Simplifying Progress

2D-LC Evaluation of Complex Samples Containing Extracellular Vesicles

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Introduction

The aim of the study was the development of a robust and high-throughput method that would enable a comprehensive evaluation of extracellular vesicles (EVs) in complex samples. Our goal was to provide an insight into the heterogeneity of the present EV- and non-EV particles while at the same time elucidating the background matrix. We designed a two-dimensional liquid chromatography (2D-LC), in which we coupled the isolation of nanoparticles employing size exclusion chromatography (SEC), with their subsequent separation using anion-exchange chromatography (AEX).
The developed 2D SEC-AEX method takes 30 minutes. The result of the analysis is a detailed sample profile, displaying: 1) sample components resolved by their size, and 2) heterogeneity of particles, resolved by their charge.
The applicability of the proposed approach is demonstrated through the evaluation of various samples, including conditioned media from different cell cultures, and EV-enriched preparations. The obtained profiles display the differences in sample compositions and indicate the possible strategies for further fractionation and purification of populations of interest.

Analytical setup

Analytical setup included a PATfix® system with incorporated absorbance, fluorescence, light scattering detectors, and upgraded with a switching valve module (Sartorius BIA Separations), a TSKGEL G4000SWXL SEC column (Tosoh Bioscience), and a CIMac™ 0.1 (6) anion-exchange monolith (Sartorius BIA Separations). A schematic of the analytical setup is presented in Figure 1.

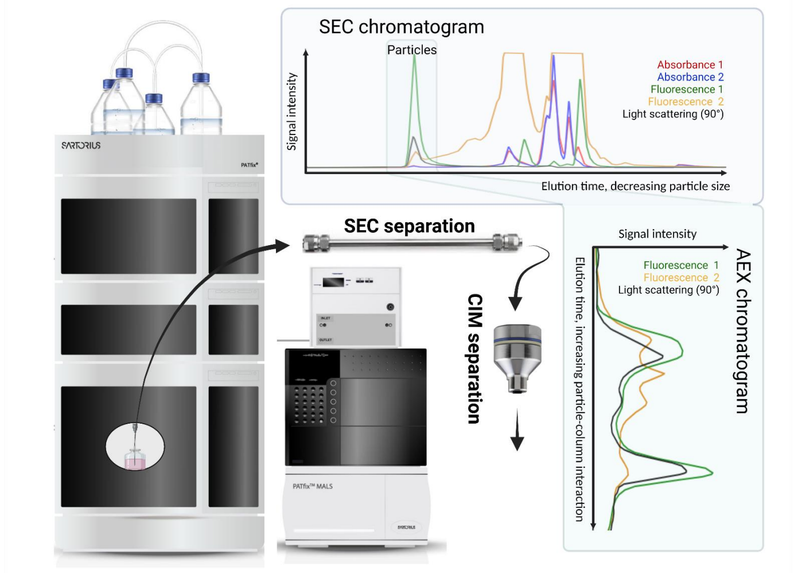

Figure 1: Schematic representation of the analytical setup (Created with BioRender.com). The sample is injected onto a size exclusion chromatography (SEC) column. The size-based elution profile illustrates the contents of nanoparticles and smaller sample constituents. The fraction eluting in the range of nanoparticles with predicted size exceeding 30 nm is directed through an anion exchange column (AEX). The charge-based AEX-elution profile reveals distinct particle subpopulations. Evaluation of resolved components is enabled through the monitored signals of absorbance, fluorescence and light scattering.

Chromatography was performed at a flow rate of 1 mL/min, with the following mobile phases (MP): MP-A: 50 mM HEPES, 75 mM NaCl, 1% sorbitol, 0.05% Poloxamer 188, pH 7.2; MP-B: 50 mM HEPES, 2 M NaCl, 1% sorbitol, 0.05% Poloxamer 188, pH 7.2; MP-C: 0.1 M NaOH, 2 M NaCl; MP-D: 0.5 M Tris, 1 M NaCl. SEC was run with 100% MP-A, and AEX was performed applying a progressive gradient of 0-100% MP-B. The method included cleaning in place (CIP) of the AEX column with MP-C and its re-equilibration with MP-D and MP-A, following each sample analysis. Figure 2 shows a schematic of the chromatographic method. Absorbance at 260 nm (A260) and 280 nm (A280), employing a 10 mm optical path length UV cell was monitored for the SEC channel with an aim to reveal biological molecules. The fraction of largest particles normally exhibited little absorbance, therefore light scattering and fluorescence were employed to maximize the detection potential. The AEX profile was conducted following light scattering at a 90° angle, and two fluorescence channels with excitation/emission wavelengths set to: 488 nm /520 nm (F488/520) to detect engineered green fluorescent protein (eGFP), and/or green-fluorescent probes (FITC-labelled antibodies), and 280 nm /348 nm to detect tryptophan (F280/348, TRP).

