Izon qEV: a new tool for rapid EV isolation

Introduction

Extracellular vesicles (EVs) are prevalent in biological fluids and are of great interest to researchers due to the compelling evidence for their vital roles in cell signalling, immune response modulation, apoptosis induction and cellular activation, as well as their altered levels in disease states[1,2]. They are expected to offer highly effective new options for diagnostics and therapeutics. EVs include exosomes, microparticles, microvesicles and apoptotic bodies, with the exosomes and microvesicles from 30 – 200 nm diameter being of particular interest. The heterogeneous nature of biological fluids often complicates measurement and analysis of individual components, in particular the presence of free molecules such as proteins, RNA, DNA, cell debris, lipids and lipoprotein HDL hamper analytical procedures. Analysis of EVs requires their isolation from complex biological fluids. However obtaining pure samples of EVs effectively has been a key limitation to research[3]. Whilst ultracentrifugation and density gradient sedimentation are the most well documented methods[4], they are known to suffer from the presence of contaminating cellular debris[5] and take a long time to achieve separation. Other commercial methods that use proprietary precipitative reagents have severe drawbacks with respect to aggregation and contamination.

There is a need for a standardised approach for EV isolation which will allow rapid and gentle purification, avoiding the common problems of aggregation and insufficient removal of contaminating biological molecules. This is currently difficult without multiple techniques or bespoke instrumentation, and so a cost effective process that is accessible to all researchers is needed. Standardisation of analysis data is important for EV research to be successfully applied to routine clinical use. Faster processing of samples will also be necessary to get practical clinical benefits from EV research.

This white paper introduces the qEV, a Size Exclusion Chromatography (SEC) separation technology specifically developed to address the existing obstacles in routine EV extraction. qEV is rapid, gentle, non-aggregating, highly purifying, cost effective and easily standardisable. The use of qEV columns leads to higher quality measurement and analysis. In particular it can be combined with Tunable Resistive Pulse Sensing (TRPS), which is now the measurement method of choice for biofluid analysis, combining rapid integral isolation of EVs with precise, standardised particle quantitation and sizing characterisation. These approaches, combined with down-stream phenotyping capabilities, will become bench-mark tools in biomedical diagnostics.

EV isolation

The quality of EV sample preparation is a critical factor affecting the quality of the downstream characterisation data collected. For instance RNA profiling of EVs is difficult if the EV preparation is highly contaminated with free RNA. Analysis of surface biomarkers can be hampered by aggregation or contaminating cell debris. The key issues for establishing a gold standard EV isolation

method requires addressing the following issues: accurate concentration and size analysis, preserving the integrity of the EVs and obtaining high levels of purity. These issues are addressed below.

EV concentration analysis

In the near future, screening of EVs for quantitative changes and biomarker profiling will be a critical component in early disease detection as well as monitoring disease progression. EVs must be purified away from the myriad of other components present in complex biological fluids before quantitative analysis can be undertaken. The concentration of EVs in biological fluids typically ranges from 10^6 to 10^{10} particles per mL[6,7]. However the true variation between EV concentration from different biofluids, between individuals, and during the development of disease is unknown, due to a scarcity of comprehensive studies, caused in part by the lack of adequate high quality isolation and measurement capabilities. It is now recognised that concentration numbers need to be referenced to a well-defined size range. Effective isolation makes that possible.

Recovery data and size distribution data for EVs before and after qEV fractionation show that together with TRPS, accurate size and concentration measurements are obtainable.

EV sample viability and purity

EVs, like other membrane vesicles are delicate structures that have limited stability and require handling with due care. Degradative processes may occur rapidly and for this reason preparation procedures should be rapid to minimize time dependent degradation. qEV is the most rapid, taking less than 15 minutes to achieve separation of EVs from many types of biological fluid. This is in contrast to centrifugation protocols that range from multiple hours to days and the precipitation kits that often require overnight incubation.

Existing methodologies for EV purification do not achieve the simultaneous goals of high purity, rapid isolation and EV integrity. To avoid having to compromise between yield and preparation time, a quick, effective and reproducible purification method is required. Izon Science's qEV size exclusion chromatography column exhibits high levels of purity, is gentle, avoids aggregation and delivers EVs into physiological buffers ready for downstream analysis[8].

