–35 nm in diameter for pORF2 VLPs and 60–100 nm for reporter-linked VLPs, while Hughson *et al.* (2010) described inactivation studies on Japanese Encephalitis Virus vaccine.

Aimed at the development of anti-cancer vaccines, work undertaken by Mayer-Sonnenfeld *et al.* (2013) compared protein content of chaperone-rich cell lysate (CRCL) anti-cancer vaccines prepared from human tumors of different histological origins to evaluate the uniformity of their protein content. Protein samples were separated and identified by SDS-PAGE and slices cut from gels for

protease digestion followed by mass spectrometry analysis, the content assessed by gene ontology and networking programmatic computation. CRCL preparations were also analyzed by NTA and TEM.

Mann *et al.* (2013) showed that pulmonary delivery of DNA vaccine constructs using deacylated PEI elicited immune responses and protected against viral challenge infection. They used NTA to verify polyplex and lipoplex formations.

Cayatte *et al.* (2013) have been working on the development of a vaccine against human cytomegalovirus (HCMV), a betaherpesvirus, which can cause severe disease in immunosuppressed patients following congenital infection. Proposing that dense bodies (DB) are complex, non-infectious particles produced by HCMV infected cells and may represent a vaccine option, they explored characterization and defined DB production methods followed by systematic evaluation of neutralization and cell mediated immune responses to the DB material in Balb/c mice. They purified DB from Towne infected cultures treated with viral terminase inhibitor 2-bromo-5,6-dichloro-1-beta-d-ribofuranosyl benzimidazole riboside (BDCRB) which were characterized by NTA, 2D fluorescence difference gel electrophoresis (2D-DIGE), immunoblotting, quantitative ELISA and other methods.

Recently, Liu *et al.* (2013), working on influenza virosomes supplemented with GPI-0100 adjuvant, have evaluated, using NTA, the adjuvant effect of GPI-0100 on a virosomal influenza vaccine formulation. In contrast to influenza subunit vaccine adjuvanted with GPI-0100, highly immunogenic virosomal vaccine supplemented with the same dose of GPI-0100 provided full protection of mice against infection at extremely low hemagglutinin antigen doses, and allows the use of very low antigen doses without compromising the protective potential of the vaccine.

Patil *et al.* (2013) have recently evaluated monophosphoryl lipid A (MPLA) as an adjuvant for pulmonary delivered whole inactivated virus influenza vaccine (WIV). Using NTA to successfully size WIV derived from A/PR/8/H1N1 before and after addition of MPLA, these studies suggested that the mucosal and systemic immune responses to pulmonary delivered influenza vaccines can be significantly enhanced by using MPLA as adjuvant and that MPLA-adjuvanted SFD vaccine was particularly effective, implying that delivery of adjuvanted vaccine powder to the lungs can be an attractive way of immunization against influenza.

Phage

In their systematic experimental exploration into how different bacteriophage (phage) traits may influence the formation of a plaque, Gallet *et al.* (2011) constructed a series of isogenic λ phages that differ in their adsorption rate, lysis timing or morphology to determine the effects these changes have on three plaque properties: size, progeny productivity, and phage concentration within plaques. NTA was successfully used to determine phage concentration to conclude that a more realistic theoretical approach to plaque formation is needed in order to capture the complex interaction between phage and its bacterium host in a spatially restricted environment.

Phage were also the subject of another study by Zhou *et al.* (2012) in which NTA was used to confirm the high purity and dispersion of the 72nm fd Y21Mphage preparation used. Similarly, Carter *et al.* (2012) demonstrated that a commercially available bacteriophage cocktail (EcoShield™) significantly reduces *Escherichia coli* O157:H7 contamination of lettuce and beef, but did not protect against recontamination.

Virus Templating

In the past decade, spherical and rod-like viruses had been used for the design and synthesis of a new kind of nanomaterials with unique chemical positioning, shape, and dimensions in the nanosize regime. Uniquely, NTA allows the average intensity of a particle's scattering to be measured at the same time as its size is being determined by its dynamic behavior. This allows particles of differing refractive index, but same size, to be discriminated by plotting these two parameters as a function of each other. This capability has been exploited in experiments where virus particles, being of highly defined structure and uniform in size, have been used as templates for the simple production of highly monodisperse metallized particles by an electrodeless deposition metallization process.

Aljabali *et al.* (2011a) demonstrated chemically-coupled-peptide-promoted virus nanoparticle templated mineralization using Cowpea mosaic virus (CPMV). The cationic polyelectrolyte, poly(allylamine) hydrochloride (PAH), is electrostatically bound to the external surface of the virus capsid; the polyelectrolyte promotes the adsorption of anionic gold complexes, which are then easily reduced, under mild conditions, to form a metallic gold coating. As expected, the templated gold nanoparticles can be further modified with thiol reagents (Aljabali *et al.*, 2011b). This work followed earlier studies in which they used NTA to demonstrate mineralization of the virus. For each of the mineralized virus particles there was a significant increase in the relative refractive index compared with wild-type and with peptide-CPMV conjugates. The analysis was also considered consistent with the mineralized particles being monodisperse as indicated by the particle size distribution; although for ZnS-CPMV there was a little non-specific aggregation under the measurement conditions employed (Aljabali *et al.*, 2010). NTA is unique in its ability to measure both the size and differences in light scattering properties of particles on an individual basis.

The plant viral nanoparticle tobacco mosaic virus (TMV) has been used for the production of a variety of metals including cobalt, nickel, iron, platinum, cobalt-platinum and nickel-iron and 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 (Bromley *et al.*, 2008). Other studies have prepared virus templated nanoparticles of silica (Steinmetz *et al.*, 2009) and iron-platinum (Shah *et al.*, 2009). To compensate for the low sensitivity of magnetic resonance imaging (MRI), nanoparticles have been developed to deliver high payloads of contrast agents to sites of disease. Bruckman *et al.* (2013) recently reported the development of supramolecular MRI contrast agents using rod-shaped TMV nanoparticles (300 × 18 nm) that were loaded with up to 3500 or 2000 chelated paramagnetic gadolinium^(III) ions selectively at the interior or exterior surface, respectively. Dynamic

Light Scattering (DLS) and NTA were used to show that interior-labelled TMV rods can undergo thermal transition to form 170 nm sized spherical nanoparticles containing $\sim 25\,000$ gadolinium chelates and a per-particle relaxivity of almost $400\,000\text{ mM}^{-1}\text{ s}^{-1}$ ($15.2\text{ mM}^{-1}\text{ s}^{-1}$ per gadolinium), thus laying the foundation for the use of TMV as a contrast agent for MRI. Other studies on biotemplating rod-like viruses for the synthesis of copper nanorods and nanowires have been reported recently (Zhou *et al.*, 2012) in which was demonstrated the controlled synthesis of copper nanorods and nanowires by electroless deposition of copper on three types of palaydium-activated rod-like viruses. The synthesis conditions described in the work were considered scalable and amenable for biological templates and the synthesized structures preserve the dimensions and shape of the rod-like viruses utilized during the study. The work opened the possibility of generating a variety of nanorods and nanowires of different lengths ranging from 300 nm to micron sizes with potential in nanoelectronics, sensing, and cancer therapy. Finally, Trifonova *et al.* (2012a and b) have investigated the antigenic properties of complexes based on structurally modified plant viruses.

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Chapter XIII: Nanoparticle Production

Nanoparticles and engineered nanoparticles (ENPs) are being increasingly exploited throughout a wide range of industry sectors in order to benefit from the frequently greatly enhanced properties exhibited by materials when they are reduced to the nanoscale. Despite the growing importance of obtaining accurate estimates of size, size distribution and concentration of such nanoscale particles in an increasingly wide range of applications, existing techniques for obtaining such information (e.g. electron microscopy, light scattering) can prove time consuming and complex with results that are difficult to interpret, particularly in samples which are heterogeneous in composition or which contain a range of particle sizes, e.g. are polydisperse.

The following chapter highlights the growth in the use of the technique of NTA as applied to nanoparticles and nanomaterials production and analysis, from the very first reports on nanoscale silver and gold (Lundahl *et al.*, 2008 and Marsh *et al.*, 2008, respectively).

NTA in Nanoparticle Design and Production

The effects of ball milling time on the synthesis and consolidation of nanostructured WC–Co composites was investigated by high energy milling in a horizontal ball mill by Hewitt and Kibble (2010) using NTA to determine particle size distribution. This showed that the number of nanosize ($<0.2\ \mu\text{m}$) particles increased with milling time. The onset of the WC–Co eutectic was lowered to $1312\ ^\circ\text{C}$ through an increase in milling time. Hewitt *et al.* (2009) had previously studied the effect of milling temperature on the synthesis and consolidation of nanocomposite WC–10Co powders using NTA.

Hennart *et al.* (2012) have also used the NTA principle to characterize the particle size distribution of sub-micron particles suspended in a liquid as well as other techniques such as imaging techniques (SEM, CryoTEM), static light scattering (SLS) and dynamic light scattering (DLS). Their conclusion was that the presence of aggregates frequently severely perturbed results in these other techniques.

Kendall (2011) discussed problems of particle aggregation in ceramics presenting three types of problem 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 microstructure and strength. The first theoretical problem with ceramic particle aggregation was to define the weak interatomic forces between spheres. The second concerned the better processing that can be applied to dispersed particles to deliver improved ceramic properties by adding polymer to ceramic dispersions to reduce particle attractions which lead to aggregation. The last was 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, Pollet *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 nonylphenoethoxylate (NP9). The aggregation behavior of the nanoparticles was studied using 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 to help them conclude that the surfactant molecule selection is vital to obtaining effective fuel cell catalyst (Newton *et al.*, 2011).

Polymeric systems have also been studied using NTA. Yang *et al.* (2011) monitored the effects of particle size matched filling of spherical silica on the flowability of epoxy molding compounds for large-scale integrated circuits packaging while Stevens *et al.* (2012) have investigated nanosponge formation from organocatalytically synthesized poly(carbonate) copolymers. Polleto *et al.* (2012) have reviewed the use of polymeric nanocapsules in nanocosmetics and nanomedicines comparing a variety of light scattering techniques, including NTA, with EM.

NTA has been used by Kucherov *et al.* (2012) to analyze the particulate nature of brittle material debris undergoing ballistic impact and proposed that failure waves can be interpreted as the result of the decay of the shock-excited phonon continuum into low frequency peaks in the phonon density of states. Experimental confirmation of this model was reported using fractured particle size analyzes and comparing their results with predicted acoustic wavelengths.

Herrington *et al.* (2012) have studied the effect of the size and size distribution of BaTiO₃ 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 used NTA, amongst other techniques, for determining nanoparticle size and number.

Homeijer *et al.* (2010) discussed polymer-induced liquid-precursor (PILP) process in the non-calcium based systems of barium and strontium carbonate and Carja *et al.* (2010) presented data on nanoparticles of nickel oxide: growth and organization on zinc-substituted anionic clay matrix by a one-pot route at room temperature.

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 75 nm with a relatively broad size distribution of sigma = 31%, but that this broad size distribution had been subsequently narrowed in a single irradiation step to sigma = 20% without a significant change of the mean nanoparticle radius utilizing selective laser tailoring. The use of NTA in nanoparticle production studies by laser pyrolysis and laser ablation had both been described earlier (Sentein *et al.*, 2009; Menéndez-Manjón *et al.*, 2009).

The kinetics of aggregation of initially formed primary particles of alkoxide complexes of rhenium was followed by NTA (Nikonova, 2011). The initial particles with the size below 10 nm aggregated uniformly to spherical particles of several hundred nanometers in size within minutes. The

aggregates could be split into initial small particles again by sonication in a standard ultrasound bath in 5 minutes. Reproducible re-aggregation subsequently followed with formation of the same type of spherical aggregates in the same time scale. This was considered important evidence for the formation of the observed oxide particles through a Micelle Templated Self- Assembly of Ligands (MTSAL) mechanism. Nikonova *et al.* (2011) then demonstrated the role of strongly coordinated inorganic anions on precursor-directed assembly of complex oxide nanobeads using NTA to follow the aggregation process.