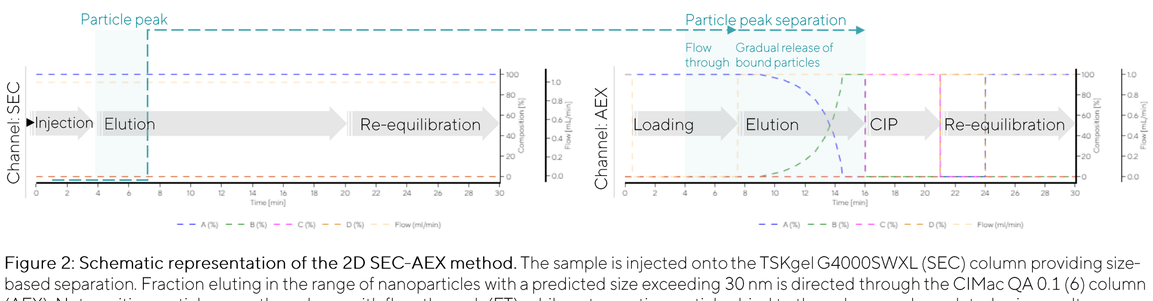

Figure 2: Schematic representation of the 2D SEC-AEX method. The sample is injected onto the TSKgel G4000SWXL (SEC) column providing size-based separation. Fraction eluting in the range of nanoparticles with a predicted size exceeding 30 nm is directed through the CIMac QA 0.1 (6) column (AEX). Net-positive particles pass the column with flow-through (FT), while net-negative particles bind to the column and are eluted using a salt gradient, revealing distinct particle subpopulations. The gradient was optimized for detection of EV subpopulations based on extensive preliminary study of various EV samples.

Samples

Conditioned media samples were collected from cell cultures of HEK293T CD63-eGFP (sample CM 1), Jurkat (sample CM 2) and bone marrow-derived MSC (CM 3). Jurkat cells are suspension cells *per se*, while the other two adherent cell lines were grown in suspension with the use of microcarriers. Cells and larger particles were removed by filtration through a PP3 capsule 1.2 µm (Sartorius). Clarified conditioned media were used for chromatography evaluation. Additionally, two EV-enriched preparations (EVP1 and EVP2) were obtained from the CM 3 sample using CIMmultus® OH [(1)]. 300 µL of each sample was injected directly with no pretreatment or after pre-incubation with a selected fluorescent probe. Immunolabelling was performed using FITC-conjugated antibodies targeting human tetraspanins CD63, CD81, CD9 (BioLegend).

2D SEC-AEX fingerprint

Complex biological samples contain numerous components at varying amounts, and the exact targets of interest may not always be predictable. A fingerprint profile based on the common properties of biological molecules (e.g., A260, A280, autofluorescence, light scattering) can provide valuable orientation, recognition of potential components of interest, and enable their monitoring even without a definite identification. Figure 3 shows a 2D SEC-AEX profile of cell culture conditioned medium CM 1 which is expected to contain green-fluorescent CD63-positive EVs (2).
Quantitative evaluation of small sample constituents such as free proteins, nucleic acids, and their small complexes can be derived from UV light absorbance followed in the SEC part of separation (Figure 3a, left chromatogram). The AEX part of the 2D method displays heterogeneity in the particle population (Figure 3a, right chromatogram). In CM 1, subpopulations of CD63$^+$ EVs were indicated by eGFP fluorescence (Figure 3a, right chromatogram, fractions 2 and 5), while three additional populations (peaks 1, 3, 4) were exposed by TRP fluorescence.

