ADVANCES IN LARGE-SCALE EXTRACELLULAR VESICLE SEPARATION

APPLICATION NOTE

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To successfully leverage extracellular vesicles (EVs) as 'nature's delivery vehicle', there is a critical need for efficient, large-scale separation solutions. However, many separation techniques used in research stages – such as ultracentrifugation – are not suited to large-scale operations and represent a major bottleneck in the development of EV applications. Over the years, Izon Science has grown its separation and analysis technology to support the growing EV research field and related industries. Izon's qEV isolation platform, which consists of a range of size-exclusion chromatography columns (qEV columns) and the Automatic Fraction Collector (AFC), is widely used in EV research.

Recognising the demand for large-scale separation solutions, Izon applies its expertise to support industry partners seeking a custom-designed, multi-component strategy for the isolation of EVs from their cell culture conditioned media. We offer this through the qEV PurePath for Therapeutics service which specialises in designing, optimising and piloting EV isolation from large volumes for therapeutics customers. Similarly, for diagnostics companies aiming to increase the throughput of EV isolation from numerous biofluid samples, we offer qEV PurePath for Diagnostics.

Here, we present the current state of EV isolation methods and outline the approach taken by Izon Science to achieve a level of recovery and purity suited to one particular large-scale operation as part of our qEV PurePath for Therapeutics service.

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1 / INTRODUCTION

Advances in extracellular vesicle (EV) research have provided invaluable information about the role of EVs in physiological and pathological conditions, and this growing body of knowledge continues to reveal the huge potential of EVs for clinical use. Native EVs have composition-dependent properties and capabilities which make them highly suited to therapeutic applications, such as efficient cell entry and cargo delivery, low immunogenicity, high bioavailability, low cytotoxicity, high biocompatibility, and tissue tropism. In addition to these natural traits, EVs can be further bioengineered; modifications in their composition may lead to improved stability, drug-loading capabilities and cell targeting.[1]

Currently, the therapeutic application of EVs is partly limited by the lack of a standardised method for isolating clinical grade EVs. While technologies have been well established for the manufacture of other lipid nanostructures such as synthetic liposomes, there is a lack of consensus for methods adjusted to more complex EV samples. In general, the clinical translation or successful transfer of low-scale methods to mass scale is challenging, and the EV field is no different. Regardless of the purpose of EV preparations, EVs must be produced and isolated in quantities that are sufficient for both mandatory EV quality assessments and the desired application. The EV-ceutical field is growing rapidly, with applications being pursued across therapeutics, including drug delivery systems, vaccines, and in cosmeceutical and nutraceutical applications. Each area will have specific regulations and requirements to be met and share a need for custom and efficient separation workflows.

qEV PurePath for Therapeutics is a full-service customisable EV isolation workflow designed to help take you from crude starting sample to a therapeutics-ready isolate. In this application note, we will take you through some of the steps involved in isolating EVs from large volumes.

2 / CLARIFICATION, A KEY STEP IN LARGE-SCALE EV SEPARATION

Before EVs can be separated from other components, the sample must undergo a process called clarification to remove the bulk part of the contaminant load. During clarification, components such as intact cells and cellular debris are removed to improve the efficiency of the subsequent EV separation method.

Common clarification methods in the EV field include the use of a sequential 'step-up' approach to low-speed centrifugation (e.g. 200 xg, 2000 xg, 20000 xg), membrane-based microfiltration or ultrafiltration with suitable pore sizes to retain EVs of interest. For samples with a particularly high contamination burden, the best results will be achieved using a combination of these methods. Some methods, such as ultrafiltration, have the added benefit of providing EV enrichment – leading to more EV counts per unit volume.

However, it should be noted some of these techniques can bias the size of isolated EVs. For example, centrifugation will lead to the pelleting of larger EVs, depleting them from your sample. As such, the correct clarification method should be chosen for your application. Our expert scientists can help you choose and test the method most appropriate for your application as part of the qEV PurePath for Therapeutics service.

