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Challenges in Analysis of Circulating Extracellular Vesicles in Human Plasma Using Nanotracking and Tunable Resistive Pulse Sensing

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Abstract

Aim: Extracellular vesicles (EV) are secreted from both healthy and diseased tissues and are detectable in most body fluids, where their measurement can be of prognostic/diagnostic value. We aimed to evaluate pre-analytical and analytical variables in the measurement of EV in human plasma using nanotracking analysis (NTA) and tunable resistive pulse sensing (TRPS).

Method: Commercial beads (200 nm and 400 nm in diameter) and human plasma from volunteer donors were used in this study. A total of 36 bead and 175 plasma measurements were undertaken by NTA and TRPS. The pre- and analytical conditions tested on plasma samples were: fresh, stored for 1 day or 1 week at 4°C or -80°C. The other variables included testing of neat or pelleted EVs and choice of diluent.

Results: The 200 nm and 400 nm beads, when tested alone or combined showed coefficient of variations (CV) of <10% at all dilutions. The CVs for triplicate results of plasma samples varied between 3-43%, with samples frozen for 1 week showing the least CV spread (5-15%). The EV counts from pellets or supernatants obtained after centrifugation of plasma at 21000*g* for 1 h, were different to the neat samples. Although both the supernatant and pellet fractions contained EVs of approximately 100 nm in size, only the pellet contained larger vesicles of 300 nm, and only the supernatant contained vesicles <100 nm.. Different EV counts were obtained for the same plasma aliquot using NTA and TRPS.

Conclusions: Measurements by NTA can be performed on fresh and/or frozen samples provided similar storage and centrifugation conditions are used. The CVs for plasma samples are high indicating the need for standardized conditions. TRPS requires at least two different nanopore filters for measurements and plasma as diluent in lower chamber for optimal results. NTA and TRPS measure EVs by different approaches and the knowledge of the size range for measurement by each method is critical.

Keywords: Extracellular vesicles; Microvesicles; Plasma; Nanotracking analysis; Tunable resistive pulse sensing

Introduction

A variety of extracellular vesicles (EVs), which are membrane bound vesicles released by cellular elements, play an important role in both normal and pathophysiology of the human body. Particles of any size in circulating plasma are now referred to collectively as extracellular vesicles [1]. The term 'microparticles' (MP), used previously in literature to denote platelet derived vesicles, is generally now re-designated as microvesicles (MV) and refers to vesicles in the size range of approximately 100 nm-1 μm. Exosomes on the other hand specifically refer to vesicles released by exocytosis and usually in the size range of 30-100 nm [2].

The existence of cell derived membrane bound particles in human circulation has been described for over two decades [3]. The clinical and pathophysiological significance of these particles has been unravelled in the last few years. These vesicles are usually derived from human blood cells such as platelets, white cells and red blood cells but more recently, EV derived from cancer cells and other tissues have also been reported [4]. The measurement of circulating EV have been described to be of prognostic and diagnostic importance for several diseases particularly in haemostatic/coagulation disorders, cancers and certain inflammatory disorders [5]. Circulating EV can be detected by analysis of human plasma with methods such as flow cytometry, enzyme linked immuno-absorption, as well as functional studies [6,7].

Standardized flow cytometry can determine the number of vesicles as well as antigenic specificity which can help determine the cell of origin. An example of flow cytometric approach includes using a fluorescently labelled anti-CD41 antibody to identify platelet derived vesicles. The challenge with this technique is the limitation posed by the wavelength of the laser light used, thus particles less 400 nm are difficult to capture by standard flow cytometry [8]. Modified and dedicated flow cytometry may be used to detect vesicles as small as 200 nm [9].

A commonly used functional approach uses a combination of phospholipid based capture on a plate followed by functional thrombin production estimation. These assays are not limited by size and hence complement a variety of ways to quantitate EV.

