Analysing Extracellular Vesicles using TRPS

Introduction

With the widespread recognition of extracellular vesicles (EVs) as diagnostic and therapeutic agents, the importance of accurate and reliable measurement techniques has become clear[1]. These membrane-bound subcellular particles are secreted by almost all cell types. EVs include exosomes, microparticles, microvesicles and apoptotic bodies, categorised by size and origin. Characteristically, EVs express markers derived from their cell of origin, and their roles in physiological processes including cell-to-cell communication, and disease progression are beginning to be uncovered. Effects of EV-mediated signalling have been seen to include antigen presentation, induction of apoptosis and cellular activation[2]. Increased concentrations of EVs have been associated with disease states including cancer, autoimmune conditions and cardiovascular disease.

This paper focuses on EV analysis by tunable resistive pulse sensing (TRPS) complemented by size exclusion chromatography (SEC) isolation. Izon Science has recently released the 2015 version of its flagship TRPS instrument now called the qNano Gold. This instrument has application protocols to guide the users, reagents to address non-specific binding to the pore, and an improved limit of detection that allows smaller particles to be analysed with larger pores, giving greater system stability. The system software suite has also had a major upgrade, considerably improving user friendliness and further reducing measurement time.

The quantification and sizing of whole vesicles is of major interest for EV diagnostics. Many measurement techniques introduce significant variability to the results as they rely on user-defined settings and prior knowledge of the sample. TRPS is the most suitable method for analysis of EVs from biological fluids due to its calibrated and consistent measurements. TRPS on qNano Gold redefines the method of measuring concentration by reporting concentration as a function of a defined size range – this feature makes comparison of results standardisable and comparable. The fully calibrated measurements and inbuilt user guides leads to results that are directly comparable across laboratories. The qEV, qNano Gold, gold-standard protocols, and pore treatment reagents have been fully tested in a large international trial proving their accuracy and reliability.

The qEV column isolates EVs in a process that typically takes less than 15 minutes. The eluted vesicles are highly purified, non-aggregated and equilibrated into physiological buffers. The qEV purification method is fully described in the white paper "Izon qEV: a new tool for rapid EV isolation" It is possible to process raw biofluids to extract and analyse EVs in under one hour. TRPS with qEV offers a high throughput measurement system to quantify particle-by-particle size, concentration and zeta potential with high accuracy and resolution[3–5]. Importantly, these results are obtainable in a directly comparable, standardised form by different

people in different labs using different instruments.

Standardisation of measurements

Until now there has been a clear absence of a standardised methodology of EV measurement, and the need for this is well recognised[1]. The polydispersity of EV samples challenges traditional techniques, as instrument settings will often need to be optimised to a narrow size range in order to record quality data. qEV purification and TRPS analysis are powerful tools for rapid and precise EV sizing, quantitation and charge measurement.

The critical factors for successful TRPS analysis of EVs include sample quality preparation via qEV SEC, calibrated measurements, pore pre-treatment to give enhanced measurement stability, and consistent measurements settings.

Sample preparation quality

EV analysis by TRPS is now routine for many biofluids, including serum, plasma, saliva, urine and cerebral spinal fluid. Sample quality is paramount with pore based technologies. The combination of centrifugation followed by qEV fractionation eliminates the bulk contaminating components of complex biological samples including free lipids, lipoproteins, cell debris and small molecules. The samples eluted from the qEV are ready for immediate TRPS analysis[1].

Calibrated measurements

As every TRPS measurement is calibrated to a known standard, the accuracy of the measurement is guaranteed and results from different days, users and pores can be directly compared.

Concentration is calculated from the rate of particles passing through the pore over time. Unlike other techniques, the calculation is independent of the biochemical composition and size of the particle, so long as it is within the detection limits of the pore. Additionally, due to its small sensing zone and lower detection limit of ~40 nm, TRPS does not suffer from measurement of multiple vesicles at the same time – the well-known issue of "swarming" in flow cytometry – and it is able to measure a wide range of particles that may otherwise be missed by other techniques[6]

Stability of measurements

The concept of non-specific binding (NSB) relates to interactions of sample components which may interfere with routine laboratory analytical procedures; NSB is a well-known issue in bioanalysis and all methodologies must deal with it adequately.

