CONSIDERATIONS FOR CUSTOMISING LARGE-SCALE EV SEPARATION

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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, and 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.

In recognition of the need for large-scale separation solutions, Izon applied its expertise to support an industry partner seeking a custom-designed, multi-component strategy for the isolation of EVs from their cell culture conditioned media. 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 this particular large-scale operation.

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 native 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 and remarkable 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, 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.

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 in order 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 with micron or sub-micron pore sizes (e.g., 0.1, 0.45, 5 µm) or ultrafiltration with pore sizes adjusted to a molecular weight cut-off (e.g. 10, 100, 300, 750 kDa). Usually, 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.

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 considering the initial EV source volume, type, and relevant complexities, and EV specificity is defined as the extent of separation of EVs from all other non-EV entities[2]. Historically, EVs have been separated and enriched by ultracentrifugation (UC); however, it is known now that the physical integrity 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 which do not use standardised speed conditions[3].

Meanwhile, density gradient centrifugation (DGC) 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, DGC is even more restricted in the volume processing capacity per run, significantly more laborious and requires a higher level of skill to operate.

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[4], introduces precipitating agents that must later be removed, and can induce protein aggregation[5].

As UC and DGC 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.

SEC, AN ATTRACTIVE APPROACH FOR EV SEPARATION

Size exclusion chromatography (SEC) has becoming highly popular in EV research[6] due to its simplicity, potential for standardisation, quick protocols, clean isolation and protection of EV physicochemical properties, and its ability to provide a high level of EV recovery[4]. SEC enables size-based separation of EVs in a column filled with a resin with defined EV-appropriate pore sizes. EVs larger than pore sizes flow down quickly around the resin and elute earlier, whilst smaller components like proteins elute later, as they enter resin pores and flow more slowly down the column. Although SEC is a size-based separation technique, it provides a significant level of interaction between the EV-sample and the resin to provide efficient separation[7]. Altogether, SEC continues to provide reliable and rapid EV isolation from complex samples like plasma and serum[8].

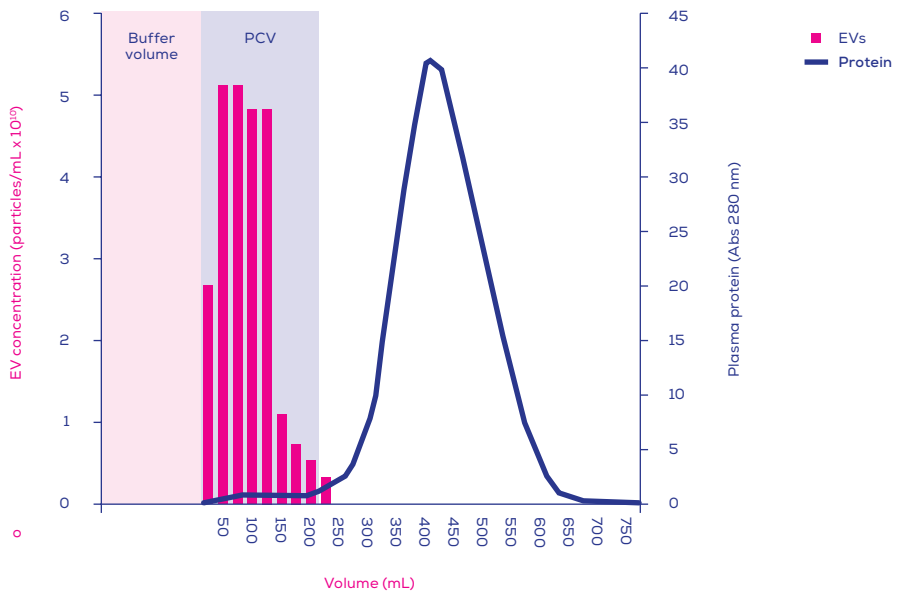

Figure 1. Typical elution profile for a qEV100/70nm column with 100 mL of plasma loaded; proteins (blue line) elute in later volumes than vesicles (pink bars). Vesicle concentration (80-300 nm range) was measured using tunable resistive pulse sensing, relative protein levels were determined by measuring absorbance at 280 nm.

SAMPLE-DEPENDENT CONSIDERATIONS FOR BIOPROCESS DEVELOPMENT

As each EV-containing sample is unique in its complexity, many factors must be considered during process development such as the initial sample volume, sample feed flow rate, elution flow rate, biophysical characteristics of contaminants and EVs of interest, expected EV yield, required EV dose (quantity, number of cycles and administration route) and storage capabilities for the EV preparation.

These variables can vary significantly, as seen by examining required yields and doses throughout the literature. For highly EV prolific cell systems such as MSCs, for example, an initial volume of 500 mL of cell culture conditioned media (CCM) could yield enough EVs to see an expected outcome in the recipient organism – as seen in mice where doses were 50-100 µg of EVs[1]. For larger animal models, however, doses might significantly increase; EV administration to non-human primates and pigs have required 1 mg or 2.7 x 10^{11}/kg of EVs per dose, respectively[1,9]. Conversely, low doses have also been used to achieve successful outcomes; for example, native MSC-EVs were used as therapeutics in phase II and III in clinical trials with intra-arterial injections of 100 µg EVs/kg/dose or approximately 10^6 EVs/kg/dose[10] or local injections of 2-4 x 10^9 MSC-EVs[11].

