Extracellular Vesicle Purification by Anion Exchange Chromatography Using Nuvia HP-Q Resin

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ANION EXCHANGE

Abstract

Extracellular vesicles (EVs) are emerging as versatile tools for disease diagnosis, drug delivery, and therapeutic interventions, prompting the development of efficient purification methods. Anion exchange chromatography (AEX) presents a promising approach for EV isolation due to the negative charge characteristics of EVs. Here, we explored the feasibility of utilizing Nuvia HP-Q Resin, an anion exchange resin developed specifically for large biomolecule purifications at scale, for EV capture from cell culture supernatants. Employing an EconoFit Nuvia HP-Q Column, we achieved high-yield EV capture, as confirmed by nanoparticle tracking analysis (NTA). The purification quality was assessed via protein assays and multiplex fluorescent western blotting, while size exclusion chromatography (SEC) was employed post-AEX to further enhance EV purity for a reproducible research- to mid-scale workflow. The study also underscores the potential of AEX using Nuvia HP-Q Resin for scalable and high-quality EV purifications.

Introduction

EVs possess significant potential for medical applications, including the diagnosis of diseases through biomarker detection, and as drug and vaccine delivery systems for therapeutic purposes. A wide range of complementary methods are available for EV isolation. The method of choice depends mostly on the sample source and anticipated downstream applications. Regardless of method or workflow, the ability to adapt it to increasing sample size is always advantageous.

Cell culture supernatants are the primary source for EV-based therapeutics. These require a robust and scalable purification platform such as chromatography, which is routinely used to produce biological material for clinical applications at high yield. Ultrafiltration (UF) is a popular method for the initial concentration of EVs from cell culture supernatants. However, this method can be cumbersome for processing larger volumes, and sample loss on membranes can be a concern. Tangential flow filtration (TFF) is a scalable alternative to ultrafiltration, but it is not readily available to most researchers due to cost.

Another scalable chromatography technique is AEX, which is frequently used to increase EV purity after TFF. However, several publications have also shown the promise of AEX for the direct capture of EVs from cell culture supernatants. AEX can provide rapid capture and decreased contamination compared to TFF-concentrated EVs, and it offers scalability from research scale to production scale.

Nuvia HP-Q Resin is a high-performance strong AEX resin engineered for the purification of large biomolecules at fast flow rates. Its optimized pore size and rigid bead structure enhance pH stability and reduce backpressure, making it a promising candidate for the development of a scalable EV capture purification method. It is also suitable for small, research-scale purifications.

Here, we demonstrate the feasibility of using Nuvia HP-Q Resin to capture EVs from cell culture supernatants at high yield. Particle recovery was tracked with NTA and purification quality was further analyzed and monitored by protein assays and multiplex fluorescent western blotting. In addition, SEC was performed after the AEX capture step to increase EV purity.

Materials and Methods

All solutions, buffers, and media were filtered through 0.2 µm membranes and checked for particle content with NTA. It is highly recommended that all buffers used for chromatography are thoroughly degassed prior to use.

BIO-RAD

Cell Culture

The human breast cancer cell line MCF-7 (European Collection of Authenticated Cell Cultures, #86012803) was cultured in Dulbecco's Modified Eagle Medium (DMEM; PanBiotech GmbH, #P04-03590) supplemented with 10% fetal bovine serum (PanBiotech, #P30-3306). Cells were seeded at a density of 3 x 104/cm^2 in T-175 flasks (Thermo Fisher Scientific Inc., #178883) and maintained in a humidified incubator at 37°C and 10% CO_2. Cells were grown to 30–40% confluency and washed with sterile-filtered 1x Dulbecco's Phosphate Buffered Saline (DPBS) w/o Calcium w/o Magnesium (Biowest, #L0615). Then, 30 ml of Dulbecco's Modified Eagle Medium/High Glucose, without phenol red (DMEM; Cytiva, #SH30284.02) was added per flask and the cells were incubated for up to 120 hr in serum-free conditions. The morphology of the cells was monitored daily. Cell viability was checked at the time of harvest via Trypan Blue (Thermo Fisher Scientific, #T10282) staining and cells were counted in a Neubauer chamber. The supernatant was centrifuged at 200 x g for 5 min and 2,000 x g for 30 min at 4°C to remove all cells and debris. The resulting conditioned media (CM) was stored in aliquots at –80°C or processed immediately.

Sample Preparation

Nucleic acids in fresh or thawed conditioned media were partly digested with 10 U/ml of Benzonase (Novagen, #71206-3) at 4°C for 18 hr in the presence of 2 mM $MgCl_2$ and then vacuum filtered (VWR, Inc., #514-0341, 0.45 µm, PES) to remove larger membranous structures. Filtration was necessary to avoid high column backpressure when more than 150 ml of CM was processed. Volume-reduced CM reference samples used for NTA measurements or SDS-PAGE were obtained by ultrafiltration using Vivaspin 20 Centrifugal Concentrator Polyethersulfone 10 or 100 kD cutoff filters (Sartorius, #VS2002 or #VS2042).