In the case of TRPS, NSB can interfere with measurements in two ways – firstly, contaminating molecules can bind to the pore, altering the membrane properties during the course of an experiment and rendering the calibration meaningless. Secondly, the EVs themselves can bind to the pore, causing it to block and prevent further measurement.

Managing NSB in TRPS is a critical part of the measurement in order to obtain meaningful results. Izon Science has developed reagents to treat the pore and render it highly resilient to modification by NSB and pore blocking, without interfering with calibration of measurement of EVs (figure 1). This

[1] Contact enquiries@izon.com for standard protocols for common biofluids

treatment achieves same pore performance before and after exposure to biofluids, increasing the stability and achievable duration of the measurements.

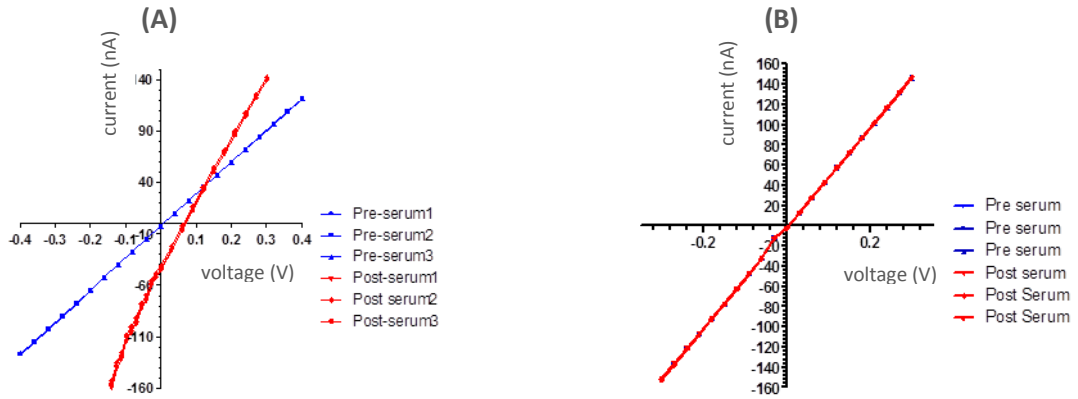


Figure 1. Contact with biological fluids alters the properties of the pore: An IV curve shows the resolving characteristic of a pore and must remain the same for the entire measurement. Analysis of carboxylate polystyrene particles before (blue data points) and after (red data points) exposure of the pore to serum proteins showed that the IV curve were significantly affected (A). When the pore is pre-treated to prevent modification, the IV curve is unaffected after exposure to serum proteins (B).

Consistent measurement settings

A considerable problem of commonly used techniques for EV characterisation is the influence of user-defined settings, and requirement of precise prior knowledge of the sample such as composition, refractive index, density and so on. This means that the results obtained will differ between users, and are therefore not comparable. With TRPS the settings for capturing polydisperse samples are independent of the sample, and can be pre-determined allowing consistent capture of target size ranges of interest (figure 2).

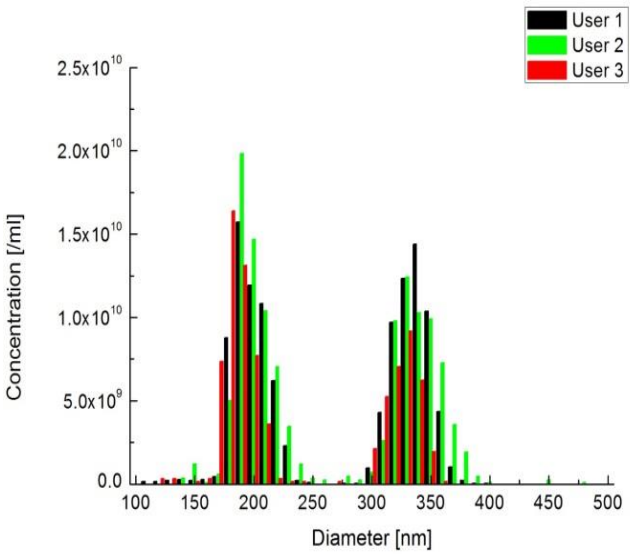


Figure 2. Repeat measurements of the same sample show high reproducibility: The same bimodal sample was distributed to and measured by three users in different groups across the world, without prior communication between the groups. The size distributions overlay very well, indicating the reliability of the data.