Speed

The time required for a qEV separation is approximately 15 minutes and this makes for a very time and cost efficient method. Equally importantly it offers a step change improvement in time to get a measurement or analysis result. Combining qEV separation with TRPS measurement enables accurate and detailed EV data to be obtained from plasma in 30-40 minutes.

The qEV principle

qEV uses size exclusion chromatography, a commonly used preparative technique to purify molecules or particles in a polydisperse solution according to their size. The technique works to separate a mixture by distributing the individual components between a stationary phase (the partially permeable gel matrix within the column), and a mobile phase (the eluent), which carries the mixture through the column. Small particles diffuse into the beads of the matrix, and are slowed in their passage through the column, whilst large particles cannot enter the beads and pass through the column quickly with the mobile phase.

SEC purification is known as a rapid, and efficient method to isolate vesicles from biological fluids. The gentle nature of the technique eliminates the problems of vesicle

aggregation that are seen during high speed centrifugation steps[9]. Furthermore, the viscosity of biological fluids does not cause a problem as it does in centrifugation-based procedures. The time to purify vesicles is rapid, reducing potential degradation over time while the sample is being purified.

Details of the Izon qEV columns

Izon currently provides a qEV column (capable of being reused approximately five times) and isolates EVs rapidly. The columns contain a gel with an approximate 70 nm pore size[10]. Theoretically, particles above 70 nm should elute in the void volume as they cannot enter the pores, and in practice it has been seen that the smallest EVs in the void volume have a 70nm diameter[8]. Proteins and other contaminating molecules are slowed in their passage through the column as they enter the stationary phase and therefore elute later. The time to perform an EV isolation using a qEV is made up of two components - preparation time to equilibrate the column, typically about 10 minutes; and sample running time of approximately 5 minutes. The current column is packed with 10 mL of gel. In the future, more varieties of column will be available, mini columns, longer columns as well as columns with smaller pore sizes. The combination of qEV + TRPS represents an advance in EV analysis, for instance in the case of plasma EVs, it is feasible to go from raw blood to EV measurement and quantitation in under an hour.

qEV efficient removal of protein

Under ideal conditions SEC purification of vesicles can result in up to 6000-fold enrichment in vesicles relative to protein[8]. Enrichment in later fractions decreases as protein begins to elute (see figure 1).

Figure 2 shows the elution profile of the EVs in fractions 7 to 9 measured by TRPS and the background proteins and components of the sample measured by UV absorption at 280nm. The enrichment for EVs is clear, they elute predominantly in fractions 7 to 9 and the serum protein is slowed eluting predominantly from fractions 11 to 30. The separation of the peaks means that collection of pure vesicles is simple.

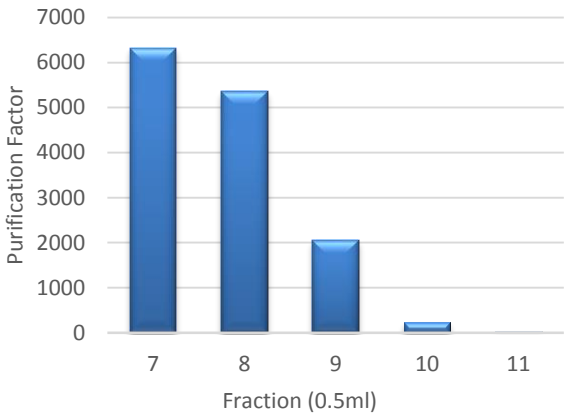

Figure 1. Typical purification of EVs relative to protein after qEV purification. The purification factor (reduction in ratio of protein after and before SEC isolation) in each fraction is shown. Fraction 7 and 8 were most enriched.

