BIA separations

Better purification. Faster.

A cornerstone platform for industrial purification of exosomes

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Introduction

There are two important prerequisites for development of an industrial purification process for any biological drug:

1) Good in-process assays to guide development.

2) Good fractionation tools.

The defining elements for good in-process assays include the ability to discriminate the product from contaminants and impurities, simplicity, short assay time/high throughput, high sensitivity, accuracy, reproducibility, and platformability.

The defining elements for good fractionation tools begin with the ability to maintain product integrity while removing contaminants. They further include simplicity, scalability, and platformability.

Introduction

Platformability refers to the ability of a standardized template approach to accommodate all the members of an extended product family.

Monoclonal antibodies are a good example. The emergence of a platform approach early in their evolution was a key enabling factor in the success of the industry as a whole.

Like antibodies, exosomes represent a fairly well defined and extended product family, making the industrial value of a platform approach potentially just as high.

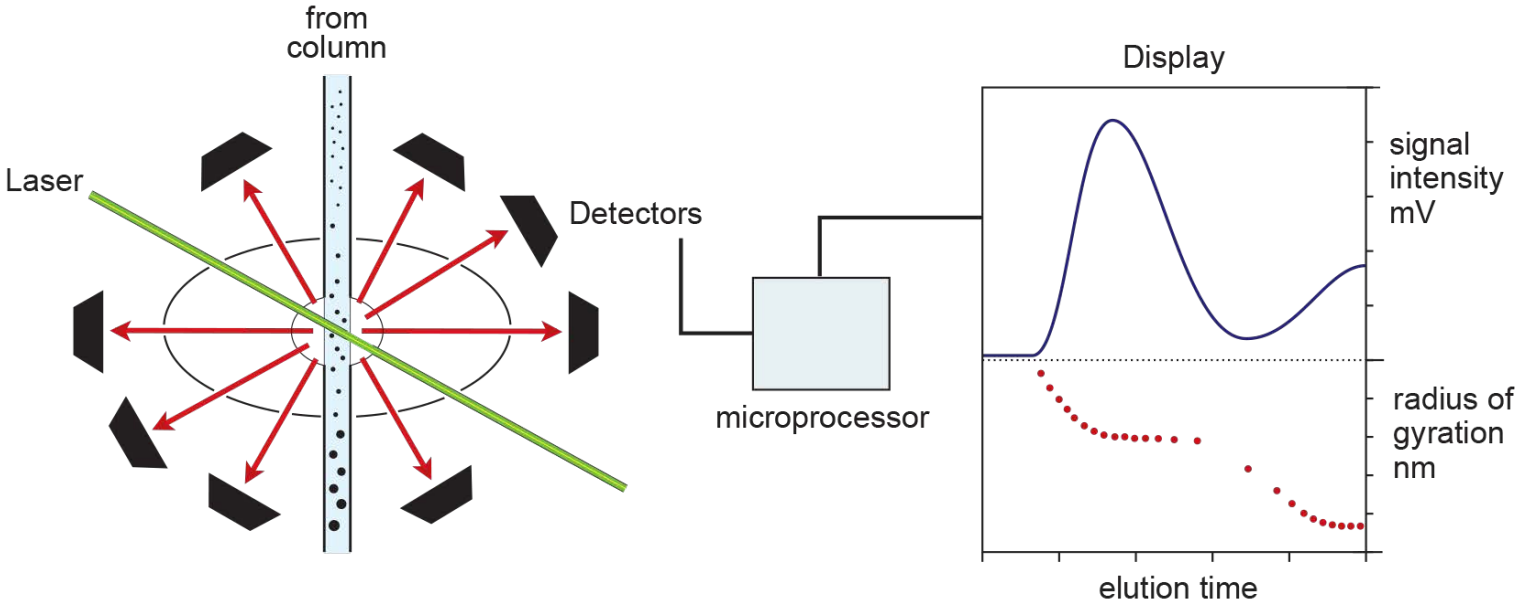
This presentation introduces new purification and analytical tools designed expressly to support industrial platform purification of exosomes.

Analytics: in-line vesicle detection with MALS

Multi-Angle Light Scattering adds a valuable dimension to chromatographic analysis of extracellular vesicles.

Scatter is proportional to particle size. Large species like EV produce more scatter than proteins and their signal is amplified proportionately.

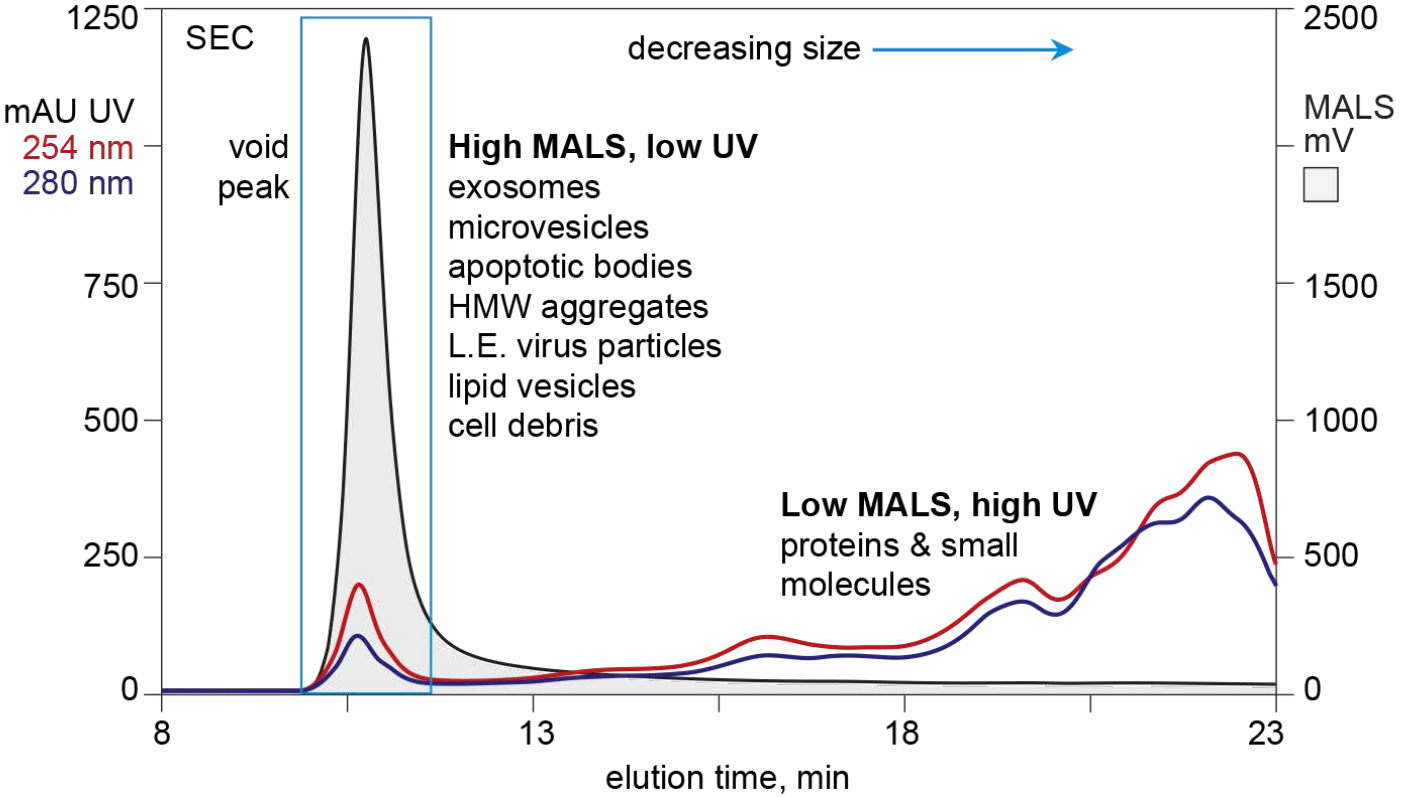
MALS can also be used to generate estimations of mass.



Analytics: in-line vesicle detection with SEC-MALS

Multi-Angle Light Scattering amplifies sensitivity for large species.

SEC. TSKgel™ G4000SWxl. 0.5 mL/min. Filtered HEK293 harvest.



The void peak contains species that are too large to diffuse into the chromatography particle pores. Size separation within the void space between the particles is extremely limited.

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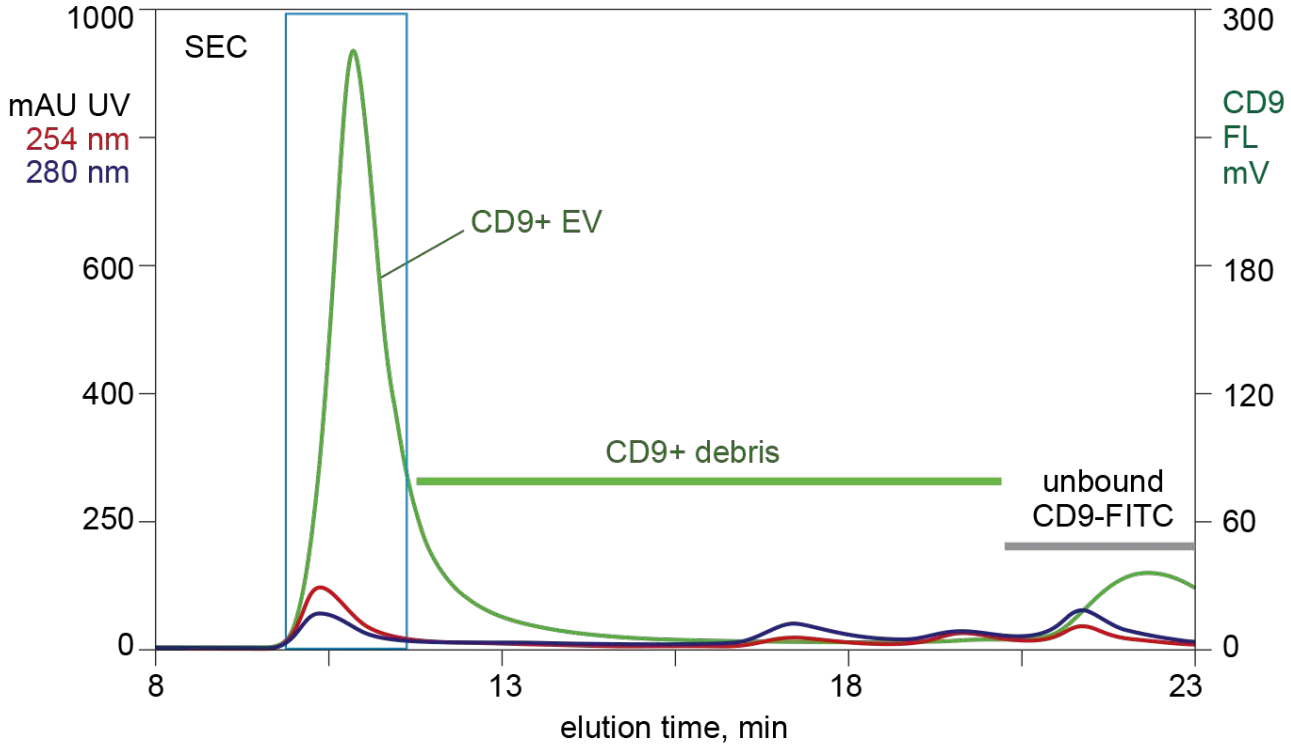
Analytics: detection by SEC-immunofluorescence

Pre-incubation of samples with a flourescent antibody conjugate enables quantitative detection of specific vesicle markers.

