Chromatography

Extracellular Vesicle Isolation by Size Exclusion Chromatography

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Overview

Extracellular vesicles (EVs) released from various cell origins are a heterogeneous assembly of diverse membrane-enclosed nanosized particles. EVs harbor specific molecule cargo, play a dynamic role in intercellular communication, and are the subject of growing interest for diagnostics and therapeutic applications. Due to their heterogeneous nature, the separation and isolation of EVs is challenging, and no single technique can achieve comparable purity levels across the many different sample matrices. There is a wealth of different technologies for the isolation of EVs. However, a general gap in the EV field is the need for more standardization and automation. Size exclusion chromatography (SEC) has gained much recognition as a valuable method in EV isolation schemes because it has the potential to be gentle, reproducible, and capable of isolating EVs with suitable and desirable purity and integrity for applications of interest. Commonly, EV isolations by SEC are conducted using a gravity flow column requiring significant experimenter involvement, contradicting efforts toward standardization and reproducibility desired by the EV research community. Here, we present the use of SEC columns packed with Bio-Gel™ A-1.5m Gel, a chromatography resin, in conjunction with an NGC Chromatography System, which delivers significantly more control and enables more detailed information to be obtained (Figure 1). Data from nanoparticle tracking analysis (NTA), electrophoresis using Bio-Rad's Stain-Free technology, and multiplex fluorescent western blotting show that Bio-Gel A-1.5m Resin can effectively enrich EVs at a high yield. Following the presentation of data, we discuss technical considerations for avoiding major pitfalls when using SEC as an EV isolation method, including cell culture, sample preparation, column packing, chromatography, and EV characterization.

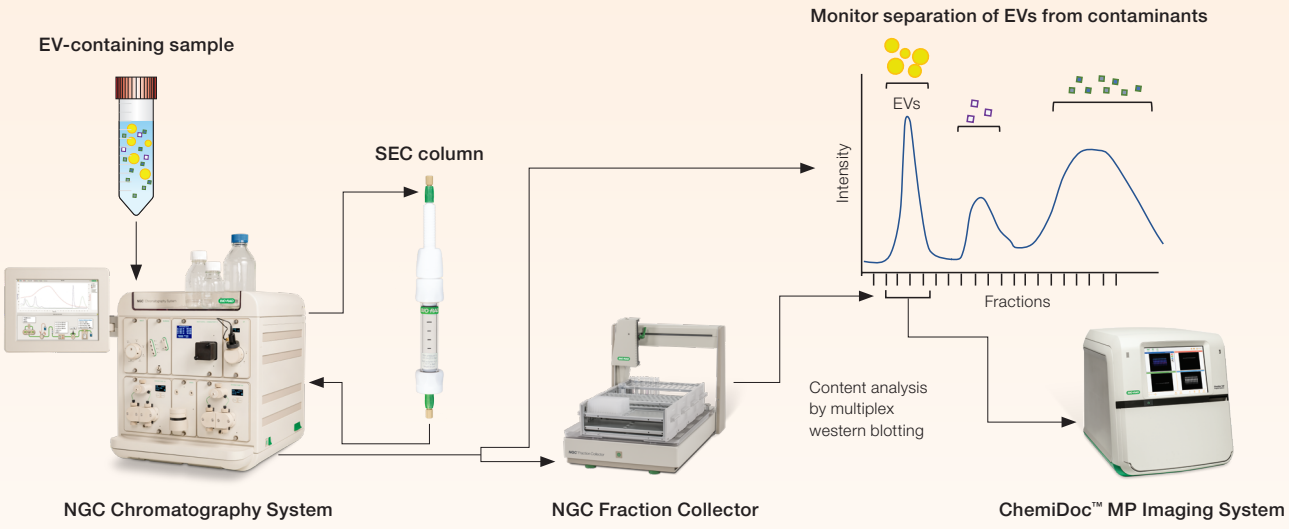


Fig. 1. SEC offers a fast and gentle method for EV isolation. SEC can be performed manually or using an NGC Chromatography System, enabling a controlled process and real-time monitoring of EV separations at different wavelengths, including those to monitor labeled EVs. Small volumes collected into 96-well plates with the NGC Fraction Collector or the BioFrac Fraction Collector can facilitate the collection of purer EV-containing fractions ready for downstream analysis. Automation of EV separations, standard sample runs, column QC, and column cleaning and maintenance can all be achieved with an NGC Chromatography System. The ChemiDoc MP Imaging System and a range of antibodies from Bio-Rad efficiently check for EV markers in collected fractions. EV, extracellular vesicle; QC, quality control; SEC, size exclusion chromatography.

