Nanosight Limited

Nanoparticle Tracking Analysis

A Review of Applications and Usage in the Analysis of Exosomes and Microvesicles

Bob Carr and Matthew Wright
Table of Contents

A Review of Applications and Usage in the Analysis of Exosomes and Microvesicles ......................................................... 1

Introduction ..................................................................................................................................................................................... 3

Nanoparticle Tracking Analysis (NTA) ........................................................................................................................................ 3

NTA Methodology ........................................................................................................................................................................... 4

Microvesicles and exosomes; definitions and nomenclature ........................................................................................................ 6

Origin, occurrence and role ............................................................................................................................................................... 7

Potential as biomarkers ..................................................................................................................................................................... 7

Isolation and purification .................................................................................................................................................................... 8

Current detection and analysis methodologies..................................................................................................................................... 9

New commercial tests ............................................................................................................................................................................ 10

NTA applied to the study of microvesicles and exosomes .................................................................................................................. 11

Research at the University of Oxford ............................................................................................................................................. 11

Other studies ....................................................................................................................................................................................... 14

Platelet-derived microparticles (PMV) ........................................................................................................................................... 14

Preparation, purification and detection protocol development ....................................................................................................... 15

Comparison of NTA to Flow Cytometry and EM ............................................................................................................................ 17

Cancer Studies and Intracellular Communication .......................................................................................................................... 19

Intracellular communication ............................................................................................................................................................... 21

Diagnostics potential ........................................................................................................................................................................... 22

Therapeutic potential ......................................................................................................................................................................... 23

Conclusion ......................................................................................................................................................................................... 24

References ......................................................................................................................................................................................... 25
Introduction

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 mini-review shows the degree to which the technique of Nanoparticle Tracking Analysis (NTA) is helping to address these problems.

Nanoparticle Tracking Analysis (NTA)

NTA is an established method for the direct and real-time visualization and analysis of nanoparticles in liquids. Based on a laser-illuminated microscopical technique, the Brownian motion of nanoparticles is analyzed in real-time by a high sensitivity scientific CMOS camera; each particle being simultaneously but separately visualized and tracked by a dedicated particle tracking image analysis programme. Because each particle is visualized and analyzed separately, the resulting estimate of particle size and particle size distribution does not suffer from the limitation of being an intensity-weighted, z-average distribution, which is the norm in conventional ensemble methods of particle sizing in this size regime, e.g. the well established method of Dynamic Light Scattering (DLS) or Photon Correlation Spectroscopy (PCS). The ability of NTA to simultaneously measure particle size and particle scattering intensity allows heterogeneous particle mixtures to be resolved and, importantly, particle concentration to be estimated directly; the particle size distribution profile obtained by NTA being a direct number/frequency distribution. The technique, its operating principles and methodology have been described fully elsewhere (Carr et al 2009).

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 (Ri). While for very high Ri particles, such as colloidal gold, accurate determination of size can be achieved down to 15 nm diameter, for lower Ri particles, such as those of biological origin (e.g. exosomes), the smallest detectable size might only be 30 nm. However this minimum size limit allows the analysis of microvesicles and exosomes of a size which would normally be far below the detection threshold of 300 nm obtained by most commercially available flow cytometers. The upper size limits are

![Diagram](image-url)
NTA Methodology

A finely focused and specially configured laser beam is passed through a prism-edged optical flat, the refractive index of which is such that the beam refracts at the interface of the glass-liquid layer placed above it, compressing to a low profile, intense illumination region in which nanoparticles can be easily visualized microscopically (Figure 1). Mounted on a C mount, a sCMOS camera, typically operating at 30 frames per second, is used to capture a video with a field of view approximately 100 μm x 80 μm.

Particles in the scattering volume are seen moving rapidly under Brownian motion. The NTA programme simultaneously identifies and tracks the centre of each particle on a frame-by-frame basis throughout the length of the video (typically 900 frames or 30 seconds). Figure 2 shows an enlarged image of two such particles and the trajectory they have taken over several frames as tracked by the NTA image analysis programme.

