Simplifying Progress

Application of PATfix® SEC and CIM® Anion Exchange Chromatography for Optimization of EV Upstream Production

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Introduction

Extracellular vesicles (EVs) are a diverse group of particles secreted by all living cells. Numerous different therapeutic applications of both native and engineered EVs are currently in different stages of clinical development. Nevertheless, considerable challenges are still present in the manufacturing, purification and analytics of EVs. Many factors can influence the final product, therefore an all-inclusive approach to development of the bioprocess is needed. Cell culture parameters and production platform selection might alter the number and composition of EVs. Furthermore, raw materials used in upstream production, such as media and supplements, can greatly impact the chromatographic purification. In this study, we evaluated EV production in different HEK293-derived cell lines. Separation on a strong anion exchange column CIMmultus®-EV was used to assess the abundance of different EV populations. Multi-detector PATfix® SEC analytics coupled with antibody labeling was then used to analyze chromatographic fractions. Furthermore, the analytical methods and performance in downstream processing were applied in the optimization of the upstream process.

1. Experimental setup

We compared EVs generated in two HEK293-derived cell lines. One of them was an adherent HEK293T, cultured with 10% fetal bovine serum (FBS). The second cell line was a suspension HEK293 (HEK293sus) adapted to growth in a chemically defined medium, i.e. without FBS. For EV production HEK293T cells were cultured in DMEM with 10% exosome-depleted fetal bovine serum (EV(-)-FBS) to avoid cross-contamination with FBS EVs. In FBS-free production of HEK293T EVs, growth media was then replaced with DMEM as production media. HEK293sus were maintained in commercially available chemically defined medium, which was also utilised for EV production. All conditioned medium samples were filtered through 0.45 µm MiniSart PES filter (Sartorius) and stored at -80 °C prior to further use.

Chromatographic separation was carried out on an ÄKTA Pure™ 25 M system (Cytiva). In brief, 100 mL of harvest sample was diluted in loading buffer and applied to CIMmultus EV column (4 mL, 2 µm channels, Sartorius BIA Separations). EVs were eluted in a salt gradient, and elution fractions were collected for further analysis [1, 2]. NanoSight 300 system (Malvern Panalytical) was used for nanoparticle tracking analysis (NTA). Additionally, to determine the tetraspanin composition of EV fractions, samples were first fluorescently labeled with FITC-conjugated antibodies for the identification of three common exosome markers (anti-CD63, anti-CD81 and anti-CD9, all from BioLegend). EV samples were then analyzed by PATfix system (Figure 1) using a size exclusion (SEC) TSK gel G4000SWXL column (Tosoh Bioscience) [3]. Total protein content in chromatographic fractions was determined using bicinchoninic acid assay (BCA).

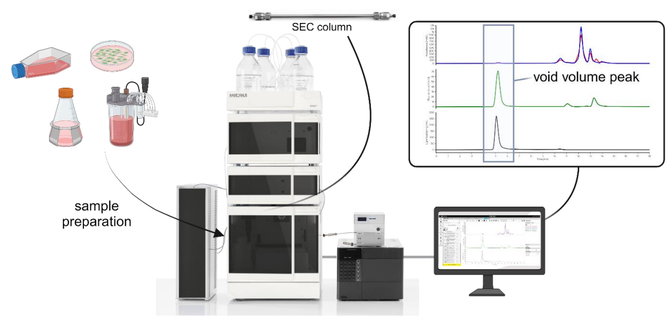

Figure 1: Overview of the PATfix system used in analysis of upstream and downstream EV preparations (created with BioRender.com). Samples are loaded on a SEC column. Absorbance, fluorescence and multi-angle light scattering (MALS) are recorded. An example chromatogram shows detected EVs in the void volume.

2. EV production in adherent and suspension HEK293 cells

In comparison with adherent HEK293T, the suspension cell line HEK293sus produced 10-25x higher concentration of EVs (Table 1). On CIMmultus EV column, different chromatographic profiles were also observed (Figures 2A and B). Four elution fractions can be separated from HEK293T EV harvest with MALS signal peaks in E1, E2 and E4. In general, HEK293sus EVs eluted at higher salt concentrations than HEK293T EVs and MALS signal peaks were observed in E3 and E4 fractions. Besides the presence/absence of SV40 large T-antigen, and one of the lines being adherent, the most prominent difference that could affect the CIMmultus EV profile is the presence of exosome-depleted FBS (EV(-)-FBS) in production medium. We therefore recorded the background of fresh DMEM + 10% EV(-)-FBS medium on CIMac analytical anion exchange column (Figure 2C) and noticed prominent UV and MALS signals at the start of the gradient, present also in the preparative chromatogram of HEK293T EVs (Figure 2A). E1 and E2 elution peaks in HEK293T EVs CIMmultus EV separation can therefore include EV(-)-FBS-derived components.