Redefining concentration measurements

The polydispersity of EV preparations demands a high resolution technique, and TRPS has been shown to be the most accurate method of determining subpopulations within a polydisperse sample[7].

Accurate measurement of the polydispersity is essential as this can be indicative of the origin of the vesicles, and has potential as a diagnostic marker. For example, tumour - derived EVs have been seen to have greater polydispersity than those from healthy cell lines[8–10]. To aid in comparison the qNano Gold from Izon Science measures the concentration against standard size ranges to give a consistent measure for polydispersity (see figure 3).

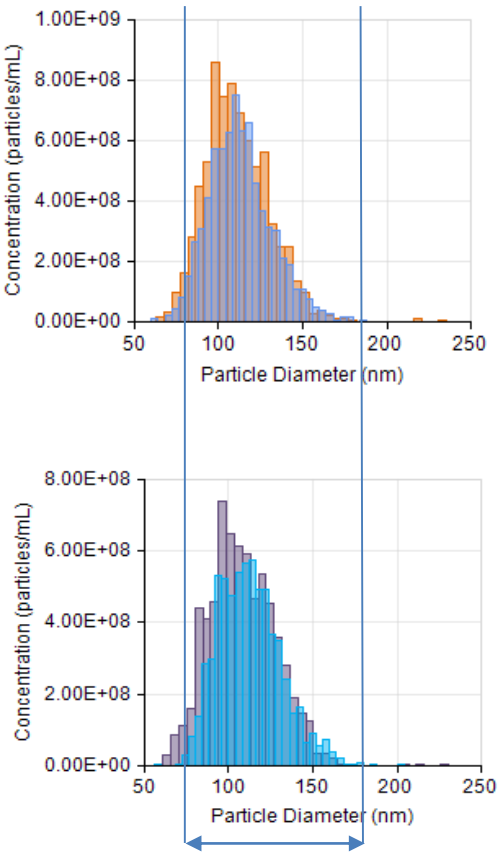
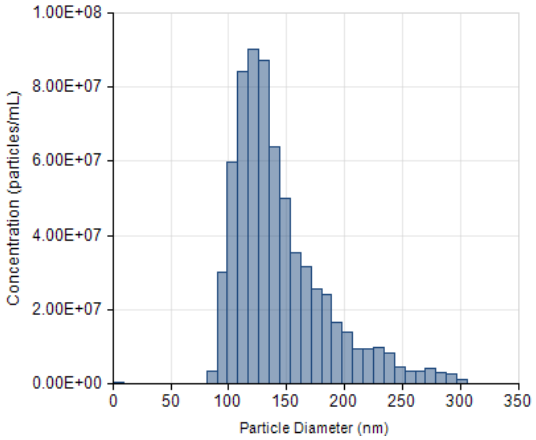


Figure 3. Two patient's data can be compared by using a standard size range to report concentration over. In the example above the blue lines illustrate comparison over 80 to 180nm.

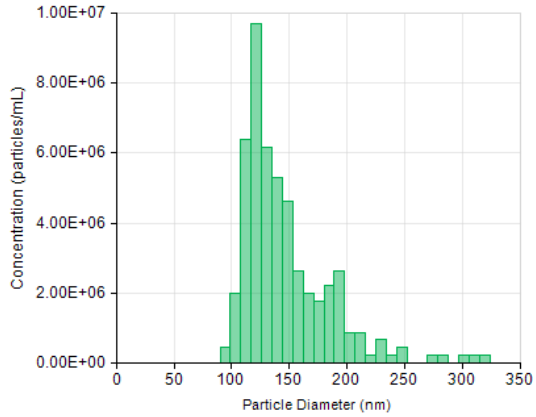
TRPS has been used to measure the size distribution of EVs from a number of sources, and standard protocols for common biofluids[2] have been developed to allow the user to obtain accurate data. The measurement of various biofluids is demonstrated for urine, cerebrospinal fluid (CSF) and plasma derived EVs, which are shown in figure 4.

