SARTORIUS

Simplifying Progress

Monolith Based Chromatography Is a Suitable Tool to Prepare and Analyze *E. coli* - Outer Membrane Vesicles

Simon Staubach[2], Agnieszka Razim[3] , Katja Vrabec[1], Darja Božič[1], Ana Mavri[1], Nastja Bizjak-Ternik[1], Andrej Raspor[1], Maja Leskovec[1], Anna Schmid[3], Irma Schabussova[3] & Aleš Štrancar[1]

[1]Sartorius BIA Separations d.o.o., Mirce 21, 5270 Ajdovščina, Slovenia
[2]Sartorius Stedim Biotech, August Spindler Str. 11, 37079 Göttingen, Germany
[3]Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Kinderspitalgasse 15, 1090 Vienna, Austria

Introduction

Intranasal administration of *Escherichia coli* O83 - outer membrane vesicles (OMVs) was found to reduce allergic airway inflammation induced by ovalbumin in mice. Production of sufficient amounts of OMVs to supply further clinical trials implies the need for scalable methods and precise analytics. Different orthogonal methods are required to provide a complete picture of the process. We present an exemplary OMV preparation, demonstrating robustness of our chromatographic platform. As shown before for various types of extracellular vesicles from animal and human cell origin, the platform was proved suitable also to isolate OMV's produced by *E. coli*. The purification process was monitored using three common analytical techniques – nanoparticle tracking analysis, flow cytometry and PATFix chromatography. The results demonstrate that chromatographic preparative methods are suitable to produce OMVs and that orthogonal analytical methods are mandatory to paint an accurate picture through the whole preparative process.

1. Experimental Setup

Preparative Protocol

Probiotic *E. coli* strain 083 was grown in Broth Medium until 1.2 turbidity was reached. Harvest was collected, centrifuged (6000 x g, 20 min) and filtered 0.22 µm, stored frozen at -80 °C until shipment to Bia Separations (Slovenia) on dry ice. After ON thawing at 4-8 °C, 100 ml of sample was processed according to cornerstone Process Development™ Pack [1,2]. Briefly, by Tangential Flow Filtration (TFF/750 kD), 3 DV buffer exchange to high salt buffer, TFF integrated salt tolerant nuclease digestion step for 1h (Blirt/Qiagen), buffer exchange by 6 DV to low salt AEX chromo step binding buffer. Half TFF-retentate (50 ml) was applied to AEX chromatography (Fig.1 a). Chromatographic separation was carried out on an ÄKTA Pure™ 25 M system (Cytiva) assembled with CIMmultus EV column (1 mL, 2 µm channels, Sartorius BIA Separations). OMVs were eluted in a salt gradient, and elution fractions were collected for further analysis.

Schematic *E. coli* OMV Preparative Protocol

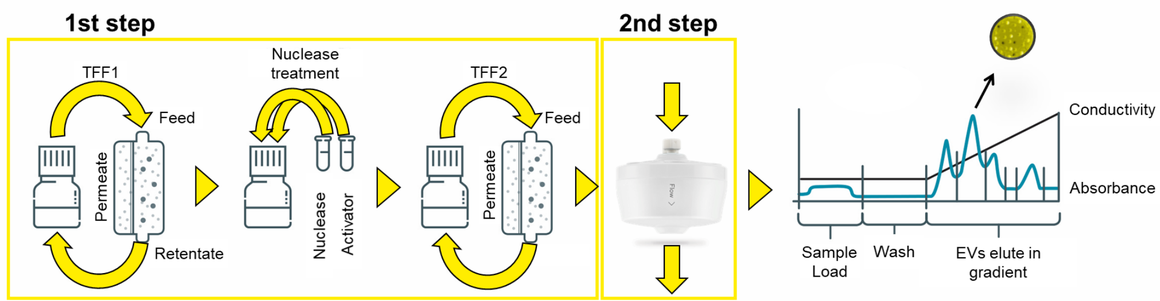

Figure 1: Tangential Flow Filtration with integrated salt tolerant nuclease digestion step followed by AEX chromatography