Hagmeyer *et al.* (2011) have demonstrated the self-assembly of calcium phosphate nanoparticles into hollow spheres induced by dissolved amino acids by multiple techniques (AFM, SEM, DLS and NTA) and in more recent work (Hagmeyer *et al.* (2011)) have gained direct experimental observation of the aggregation of α -amino acids into 100-200 nm clusters in aqueous solution. Their presence was shown by NTA, AFM, and ESI mass spectrometry. Domingos *et al.* (2010) explored the role of calcium and phosphate in the aggregation of titanium dioxide nanoparticles.

Zhou *et al.* (2011) have used NTA to determine the hydrodynamic diameters of the nanoparticles suspended in water in their attempts to tune the mechanical properties of liquid crystalline nanoparticles. They reported the synthesis of colloidal nanoparticles with an internal structure forming a gel-like matrix. These nanoparticles were composed of low molecular weight liquid crystal (LC) 4-pentyl-4-cyanobiphenyl (5CB) encapsulated in an LC-based polymer network. Using nanoscopic mechanical analysis, they demonstrated the ability to independently tune the shape anisotropy and stiffness by varying respectively the 5CB concentration and the extent of the polymer cross-linking.

Thermosensitive hydrogels were the subject of another study by de Graaf *et al.* (2012) in which they developed a micelle-shedding thermosensitive hydrogel based on poly(N-isopropylacrylamide)-poly(ethylene glycol)-poly(N-isopropylacrylamide) as a sustained release formulation for the delivery of the cytostatic agent paclitaxel (PTX). They showed that, at the highest dose, PTX completely inhibited tumor growth for at least 3 weeks with a single hydrogel injection. This promising concept may find application as a depot formulation for sustained, metronomic dosing of chemotherapeutics.

Pinheiro *et al.* (2012) have reported the preparation and characterization of low dispersity anionic multi-responsive core-shell polymer nanoparticles. The nanoparticles had a glassy poly(methyl methacrylate) (PMMA) core of ca. 40 nm radius and a crosslinked PNIPAM anionic shell with either AA or MA co-monomers, as determined by NTA.

Bewernitz *et al.* (2012) have studied the same material in a meta-stable liquid precursor phase to investigate its interactions with polyaspartate and proposed that charged polyelectrolytes, like acidic proteins, may be employed by invertebrate organisms to direct crystal growth through an intermediate liquid phase in a process called the polymer-induced liquid-precursor (PILP) process. More recently, Dressick *et al.* (2012) used NTA for detection and analysis of polyelectrolyte aggregates in their study on divalent-anion salt effects in polyelectrolyte multilayer depositions,

while Kim *et al.* (2012) used DLS to size, and NTA to measure concentration, of the chitosan-lignosulfonates sono-chemically prepared nanoparticles they described.

Hamed *et al.* (2012) described the synthesis, characterization and surface modification of ZnCrFeO_4 nanoparticles using the sol gel technique with nanoparticle size controlled through a two-stage annealing process. The resulting nanoparticles were found by EM, AFM and X-ray diffraction studies to have excellent crystal quality while NTA was used to estimate the degree of aggregation present.

During room-temperature synthesis of nanocrystalline and monodisperse titanium dioxide Seisenbaeva *et al.* (2013) measured the NTA size distribution of the particles redistributed in ethanol by sonication.

While producing amphiphilic copolymers based on polyoxazoline and grape seed vegetable oil derivatives, Travelet *et al.* (2013) pointed out that DLS results in terms of characteristic size were corroborated using NTA, and also by AFM and TEM imaging, where well-defined spherical and individual nanoparticles exhibited a very good mechanical resistance upon drying

In synthesizing a series of the highly crystalline MFe_2O_4 ferrite spinel, via a modified Bradley reaction using microwave stimulation, particle size was estimated using theoretical calculations from X-ray data as well as by direct experimental techniques such as TEM, DLS and NTA (Wiglusz *et al.*, 2013)). Polycrystalline MgAl_2O_4 spinels had also been studied using NTA in earlier work (Goldstein *et al.*, 2009) and Goldstein *et al.* (2010) had also described the influence of powder type on the densification of transparent MgAl_2O_4 spinel.

Panda *et al.* (2013) used NTA to analyze hydroxyapatite (HAp) prepared from fish scale and synthetic body fluid (SBF) solution to confirm that HAp bio-materials from fish scale are physico-chemically and biologically equivalent to the chemically synthesized HAp from SBF.

For the characterization of phase inversion and emulsification properties pre- and post-inversion, Lefsaker (2013) employed several techniques to investigate the properties of his samples including the conductivity, the e-critical cell, the near-infrared spectroscopy (NIR) and a cone and plate rheometer, but also used NTA for determination of the diffusion coefficient of his sample.

The agglomeration of nanoparticle suspensions has been the subject of significant study and NTA has been used to support other techniques (e.g. hydrodynamic chromatography and single particle-inductively coupled plasma mass spectrometry; Rakcheev *et al.*, 2013) in establishing the size and size distribution of both calibration particles and the agglomerates formed in different nanoparticle systems (Otanicar *et al.*, 2013).

Using a modified NTA system Jakobi *et al.* (2011) have determined the stoichiometry of alloy nanoparticles from laser ablation of PtIr in acetone and their electrophoretic deposition on PtIr electrodes. Hartmann *et al.* (2012) have considered the challenges of testing metal and metal oxide nanoparticles in algal bioassays using titanium dioxide and gold nanoparticles as case studies. Schrittwieser *et al.* (2012) modelled and developed a biosensor based on optical relaxation

measurements of hybrid nanoparticles using NTA to characterize their core asymmetric and magnetic nanoparticles. Using the reprecipitation method, Zhou *et al.* (2013) synthesized poly(N-vinylcarbazole) nanoparticles (PVK NPs) as a model system. Electrochemical sticking and sensing experiments were then conducted, which involve PVK nanoparticle immobilization on the electrode surface and subsequent oxidative sensing, to enable rapid detection of polymer nanoparticles in aqueous solution. They suggested their technique was better than the other techniques they tried, namely DLS and NTA.

Nano-Silica

Mesoporous silica is a form of silica and a recent development in nanotechnology. The most common types of mesoporous nanoparticles are MCM-41 and SBA-15. Research continues on these particles, which have applications in catalysis, drug delivery and imaging. Despite their low refractive index and the resultant difficulty in visualizing them when present at small size (e.g. <40 nm) NTA has been used in their detection, analysis and characterization in many applications.

Monodisperse spherical silica particles are potentially 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 microsphere of a layered silicate produced by using monodisperse silica particles, showing that silica spheres of submicrometer size were covered by a swellable layered silicate, which plays a role in accommodating cationic species.

Luminescence and imaging studies of 500 nm diameter colloidal silica stained with the transition metal complex $[\text{Ru}(\text{bpy})_3\text{Cl}_2]$, $[\text{Ru}(\text{bpy})_3\text{SiNP}]$, have been detailed and suggest that such particles are ideal for particle tracking velocimetry (PTV) or particle imaging velocimetry (PIV) for analysis of fluid flow in microchannels according to Lewis *et al.* (2012). They used NTA to determine the number distribution of particles in the generated sample of $[\text{Ru}(\text{bpy})_3\text{SiNP}]$ of a certain diameter.

Yang *et al.* (2011x) obtained relevant particle size distribution 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.

Yip *et al.* (2012) have investigated the fluorescence anisotropy metrology of electrostatically and covalently labelled silica nanoparticles by comparing the size of silica nanoparticles using the time-resolved fluorescence anisotropy decay of dye molecules when electrostatically and covalently bound to stable silica nanoparticles. Silica nanoparticles produced using Stöber synthesis of tetraethylorthosilicate (TEOS) are found to be controllable between ~3.1 and 3.8 nm radius by adjusting the relative water:TEOS concentration. While the primary particle size was not detectable by NTA, nanoparticle aggregates in LUDOX® colloids were investigated by tracking analysis of particle diffusion via NTA.

Zu *et al.* (2012) described the preparation of ultrafine polyethylene-silica composite particle with a core-shell structure, using SEM observation and NTA to determine that the composite particles

possess a spherical morphology and the mean size is about 160 nm respectively. Bell *et al.* (2012) have discussed optical methods for the characterization of nanoparticles with the latter study being focused on silica. In exploring the concept of fumed silica nanoparticle-mediated biomimicry for optimal cell–material interactions for artificial organ development, de Mel *et al.* (2013) used NTA to determine the respective size of the particles under development.

Finally, Jing *et al.* (2013) have investigated the formation of supported lipid bilayers on silica in relation to lipid phase transition temperature and liposome size. DPPC liposomes ranging from 90 nm to 160 nm in diameter, as measured by NTA, were prepared and used for studies of the formation of supported lipid membranes on silica (SiO₂) at temperatures below and above the gel to liquid-crystalline phase transition temperature (T_m). It was found that liposomes smaller than 100 nm spontaneously rupture on the silica surface when deposited at a temperature above T_m and at a critical surface coverage, following a well-established pathway. In contrast, DPPC liposomes larger than 160 nm do not rupture on the surface when adsorbed at 22 °C or at 50 °C. However, when liposomes of this size are first adsorbed at 22 °C and at a high enough surface coverage, after which they are subject to a constant temperature gradient up to 50 °C, they rupture and fuse to a bilayer.

Nano-Silver

Nanoparticles made of silver are increasingly used as additives for materials and coatings with special biological, optical, and electrical properties. Nano-silver absorbs light at a characteristic wavelength (due to metallic surface plasmons), which leads to a yellow color. This was first applied in the coloring of glassware hundreds of years ago. Today, the constant improvement of methods for the production and characterization of nanoparticles allows a better understanding and utilization of nanotechnology. As regards to optical properties, the embedding of nano-silver and nanoparticles from other metals in transparent materials can be tuned to create optical filters that work on the basis of nanoparticle absorption. Another application of nano-silver that is currently established involves conductive nano-inks with high filling degrees that are used to print highly precise continual conductive paths on polymers.

However, the most relevant characteristic of nano-silver is its chemical reactivity. This leads to an antimicrobial effect of silver that is based on strong bonds between silver ions and groups containing carbon monoxide, carbon dioxide, or oxygen and which prevents the spreading of bacteria or fungi. Nano-silver provides a large number of surface atoms for such antibacterial interaction. This has led to many medical applications of nano-silver, such as in catheters or wound dressings. Meanwhile, there are now many consumer products on the market that contain nano-silver, which has partly raised skepticism regarding product safety.

Khaydarov *et al.* (2012) 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 synthesized colloidal dispersions showed 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 her dispersions with NTA.

Kosmala *et al.* (2011a) have also reported the development of high 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 dependence 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 the reduction of the costs, and high efficiency of the printing process (Kosmala *et al.*, 2011). Kosmala *et al.* (2012) also developed a method for the synthesis of silver nano particles and fabrication of aqueous Ag inks for inkjet printing using the combination of a triblock copolymer and high intensity focused ultrasound (HIFU) while Yosef and Avnir (2011) entrapped dye molecules within submicron silver particles using NTA to show the existence of silver particle-clusters 150–200 nm in size.

The anti-microbial effect of *Murraya koenigii*-mediated synthesis of silver nanoparticles against three human pathogenic bacteria was explored by Bonde *et al.* (2012) using NTA to determine particle size distribution and number concentration during the synthesis. Sable *et al.* (2012) similarly undertook the phytofabrication of silver nanoparticles by using aquatic plant *Hydrilla verticillata* with the help of UV-Vis spectroscopy, FTIR, NTA, Electrophoretic Light Scattering (ELS) and SEM.