The plasma standard has been tested in an international trial involving 6 different research groups used who completed 69 measurements obtain a variance of 56% for the isolation and measurement steps. This is the first such standardised measurement trial of its type. Trials for urine and CSF are being planned.

Urine



CSF



Plasma

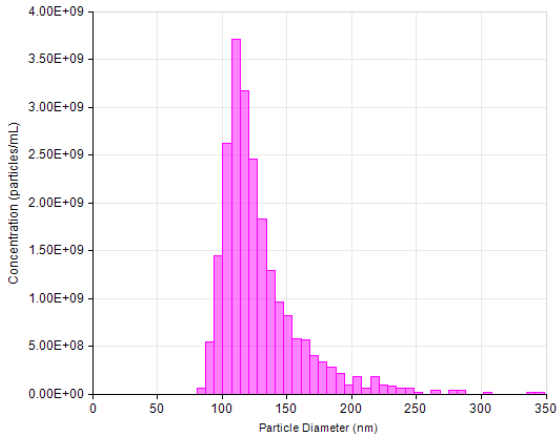


Figure 4. TRPS is able to measure EVs from a variety of sources: The histograms above represent the size distributions of EV samples purified from three different human biofluids.

[2] Available for download from www.izon.com

Biomarker identification (phenotyping)

Phenotyping refers to assays that reveal protein biomarkers on EVs and relates those biomarkers to EV source or disease state. Despite their recognition as potential diagnostic agents, the ability to detect surface markers of EVs remains limited. The current methods of immunoblotting or flow cytometry require large numbers of EVs, and have an insufficient detection limit[11]. Rapid and simple methods to define the phenotype of EVs will be essential if they are to be used in diagnostic tests.

The particle by particle analysis of TRPS allows for unique analysis and characterisation of the EV surface markers, and sensitive quantification of the EVs displaying particular markers.

Phenotyping using aptamers

The ability to analyse individual particle charge is a powerful feature of TRPS for phenotyping EVs. As they are typically weakly negatively charged, the addition of a highly charged probe will significantly shift the charge of the EVs. This shift can be detected on a single particle basis using TRPS.

Aptamers are single-stranded DNA molecules that bind to target molecules with high specificity. Measurement of the binding of aptamers to particles using TRPS has been demonstrated recently[12]. The highly negative charge of the aptamer alters the zeta potential of the particle displaying the target upon binding. EVs from the B16 tumor cell lines express Annexin A2, and so were incubated with an aptamer targeted to this protein to investigate binding.[13] A significant shift in EV charge was seen after incubation (figure 5), which did not occur when EVs were incubated with a non-specific aptamer (data not shown).