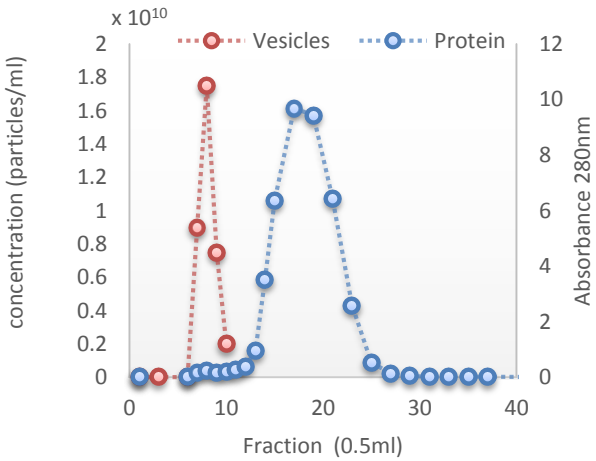

Figure 2. Typical elution profile for a qEV column, proteins elute in later fractions than vesicles

qEV removal of lipids

A significant problem with ultracentrifugation and density gradient sedimentation is that the similar density of lipoprotein HDL and LDL particles to EVs means that the particles often co-isolate with EVs[11]. Collection of appropriate fractions from SEC isolation

results in up to 95% removal of contaminating HDL from EV samples[8]. Often, contamination from lipids is assessed by visual examination and if deemed to be unacceptable, lipids are removed by refrigeration and precipitation after centrifugation[12]. This prolongs the purification by several hours, and as it is recommended that EVs be characterised as soon as possible after collection[13], this may be an undesirable step. The mechanism of removal of lipids from solution by SEC is not completely understood but is thought to rely on adsorption[14].

Reproducibility of purification

Quantification of EVs before isolation to estimate the yield of purification methods is prone to error due to the high levels of contaminants

A formal methodology has been adopted that uses a known sample and measures it multiple times to give an estimate of processing and measurement variability. This can then be used to evaluate different techniques in a comparative way. It also gives valuble information for practical use to evaluate samples from different sources, as the variance of isolation and measurement is quantified and can be accounted for in the comparison.

An international study[15] has been carried out by six researchers at different research institutes whereby an indentical plasma sample was isolated and analysed using qEV columns and TRPS. This showed that the variation of measurements, expressed as coefficient of variance (CV) was 56%, this number includes the isolation method variance and the measurement variance. The 95% confidence level (normalised to mean) was 0.86 to 1.14

Purity and sample loading on the qEV

Size exclusion chromatography always results in some dilution of the sample that is being fractionated. The dilution will depend on the input volume and the number of fractions the target particles elute within. Optimal loading volume on the qEV is between 100-500 µL of sample and the dilution factor is typically 2 to 4 fold. Higher loading volumes broaden the distribution profile and the EVs will elute later (see figure 3, 2000 µL loading). Higher volumes of sample result in EVs of lower purity, contaminated with background proteins which are eluting from the column.

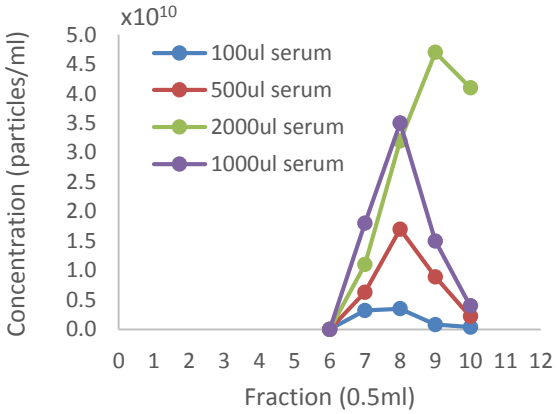

Figure 3. Vesicle elution profiles for loading of serum from 100 to 2000 µL. Loading greater than 500 µL extends the elution peak into fractions 9 & 10 where the protein is beginning to elute. High purity is maintained by collecting fractions 7 and 8. Particle concentration is measured in the size range 70-300 nm.

