Simplifying Progress

Characterization of EVs Subpopulations From CIMmultus® EV Using PATfix® System

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Introduction

Cells release extracellular vesicles (EVs) of different sizes and intracellular origin. Due to their heterogenicity, the isolation of the target EV population from a mixture of supernatant-derived particles can be challenging. Anion exchange chromatography (AEX) exploits the negative charge on EV surface molecules for binding to the positively charged solid phase. CIMmultus® EV, an AEX chromatography monolith column, can separate EVs in subpopulations based on charge and offers insight into the heterogenicity of particles.

Besides the availability of preparative tools for separation, combining multiple orthogonal and complementary characterization tools is crucial for defining the EV product of interest. In this work, we used a multiple-detector PATfix® system for the analysis of CIMmultus EV-fractionated samples. Samples were analyzed for the presence of EV-related tetraspanins using the fluorescence detector. PATfix MALS 3609 detector was used for the analysis of particle-containing samples and calculation of particle sizes.

1. Sample preparation

Native HEK293T cells and engineered HEK293T CD63-eGFP cells were cultivated on FACT III (Sartorius) microcarriers in Ambr250 bioreactor (Sartorius) in DMEM growth media containing 10% FBS, then switched to production media without FBS for 2 days. For harvesting, the vessels were removed from the system and microcarriers were allowed to settle by gravity. Conditioned media was collected and filtered through Sartopure PP3 capsule 1.2 µm (Sartorius).

100 mL of each conditioned media sample was treated with nuclease and re-buffered, then applied to a CIMmultus EV column with a column volume (CV) of 1 mL and 2 µm channels. The chromatographic separation was performed on an Akta Pure 25 M system (Cytiva), equipped with a MALS detector. The loading buffer composition was 25 mM BTP 100 mM NaCl 2% sorbitol pH 7.5 and the elution buffer 25 mM BTP 2 M NaCl 2% sorbitol pH 7.5. The columns were loaded at 10 CV/min flow rate and eluted at 2 CV/min. EVs were eluted at different salt concentrations and collected as shown in Figure 1.

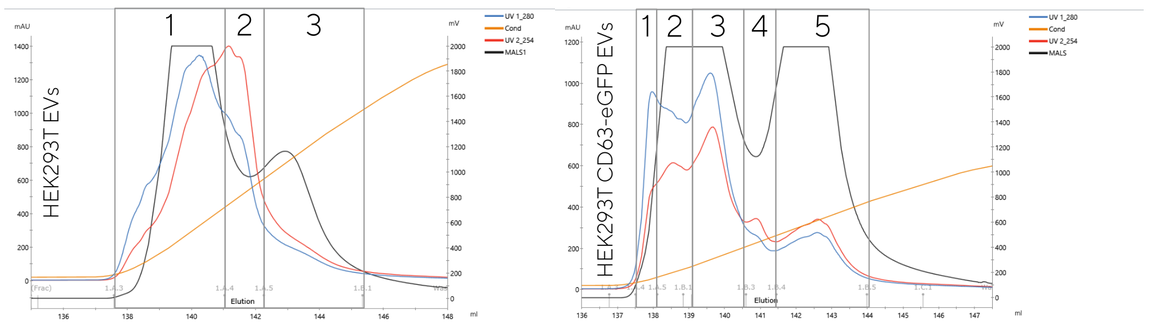

Figure 1: Preparative chromatograms from CIMmultus EV column. Vesicles were separated in salt gradient and collected in fractions labeled HEK293T EV fraction 1-3 and HEK293T CD63-eGFP EV fraction 1-5.

2. PATfix analytical setup

Samples were analyzed using the PATfix system (Sartorius BIA Separations) and size exclusion chromatography (SEC). HEK EVs, engineered to express CD63-eGFP on the surface were analyzed unlabeled. Native HEK EVs were labeled with anti-CD9, anti-CD63, and anti-CD81 FITC-conjugated antibodies (BioLegend). Each sample was injected to a SEC column TSKGEL G4000SWXL (Tosoh Bioscience) where the labeled EVs were separated from the excess antibody. 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 and 280 nm, fluorescence was set to excitation and emission peaks of the used fluorophores, and light scattering was monitored at 9 angles. The running buffer was 50 mM MES, 150 mM NaCl, 0.05 % Poloxamer, pH 6.5, and the flow rate was 1 mL/min.