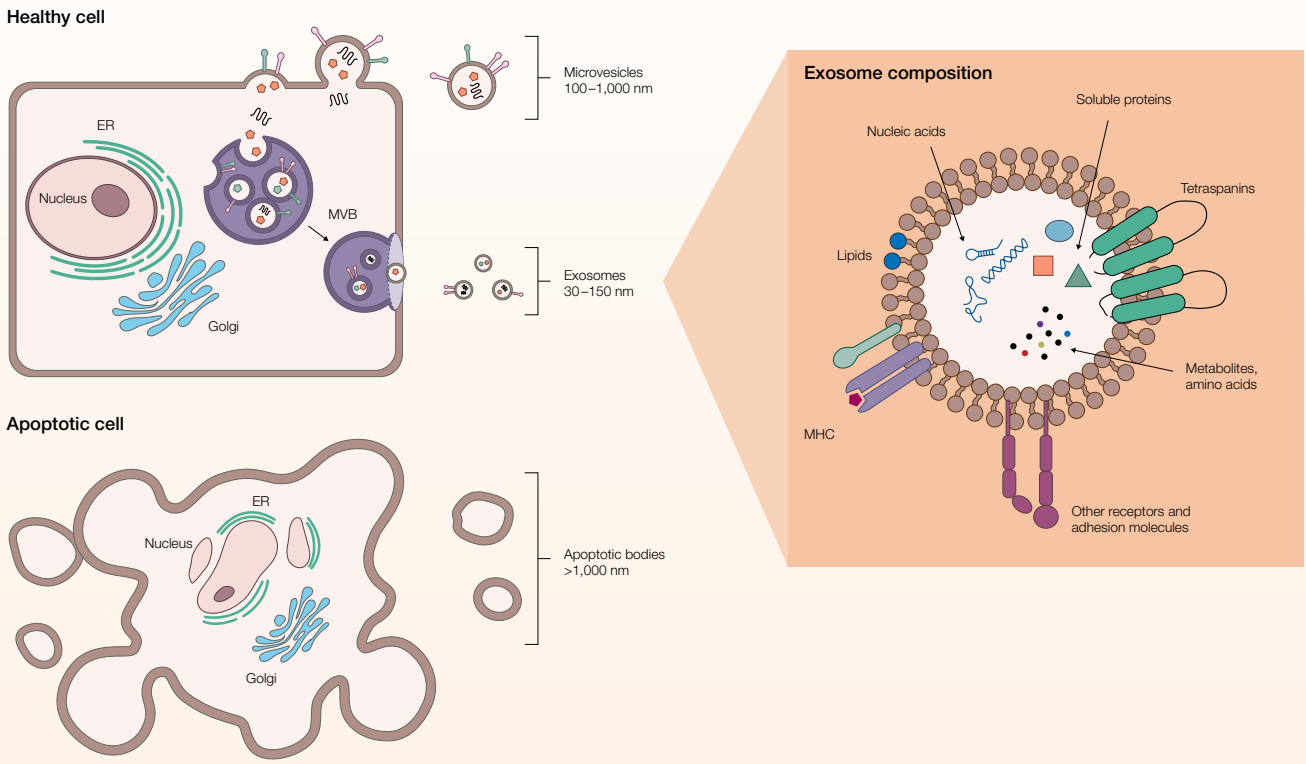


Fig. 2. Subcellular origins of the major classes of extracellular vesicles. Exosomes are released via exocytosis and have a size range of 30–150 nm. Microvesicles are released through budding from the plasma membrane and range in size from 100 to 1,000 nm. Apoptotic bodies are released through blebbing from cells undergoing apoptosis and are generally >1,000 nm in size. Exosomes are composed of a complex set of molecules, some of which are shown here. ER, endoplasmatic reticulum; MHC, major histocompatibility complex; MVB, multivesicular body.

Introduction

EVs are a very heterogeneous assembly of diverse membrane-enclosed nanosized particles of approximately 30–5,000 nm. The three major types of EVs, characterized by their different subcellular origins (Figure 2), are called apoptotic bodies, microvesicles, and exosomes. Apoptotic bodies (>1,000 nm) are released by apoptotic cells, microvesicles (100–1,000 nm) are shed from the plasma membrane, and exosomes (30–150 nm) stem from the endosomal pathway (Martínez-Greene et al. 2021). Despite their differences in biogenesis, microvesicles and exosomes have overlapping biophysical properties, such as density, size, and transmembrane protein markers including CD9, CD63, and CD81. These overlaps render their analytical differentiation very difficult. Current EV research focuses on microvesicles and exosomes to elucidate their potential as novel biomarkers due to their important roles in intercellular communication. Many reports indicate that exosomes can influence various physiological and pathological signaling pathways by delivering information to neighboring and remote cells through their cargo. Exosome cargo partly mirrors the transcriptome/proteome of their parental cells and consists mainly of proteins, nucleic acids, and lipids. In other words, exosomes are endogenous nanocarriers. Thus, the therapeutic use of EVs as drug delivery vehicles has been investigated in a growing number of publications (Johnsen et al. 2014).

Due to their heterogeneous nature, the isolation and purification of EVs is very challenging, and no single technique can achieve comparable purity levels across the many different sample matrices. The absolute purification of EVs is an unrealistic goal (Théry et al. 2018), and the terms isolation and purification are frequently used in the literature even in contexts where EVs are merely enriched or concentrated. However, EV purity can be increased by combining various purification methods (Liangsupree et al. 2021). These methods can be roughly divided into five categories—density, solubility, size, charge, and affinity-based techniques. Many of these methods are difficult to standardize because they are not instrument controlled, lack automation, and may require significant manual user intervention, all contributing to diminished process control. In addition, popular methods such as density ultracentrifugation are time consuming, result in low yields, and have limitations for scaling up.

SEC has gained much recognition as a valuable method in EV isolation schemes because it has the potential to overcome many of the shortcomings

mentioned above. SEC is a gentle, reasonably scalable method, and resins with diverse pore characteristics are available to address different purification challenges. Moreover, a high degree of automation and reproducibility can be obtained when a chromatography system operates SEC columns.