Chromatography Equipment

All equipment for chromatography experiments was from Bio-Rad Laboratories, Inc. Chromatography was monitored at standard wavelengths of 260 and 280 nm and performed at room temperature using an NGC Quest 10 Plus Medium-Pressure Chromatography System (#7880003). The instrument was configured with a sample pump, sample inject valve, sample loops of different sizes, two buffer inlet valves, a column switching valve, and a multi-wavelength UV detector (10 mm path length) with an integrated conductivity meter. A typical fraction size was 1 ml, which was collected in standard 96-well plates with the small-volume BioFrac Fraction Collector equipped with a Microplate Drop Head Kit (#7410088) and Ice Bath/Microplate Rack (#7410017).

Chromatography Resins, Columns, and Samples

Spin-column experiments were performed with Nuvia HP-Q Resin (Bio-Rad, #12007022) to screen and evaluate static particle recovery at several buffer conditions to guide NGC-assisted chromatography using EconoFit Nuvia HP-Q Columns. The protocol was adapted from similar work to conduct the initial screening of chromatographic conditions for the purification of recombinant proteins (Bio-Rad bulletin 7128). A 1 ml EconoFit Nuvia HP-Q Column (Bio-Rad, #12009282) was used for AEX chromatography with a phosphate buffer system (Buffer A: 50 mM phosphate, pH 7.2; buffer B: buffer A with 2 M NaCl) and operated at 2 ml/min during sample application and 1 ml/min during the elution phase. Specifically, moderately nuclease-treated and filtered CM harvested after 120 hr from MCF-7 cells (~210 ml) was subjected to AEX with a 1 ml EconoFit Nuvia HP-Q Column. The CM feed with a total particle number of about 6.4E + 11 was loaded onto the column at a sample pump speed of 2 ml/min. The consecutive wash and elution steps were performed at 1 ml/min with 5% buffer B (100 mM salt). EVs were step eluted with 1 M NaCl over 15 column volumes (CVs) and step elution was continued with 1.25 M and 2 M NaCl over 10 and 7 CVs, respectively. After each run, the column was thoroughly cleaned with 0.5 M sodium hydroxide, washed with water, and re-equilibrated with buffer.

For size exclusion chromatography, the resin Bio-Gel™ A-1.5m, fine (Bio-Rad, #151-0450) was used to pack an empty glass Econo-Column™ (2.5 × 20 cm; Bio-Rad, #7372522) fitted with a Flow Adaptor (Bio-Rad, #7380017). Approximately 73 ml of resin was used for column packing at a speed of 1.6–1.7 ml/min. The column was operated at 1.3 ml/min with phosphate buffered saline (PBS) consisting of 10 mM phosphate, 150 mM NaCl, pH 7.2.

BCA Protein Assay

The protein concentration of samples was determined by using Micro Bicinchoninic Acid (BCA) Protein Assay kit (G-Biosciences, #786-571) following the manufacturer's protocol. BSA (Quick Start Bovine Serum Albumin Standard Set; Bio-Rad, #5000207) was used for standard curve generation.

Protein Electrophoresis and Western Blotting

All equipment and materials were from Bio-Rad Laboratories unless specified otherwise. The quality of chromatographic purification steps was monitored by SDS-PAGE with precast 4–20% Criterion TGX Stain-Free Protein Gels (12+2 well, 45 µl; #5678093). Precision Plus Protein All Blue Prestained Protein Standards (#1610373) and Precision Plus Protein Unstained Protein Standards (#1610363) were used for molecular weight calibration. 2x or 4x Laemmli Sample Buffer (#1610737; #1610747) supplemented with 2 or 4% Dithiothreitol (DTT, #1610610) was used for the preparation of samples for SDS-PAGE under reducing conditions. After electrophoresis, the SDS-PAGE gel was UV-activated for 45 sec and the resulting Stain-Free image was recorded on a ChemiDoc™ MP Imaging System (#12003154). The activated gel was transferred to an Immun-Blot™ Low Fluorescence PVDF Membrane (#1620262) in 7 min using the Trans-Blot™ Turbo Transfer System (#1704150) with Trans-Blot Turbo Midi 0.2 µm PVDF Transfer Packs (#1704157). The PVDF blotting membrane was blocked for 60 min at room temperature with gentle agitation using Intercept (TBS) Blocking Buffer (LI-COR, #927-60001). After blocking, the PVDF membrane was cut into two pieces between the 37.5 and 50 kD molecular weight markers. Each membrane piece was incubated with a pool of primary antibodies

Table 1. Product information for antibodies used to detect EV-specific targets by multiplex fluorescent western blotting.

