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Simplifying Progress

Development of an End-to-End Scalable Purification Platform for Extracellular Vesicles

RoosterBio®

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Introduction

Extracellular vesicles (EVs) are being evaluated as novel therapeutics for a variety of diseases. EVs derived from human mesenchymal stromal cells (hMSC-EVs) constitute most clinical investigations. Despite the growing interest, purification and analytical characterization are challenging, and have only recently starting to be explored. Sartorius, a frontrunner in EV process development, provides end-to-end solutions for EV production, purification, and analytical characterization. Here, we demonstrate an optimized purification platform (from harvest to the final step before fill and finish) with the goal to maximize yield and purity of EVs. Furthermore, a detailed analytical platform was established for EVs, where purified EVs were systematically tested for hMSC-EV-specific membrane protein markers and cytosolic protein. Finally, the biofunctionality of EVs was confirmed by closure of a scratch wound.

Experimental Approach

Sartorius, a leading provider of futuristic downstream technologies along with state-of-the-art analytical tools, developed a platform purification process from clarification to the sterile filtration (Figure 1) for EV in collaboration with RoosterBio, an industry-leader in manufacturing high-quality hMSCs along with paired bioprocess medium formulations for cell growth and EV production. Conditioned media containing EVs was harvested from a microcarrier-based suspension culture using RoosterBio xeno-free hMSCs (RoosterVial™ hMSC) coupled with RoosterBio MSC expansion media (RoosterNourish™), and RoosterBio EV collection media (RoosterCollect™).

Figure 1: *Schematic Process Flow Representing Unit Operations Involved in the EV Purification Process*

Clarification filters were used to remove large particulates and reduce turbidity. The obtained clarified harvest was subjected to tangential flow filtration (TFF) on the Sartoflow® Smart for subsequent concentration (5x) and diafiltration optimisation (Figure 2). The first diafiltration was performed to change the buffer for the enzyme reaction (DNase) which aimed to reduce DNA. The second diafiltration was carried out post-enzyme reaction into chromatography (Chrom) buffer to prepare the EV load. For the end-to-end run, Sartoflow® Advanced was used for execution of the TFF process.
Post TFF, after passing through a finer filter, the filtrate was loaded onto a chromatography column at a total dynamic binding capacity (DBC) of 7.00E+11 particles/mL of column matrix, determined by performing breakthrough curve analysis (data not shown here). Following a salt wash, the bound EVs were eluted with a step gradient of salt.
Finally, another TFF step (TFF 2) was performed to formulate EVs to the target concentration (>1.00E+10 particles/mL). Vials of purified EVs were frozen for further analysis. The Process was initially carried out in parts for process development and optimisation. Once optimised, an end-to end run was carried out by generating 15 L of conditioned media containing EVs in Biostat® STR Gen3 50 L and processing it through the scaled-up DSP operations for a proof of concept for the platform.

Figure 2: ***A)*** *Sartoflow® Smart Benchtop TFF System for Process Development.* ***B)*** *Sartoflow® Advanced for the End-To-End Run*

Particle recovery | concentration for individual process steps were measured using Particle Metrix Zetaview®, a nanoparticle tracking analysis (NTA) in the scatter-mode. EV integrity was confirmed via measurement of the intact lipid bilayer particle through fluorescent labeling and interpreted by Particle Metrix Zetaview® (fluorescence mode) throughout the purification process. Furthermore, EV integrity was confirmed via size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) based separation measurements, where overlapping fluorescent and scattering EV signals were measured using a PATfix® biochromatography instrument (Figure 3).

Figure 3: *Patfix® Biochromatography System (Sartorius)*

Protein impurities were measured using a Bradford analytical assay to determine EV purity (EVs/mg protein) and DNA impurities were quantified using a PicoGreen assay. Classical EV Tetraspanin markers (CD63, CD9 and CD81) and cytosolic cargo protein (TSG101) were confirmed by Western blot analysis. The potency of purified EVs was measured through scratch wound closure analysis through the Incucyte® Live-Cell analysis system (Figure 4).

