Simplifying Progress

Utilizing CIMmultus® OH for Efficient Purification of MSC EVs

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Introduction

Mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) have promising therapeutic potential in diverse applications, such as tissue regeneration and immunomodulation. On the pathway to the clinic, there is a substantial need for development of scalable methods for EV production and isolation. Moreover, advancements in analytical tools for characterization of EVs are necessary to ensure a consistent product. Here, we showcase an integrative approach combining upstream, downstream and analytical processes for successful generation of MSC EVs (Figure 1).

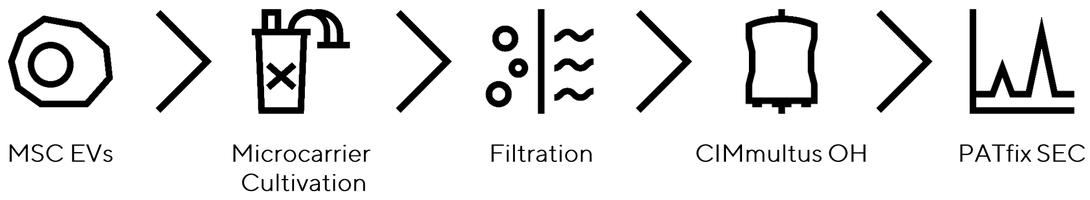

Figure 1: Overview of the EV bioprocess described in this study. We optimized MSC EV production and scaled-up the process from 2D system in flasks to 3D microcarrier-based cultivation in a stirred tank bioreactor. Conditioned media EVs were then filtered and captured by preferential exclusion chromatography on the CIMmultus OH preparative column using pre-optimized conditions. Samples from different stages of the EV bioprocess were analyzed by multi-detector PATfix® SEC analytics, which guided both upstream and downstream process development.

1. Experimental setup

Bone marrow-derived MSC cells (ATCC) were cultivated in complete MSC NutriStem XF Medium (Sartorius) with added 2.5% human platelet lysate (hPL, PL BioScience). For EV production, growth media was removed after 4-day growth phase and replaced with production media Advanced DMEM (Gibco). 2D EVs were produced in Hyperflasks (Corning), while 3D production was performed in Ambr250 mini-bioreactor system (Sartorius) using FACT III microcarriers (Sartorius). Conditioned media samples were harvested after 3-day EV production phase, filtered through a 1.2 µm Sartopure PP3 filter (Sartorius) and stored at -80 °C.

An ÄKTA Pure 25M with a CIMmultus OH column (Sartorius) was used for preparative chromatography. Screening of following binding variables was performed: pH, cosmotrophic salt type and concentration. Based on experimental results, MODDE software was employed to identify optimal binding conditions, that were then applied to CIMmultus OH 1 (6) binding buffer. EVs were eluted in a linear gradient and fractions were collected based on peak positions.

Particle concentration was determined by nanoparticle tracking analysis (NTA; NanoSight 300, Malvern Panalytical). To determine the abundance of tetraspanin exosome markers, samples were labeled with FITC-conjugated antibodies targeting CD63, CD81 and CD9 (all from BioLegend). Samples were then analyzed using the PATfix system with a size exclusion column (Tosoh Bioscience) [1].

To determine the reduction of impurities in the OH capture step, we performed PicoGreen total DNA and Bradford total protein plate assays of starting material and elution fractions.

2. Optimization and scale-up of MSC EV production

2D MSC EV production was first optimized in small-scale experiments in T25 flasks, where the effects of cell density and length of the EV production phase were explored (Figure 2A). The highest particle concentrations and exosome marker presence were found in samples produced with high cell density and an extended EV production phase. Longer production periods and increased cell density have led to EV particles with higher CD63 and lower CD9 signals. To further increase EV output and ensure scalability, the process was subsequently adapted to a microcarrier-based culture in a stirred tank bioreactor. Production was followed by daily sampling (Figure 2B). Similarly to the 2D method, we observed a relative decrease in CD9 signal with longer production time, whereas particle concentration increased.