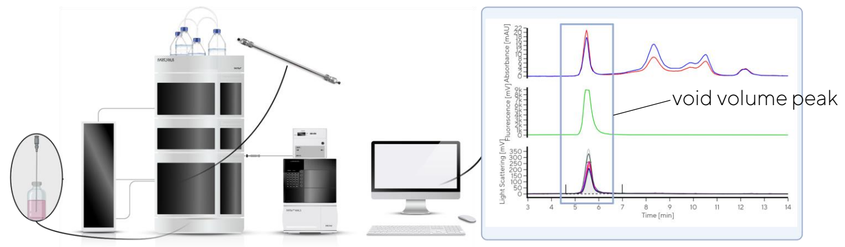

Figure 2: PATfix system setup used in these experiments (Created with BioRender.com). Samples are contained in an autosampler, before being injected into a SEC column. Absorbance, fluorescence, and light scattering are monitored and the data is interpreted in PATfix software. An example SEC chromatogram shows the void volume peak where particles are eluted.

3. Evaluation of EV distribution in chromatographic samples

SEC chromatograms were analyzed in PATfix software. Fluorescence (FL) and light scattering (LS) peak area were calculated from SEC void volume peak area, sample volume, and dilution. The distribution of FL and LS peaks in chromatographic samples was plotted in Figure 3. Light scattering shows the particles are distributed in two main peaks in both the native and engineered cell lines. The EVs with the highest fluorescence signal response were eluted in the first fraction.

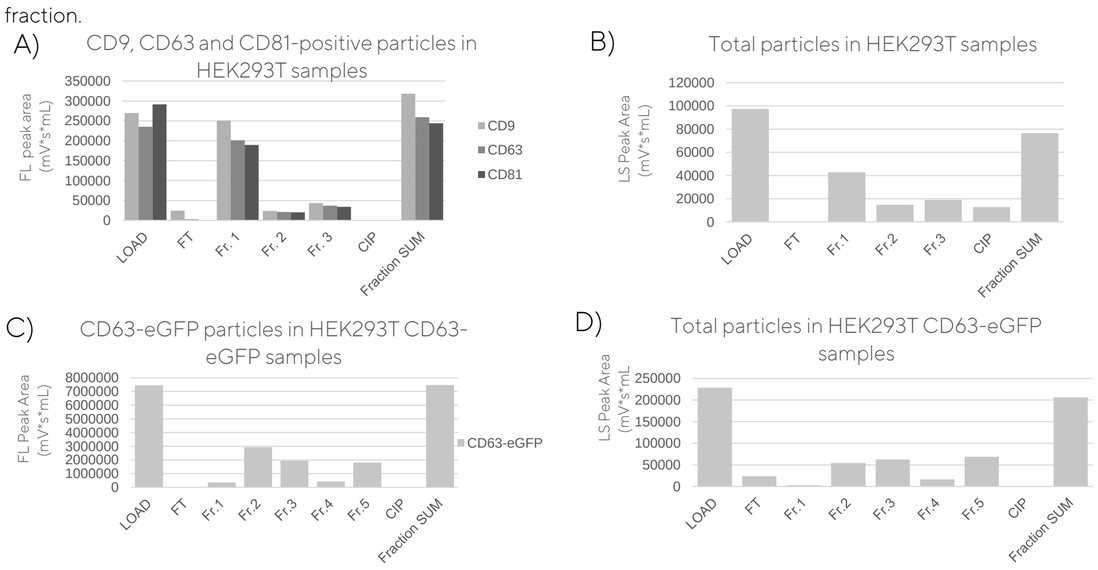

Figure 3: Distribution of FL and LS peak area in representative samples LOAD (total sample loaded to CIMmultus EV column), FT (flow-through, the unbound fraction), Fr. 1-5 (elution fractions containing EVs) and CIP (cleaning-in-place): A) and B) HEK293T samples, C) and D) HEK293T CD63-eGFP samples.