Based on these results we revisited the production strategy for the adherent HEK293T cell line. Once the cells grew to sufficient confluency, media was exchanged to DMEM only to completely exclude FBS from the EV production phase. Compared to previous results, we observed that cells in FBS-free production media produced fewer EVs with somewhat higher particle size (Table 1). Contrary, no significant change in EV marker expression was observed.

Cell line	production media	NTA [particles/mL]	NTA size mode [nm]	EV productivity [particles/cell]	FL peak area ratio CD63 : CD81 : CD9
HEK293T (adherent)	DMEM + 10% EV(-)-FBS	1.51E+09	121.0	2032	19 : 53 : 28%
	DMEM	6.42E+08	139.6	n.a.	14 : 56 : 30%
HEK293sus (suspension)	CDM	1.60E+10	150.8	6107	11 : 69 : 20%

Table 1: Comparison between quantity, size and marker expression of EVs obtained with different HEK293-based production strategies. CDM – chemically defined medium, n.a. – not assessed.

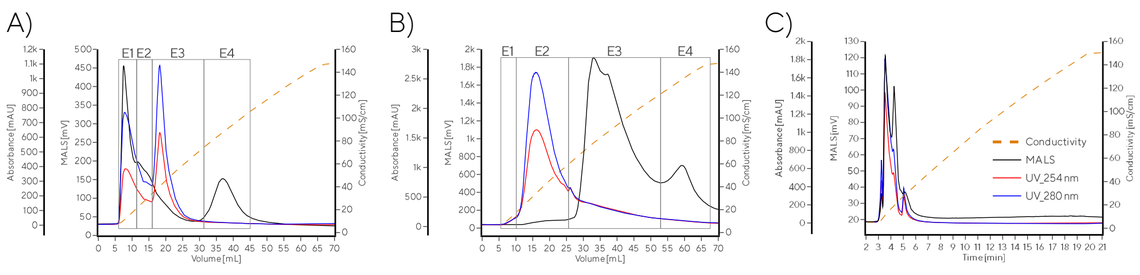

Figure 2: EV populations separated on CIMmultus EV preparative monolith column. A) HEK293T EVs, B) HEK293sus EVs, C) Analytical chromatogram of DMEM + 10% EV(-)-FBS medium recorded with PATfix on CIMac anion exchange monolith column. Preparative and analytical chromatograms are zoomed-in on the salt gradient, as seen from conductivity (orange dashed line).

3. Analysis of CIMmultus EV elution fractions on PATfix SEC

After separation on anion exchange (Figures 2A and B), all elutions were analyzed with PATfix SEC. For the purpose of marker abundance calculation, the void volume peak was integrated for each fluorescent signal.

In HEK293T EV harvest CIMmultus EV separation (Figures 2A and 3A) the main exosome-containing fraction was E2, which eluted from 20 to 35 mS/cm^2. It had the highest particle concentration and expression of all three tested exosome markers. Total protein assay (Figure 3C) confirmed high protein content in the E1 fraction, which is responsible for the high MALS signal on the CIMmultus EV chromatogram, in the peak that elutes from 5 to 40 mS/cm^2 (Figure 2A). As seen in Figure 2C, this fraction largely originates from EV(-)-FBS in the production medium that was used.

In comparison, HEK293sus harvest separation (Figures 2B and 3B) showed only low particle count in E2 fraction, but this sample was tetraspanin-rich. The main exosome-containing fraction in this case was E3, high in both particle count and exosome marker expression, eluting from 60 to 120 mS/cm^2. As expected, total protein concentrations in all elution fractions were lower, due to the use of chemically defined FBS-free production medium (Figure 3D).

In both adherent and suspension HEK293 EV separation, a late eluting E4 fraction was observed. In this fraction, a high particle concentration with a larger average diameter was observed. Nonetheless, only a low portion of these particles was positive for exosome markers. Ratios between EV marker fluorescence in load and elution fractions were consistent for both HEK293T and HEK293sus EVs, with CD81 being the predominant signal in all cases.