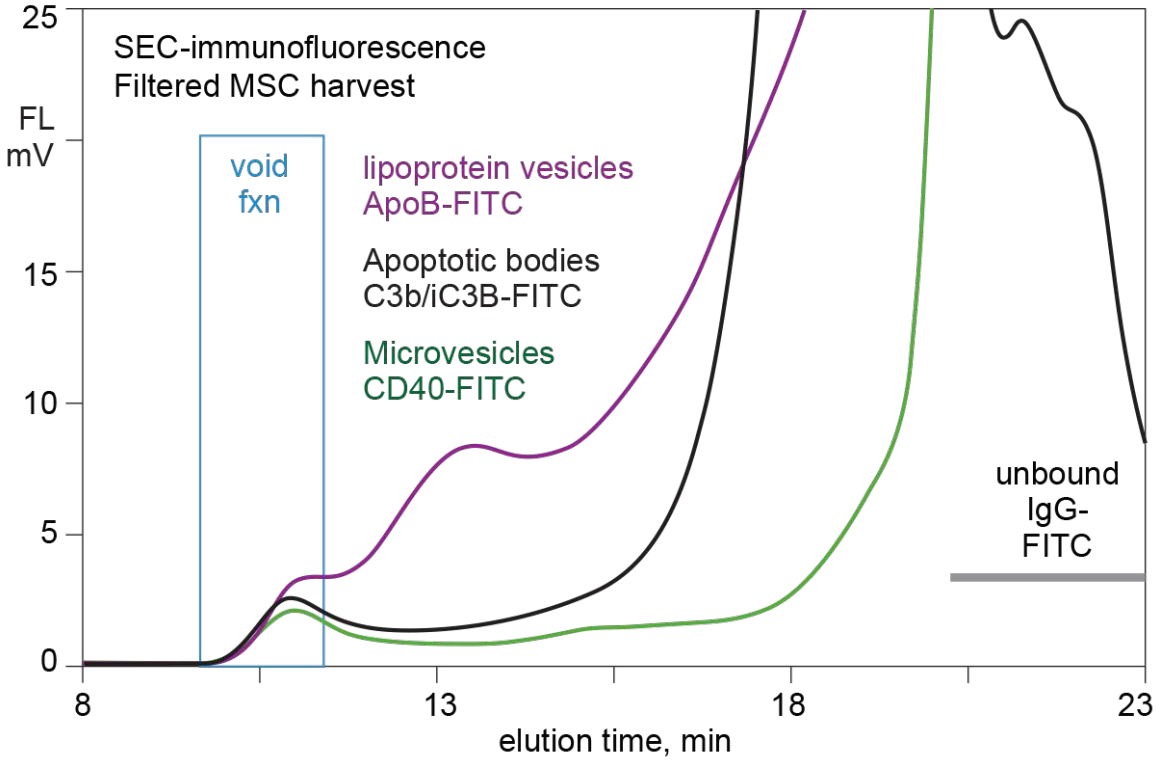
SEC-IF with CD9-FITC. Partially purified HEK293 harvest.



This method can be performed with any fluorescent immunoconjugate: any antibody, any fluorophore, but it works only with SEC because the conjugate chemistry affects vesicle retention with adsorptive methods.

Analytics: detection by SEC-immunofluorescence

SEC-IF can also be used to map size distribution of other vesicle types in cell cultures and purification fractions.



All vesicle types co-elute partially with exosomes in the void volume. Despite being typically described as much larger than exosomes, the majority of microvesicles and apoptotic bodies are actually smaller. The same pattern is evident with lipoprotein vesicles.

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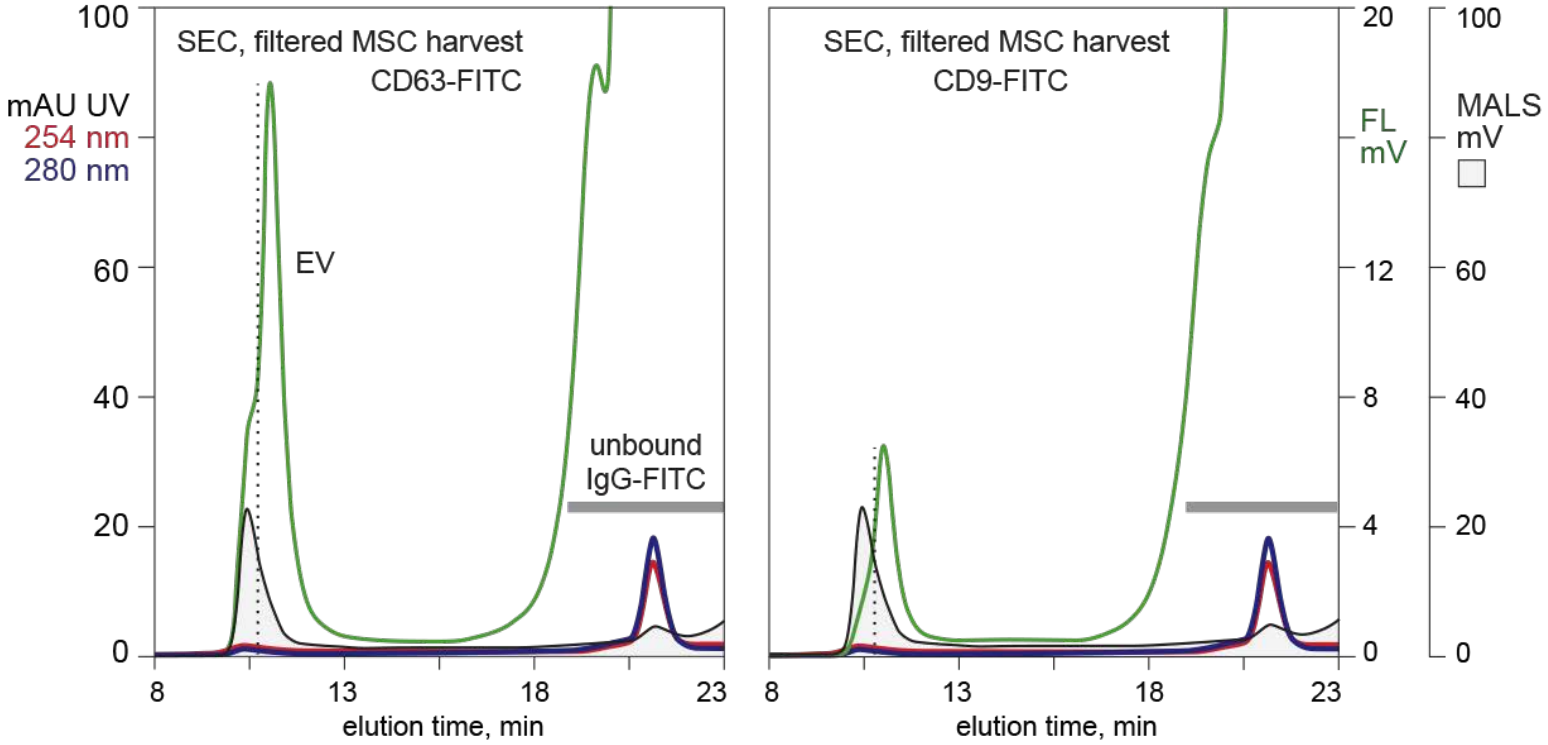
Analytics: combined detection by SEC-MALS/IF

MALS and IF are orthogonal and provide complementary insights.

SEC. TSKgel™ G4000SWxl, 0.5 mL/min. Filtered MSC harvest (Rooster Bio).

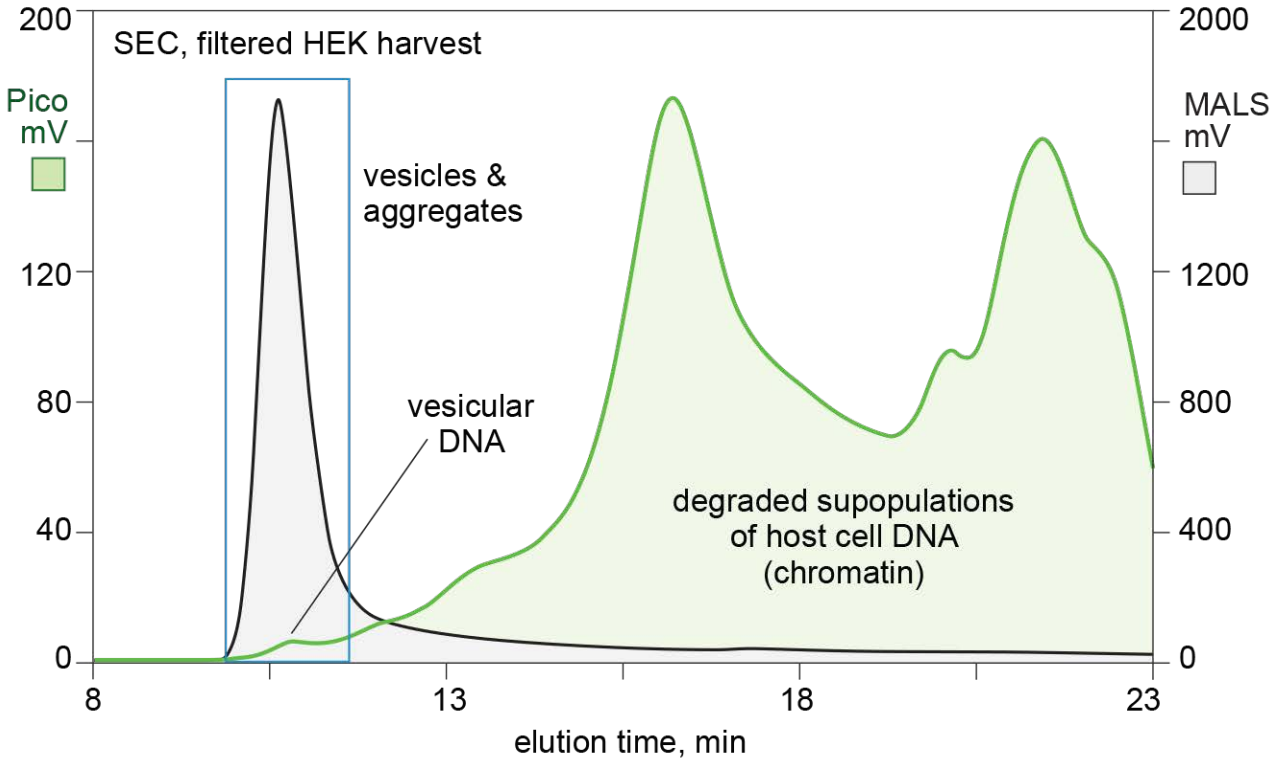


Combined MALS /IF shows a void population of large EVs with moderate marker density and later-eluting smaller species with high marker density. The same trend is apparent for both CD9 and CD63.