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Evolving technologies to measure EVs now include nanotracking analysis (NTA) and tunable resistive pulse sensing (TRPS). Nanotracking (commercialized as Nanosight NS500 and other versions, Malvern Instruments Limited, Worcestershire, UK) specifically utilizes a combination of microscopic capture of laser light scatter with a charge device coupled camera. A dedicated software program is able to detect dynamic light scattering due to the Brownian motion of individual particles in solution which allows mathematical determination of the size and number of particles [10].

The tunable resistive pulse sensing (TRPS, commercialized as qNano and other versions (Izon Science UK) is a non-optical technique based on the passage of particles through a nano-scale pore. As a vesicle moves through the pore it creates an increase in electrical resistance, which is measured as a blockade height. Mathematical derivations allow the calculation of the vesicle volume from this resistive pulse. Particle by particle detection occurs, enabling size distribution within a sample to be determined. The calculation of particle size by TRPS is dependent on several variables such as pore size, electrolyte used, as well as the stretch, voltage and pressure applied [11].

This article explores the technical challenges as well as advantages of using these two promising techniques for detection and measurement of circulating EV in human plasma.

Methods

General approach to pre-analytical variables for NTA and TRPS

Sample collection: Blood samples were collected in 3.2% sodium citrate from volunteer donors after informed consent. The plasma was immediately separated by centrifugation at 1500*g* for 15 minutes and either processed for immediate EV enumeration or stored at 4°C or -80°C for future experiments.

Processing and analysis: A total of 36 bead and 175 plasma measurements were undertaken with triplicate runs. Commercial beads (200 nm and 400 nm, Izon sciences UK) were analysed in various serial dilutions. The pre-analytical variables for plasma included analysis within 2 h of collection (Fresh), and after storage at 4°C or -80°C for 24 h or 1 week. Other variables tested included measuring neat (no dilution), supernatant and pellets after samples were centrifuged at 21000*g* for 1 hour. The centrifugation conditions were chosen based on commonly employed methods for isolating EV [12]. The statistical analysis was performed using Prism 6 software (Graphpad, USA). Ethics approval for using human plasma samples for this project was obtained prior to commencement of the experimental work (Hunter New England ethics committee approval number 06/12/13/5.05).

NTA for EV in human plasma

Specific pre-analytical variables

Dilutions and linearity for beads and plasma: *Two* bead sizes 200 nm and 400 nm were tested in dilutions ranging from 1:100 to 1:1000 in triplicate measurements. A mixed sample (1:1) of the two bead sizes was also tested in such serial dilutions.

The neat, supernatant or pellet fractions from plasma were tested in triplicate. The nanotracking was performed in both scatter and fluorescent modes in order to distinguish EV from background. Qdot 625 stain (Life Technologies/ThermoFischer Scientific, MA, USA) was diluted 1:100 and incubated with samples at 37°C for 1 h. A negative control (stain in buffer) and unstained plasma was used with every experiment. Events were collected from the samples at the entire range of camera settings from levels 9-16 (based on camera shutter and gain) both for scatter and fluorescent modes with fine tuning of the camera adjusted to best visualize the EVs at the camera setting. The detection threshold was set at 10 and events were captured for 90 s. Results were analysed using the NTA software (Nanosight NTA 2.3 software).

TRPS (qNano) for EV in human plasma

Sample preparation by pre-filtration: A filtration step was required for analysis using TRPS in order to prevent clogging of the nanopore. The 0.8 μm filter and 0.45 μm filters efficiently prepared the sample for analysis on 800 nm and 200 nm nanopores respectively.

Fluid composition in the top and bottom chambers: A combination of Plasma/PBS and plasma/plasma was tested across the nanopore in the qnano instrument to check for optimal results. Dilutions ranging from 1:2 to 1:50 were tested for linearity.

Linearity across dilutions and optimal dilution for plasma samples: We also investigated if plasma dilution was a viable option without impacting on the actual particle count. We used a 200 nm nanopore for the experiments and compared the current (nA) and particle rate with neat, 1:10 and 1:20 plasma samples diluted in PBS.