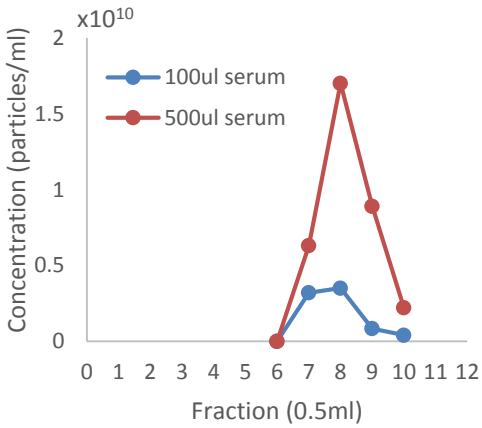

Figure 4. Elution profile for 100 µL and 500 µL of bovine serum from qEV, the optimal loading range for these columns. EVs concentrations are in the 70-300 nm range

Figure 4 shows the qEV elution profiles for the recommended loading volumes of 100 and 500 µL serum. With 100 µL, the total combined particle concentration that eluted in fractions 7 to 10 was 7.5 x 10^9/mL. The total combined particle concentration that eluted in fractions 7 to 10 when 500 µL sample volume was loaded was 34 x 10^9/mL. The recovery from 500µL sample volume was therefore 91% when compared to the yield for the 100 µL. Loss occurs with higher loading volumes as the elution peak is broadened and some particles elute in fraction 11 and are of lower purity.

Recovery studies with the qEV

Studies were carried out with known concentrations of both polystyrene nanoparticle standards, and liposomes designed to mimic the size and composition of EVs.

The dilution factor depends on which fractions are pooled. Using a 500 µL starting volume of polystyrene standard particles (mode diameter 400 nm), the total particle recovery was ~100%. Figure 5 illustrates that fractions 5 to 11 need to be pooled to recover 100% of the particles. This results in a dilution factor of 7 and is therefore not practical.

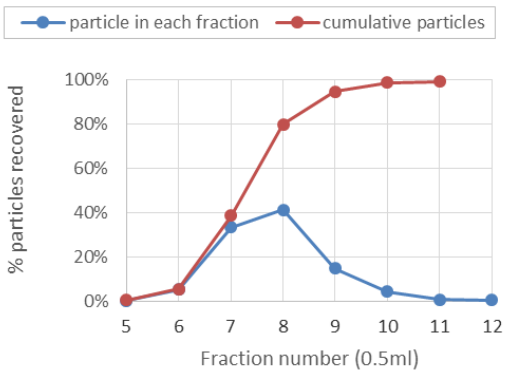

Figure 5 Elution profile for 400 nm polystyrene particles

Table 1 shows the dilution factor, and the recovery rate for various fraction collections. Liposome trials have shown recovery rates of ~50%, when the two fractions 7 and 8 were pooled, which is consistent with the polystyrene example above.

Fractions pooled	Volume pooled (mL)	Percent of particles recovered	Dilution factor of sample
7	0.5	33%	3.0
8	0.5	41%	2.4
7 to 8	1.0	74%	2.7
7 to 9	1.5	89%	3.4
6 to 9	2.0	94%	4.2
6 to 10	2.5	98%	5.1

Table 1. Dilution effect as a parameter of fractions pooled and recovery rate. Whilst it is possible to recover 100% of particles the highest concentration obtained is by measuring each fraction and using the two fractions with highest concentration. Thus a compromise between concentration and purity of the EVs that are collected is needed.

Size distribution

A concern with any sample preparative technique is that bias may be inadvertently introduced, resulting in a preparation that is not representative of the natural state. Whilst it has been demonstrated that typical methods of EV purification do not usually influence the size distribution profile of a model liposome system, comparison of the distribution in EV samples has been hindered by the difficulty in measuring raw samples. EVs in cerebrospinal fluid (CSF) and serum are able to be measured using TRPS prior to qEV purification. Figure 6 demonstrates that qEV purification does not affect the size distribution profile of EVs in CSF or serum.