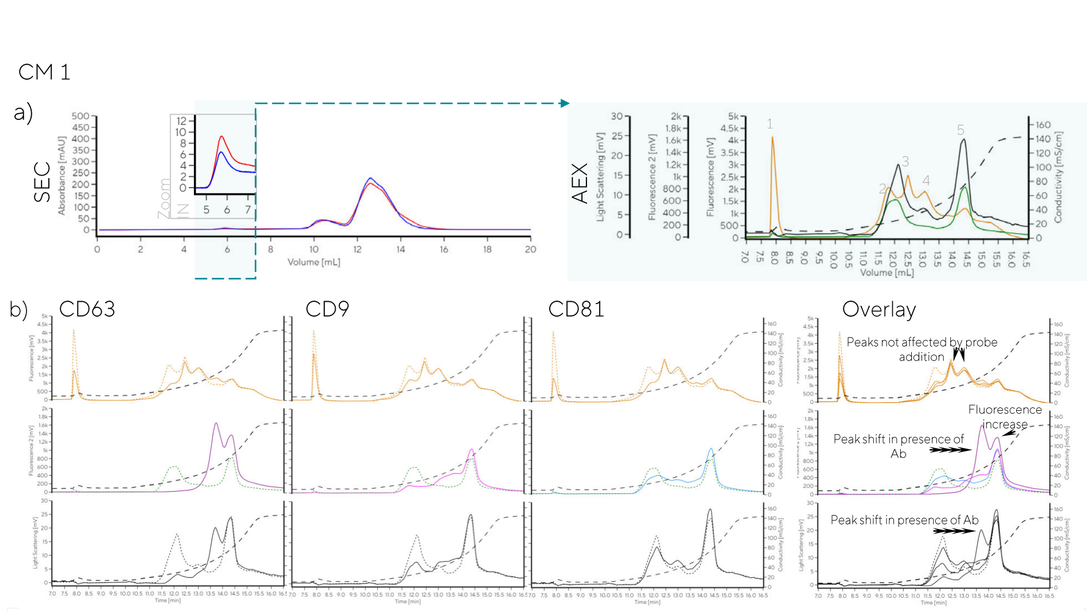

Figure 3: 2D SEC-AEX fingerprint of a conditioned medium sample CM 1. a) Evaluation of CM 1 with no probe addition. Absorbance profile of SEC elution. The A280-prevalent peaks can be related to the presence of proteins, while A260-prevalent peaks may indicate the presence of nucleic acids. A small peak eluting at 5-7 mL (a close-up is presented in a frame) is attributed to the components larger than approximately. 30 nm. Smaller components elute with larger volumes. The highlighted fraction is directed on the AEX column. The AEX-resolved particle profile is presented on the right chromatogram. The distinct fluorescence and light scattering peaks indicate several particle subpopulations. A few fractions of possible interest are marked with numbers: 1 – non-binding fraction, 2-5 – bound fractions, in order of increasing strength of interactions with the AEX column. b) The AEX part of the 2D SEC-AEX profiles obtained for immunolabelled CM 1. Labelling-related fluorescence signals are drawn in different colours for different targets. The profile of the non-labelled sample is shown in overlays (dotted lines) to highlight the changes in the CM 1 profile induced by probe addition.

Green fluorescent peaks (fraction 2) largely aligned with light scattering peaks, implying that a large proportion of the present particles ware CD63-eGFP$^+$. This was further supported by the analysis of the immunolabelled sample (Figure 3b), where fraction-2-related LS and green fluorescence shifted in the presence of CD63 antibody. The observed shift can be attributed to the changed particle surface properties due to the bound antibodies. Increased fluorescence in immunolabelled samples also indicated the presence of some CD9$^+$ and CD81$^+$ particles. Correlations between the measured signals suggested that these may represent a subset of the CD63-eGFP-positive population.