The average distance each particle moves in x and y in the image is automatically calculated. From this value, the particle diffusion coefficient, $D_t$, can be obtained and, knowing the sample temperature $T$, and solvent viscosity $\eta$, the particle hydrodynamic diameter $d$ is identified. That 3-dimensional Brownian movement is tracked only in 2-dimensions (x and y) is accommodated by use of the following variation of the Stokes-Einstein equation (Equation 1):

$$\frac{(x, y)^2}{4} = D_t = \frac{TK_B}{3\pi\eta d}$$

where $KB$ is Boltzmann’s constant.

To enable a sufficient number of particles to be analyzed within an acceptable time period (e.g. <60 seconds) from which a statistically meaningful and reproducible particle size
distribution profile can be obtained, samples should contain between $10^7$ and $10^9$ particles/ml. Thus, dilution of a sample is often required to achieve this concentration.

The benefit of being able to simultaneously measure two independent parameters, such as particle scattering intensity and particle diameter (from dynamic behaviour), can prove valuable in resolving mixtures of different particle types (e.g. distinguishing between inorganic and polymer particles of the same diameter). Similarly, small differences in particle size within a population can be resolved with far higher accuracy than would be achieved by other ensemble light scattering techniques, Figure 3.

The laser with which the nanoparticles are illuminated can be exchanged for one with which fluorescence can be excited, allowing nanoparticles labeled with fluorescent molecules to be visualized, tracked, and thus sized and counted 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 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 labeling 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)-labeling, while simultaneously measuring the size of the exosome by analysing its Brownian motion, the two measurements being independent of each other. Note also that the concentrations of such labeled exosomes can still be recovered and compared to the total number of similar sized structures whether labeled or not.

Finally, electric fields can be applied across the nanoparticle suspension such that charged particles will undergo electrophoresis, their electrophoretic velocity and direction indicating their zeta potential and polarity respectively. This information can be gained simultaneously whilst measuring the particle size and optical properties (e.g. relative scattering intensity or fluorescence). These measurements are independent of each other yet can be made simultaneously, on each particle tracked and in real time, potentially allowing high resolution 3-dimensional discrimination of sub-populations within a complex mixture. In Figure 4, two particle types can be resolved based on a combination of three such independent measurements.
Microvesicles and exosomes; 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 it’s still the subject of much debate.

In a recent and comprehensive review, Gyorgy discusses the technical pitfalls and potential artefacts in the rapidly emerging field, compares results from meta-analyses of published proteomic studies on membrane vesicles, and summarises the clinical implications of membrane vesicles. Following an emerging consensus in terms of nomenclature, he describes exosomes as being 50-100 nm in diameter and microvesicles as 100-1000 nm and lists those techniques which have been used most frequently in their isolation and purification and in their 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 et al. (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 substantial biological diversity of the underlying biological process.

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.
Origin, occurrence and role

MV \(\text{s}\) 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 and 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).

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 (miRs) 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.

Most 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.

Potential as biomarkers

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 overexpression of certain tumor cell biomarkers, suggests an important role of exosomes in diagnosis and biomarker studies. 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, tumour 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 tumour 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
Applications of NTA in Exosomes Research

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 (50–100 nm) 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 bicinchoninic 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 (\(\lambda_{em} = 405\) nm and \(\lambda_{em} > 430\) 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 ± 47 \(\mu\)g/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.

The subject of microparticles and exosomes as biomarkers has been recently reviewed by Burger et al (2012) in which they summarise approaches for the detection of microparticles and examine novel concepts relating to the formation of microparticles and their biological effects and as well as the evidence for microparticles as both biomarkers of, and contributors to, the progression of disease.

**Isolation and purification**

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) describes 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 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.

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 count.