Antibody Target, Human	MW, kD	UniProt Accession Number	Subcellular Location, UniProt	ISEV Category	Ab Host, Clonality	Vendor	Product Code	Secondary Antibody	Vendor, Product Code
Alpha-actinin-4	100	O43707	Cytoplasm, cytoskeleton, nucleus	4d	Rabbit, polyclonal	Bio-Rad	VPA00686	StarBright™ Blue 700, GAR	Bio-Rad, 12004162
Programmed cell death 6-interacting protein (ALIX)	96	Q8WUM4	Cytoplasm, cytoskeleton, extracellular (secreted)	2a	Mouse, monoclonal	Bio-Rad	VMA00273	DyLight 800, GAM	Bio-Rad, STAR117D800GA
Transitional endoplasmatic reticulum ATPase (VCP)	89	P55072	Cytoplasm, endoplasmic reticulum, nucleus	n/a	Mouse, monoclonal	Bio-Rad	VMA00307	DyLight 800, GAM	Bio-Rad, STAR117D800GA
Calnexin	67	P27824	Endoplasmic reticulum	4c	Goat, polyclonal	Bio-Rad	VPA00096	DyLight 800, DAG	Agrisera AB, AS122377
Actin, beta	41	P60709	Cytoplasm, cytoskeleton, nucleus	2b	HuCAL® hFAB, rhodamine-labeled	Bio-Rad	12004164	Not required	n/a
Annexin A5 (ANXA5)	32	P08758	Cytoplasm, plasma membrane, extracellular (secreted)	2a	Rabbit, polyclonal	Bio-Rad	AHP1924	IRDye 800, GAR	LI-COR, 926-32211
CD81 antigen	26	P60033	Cell membrane	1	Mouse, monoclonal	Bio-Rad	MCA1847	StarBright Blue 700, GAM	Bio-Rad, 12004159
CD9 antigen	24	P21926	Cell membrane	1	Mouse, monoclonal	SCBT	sc-13118	StarBright Blue 700, GAM	Bio-Rad, 12004159
Histone H3	17	P68431	Chromosome, nucleus	4a	Rabbit, polyclonal	Bio-Rad	VPA00826	IRDye 800, GAR	LI-COR, 926-32211

Ab, antibody; FAB, fragment antigen binding; DAG, donkey anti-goat; GAM, goat anti-mouse; GAR, goat anti-rabbit; HuCAL, Human Combinatorial Antibody Library; ISEV, International Society for Extracellular Vesicles; LI-COR, LI-COR, Inc.; MW, molecular weight; SCBT, Santa Cruz Biotechnology, Inc.; UniProt, Universal Protein Resource Database.

(diluted 1:1000) to detect a corresponding set of targets based on their molecular weights (Table 1). After incubation with primary antibodies for 18 hr at room temperature, the blotting membrane was washed 4 x 10 min with 1x Tris-Buffered Saline (TBS) (#1706435) containing Tween 20 (#1662404) at 0.1% (TBST) and further incubated for 1 hr at room temperature with the respective host-matched fluorescently labeled secondary antibodies, diluted 1:10,000 in blocking buffer. Before imaging with the ChemiDoc MP Imaging System, the blotting membranes were washed again 4 x 10 min with TBST. Gel and blot analysis were performed with Image Lab Software (version 6.1.0).

Nanoparticle Tracking Analysis (NTA)

The concentration and size distribution of particles in CM and chromatography samples were measured at 520 nm with a ZetaView PMX220 instrument (Particle Metrix GmbH, Germany). Polystyrene beads, 100 nm in diameter, were used for instrument calibration. Ideal measurement concentrations were found by pre-testing the ideal particle per frame value (100–200 particles/frame). Each sample was measured three times at 11 different positions, capturing 30 frames per position at 25°C under the following settings: camera sensitivity: 93; shutter: 100; frame rate: 30/s. After measurements, video recordings were evaluated using ZetaView Software (version 8.05.16 SP3) with parameters set at maximum area: 1,000; minimum area: 10; maximum brightness: 255; minimum brightness: 30; particle size distribution nm/class: 5. Only particles tracked in 15 consecutive video frames were included in the final analysis and statistics. For further analysis, data were converted into an FCS file and analyzed using FCS Express 7 Software (De Novo Software).

Results and Discussion

AEX has been successfully applied for the purification of EVs from cell culture supernatants in flow-through mode, and more frequently in bind-elute mode (Bonner et al. 2024, Malvicini et al. 2023, Heath et al. 2018). Here we report proof-of-concept results with Bio-Rad´s Nuvia HP-Q Resin operated in standard bind-elute mode to capture EVs as part of a multistep research-scale purification workflow by processing 210 ml of raw CM feed with a 1 ml EconoFit Nuvia HP-Q Column.