Agarose-based resins and derivates are usually the primary choice as stationary phase material for SEC in EV enrichment; however, corresponding prepacked columns of the appropriate size are not always available, especially when considering scale-up. Carefully chosen column dimensions are important to the success of a particular purification problem, and self-packing of SEC columns may be required. The performance requirements for SEC columns in EV research are significantly lower than those applied in protein purification. SEC of EV samples is performed under gentle pressure conditions, and packing low-pressure columns is simple, even for inexperienced users. Self-packed low-pressure columns are economical compared to prepacked single-use gravity flow or spin columns. Provided that a self-packed column is carefully maintained, it can be expected to give reproducible, high-resolution results over many run cycles.

Here, we present an EV isolation and characterization workflow (Figure 1) using Bio-Gel A-1.5m Resin to enrich EVs obtained from serum-free conditioned cell culture media using an NGC Chromatography System. Bio-Gel A-1.5m Resin with a fractionation range for globular proteins of 10,000–1,500,000 Da has technical characteristics such as bead size, pH stability, and flow characteristics that make the resin a promising candidate for EV isolation. The quality of the corresponding SEC separations using self-packed columns was assessed by nanoparticle tracking analysis (NTA), electrophoresis using Stain-Free technology, and multiplex fluorescent western blotting. Technical considerations for avoiding major pitfalls during the isolation and characterization of EVs when using SEC columns are also presented.

Application Data

Materials and Methods

All buffers and media for EV experiments were filtered through 0.2 µm or 0.45 µm membranes. In addition, all chromatography solutions were degassed before use.

Cell Culture and Sample Preparation

The human breast cancer cell line MCF-7 was cultured in DMEM, w: 4.5 g/L Glucose, w: L-Glutamine, w: Sodium pyruvate (PAN-Biotech GmbH, catalog #P04-03590) supplemented with 10% FBS (fetal bovine serum, PAN-Biotech, #P30-3306). Usually, 3 x 10^4 cells were seeded/cm^2 in T-175 Nunc Cell Culture Treated Flasks with Filter Caps (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 phosphate buffered saline (PBS) solution. Then, 30 ml of 1x RPMI 1640, w: stable Glutamine, w: 2.0 g/L $NaHCO_3$ (PAN-Biotech, #P04-18500) 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 by staining cells with Trypan Blue Stain (Thermo Fisher Scientific, #T10282), and cells were counted in a Neubauer chamber. The percentage of living cells was always at least 97%. The conditioned cell culture media was collected and centrifuged at 2,000 x g for 30 min at 4°C to remove cells and debris. The resulting conditioned media was stored at –80°C or processed immediately. Fresh or thawed conditioned media was centrifuged at 10,000 x g for 30 min to remove larger membranous structures such as apoptotic bodies. Usually, conditioned media was concentrated 100-fold by ultrafiltration (UF) using a Vivaspin 20 Centrifugal Concentrator Polyethersulfone, 100 kD cutoff (Sartorius AG, #VS2041), and the resulting concentrated conditioned media (CCM) was used immediately for NTA analysis and SEC.

Cell culture conditions and sample preparation steps are critical for optimal results and reproducible data. **Jump to section Sample Collection and Preprocessing.**

Extracellular Vesicle (EV) Precipitation with Polyethylene Glycol (PEG) 6000

To confirm the EV elution zone of SEC columns (see Figure 10), the CCM sample was further concentrated after UF by precipitation with PEG. The CCM sample was diluted with 40% Polyethylene Glycol 6000 (MilliporeSigma, #807491) to a final PEG concentration of 8%. The sample was gently mixed by inverting the tube three times. Precipitation was performed at 4°C for 1 hr or overnight. Precipitated EVs were collected by centrifugation at 1,500 x g for 30 min at 4°C. The supernatant was carefully removed, and the tube was centrifuged for a second time at 1,500 x g for 5 min to remove any residual liquid. The pellet was carefully resuspended with 60 µl sterile-filtered PBS per mg pellet weight.

Chromatography System

Chromatography was monitored at room temperature at standard wavelengths of 220, 260, and 280 nm using an NGC Quest 10 Plus Chromatography System (Bio-Rad Laboratories, Inc., catalog #7880003). The instrument was configured with a sample inject valve, sample loops of different sizes, two buffer inlet valves (Bio-Rad, #7884006), a column switching valve (Bio-Rad, #7884012), and a multi-wavelength UV detector (5 mm path length) with an integrated conductivity meter (Bio-Rad, #12010343). The typical fraction size was 250 µl, and fractions were collected in 96-well plates with the BioFrac Fraction Collector equipped with a BioFrac Microplate Drop Head Kit and BioFrac Ice Bath/Microplate Rack (Bio-Rad, #7410002,

Table 1. Overview of resin characteristics and column running conditions utilized for EV enrichment by low-pressure SEC in self-packed Bio-Gel A-1.5m and Sepharose CL-2B columns.

Resin	Econo-Pac™ Column Diameter, cm	Column Name	Packing Volume, ml	Bed Height, cm	Peak Asymmetry Factor	Bead Size, µm	Exclusion Limit, kD	Flow Rate, cm/hr or ml/min	Fractionation Range for Globular Proteins, kD	Maximum Pressure, psi	pH Stability Range	Mobile Phase
Bio-Gel A-1.5m, Fine	1.5	BGA	15.5	8.8	1.65	37–75	1,500	16 or 0.5	10–1,500	15	4–13	PBS, pH 7.8
Sepharose CL-2B	1.5	SCL2B	16	9	1.57	60–200	40,000	16 or 0.5	70–40,000	15	3–13	PBS, pH 7.8

BGA, Bio-Gel A-1.5m Resin; Econo-Pac, Econo-Pac Chromatography Column; EV, extracellular vesicle; PBS, 1x phosphate buffered saline; SCL2B, Sepharose CL-2B; SEC, size exclusion chromatography.