Stretch and voltage

A fixed blockade height approach and a fixed stretch/voltage approach was compared.

Results: NTA for EV in human plasma

Reproducibility and linearity for beads: A CV of <10% for all dilutions of both 200 nm and 400 nm sized beads was observed and linearity in measurements demonstrated as shown in Figure 1. The bead counts calculated by NTA, upon dilution, varied by two to six-fold compared to expected bead concentrations provided by the manufacturer (data not shown).

Centrifugation and storage conditions for plasma samples: There was no significant difference between EV results for fresh or frozen stored samples under different storage conditions (2-way ANOVA $p>0.05$, Figure 2). A trend for higher EV levels in frozen samples was observed. The CVs for triplicate results of plasma samples varied between 3-43%, with samples frozen for 1 week showing the least CV spread (5-15%).

The EV counts from plasma pellets or supernatants obtained after centrifugation at 21000*g* for 1 h, were reduced compared to the neat samples. However, there was no significant difference between the EV counts in the pellet and supernatant fractions, with particles averaging 5.08 and 7.46 x 10^8/L respectively. To investigate this surprising finding, the vesicle sizes between the fractions were compared by further analysis of the peaks on the nanosight graphical histogram output. The major (first) peak on the NTA histogram was similar for both fractions and measured 107.1 nm and 101.1 m respectively. However, both fractions contained a minor peak, and the median size for this minor (second) EV size peak from supernatant was 94.3 nm, which was significantly smaller compared to the 312.4 nm for the pellet (Figure 3).

Sample dilution: Ideal dilution for plasma samples was determined to be between 1:50 to 1: 100 depending on whether fluorescent or scatter events were captured. In lower dilutions, it was easier to detect fluorescent vesicles/particles, as there tended to be fewer events than in the scatter detection mode. When the number of tracks captured was between 40-200, the CVs were tighter. Dilutions in the range of

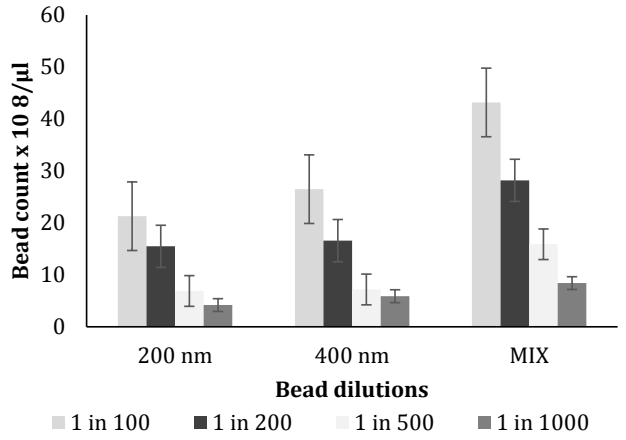

Figure 1: Bead counts for 200 and 400 nm beads in various dilutions with mean and error bars (±2 SD).

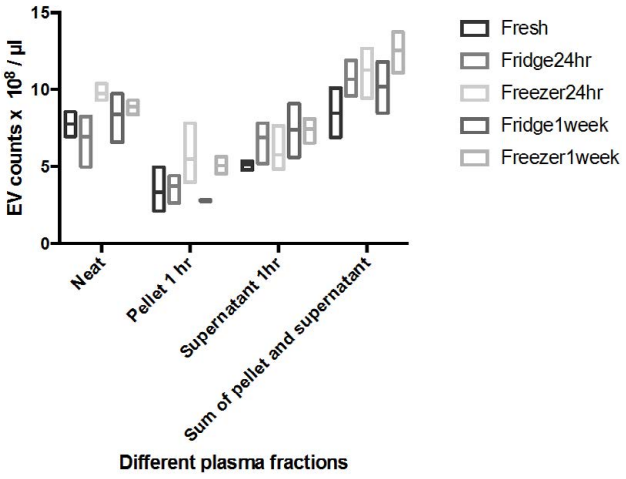

Figure 2: Comparison of EV counts obtained from neat, pellet and supernatant fractions obtained after centrifugation at 21000*g* for 1 h.