Cell Culture and Sample Preparation

Standard 2-D cell culture was used to produce CM in serum-free DMEM. It is worthwhile noting that other cell culture platforms, such as bioreactors, may produce different EV subtypes and challenge the purification of EVs with higher concentrations of protein and nucleic acid impurities. Recently, Bonner et al. (2024) reported a 1,000-fold increase in nucleic acid contamination for HEK 293T cell supernatants obtained from 3-D bioreactor cultures compared to 2-D cell culture.

Chromatin and nucleic acids are negatively charged and may occupy a significant portion of the binding sites of AEX resins. Therefore, in research-scale purifications, it is often recommended to pretreat CM with nucleases (1–2 hr at 37°C) in concentrations up to 150 units/ml CM. However, DNA association with EVs may also have biological significance (Liu et al. 2022). Here, we have chosen a moderate overnight nuclease treatment with 10 units/ml CM at 4°C, which was sufficient to lower nucleic acid content to concentrations not interfering with our workflow.

EV Capture by AEX Using Nuvia HP-Q Resin

Instrumentation

The NGC Chromatography System was used in standard configuration/tubing for EV capture by AEX and purification with SEC. According to Evtushenko et al. (2020), EVs tend to get absorbed on plastic surfaces, leading to significant particle loss, especially during storage at 4°C in standard plastic tubes. Recently, Koch et al. (2024) reported a loss of 20–30% of EVs in their chromatography system.

We tested column-independent particle loss of our setup by injecting 2 x 8 ml of a preconcentrated (5x with UF, 100 kD) CM sample with a column in bypass and collected 1 ml fractions in a standard deep-well plate. The comparison of the total particle numbers before and after the runs revealed a particle loss of about 15%. The causes — for example, instrument-specific losses versus losses related to fraction collection and pooling — were not investigated. Nevertheless, for maximum particle recovery, it is highly recommended to keep the flow-path of the tubing as short as possible and use low-bind plates and tubes for sample collection and storage. If purification experiments with small volumes of plain CM samples (<20 ml) are planned, refer to bulletin 3229 for NGC System and tubing reconfiguration. It is advisable to clean chromatography systems to remove endotoxin contaminants (bulletin 7260) that may mask or skew downstream assays or experiments with purified EVs.

EV Capture Using EconoFit Nuvia HP-Q Column

Small-scale experiments using spin columns demonstrated that Nuvia HP-Q Resin has promising binding and elution characteristics for EVs, with ~95% total particle recovery (data not shown). Slightly modified buffer conditions were readily applied to capture EVs from unconcentrated CM samples on the NGC Chromatography System using EconoFit Columns packed with Nuvia HP-Q Resin. Chromatograms were recorded at two standard wavelengths (260 and 280 nm) and a representative profile is displayed in Figure 1. Flowthrough (FT), wash, and eluate samples were pooled and the amount, median size X50 (nm), and size distribution of particles in all collected sample pools were measured by NTA. These datasets were used to calculate overall particle recovery, which typically was ~85%.

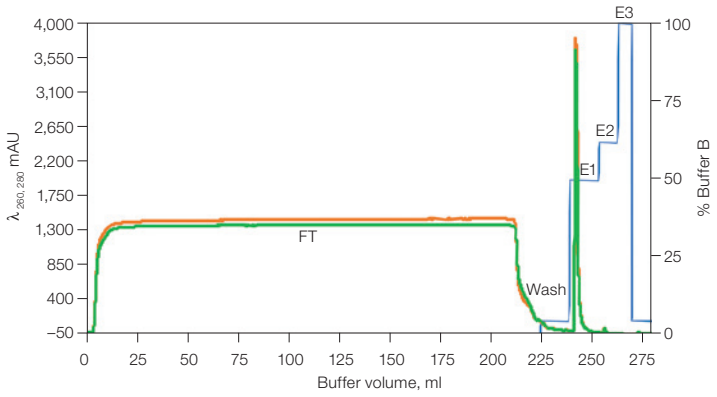


Fig. 1. AEX chromatogram of EV capture from 210 ml CM sample and stepwise elution on a 1 ml EconoFit Nuvia HP-Q Column. Flowthrough, wash, and AEX eluate samples E1–E3 were further characterized by NTA for particle size and concentration. (—), 280 nm; (—), 260 nm. Buffer A: 50 mM phosphate, pH 7.2; buffer B: 50 mM phosphate, 2 M NaCl, pH 7.2. AEX, anion exchange chromatography; CM, conditioned media; EV, extracellular vesicle; FT, flowthrough; NTA, nanoparticle tracking analysis.