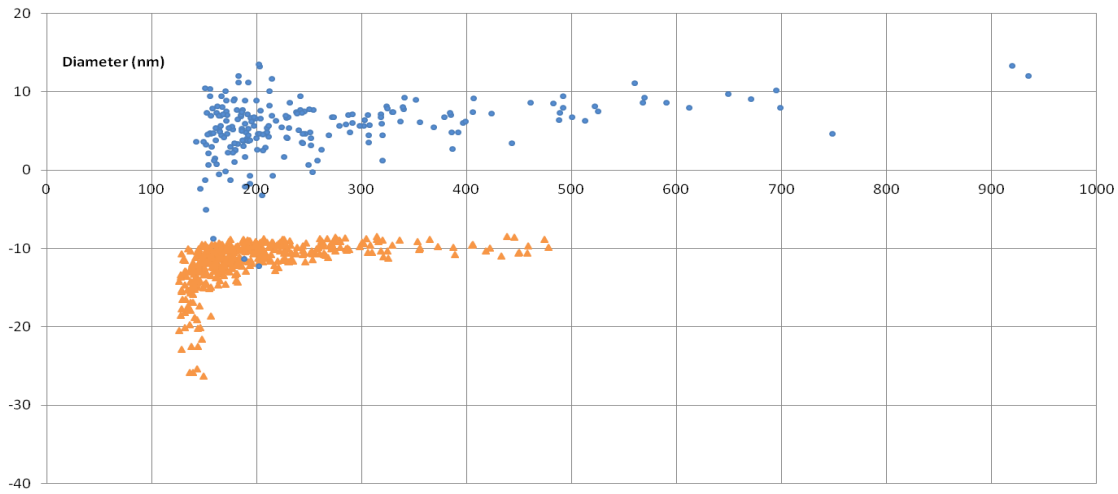


Figure 5: Binding of a targeted aptamer induces a detectable change in EV zeta potential: Before incubation with the aptamer (blue data points), EVs were found to have a zeta potential of 5.5 mV in PBS buffer. After incubation (orange data points), the zeta potential of the EVs was shifted to a value of -12 mV in PBS buffer, indicating that the aptamer had bound and altered the surface charge.

Particle-by-particle zeta potential quantification distinguishes EV populations

Individual particle charge measurement also opens up the possibility of characterising EV populations by charge differences only.

A calibration based zeta potential method was recently developed (Vogel *et al.,* to be published), based on the measurement of signal durations of translocation events as a function of voltage and applied pressure. This provides a very reproducible technique, which is consistent across different instrument settings and users. The particle-by-particle nature of TRPS is a unique feature for zeta potential analysis as most other techniques report an average value. Zeta potential analysis may become particularly useful for identification of EV identity and origin, for example, some cancer cell-derived EVs are known to show phospholipid reorganisation, and the alteration of surface charge through the externalisation of different lipids may be able to be detected as a diagnostic marker [14].

While the physiological relevance of EV zeta potential is currently unknown, as more researchers adopt TRPS for EV measurement, trends may be identified. Examination of EV zeta potential has been used to identify subpopulations within a sample. Figure 6A shows data recorded from a human CSF sample. When the particle size distribution is analysed, the sample shows a fairly monodisperse profile. The full width half maximum (FWHM) value for a particular particle is related to its zeta potential. A larger FWHM indicates that it traverses the pore more slowly, and is therefore less negatively charged. When the FWHM values of the EVs in the human CSF sample are examined (figure 6B), two populations can be seen. This indicates that there are in fact two distinct EV types in the sample, which would not be identified through size analysis alone. An advantage of TRPS is the ability to obtain large amounts of information about individual particles from one recording.

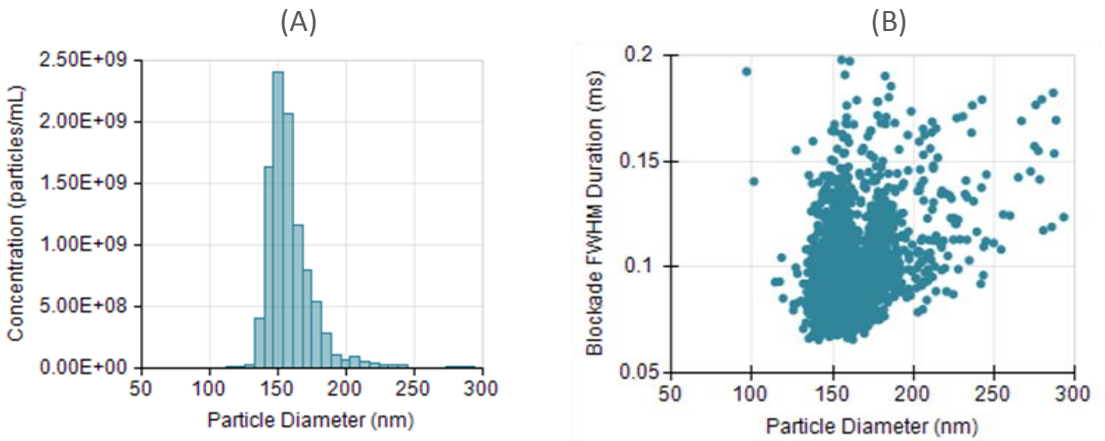


Figure 6: A: The size distribution profile for a single sample shows a fairly monodisperse profile. B: When the zeta potential of the EVs is examined (represented by particle FWHM), two distinct populations can be seen.