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. They 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 they 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 labeling, 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 of obtaining biochemical information by applying
Applications of NTA in Exosomes Research

fluorescent antibody labeling, but it was recognized that this was not easy to perform and involved several practical and optical problems. InNTA 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 citing numerous suitable technologies for analysis of the size, density and molecular composition of exosomes and microvesicles (EMVs) together with methods for their improved isolation and purification out of heterogenous 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 and accordingly “many of the problems and disadvantages associated with current single-parameter technologies could be overcome by the recently introduced method of NTA. This 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.

- 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.
Applications of NTA in Exosomes Research

- 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, 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. have 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 (2012), using NTA as proof that their reagent is as effective as ultracentrifugation for the isolation of exosomes (http://www.lifetechnologies.com). Similarly, Zeringer (2012) has described the use of this reagent for the concentration of exosomes from different sample types for downstream analysis.

It should be recognized, however, 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.

**NTA applied to the study of microvesicles and exosomes**

**Research at the University of Oxford**

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 >500 nm 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 showing that while both systems gave similar results on calibration quality beads over the size range 50–650 nm, measurement of either purified MPs or diluted normal Platelet Free Plasma (PFP) NTA gave a polydisperse MP distribution (up to 1000 nm) but with a predominant population from <50 nm to above 300 nm. Analysis of diluted PFP in PBS (1:40–1:160) suggested that the concentration of particles was 200–260×10^9/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. 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.

The latter group was interested in the use of exosomes as potential diagnostics for the condition of pre-eclampsia in pregnancy (a common disorder characterised by hypertension, proteinuria, endothelial dysfunction and systemic inflammation) (Sargent 2010a and 2010b, Mincheva-Nilsson and Baranov 2010). Circulating microvesicles shed by the placenta during pregnancy include syncytiotrophoblast microvesicles (STBM) and exosomes which have the potential to interact with maternal immune and endothelial cells and may have both proinflammatory and immunoregulatory effects and it was suspected that increased shedding of STBM was associated with pre-eclampsia. NTA was used alongside flow cytometry and western blotting to confirm that excess shedding of syncytiotrophoblast vesicles in pre-eclampsia is a cause of the maternal syndrome.

However, the number of STBM observed in the peripheral blood is much lower than predicted by the rate of shedding. Gardner 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 (100 nm - 1 μm) and nanovesicles (< 100 nm; exosomes) isolated from the placenta as they have major potential as novel biomarkers for the condition of pre-eclampsia. Such microvesicles 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 ≥ 290 nm but nothing smaller. However, they showed that NTA could measure cellular vesicles down to approximately 50 nm.

Sheldon et al. (2010), in their study on notch 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 114 nm for HUVECs and 120 nm for U87 cells, compared with published sizes of 50-100 nm). They stated that, while sizing of exosomes by electron microscopy 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 labeled anti-placental alkaline phosphatase antibody (NDOG2-Qdot605), flow cytometry showed that 93.5% of the vesicles labeled 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 2011b). 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 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 have 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, NTA analysis of PMV, achieved by labeling with a quantum dot-conjugated cell-tracker peptide, produced vesicle counts of 1.49x10^7/μl for PFP and 1.20x10^7/μl for the reconstituted pellet. >95% of all pelleted particles were labeled with the cell tracker, compared to <0.1x10^7/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 suggests that PFP comprises a large population of low density vesicles that are not cellular derived. The presence of lipoproteins will become problematic for flow-cytometry as particle size detection limits continue to fall. The mean PMV (pelleted) count was 1.82x10^7/μl (SD 0.78), with a mean modal size of 92.7 nm (SD 6.9 nm) and a mean median size of 107.3 nm (SD 9.8). The size distribution showed that 75% of PMV were <150 nm, while <2% were greater than 300 nm; 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 50 nm to 1 μm with the majority being <500 nm (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 50 nm in size, and that NTA of platelets, RBC and endothelial-derived vesicles revealed that their size distribution differed, ranging
from 50-900 nm, 50-400 nm and 50-650 nm respectively. They showed that vesicle counts, 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.