Figure 4: *Incucyte®, a Live Cell Analysis System (Sartorius)*

Results

Percentage Recoveries and Purity

The optimized process was able to maintain high particle count and EV recovery throughout purification process (Figure 5). Impurity removal was effective, enabling over >98% reduction in protein and >99% reduction of DNA post-TFF 2 (Table 1). The purity of EVs was reduced post-TFF 2 due to the selection of a formulation buffer with a higher protein content to stabilize EV. Therefore, chromatography should be regarded as a real presentation of purity for direct-to-formulation applications. A purification factor of 23-fold was achieved via chromatography. The cumulative recovery of EVs after chromatography step was >34%.

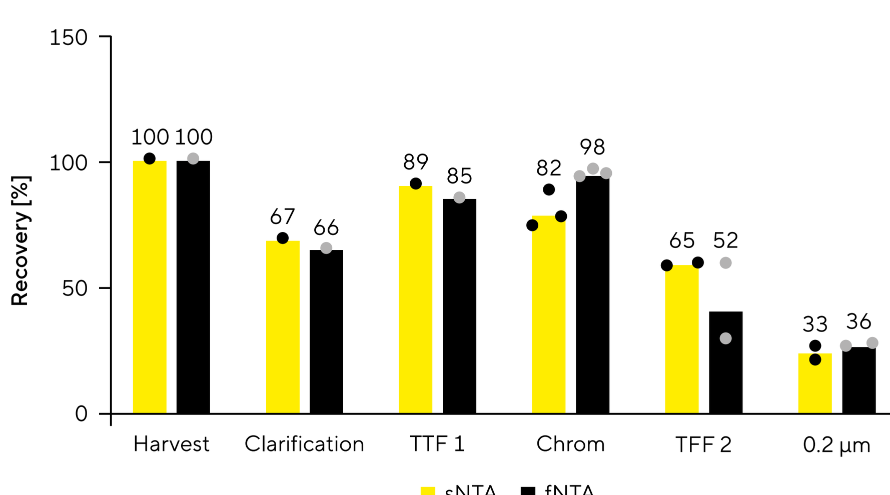

Figure 5: *Comparison of Particles and EV Step Recoveries Across the Platform*

Purification Step	EV/mg Protein	DNA [ng/mL]
Feed	8.54E+10	94.5
Clarification	6.49E+10	56
TFF 1	1.37E+11	18.8
Chrom	1.95E+12	4.5
TFF 2	1.44E+12	6.85

Table 1: *Particle Purity and DNA Impurity Level Across the Purification Process*

EV Size Distribution

EV size distribution was analyzed using NTA fluorescence mode. The size range observed was 50-400 nm and median EV size was maintained at 180 nm throughout the purification process (Figure 6). Our purification platform is targeted to purify the EVs of all sizes; however, if only 50-200 nm sized EVs are targeted for the therapeutic application, EV recovery for the complete process was found to be 29% instead of 23% for all size ranges. The final product had 83% EVs between 50-200 nm and 15% >200 nm.