Chakraborty *et al.* (2012) investigated the effect of Ag nanoparticle addition and ultrasonic treatment on a stable TiO₂ nanofluid to help the separation and recycling of nanoparticles from fluid waste. NTA was used to determine particle size distribution of TiO₂ nanoparticles.

In discussing the challenges for physical characterization of silver nanoparticles under pristine and environmentally relevant conditions, MacCuspie *et al.* (2011) undertook a rigorous physico-chemical characterization by consensus methods and protocols (where available) which enabled an understanding of how the underlying measurement method impacts the reported size measurements, which in turn provided a more complete understanding of the state (size, size distribution, agglomeration, etc.) of the AgNPs with respect to the dispersion conditions. However, the lower sensitivity NTA instruments that was available at the time was found to be incapable of measuring 10, 20 and 40 nm Ag.

To improve the characterization of nanoparticles, including silver, Klein *et al.* (2011) have, using their expertise in the production and analysis of reference materials, generated a European Commission Joint Research Centre report on a EUR 24693 EN NM-series of representative manufactured nanomaterials in which they assessed '300' silver characterization, stability and homogeneity. Key properties of size and size distribution were studied in an inter-laboratory comparative study using SEM as well as TEM and NTA. As in the work of McCuspie, the sizes of nanosilver tested were found to be outside of the range of NTA instrumentation used.

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; TiO₂, CeO₂, ZnO, Ag nanowires, and Carbon Nanotubes (CNTs). Their data comparing Sp ICP-MS to Disc Centrifuge and NTA revealed a broader size distribution when measured by NTA 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 found to be 32 nm with a concentration of 17.4×10^{10} particles/mL. No aggregations or debris were detected on NTA measurements.

The natural synthesis of Ag nanoparticles was further explored by Meshram *et al.* (2013), who claimed a method that was cost-effective, energy-efficient and easy by using white sugar and sodium hydroxide (NaOH) in the presence of sunlight. They employed visual observation, ultraviolet–visible spectrophotometry, Fourier transform infrared (FTIR), NTA and TEM in their analysis. NTA revealed the polydisperse nature of nanoparticles, 15–30 nm in diameter, while TEM demonstrated the presence of spherical AgNps in the range of 10–25 nm. Similarly, Vezina *et al.* (2013) have extended this work using white sugar and sodium hydroxide (NaOH) in the presence of sunlight to prepare silver nanoparticles (AgNps) in a simple, eco-friendly and economically sustainable way, making it amenable to large-scale industrial production of AgNps. NTA revealed the polydisperse nature of nanoparticles, 15–30 nm in diameter, while FTIR showed the presence of gluconic acid as capping agent, which increases the stability of AgNps in the colloids. TEM demonstrated the presence of spherical AgNps in the range of 10–25 nm.

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 20-50 nm, with the average size of 30 nm.

Raheman *et al.* (2011) used NTA and TEM to show that the silver particles synthesized extracellularly by an endophytic fungus were in the range 10-40 nm and exhibited antibacterial activity against human pathogenic bacteria, while Yadav and Rai (2012) described the bioreduction and mechanistic aspects involved in the synthesis of silver nanoparticles using *Holarrhena antidysenterica*.

Birla *et al.* (2013) reported the rapid synthesis of silver nanoparticles from *Fusarium oxysporum* by optimizing physico-cultural conditions and Bonde *et al.* (2012), from the same group, have similarly described some comparative studies on synthesis of silver nanoparticles by *Fusarium oxysporum* and *Macrophomina phaseolina* and its efficacy against bacteria and *Malassezia furfur*. This group have most recently screened eighteen *Phoma* *sp.* for the mycosynthesis of silver nanoparticles (AgNP's). Out of eighteen, seventeen *Phoma* *sp.* demonstrated mycosynthesis of AgNP's, which were characterized by UV-Vis spectrophotometry, FTIR, XRD, TEM, SEM, NTA and ELS measurement

(Gade *et al.*, 2013a). Gade has also recently described a 'green' extracellular synthesis of silver nanoparticles (SNPs) by *Phoma glomerata* (MTCC-2210) which showed rapid synthesis in bright sunlight. NTA and EM were used to demonstrate the synthesis of polydisperse and spherical SNPs while FTIR revealed the presence of a protein cap on the silver nanoparticle, which led to increase stability of SNP in the silver colloid (Gade *et al.*, 2013b).

The rapid biosynthesis of silver nanoparticles by exploiting the reducing potential of *Trapa bispinosa* peel extract has been described by Pandey *et al.* (2013) using NTA to determine the particle size distribution obtained. This produced monodisperse silver nanoparticles (SNPs) within 120 seconds at 30 °C, which is the shortest tenure reported for SNP synthesis using plants. Gudadhe *et al.* (2013) have recently synthesized silver nanoparticles (Ag-NPs) using an extract of *Ocimum sanctum* leaves that was mixed with agar-agar to prepare an agar-silver nanoparticle (A-AgNp) film. This film was surface-coated over the fruits, *Citrus aurantifolium* (Thornless lime) and *Pyrus malus* (Apple), and evaluated for the determination of antimicrobial activity of A-AgNp films using disc diffusion method, weight loss and shelf life of fruits. Their study demonstrated that A-AgNp films possessed antimicrobial activity and also increased the shelf life of fruits used.

Recently, Neumann *et al.* (2013) have used NTA to investigate the performance of silver nanoparticles in the catalysis of the oxygen reduction reaction in neutral media and generated data showing the nanoparticles produced to be 9 nm in radius. Milczarek *et al.* (2013) reported a one-step synthesis of softwood lignosulfonate-stabilized silver nanoparticles in an aqueous solution at room temperature. As no particles of diameter greater than 100 nm were detected using NTA, the formation of aggregates that was observed by TEM was considered likely to be an artefact of the TEM sample preparation.

Recently, Luque and his colleagues (Luque *et al.*, 2013) have evaluated biomass-derived stabilizing agents for colloidal silver nanoparticles via NTA and concluded that NTA has "been proved to be a highly useful, simple and efficient characterization tool to differentiate between capping efficiencies of various biomass-derived stabilizing agents (e.g. starch, alginic acid and a waste-derived hemicellulosic syrup) of aqueous colloidal silver suspensions".

Electrochemical studies involving the immobilization of nanoparticles from solution at a solid surface followed by anodic stripping voltammetry as a simple technique allowing the analysis of nanoparticle concentrations and identity. Stuart *et al.* (2013) improved the rate of silver nanoparticle adhesion to 'sticky electrodes' in stick and strip experiments at a meso-2,3-dimercaptosuccinic acid (DMSA)-modified gold electrode using NTA to size the adhering particles. The same group also wrote a perspective and guide for experimentalists undertaking electrochemical studies of silver nanoparticles (Tschulik *et al.*, 2013). This latter report summarized four different electrochemical techniques that have been established and frequently used to characterize various properties of silver nanoparticles. These were based on drop casting, in situ nanoparticle sticking and stripping, transfer sticking and stripping or nanoparticle impacts. NTA was used throughout to confirm nanoparticle size, distribution and concentration.

Gold

In applications in medicine and more specifically drug delivery, the dispersion stability of gold nanoparticles plays a significant role on their final performances. With the use of two laser technologies, DLS and NTA, Du *et al.* (2012) reported a simple method to estimate the stability of nanoparticles dispersed in phosphate buffered saline (PBS). By investigating the effects of sonication treatment and surface modification by five types of surfactants, including nonylphenol ethoxylate (NP9), polyvinyl pyrrolidone (PVP), human serum albumin (HSA), sodium dodecyl sulphate (SDS) and citrate ions on the dispersion stability, the varying self-aggregation and adhesion of gold nanoparticles dispersed in PBS were demonstrated. The results showed that PVP effectively prevented aggregation, while HSA exhibited the best performance in avoiding the adhesion of gold nanoparticle in PBS onto glass and metal. Similarly, Treuel *et al.* (2012) quantified the influence of polymer coatings on the serum albumin corona formation around silver and gold nanoparticles employing DLS, TEM, SEM, NTA and/or differential centrifugal sedimentation in their study. Aljabali *et al.* (2011) produced virus-polyelectrolyte-templated gold nanoparticles, his results being supported by NTA data.

Recently, Otsuka *et al.* (2013) described the self-assembly of maltoheptaose-block-polystyrene (MH1.2k-b-PS4.5k), into micellar nanoparticles and the subsequent encapsulation of gold nanoparticles. The mean hydrodynamic radii (R_h) of the nanoparticles were determined by DLS to be ca. 30 and 80 nm depending on the method of preparation. These results were clearly visualized by TEM, AFM and field emission gun-scanning electron microscope imaging, and complemented by NTA.

Mahl *et al.* (2011) reported on the possibilities and limitations of different analytical methods for the size determination of a bimodal dispersion of metallic nanoparticles (silver nanoparticles (about 70 nm) and gold nanoparticles (about 15 nm)). Using SEM, TEM, DLS NTA and analytical disc centrifugation, the differences between the methods were highlighted and their ability to distinguish between silver and gold nanoparticles in the mixture demonstrated. The size distribution data from the different methods were clearly different, therefore it was recommended to apply more than one method to characterize the nanoparticle dispersion. In particular, the smaller particles were undetectable by DLS and NTA in the presence of the large particles. For the 1:1 mixture, only electron microscopy and analytical disc centrifugation were able to give quantitative data on the size distribution. On the other hand, it is not possible to make statements about an agglomeration in dispersion with electron microscopy because an agglomeration may also have occurred during the drying process.

Pettibone and Hudgens (2011) explored gold cluster formation with phosphine ligands: suggesting etching as a size-selective synthetic pathway for small clusters and Yuan *et al.* (2012a) advocated plasmonic gold nanostars as a potential agent for molecular imaging and cancer therapy reporting also on the spectral characterization and intracellular detection of Surface-Enhanced Raman Scattering (SERS)-encoded plasmonic gold nanostars (Yuan *et al.*, 2012b and 2012c). The particle hydrodynamic size distribution, concentration and zeta potential were determined by NTA and ELS. Intracellular detection of silica-coated SERS-encoded nanostars was also demonstrated in breast

cancer cells. The non-aggregated field enhancement makes the gold nanostar ensemble a promising agent for SERS bioapplications.

Yuan *et al.* (2012) further described *in vivo* particle tracking and photothermal ablation using plasmon resonant gold nanostars again using NTA to measure the nanoparticles' hydrodynamic radius, zeta potential, and concentration.

Xie *et al.* (2012a and 2012b) have used NTA to measure the size and number of hollow gold particles in their study of both SERS investigation of hollow gold nanospheres and synthesis, and NIR optically probed properties of hollow gold nanospheres with localized surface plasmon resonance greater than one micrometer.

The development of gold nanostars was also explored by the group of Vo-Dinh in which a variety of analytical techniques, including NTA, was used to investigate the synthesis of gold nanostars which were tagged with a SERS reporter and linked with an MRI contrast agent Gd^{3+} (Liu *et al.*, 2013). *In vitro* experiments demonstrated the developed nanoprobe to be a potential theranostics agent for future biomedical applications. In an expansion of this work Wang *et al.* (2013) described a SERS-based detection approach, referred to as "molecular sentinel" plasmonic nanoprobe, to detect an RNA target related to viral infection. This work arose from their earlier work on the production of silica-coated gold nanostars for combined SERS detection and singlet oxygen generation as a potential nanoplatform for theranostics (Fales *et al.* (2011)).