4. Determination of EV size using PATfix MALS

The radius of particles was determined from SEC chromatograms in PATfix software (Figure 4). Additionally, NTA analysis was performed on Nanosight 300 (Malvern Panalytical) (Figure 5). Despite the fact that fractions vary in particle charge and surface antigens, all fractions contained particles in the size range of EVs, emphasizing the importance of using multiple approaches for the characterisation these particles.

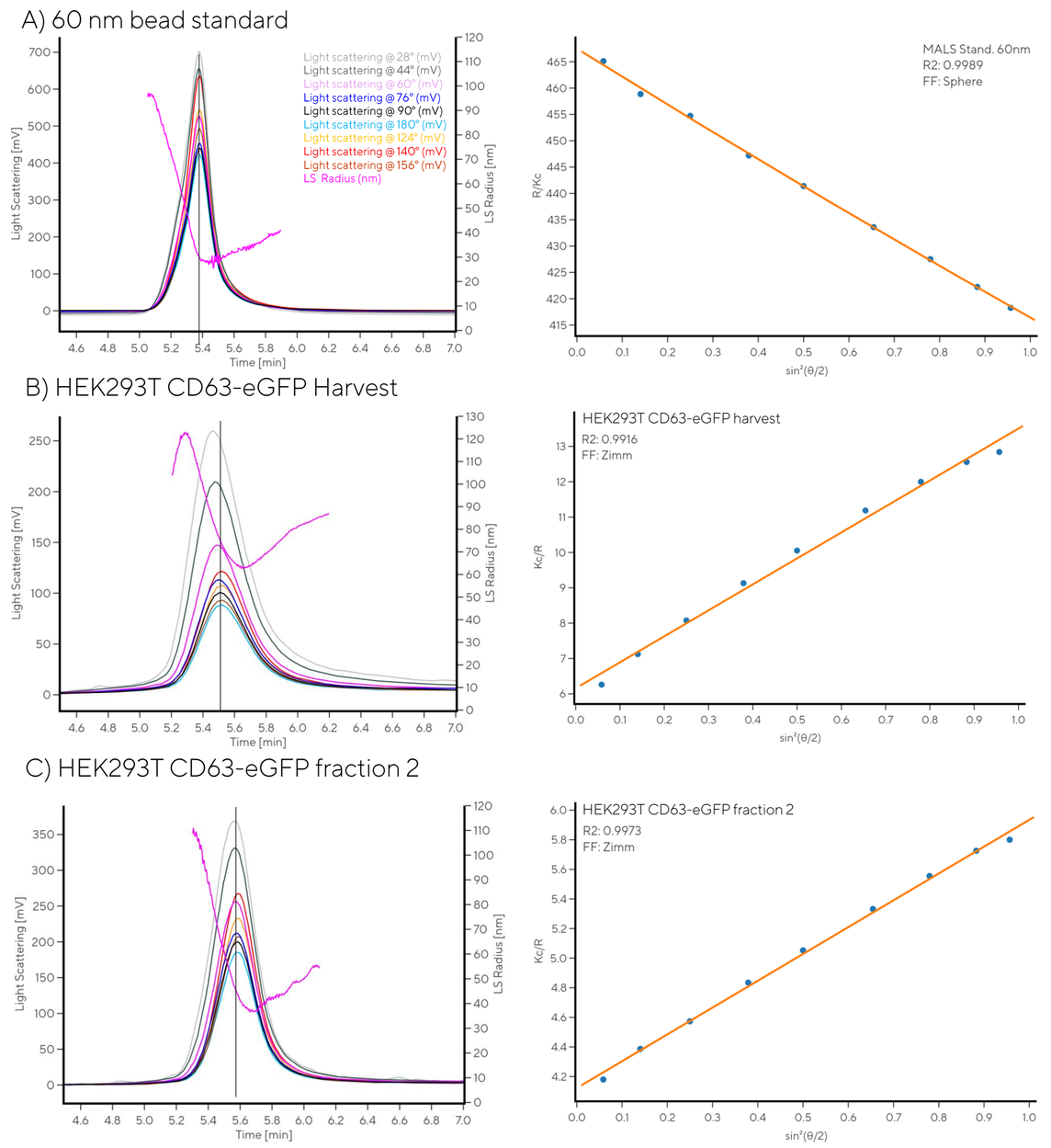

Figure 4: Light scattering chromatograms of the SEC void volume and corresponding LS charts. The particle radii were measured at the LS 90° peak. (A) SEC chromatogram of 60 nm polystyrene spherical bead standard (Postnova, Part No. Z-PS-POS-000-0,06) and the corresponding LS chart using form factor sphere. The standard was used for detector normalization. B) SEC chromatogram of HEK 293T CD63-eGFP EV Harvest and the