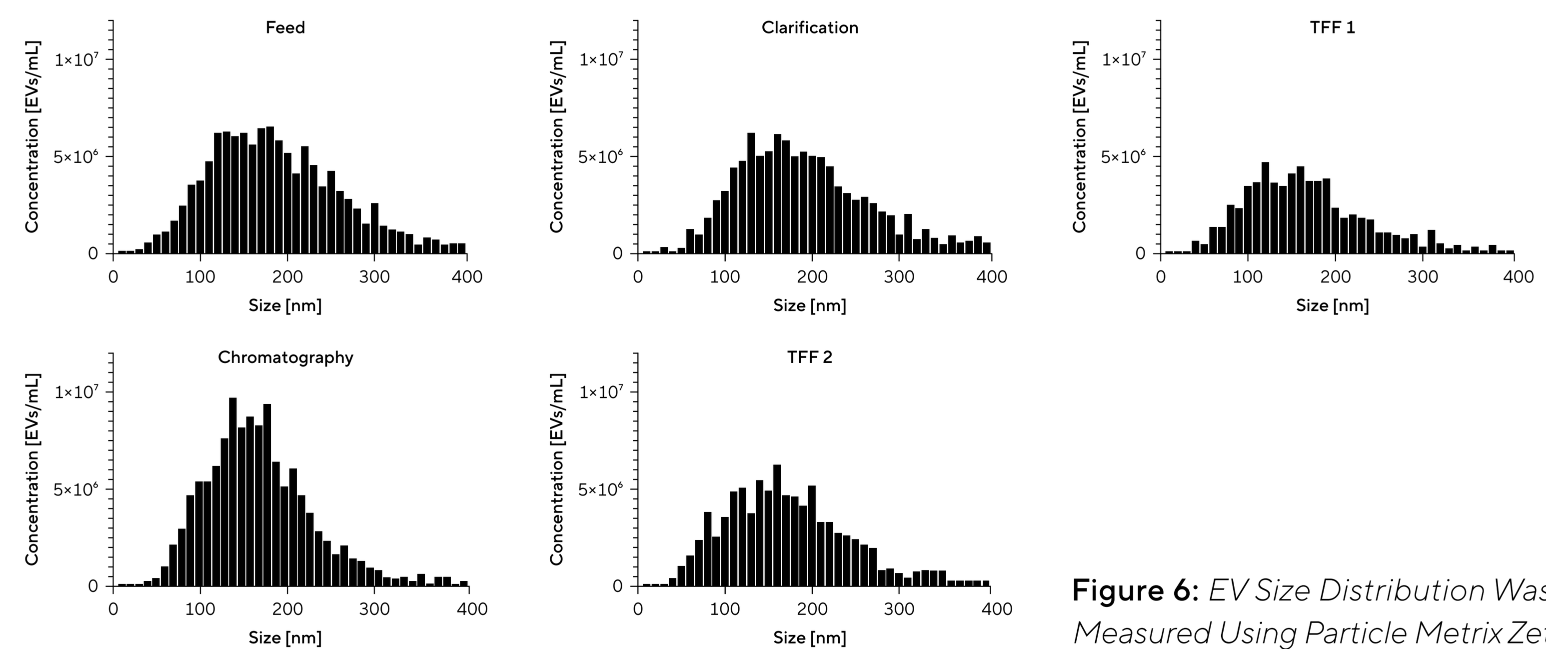

Figure 6: *EV Size Distribution Was Measured Using Particle Metrix Zetaview®*

EV Integrity via PATfix® Biochromatography

EV integrity was measured and confirmed by a second orthogonal technique using a SEC column. Here, overlapping MALS, UV, and fluorescence signals for EV remained consistent as EV elution peaked at ~10 minutes, and the peak became larger and sharper throughout, representing increased purified EV concentration (Figure 7). The difference between MALS and fluorescence signals in the TFF 2 step is attributed to the presence of stabilizing protein used in the formulation buffer.