The administration of EVs to human subjects requires the production, separation, and readiness of highly enriched EV preparations, most likely derived from litres of EV-containing matrices. Here, EV production is an important and optimisable aspect of scaling up, involving the manipulation of cells sources, cell culture format (e.g. high density bioreactors) or the use of EV-release stimulants to improve EV yields[3,12,13].

COMMON BIOPROCESSES RELEVANT TO EV-RELATED APPLICATIONS

Large-scale separation processes aim to produce a product that is of sufficient purity, in an appropriate volume. The development of large-scale processes for EV separation, therefore, is critical to scaling up any EV-based application. Achieving this requires more than one EV separation method, allowing cost-effective optimisation and scalability of independent operational units, as a sequence or in different combinations.

Within the biopharmaceutical industry, biotherapeutics such as antibiotics and antibodies are commonly purified using variations of ultrafiltration methods, including cross flow filtration (CFF, also known as tangential flow filtration, TFF). The separation principle behind CFF is that pressure-driven fluid is passed in parallel to the filter/membrane, allowing re-circulation of the retentate fluid and continuous filtration or removal of small particles from large volumes (Figure 2). This allows CFF to concentrate samples, with concentration factors ranging from approximately 2X to 40X, for example. Compared to traditional, dead-end ultrafiltration (where flow is perpendicular to the membrane), membrane damage from fouling and clogging is minimised in CFF. However, CFF by itself does not assure effective protein separation from EVs, although it is an effective way to concentrate EVs by reducing sample volume with minimal EV interference[4]. Once this is complete, the resulting EV slurry can undergo a more specific separation process.

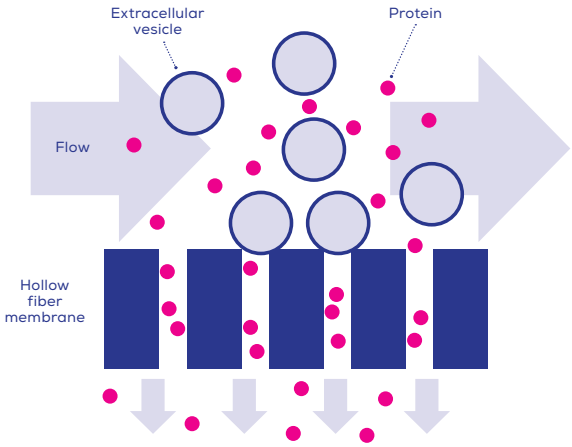

Figure 2. Cross-flow filtration (CFF). Pressure-driven fluid is passed in parallel to the filter/membrane, allowing re-circulation of the retentate fluid and continuous filtration or removal of small particles from large volumes.

SEC has rapidly become positioned as a reliable and scalable EV separation method, made popular amongst researchers due to its accessibility (easy, one-step bench-top use), short processing times which allows stability for labile products like EVs, and relatively low costs. Additionally, SEC does not induce protein or EV aggregation and critically, maintains EV integrity – an excellent indicator of EV functionality important to applications downstream[4]. This has been ratified in few studies, where SEC-isolated umbilical cord MSC-EVs, and not UC-isolated matching EVs, had a significant reduction of T cell proliferation and pro-inflammatory cytokine production[14], and SEC-isolated EVs presented different biodistribution in mice compared to UC-isolated EVs[15].

In summary, clarification is implemented to remove very large particulates, while concentration approaches can be applied to remove smaller contaminants[16] and reduce the sample to a volume that is suited to SEC separation. Overall, the process balances out the small amount of dilution that may occur during the consecutive SEC purification step. The ultrafiltration-SEC combination has been shown to outperform other EV isolation methods previously[15,17] and together, provide an effective way forward for large-scale EV separation.

IZON IDEALLY POSITIONED FOR LARGE-SCALE SEPARATION STRATEGY DEVELOPMENT

Given the complexity of EV-based production and separation, the EV-ceutical industry must develop controlled manufacturing processes, ideally involving sequential and automated steps. With a strong background in the EV separation business, Izon Science is ideally suited to providing support and strategy development for the large-scale bioprocessing and separation of EVs. Not only does Izon have an isolation platform that is well established in the EV research field (the qEV isolation platform, with AFC-compatible columns available from 150 µL to 10 mL); Izon also offers the qEV100 – a column capable of withstanding the pressure and compression associated with loading 100 mL of an EV-containing sample.

Izon's experience in EV analytics and quantification is also highly relevant in this context. The Exoid, Izon's tunable resistive pulse sensing (TRPS)-based technology for single-particle analysis, can be applied to assist process optimisation by providing particle-by-particle measurements (particle size, concentration and charge) with unmatched precision, resolution, and accuracy. Together with its separation and analysis experience, Izon has the expertise required for navigating the many challenges associated with large-scale EV separation, including engineering, pre-processing requirements, and downstream analysis.