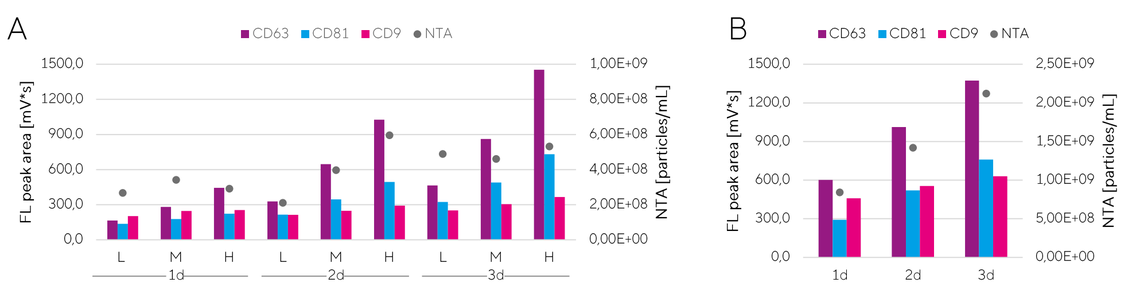

Figure 2: A) Effect of cell density and length of EV production on particle concentration and exosome marker abundance in small-scale 2D production optimization. B) Timeline of EV production in 3D microcarrier-based system. d – day, L – low, M – mid, H – high cell density.

Comparison of 2D and 3D large-scale production strategies (Table 1) demonstrates similar size of produced EVs, but more than 4× higher particle concentration and per cell productivity was obtained in the 3D system. This effect could be explained by higher shear stress in 3D conditions [2]. Examination of exosome marker presence shows that EVs produced in 3D have higher relative CD9 signal in comparison to 2D production. This could indicate that the EV composition changes with 2D to 3D transition, presumably due to differences in cell density per surface area. Alternatively, more residual highly CD9-positive EVs from hPL in the growth media may have remained in the 3D harvest..

Production system	Surface area/volume ratio [cm^2/mL]	EV productivity [particles/cell]	NTA [particles/mL]	NTA size mode [nm]	FL peak area ratio CD63 : CD81 : CD9
2D (flask)	3.1	4.29E+03	4.80E+08	163.6	55 : 31 : 14%
3D (microcarrier)	5.0	2.10E+04	2.12E+09	153.7	47 : 27 : 26%

Table 1: Comparison of surface-to-volume ratio, EV productivity, EV concentration, size and relative exosome marker abundance for 2D and 3D MSC EV production systems.

3. Screening of OH EV capture conditions

Downstream capture screening was done with the following parameters: pH range from 6.5 to 8.0, chaotropic salt type and concentration: citrate (1.0-0.65 M) and phosphate (1.5-1.0 M). MODDE software (Sartorius) was used to design the screening study and evaluate results, based on the recoveries from NTA particle count and CD81 abundance. We focused on the analysis of CD81 marker, since it had the most stable signal in all sample types. 3D harvest was used as study material. Analysis showed that optimal conditions differ between citrate and phosphate. Capture with citrate optimal conditions yielded higher NTA and fluorescent marker-based recovery. Based on this, the citrate optimal binding setpoint was chosen for the CIMmultus OH standard conditions.

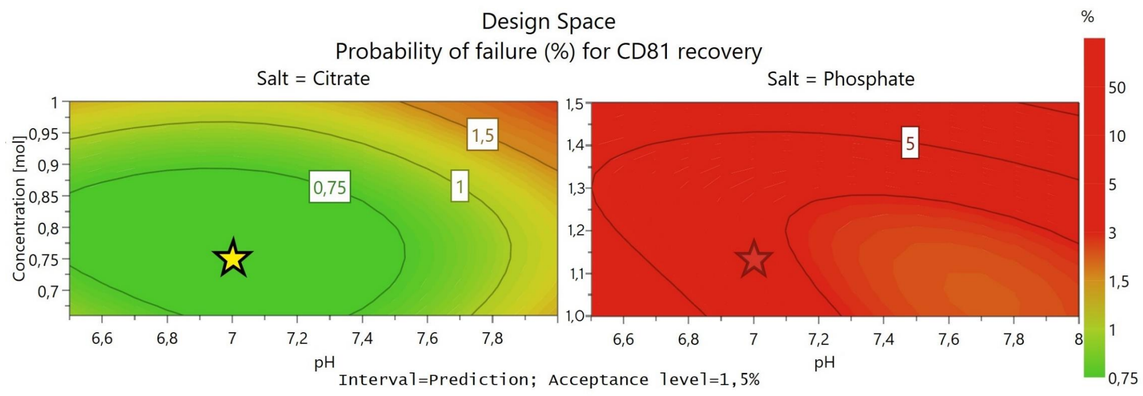

Figure 3: Optimization color gradient map. Citrate and phosphate were screened for pH (6.5-8) and concentration (1.0-0.65 M for citrate, 1.5-1.0 M for phosphate) binding conditions. MODDE software generated a probability of failure map to recover at least 50% of CD81 marker from all screened conditions. Based on these predictions, we chose 750 mM citrate pH 7.0 (marked with star) as our binding conditions for EVs on the OH column.