Analytics: DNA size distribution by SEC-picogreen

Fluorescence can also be used to increase sensitivity of DNA detection.

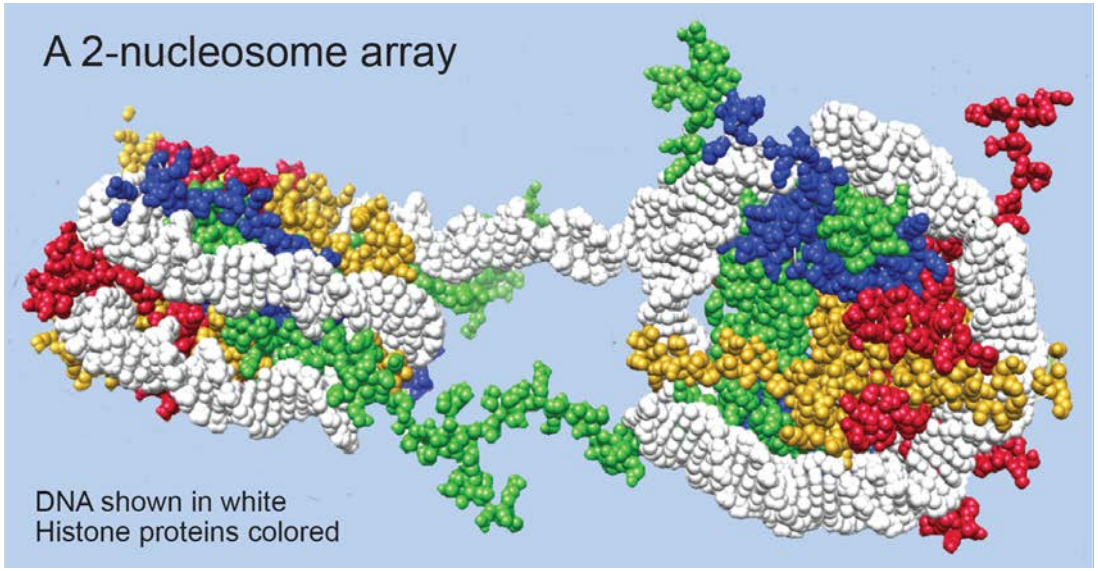
Picogreen™ incubated with HEK293 harvest before application to column.

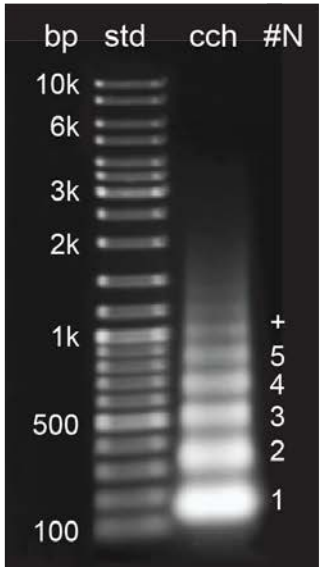


Combining Picogreen with SEC reveals the size distribution of DNA, providing an orthogonal extension to the basic selectivity of both. Off-line analysis is still recommended for absolute quantitation.

Host cell DNA exists in the form of chromatin

Chromatin in cell culture harvests is the remnant of the chromosomal mass from host cells. Its basic structural subunit is a nucleosome.





DNA is highly electronegative (pKa 2.6). The minor groove is hydrophobic. It also participates in hydrogen bonding, metal affinity, and van der Waals interactions.

Histones are highly electropositive (pI 9–11) and extremely hydrophobic. They also participate in metal affinity, hydrogen bonding, and van der Waals interactions.

These chemical potentials promote chromatin binding to every surface it contacts.

Nucleosomal arrays in cell harvests range in size from about 12 nm to 500 nm. They persist for months after harvest.

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Why is chromatin an issue for exosome purification?

1. Chromatin interferes with *all* purification methods: filtration, precipitation, chromatography.

   It reduces capacity by 10–75%, depending on the method.

   It inflates host cell protein contamination by 100-fold.

   It inflates host DNA contamination by 10,000-fold.

   It reduces product recovery at each step by 10–50%.

2. Chromatin *protects* host-cell DNA.

   The association of DNA with histone proteins is so strong that it limits access by nuclease enzymes and prevents complete lysis.

3. Extracellular chromatin is *antigenic*.

[1] J. Chrom. A 1291 (2013) 33. [2] J. Chrom. A 1340 (2014) 68. [3] J. Chrom. A 1374 (2014) 145.
[4] J. Chrom. A 1408 (2015) 151. [5] Anal. Bioanal. Chem. 407 (2015) 4173.
[6] J. Chrom. A 1431 (2016) 1. [7] J. Chrom. A 1453 (2016) 54.

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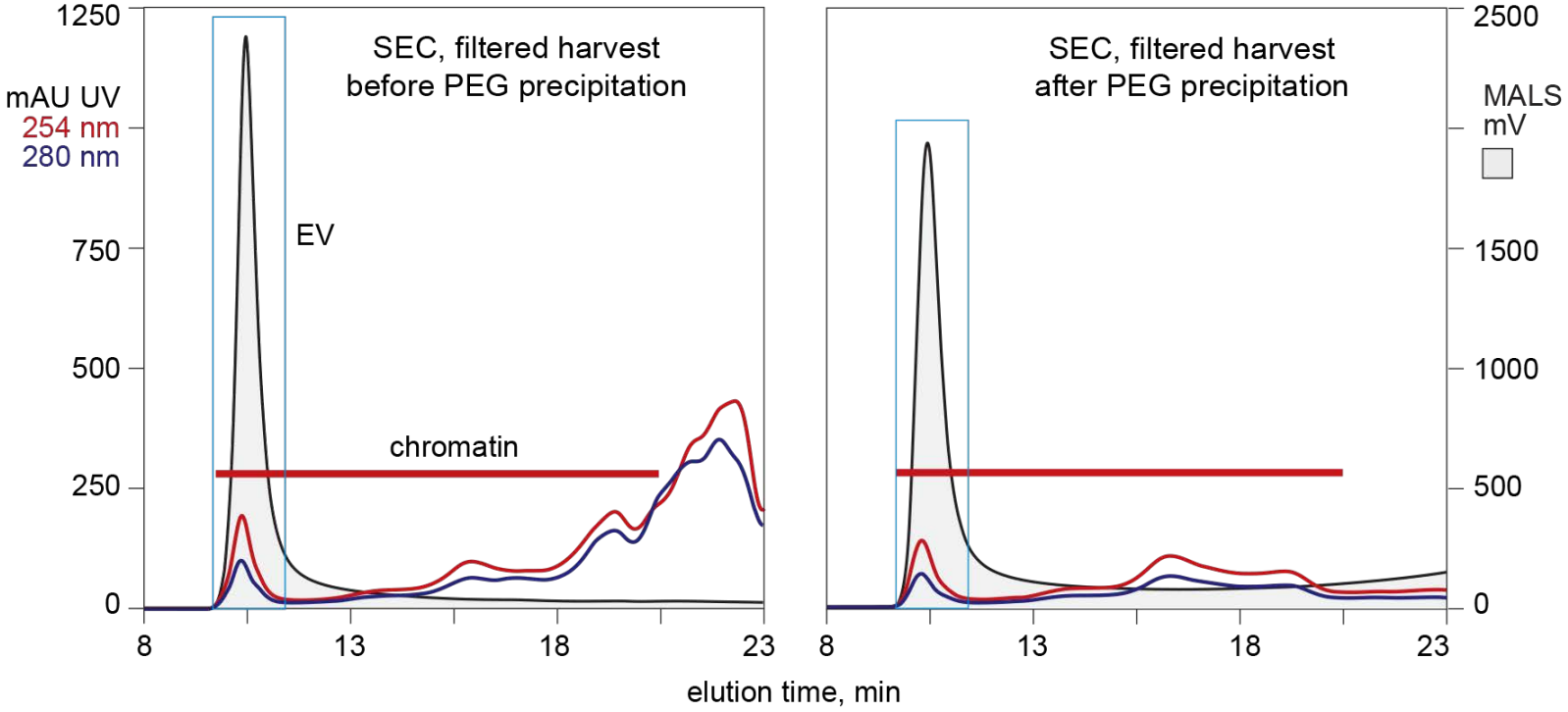
Impact of chromatin on exosome purification

Precipitation with polyethylene glycol (PEG, commercial kit).

SEC-MALS/UV. Filtered, serum-free HEK293 harvest versus PEG-purified.



Chromatin co-precipitates with EV during PEG precipitation but small contaminants are reduced substantially.

Residual PEG can be eliminated by anion exchange chromatography.

Exosomes bind. PEG flows through. TFF with large pores also works.

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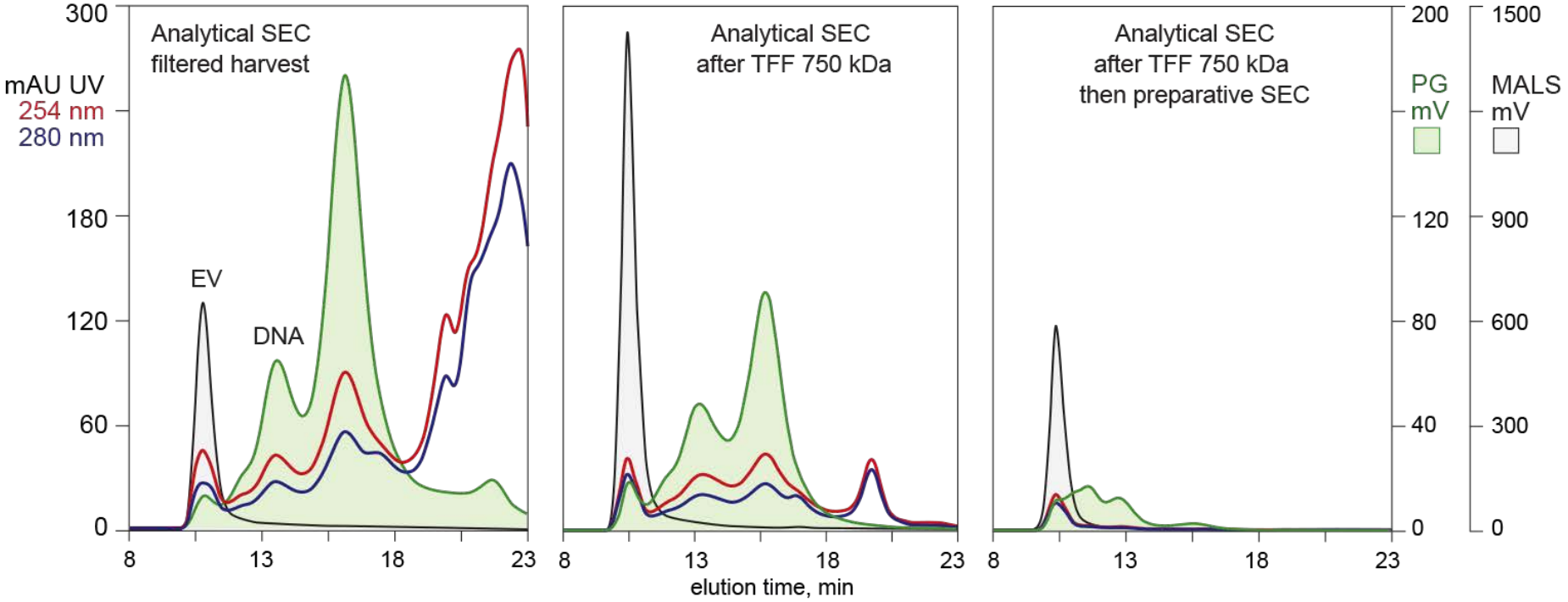
Impact of chromatin on exosome purification

TFF followed by preparative size exclusion chromatogaphy.