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. While their protocol covers the generation of targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand; how to purify and characterize exosomes from transfected cell supernatant; crucial steps for loading siRNA into exosomes and finally, 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 30 minute 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.

Other studies

**Platelet-derived microparticles (PMV)**

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 electron microscopy (EM), confirming that size distributions by the two techniques correlated well showing that over 90% of PMVs were <500 nm and over 70% were <250 nm, irrespective of the method of activation by various physiological stimuli in comparison to Ca2+-ionophore (A23187). 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 multitaled participants in intercellular communication (Aartonen 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.

The development of standardized methods for the analysis of platelet-derived extracellular vesicles (PL-EVs) in human platelet hemapheresis products was described by Orsó 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 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.

**Preparation, purification 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 dynamic light scattering (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, but larger particles reduce the number of small particles detected by the software, and 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).

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 Scanning Electron Microscopy 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 Light Scattering Methods” suggesting it offers many advantages to particle size distribution characterization. In addition to 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 2012a) employed NTA, which 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-labeling of the exosomes with the detection antibodies (CD9, CD63 and CD81).

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 by 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 set up of a protocol for exosomes isolation from urine, and the quantification and analysis of surface markers and microRNA (miRNA) content, Dimuccio et al (2012) compared and tested four protocols of exosome isolation, based on 1) ultracentrifugation (100,000 g at 4°C for 1h); 2) nanomembrane concentrator Amicon (100k); 3) nanomembrane concentrator Vivaspin 500 (Sartorius); and 4) 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 for count. 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 exosomes concentration for protocol No. 2 but NTA analysis showed high concentration of exosomes in samples obtained using protocols No.1-2 (4.7 x 10^8 and 3.5 x 10^8 exosomes/ml). Their study identified a protocol based on ultracentrifugation as the most
suitable to obtain exosomes from urine and in which exosome count using the NTA analysis 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 noninvasive 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, concluding 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, Shibata and Suga (2012) described their studies on the interaction between the isolated exosomes (from cell culture) and solid materials (including SiO$_2$, Al$_2$O$_3$) 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-electron microscopy, 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 × g centrifugation can introduce a biased evaluation of the genuine size distribution of the vesicles in the extracellular medium.

Very recently, Webber and Clayton (2013) have proposed a straightforward method to estimate the purity of vesicle preparations by comparing the ratio of nanovesicle counts to protein concentration, using NTA and a colorimetric protein assay such as the BCA-assay. They suggested their approach may aid in comparing/standardizing vesicle purity across diverse studies, and may be of particular importance in evaluating vesicular biomarkers.

**Comparison of NTA to Flow Cytometry and EM**

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 (normally 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 in order to calibrate the system to the calcein-labeled liposome preparations and CFSE-labeled mouse hepatitis virions with which the system capabilities were demonstrated, though wide angle flow cytometric forward scattering could be used for larger and higher refractive index 100 nm and 200 nm fluorescently labeled 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 calibration of flow cytometers 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 than 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 but 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 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.

The use of flow cytometry for the study of microparticles and exosomes has recently been comprehensively reviewed by Baj-Krzyworzeka et al (2012a).

More specifically, György et al (2012b) compared an improved flow cytometric 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 that 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 counts, measured by NTA, were two orders of magnitude higher than the...
total (AX+) 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 particle, whereas by FC they enumerated only the true (AX-positive, Triton sensitive) vesicle-related events. However, they pointed out that using the fluorescence capability of the NTA system and specific labeling, individual populations may also be analyzed.

**Cancer Studies and Intracellular Communication**

Given it is now accepted that microvesicles (MVs) and exosomes play a pivotal role in cell-to-cell communication and that tumour cells have specifically been demonstrated to release such membranous structures, and that, these cell-derived vesicles can exhibit an array of proteins, lipids, and nucleic acids derived from the originating tumour, 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.

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, 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 (PCa-MVs), which was shown by electron microscopic study and NTA for the first time.