To unlock the full potential of SEC, use a chromatography system rather than gravity-driven columns. **Jump to section SEC as an Isolation Method in EV Research.**

#7410088, #7410017). The NGC fraction collector with microplate rack can also be used (Bio-Rad, #17002070, #12003754). 10x Phosphate Buffered Saline (Bio-Rad, #1610780) was diluted with ultrapure water to 1x PBS (PBS) and used as a mobile phase in all experiments.

Resins and Column Packing

Bio-Gel A-1.5m Gel, Fine, a chromatography resin (BGA, Bio-Rad, #1510450), and Sepharose Cl-2B agarose-based gel filtration matrix (SCL2B, Cytiva, #17014001) were used to pack empty 1.5 cm diameter Econo-Pac™ Chromatography Columns (Bio-Rad, #7321010) that support bed volumes of 1–20 ml. Bio-Rad offers a wide selection of empty chromatography columns for various bed volumes and bed heights, summarized in bulletins 2289 and 7317. Detailed packing instructions for Bio-Gel A-1.5m Resin can be found in the Bio-Gel A Gels Instruction Manual #4006139. Additional information on SEC resins and running conditions is presented in Table 1. Resin cleaning was performed weekly with 0.1 N sodium hydroxide, and columns were stored at 4°C after re-equilibration with 0.02% sodium azide in degassed PBS.

An Econo-Pac Flow Adaptor (Bio-Rad, #7380019) was used to pack and operate the columns connected to an NGC Chromatography System. Packing and operating low-pressure columns such as Econo-Pac or Econo Columns with a flow adaptor connected to a peristaltic pump or a chromatography system are recommended. In principle, low-pressure SEC columns can be operated under gravity conditions, but this is not recommended because when connected to flow adaptors, they show improved column performance. Flow adaptors eliminate the headspace above the resin bed and protect it from disruption during sample loading.

Packing SEC columns for EV separations is relatively easy. Learn about some key considerations and how to test column performance. **Jump to section Resin, Column Packing, and Performance Testing.**

Characterization of Packed Columns

The self-packed columns were characterized with acetone (2.5% v/v in degassed water), Blue Dextran MW 2,000,000 Da (MilliporeSigma, #D5751), and Gel Filtration Standard (Bio-Rad, #1511901) consisting of thyroglobulin (670,000 Da), bovine gamma globulin (158,000 Da), chicken ovalbumin (44,000 Da), equine myoglobin (17,500 Da), and vitamin B12 (1,350 Da). Blue Dextran was dissolved in degassed PBS at a concentration of 1–2 mg/ml, and each vial of Gel Filtration Standard was dissolved with 1 ml of degassed water. Both solutions were thoroughly vortexed and centrifuged for 10 min at 10,000 x g to remove any fine particulates.

Protein Assay

EV-containing samples in PBS were lysed by sonication in the presence of 0.1% (w/v) Triton-X 100 Detergent (Bio-Rad, #1610407). The protein concentration of samples was determined by the *DC* Protein Assay Kit I (Bio-Rad, #5000111), which tolerates a wide range of detergents.

Nanoparticle Tracking Analysis

The concentration and size distribution of particles in samples before and after SEC were measured by NTA at 520 nm with a PMX 220 ZetaView TWIN Laser instrument from Particle Metrix GmbH. The instrument is equipped with two lasers (405 and 520 nm) and appropriate filters (410 and 550 nm) for fluorescence measurements (F-NTA). Particle movement is captured by a laser-scattering microscope connected to a video camera. Polystyrene beads, 100 nm in diameter, were used for instrument calibration. Ideal measurement concentrations were found by pretesting the optimal particle per frame value (100–200 particles/frame). Each sample was measured three times at 11 different positions and captured at 30 frames per position at 25°C under the following camera settings: sensitivity, 93; shutter, 100; frame rate, 30/sec. After measurement, video recordings were evaluated with ZetaView Software, version 8.05.16 SP3 (Particle Metrix GmbH), and parameters were set at maximum area, 1,000; minimum area, 10; maximum brightness, 255; minimum brightness, 30; particle size distribution (PSD) nm/class, 5. The final analysis and statistics included only particles tracked in 15

Table 2. Product information for antibodies used to detect EV-specific targets by fluorescent multiplex western blotting.