The NTA data showed a moderate particle loss in FT and wash pools (total of ~4%), while ~70% of the total particle amount was recovered in eluate pool E1 obtained with 1 M NaCl (Figure 2). The median particle size varied between feed, wash, and eluate pools E1 and E2. The particles in the wash pool, which represent about 3% of the total recovered particle number, had a median diameter of 88 nm. The median particle size increased to 115 nm when measuring eluate pool E1. Particles found in eluate pool E2 displayed even larger particles having a median size of 133 nm. The increase of median particle size from wash to E2 can likely be explained by the increased surface area of larger particles and thus higher net negative charge, leading to a stronger binding to the resin. Recently, Koch et al. (2024) reported on the ability of AEX resins to separate EVs according to size. If those very small particles found in the wash solution are of particular interest, one can attempt to improve their capture by adjusting the feed buffer pH toward 8 or decreasing the wash buffer conductivity.

Protein assays were performed and the total protein content for the feed (210 ml), FT (220 ml), and eluate E1 (15 ml) were calculated to be 5.7, 2.5, and 2.7 mg, respectively. The data suggest that about 45% of total protein was removed during EV capture with Nuvia HP-Q Resin, leading to increased overall purity of the collected EV sample.

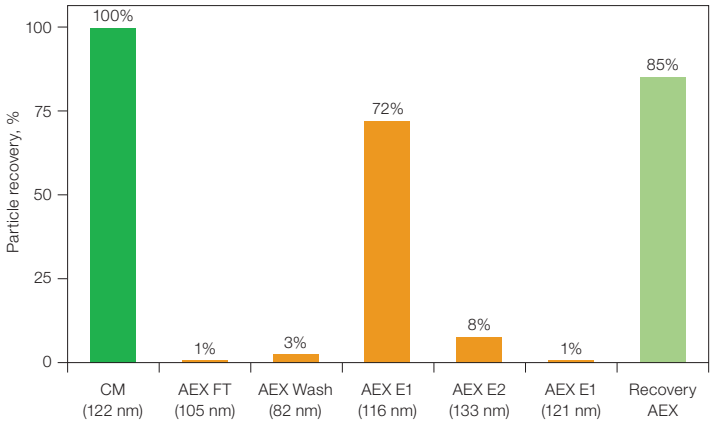


Fig. 2. Median particle size (nm) and particle recovery in individual chromatographic steps after AEX with Nuvia HP-Q Resin based on NTA measurements. The total particle amount in 210 ml CM was set to 100%. Total particle recovery was 85%. AEX, anion exchange chromatography; CM, conditioned media; FT, flowthrough; NTA, nanoparticle tracking analysis.

SEC Purification Following EV Capture Using Nuvia HP-Q Resin

When using Nuvia HP-Q Resin, SEC after AEX offers the additional benefit of removing the high salt levels that result from using 1 M NaCl for EV elution. Short-term storage (up to 48 hr) of EVs in high-conductivity solutions is usually not harmful to EV integrity. For optimal SEC column performance using Bio-Gel A Resin, it is recommended to apply sample volumes in the range of 1–5% of the total column bed volume. Here, 14 ml of the Nuvia HP-Q eluate pools was tenfold concentrated with ultrafiltration (100 cutoff), and 1 ml of sample was applied with a static loop to a glass Econo-Column (2.5 cm in diameter) packed with ~73 ml of Bio-Gel A Resin.

The Bio-Gel A column was pre-equilibrated with PBS, pH 7.2. The corresponding elution profile is presented in Figure 3A. Two sample pools (SEC P1 and P2) were generated and analyzed for total particle amount and median size (Figure 3B). Total particle recovery was ~83%, which is an excellent value for SEC. Zhang et al. (2020) reported particle losses of up to 50% during SEC with other resins or prepacked columns. As already outlined in Bio-Rad bulletin 3540, the use of the Bio-Gel A Resin results in the bulk-like elution of EVs in the void volume of the column. However, due to its exclusion limit of 1,500 kD, the removal of very high molecular weight protein contaminants is more challenging. The purity of EV preparations after enrichment by SEC can be demonstrated individually, for example, by western blotting (see section 3.4 of the bulletin), or in a more generic way by comparing the total number of particles to the total protein amount in samples. Here, the ratios of particles to µg of protein were ~1.1E + 8 for CM, ~1.7E + 8 for sample E1 after AEX, and ~1.6E + 9 in the final sample SEC P1, denoting a successful purification workflow and a significant increase in generic EV sample purity.