SEC-UV/MALS/picogreen. Stages of purification from HEK293 harvest.



A subset of smaller chromatin species is removed by TFF with wide-pore membranes but larger species are concentrated with EV. SEC substantially reduces the remainder but chromatin in the same size class as EV remains, along with more than expected smaller species. This is because of the chemical stickiness of chromatin (see ref. 5).

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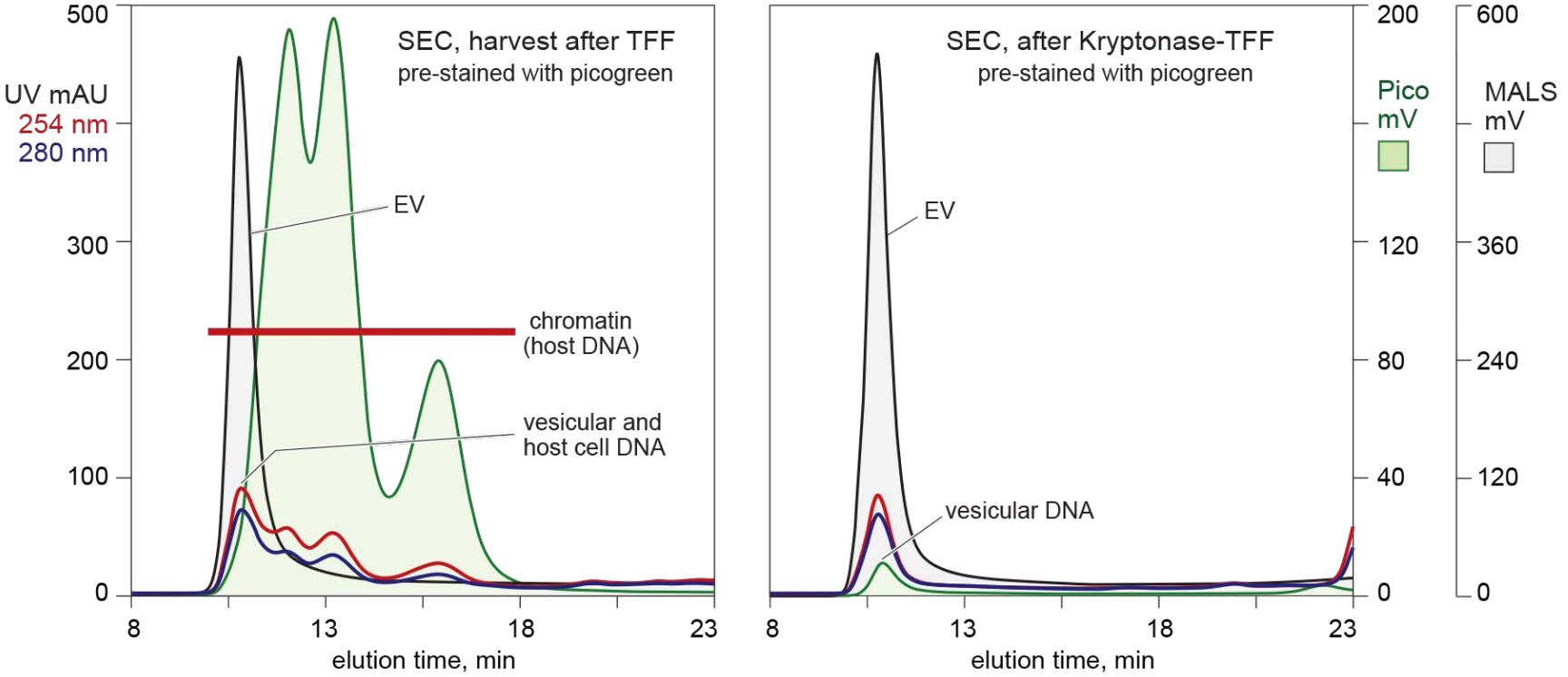
Chromatin reduction enables better purification

Kryptonase™ represents an integrated reagent/treatment method.

SEC profiles before and after Kryptonase treatment at room temperature.



Kryptonase digests the majority of nucleic acids in 1 hour. The integrated TFF step removes the liberated histone proteins and residual enzyme along with DNA fragments and nucleotides. Histone removal is important because their high electrostatic affinity for exosomes promotes complex formation.

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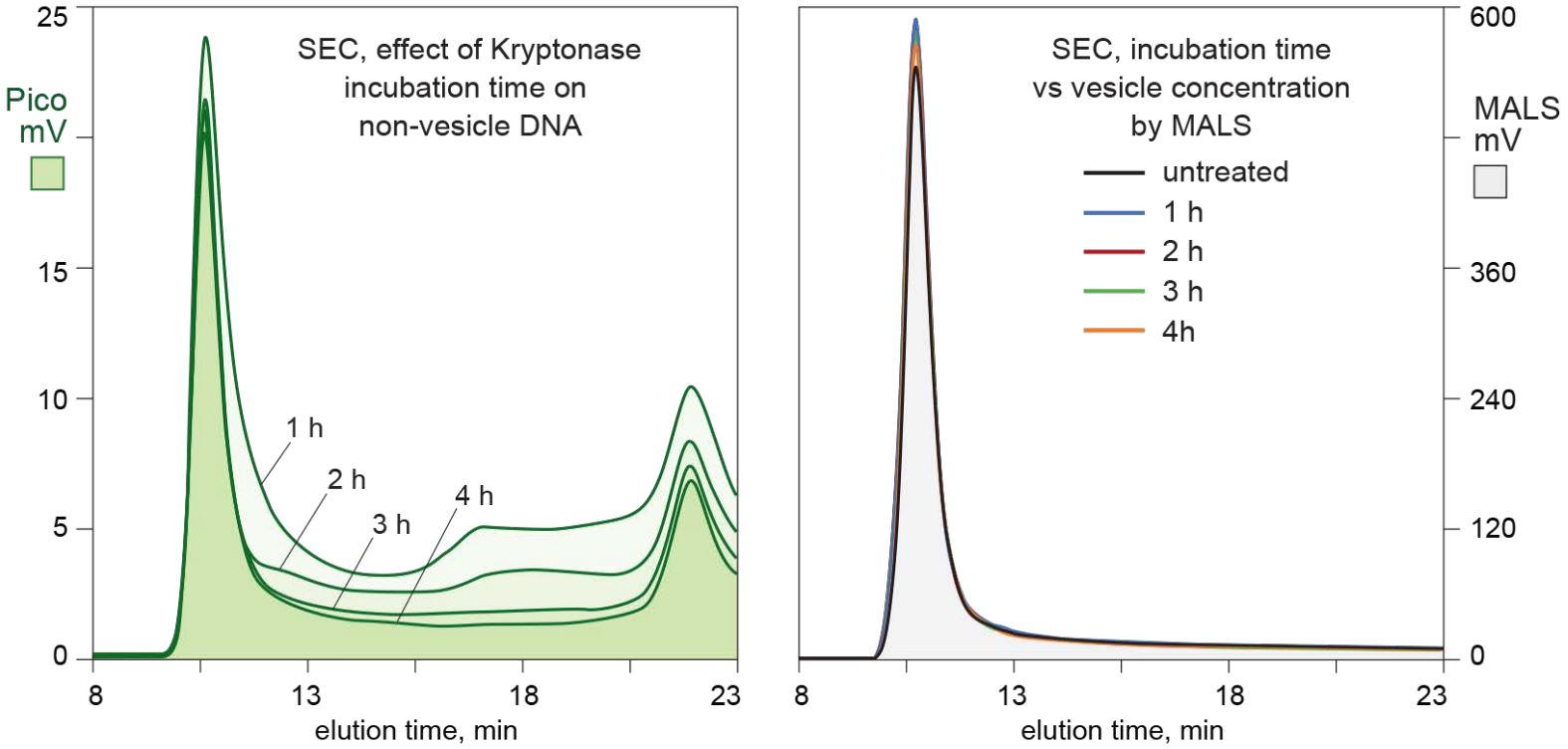
Chromatin reduction enables better purification

Conservation of vesicles during treatment with Kryptonase.

SEC profiles before and after treatment at room temperature.



Extended incubation with Kryptonase removes more DNA, especially in the region close to the vesicle fraction. A subset remains despite long incubation because it is protected by its strong association with histone proteins. MALS shows that vesicle recovery is conserved at all time intervals.