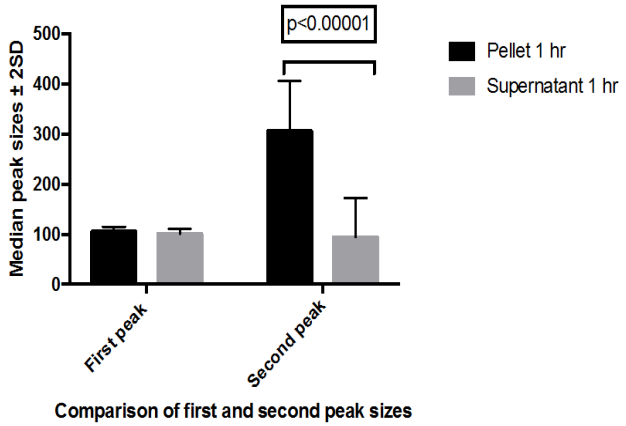

Figure 3: Comparison of largest and second largest peak sizes in EV from pellet and supernatant after centrifugation at 21000*g* for 1 h (n=6), frozen samples.

1:1000 or 1:10000 were required for samples where the number of tracks exceeded 200. The instrument defaults to water's viscosity (0.97 cP at 23.3°C) in its custom settings and may need to be adjusted to the viscosity of the sample being run.

Complex samples-plasma at different camera levels: As the camera level and filter varied from fluorescent to light scatter, adjustments on the instrument were required to be made before the events were captured. Most bead or plasma events appeared to be best detected at camera levels of 13-15, with background scatter increasing with camera level (Figure 4). Often camera levels at 16 (highest possible level) impeded any detection of vesicles or particles due to significant background scatter. With each camera level fine tuning of the focus was also critical to achieving maximum capture.

Results

Technical aspects of TRPS (qNano) for EV in human plasma

Electrolyte and conductivity: The electrolytes used in the experiments directly influence the conductivity and therefore the rate of particle movement across the nanopore. For example, the particle rate will be higher flowing from a solution that is more charged to a solution with less charge than if both chambers have the same solution. Table 1 shows the comparison of plasma in both the chambers as compared to plasma in the top and PBS in the bottom chamber.

Linearity across dilutions and optimal dilution for plasma samples: The particle rate was high when plasma was used undiluted in the upper chamber (Table 2), leading to repeated pore clogging and interruptions to the experiments. This problem was ameliorated when the plasma was diluted, which also increased the current across the nanopore. Longer acquisition times and different settings were required for diluted samples and up to 90 s was used to capture more events; viscosity settings needed to be adjusted to the medium of suspension (e.g. changed to PBS=1.03. cP).

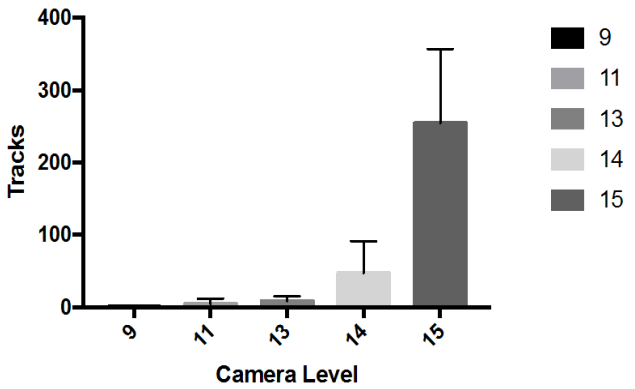

Figure 4: Tracks captured in fluorescent mode for plasma EV at various camera levels (in triplicate, with error bars showing SD).