Antibody Target, Human	MW, kD	Accession Number, UniProt	Subcellular Location, UniProt	ISEV Category*	Ab Host, Clonality	Vendor, Product Code**	Fluorescent 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	Alix (1): Mouse, monoclonal,	Bio-Rad, VMA00273	DyLight 800 GAM	Bio-Rad, STAR117D800GA
					Alix (2): rabbit, polyclonal	Bio-Rad, VPA00765	Starbright Blue 700 GAR	Bio-Rad, 12004162
Transitional endoplasmic reticulum ATPase (VCP)	89	P55072	Cytoplasm; endoplasmic reticulum; nucleus	4c	Mouse, monoclonal	Bio-Rad, VMA00307	DyLight 800 GAM	Bio-Rad, STAR117D800GA
Tubulin beta chain	50	P07437	Cytoplasm; cytoskeleton; microtubule	2b	HuCal® hFAB, rhodamine-labeled	Bio-Rad, 12004165	Not required	n/a
GAPDH	36	P04406	Cytoplasm; cytoskeleton; membrane; nucleus	2b	HuCal hFAB, rhodamine-labeled	Bio-Rad, 12004167	Not required	n/a
Annexin A5 (ANXA5)	32	P08758	Cytoplasm; plasma membrane; extracellular (secreted)	2a	Rabbit, polyclonal	Bio-Rad, VPA00077	IRDye 800 GAR	LI-COR, 926-32211
CD81 antigen	26	P60033	Cell membrane	1	Mouse, monoclonal	SCBT, sc-166029	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 H2B	17	Q99879	Chromosome; nucleus	4a	Rabbit, polyclonal	Bio-Rad, VPA00538	IRDye 800 GAR	LI-COR, 926-32211

* For information on ISEV categories see Table 5.

** For Bio-Rad antibodies visit bio-rad-antibodies.com.

Ab, antibody; FAB, fragment antigen binding; GAM, goat anti-mouse; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; 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.

consecutive video frames. For further analysis, data were converted into an FCS file and exported to FCS Express 7 Software (De Novo Software).

Direct Fluorescent Membrane Labeling

CellMask Orange Plasma Membrane Stain (CMO dye, Thermo Fisher Scientific, #C10045) was used for fluorescent membrane labeling of EV-containing samples. A CMO dye stock solution (1:100) was prepared in ultrapure water and stored at –20°C. Prior to use, aliquots were thawed for single use only and further diluted to a working concentration of 1:500 in ultrapure water. The labeling reaction, performed in the dark, was started by mixing 2 µl CMO dye (1:500), 2 µl sample, and 16 µl PBS. After 5 min, 1,980 µl of ultrapure water was added to the reaction mix and immediately measured by NTA in fluorescent mode at 520/550 nm. It is highly recommended to perform a titration experiment to optimize the dye/particle ratio.

Protein Electrophoresis and Multiplex Western Blotting

The quality of the chromatographic purification steps was monitored by SDS-PAGE with 4–20% Criterion TGX Stain-Free Precast Gels, 12+2 well, 45 µl (Bio-Rad, #5678093). Precision Plus Protein All Blue Prestained Protein Standards (Bio-Rad, #1610373) and Precision Plus Protein Unstained Protein Standards, *Strep*-tagged recombinant (Bio-Rad, #1610363) were used for molecular weight calibration. UF CCM samples were prepared for SDS-PAGE under reducing conditions with 2x or 4x Laemmli Sample Buffer (Bio-Rad, #1610737; #1610747) supplemented with 2 or 4% Dithiothreitol (Bio-Rad, #1610610). After electrophoresis, the Criterion TGX Stain-Free Precast Gel was UV activated for 45 sec, and the resulting Stain-Free technology image was recorded on a ChemiDoc MP Imaging System (Bio-Rad, #12003154).

Monitor EV markers and contaminants using multiplex western blotting. Learn about commonly assayed biomolecules and how to prepare samples for multiplex western blotting. **Jump to section Characterization.**

After imaging, the gel was immediately subjected to multiplex western blotting. Here, the activated gel was transferred to an Immun-Blot™ Low Fluorescence PVDF Membrane (Bio-Rad, #1620262) with the Trans-Blot™ Turbo Midi Transfer Packs (Bio-Rad, #1704157), using the 7 min protocol on the Trans-Blot Turbo Transfer System (Bio-Rad, #1704150). The 0.2 µm PVDF membrane that comes in Trans-Blot Turbo Midi Transfer Packs (Bio-Rad, #1704157) is removed, discarded, and replaced with the activated Immun-Blot Low Fluorescence PVDF membrane in its place for optimal blotting sensitivity. The PVDF blotting membrane was blocked using Intercept (TBS) Blocking Buffer (LI-COR, Inc., #927-60001) for 60 min at room temperature with gentle agitation. 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 (diluted 1:1000) to detect a corresponding set of targets based on their molecular weights (see Table 2 for product information).

After incubation with primary antibodies for 18 hr at room temperature, the blotting membrane was washed four times for 10 min with 1x Tris Buffered Saline (Bio-Rad, #1706435) containing Tween 20 (Bio-Rad, #1662404) at 0.1% (TBST) and further incubated for 1 hr at room temperature with the respective host-matched fluorescently labeled secondary antibodies (see Table 2) diluted 1:10,000 in Blocking Buffer. Each of the secondary antibody solutions was supplemented with either hFAB Rhodamine Anti-Tubulin Primary Antibody (50 kD, Bio-Rad, #12004165) or hFAB Rhodamine Anti-GAPDH Primary Antibody (37 kD, Bio-Rad, #12004167). Before imaging with the ChemiDoc MP Imaging System, the blotting membranes were washed four times for 10 min with TBST. Gel and blot analysis was performed with Image Lab Software, version 6.1.0 (Bio-Rad, #12012931).