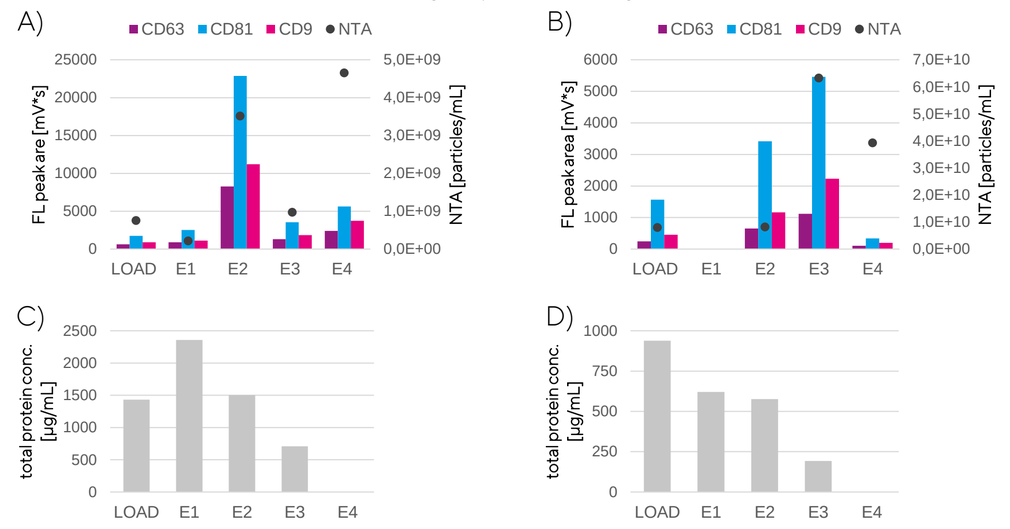

Figure 3: Comparison of exosome marker fluorescence, NTA particle concentration and total protein concentration between load and elution fractions of CIMmultus EV: A) and C) HEK293T, B) and D) HEK293sus, respectively.

4. Optimization of cell density and duration of HEK293T exosome production in FBS-free medium

To eliminate the use of animal-derived components in EV production media, the FBS-free upstream production in HEK293T cells was further optimized by testing different cell densities and periods of conditioned media collection. The highest particle concentration was obtained after three days of production at high cell density (Figure 4A). The MALS90° signal in the SEC void volume peak was well aligned with particle concentration measured by NTA. Considering only particle number, long EV production at high cell density is optimal (Figure 4B).

EVs generated by this cell line are primarily CD81-positive, as previously shown (Figure 4C). Longer production and increased cell density resulted in a minor relative increase of CD9 and CD63 markers in comparison to CD81. We noticed a decrease in cell quantity and viability with longer production times, because FBS-free media did not contain any components that promote cell attachment (Figure 4D). Thus, 2-day production at high cell density was best for achieving high EV concentration while maintaining cell viability over 95% to prevent possible formation of apoptotic bodies..

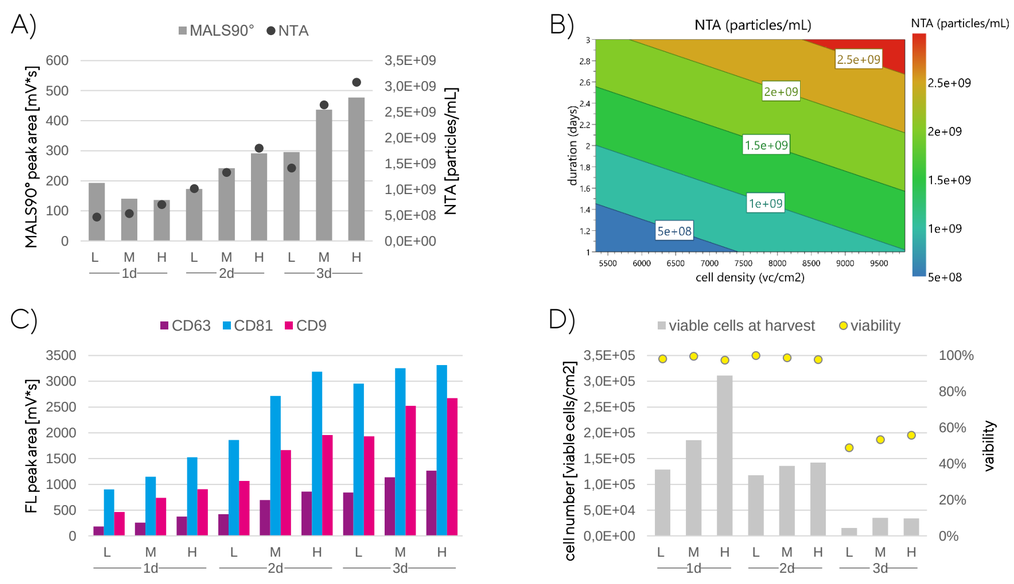

Figure 4: Effect of cell density and length of EV production. A) Correlation between NTA particle concentration and MALS signal. B) Analysis of optimal parameters in MODDE. C) Exosome marker expression in samples. D) Cell culture parameters. d – day, L – low, M – mid, H – high cell density.

5. Conclusions

- Adherent and suspension HEK293-derived cell lines produce EV populations with different characteristics.
- PATfix triple detector setup allows monitoring of EV concentration and exosome marker presence in upstream samples.
- Production media composition affects the chromatographic step of EV purification and requires careful consideration.
- In the HEK293T cell line, a short FBS-free EV production process with high starting cell density is optimal.

Literature

[1] Instruction Manual for Purification of Exosomes (2023). Available at: https://www.biaseparations.com/en/library/guidelines/1200/

[2] Vrabec K. *et al.* Exosome purification with CIMmultus EV-1 Advanced Composite Column (2019). Available at: https://www.biaseparations.com/en/library/posters/1050/

[3] Vrabec, K. *et al.* Characterization of EVs Subpopulations From CIMmultus ® EV Using PATfix® System (2023). Available at: https://www.biaseparations.com/en/library/posters/1220/