Combination of fluids in upper and lower chambers	PBS/Plasma (bottom/top)	Plasma/Plasma (bottom/top)
Avg Current (nA)	102.3 ± 3.54	109.58 ± 2.1
RMS noise (pA)	11.93 ± 0.51	11.8 ± 0.4
Particle Rate (particles/min)	3257.63 ± 1589.11	2395.9 ± 1433.67

Voltage=0.6V Stretch=45.67 Pressure=7.

Table 1: Electrolyte and conductivity across chambers (n=3).

Using a combination of pre-filtration for polydisperse plasma samples: A pre-filtration step with a 0.8 μm syringe filter for the 800 nanopore and a 0.45 μm syringe filter for the 200 nanopore was employed to detect particles of various size ranges based on the upper limit of detection for the respective nanopore. The stretch (42.8 nm) and voltage 0.32 V was kept constant across all measurements as per manufacturer's instructions and published literature [11,13].

Nanotracking and TRPS - Do they measure the same vesicles?

Nanotracking and TRPS employ different technologies and hence measurements made with either instrument may reflect different populations of particles. The table below demonstrates that the respective optical and non-optical measurements by nanotracking and TRPS can vary on the same sample and pre-analytical preparation combination (centrifugation, pellet vs. supernatant and filtration) as shown in Table 3.

Discussion

Emerging techniques of NTA and TRPS have been increasingly used in detection and quantitation of EV of biological origin [14]. Although there is a significant body of literature looking at beads and liposomes potentially simulating body fluids there is a dearth of published literature estimating EVs in human plasma samples [13]. We investigated the pre-analytical as well as analytical variables for human plasma measurements by NTA and TRPS, hypothesizing that this would be more complex than bead or liposome measurements. The key findings indicate that standardized pre-analytical processing such as storage and centrifugation as well as knowledge of particle size range is critical for measurement of EVs in human plasma. Some of the specific findings are discussed in the sections below.

Pre-analytical variables for measuring EV in plasma using NTA or TRPS

Pre-analytical variables for both techniques can significantly impact on the results. Sampling of blood for functional and numerical assessment of circulating cell-derived vesicles has been shown to be best achieved by citrated or heparinised samples [15]. Though EDTA may be sufficient as an anticoagulant and preferable for RNA based experiments, it is likely that any concomitant EV testing involving coagulation, thrombin generation or functional experiments cannot be undertaken on the same sample.

	PBS/Plasma neat (bottom/top)	PBS/Plasma 1:10 (bottom/top)	PBS/Plasma 1:20 (bottom/top)
Avg. Current(nA)	97.26	118.27	122.65
RMS noise (pA)	10.46	7.42	7.74
Particle Rate (particles/min)	360.2	190.8	145.1
CV (%) for Avg. current	30.3%	4.5%	1.0%

Table 2: Plasma samples showing differences in CV with various dilutions, n=3.

Sample	TRPS particles × 10^8	NTA particles × 10^8
Neat	23	4.4
Pellet 1 h	14	8.1
Supernatant 1 h	17	5.78
Pellet 10 mim	11	2.87
Supernatant 10 min	14	7.38

P<0.0001 for the two sets of results in all rows.
All plasma samples subjected to 800 μm pre-filter.

Table 3: Comparison of NTA and TRPS, on pre-filtered plasma, for varying pre-analytical conditions.

A neat (i.e. not centrifuged) sample is theoretically sufficient for measuring all EVs in the plasma sample, however, we observed that for analysis by NTA and TRPS, diluted plasma samples provided more optimal measurement conditions. The quantitation of a polydisperse mix of vesicles within a given sample is challenging given the finite size range within which each technique operates. Bench top centrifuges achieve a force of up to 21000*g* which is enough to achieve pelleting of a significant proportion of larger EV (mostly microvesicles and some exosomes), however small <100 nm and/or less dense EV are not pelleted efficiently. The examination of MV, the fraction of EV that lie between 100 nm-1000 nm, as seen by the size of the second peak by NTA in our experiments, is potentially facilitated by centrifugation at 21000*g* for 1 hour to pellet these vesicles. Centrifugal forces greater than 100000*g* will achieve isolation of the exosome and small vesicle pellet (vesicles in the size of 100 nm or smaller) [16].