Analytical Platform Setup

Samples were analyzed using the PATfix system (Sartorius BIA Separations) and size exclusion chromatography (SEC)[3]. *E. coli* OMV samples were stained with MemGlow488 dye/ThermoFisher according to the manufacturers protocol. Each sample was injected to a SEC column TSKGEL G4000 SWXL (Tosoh Bioscience) where the labeled OMVs were separated from the excessive dye. The analysis was performed on the PATfix system with the following detectors UV cell with 50 mm optical path length, fluorescence detector, and MALS 3609 detector. Absorbance was monitored at 260 nm and 280 nm, OMVs fluorescence stained by MemGlow was measured at 520 nm emission peak, 488 nm excitation, and light scattering was monitored at 6 angles (60°,76°,90°,180°,124°,140°). The running buffer was 50 mM MES, 150 mM NaCl, 0.05 % Poloxamer, pH 6.5 and the flow rate was 1 mL/min. Applied orthogonal analytical methods were Nanoparticle Tracking Analytic (NanoSight 300/Malvern Panalytical), sample preparation according to manufacturers protocol and flow cytometry by Cell Stream/ Cytek [3]. Flow cytometry samples were stained by MemGlow, different sample dilutions were prepared to increase accuracy of measurement.

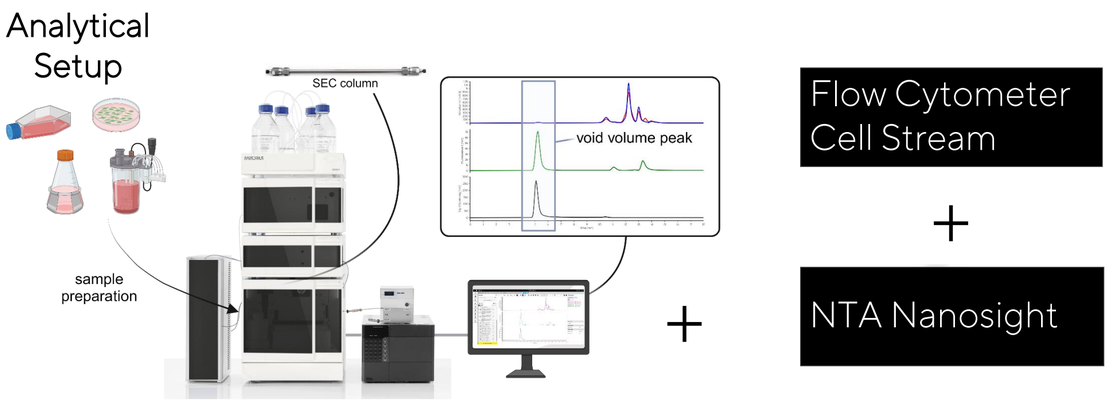

Figure 2. Schematic SEC-MALS OMV analytics of *E. coli* cell culture supernatant and preparative fractions. Multi-angle light scattering amplifies sensitivity for large species like OMVs. SEC is applied to separate OMVs and smaller sized sample constituents. This enables detection and quantification of residual contaminants in the processed samples. Peak area of MemGlow488 fluorescence correlates to stained OMV amounts. MemGlow 488 was measures ex 488 nm / em 520 nm. The same stained samples were additionally measured by flow cytometry. Samples measured by NTA were unstained.

1. Result - Preparative AEX Chromatography – Elution Profile

OMVs elute in two MALS peaks by application of a continious gradient. MALS 1 peak was collected in three elution fractions (E1+E2+E3) and MALS Peak 2 in two elution fractions (E4+E5) . For further analytics single or combined fractions were analyzed by PATfix, NTA and FCM