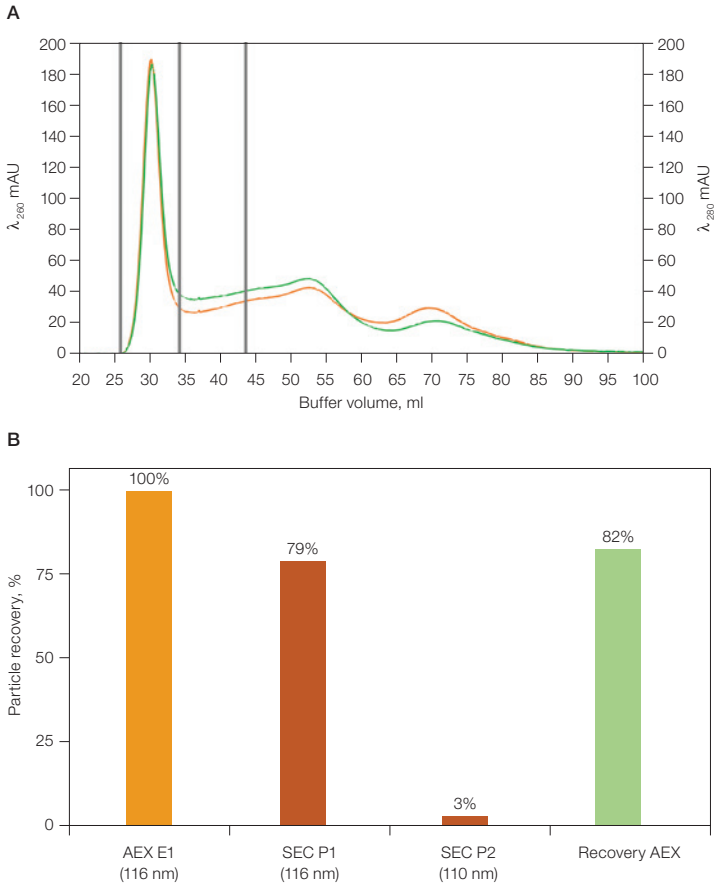
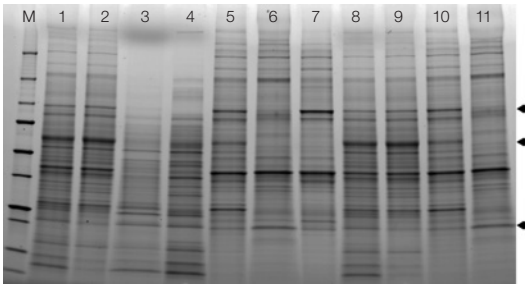


Fig. 3. EV purification with SEC of step-eluate sample AEX E1 obtained with a 1 ml EconoFit Nuvia HP-Q Column. Two sample pools, P1 and P2, were generated to measure median particle size (nm) and particle concentrations with NTA. **A**, EV purification chromatogram. (—), 280 nm; (—), 260 nm. **B**, NTA analysis results. The total particle amount in 1 ml AEX E1 sample was set to 100%. Total particle recovery was 82%. Chromatography buffer: 10 mM phosphate, 150 mM NaCl, pH 7.2. AEX, anion exchange chromatography; NTA, nanoparticle tracking analysis; SEC, size exclusion chromatography.

Sample Characterization by Western Blotting

Western blotting was used for protein content–based characterization of the chromatography samples by monitoring a few marker proteins to verify the presence and estimate relative enrichment of EVs in comparison to the feed, after both AEX and SEC. EV-positive protein targets include CD9, CD81, ALIX, and annexin 5, while primary antibodies raised against histones, calnexin, beta-actin, and actinin 4 were used as examples for the detection of common (generally accepted) contaminating proteins (see Table 1). The high molecular weight protein VCP was included in the analysis because Bosque et al. (2016) reported enrichment of VCP in small EVs obtained from tumoral cells, and VCP is listed in the ExoCarta database. On the other hand, Jeppesen et al. (2019) reported VCP as nonvesicular when studying a few cancer cell lines. According to the International Society for Extracellular Vesicles (ISEV) (Welsh et al. 2024), the classification of individual proteins being generic contaminants, and thus used as negative markers in EV purification, is sometimes difficult because they may be present in some subtypes of EVs or under certain conditions, with no general rule.