corresponding LS chart using Zimm plot. All chromatographic samples were characterized using this plot. C) SEC chromatogram of HEK 293T CD63-eGFP fraction 2.

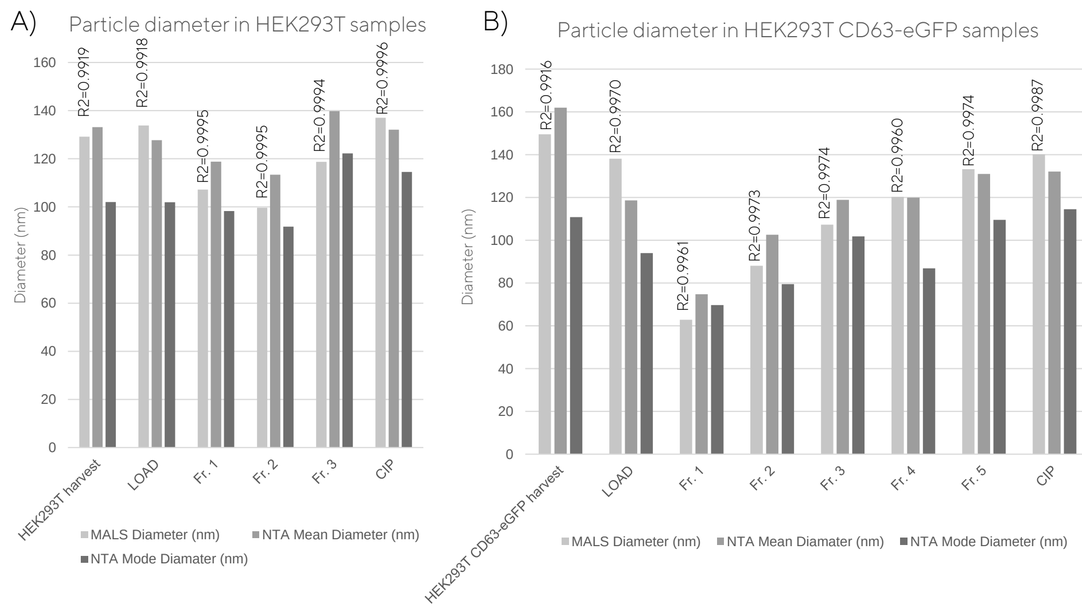

Figure 5: Particle diameter determined by MALS and NTA for A) HEK293T samples and B) HEK293T CD63-eGFP samples.

5. Conclusions

- CIMmultus EV chromatographic column fractionates supernatant-derived vesicles in multiple populations that differ in size and surface antigen composition
- PATfix triple detector setup enables rapid sample characterization for both native and engineered EV samples
- Fluorescence detector allows for relative quantification of fluorescent EVs or EVs labeled with a fluorescent antibody
- Combining MALS detector with SEC analytics enables relative quantification of particles in samples and determination of particle size

6. Acknowledgements

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