4. CIMmultus OH MSC EV capture

We first tested the column capacity for 3D harvest (not shown) and then loaded the CIMmultus OH 1 mL column to 70% capacity (Figure 4A). Elution fractions were obtained (Figure 4B) and analyzed using fluorescent markers and NTA. Recoveries calculated based on fluorescent markers (Figure 5A) ranged from 50% (CD9) to 90% (CD81). Total particle content measured by NTA correlated with observed exosome marker presence in fractions. The total dsDNA reduction was 97.5% and the reduction of total protein content was 94.0% (Figure 5B). It is important to consider the limitations of the performed assays. Total protein analysis detects both protein contaminants and protein content in EVs, while NTA measures both EVs and other particles.

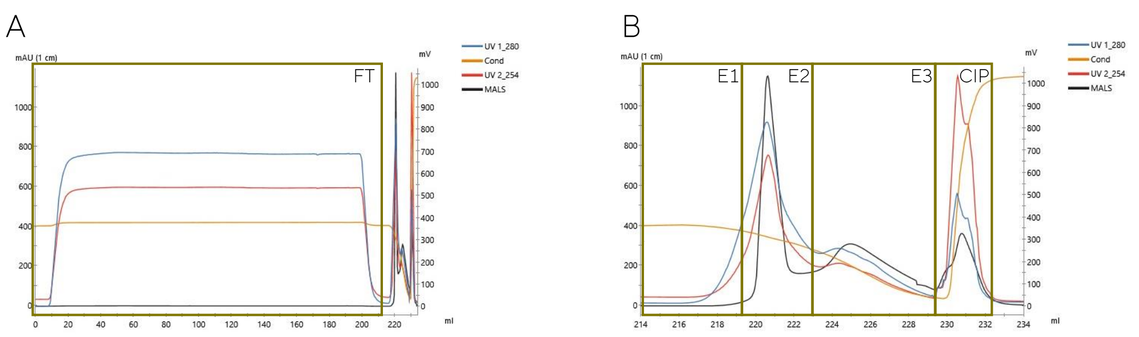

Figure 4: A) CIMmultus OH chromatogram. 90 mL of 3D material was loaded on the column. B) Elution zoom-in with marked fractions. FT- flow-through, E1, E2, E3 – elution fractions, CIP – cleaning in place.

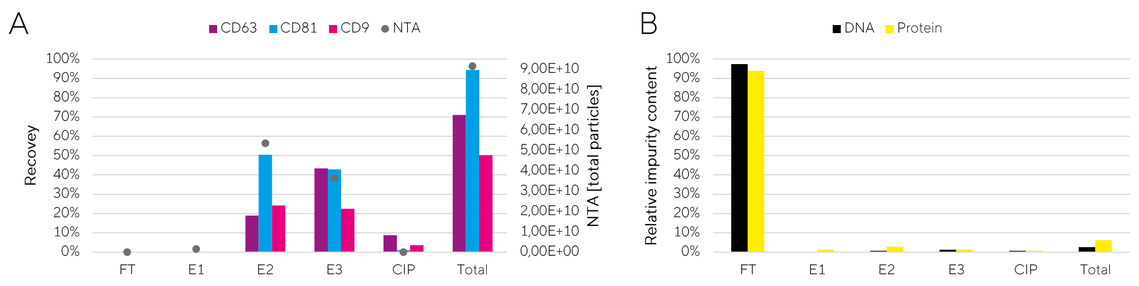

Figure 5: Analytics of OH capture fractions. A) Recovery of fluorescently labeled particles was calculated from harvest (100%). Particle concentration was measured by NTA and expressed as total particles per fraction. B) Protein and DNA reduction measured by Bradford and PicoGreen assays, respectively. FT – flow-through, E1, E2, E3 – elution fractions, CIP – cleaning in place.

5. Conclusion

- MSC EV yields in 2D production correlate with higher cell concentrations and longer production times.
- Transition to 3D production system can elevate EV particle concentration and per cell productivity.
- We demonstrated the value of MODDE software in development of chromatographic conditions for EV capture.
- CIMmultus OH is used to capture EVs directly from conditioned media, while removing more than 90% of impurities.

Literature

[1] Vrabec K. *et al.* Characterization of EVs subpopulations from CIMmultus EV using PATfix system. 2023. Available at: https://www.biaseparations.com/library_items/characterization-of-evs-subpopulations-from-cimmultus-ev-using-patfix-system/
[2] Jeske R. *et al.* Upscaling human mesenchymal stromal cell production in a novel vertical-wheel bioreactor enhances extracellular vesicle secretion and cargo profile. *Bioactive Materials.* 2022; 25: 732-747. https://doi.org/10.1016/j.bioactmat.2022.07.004