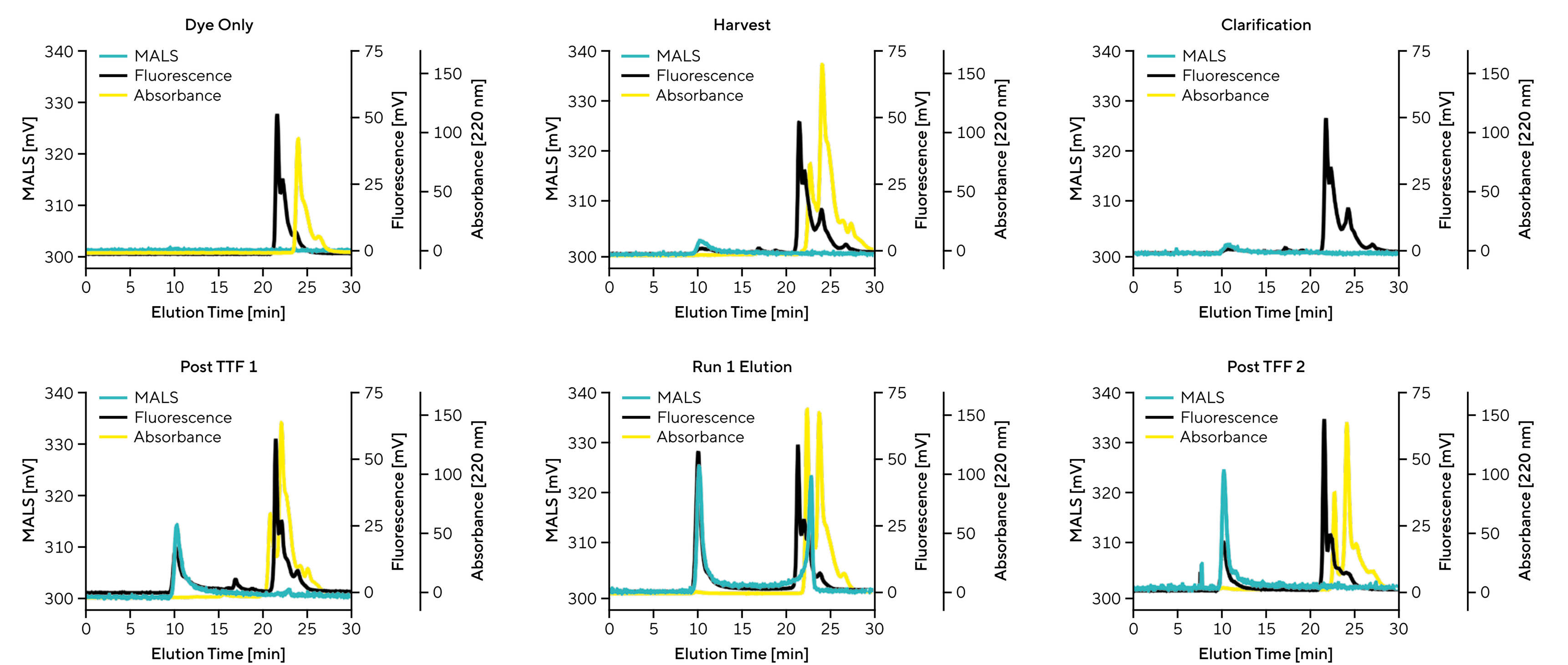

Figure 7: *Purified EV Particles at the End of Each Unit Operation Were Profiled Through PATfix® Biochromatography System and, EV Integrity Was Confirmed by Overlapping MALS (Teal), UV (Yellow), And Fluorescence (Black) Signals*

Particle Identity and Potency

Purified EVs maintained hMSC-specific EV Tetraspanin transmembrane markers and the presence of cargo protein, confirmed by Western blot (Figure 8). Along with this NanoFCM was also used to confirm the presence of CD81 surface markers and showed presence throughout the operation (Figure 9). The functionality of the purified EV post-TFF 2 was confirmed by measuring the wound closure density compared to the positive and negative control using the Incucyte® live-cell analysis system (Figure 10).

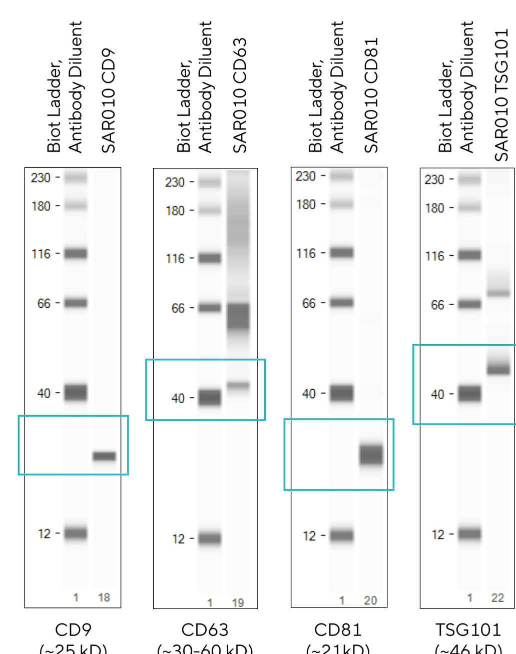

Figure 8: *hMSC-EV Markers Presence Was Confirmed by the Clear Band at the Respective Size (Box) Post-purification Process*