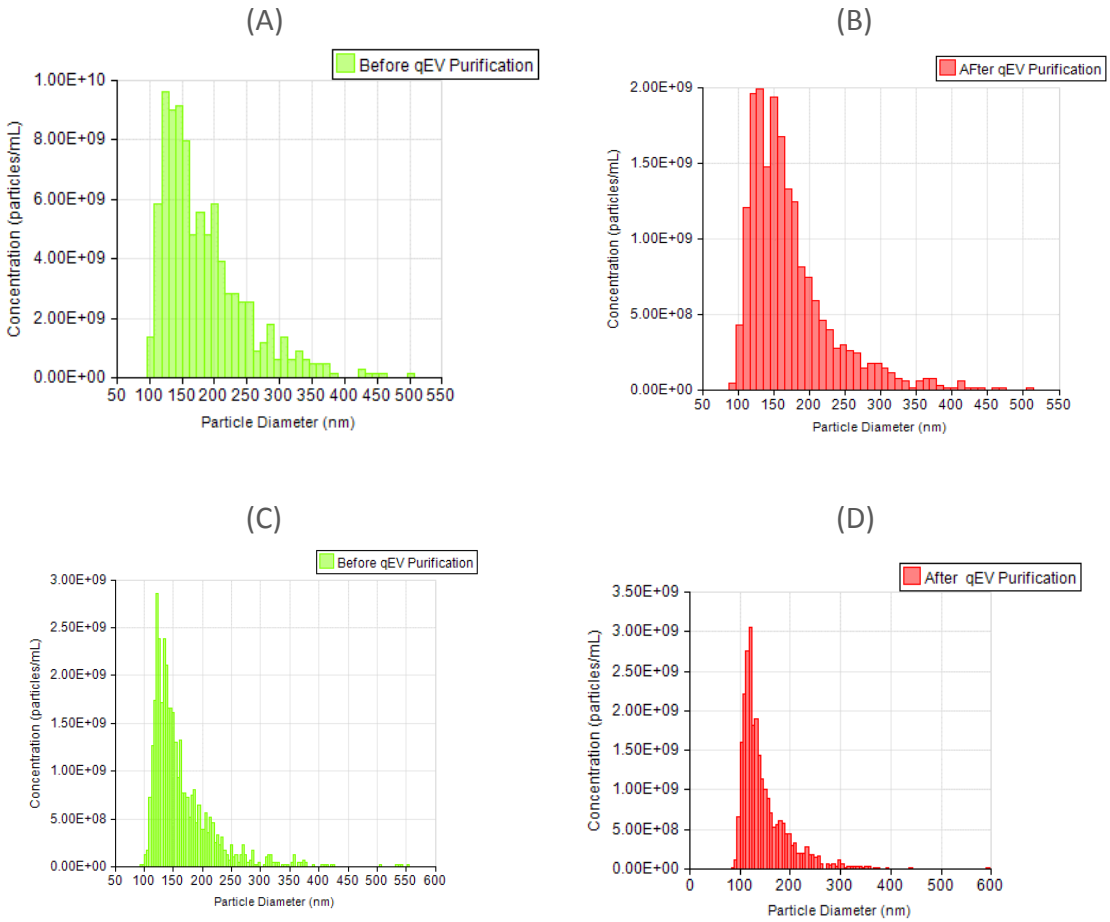

Figure 6. Effect of qEV purification on particle size distributions. Comparison of the two size distribution profiles shows that the qEV purification causes no significant change to the size distribution profile of EVs, though there is some decrease in concentration as would be expected. A) and B) are for a CSF sample; C) and D) are for a serum sample.

Fraction collection volume

Collection of 0.5 mL fractions is recommended, with the highest EV concentration typically found in the 7th and 8th fraction. The choice of fraction volume will determine the purity, dilution, and ultimately the yield of EVs collected. Beyond the void volume (that is, beyond about 4.5 mL elution volume), the complex mix of smaller components in the original sample that were not excluded from the resin will begin to elute. This will include proteins and other non-EV related solutes from the sample. Collecting later fractions could mean that the EVs are not completely isolated from these particles, decreasing the purity of the sample. A large fraction volume will also mean that the tested sample is more dilute, and may hinder measurements.

TRPS analysis of EV samples and non-specific binding

Biologicals contain a complex mixture of molecules, e.g. proteins, peptides, lipids and carbohydrates. Purification greatly reduces the quantity of these, but small amounts may remain, and could interfere with TRPS measurements through non-specifically binding (NSB) to the pore. As with many analytical procedures, dealing with NSB is important for successful analysis. For instance, Western blots, Southern blots and *in-situ* hybridisation experiments all utilise reagents to eliminate NSB issues. For EV measurements the use of Izon Science Coating

Reagent (ICR) for pre-treating the pore prior to measuring the biologicals is essential. ICR has been especially developed for TRPS analysis and contains pH and electrolyte balanced components to ensure reliable and consistent EV measurements.