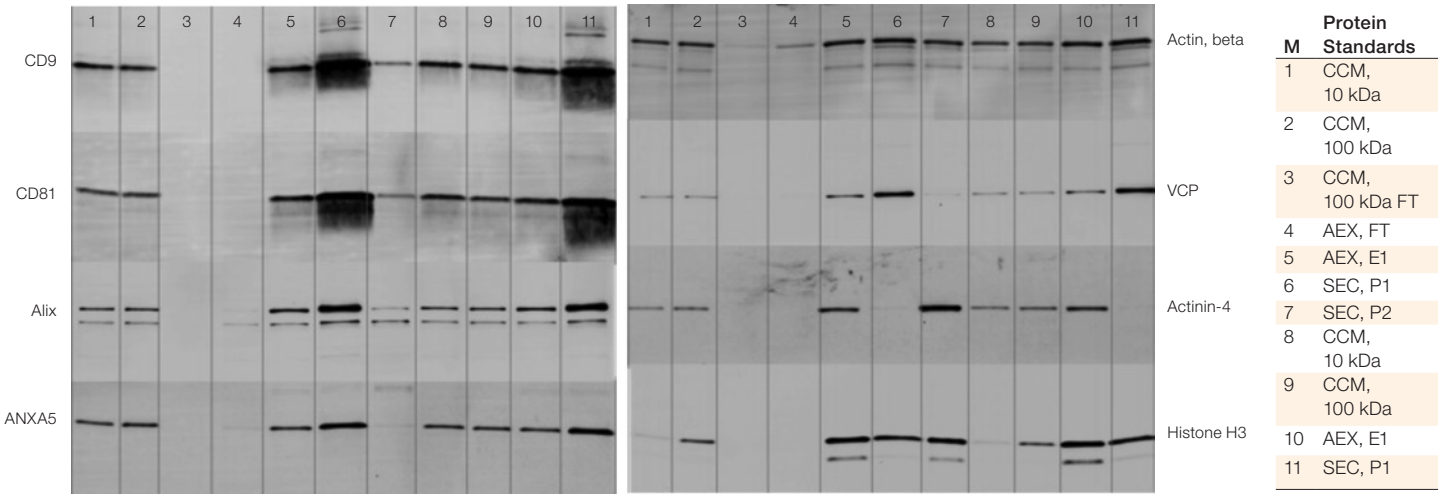
Two differently concentrated CM samples (UF with 10 and 100 kD cutoff) along with samples from both the capture step with Nuvia HP-Q Resin and further purification with SEC were analyzed by SDS-PAGE with Stain-Free technology. Each lane was loaded with approximately 6 ± 1 µg of total protein and the Stain-Free gels were of high quality (Figure 4). Some qualitative and quantitative differences were readily apparent between the two UF-concentrated feed samples and samples obtained after AEX and SEC (arrows in Figure 4). Lane 4 depicts the FT sample obtained after AEX and most protein bands show molecular weights from 10–150 kD.



M	Protein Standards
1	CCM, 10 kDa
2	CCM, 100 kDa
3	CCM, 100 kDa FT
4	AEX, FT
5	AEX, E1
6	SEC, P1
7	SEC, P2
8	CCM, 10 kDa
9	CCM, 100 kDa
10	AEX, E1
11	SEC, P1

Fig. 4. Analysis, using SDS-PAGE with Stain-Free technology, of fraction pools from total workflow combining AEX with Nuvia HP-Q and SEC with Bio Gel A. Criterion TGX Stain-Free Precast Gels, 4–20% gradient, with a total protein load of 6 (±1) µg/lane. CCM, 10 kD: CM concentrated with UF (10 kD cutoff); CCM, 100 kD: CM concentrated with UF (100 kD cutoff); CCM, 100 kD FT: flowthrough from CCM, 100 kD, concentrated with UF (10 kD cutoff). AEX, anion exchange chromatography; CM, conditioned media; CCM, concentrated conditioned media; SEC, size exclusion chromatography; UF, ultrafiltration.

The results of the western blotting experiments are presented in Figure 5, and most of the chosen primary antibodies yielded robust signals throughout. The fluorescent recordings were normalized against the total protein load of each lane with Image Lab Software and fold-changes relative to the CM sample (100 kD UF) were calculated and are presented in Figure 6. The Nuvia HP-Q eluate



M	Protein Standards
1	CCM, 10 kDa
2	CCM, 100 kDa
3	CCM, 100 kDa FT
4	AEX, FT
5	AEX, E1
6	SEC, P1
7	SEC, P2
8	CCM, 10 kDa
9	CCM, 100 kDa
10	AEX, E1
11	SEC, P1

Fig. 5. Multiplex fluorescent western blotting. Stain-Free SDS-PAGE gels were subjected to multiplex fluorescent western blotting and probed with various primary antibody combinations from Table 1. The fluorescent signals were recorded with the ChemiDoc MP Imaging System at different excitation wavelengths and filter combinations. AEX, anion exchange chromatography; CCM, concentrated conditioned media; SEC, size exclusion chromatography.