Given protein-conjugated gold nanoparticles (AuNPs) have been extensively explored for the development of many novel protein assays, James and Driskell (2012) demonstrated that NTA can be used as a rapid and simple analytical tool to monitor bioconjugation and to study protein-protein interactions. Firstly the adsorption of protein A onto gold nanoparticles was analyzed using NTA resulting in a measureable increase in hydrodynamic radius that correlated with protein A concentration. NTA was then used to investigate the binding of mouse IgG to Protein A-conjugated AuNPs and the K_a was measured as $2.00 \times 10^7 M^{-1}$. Furthermore, an assay for the detection of mouse IgG was developed using NTA to detect the binding to antibody-AuNP conjugates exhibiting a detection limit of 3.2 ng/mL. However, the formation of aggregates resulting from the use of a polyclonal antibody and multiple binding sites on the antigen prevented the determination of binding affinity for this antibody-antigen system. To measure the binding affinity for this antibody-antigen system the IgG antigen was conjugated to the AuNPs and NTA was used to monitor the binding of the antibody. In this configuration aggregation of conjugates was not detected and a binding affinity constant of $2.80 \times 10^8 M^{-1}$ was measured. NTA results obtained in this work were validated by comparison to DLS. This work represented the first evaluation of NTA as an analytical tool for characterizing AuNP bioconjugates, investigating protein-protein binding, and detecting low levels of antigen in a bioassay.

In their work on the generation of representative nanomaterials (e.g. silver) for subsequent use in toxicological studies, the European Commission Joint Research Centre have recently reported their characterization of 'NM-300', (a representative manufactured nanomaterial) by a number of sophisticated techniques which resulted in the development and validation of a dedicated method

according to ISO17025 principles. They reported that key properties of size and size distribution were studied in an inter-laboratory comparative study using SEM, TEM and NTA (Klein *et al.*, 2011).

Jiang *et al.* (2013) used multiple techniques, namely NTA, differential centrifugal sedimentation (DCS), UV-visible spectroscopy (UV), second order spectroscopy (SOS) and TEM to follow the slow agglomeration of gold colloids of approximate diameter 30 nm in the presence of a small concentration of L-cysteine·HCl. This work was described more fully by Jiang (2013).

In a similar vein, Engelbrekt *et al.* (2013) investigated the complexity associated with the time-dependent physical and chemical properties in aqueous solution during the chemical synthesis of gold nanoparticles (AuNPs) synthesized from gold salt (HAuCl₄). Chemical synthesis of AuNPs is a reduction process accompanied by release of ions and protons and formation of solid particles. Dynamic information from redox potential, pH, conductivity, and turbidity of the solution enables distinct observation of reduction and nucleation/growth of AuNPs phases with NTA being used to monitor, in real time, the formation of gold nanoparticles.

Iron Oxide

The synthesis of iron oxide (magnetic) nanoparticles by a filtrate of *Phoma glomerata* (a plant pathogen) has been reported by Gudadhe *et al.* (2012), NTA being used to reveal polydisperse nanoparticles with average size of 56 nm.

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 display excellent acidic resistance and adsorption properties even in an acidic solution (pH = 3).

The synthesis, solution stability and ⁶⁴Cu²⁺ labelling of magnetite nanoparticles (NPs) coated with different macrocycles has been reported by Barreto *et al.* (2011) using NTA to demonstrate that the NPs formed a stable colloidal suspensions in 0.05 M aqueous 2-(N-morpholino)ethanesulfonic acid (MES) buffer, which consist 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 behavior of nanoparticles of zero valent iron (NZVI), Cirtiu *et al.* (2011) measured the size, surface characteristics and colloidal stability of zero valent iron nanoparticles post and pre-treatment. TEM images and NTA revealed that iron nanoparticles synthesized in the presence of the polymers were larger in diameter, with TEM mean diameters ranging from 84.5 to 189 nm, than the bare-NZVI (59.1 nm), 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 analyzes to investigate the possible formation of nanoparticles in solution. Experiments performed include DLS and NTA. Catalytic reactions had very

low concentration of nanoparticles in solution (< 0.1 ppm), that was below the limit of detection for DLS and accordingly it was not possible to have a reliable size distribution measurement. NTA experiments were shown to be more sensitive in the range of 10 nm to 2000 μm , and measured values of particles/mL were in the same magnitude order 0.76×10^8 particles/mL as the blank experiments. Finally, Kadar *et al.* (2011) have shown the stabilization of engineered zero-valent nanoiron with Na-acrylic copolymer enhances spermotoxicity using NTA to detect aggregation behavior.

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Chapter XIV: Materials and Misc

Miscellaneous Materials

Binns *et al.* (2012) have reported on a new method to produce liquid suspensions of hydrosol suspensions of elemental and core-shell nanoparticles by co-deposition with water vapor from the gas-phase in ultra-high vacuum conditions. They extended the method to include core-shell nanoparticles, in which there was flexible control over the core size and shell thickness and free choice of the material in either. NTA was used to check for aggregation while DLS measured the primary particle size of approximately 5nm. Using a modified NTA system, Jakobi *et al.* (2011) have determined the stoichiometry of alloy nanoparticles from laser ablation of PtIr in acetone and their electrophoretic deposition on PtIr electrodes.

A model for metal spherical particle formation was proposed by Irizarry (2010) guided by optical kinetic data of monodisperse metal colloid synthesis. Using a new strategy, called simulated dynamic optical response, it was found that autocatalytic formation of primary particles followed by a zone of very fast aggregation mechanism can describe the dominant dynamics during early stages. In later stages, the dominant mechanism switches to slower aggregation modulated by a stability factor.

Capretto *et al.* (2011), through experimental and computational analysis, worked on the continuous-flow production of polymeric micelles in microreactors demonstrating that microfluidic reactors provide a useful platform for the continuous-flow production of polymeric micelles with improved controllability, reproducibility and homogeneity of the size characteristics. Capretto has also looked at the mechanism of co-nanoprecipitation of organic actives and block copolymers in a microfluidic environment (Capretto *et al.*, 2012).

In their study on nanoscale clustering and nucleation in aqueous glycine solutions, Jawor-Baczynski *et al.* (2012) used several analytical techniques including small angle x-ray scattering and static and dynamic light scattering, NTA was used specifically to detect much larger clusters on the order of hundreds of nanometres, their presence confirmed by both SAXS and DLS. Wark *et al.* (2011) investigated the dynamic multimodal surface plasmon enhanced imaging of single nanoparticles and assembled clusters in suspension also using NTA for aggregate analysis.

Liposomes are artificially prepared vesicles consisting of natural and synthetic phospholipids that are widely used as a cell membrane mimicking platform to study protein-protein and protein-lipid interactions, monitor drug delivery and encapsulation and Morton *et al.* (2012a) have developed a constant pressure-controlled extrusion method for the preparation of nano-sized lipid vesicles. Manual extrusion using gas-tight syringes and polycarbonate membranes is a common practice but heterogeneity is often observed when using pore sizes <100 nm due to variability of manual pressure applied. They employed a constant pressure-controlled extrusion apparatus to prepare synthetic liposomes whose diameters range between 30 and 400 nm. DLS, EM and NTA were used to quantify the liposome sizes as described in their protocol, with commercial polystyrene beads used as a calibration standard.

Complexes of dyes and polyelectrolytes have found widespread use in a variety of functional materials and interfaces and Helseth (2012), in a study of pyranine-induced self-assembly of colloidal structures using poly(allylamine-hydrochloride), found that upon mixing the anionic dye pyranine and a cationic polyelectrolyte, poly(allylamine-hydrochloride), two different colloidal structures may form. NTA was used to follow changes in particle sizes with time. Combining fluorescence measurements with studies of the particle size with time, it was found that red-shift was related to the crosslinking of the dye and the polyelectrolyte and was not influenced significantly by the aggregation and particle growth.

In a most interesting variation on the conventional use of NTA, Wilson *et al.* (2012a) have tracked the autonomous movement of platinum-loaded stomatocytes. Polymer stomatocytes are bowl-shaped structures of nanosize dimensions formed by the controlled deformation of polymer vesicles. The stable nanocavity and strict control of the opening are ideal for the physical entrapment of nanoparticles which, when catalytically active, can turn the stomatocyte morphology into a nanoreactor. They reported the generation of autonomous movement of the polymer stomatocytes by selectively entrapping catalytically active platinum nanoparticles within their nanocavities and subsequently using catalysis as a driving force for movement. Hydrogen peroxide is free to access the inner stomatocyte cavity, where it is decomposed by the active catalyst (the entrapped platinum nanoparticles) into oxygen and water. This generates a rapid discharge, which induces thrust and directional movement. The design of the platinum-loaded stomatocytes resembles a miniature monopropellant rocket engine, in which the controlled opening of the stomatocytes directs the expulsion of the decomposition products away from the reaction chamber (inner stomatocyte cavity). NTA was used to detect and visualize the population of nanoparticles. Wilson *et al.* (2012b) have further extended this work to study, using NTA, the way in which the speed of these nanomotors can be controlled by varying the fuel concentration.

Finally, Jornada *et al.* (2012) have recently established the mechanism of self-assembly, control of size and loading capacity of lipid-core nanocapsules for use in drug delivery. Nanocarriers have been developed as drug delivery systems to be administered by different biological routes. To ensure the nanotechnological properties, pre-formulation studies are especially critical in determining the influence of the process parameters on the size and polydispersity of particles. Thus, the objective of this work was to establish the mechanism of self-assembly, by determining the influence of the critical aggregation concentration of the materials in the organic phase on the final average particle size and polydispersity of polymeric lipid-core nanocapsules obtained by interfacial deposition of polymer using NTA. Measurements of the surface tension and viscosity of the organic and aqueous phases were correlated with the particle size and the concentration of raw materials. They demonstrated that the lipid-core nanocapsules are formed on the nanoscopic scale as unimodal distributions but only if the aggregation state of raw materials in the organic phase tends to infinite dilution. The strategy for controlling the particle size distribution is a valuable tool in producing lipid-core nanocapsule formulations with different loading capacities intended for therapeutics.

Composite materials

Green *et al.* (2012) have recently developed multicomponent degradable cationic polymers that self-assemble with DNA to form particles that are effective for gene delivery while formulations of lipid-core nanocapsules, stabilized with polysorbate 80-lecithin and uncoated or coated with chitosan (LNC and LNC-CS), were prepared and characterized by laser diffraction ($D_{[4,3]}$: 129 and 134 nm), DLS (119 nm and 133 nm), NTA (D_{50} : 124 and 139 nm) and particle mobility analysis (zeta potential: -15.1 mV and $+9.3$ mV).

Combining several different materials types, Fattisson *et al.* (2010) established the roles of solution chemistry and organic molecules on deposition of carboxymethylcellulose-coated zero-valent iron nanoparticles onto silica and Pazik *et al.* (2011) used BaTiO_3 as a case study to investigate the surface functionalization of the metal oxide nanoparticles with biologically active molecules containing phosphonate moieties. Donati *et al.* (2011) have filed patents on nanocomposite materials based on metallic nanoparticles stabilized with branched polysaccharides.

Stevens *et al.* (2012) described nanosponge formation from organocatalytically synthesized poly(carbonate) copolymers and Guerrini *et al.* (2012), in tuning the interparticle distance in nanoparticle assemblies in suspension via DNA-triplex formation, established a correlation between plasmonic and SERS responses. They exploited the triplex-assembling ability of DNA-conjugated silver nanoparticles to engineer interparticle junctions with controlled interparticle distance and tuned the aggregation rate to allow accurate investigation into the correlation between the averaged time-dependent plasmonic and SERS responses within a complex ensemble of nanoparticles in suspension.

In reporting recent modelling and design work indicating that mixtures of nanoparticles in liquids can be used as an alternative to conventional optical filters Taylor *et al.* (2013) used NTA to show that the commercially available nanofluids they were assessing as the basis for producing long-pass, short-pass and band-pass optical filters contained larger particles than the manufactured stated mean.

