Exosomes and Microvesicles: Characterization by Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA) Overview

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

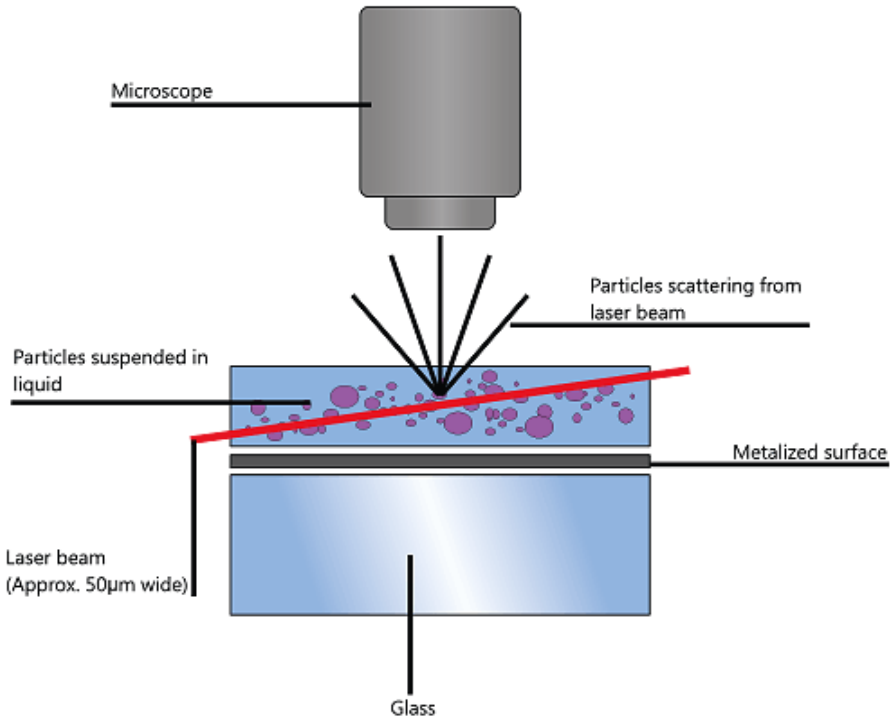

Figure 1: Schematic of the optical configuration used in NTA.

The movement of the particles is captured on a frame-by-frame basis. The proprietary NTA software simultaneously identifies and tracks the center of each of the observed particles, and determines the average distance moved by each particle in the x and y planes. This value allows the particle diffusion coefficient (Dt) 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 (Equation 1).

$$Dt = \frac{TK_B}{3\pi\eta d}$$

where KB is Boltzmann's constant.

NTA is not an ensemble technique interrogating a very large number of particles, but rather each particle is sized individually, irrespective of the others. An example of the size distribution profile generated by NTA is shown in Figure 2.

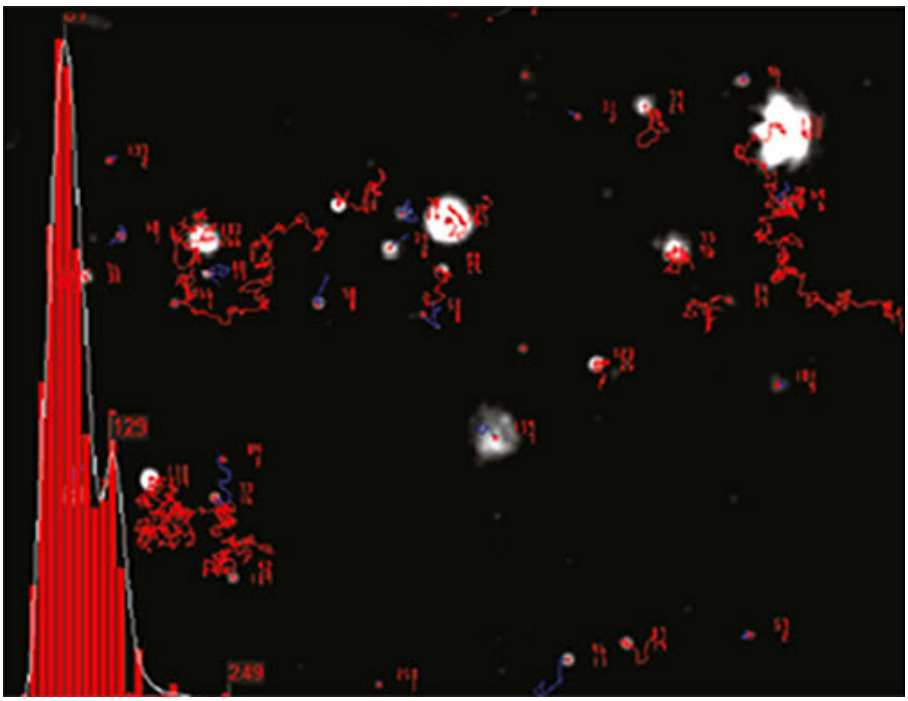

Figure 2: An example of the size distribution profile generated by NTA. The modal size for this sample is found to be approximately 70 nm, with larger sized particles also present.

In addition, the particles' movement is measured 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; 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.

Exosome and Microvesicle Characterization

While the principles underpinning the Nanoparticle Tracking Analysis (NTA) technique have been described in an earlier white paper, 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 Dynamic Light Scattering (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 ºC".

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 characterisation

Following early work on the application of 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 analyses 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 (Aβ) in the brain affected by Alzheimer's disease (AD). On the premise that Neprilysin (NEP) is the most important Aβ-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-a (@ 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-a 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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