3 / MANY SEPARATION METHODS UNSUITED TO LARGE-SCALE OPERATIONS

EV separation techniques are assessed by measuring recovery and specificity; where EV recovery is the final EV yield obtained, and EV specificity is the extent of separation from all other non-EV entities.[2] Historically, EVs have been separated and enriched by ultracentrifugation (UC); with UC however, the physical integrity and functionality of EVs may be damaged with exposure to high gravitational forces. Furthermore, on a practical level, UC is a method severely limited in its processing capacity (e.g., tube and rotor capacity) and equipment requirements, leading to low overall productivity. By the nature of the method, UC can also lead to a high level of variation between batches, or between laboratories.[3]

Meanwhile, density gradient ultracentrifugation (dgUC) makes use of differences in EV size and density to remove non-EV contaminants and has been reported as a "gold standard" method for EV separation in the research field. Compared to UC, however, dgUC is even more restricted in the volume processing capacity per run, significantly more laborious and requires a higher level of skill to operate. This skill requirement will necessarily increase the variation between users and batches.

Another approach includes the use of precipitating agents; however, it is not a true separation technique as no principle is applied to separate EVs from non-EV entities. Precipitation also has other major disadvantages; it involves potential unwanted chemical interactions that might disrupt EV markers, introduces precipitating agents that must later be removed as they alter EV functionality, are cytotoxic, and can induce protein aggregation.[4,5]

As UC and dgUC are limited in their potential for scalability and precipitation does not provide any separation from non-EV impurities, the use of these techniques at any stage of EV-ceutical manufacture would be highly unfavourable – resulting in extended processing times, high costs, and avoidable product loss.

4 / SEC, AN ATTRACTIVE APPROACH FOR EV SEPARATION

Size exclusion chromatography (SEC) has becoming highly popular in EV research[6] due to its simplicity, speed, gentle nature, potential for standardisation, clean isolation, and its ability to provide a high level of EV recovery.[4]

SEC facilitates the separation of EVs based on size using a column packed with a resin featuring specific pore sizes suitable for EVs. EVs, which exceed the resin's pore size, flow around the resin and elute earlier. In contrast, smaller components such as proteins take longer to elute because they can enter the resin pores and therefore travel more slowly down the column. Altogether, SEC continues to provide reliable and rapid EV isolation from complex biofluids like plasma and serum[8] as well as conditioned media.