Other isolation techniques

Ultracentrifugation

Ultracentrifugation (UC) is the most commonly used method for isolating vesicles from biological fluids[16]. Purification of vesicles is typically achieved through multiple centrifugation steps[17], which sequentially increase in speed and duration, thus sedimenting smaller particles. A number of issues are associated with UC, as the quantity and quality of the vesicles isolated is influenced by a number of factors including the type of rotor that is used, the rotor angle, and the solution viscosity[18]. Furthermore, numerous studies have indicated that UC results in the sedimentation of non-vesicular materials and so further downstream methods will be required to remove these particles[19]. A major problem with UC is that when centrifugal force reaches ~100,000 g (which is required to pellet vesicles) proteins in solution start to aggregate[9]. This will result in large protein aggregates being present in the sample which may be mistaken for vesicles. In addition the clumping of vesicles may occur through surface protein aggregation, and will result in inaccurate size analysis or loss of EVs. Centrifugation alone is often inadequate for the removal of soluble proteins from the sample[16] as an inadequate number of wash steps results in apparent decreases in protein concentration, which are in fact due to incomplete recovery of available material. Recovery of EVs from ultracentrifugation purification is reported to be 2 – 80 %, which indicates that serious variability can occur[8] and suggests that comparisons of samples isolated using this technique should be considered with caution.

Density gradient centrifugation

Particles are centrifuged in a tube containing a density gradient created by layering different concentrations of a liquid, typically sucrose. A sucrose gradient has the benefit that it relies on the low density of the vesicles and purification may, therefore, be more specific than a centrifugation-based approach. In comparisons of typical techniques, including ultracentrifugation and density gradient, the latter was shown to result in the purest final sample. However, where particles with similar densities are present, they will co-isolate with EVs. This has been found to be a problem in fluids such as plasma, with have high levels of HDLs which are seen to co-isolate[20]. EVs have been characterised to have densities of 1.1 – 1.9 g/mL – this allows the separation from protein and protein-RNA aggregates, but the similarity to the density of water means that some EVs take up to 90 hours to reach equilibrium density[21]. Therefore, the traditional approach of a 16 hour centrifugation time will be insufficient for isolation, and density gradient centrifugation only suggested as a final refinement step rather than a complete isolation technique[20].

Precipitation

Precipitation of vesicles from bio-fluids is based on polymer-base precipitation techniques that have been used to isolate viruses for the last 50 years. Disadvantages of these techniques include the precipitation of non-vesicular particles, and the resulting buffer may not be compatible with further analyses. In addition precipitation techniques usually require overnight incubation of the sample[16]. Pre-isolation steps have been developed to remove sub-cellular particles including LDLs and HDLs[20], and further SEC processing is usually required to remove the polymer, somewhat complicating the technique. The non-specific nature of

precipitation techniques means that all particles in a sample will be "purified", and so lipid particles, protein aggregates, or other cellular particles will remain, and further analytical steps will be required to discriminate EVs from other particles.

Conclusion

Any EV isolation method requires careful handling of the sample to preserve its biological integrity. For correct analysis of biomarkers and EV functions high levels of purification are needed to remove proteins, lipids, RNA, and other material that will hinder downstream assays. Size exclusion chromatography using an Izon Science's qEV meets these challenges of high purity and preservation of the biological and morphological characteristics of the EVs and is superior to other techniques in both speed of separation, quality of resultant sample and cost. Furthermore, due to its speed of separation (of less than 15 minutes) it increases the throughput and minimises EV degradation in situations where the EVs in their native fluid may change over time. In order to maximise the recovery of EVs from biological fluids the protocol detailed in the Izon Sciences qEV Application Notes will help to optimise your results.

Contact Izon Science at enquires@izon.com to discuss how we can support you.

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