Mariz *et al.* (2013) used NTA-analyzed hybrid nanoparticles dispersed in water prepared from selected polymers with two-photon excited fluorescence emission that competed with those of the best performing quantum dots during their study of the molecular architecture effect in two-photon absorption. In studying VI semiconductors as promising nanomaterials for applications as window layers in low-cost and high-efficiency thin film solar cells Tripathi (2013) reviewed the present status of nanoparticle-doped polymers as examples of types of inorganic/organic hybrid nanocomposite materials. He used NTA to characterize these nanocomposites.

In their description of a one-pot phase transfer and surface modification of CdSe/ZnS quantum dots by a synthetic functional copolymer which did not require coupling agents and multistep reactions, Finetti *et al.* (2013) used NTA to highlight that the particle distribution of in-house and commercial phase transferred QDs were very similar though they underlined that, for very small nanoparticles, such as those reported, the absolute size value measured with NTA is less accurate than that

measured with DLS. However, they did employ NTA to generate concentration data which was not available from DLS and could only be surmised from an absorbance curve.

While colloidal scale mesospecies (nanodroplets) were previously reported in supersaturated solutions of glycine and DL-alanine amino acids and were implicated as intermediates species on a non-classical crystallization pathway, Jawor-Baczynska *et al.* (2013) used NTA, amongst other techniques, to show that the mesospecies are also present in significant numbers in undersaturated solutions even when the solute concentration is well below the solid-liquid equilibrium concentration.

Sensors

Nanoparticles can be used for detection purposes for the quantification of nucleic acid. Thus, Wang and Vo-Dinh (2011) described using plasmonic coupling interference nanoprobe for nucleic acid detection using SERS in which NTA was needed to show the potential of nucleic acid diagnostic tools for biomedical diagnostics and biosensing applications. Similarly, Kell *et al.* (2011) developed a silica nanoparticle-based DNA biosensor capable of detecting *Bacillus anthracis* bacteria through the use of unlabelled ss-oligonucleotides. The biosensor makes use of the optical changes that accompany a nanoparticle-immobilized cationic conjugated polymer (polythiophene) interacting with single-stranded vs. hybridized oligonucleotides, where a fluorescence signal appears only when hybridized DNA is present (i.e. only when the ss-oligonucleotide interacting with the polymer has hybridized with its complement). NTA was used to show that the silica nanoparticle scaffold employed in this investigation was 188 ± 30 nm in diameter as measured by TEM and 196 ± 36 nm in diameter measured by NTA.

Schrittwieser *et al.* (2012) modelled and developed a biosensor based on optical relaxation measurements of hybrid nanoparticles using NTA to characterize their core asymmetric and magnetic nanoparticles. A range of different nanoparticles have been proposed as sensing structures. Thus Kumar *et al.* (2011) have reported the bioconjugation of InGaP quantum dots for molecular sensing, while Eremenko *et al.* (2012) describe the use of manganese dioxide nanostructures as a novel electrochemical mediator for thiol sensors. Finally, Jayapaul *et al.* (2012) described the preparation of riboflavin carrier protein-targeted fluorescent USPIO for the assessment of vascular metabolism in tumors. NTA was used in all of these studies to characterize the materials employed.

Membrane curvature and lipid composition regulates important biological processes within a cell. Currently, several proteins have been reported to sense and/or induce membrane curvatures such as synaptotagmin-1 and amphiphysin (Saludes *et al.*, 2012) and Morton *et al.* (2012) have identified a 25-mer peptide, MARCKS-ED, based on the effector domain sequence of the intracellular membrane protein myristoylated alanine-rich C-kinase substrate, that can recognize PS with preferences for highly curved vesicles in a sequence specific manner. These studies further contribute to the understanding of how proteins and peptides sense membrane curvature, as well as providing potential probes for membrane shape and lipid composition, NTA being used to monitor vesicle size.

Calò *et al.* (2012) have exploited natural vesicles produced from genetically engineered cells with tailored membrane receptor composition as promising building blocks for sensing biodevices. Using NTA to establish vesicle size, they then employed AFM to show that nanovesicles deposit and flatten without rupturing on glass substrates claiming this to be an important step in the practical realization of biosensor devices based on natural nanovesicles integrating G-protein coupled membrane receptors.

More recently, Sigolaeva *et al.* (2013) reported the use of co-assemblies of micelle-forming diblock copolymers and enzymes on a graphite substrate for an improved design of biosensor systems using NTA to characterize the diblock copolymers in aqueous solution which formed star-like micelles with a hydrophobic PB core and a cationic corona built up from either strong cationic PDMAEMAq or pH-sensitive PDMAEM.

In presenting a total internal reflection fluorescence microscopy based bioanalytical assay for the detection of whole viral particles with single virus sensitivity and specifically focussing on the detection of human norovirus, a highly infectious virus causing gastroenteritis, Bally *et al.* (2013) showed that NTA-estimated number concentrations of their virus samples matched well the titre reported by other methods.

Yang *et al.* (2013) have assessed transthyretin as both sensor and scavenger of abeta oligomers. Transthyretin (TTR) is a homotetrameric transport protein, assembled from monomers that each contains two four-stranded β -sheets and a short α -helix and loop. In the tetramer, the 'inner' β -sheet forms a hydrophobic pocket while the helix and loop are solvent-exposed. Beta-amyloid ($A\beta$) aggregates bind to TTR, and the binding is significantly reduced in mutants L82A (on the loop) and L110A (on the inner β -sheet). They exploited NTA, as a "novel technique", to show that TTR arrests $A\beta$ aggregation by both preventing formation of new aggregates and inhibiting growth of existing aggregates.

In another biosensor application, di Gennaro (2013) studied the tryptophan-terbium FRET pair interaction coupled to silver nanoprisms supporting the observation that while plasmonic coupling between fluorophores and metal surfaces has become a focal point of optical research during the last two decades, the interactions of FRET couples with metal surfaces remain relatively unexplored. NTA was used to measure silver nanoprism size and concentration. Silver nanoparticles were also used in the electrochemical detection of chloride levels in sweat as a basis for the preliminary screening for cystic fibrosis, NTA being used to size the silver nanoparticles in the solution-phase (Toh *et al.* (2013).

Similarly, Wang *et al.* (2013) have employed silver nanoparticles and Raman dye-labelled DNA hairpin probes as a SERS-based detection technique, referred to as "molecular sentinel" plasmonic nanoprobe, to detect an RNA target related to viral infection, the hydrodynamic size distribution of the bare nanoparticles being measured by NTA. They claimed that, with the use of a portable Raman spectrometer and total RNA samples, they had demonstrated for the first time the potential of the MS nanoprobe technology for detection of host-response RNA biomarkers for infectious disease diagnostics.

Nanoparticulate gold has also found increasing application in the sensing field and recent reports in which NTA has been used to help characterize such materials are summarized here.

The very strong optical resonances of plasmonic nanostructures can be harnessed for sensitive detection of chemical and biomolecular analytes in small volumes and Werts *et al.* (2013) have recently described an approach towards optical biosensing in microfluidic systems using plasmonic structures (functionalized gold nanoparticles) in colloidal suspension using NTA to address aspects of nanoparticle functionalization. Particle concentration was measured directly using NTA in the study by Lui *et al.* (2013) on quintuple-modality (SERS-MRI-CT-TPL-PTT) plasmonic gold nanostar nanoprobe for theranostics.

McLintock *et al.* (2013) described the preparation and characterization of stable and non-aggregated colloidal suspensions of gold nanorod–molecular dye complexes which exhibit very bright SERS signals. The polymer stabilized nanorod–dye conjugates were prepared without the added complexity of nanoparticle aggregation as well as having good control over the surface coverage and orientation of the dye molecules. Furthermore, they demonstrated that this new class of Raman nanotags greatly outperformed an approach based on quasi-spherical gold nanoparticles. Additional characterization of the particle concentrations and aggregation state of the NR–dye conjugate concentrations was performed using NTA.

More recently, Morasso *et al.* (2013) have undertaken the one-step synthesis of star-like gold nanoparticles for SERS. Using NTA to determine particle size and size distribution they showed that the particles exhibited excellent properties for SERS and, when compared with spherical nanoparticles with similar size and concentration, showed enhancing factors from 10 to 50 times higher depending on the dye and on the wavelength employed.

Gold was further employed for single particle luminescence imaging in cells when modified with high coatings of Ru(II) complexes. Using NTA to confirm sample uniformity, Pikramenou *et al.* (2013) showed that single 100 nm particles could be observed in whole cell luminescence imaging and which revealed their biomolecular association with chromatin in the nucleus of cancer cells.

In attempting to overcome the limitations inherent in *in vivo* monitoring system designs through invasive implantation procedures and biofouling, Cash and Clark (2013) recently demonstrated the first success in optically tracking histamine levels *in vivo* using a modular, injectable sensing platform based on diamine oxidase and a phosphorescent oxygen nanosensor in which NTA was used specifically to estimate particle concentration.

Olsson *et al.* (2013) resorted to NTA and TEM to confirm that their technique, based on quartz crystal microbalance with dissipation (QCM-D) monitoring, was capable of evaluating the size of nanoparticles deposited on surfaces. They showed that the mean nanoparticle sizes obtained by QCM-D were generally in closer agreement with the primary particle size determined by TEM and NTA than with the sizes obtained by DLS. Díaz (2013) also used NTA as well as DLS to confirm polymersome formation with the particular polymer in his production of water soluble

photochromic fluorescent nanoprobe based on diheteroarylethenes and polymer coated quantum dots.

In characterizing the nonlinear optical properties of nanocrystals by hyper-Rayleigh scattering (HRS), Joulaud *et al.* (2013) investigated by HRS measurements the second harmonic properties of BaTiO₃, KNbO₃, KTiOPO₄, LiNbO₃ and ZnO nanocrystals (NCs). It proved necessary to carefully analyze the nanocrystal suspensions and both DLS and NTA were used in this capacity. The data on two types of LiNbO₃ NCs is of interest because the NTA plots match the DLS data only when it was plotted by a number distribution format. The usual, and recommended, format for presenting DLS data is to employ the intensity profile only but significant problems and misinterpretations frequently arise otherwise. The fact that, in this case, the extrapolation from intensity distribution to number distribution appears sound is probably due to the relative monodispersity of the sample in the first place. Even then, NTA data shows more structure in the distribution than is possible to obtain from the low resolution methods of DLS.

In a recent report, Jang *et al.* (2013) introduced a new surface-based sandwich assay for the direct detection of B-type natriuretic peptide (BNP), an important biomarker for cardiac failure, at concentrations ranging from 1 nM to 500 nM. NTA was used to establish the particle density of 1.07×10^{14} particles/L for a nanocube sample exhibiting a UV-vis extinction suitable for nanoparticle-enhanced surface plasmon resonance where a DNA aptamer is immobilized on a chemically modified gold surface in conjunction with the specific adsorption of antiBNP coated gold nanocubes in the presence of the biomarker target.

Finally, Rho *et al.* (2013) have described a magnetic nanosensor for detection and profiling of erythrocyte-derived microvesicles, using NTA to confirm the size of filtered MVs to be an average size of 167 nm. This work was undertaken in an attempt to overcome the lack of sensitive, standardized MV assays which pose a significant barrier to implementing MV analyzes into clinical settings.

Carbon and Carbon Nanotubes

Following earlier and preliminary use of NTA to characterize various carbonaceous nanomaterials such as carbon nanotube-nematic liquid crystal composite materials (Trushkevych *et al.*, 2007 and 2008) and the oxidative potential of a panel of carbonaceous and metallic nanoparticles (Hohl *et al.*, 2009), more recent work using NTA has focused on carbon nanotubes and nanocolloids.

To assess the removal efficiency of formaldehyde using nano-size carbon colloid, which was produced by a comparatively easy and cheap method, Kim *et al.* (2011) produced nano-size carbon colloid based on water by an electro-chemical method. 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 behavior in different matrices. Thus, Schwyzer *et al.* (2011) 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 more extended study on the long-term colloidal stability of 10 carbon nanotube types in the absence/presence of humic acid and calcium.