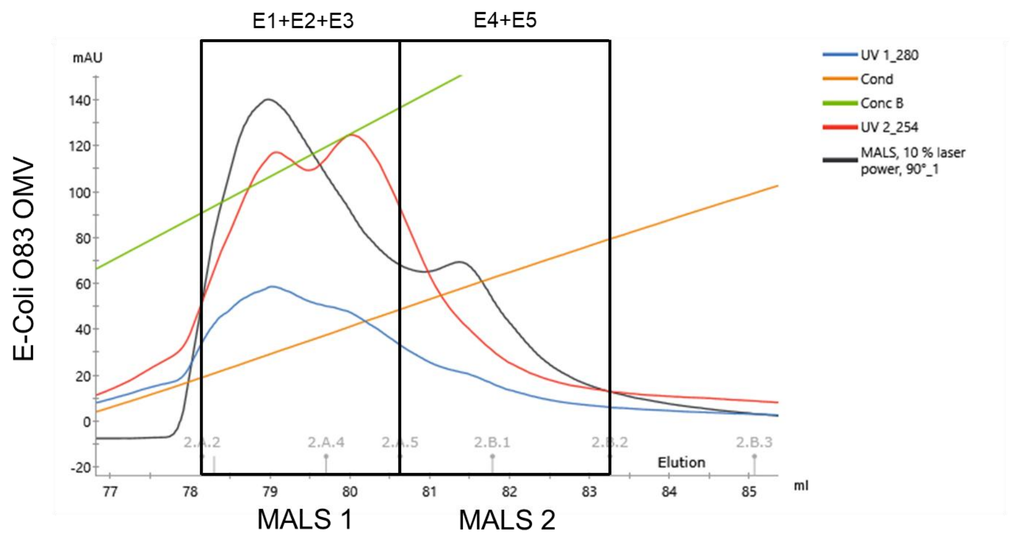

Figure 3. Elution profile *E. coli* O83 OMV - AEX chromatography preparative run. Gradient: 20 CV from low salt buffer A (20 mM NaCl 50 mM HEPES pH7.0) to high salt buffer B (2M NaCl, 50 mM HEPES, pH7.0). Multi detector setup 260 nm (red) and 280 nm (blue) absorbtion to reflect nucleic acids and protein impurities and MALS detector to detect scattering events like OMVs (black). Conductivity (orange) increases correspondent to increase of salt concentration (% of buffer B is shown in (green).

2. Results – EV Detection by MALS and Dye-SEC-MemGlow

Stained OMVs elute in the void volume. Concurring evidence by fluorescence and MALS signal. Unspecific stained material was retained in silica gel pores according to size and elute later as seen in "Starting Material". OMVs are enriched in fractions E1-E5. There is no loss detectable in TFF-FLT, in CIP scattering events are eluted but no fluorescence was detected.