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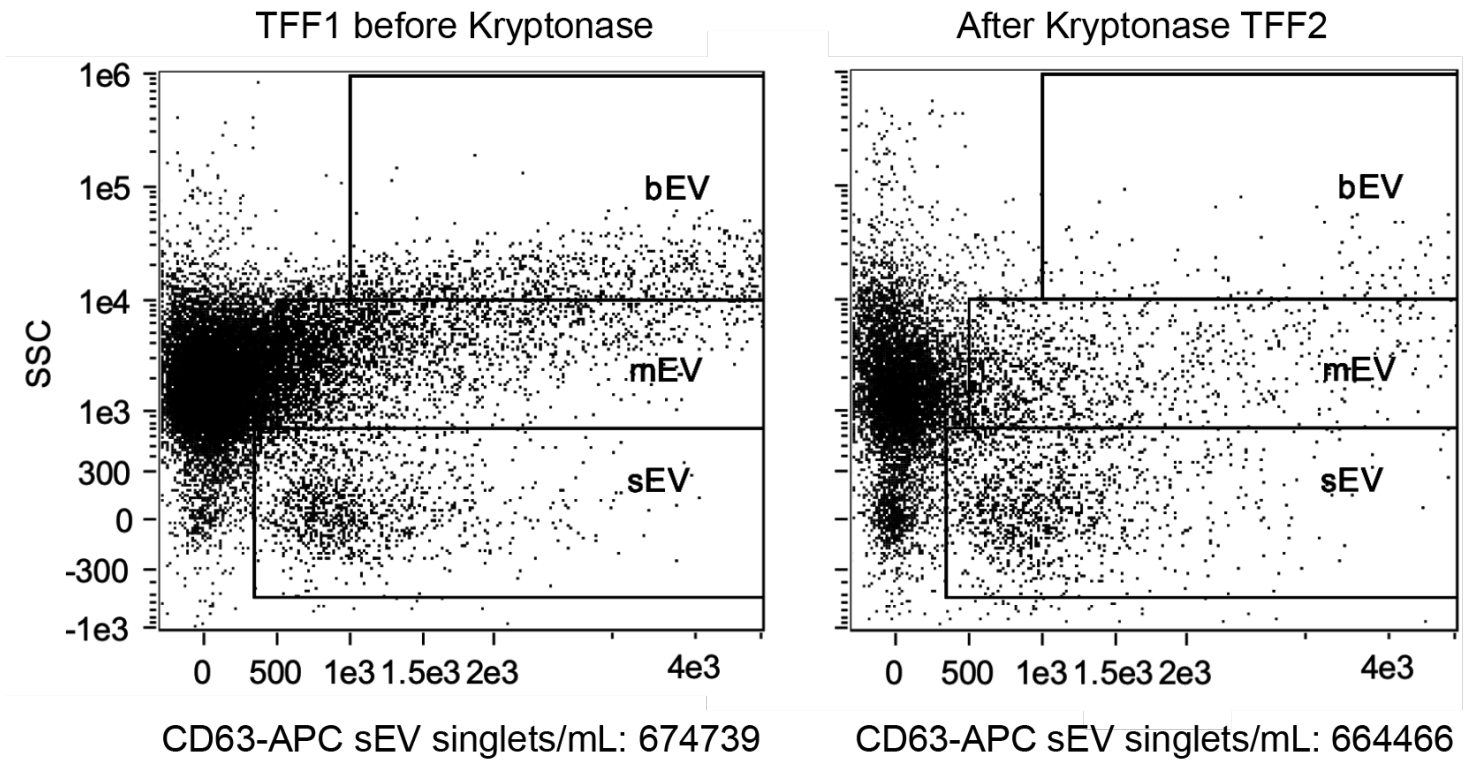
Chromatin reduction enables better purification

Flow cytometry (FCM) evaluation of vesicle size distribution.

Amnis ImageStream Mk II Imaging Flow Cytometer (Luminex).



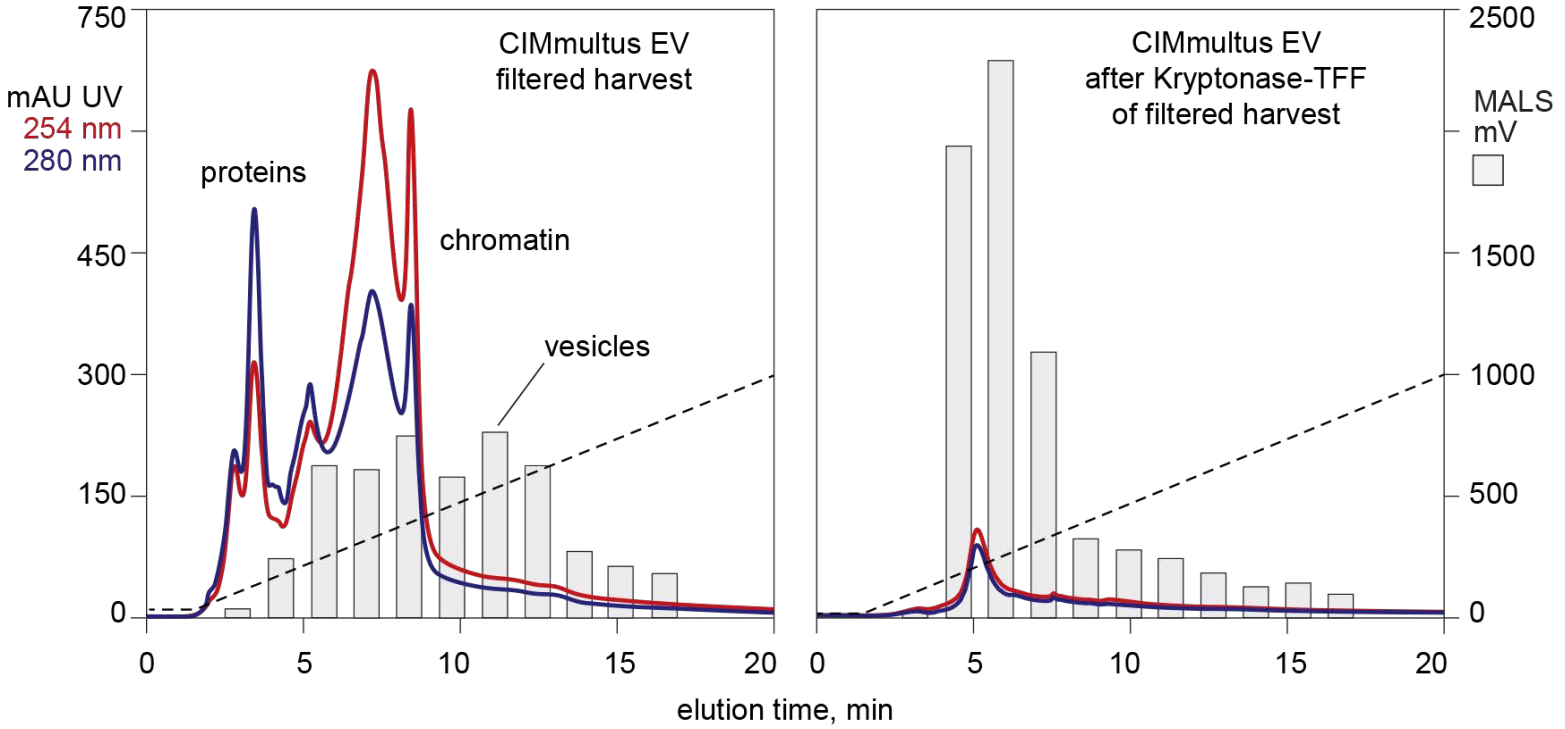
Kryptonase reduces non-specific noise. This may be an indication that debris from degraded EV form stable complexes with chromatin heteroaggregates.

Thanks to Simon Staubach and Bernd Giebel, University Hospital, Essen, GDR for FCM analysis.

Chromatin reduction enables better purification

The benefits of treatment by Kryptonase-TFF are also apparent with anion exchange chromatography.

CIMmultus™ EV. 1 mL monolith, 2 µm channels. NaCl gradient. pH 7.0.



Advance chromatin removal makes the full capacity and separation potential of anion exchange chromatography methods available for exosome capture and contaminant removal.

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Monolithic columns are important for exosomes

The internal architecture of monoliths was developed specifically to meet the unique requirements of purifying large, labile, lipid-enveloped biologics like exosomes.

For lipid enveloped viruses in the same size range, monoliths are documented to support 10–100 times higher binding capacity than porous particle chromatography media, and 2–10 times higher capacity than membrane adsorbers.

Product recovery is often 2–3 times higher than other media.

Flow rates are 10–50 times higher than traditional columns, with no compromise to capacity or separation performance.

Monoliths are also noteworthy because *internal flow through monoliths is laminar*. This eliminates the turbulent shear stress caused by eddy-vortex formation in other media formats.

Bandiera et al, Hum. Gene Ther. Met. 23 (2102) 255.
Fernandez et al, Gene Ther. 20 (2013) 353.

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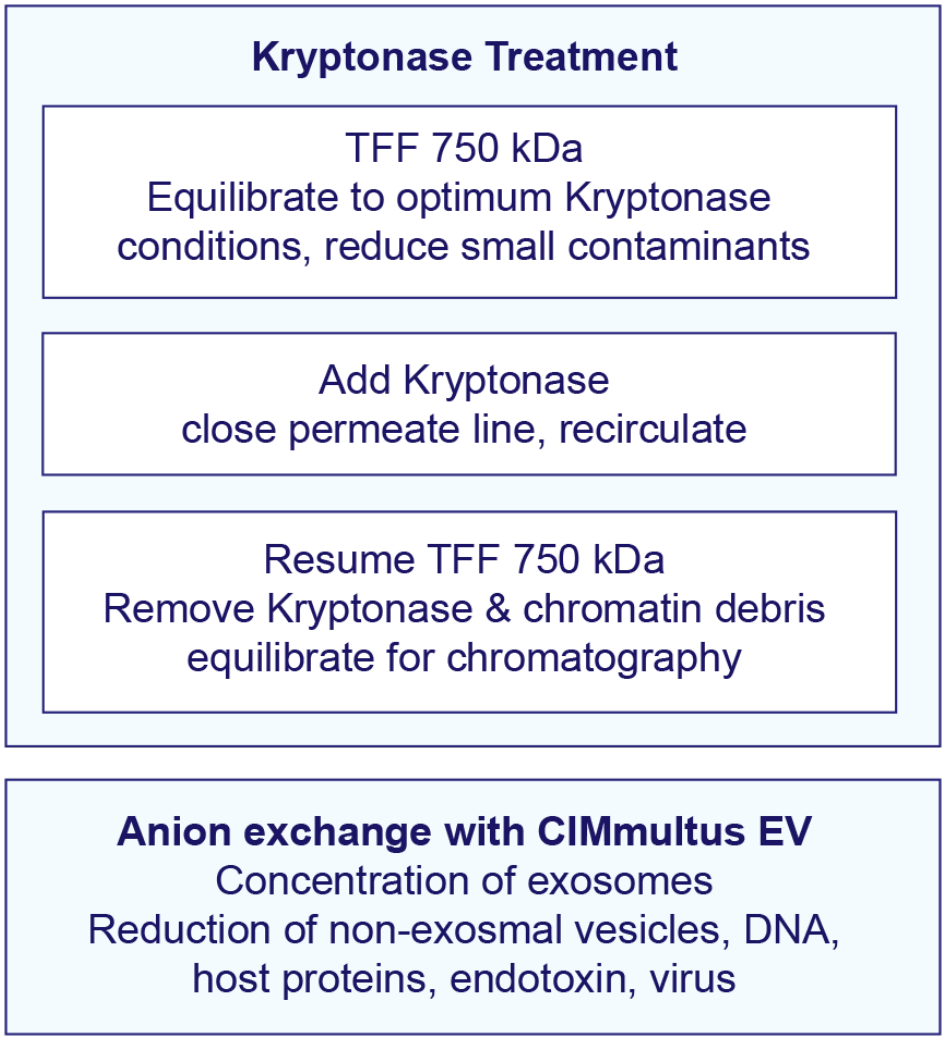
BIA's Cornerstone™ exosome purification platform



The entire Kryptonase process takes place within a single piece of equipment.

Concentration at the first TFF step reduces the amount of Kryptonase required to digest and dissociate chromatin.

Process duration for this step varies from about 2 hours up, depending on incubation time.