NTA

We evaluated the pre-analytical variables of storage and centrifugation. Lower counts of vesicles in the pellet compared to the supernatant fraction or neat plasma may reflect the fact that a centrifugation speed of 21000*g* results in isolation of all large (which would generally fit the description of endovascular or platelet derived MV) and some smaller, perhaps denser EVs, however, smaller vesicles, including exosomes, and plasma lipoproteins are not likely to be pelleted. Commercial beads can be used to simulate conditions for microvesicle estimation, however plasma samples are more challenging to analyse due to heterogeneity in EV size. EV measurement on Nanosight can be performed on fresh and/or frozen samples whilst ensuring that the pre-analytical variables are consistent. The CVs for plasma samples are high (up to 43%) indicating the need to run them in replicates. This will help reduce the high inter-run variability and average value could be considered as close to the true measurement. Solvent parameters need to be adjusted for NTA to ensure the conditions for temperature and viscosity are maintained in all experiments.

Freeze-thaw may interfere with vesicle integrity, as shown by higher EV counts in the frozen preparations. The size range detected in the pellet fractions suggest that both small and larger EV in the nm range is pelleted. The presence of EVs in the supernatant suggests some vesicles or plasma proteins cannot be pelleted and will be detectable on nanotracking. It is also likely that the exosome fraction is incompletely pelleted by 21000*g* for 1 h as shown by the presence of <100 nm EV in the supernatant fraction. It is therefore important to treat all samples in a similar fashion with respect to anticoagulant, centrifugation and storage conditions in order to minimize pre-analytic variability.

TRPS

One of the challenges using TRPS for plasma microvesicle measurement is the limitation imposed by the size of nanopores and the corresponding particle size range that can be measured. Nanopores (NP) are conical holes formed in flexible polyurethane membranes which are stretched to optimise the nanopore size on the qnano instrument according to the particles being measured. For example the 200 nm nanopore will measure a size range between 85-500 nm whereas a 800 nanopore will measure a size range between 385-2050 nm [13]. Analysis of an exosome population will require a nanopore of 70 nm (analysis range size 40-255 nm), 100 nm (analysis size range=80 to 255 nm) or 150 nm (analysis size range=70 to 420 nm). Usually, the manufacturer quoted particle range cannot be detected at single stretch of the nanopore [17].

Another technical challenge with using nanopores for analysis of heterogeneous samples such as plasma is that the smaller nanopores tend to get clogged with large particles. A filtration step was required for analysis using TRPS to detect vesicles of a certain size. The 0.8 μm and 0.45 μm syringe filters efficiently prepared samples for runs on the 800 nm and 200 nm nanopores without causing clogging during analysis. The particle rate was high when plasma alone was used undiluted in the upper chamber with the average current that flows across the pore being lower. This can also lead to repeated pore clogging which could interrupt the experiments. High dilutions resulted in very low particle rate and incorrect or inaccurate concentration estimates. Therefore, based on our observations a 1:10 or 1:20 dilution appears optimal. Stretch and voltage was fixed for our TRPS experiments based on trial runs and published literature from urinary vesicles [14].