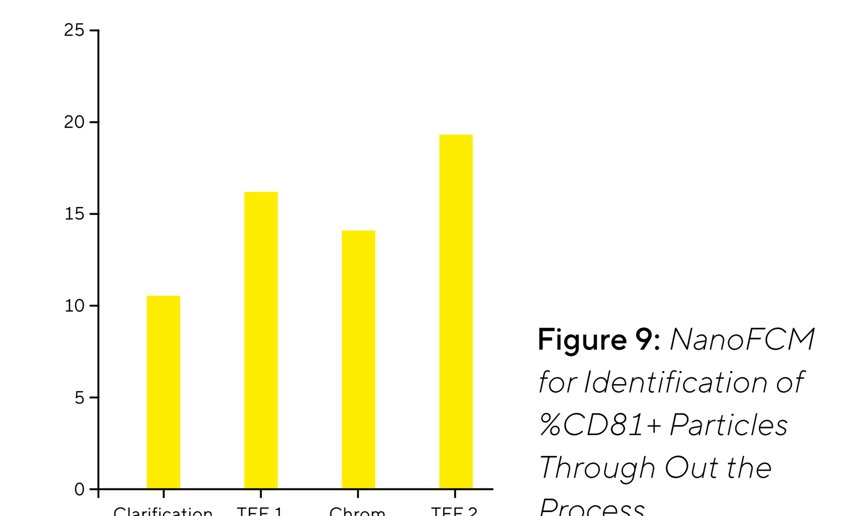

Figure 9: *NanoFCM for Identification of %CD81+ Particles Through Out the Process*

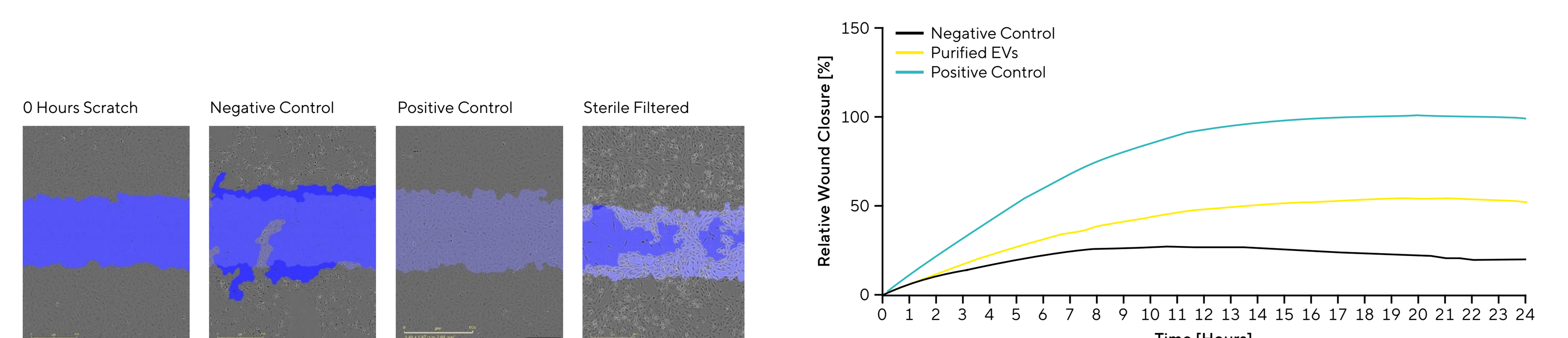

Figure 10: *Purified EVs Post-TFF 1 Unit Operation Permitted >50% Wound Closure Density in 18 Hours Representing Potent EV Post-purification Process*

Conclusions

We successfully established an end-to-end EV purification process along with a robust analytical platform to obtain highly pure and concentrated EVs while maintaining their critical quality attributes. The purified EVs were identified successfully for the presence of cell surface markers. The EVs were also tested for the wound healing assay and showed consistent wound healing characteristic proving the potency and structural integrity. The promising results showcased here were achieved using established Sartorius technologies, which are easy to integrate and scalable. These findings serve as a baseline for an effective and accelerated drug substance manufacturing for EVs.