Recently, Zemanova has investigated the cytotoxicity of a water-soluble, radioprotective C₆₀ fullerene derivative (DF) which had been obtained by a reaction of C₆₀ fullerene with peracetic acid and subsequent hydrolysis. She used NTA to show monodisperse DF was less cytotoxic to cell cultures than an unfiltered, polydisperse equivalent which coagulated on cell surfaces (Zemanova *et al.*, 2011). Clements (2013) has shown that while DLS data for a sample of a C₆₀ colloids indicated a bimodal distribution and that the larger particles detected by DLS are beyond the range of the NTA instrument, the NTA particle size distribution for this sample picked up mainly the particles slightly larger than 100nm. The mode of the NTA particle size distribution for C₆₀ agreed quite well with the number distribution data given by DLS.

The cellular toxicity of C₆₀ fullerenes in RAW 264.7 immortalized macrophages has been studied by Russ (2013) and showed that exposure of immune cells to C₆₀ fullerenes results in uptake of the nanoparticles and alterations in the normal functions of the cell. NTA was used to analyze the size of C₆₀ Fullerene and terbium endohedral Fullerene aggregates.

In a study of the photoacoustic contrast imaging of biological tissues with radiation-damaged nanodiamonds fabricated for high near-infrared absorbance, Zhang *et al.* (2013) used NTA to carry out size determinations.

Reed *et al.* (2013) used using single particle-inductively coupled plasma-mass spectrometry (spICPMS) to detect single walled carbon nanotubes by monitoring embedded metals using trace catalytic metals intercalated in the CNT structure as proxies for the nanotubes. Interestingly, analysis of split samples by both spICPMS and NTA showed the quantification of particle number concentration by spICPMS to be *several orders of magnitude lower* than by NTA. They postulated that this was a consequence of metal content and/or size, caused by the presence of many CNTs that do not contain enough metal to be above the instrument detection limit, resulting in undercounting CNTs by spICPMS. However, they claimed that since the detection of CNTs at low ng L⁻¹ concentrations is not possible by other techniques, spICPMS was still a more sensitive technique for detecting the presence of CNTs in environmental, materials, or biological applications. In a recent patent filing Fahmy *et al.* (2013) have described carbon nanotube-based compositions for activating cellular immune responses supporting their claims with NTA data on analysis of their magnetite and CL-2 loaded PLGA nanoparticles.

Finally, Sun *et al.* (2013) have investigated the adsorption of size-selected Pt colloidal nanoparticles on high-surface-area graphene powders for methanol oxidation reaction given graphene-supported nanoparticles are of tremendous interest for a variety of applications recently. They found that the adsorption of Pt colloidal nanoparticles on graphene surfaces is dramatically influenced by the

process parameter in the mixing process and, specifically, the different solution volumes during the mixing process result in various catalyst morphologies.

Most recently, Chen *et al.* (2013) have used NTA to characterize the interactions between protein and carbon black (CB) in which they revealed that the CB can react with proteins (55kDa and 70kDa) after inhalation and may modify the functional structures of lung proteins, leading to the activation of acute-inflammatory responses in the lungs.

Magnetics

Superparamagnetic nanoparticles have potential applications in targeted drug delivery and as magnetic resonance imaging contrast agents. Magnetite clusters are of particular interest for these applications because they provide higher magnetic flux (under a magnetic field) than individual magnetite nanoparticles, are biocompatible and their size and compositions can be controlled. Mejia-Ariza (2010) described the design, synthesis, and characterization of magnetite clusters using a multi inlet vortex mixer. Following earlier work on superparamagnetic nanoparticles and non-oxidic iron core-shell nanomagnets (Yiu *et al.*, 2008 and Herrmann *et al.*, 2009 respectively), in which NTA was used to characterize nanoparticle suspension purity, the technique has found increasing use in this field of magnetic nanoparticle research.

Etgar *et al.* (2010) reported the trajectory control of PbSe- γ -Fe₂O₃ nanoplateforms under viscous flow and an external magnetic field. The flow behavior of nanostructure clusters, consisting of chemically bonded PbSe quantum dots and magnetic gamma-Fe₍₂₎O₍₃₎ nanoparticles, was investigated. The clusters are regarded as model nanoplateforms with multiple functionalities, where the gamma-Fe₍₂₎O₍₃₎ magnets serve as transport vehicles, manipulated by an external magnetic field gradient and the quantum dots act as fluorescence tags within an optical window in the near-infrared regime. The clusters' flow was characterized by an NTA instrument to visualize their trajectories within a viscous fluid (mimicking a blood stream). The trajectories were examined under various flow rates, viscosities and applied magnetic field strengths and the results revealed a control of the trajectories even at low magnetic fields (<1 T), validating the use of similar nanoplateforms as active targeting constituents in personalized medicine.

Paquet *et al.* (2011) developed a new form of particle generating a synergistic enhancement of the T2 relaxation using clusters of superparamagnetic iron oxide nanoparticles (SPIONs) encapsulated in a hydrogel using NTA to follow increase in particle diameter with progressive coatings and Song *et al.* (2011) prepared PANI/nano-ZnO composites prepared by *in-situ* polymerization under a magnetic field. NTA has also been used in to follow intermetallic magnetic nanoparticle precipitation by femtosecond laser fragmentation in liquids (Yamamoto *et al.* 2011).

The field of magnetic nanoparticles and their biomedical applications has been reviewed by Banerjee *et al.* (2010) and Rieger *et al.* (2012) have developed antibody-labelled superparamagnetic nanoparticles for the visualization of benzo[a]pyrene in porous media with magnetic resonance imaging in an attempt to achieve advanced visualization and quantification tools to link *in vitro* experiments with natural systems. The surface coatings of proteins on superparamagnetic iron

oxide nanoparticles (SPIONs) that form immediately on contact with a biological milieu were assessed using a variety of techniques, including NTA, following stabilisation of the SPION with citric acid, poly(acrylic acid) or double layer oleic acid (Jedlovsky-Hajdú *et al.*, 2012).

More recently, Bruckman *et al.* (2013) have reported on the development of supramolecular high-relaxivity MRI contrast agents using the plant viral nanoparticle tobacco mosaic virus (TMV). Rod-shaped TMV nanoparticles measuring 300×18 nm were loaded with up to 3500 or 2000 chelated paramagnetic gadolinium(III) ions selectively at the interior or exterior surface, respectively. Spatial control is achieved through targeting either tyrosine or carboxylic acid side chains on the solvent exposed exterior or interior TMV surface. Further, they showed that interior-labelled TMV rods can undergo thermal transition to form 170 nm-sized spherical nanoparticles containing ~25 000 Gd chelates and a per particle relaxivity of almost 400 000 mM⁻¹ s⁻¹ (15.2 mM⁻¹ s⁻¹ per Gd). NTA was used to size the nanoparticles.

Finally, in showing that induced clustered nanoconfinement of superparamagnetic iron oxide in biodegradable nanoparticles enhances transverse relaxivity for targeted theranostics, Ragheb *et al.* (2013) used NTA to both visualize and analyze the sample allowing them to conclude that fatty acid modified iron oxide prolonged retention of the contrast agent in the polymer matrix during degradative release of drug. Antibody-fatty acid surface modification facilitated cellular targeting and subsequent internalization in cells while inducing clustering of encapsulated fatty-acid modified superparamagnetic iron oxide during particle formulation and that, accordingly, clustering of superparamagnetic iron oxide in poly(lactide-co-glycolide) did not affect the controlled release of encapsulated drugs such as methotrexate or clodronate and their subsequent pharmacological activity.

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Chapter XV:Industrial Applications

Nanomaterials in industry – a measurement requirement.

The increasing number of nanomaterial based consumer products raises concerns about their possible impact on the environment. Suitable methods for their analysis are a particular problem in this regard. As discussed by Paterson *et al.* (2011) there is an urgent need for standardized methods and environmental monitoring programs for anthropogenic nanoparticles in order to appropriately assess the risks to biological species due to potential nanoparticle exposure. In doing so they issued a challenge to national and international regulatory and research agencies to help develop standard methods, quality assurance tools, and implement environmental monitoring programs for this class of pollutants thereby generating baseline data that could facilitate the environmental risk assessment evaluations that are currently virtually absent.

In a similar vein, Dean (2012) emphasized the need to produce stable reference materials while preventing agglomeration such that the behavior of the nanomaterials needs to be controlled by modifying the surface of the particles. He suggested that such modified particles could then be “characterized in biological solutions using instrumentation such as Nanoparticle Tracking Analysis (NTA), which offers a unique method for visualizing and analyzing particle size and size distribution by relating the rate of Brownian motion to particle size”. He advocated combining NTA with a label-free, real-time, cell-electronic sensing system to measure changes in cell number following nanoparticle exposure so that nanoparticles in complex suspensions could be characterized in terms of size, distribution, and toxicity

NTA has recently become an ASTM method for the analysis of particle size distribution of nanomaterials in suspension being one of the very few techniques that are able to deal with the measurement of particle size distribution in the nano-size region, The ASTM (2012) guide describes the NTA technique for direct visualization and measurement of Brownian motion, generally applicable in the particle size range from several nanometers until the onset of sedimentation in the sample and is acknowledged as being capable of being routinely applied in industry and academia as both a research and development tool and as a QC method for the characterization of submicron systems.

Given the recognized importance of the subject 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 represent a particularly difficult challenge, the European Commission published in October 2011 its recommendation on the definition of ‘nanomaterial’. This will have an impact in many different areas of legislation, such as the European Cosmetic Products Regulation, where the current definitions of nanomaterial will come under discussion regarding how they should be adapted in light of this new definition. This new definition calls for the measurement of the number-based particle size distribution in the 1–100nm size range of all the primary particles present in the sample independently of whether they are in a free, unbound state or as part of an aggregate/agglomerate. Recently, Linsinger *et al.* (2012) have

analyzed the requirements on measurements for the implementation of the European Commission definition of the term 'nanomaterial'.

Calzolari *et al.* (2012) have subsequently reviewed methods for measuring nanoparticles 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 used techniques for the size measurement of nanoparticles when addressing this new definition of nanomaterials illustrating the problems to be overcome in measuring nanoparticles in food and consumer products with some practical examples. In assessing NTA and in comparison the other such techniques, they acknowledged that NTA was effective in overcoming the inherent weaknesses of the DLS and static light scattering methods when confronted with mixtures of relatively similarly sized particles and had a number of important advantages including relatively low instrument cost and high sensitivity which can detect nanoparticles at concentrations as low as 10^6 particle/cm³. They did point out however, the inherent limitation of the technique in not being able to detect nanoparticles below 10-20nm meant it did not meet the full requirements of the EU definition and was, furthermore, a technique which required expertise on the part of the operator. In analyzing foodstuffs, Famelart *et al.* (2013) recently used NTA to determine heat-induced effects on the particle size distribution of casein micelles through the formation of disulphide bonds formed during acid gelation of preheated milk in the presence and absence of N-ethylmaleimide (NEM), a thiol-blocking agent.

Paper, Inks, Printing and Coatings.

The use of engineered nanoparticles as additives to papers and as coatings materials and inks has been increasingly studied over the last few years.

Lamminmäki and her co-workers have described studies using NTA into the reported short timescale inkjet ink component diffusion as an active part of the absorption mechanism into inkjet coatings (Lamminmäki *et al.*, 2011a) 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 is 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 4msecs from the time of ink application on a high-speed inkjet printing press (Lamminmäki *et al.*, 2011b). She described in detail the various parameters associated with the comparative dynamics of bulk liquid flow and interpolymer diffusion during inkjet ink imbibition in porous coating structures (Lamminmäki, 2012).

Kosmala *et al.* (2011) have also reported the development of high 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 in dependence 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 the reduction of the costs and higher efficiency of the printing process.