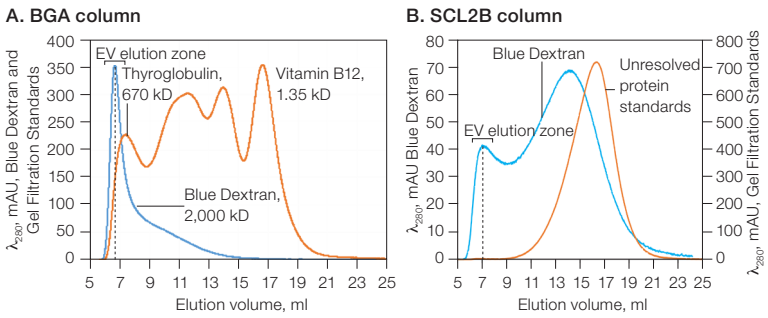


Fig. 3. Characterization of SEC columns described in Table 1. **A**, BGA column; and **B**, SCL2B column, were individually characterized with Blue Dextran 2000 (—) maximum MW ~2,000 kD and Gel Filtration Standard (—), a mixture of five standards, MW 1.35–670 kD. The elution start of Blue Dextran 2000 marks the approximate void volume (**---**) for both columns and, thus, a putative EV elution zone. The BGA Resin has a smaller pore and particle size than the SCL2B Resin and hence shows a better resolution of the gel filtration standard mixture used here. The standard is not resolved with the SCL2B column. A higher resolution to obtain well-separated, distinct protein peaks may be achieved by increasing the column length, however, this was not the focus here. AU, absorbance unit; BGA, Bio-Gel A-1.5m Resin; EV extracellular vesicle; MW, molecular weight; SCL2B, Sepharose CL-2B; SEC, size exclusion chromatography.

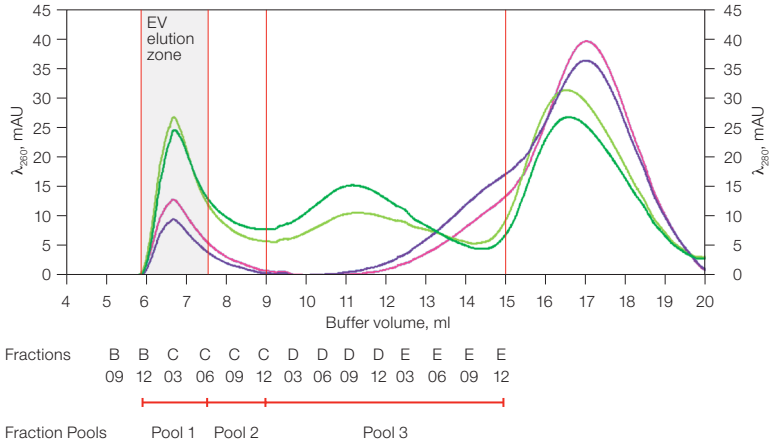


Fig. 4. Chromatograms of EV separations of CCM samples on a BGA or SCL2B column. Chromatography performed on an NGC Quest 10 Plus Chromatography System. Elution profiles were recorded at 260 nm and 280 nm. Fractions of 250 µl were collected with the BioFrac Fraction Collector in a 96-well plate. Three fraction pools were generated and analyzed for EV enrichment by multiplex fluorescent western blotting (see Figure 7). SCL2B, λ_{260} (—), λ_{280} (—), BGA, λ_{260} (—), BGA, λ_{280} (—). AU, absorbance unit; BGA, Bio-Gel A-1.5m Resin; CCM, concentrated conditioned medium, EV, extracellular vesicle; SCL2B, Sepharose CL-2B.

Results and Discussion

SEC has been successfully applied for the separation of EVs originating from a variety of different biofluids and cell culture supernatants, either as a single-step method (Böing et al. 2014) or in combination with orthogonal or prefractionation methods such as polymer-based precipitation or differential ultracentrifugation (Martínez-Greene et al. 2021; Baranyai et al. 2015). This study was not intended to address a biological question but rather to present the advantages of instrument-controlled SEC for the separation of EVs and guidance for adopting this method.

Blue Dextran 2000 is a common void volume marker that can be used to help define EV elution zones. **Jump to section Column Void Volume Determination.**

Determination of EV Elution Zone of Size Exclusion Chromatography Columns

An important goal in EV isolation experiments using SEC is the removal of high molecular weight proteins or protein complexes. It is recommended to compare the elution profile of purified proteins of known molecular weights with the elution profile of test substances for void volume determination. Here, both a Blue Dextran 2000 sample and Bio-Rad´s Gel Filtration Standard mixture were individually analyzed on SEC columns BGA and SCL2B, and the results are shown in Figure 3. The elution of Blue Dextran 2000 begins at about 6 ml and can be regarded as the putative elution start zone of EVs for both columns. Given that the putative EV elution zone overlaps with the elution area for the highest molecular weight standard (670 kD) for the BGA column, the SCL2B column is likely to be more efficient at removing high molecular weight contaminants (presumably over 500 kD) from EV preparations.