Variability in measurements and techniques

The EV concentration and detectable size range can vary between techniques and instruments used. Variability in vesicle concentrations is primarily caused by differences between the minimum detectable vesicle sizes. The minimum detectable vesicle sizes reported in literature are 70-90 nm for NTA, 70-100 nm for TRPS, 150-190 nm for dedicated flow cytometry, and 270-600 nm for conventional flow cytometry. Transmission electron microscopy (TEM) has the potential to detect vesicles which are smaller in size, however only after they are adhered on to a surface. Dedicated flow cytometry is more accurate in determining the size of reference beads, but may have less accuracy when measuring biological vesicles, owing to heterogeneity of the refractive index of vesicles from body fluids or cells [18]. The main role of NTA and TRPS is to facilitate the measurement of EVs in the size range of 100 nm-300 nm which are usually not measured using standard techniques such as flow cytometry [1,14]. It may be critical to accurately measure vesicles in experiments where a correlation between particle count and function of EV is being ascertained [19]. One recommendation is to ensure that any reported concentration is accompanied by the minimum detectable vesicle size. Alternatively, mathematical modelling using a power-law fit can also be performed based on detectable minimum size [1].

Other authors have trialled spiking biological fluids with polystyrene beads of known size and concentration to improve EV measurement accuracy [19,20]. For NTA, a similar approach using silica beads would be less useful as the scatter mode does not discriminate between particles of similar size. We observed in our experiments that calculated bead counts show 2 to 6-fold variance from expected counts, particularly in diluted samples where the concentration of beads may not be optimal for the method employed. In fact, most manufacturers of calibration beads do not recommend dilutions given the errors that can be introduced in the process [17]. Also, for either mode, spiking samples with large (>500 nm) silica beads could lead to blockages of nanopores on TRPS and over scattering of the EVs as well as skew the characterization by NTA [18,21]. Applying correction factors for calibration beads mixed into biological samples is easier to apply in homogenous samples but more challenging in a complex sample such as plasma. Assessing subpopulations of varying sizes may be challenging by either method unless a complex mix of calibration beads of varying sizes can be independently applied to accurately quantitate. Such a process needs extensive validation before it can be applied to biological samples. However, measuring one particular narrow size range, by either technique, is possible based on the analytical limits of the process applied, e.g. pre-filtration and selecting nanopore size for TRPS, pre-filtration and focusing on one peak size for NTA.

Concluding notes on applications of nanotracking and TRPS for assessment of circulating MV or EV in plasma

The focus in vascular biology and haemostasis research is usually to evaluate the contribution of vesicles derived from platelets, as well as white, red and endothelial cells to the haemostatic process. Endothelial integrity and function can also be evaluated by measuring endothelial derived EV in circulation. The size range for studying endovascular vesicles in circulation is quite wide -ranging from a few nanometers up to 1 μm [22]. There are several reports of cardiovascular risk factors as well as cardiovascular disease being linked to circulating MV or EV - however, the studies have often used widely disparate technologies making it challenging to compare the results [2].

Flow cytometry techniques have been very popular in clinical physiology and vascular biology EV research, however the limitations of flow cytometry are now well recognized [8]. Both nanotracking and TRPS provide alternative techniques which may have potential to overcome some of these drawbacks [23]. Whilst nanotracking has potential for identifying specific EVs labelled with Qdots, TRPS has the potential of being able to mark EVs with aptamers. TRPS also has potential for particle by particle measurement of surface charge (known as zeta-potential) which may provide a discrimination between particles of varied surface charge and surrogate evidence for different cells of origin for the vesicles [11,14].

The potential benefits for sizing biological nanoparticles via resistive pulse sensing with a tunable nanopore has been recently demonstrated in experiments using adenovirus for chemotherapy delivery. The relation of size to physiological or pathophysiological role of the EVs is not clear; though it is possible that certain sized biological vesicles have greater capacity for cargo (either RNA or protein) transfer [12,23]. It is important to understand that biological fluids such as plasma are complex and heterogeneous. Repeated measures using several settings or analytical parameters may be required to capture the heterogeneity in a single sample and may still be unable to accurately measure vesicles in all size ranges. Understanding the advantages as well as limitations of newer approaches such as NTA and/or TRPS will enable focused, more accurate and comprehensive assessment in a specific size range of EV in circulation.

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