sample (lanes 5 and 10) was clearly positive for the typical EV marker proteins (CD9, CD81, ALIX, ANXA5), while only very weak signals for ALIX and ANXA5 were recorded for the respective FT sample (lane 4). This indicates a successful binding step of EVs with Nuvia HP-Q Resin. After further sample concentration with a 100 kD UF device following AEX and subsequent SEC, the EV-specific western blot signals in lanes 5 and 11 are increased by a factor of 5 or more. Here, calnexin, histones, beta-actin, and actinin-4 are discussed as possible copurifying protein contaminants. The widely used ER-marker protein calnexin was detectable in CM and AEX E1 samples but gave no signal after purification with SEC (blot data not shown). The 100 kD protein actinin-4 has a pI of ~5.3 and is therefore clearly bound by AEX. However, actinin-4 was effectively removed by SEC and not present in SEC P1 sample. The very basic histone proteins will likely not interact with an AEX resin at a pH of 7.2, unless attached to negatively charged nucleic acids. The AEX eluate sample (lane 5) shows an increase of the histone signal over the feed, indirectly indicating the presence of nucleic acids in the sample. The blot signal for the acidic protein VCP, which can assemble as a homohexamer (540 kD), is increased in both AEX E1 and SEC P1 eluate samples. Data interpretation of VCP presence in our samples is difficult due to the above-mentioned conflicting data in the literature. However, we previously also indicated that VCP may be separated away from EVs if the exclusion limit of the SEC resin is sufficiently high to enable entry and migration through the SEC resin while EVs bypass in the void volume (bulletin 3540).

The cytoskeleton protein beta-actin showed a moderate signal increase in all chromatographic samples (AEX FT, AEX E1, SEC P1, SEC P2) compared to CM, 100 kD. Very often, the detection of beta-actin in EV samples is explained through promiscuous incorporation processes. However, Jeppesen et al. (2019) did not find evidence that classical CD63-, CD81-, and CD9-positive exosomes possess cytoskeletal constituents that make up actin filaments, microtubules, or intermediate filaments.

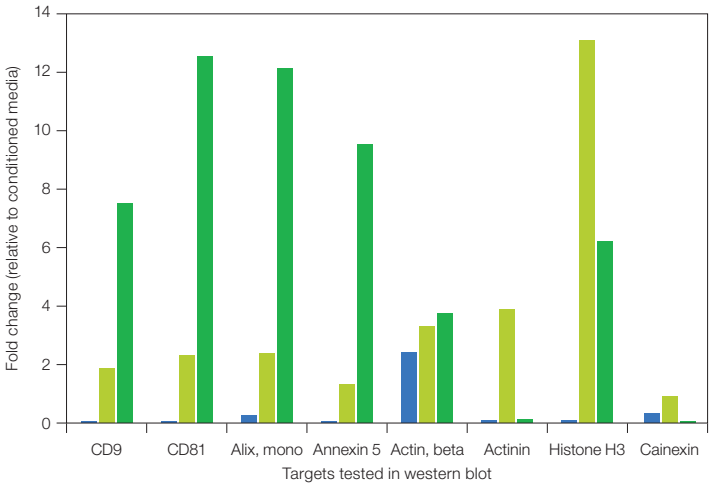


Fig. 6. Relative fold-change analysis of multiplex fluorescent western blotting signals. Stain-Free signals from Figure 5 were used as a loading control and fluorescent western blot signals of various targets were normalized against CCM (CM concentrated by UF with 100 kD cutoff). All relevant EV-specific targets (for example, CD9, CD81, ALIX) are increased after AEX and SEC and indicate successful EV enrichment for both columns. (■), Nuvia HP-Q flowthrough; (■), Nuvia HP-Q eluate; (■), after SEC. AEX, anion exchange chromatography; CCM, concentrated conditioned media; CM, conditioned media; SEC, size exclusion chromatography; UF, ultrafiltration.

Conclusion

The development of scalable purification platforms based on chromatographic techniques is widely considered a useful strategy for the isolation and purification of EVs from cell culture supernatants. AEX chromatography is a well-established method and is thought to be useful in gently capturing EVs at high yields. Although commonly applied as a polishing step following, for instance, UF or TFF, AEX can also be used as a capture step. Here, we have shown that Nuvia HP-Q Resin efficiently binds EVs from CM, and bound EVs can be efficiently eluted using NaCl. The calculated total particle recovery rate after AEX was

around 85% and the main eluate sample contained around 70% of the total processed particle number. During the capture step, approximately 45% of the total protein of the CM feed obtained from 2-D cell culture was removed. Nuvia HP-Q Resin can be used to capture and concentrate EVs as a preliminary step in the purification workflow and can also be used as a polishing resin. Overall, the data show that the strong AEX resin Nuvia HP-Q is an excellent tool for the development of EV purification workflows. It is worthwhile noting that resin screening is a critical step in method development. Nuvia HP-Q Resin demonstrated strong performance for the target EVs in this study. However, other AEX resins may be better suited for your specific EV target. If you need assistance, reach out to our experts. Contact us at bio-rad.com/ChromContact to get started.

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