Chromatography and Nanoparticle Tracking Analysis

According to a study published in 2019, more than 45% of all publications using SEC for EV isolation mention SCL2B as their preferred SEC resin (Monguió-Tortajada et al. 2019); here, SCL2B is compared to BGA for EV isolation. The conditioned media harvested after 120 hr from MCF-7 cells (230 ml) was concentrated as described, and the resulting CCM sample subjected to SEC with columns packed with BGA or SCL2B Resin, which differ significantly in their exclusion limits (see Table 1). The particle concentration of the CCM starting material was 1.6 x 10^{11}/ml, and each of four runs at

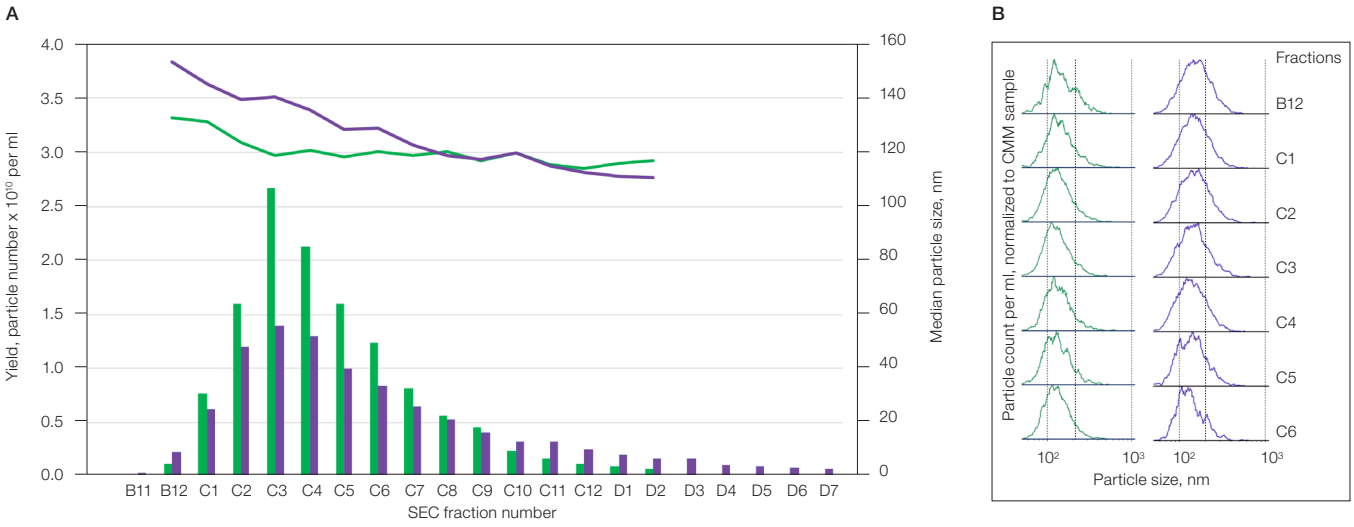


Fig. 5. Particle size and concentration measurements by NTA of individual 250 µl fractions obtained after SEC with BGA (■) and SCL2B (■) columns as shown in Figure 4. **A**, particle concentration measurements (bar chart) performed on fractions B11–D7 represent the SEC separation range of 5.5–10.75 ml. Total particle yield for BGA, 80%, and SCL2B, 63%. Median particle size, (line plot) is displayed for fractions B12–D2, representing the SEC separation range of 5.75–9.5 ml. **B**, particle size distribution of fractions (log scale) B12–C6 represents pool 1 obtained after SEC with either the BGA or SCL2B column. Vertical black lines (---) represent 100, 200, and 1,000 nm, respectively. BGA, Bio-Gel A-1.5m Resin; CCM, concentrated conditioned medium; NTA, nanoparticle tracking analysis; SCL2B, Sepharose CL-2B; SEC, size exclusion chromatography.

Table 3. Particle counts in pooled fractions and particle-to-peak area ratios. Fraction pools were generated after SEC with BGA and SCL2B columns and analyzed for particle concentration. The fraction pool volumes were further reduced by ultrafiltration to obtain highly concentrated samples for multiplex western blotting analysis. Relative generic sample purity can be estimated by comparing particle concentration to SEC peak area at 280 nm wavelength.

Column	Sample	Particles, ml	Volume, ml	Concentration Factor after UF	Protein Concentration, µg/µl	Peak Area, 280 nm	Ratio, Particle Number/Peak Area
	CCM	1.60 x 10^{11}	n/a	n/a	0.6	155	2.6 x 10^{8}
BGA	Pool 1	1.40 x 10^{10}	5.0	59	n/a	30	4.7 x 10^{8}
	Pool 2	4.60 x 10^{9}	4.5	56	n/a	15	3.1 x 10^{8}
	Pool 3	n/a	18.0	45	0.6	–	n/a
SCL2B	Pool 1	6.20 x 10^{9}	5.3	88	n/a	13	4.8 x 10^{8}
	Pool 2	3.10 x 10^{9}	5.0	100	n/a	3	1.0 x 10^{9}
	Pool 3	n/a	18.0	45	0.4	–	n/a

BGA, Bio-Gel A-1.5m Resin; CCM, concentrated conditioned medium; n/a, not applicable; SCL2B, Sepharose CL-2B; UF, ultrafiltration.

0.5 ml/min was performed with a sample and fraction size of 250 µl. The chromatograms were recorded at two standard wavelengths (260 and 280 nm), and the typical elution profiles are shown in Figure 4. As expected, EV elution starts for both columns at ~6 ml but the chromatograms show qualitative and quantitative differences along the elution path. The amount and median size (x50, nm) of particles in individual consecutive fractions was measured by NTA (Figure 5A). Measurements were stopped once particle concentration was in the range of 2 x 10^{8}/ml to 5 x 10^{8}/ml. The BGA column shows a denser particle elution profile, documented from fraction B12 (5.75 ml) to D2 (9.5 ml), while particle concentrations for the SCL2B column above the 5 x 10^{8}/ml cutoff were from B12 (5.5 ml) to D7 (10.75 ml). This dataset was used to calculate total particle recovery for both elution ranges, approximately 80% for BGA and 63% for SCL2B columns, respectively. Size histograms for fractions representing pool 1 (see Figure 4) are shown in Figure 5B and suggest a slightly shifted size distribution of both workflows, as already indicated through the x50 (nm) values.