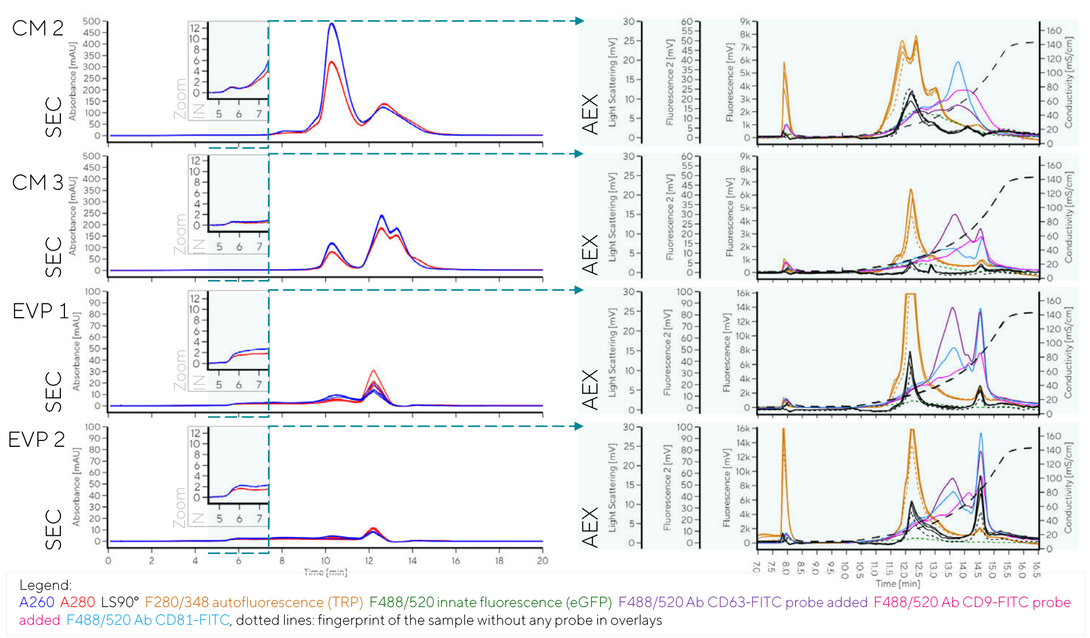

Figure 4: 2D SEC-AEX fingerprints of conditioned media samples CM 2 and CM 3; and two EV-enriched purified preparations EVP 1 and EVP 2 that were obtained from CM 3. Chromatograms obtained in analysis of unlabelled and the corresponding immunolabelled replicates are shown overlaid to manifest the changes in profile induced by probe addition. The unlabelled sample profile is denoted by dotted lines. SEC elution displays evident differences in sample matrices (left chromatograms). Particles eluting at 5-7 mL (a close-up is presented in a frame) were resolved on AEX. Their profile was constructed based on fluorescence and light scattering (right chromatograms). Distinct signal intensities and/or signal-signal rations indicate several particle subpopulations. Labelling-related fluorescence signals are presented in different colours for different targets. Note the different scaling for absorbance and fluorescence signals in different samples.

Figure 4 presents the results of the analysis of two other (completely different) conditioned media samples and two EV-enriched preparations. CD9/CD63/CD81-positive peaks suggested the presence of EVs in all samples, with evident differences in their heterogeneity and quantity (note the different scaling for CM and EVP samples). In all samples, the addition of antibody probes resulted in additional fluorescent peaks that deviated from those exposed by light scattering and tryptophan fluorescence. Furthermore, changes in sample profiles induced by probe addition were minor, which may suggest that a significant proportion of particles in these samples were negative for the tested markers CD9, CD63 and CD81. The affinity conditions of distinct subpopulations, as read from the resulting chromatogram, offer a foundation for designing further purification strategies. In the case of well-established probes, some possible contaminations with overlapping properties can be foreseen, as demonstrated in the analysis of immunolabelled samples included in the present study.

Contribution to the EV research toolbox

- Sample profile in less than 1h
- Minimal sample preparation
- Applicable to a broad range of samples
- Identification of different sub-populations of EVs and non-EV nanoparticles that naturally overlap in size
- Preposition of potential purification strategies

References

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2. Ludwig, A. K., et al (2018). Precipitation with polyethylene glycol followed by washing and pelleting by ultracentrifugation enriches extracellular vesicles from tissue culture supernatants in small and large scales. *Journal of Extracellular Vesicles, 7*(1).

Acknowledgments

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If you would like us to analyze your samples by the presented approach, please contact info@biaseparations.com or the corresponding author.