Baj-Krzyworzeka et al (2012b) have focused on the interactions of tumour-derived microvesicles (TMV) with human monocytes, which are precursors of tumour associated macrophages (TAM). Their work has shown that monocytes pre-exposed to TMV and restimulated with tumour cells show M2-like cytokine secretion and that TMV significantly modulate biological activity of monocytes and may affect their function during tumour progression, thus TMV mimicking the effect of tumour cells on monocytes. They postulate that TMV should be considered as a modulator of monocyte/macrophage functions in the tumour bed and in peripheral blood.

Mizrak and his co-workers reported the first use of a therapeutic mRNA/protein via MVs for the 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
Applications of NTA in Exosomes Research

prodrug (5-fluorocytosine (5-FC)), which is converted within tumor cells to 5-fluouracil (5-FU)—an anticancer agent. Excitingly, these studies suggest that MVs can serve as novel cell-derived “liposomes” to effectively deliver therapeutic mRNA/proteins in the treatment of diseases. Cicero and Raposo (2012) have reviewed the general area of the cell biology of exosomes from a historical perspective and Taylor and Gercel-Taylor (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 remodeling, 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.

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 500 ng/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. Bruno et al (2012) have shown that microvesicles derived from human bone marrow mesenchymal stem cells inhibit tumor growth. The 145 nm (NTA-measured) microvesicles, when administered intra-tumour 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 tumour treatment.

Attempting to define the mechanisms by which fetuin-A mediates the adhesion of tumor cells, Watson et al (2012) used the counting 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 heparanase (Thompson 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 tumours which promotes tumour progression, angiogenesis and metastasis, potentially through exosome-mediated signaling, they 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.

Finally, Morton et al (2012b) have described microvesicles as indicators of cancer progression using biomarkers in a further methodology building on their more familiar role in proteomics and genomics.

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 has not been studied. Garnier et al (2012) have 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.
Applications of NTA in Exosomes Research

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.

Finally, Cantaluppi et al (2013) have described the isolation, characterization and pro-angiogenic activity of microvesicles (MVs) derived from human pancreatic islets in which they used NTA, FACS, western blot, bioanalyzer, RT-PCR for specific islet-associated genes and miRNAs.

**Intracellular communication**

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.

A number of studies have begun to utilize NTA for the detection and counting 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 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.

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 extracellular heat shock protein 72 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-1β 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 responses (p < 0.05). This data suggested that exposure to stress can alter the proteomic composition of circulating exosomes, thereby enhancing the innate immune response. Iglesias et al (2012) have that shown that human mesenchymal stem cells, from amniotic fluid or bone marrow, reduce pathologic cystine accumulation in co-cultured CTNS 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 cystinosin 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.
Finally, 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 100-400 nm though the LDL particles might have exhibited, in part at least, a common size range.

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.

### Diagnostics 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 (MVs) of multiple sclerosis (MS) patients under treatment of interferon-beta-1b, in which they confirmed the presence of MVs in their preparation using two laser-based detection systems: 1) Fluorescence-activated cell sorting (FACS) analysis and 2) 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), dynamic light scattering (DLS) and electron microscopy (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-labeled 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 microRNAs (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, e.g., cancers and infectious disease. The influence of food intake and fasting on circulating biological nanoparticle carriers of miRNAs was assessed by nanoparticle tracking analysis (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

**Therapeutic potential**

Tumour microvesicles isolated from a variety of cell lines were analyzed for exoRNA content as a function of exosome particle size distribution profile (Balaj *et al* 2011), from which they proposed that tumour microvesicles also carry DNA in addition to a selected set of proteins and RNAs, thus expanding the nucleic acid content of tumour microvesicles to include: elevated levels of specific coding and non-coding RNA and DNA; mutated and amplified oncogene sequences; and transposable elements. Thus, tumour 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 tumour 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 30-150 nm 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 $5.2 \times 10^8$/ml with a mean size of 180 nm 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 labeling 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.

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. This paracrine action may be accounted for, at least in part, by microvesicles (MVs) released from mesenchymal stem cells, resulting in a horizontal transfer of mRNA, microRNA and proteins. They discuss 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.