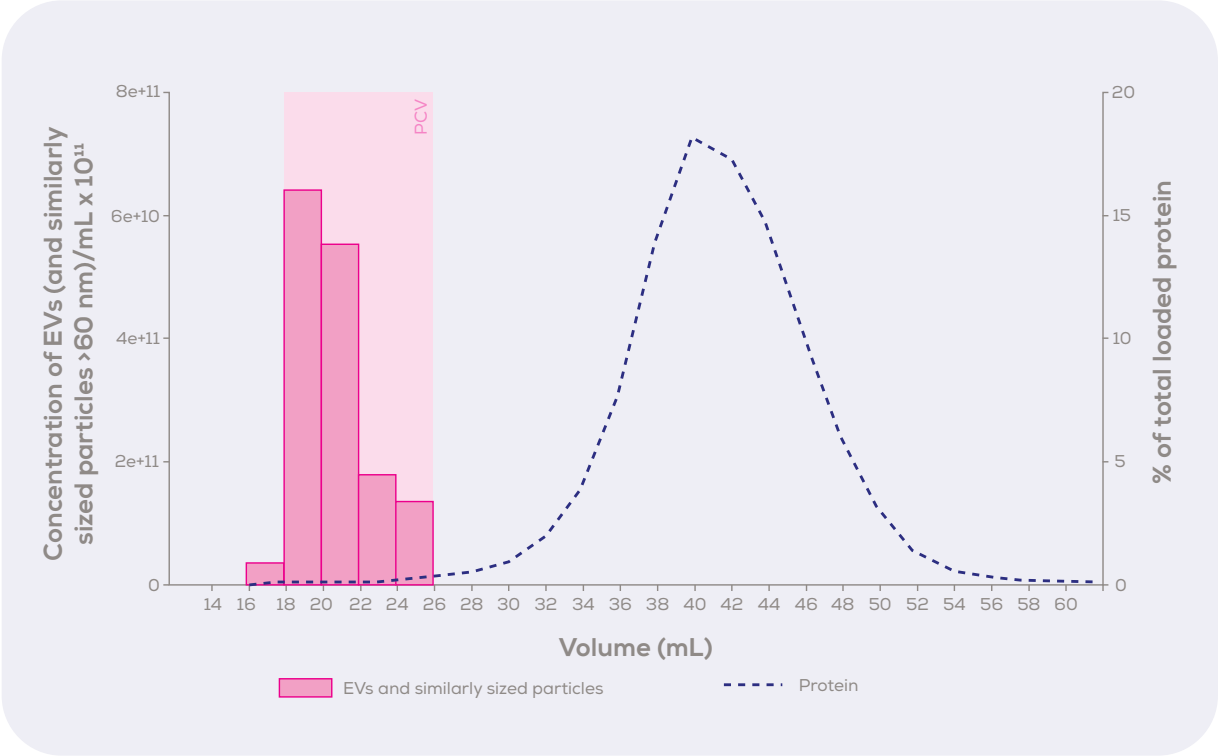

Figure 1. Typical elution profile for a qEV2 Gen 2/35nm column with 2 mL of plasma loaded; proteins (blue line) elute in later volumes than vesicles (pink bars). Pink shaded area is the EV-containing volume (PCV: Purified Collection Volume) optimised for the qEV Gen 2 column. Vesicle concentration was measured via Tunable Resistive Pulse Sensing using the Exoid, relative protein levels were determined by bicinchononic assay.

5 / HOW DOES SEC COMPARE TO PRECIPITATION AND ULTRACENTRIFUGATION?

Our SEC columns, known as the qEV range, are available in different sizes to accommodate sample inputs ranging from 150 µL to 100 mL. Whilst we have also created substantially larger custom columns for customers (currently for starting sample volumes of around 2000 L!), the principles of EV isolation remain the same between columns. In the literature, many independent researchers have compared qEV columns to other methods, allowing an unbiased view of how qEV columns compare to precipitation and ultracentrifugation.

When it comes to isolating EVs at scale for therapeutics, there are two key measures used to compare isolation methods: yield (particles per mL) and purity (yield per unit of protein). As you can see in Figure 2, a cross-study comparison showed no statistical difference in yield between the three techniques, making this an unimportant consideration for method choice. However, there is a significant difference in purity; EV isolates produced by qEV columns are significantly purer than either precipitation or ultracentrifugation methods (Figure 2). As such, qEV columns are more suited to EV therapeutics and scaled applications where purity is essential to prevent contaminant-driven side effects.

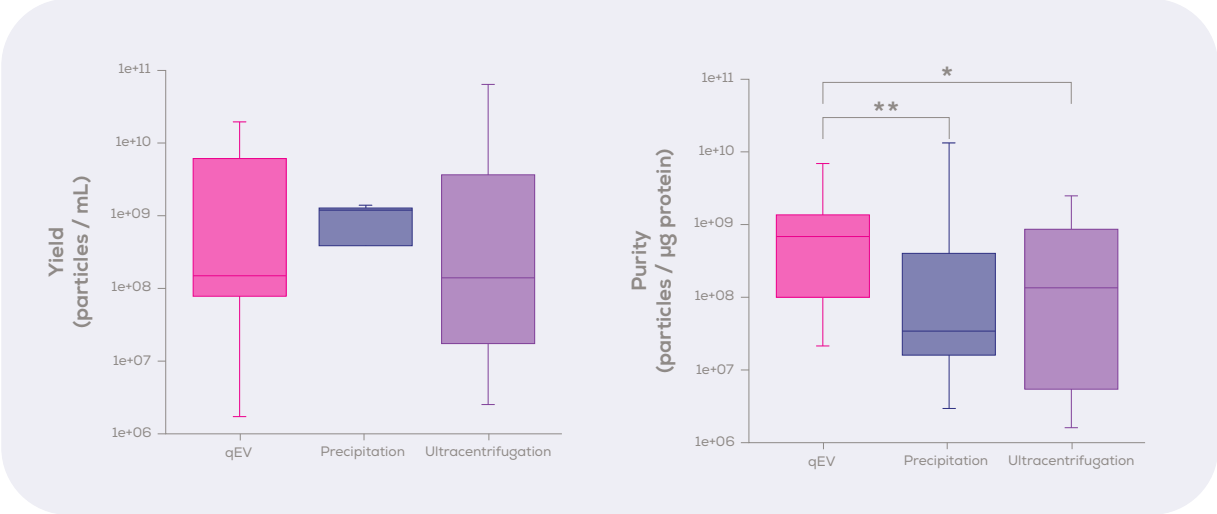

Figure 2. Comparison of isolate yield and purity for EVs isolated using qEV SEC columns, precipitation or ultracentrifugation. Data from the literature.[9-20] Statistical analysis was by Kruskal-Wallis Test with Dunn's post-hoc test. * $p<0.05$, ** $p<0.01$.