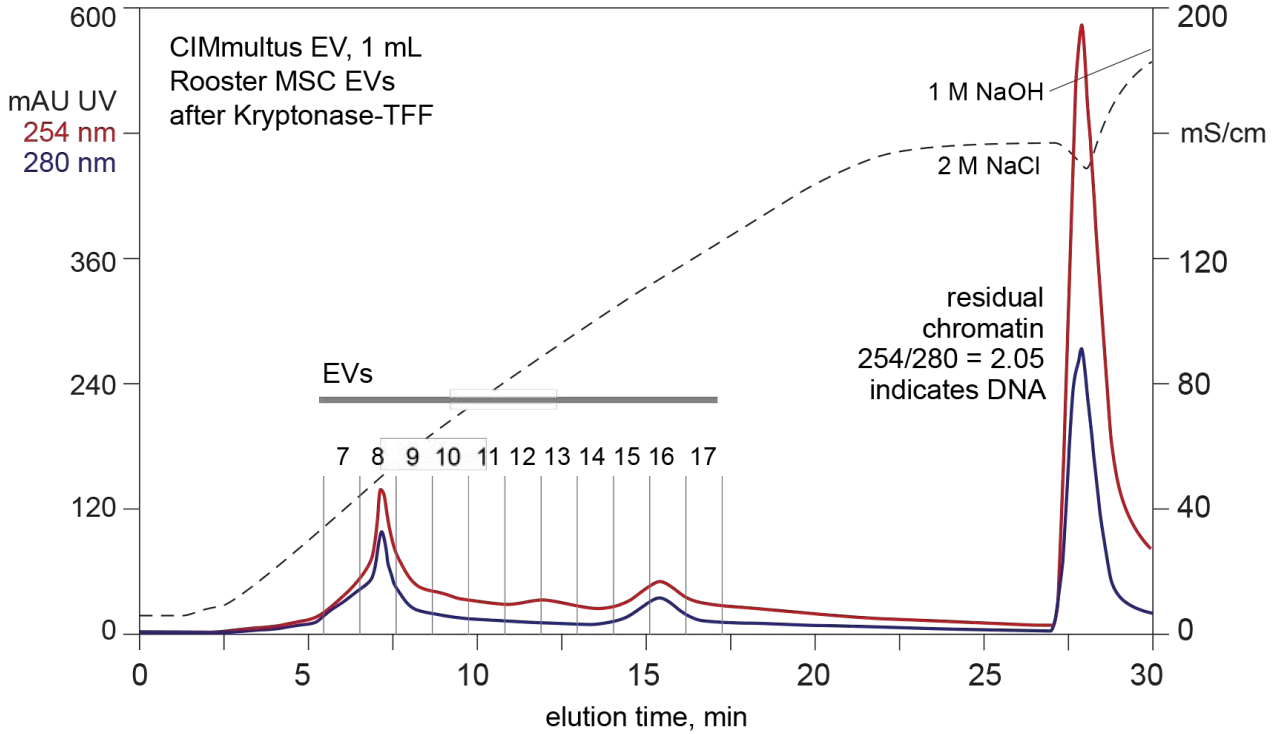
The monolith step can be performed in about an hour, depending on sample volume.

Overall process recovery is 50–90% depending on the starting material.

Anion exchange chromatography after Kryptonase-TFF

Anion exchange chromatography fractionates EV and removes most of the chromatin that remains after Kryptonase-TFF treatment.

CIMmultus™ EV, NaCl gradient, pH 7.0. Rooster Bio MSC EV.

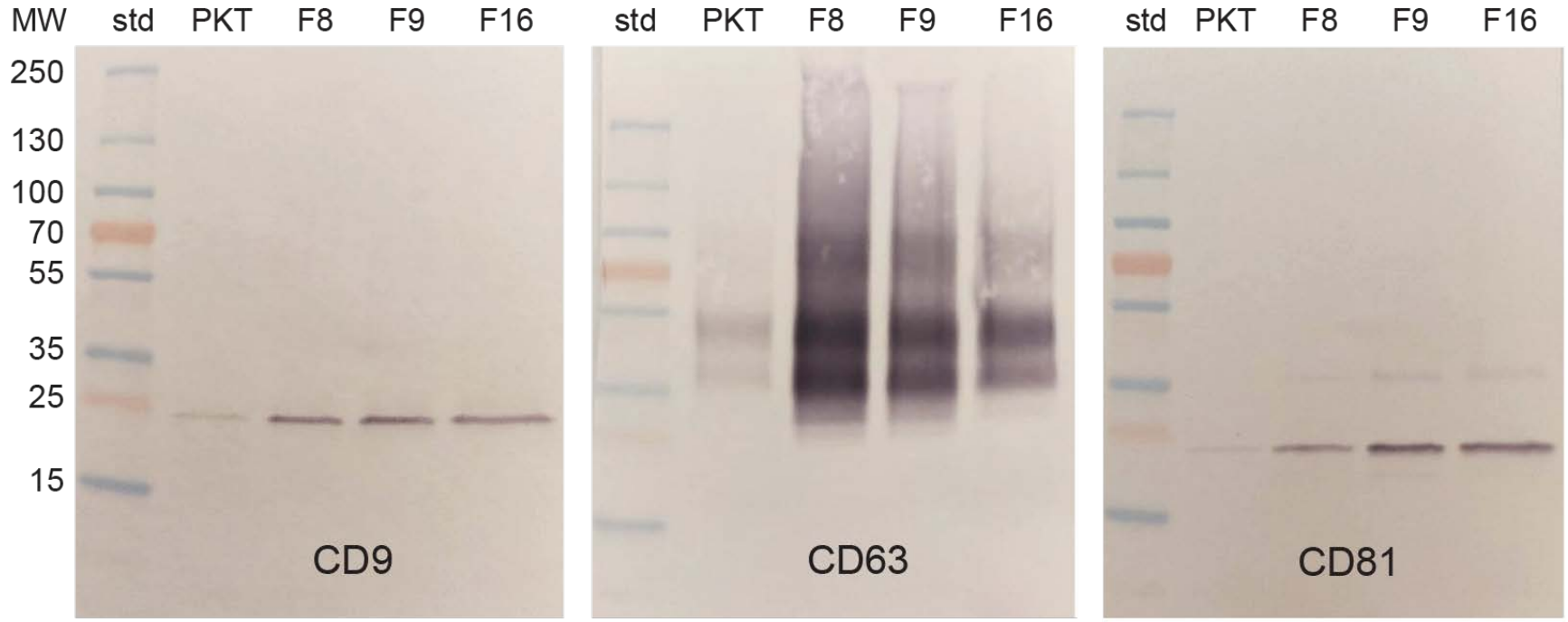


The UV wavelength absorption ratio of pure DNA at 254 and 280 nm is 2:1. This matches the NaOH clean-in-place peak after the sodium chloride gradient. Proteins are 280 dominant.

Anion exchange chromatography after Kryptonase-TFF

Western blot confirmation of vesicle markers in key fractions.

Fraction numbers correspond to the previous slide. Rooster Bio MSC EV.

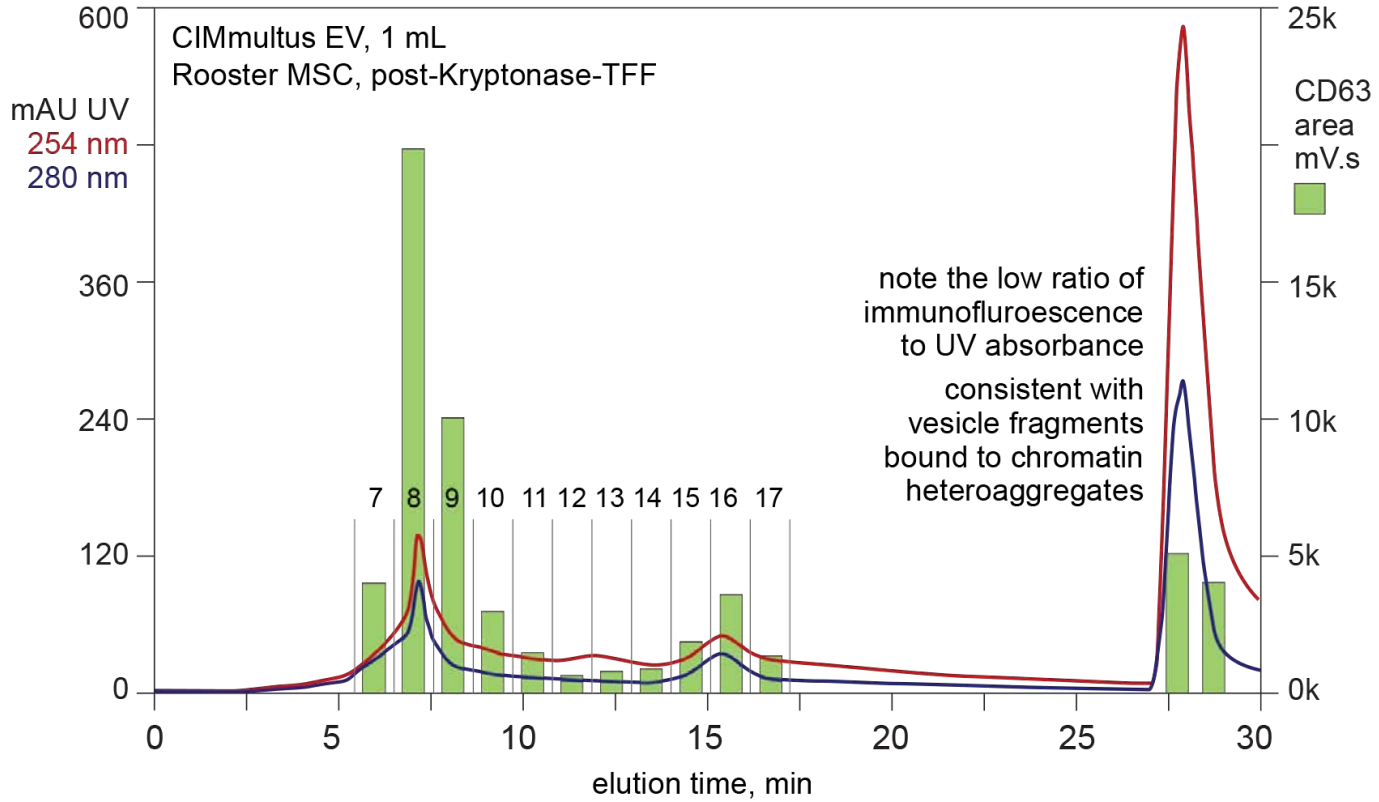


PKT: post-Kryptonase TFF. Note the concentration factor of the elution fractions compared to the KT-treated sample. The sample could be concentrated more at the TFF step but concentrating on the monolith is faster, more efficient, and exposes the exosomes to less shear stress.

Anion exchange chromatography after Kryptonase-TFF

SEC-IF provides a quantitative enhancement over Western blots.

CIMmultus EV, NaCl gradient, pH 7.0. CD63 values from SEC-IF.