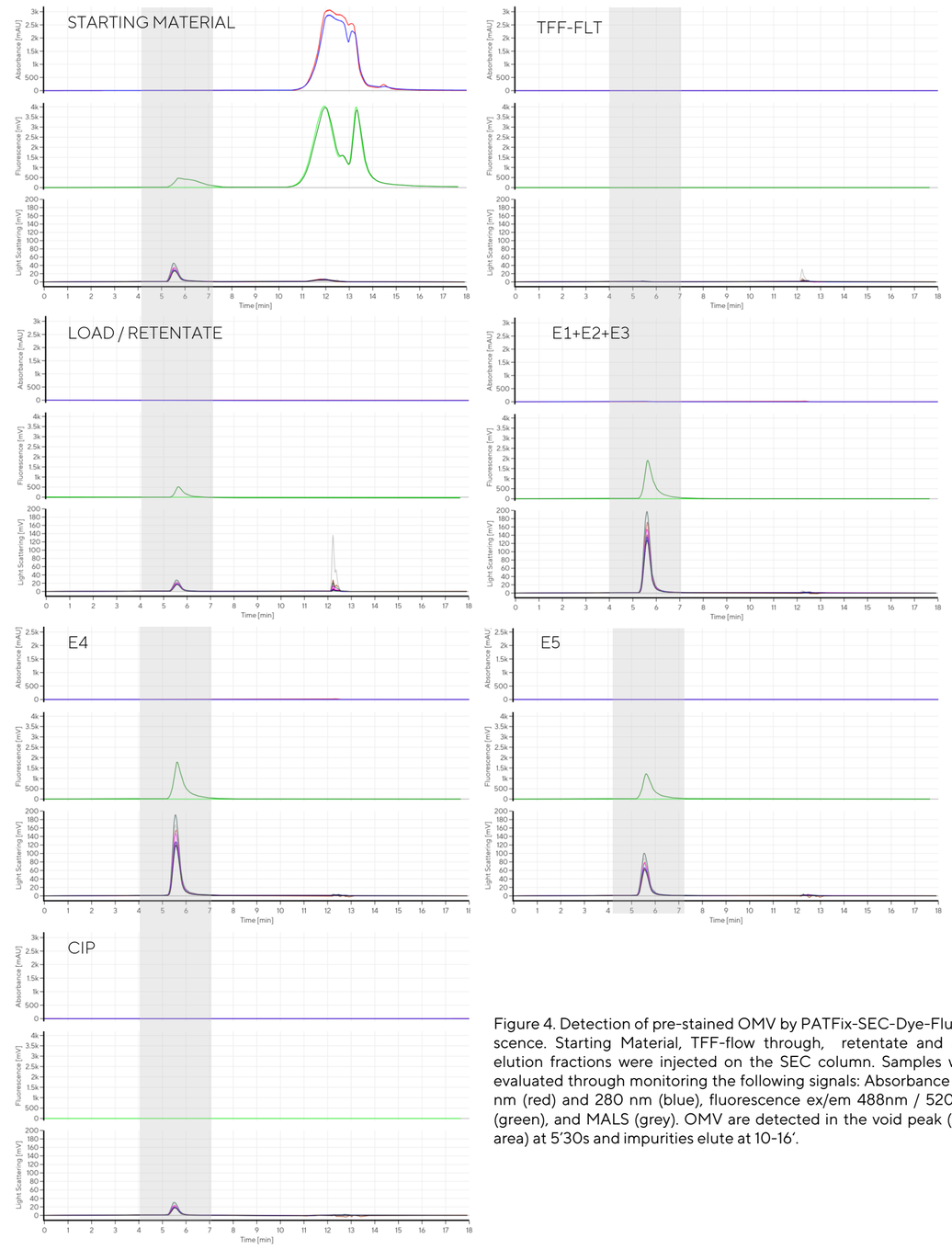

Figure 4. Detection of pre-stained OMV by PATFix-SEC-Dye-Fluorescence. Starting Material, TFF-flow through, retentate and AEX elution fractions were injected on the SEC column. Samples were evaluated through monitoring the following signals: Absorbance 260 nm (red) and 280 nm (blue), fluorescence ex/em 488nm / 520 nm (green), and MALS (grey). OMV are detected in the void peak (grey area) at 5'30s and impurities elute at 10-16'.

3. Results – Comparison of Dye-SEC, NTA, Dye-FCM

Fluorescence peak areas were calculated from SEC void volume peak areas determined by PATFix software multiplied by sample volume, and dilution. NTA total particle amount was calculated by measured particles and dilution and FCM events by measured events and dilution. Ca 60% recovery is reached adding up MALS1 & MALS2 fractions content

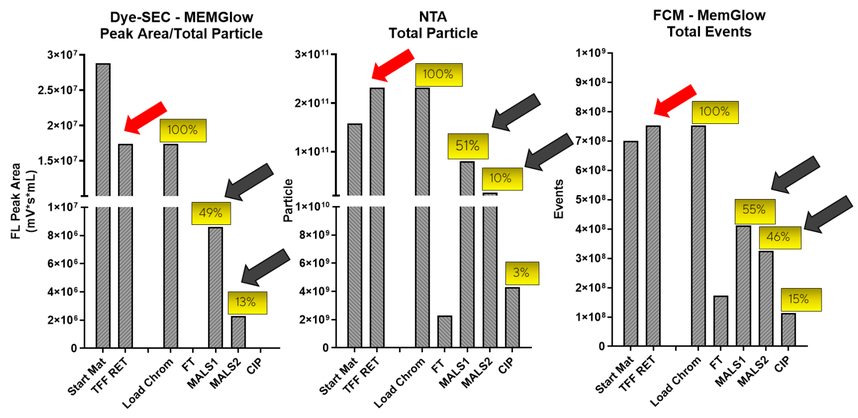

Figure 5: Tracking OMV's through the purification process with Dye-SEC, NTA and FCM. Dye-SEC and FCM samples, were measured after incubation with MemGlow 488, NTA particles were measured with unstained samples. Red arrows point to calculated OMV amount in TFF-Retentate a value unreliable high (more than 100%) observed in NTA particles and FCM events diagram. This is a common and often appearing error after TFF application caused by aggregate formation (data not shown). Misleading overestimation of vesicle content occurs. Dye-SEC seems to be unaffected by this error and is more specific and reliable. Black arrows point to percentage recovery, Dye-SEC and NTA are in concordance but FCM MALS2 measurment seems too high.

4. Conclusion

- The basic Cornerstone Process development Pack procedure comprising TFF, Nuclease Digestion and AEX chromatography was suitable to prepare OMV from cell culture supernatant with an overall process recovery of ca 60%
- The gained insights provide valuable guidance for process development
- The process is fully scalable
- Orthogonal methods are needed to proof reliability of results, NTA and FCM overestimated TFF recovery but Dye-SEC not. Recovery of ~60% was matched by Dye-SEC and NTA but FCM not.- 2nd MALS appears too high value in diagram
- MALS is an important tool that enables to identify OMVs during the whole process in accurate correlation with Dye-SEC results without necessity of staining
- Dye-SEC provides, specific, quantitative results and enables tracking of recovery through the purification processes.

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/