EVs that were measured from CSF of both healthy and diseased individuals appeared to show some differences in their zeta potential (figure 7). Although a large difference in mean value was not seen in the disease state, the charge profile of the healthy sample showed a broader spread than that of the diseased sample.

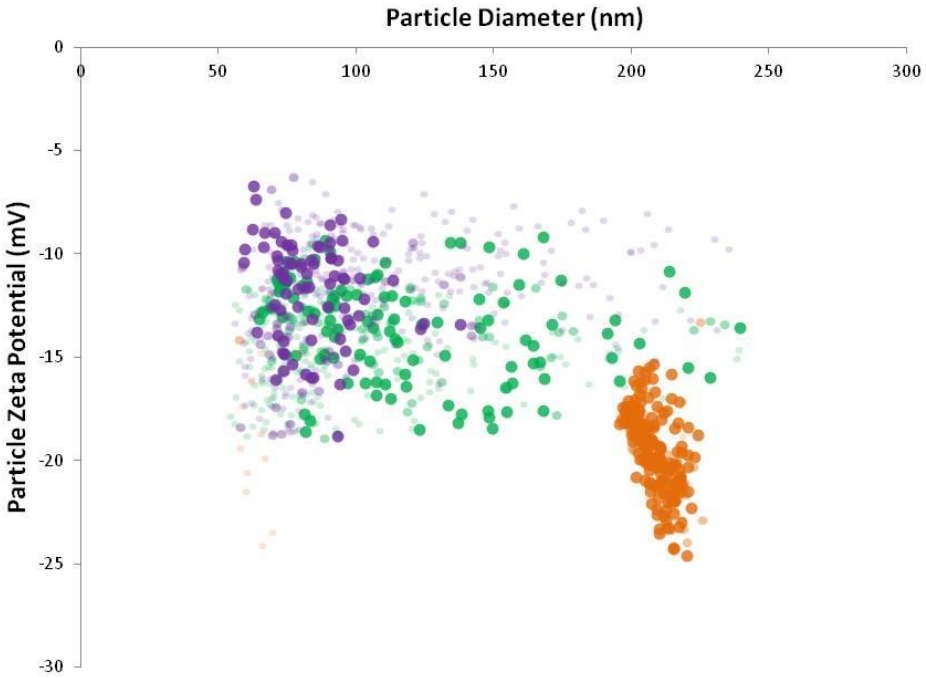


Figure 7 Charge profiles of healthy and diseased samples: Particles from healthy samples (green dots) have a mean zeta potential of -17 mV, whereas particles from diseased samples (purple dots) have a mean zeta potential of -15mV in PBS buffer. Calibration particles are shown in orange for reference (diameter = 210 nm; zeta potential = -21 mV in PBS).

Conclusion

TRPS provides EV researchers with a valuable tool to analyse the size distribution, concentration and zeta potential of vesicles with high resolution and accuracy. The standardised protocols and reagents developed by Izon allow users to generate reliable and reproducible data, meaning that results from groups across the world can be meaningfully compared. The particle-by-particle approach of TRPS analysis builds an accurate size distribution profile for the sample with minimal prior knowledge of the sample required. This overcomes many of the limitations that are currently hindering researchers with traditional bulk analysis techniques.

The application of TRPS analysis to EVs has been demonstrated with EV samples purified from urine, plasma, saliva, cell culture media and cerebrospinal fluid. The ability to analyse EVs from diverse samples with TRPS will enable researchers to build up an accurate, comparable picture of the EV "signature" of various biofluids, and begin to identify the changes that occur in disease states. This will be an essential step toward the use of EV analysis as a diagnostic technique. The measurement of zeta potential in combination with particle size will lead to a more detailed understanding of EVs, and could have implications for their use as therapeutic agents.

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