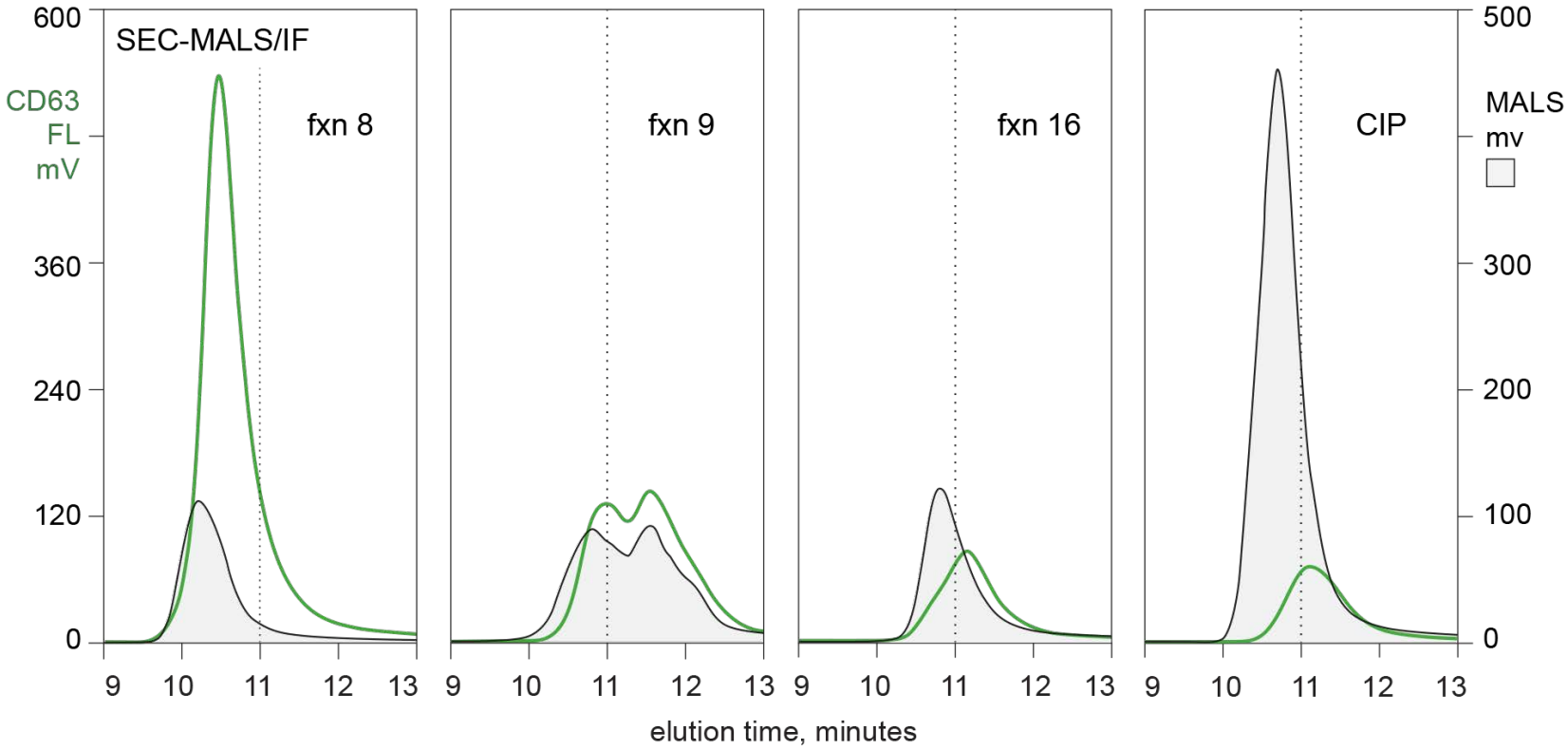


UV and IF show the same high and low fractions but their quantitative correlation is poor. This implies anion exchange separates different EV types. Note that SEC is unable to separate these populations.

Anion exchange chromatography after Kryptonase-TFF

SEC-MALS/IF suggests anion exchange separates distinct EV subsets.

MALS/CD63. Rooster MSC, fractions from CIMmultus EV.



MALS and IF show that fraction 8 represents predominantly large CD63+ EV while other fractions represent mixes of large and small species. MALS/IF ratios further suggest EV composition in fraction 8 is distinct from later fractions.

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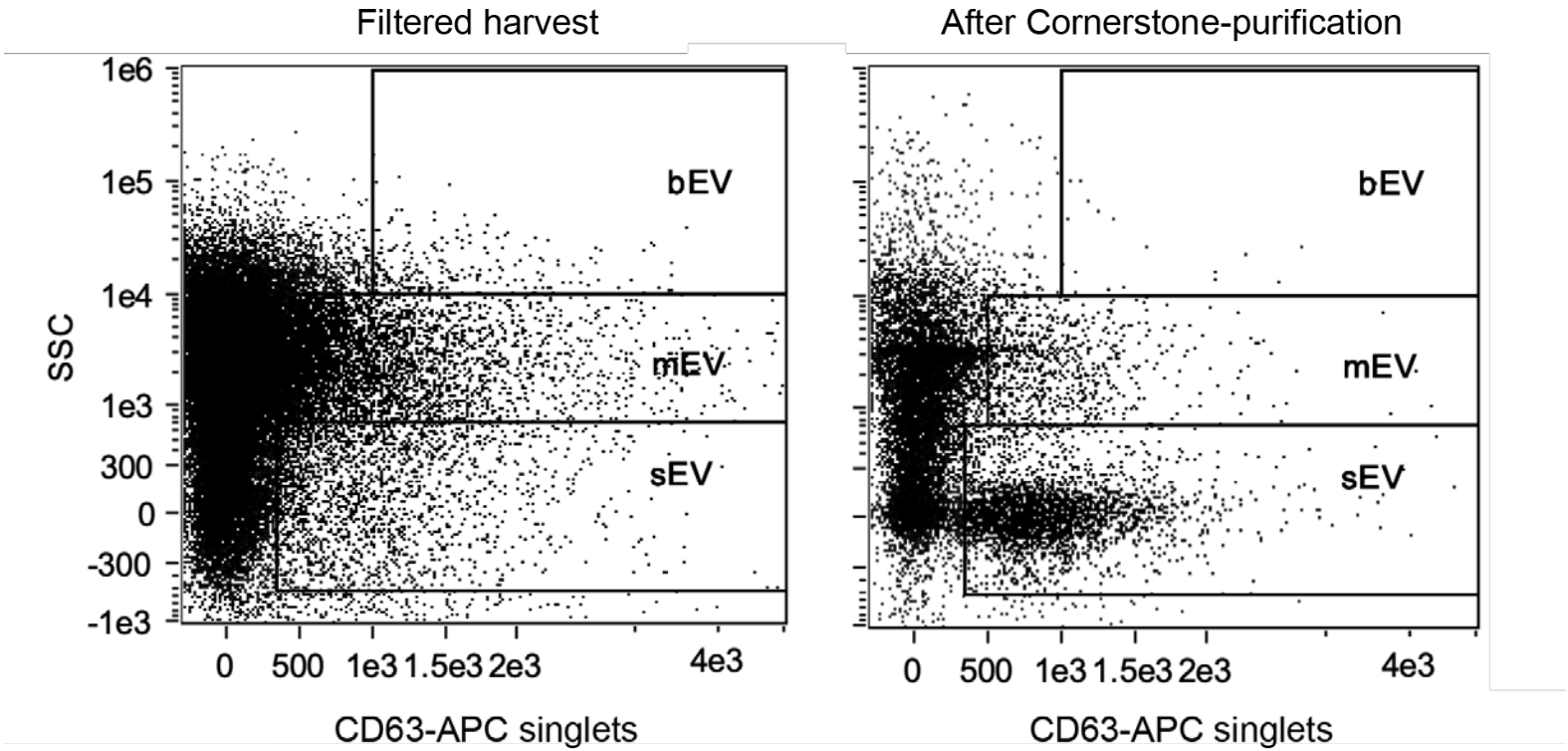
Analytical results from the Cornerstone platform

Flow cytometry, MSC harvest versus Cornerstone-purified.

Concentration of exosomes with reduction of non-exosomal vesicles.



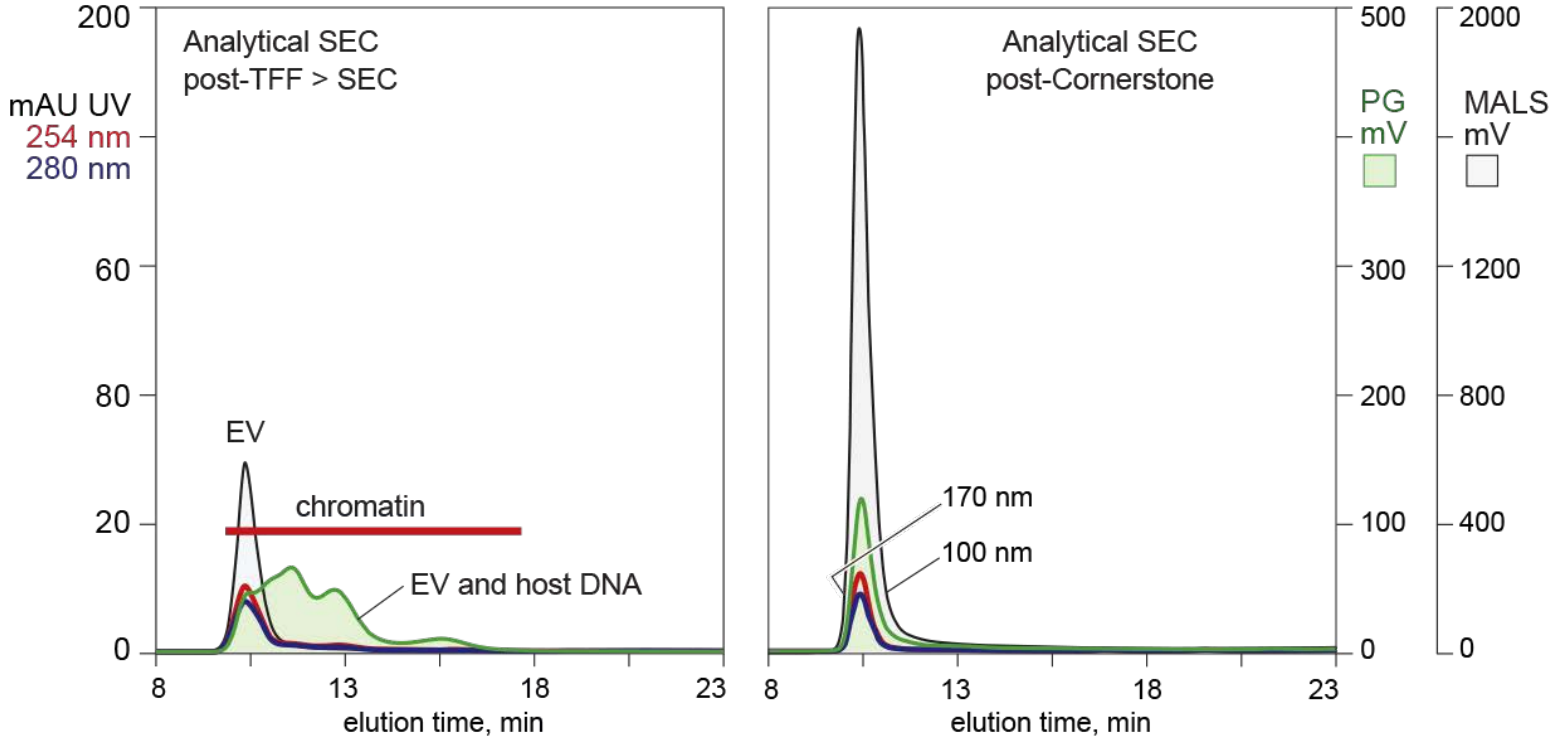
The Cornerstone platform selectively concentrates exosomes from dilute source materials. Similar purification results are achieved with serum-supplemented and serum/platelet lysate-supplemented cell cultures.