Laitinen has also described the preparation and characterization of α -methylstyrene–butadiene latexes for paper coating applications (Laitinen *et al.*, 2012), showing that coating colors containing α -methylstyrene seems to have an improved water retention compared to the commercial reference styrene-butadiene latex coating color and the laboratory prepared styrene-butadiene coating color. The particle size of the latex samples was measured using NTA.

Nanocelluloses can be used to fabricate and reinforce hemp fibers. Thus, Dai *et al.* (2012) developed a novel fabrication which was employed to produce nanocelluloses from natural fibers (hemp) and the developed nanocellulose was then used as a “coupling agent” to modify hemp fibers themselves. The size distribution of nano-particles (nanocellulose) was measured by NTA and results showed that oxidation–sonication developed nanocellulose had wider size range (29–281 nm) than the average size (100–112 nm). Mechanical testing showed that the nanocellulose modification could improve the mechanical properties of natural fibers significantly. The modulus, tensile stress and tensile strain of nanocellulose modified hemp fibers were increased by 36%, 72% and 68%, respectively. Curable biopolymer nanoparticle latex binders have recently been patented for mineral, natural organic or synthetic fiber products and non-woven mats (Tseitlin *et al.*, 2012)

Treatment of Wastes and Contamination

As nanoparticles become more widely spread throughout industry and consumer products, release from, and exposure to, such nanoparticle-containing materials becomes of increasing concern and the subject of intense study. While the toxicity and environmental fate of nanoparticles has been described elsewhere in this document (See Chapter III and IV), specific examples of the use of NTA for sizing and concentration measurement of nanoparticles in development of monitoring protocols as might be applied to industrial products and manufacturing processes are described here.

Thus, 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. While 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, in terms of dust generation, has been reported with more particles in the size range between 175 and 350 nm being generated during drilling of the nanocomposites, these particles deposit in a shorter time. In a similar type of application, Njuguna *et al.* (2011) have investigated the nanoparticles generated from nanofiller reinforced polymer nanocomposites during structural testing.

Künniger *et al.* (2010) investigated the consequences for functionality and the aquatic environment of the release of conventional and nano-sized biocides from coated wooden façades during weathering. Extending these studies to show that Ag-NPs are likely transformed to silver complexes, which are considerably less toxic than ionic silver, Künniger *et al.* (2013), in her comparative study of metallic silver nanoparticles (Ag-NP), most recently compared conventional organic biocides used as transparent, hydrophobic coatings of wooden outdoor façades.

Cabot *et al.* (2012) have used NTA to monitor changes in tobacco smoke particle size when measured over a series of different time points. The health effects of automotive particulate pollution, specifically related to engineered Pd-nanoparticles, were studied by Wilkinson *et al.* (2011) using NTA and DLS to track particle aggregation in cell growth media. The measurement of soot-in-oil agglomerates from automotive engines was recently carried out by NTA and compared to TEM (La Rocca *et al.*, 2013). Diluting used sump oil in heptane, both techniques showed that soot-in-oil exists as agglomerates with average size of 120nm but that NTA was able to measure particles in polydisperse solutions and report the size and volume distribution of soot-in-oil aggregates with the advantage of being fast and relatively low cost compared with TEM.

A new SAE Standard (equivalent to MIL-L-21260) covering military engine oils suitable for preservation, break-in, and lubrication of reciprocating internal combustion engines in equipment used in combat/tactical service has recently been proposed in which NTA was used to establish protocols for measuring soot agglomerates size distribution in used automotive lubricant oils (SAE Standard (2013).

Peetsch and Epple (2011) employed DLS, NTA, SEM, energy-dispersive X-ray spectroscopy (EDX), X-ray powder diffraction (XRD), atomic absorption spectroscopy (AAS), thermogravimetric analysis (TG), and elemental analysis in their characterization of the solid components of three desensitizing toothpastes and a mouth wash.

Having established that, to March 2011, there existed over 100 food and food-related nanoproducts, Chen *et al.* (2012) investigated and developed a simple test for the characterization and preliminary toxicity assay of nano-titanium dioxide additive in sugar-coated chewing gum. Using NTA their results surprisingly showed that the number of food products containing nano-TiO₂ (<200 nm) is much larger than known, and consumers have already often been exposed to engineered nanoparticles in daily life and that over 93% of TiO₂ in gum is nano-TiO₂ and it is unexpectedly easy to come out and be swallowed by a person who chews gum. Similarly, van Landuyt *et al.* (2013) showed by NTA that nanoscale particles exist in dental abrasives (up to 60vol %) and that dental personnel (and patients) may inhale nano-sized dust particles (<100 nm) during abrasive procedures to shape, finish or remove restorations.

Recognizing that no standard test method is currently available for evaluating the efficiency of personal protective equipment against nanoparticles, in particular in the case of gloves, Dolez *et al.* (2011) used NTA and other techniques to determine the rate of nanoparticle penetration through protective gloves in conditions simulating glove-nanoparticle occupational interaction. They reported on commercial 15nm TiO₂ nanoparticles-powder and colloidal solutions in 1,2-propanediol, ethylene glycol and water and for four types of protective gloves (disposable nitrile and latex as well as unsupported neoprene and butyl rubber gloves) they showed that mechanical deformations and contact with colloidal solution liquid carriers may affect glove materials. Preliminary results obtained with TiO₂ powder indicated a possible penetration of nanoparticles through gloves following mechanical deformations.

Textile materials with engineered nanoparticles (ENPs) have excellent properties as they are antibacterial, antimicrobial, water resistant and protective. The textile industry has recognized the importance and the advantages of ENPs, so they now comprise one of the fastest developing branches of processing and are the subject of significant patent activity, some of which employs NTA analysis in the description (Corona *et al.*, 2013). The most important sources of ENPs released to the environment from textiles are textile-industry wastewaters and waters from large hospital or hotel laundries. Rezić (2011) has reviewed analytical techniques for the characterization of ENPs on textiles. In this context, the increasing number of nanomaterial-based consumer products raises concerns about their possible impact on the environment. In assessing the effluent from a commercially available silver nanowashing machine, Farkas *et al.* (2011) used inductive coupled mass spectrometry (ICP-MS) and TEM to confirm the presence of an average of 10nm silver nanoparticles but employed NTA to determine that 60–100nm particles were also present. The effluent was shown to have negative effects on a natural bacterial community as its abundance was clearly reduced when exposed to the nanowash water and they 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 might be released into the environment. Ling and Pui (2013) also used NTA to characterize nanoparticles from abrasive waterjet machining (AWM) and electrical discharge machining processes showing a peak size of 100-200 nm and that while the filtration systems of the cleaning systems were found to remove 70 and 90 % the nanoparticles present, the particle concentration of the filtered water from the AWM was still four times higher than that found in regular tap water.

Nanoparticle-containing matrices are being increasingly investigated for the removal of environmental pollutants from industrial process wastewaters. NTA was employed by Prasad *et al.* (2012) in their study of the adsorption of arsenite (As^{3+}) 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.

Mallampati *et al.* (2012) demonstrated, in part by employing NTA, the enhanced heavy metal immobilization in soil by grinding with addition of a nanometallic Ca/CaO dispersion mixture. Raychoudhury *et al.* (2011) assessed the transport of two polyelectrolyte-stabilized zerovalent iron nanoparticles in porous media for the remediation of contaminated subsurface environments. Using DLS, NTA and laser Doppler velocimetry, they measured the aggregate size and surface charge of bare and carboxymethylcellulose-coated nZVI particles.

Similarly, 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, the high efficiency for different metals (>90%), 79 mg g⁻¹ adsorption capacity for Pb^{2+} and ultrafast removal process (Pb^{2+} , 99.57%) within 1 minute).

In developing a simple and rapid room-temperature aerosol deposition method to fabricate TiO_2 films for photokilling/photodegradation applications, Park *et al.* (2012) used NTA to demonstrate a

mean size of approximately 1 μm on fracturing following impacting a glass substrate to form a functional thin film, a process known as aerosol deposition.

Investigating new techniques for enhanced oil recovery (EOR) Hendraningrat *et al.* (2012a) have undertaken a glass micromodel experimental study of hydrophilic nanoparticles retention for EOR, in which NTA was used to enumerate particles in both the influent and effluent in a glass micromodel. Further work reported an evaluation of oil recovery using nanofluid injection onto several water-wet Berea sandstone core plugs (Hendraningrat *et al.* (2012b). Hendraningrat and his colleagues have subsequently carried out and reported numerous further studies in this area in which NTA was used to determine the size, size distribution and concentration of nanoscale particles used in the field of EOR. Li *et al.* (2013) showed that a hydrophilic silica nanoparticles suspension enabled improved oil recovery by a 2-phase flow system. Hendraningrat also reported a coreflood investigation of nanofluid enhanced oil recovery again in low-medium permeability Berea sandstone (Hendraningrat *et al.*, 2013a and 2013b) as well as comparing the effect of some parameters influencing enhanced oil recovery process using these silica nanoparticles (Hendraningrat *et al.*, 2013c). The latest data regarding these studies has been reported recently: the retention of nanoparticles during flooding experiment in several water-wet Berea cores was investigated in 3 different ways involving continuously increasing pressure during single-phase coreflood experiment with microscopic visualization under SEM integrated with Energy Dispersive X-Ray Spectroscopy (EDX) to distinguish nanoparticles with other elements and NTA particle measurement between influent and effluent (Hendraningrat *et al.*, 2013d).

Filtration

The ability of the NTA technique to generate high resolution particle size distribution data as well as nanoparticle concentration data makes the technique ideally suited to the testing of filters and filtration processes.

Ling *et al.* (2011) have used NTA to measure particle (50–500 nm) concentration upstream and downstream of the filter to determine the filtration efficiency of a model membrane filter, the Nucleopore® filter, for application in the purification and disinfection of drinking water as well as removal of NPs in highly pure chemicals used in the industries. NTA measurements were found reliable within a certain concentration limit (about 10^8 – 10^{10} particles/ cm^3) and they stated that experimental results are comparable with previously published data obtained using an aerosolization method, thus validating the capability of the NTA technique.

Co-workers Boulestreau and Schulz have 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 the 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 device as well as the impact of the prefiltration on the measurements made. They showed that NTA was able to measure the particle size distribution and the absolute particle concentration of particles between 100 and 1000 nm in secondary effluent. Their results showed clearly a relationship between the amount of nanoparticles below 200 nm and the filtration behavior. Further such work by Schultz *et*

al. (2011) on improving understanding and prevention of membrane fouling in treated domestic wastewater used NTA to demonstrate that a combination of ozonation/coagulation showed synergistic effects and which led to an additional decrease of submicron particle content and further improvement of the filtration performance.

More recently Boulestreau and co-workers have described the on-line use of NTA in which it was used to optimize the ozonation and the 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 thus allowing users to detect the effect of ozonation and coagulation on the nanoparticles and that the NTA instrument is “a highly capable device to analyze the nanoparticles” (Boulestreau *et al.*, 2012).

Schulz (2012) described his work on submicron particle analysis to characterize fouling in tertiary membrane filtration in which he tested a combination of pre-ozonation, coagulation and subsequent low-pressure membrane filtration as an option for tertiary wastewater treatment. He showed that “by Nanoparticle Tracking Analysis (NTA) a reliable and reproducible detection of the colloid content in treated domestic wastewaters is possible. The effects of the pre-treatments on submicron particle size distribution and on the absolute concentration can be detected”. The results of his work demonstrated that ozonation and coagulation were found to reduce the content of small colloids < 200 nm by forming larger agglomerates, resulting in a better filterability of the water. A combination of both treatments shows synergetic effects and a further reduction of the particle content as well as of the total fouling resistance was observed. More recently, Boulestreau and Miehe (2013) have published guidelines for the use of online fouling monitoring in tertiary treatment; work carried out under a Project entitled OXERAM 2. In order to improve performance of both polymeric membrane and a microsieve pilot scale process, on-line monitoring was implemented. After a literature review and extensive laboratory testing at the Technical University of Berlin, two instruments were selected as being ideal for this purpose. The first was on-line NTA which was used to give “reliable and reproducible information about the concentration and size distributions of the colloidal fractions in the tested treated domestic wastewater”. The other instrument was a simple turbidometer but which was found to be less informative than NTA. As part of the same project, Godehardt *et al.* (2013) also used NTA in their study on the role of organic substances in tertiary treatment via oxidation and membrane filtration.