6 / INTRODUCING qEV PUREPATH FOR THERAPEUTICS

qEV PurePath for Therapeutics is a collaborative, customisable partnership aimed at conceiving, fine-tuning, testing, and implementing a tailored, high-throughput EV isolation workflow. In doing so, we aim to lessen the burden of EV isolation problem-solving, so that you can direct more of your resources towards refining your EV therapeutic. With the high costs of R&D being a universal truth, we're here to alleviate some of that time pressure and financial strain, propelling you more swiftly and efficiently toward your market objectives – for a fee dwarfed by the costs of you going it alone.

The qEV PurePath for Therapeutics service revolves around customisation, which is made possible by our deep-rooted expertise in EV isolation. Converting vast amounts of cell culture media into a consistent, concentrated, and pure EV isolate is a monumental challenge. Ensuring the sterility of the product is another formidable hurdle, making automated processing within a sterile environment practically indispensable for safe product generation.

Our journey to find a solution started with an exciting collaboration with a company honing in on harnessing functional properties of EVs. To meet their need for large-scale separation, we conceptualised and created a qEV column of staggering proportions, one over 400 times larger than our qEVoriginal columns. As these colossal columns must be tailored to every customer, this behemoth could not be offered as a standard, off-the-shelf product. It is also just one part of a successful therapeutics EV isolation workflow. Thus, the qEV PurePath for Therapeutics was conceived – an innovation which requires us to deliver far beyond a simple 'add to cart' solution, for what is a highly intricate problem.

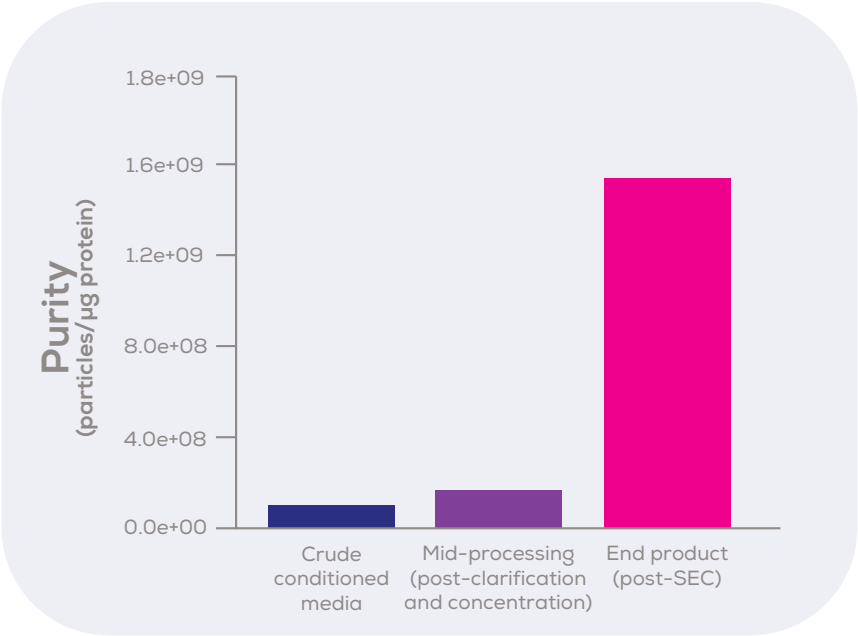

Figure 3. Case study data showing how our customisable workflow improved the purity of EVs for a qEV PurePath for Therapeutics Customer.

7 / THE PROCESS OF qEV PUREPATH FOR THERAPEUTICS

We have designed a workflow that can be tailored to the requirements of your starting sample and end goals. Our expertise spans multiple options at each stage of the process. We will work in tandem with you to develop and test a workflow that is optimised to transform your raw starting sample into a therapeutic-grade isolate. This process includes automation systems tailored to your needs, designed to facilitate scaling with ease. An exemplar workflow is shown in Figure 4.

To start your qEV PurePath for Therapeutics journey, you can read more about how the process works or book a consultation with one of our experts.

Figure 4. An exemplar, conceptual qEV PurePath workflow for therapeutics. Step 1 is clarification, which for automated systems is most likely to be in the form of serial filtration steps to eliminate larger contaminating particles. Next we have concentration, for which we recommend our tried and tested tangential flow filtration solution. After concentration, it is time for isolation in the form of a fully customised size exclusion chromatography column based upon qEV technology. Next come quality control steps, where particle size, concentration and zeta potential can be measured using Tunable Resistive Pulse Sensing, followed by assessments of potency and reproducibility (using assays built in partnership). Finally, you store the final, well-characterised and pure product. Pink = EVs; blue = protein; purple = large contaminants.

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