A THREE-PRONGED, COMPLEMENTARY WORKFLOW: CLARIFICATION, CONCENTRATION, SEPARATION

Recently, Izon Science partnered with a cosmeceutical company and developed a strategy for their processing and separation of EVs from large volumes of CCM.

Given the importance of clarification and concentration to the efficiency of the EV separation process, these essential steps were implemented to ensure SEC separation was preceded by an efficient cleaning process. This was particularly critical, given that the CCM provided was already concentrated and therefore contained a high load of protein contaminants. Batches of CCM were processed as outlined in Figure 3:

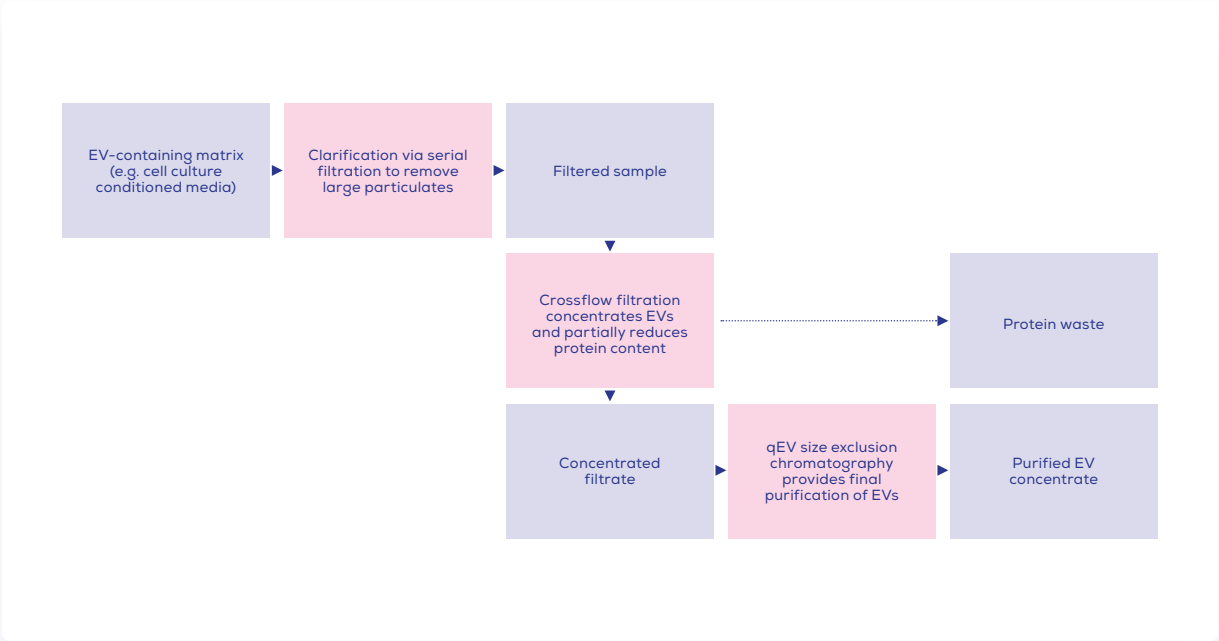

Figure 3. Flowchart of processes used by Izon Science during pilot studies in a recent partnership, to separate extracellular vesicles from a concentrated cell culture conditioned media. The initial workflow was executed using 400 mL batches of CCM; plans for scale-up are now underway.

Using this approach, Izon Science produced a final preparation containing an EV concentration in the order of 1 x $10^{12\text{-}14}$ particles/mL. Total protein was reduced by at least 95%, which meant the number of EVs per µg of protein increased by 5- to 10-fold. Following this initial work, Izon is proposing a pathway towards full-scale processing of EV isolate for batch sizes from 5 to 250 L and beyond, and is therefore planning further optimisation studies for the near future. Alongside this, Izon is also exploring potential industry partnerships that align with this goal, such as automated chromatography systems from Sepragen, methods for concentration post-SEC, and gamma-ray irradiation of columns to enable clinical use.

SAMPLE-DEPENDENT PROCESSES REQUIRES A FLEXIBLE, CUSTOMISED APPROACH

As carriers of an incredibly diverse molecular cargo, EVs are set to become a powerful platform for therapeutics in the coming years. To fulfil its potential, the EV-ceutical field requires a conjoined effort to standardise the many aspects leading up to an EV product, such as EV production, EV isolation, and EV quality/potency assessments. While continued research advancements are needed to define guidelines, criteria, and baselines underlying a robust consensus for working with EVs, the industry now needs relevant technology and support for the design and operation of large-scale EV processing.

Compared to other EV isolation methods, SEC-based EV separation is a highly cost- and time-effective approach that provides excellent EV recovery. Through the development of the qEV isolation platform, i.e., qEV columns and the AFC, Izon has built up its separation expertise and technological capabilities. This accumulated experience has shown to be highly transferrable to settings of large-scale separation, as demonstrated in a recent collaborative industry effort. The accumulated experience in SEC and EV analysis means that Izon is now ideally positioned to explore new partnerships aimed at designing and optimising processes for large-scale EV separation.

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