In an unrelated filtration problem, that of fractionation of nanocellulose by a foam filter, NTA was used in an attempt to measure bacterial nanocellulose in a sample of enzymatically pretreated nano-fibrillated cellulose from softwood. The length of nanofibres (many 10s microns) often precluded the analysis of such material though sub-micron nanocrystalline cellulose was accessible to NTA (Tanaka *et al.*, 2012).

Luechinger *et al.* (2010) earlier described a facile, broadly applicable method to prepare nanoporous silver films between 0.5–5 μm and 30–300 nm using soluble salt nanoparticles as pore templates testing them with filtration of aqueous dispersions of carbon nanoparticles (20 nm primary particle size) at a filtration efficiency of >99.6%.

In a study of the significance of electrostatic protein-polysaccharide interactions using bovine serum albumin (BSA) and sodium alginate (Na-Alginate) to specifically illustrate the contribution of this form of non-covalent network to membrane fouling, NTA was used to help demonstrate that soluble complex formation is governed by lowering zeta-potential sufficiently to enable positively charged micro-regions on the protein to bridge between negatively charged carboxyl groups on the alginate. Neemann *et al.* (2013).

In his development of a recirculating aquaculture system in which accumulation of fine suspended solids and colloids can be avoided by integrating a membrane filtration unit into the system, Holan *et al.* (2013a) used NTA to identify how the feeding regime affected membrane performance and fouling phenomena caused by dissolved and submicron colloidal particles in the system and how the membrane impacted general water quality and particle characterization. He further reported on this system in his extended work on the Intensive rearing of cod larvae, *Gadus morhua*. Holan *et al.* (2013b) thus showed there is a great potential of implementing a membrane filtration system in aquaculture recycling systems.

Nanobubbles

The generation, measurement, and applied technologies of extremely small bubbles, so-called nano- and micro-bubbles, with diameter ranging from tens of nanometer to tens of micrometer, are evolving innovatively in recent years. Nano-bubble technologies have already been implemented in actual applications such as facility cleaning, solar cell manufacturing process, plant growth, etc., and its application is considered to have the possibility to expand to wider range of fields, such as water treatment processing, environment, civil engineering, beverage, food, pharmaceutical, medical, cosmetic, plant cultivation, agriculture, fisheries, cleaning, decontamination, and also manufacturing of future functional materials. Therefore nano- and micro-bubble technology is expected to become one of the key players in major industries of the future. The existence of surface nanobubbles is becoming established following many different investigations from a number of groups. Far less common are reports of the existence of bulk nanobubbles. It has been argued that this is because they are considered less stable in bulk or that appropriate techniques for their investigation have not yet been developed.

However, NTA is proving to be particularly adept at the detection and analysis (size, size distribution, number concentration) of these relatively low concentration structures of extremely small size (compared to 'conventional' bubbles).

Seddon has recently and 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 bubble surface 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 and 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 SEM and were produced in such large quantities that the bulk density of the solution was substantially reduced.

It is noteworthy, however, that questions still remain over whether deeply sub-micron bubbles are what they are assumed to be. In a recent thought-provoking study Sedlak and Rak (2013) have shown that in solutions of low molar mass compounds and mixtures of liquids, large-scale inhomogeneities exist but which are not nanobubbles in all cases. Thus, despite the fact that in textbooks, undersaturated solutions of low molar mass compounds and mixtures of freely miscible liquids are considered as homogeneous at larger length scales exceeding appreciable dimensions of individual molecules, growing experimental evidence reveals that it is not the case. Large-scale structures with sizes on the order of 100 nm are present in degassed solutions and mixtures used in everyday life and research practice (e.g. atmospheric pressure), especially in aqueous systems. These mesoscale inhomogeneities are long-lived and their (relatively slow) formation kinetics can be monitored upon mixing the components using NTA. These results support experimental results obtained in earlier light scattering studies and, indeed, such results have been obtained (especially in 50:50 mixtures of water and ethanol) by the scientists responsible for the development of NTA (data not published).

Most of the work to date involving NTA analysis of nanobubbles has been carried out in Japan. Thus Takaya *et al.* (2011) described the formation of nanobubbles by water electrolysis and their analysis with NTA, while Kikuchi *et al.* (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 O₂ gas into the ultra-high purity water with a micro/nano bubble generator and used NTA to measure the resulting number concentration, estimated to be on the order of 10⁷ cm⁻³ of solution under the same sample preparation conditions. Uchida also investigated the efficiency with which nanobubbles could replace detergents in the washing of laundry given 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 (Uchida *et al.*, 2011). Uchida *et al.* (2012) have recently investigated the drag reduction effect of nanobubble mixture flows through micro-orifices and capillaries in which the nanobubble-containing mixture was shown to contain 1.0 vol% nanobubbles by NTA. The 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. Uchida also extended this work to include several types of nanobubble mixtures (nanobubble/water, nanobubble/surfactant and nanobubble/polymer) and discussed factors including slip wall, interfacial tension effect, electric interface phenomenon and elasticity (Uchida *et al.*, 2013).

Uehara and Yano (2011) have reported magnetized nanobubble water formed under a pulsed-magnetic field and Liu *et al.* (2013) have recently investigated the mechanism of nanobubbles' physiological activity promotion with proton nuclear magnetic resonance (pNMR) relaxation time measurements. According to the experiment results, the number of nanobubbles had a positive correlation with the spin-spin relaxation time (T₂) value of the water, which meant introducing nanobubbles could increase the mobility of water in bulk. These results suggested that the nanobubbles in water could influence the physical properties of water and that it could contribute to one of the explanations for the mechanism of nanobubble's promotion effect on physiological activity of living organisms. The hydroponic experiment showed that the nanobubbles themselves could greatly promote the growth of barley and that nanobubble technology was possibly feasible to be used in hydroponic cultivation of vegetables as a new technology in agriculture applications. NTA was used to measure the bubble size diameters, a crucial parameter in understanding the effects they exhibited.

It is interesting to note that methods for the production and apparatus for the generation of nanobubbles, and in which NTA is used for analysis for supporting data, is currently the subject of recent patent activity (e.g. Ryu, 2012; Tsuji, 2012 and Tsuji *et al.*, 2013; Lynn, 2013a and 2013b).

Numerous industrial applications of the use of nanobubbles are beginning to appear in the rapidly growing body of literature on the subject of nanobubbles. Those in which NTA is central to their analysis include studies on applications as diverse as petrochemicals and fuels, building materials and remediation of contaminated land sites and aquaculture. Ueda *et al.* (2013) described the use of water containing air bubbles with a diameter around 100 nm (nanobubbled water) on removal of radioactive carbon from granule conglomerate, asphalt and concrete contaminated sites in Fukushima, Japan. In a wide ranging study of the efficacy of water containing nanobubbles of air or oxygen gas as generated using a nanobubble aerator, Ebina *et al.* (2013) showed significant (compared to normal water) increases in growth (plant height, leaf length and fresh weight) of *Brassica campestris* grown using nanobubbled water; weight and length of DBA1/J mice free-fed nanobubbled water, as well as sweetfish and rainbow trout grown in nanobubbled water.

Nanodroplets that encapsulate a perfluoropentane (PFP) core will transition upon exposure to ultrasound pulses into gas microbubbles, which will rapidly expand and collapse resulting in disruption of cells similar to the histotripsy process but at a significantly lower acoustic pressure. Thus, in attempting to develop an image-guided, targeted ultrasound ablation technique by combining histotripsy with nanodroplets that can be selectively delivered to tumor cells, Vlasisavljevich *et al.* (2013) used NTA in the preparation of nanodroplets with an average diameter of 204 nm at 37 °C by self-assembly of an amphiphilic triblock copolymer around a PFP core, followed by cross-linkage of the polymer shell forming stable nanodroplets. Using a high speed camera to monitor microbubble generation, the peak negative pressure threshold needed to generate bubbles >50 µm in agarose phantoms containing nanodroplets was measured to be 10.8 MPa, which is significantly lower than the 28.8 MPa observed using ultrasound pulses alone. High speed images also showed that cavitation microbubbles produced from the nanodroplets displayed expansion and collapse similar to histotripsy alone at higher pressures. Nanodroplet-mediated

histotripsy created consistent, well-defined fractionation of red blood cells in agarose tissue phantoms at 10 Hz pulse repetition frequency; similar to the lesions generated by histotripsy alone but at a significantly lower pressure. These results support their hypothesis and demonstrate the potential of using nanodroplet-mediated histotripsy for targeted cell ablation.

Finally, nanobubbles of air have been introduced into gas oil for energy saving and environmental load reduction of diesel engines. After the micro air-bubbles were separated from the nano air-bubbles in a mixing tank, diesel engine performance test with a common-rail injection system was tested. The results showed a 3% reduction in a brake specific fuel consumption (BSFC), 1% rise in charging efficiency and a slight reduction in the density of exhaust smoke (Nakatake *et al.*, 2013). Similarly, Oh *et al.* (2013) investigated the effect of hydrogen nanobubble addition on the combustion characteristics of a gasoline engine. Using NTA to demonstrate a mean diameter and concentration of hydrogen nanobubble in the gasoline blend of 149 nm and about 11×10^8 particles/mL, respectively, the results showed that the power of a gasoline engine with hydrogen nanobubble gasoline blend was improved by 4.0 % in comparison with conventional gasoline at an engine load of 40 %. Also, BSFC was improved, from 291.10 g/kWh for the conventional gasoline, to 269.48 g/kWh for the hydrogen nanobubble gasoline blend, at the engine load of 40%.

Tribology of orthopaedic implant wear particles

Unsworth *et al.* (2010) first reported the use of NTA in studying the tribology of CFR-PEEK in hips and knees when generated at 0.5, 10 and 25 million wear test cycles. As the test progressed, the number of particles reduced and the dominant particle size increased from about 40nm to approx. 200 nm. AFM showed some particles as large as $3 \mu\text{m}$ to co-exist. The same group also reported on a tribological and particle debris study of as-cast and heat treated CoCrMo alloy (Kinbrum *et al.*, 2010).

More than 400,000 primary hip and knee replacement surgeries are performed each year in the United States. From these procedures, approximately 0.5–3% will become infected and when considering revision surgeries, this rate has been found to increase significantly. Sinclair *et al.* (2012) accordingly developed a broad spectrum polymer-released antimicrobial coating (Cationic Steroidal Antimicrobial-13 (CSA-13)) for the prevention of resistant strain bacterial infections. Following manufacturing, CSA-13 was micronized using a jet mill and the resultant particle size distribution was measured using NTA.

Patel *et al.* (2012) have studied cobalt-based orthopaedic alloys and explored the relationship between the forming route, microstructure and tribological performance using NTA to generate data on the mode of wear particle debris size distribution.

Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is a bioactive ceramic which is found in the mineral phase of bone tissue and is known for its great potential in tissue engineering applications. For this reason, this material can be applied as particle aggregates on ceramic slurry, coating or film on materials with a poorer biological response than hydroxyapatite. Rodrigues *et al.* (2012) obtained hydroxyapatite gel by the sol-gel process and applied it as nanoparticle aggregation in a mixture of hydroxyapatite and

tricalcium phosphate to form a ceramic slurry. This process, the polymer foam replication technique, was used to produce scaffolds which are used in tissue engineering. While the nanoparticles size before firing was approximately 5nm, NTA showed the crystallite size after calcination was approximately 63nm.

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