Thanks to Simon Staubach and Bernd Giebel, University Hospital, Essen, GDR for Amnis analysis.

Analytical results from the Cornerstone platform

Analytical SEC-MALS/Picogreen comparison of purification platforms.

MALS/CD63. Final product pools. Rooster Bio MSC.



The Cornerstone process concentrates exosomes and also removes small contaminants more effectively than SEC. Note the UV/MALS and PG/MALS ratios, both showing lower protein and DNA contamination of the EV fxn compared to purification by TFF > SEC. Vesicle size by MALS.

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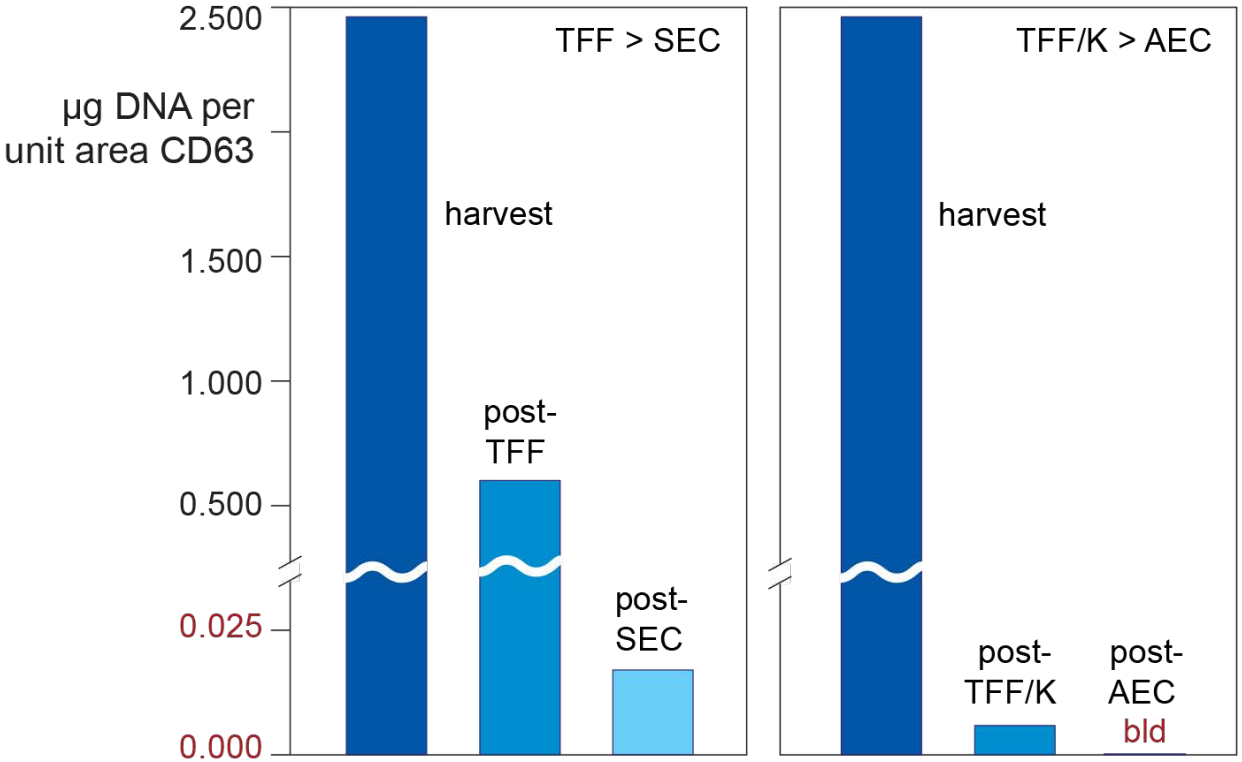
Analytical results from the Cornerstone platform

Comparison of DNA reduction by different purification platforms.

DNA values by picogreen standard plate assay (not SEC).

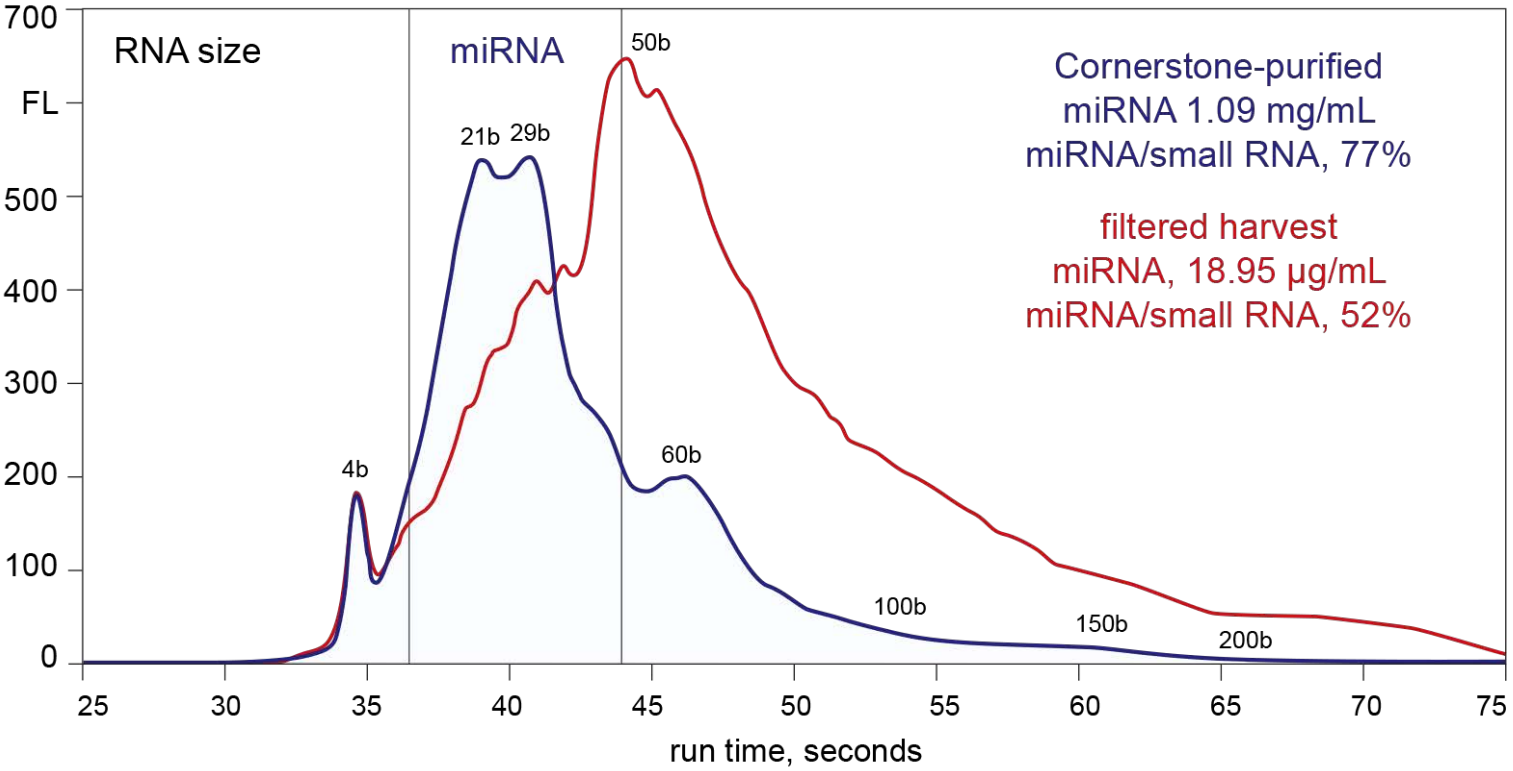


DNA after Kryptonase-TFF is already lower than after TFF > SEC. Anion exchange chromatography with CIMmultus EV is then able to reduce DNA below the limit of detection.

Analytical results from the Cornerstone platform

Size characterization of vesicular RNA.

Bioanalyzer profiles after extraction with miRNeasy™ Mini Kit.



The Cornerstone platform selectively concentrates vesicles that carry miRNA. These results also show that CIMmultus EV concentrates exosomes about 50-fold over harvest.

Are additional steps desirable or necessary?

Ultracentrifugation is an option at laboratory scale but imposes significant cost and reproducibility limitations at industrial scale.

Affinity, cation exchange, and hydrophobic interaction chromatography involve conditions that may adversely affect exosome stability. Some maybe manageable. Some maybe not.

Precipitation methods are difficult to scale up reproducibly.

Combinations with SEC may enhance removal of host cell contaminants and also buffer-exchange the sample.

- SEC might be substituted for the post-Kryptonase TFF step, then proceeding to anion exchange, or...
- Since anion exchange concentrates EV, SEC could follow anion exchange as a final polishing/formulation step.

Conclusions

The Cornerstone platform provides a good foundation for industrial purification of exosomes from all cell cultures.

Advance chromatin reduction by Kryptonase-TFF is an important contributor to its overall performance.

Anion exchange with CIMmultus EV selectively concentrates exosomes while separating them from other EV types.

Anion exchange is documented among other product classes to also reduce DNA, endotoxin, and virus contamination levels.

The process is fully and easily scalable with standard industrial reagents and equipment, from lab volumes as small as 50 mL to manfacturing volumes greater than 1000 L.

These features all contribute to the suitability of the Cornerstone platform for preparation of clinical quality exosomes.

Acknowledgements

We thank our collaborators for providing cell cultures, sharing analytical results, and valuable practical insights: Bernd Giebel and Simon Staubach from Unversity Hospital, Essen, GDR; Rooster Bio Inc, Frederick, MD, USA; and FiberCell Systems Inc, Frederick, MD, USA. From BIA Separations we thank Sebastijan Peljhan, Blaz Goricar, and Romina Zabar for guidance with integrating MALS; Nina Mencin for support with integrating fluorescence assays; Jasmina Puc and Spela Persic for miRNA extraction and analysis; Maja Leskovec and the applications development group at BIA Separations for many valuable insights and suggestions.

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