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 count seminal exosomes and found them to number between $4.7 \times 10^{11}$ and $1.2 \times 10^{12}$/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.

**Conclusion**

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 practise of some 300 nm 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 counted, 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.

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 2012). 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 showing 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.

References


Balaj L (2012) BEAMing qRT-PCR analysis of mutant IDH1 mRNA in tumor microvesicles, Exosomes and Microvesicles 2012, Sept 30, Orlando, FL., USA

Applications of NTA in Exosomes Research


Fang DYP, HW King, JYZ Li, JM Gleadle (2012) Exosomes and the Kidney: Blaming the Messenger, - Nephrology, Methods in Renal Research Online ISSN: 1440-1797

Fonsato V, Federica Collino, Maria Beatriz Herrera, Claudia Cavallari, Maria Chiara Deregbus, Barbara Cisterna, Stefania Bruno, Renato Romagnoli, Mauro Salizzoni, Ciro Tetta and Giovanni Camussi (2012) Human Liver Stem Cell7-Derived Microvesicles Inhibit Hepatoma Growth in SCID Mice By Delivering Anti-tumor microRNAs Stem Cells, Accepted manuscript online: 26 JUN 2012 , DOI: 10.1002/stem.1161


Graner M W (2012) Introduction to Extracellular Vesicles in Cancer and EMV-Target Cell Interactions, Exosomes and Microvesicles 2012, Sept 30, Orlando, Fl., USA


Harrison, P. (2008), The Application of Dynamic Light Scattering To Measuring Microparticles, ISTH SSC, Vienna, 5th July 2008

Applications of NTA in Exosomes Research

Harrison, P., Dragovic, R., Albanyan, A., Lawrie, A., Murphy, MF and Sargent, I (2009b) Application of dynamic light scattering to the measurement of microparticles; 55th Annual Meeting of the Scientific and Standardization Committee of the ISTH, The XXII Congress of The International Society on Thrombosis and Haemostasis, July 11 - 18 2009, Boston, USA, in press


Applications of NTA in Exosomes Research


Orsó E, Anne Black, Annika Pienimäki-Römer, Susanne Heimerl, Gerhard Liebisch, Oliver Kenyon2, & Gerd Schmitz (2012) Characterization and standardized analysis of platelet-derived extracellular vesicles (PL-EVs) in human platelet hemapheresis products, Exosomes and Microvesicles 2012, Sept 30, Orlando, Fl., USA

Applications of NTA in Exosomes Research

(2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET, Nature Medicine, (2012), DOI:10.1038/nm.2753


Ronquist G K, Larsson A, Stavreus-Evers A, Ronquist G (2012) Prostasomes are heterogeneous regarding size and appearance but affiliated to one DNA-containing exosome family, The Prostate, Article first published online: 26 APR 2012, DOI: 10.1002/pros.22526


Sargent I L (2010b) Placental microvesicles and exosomes and systemic inflammation in pre-eclampsia, Journal of Reproductive Immunology, Volume 86, Issue 1, Pages 21-22 DOI:10.1016/j.jri.2010.06.033


Thompson C A (2012) Secretion, composition and biological activity of tumor derived exosomes are regulated by heparanase, Exosomes and Microvesicles 2012, Sept 30 , Orlando, Fl., USA


Vojtech L, Sean Hughes, Florian Hladik, (2012) Effect of exosomes in semen on mucosal immunity to viral pathogens, Exosomes and Microvesicles 2012, Sept 30, Orlando, Fl., USA


Yuana Y; Bertina R M; Osanto S (2011) Pre-analytical and analytical issues in the analysis of blood, Microparticles, Thrombosis and Haemostasis, 105.3


Zeringer E (2012) Concentration of Exosomes from different sample types for downstream analysis using Total Exosome Isolation reagents, Exosomes and Microvesicles 2012, Sept 30, Orlando, Fl., USA