EV samples are generally a heterogeneous mixture of differently sized particles. The SCL2B column showed an increased size-based separation tendency compared to the BGA column. For BGA, a bulk-like particle elution behavior was more typical. These results agree with the characteristics of both resins. SCL2B Resin has a larger pore size than BGA, likely allowing a higher percentage of smaller-sized EVs to enter the resin and be separated from the largest EV. A more pronounced size-based particle separation can likely be achieved with both resins, for which significantly longer SEC columns are more appropriate.

The results show that more particles have been recovered after SEC with the BGA column compared to the SCL2B column. Since NTA in scatter mode cannot discriminate between relevant and background or non-EV membranous particles, SEC fractions of the main EV peak were membrane-stained with CMO dye and measured by F-NTA. The data were normalized against the total particle concentration shown in Figure 5A and expressed

as a percentage ratio of CMO dye–sensitive particles to total particles. The percentage of CMO dye–sensitive particles was reasonably uniform across BGA column fractions (46 ± 4) while slightly more variable with SCL2B (41 ± 6). However, the informative value of such a measurement is presumably limited without accompanying experiments, for example, cryo-electron microscopy or other imaging techniques. Overall, the percentage of CMO dye–positive particles is comparable for both resins, and there is no strong indication that the random accumulation of CMO dye–negative particles causes the higher particle recovery rate for the Bio-Gel A-1.5m Resin.

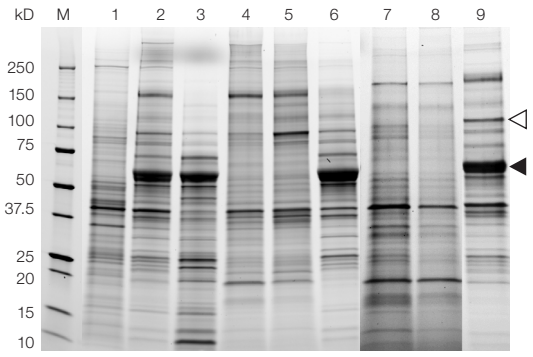
Multiplex Western Blotting Analysis

Three fraction pools were generated after each of the SEC runs (see Figure 4) and concentrated by ultrafiltration at a cutoff of 3 kD (Table 3). Usually, a 100 kD cutoff is applied for volume reduction of EV-containing fractions, but this would bias the detection of lower molecular weight protein contaminants. Fraction pooling was done equally for both resins but executed to match the elution characteristics of column BGA.

Pool 1 represents the main peak of the BGA column, pool 2 is its peak extension, and pool 3 represents the major protein contamination area with particle concentrations below ~5 x 10^8/ml.

Those fraction pools, along with MCF-7 lysate, CCM sample, and CCM sample flowthrough after ultrafiltration, were analyzed twice by SDS-PAGE with Stain-Free technology, and the gels were of high quality (Figure 6). A suitable total protein load for complex protein samples is about 5–10 µg/lane, which was achieved for all sample pools from the BGA column. Pools 1 and 2 from the SCL2B column, although provided with a higher volume reduction factor to compensate for the differences in total particle count compared to the pools of column BGA, showed a weaker total lane signal in the Criterion TGX Stain-Free Precast Gel. Some commonalities and differences between pools obtained with BGA and SCL2B columns are apparent. For example, an abundant ~60 kD protein found in the starting material CCM is absent in pool 1 but is present in pool 3 for both resins. The BGA pool 2 sample shows some differences relative to the SCL2B pool 2 sample. For example, a ~100 kD protein appears to be absent in the SCL2B pool 2 sample but is present in the BGA pool 2 sample. Overall, for both resins, the data show a similar banding pattern for the pooled fraction 1, which contains the highest number of EVs; however, as mentioned previously, EV particle yields are higher with the BGA column.

The results of the multiplex western blotting experiments are presented in Figure 7, and the chosen targets (Table 5) and primary antibodies (Table 2) yielded robust signals throughout. The fluorescence recordings obtained from both workflows were normalized against the total protein load with Image Lab Software, and fold-change ranges relative to the CCM sample were calculated (Table 4). The signal intensity of the chosen EV marker proteins (see Table 5) of category 1 (CD9, CD81) and subcategory 2a (Alix) in fraction pools 1 and 2 of the BGA and SCL2B columns are strongly increased compared to the starting material CCM, indicating successful EV enrichment with both workflows. ANXA5 (subcategory 2a) is present in all three fraction pools, but a different qualitative pattern for both columns is obtained. GAPDH and tubulin signals, which belong to subcategory 2b (promiscuous incorporation in EVs), do not show any meaningful readings at first sight. Histone H2B and actinin-4 are members of category 4 but cannot be regarded as "true" contaminants. This renders their interpretation as contamination markers in a purification scheme difficult because they have already been detected in EVs (Holliday et al. 2019; Singh et al. 2022). Histone H2B is found at slightly



Lane	Sample
M	Protein standards
1	MCF-7 cell lysate
2	CCM sample
3	CCM sample flow-through
4	Pool 1 (BGA)
5	Pool 2 (BGA)
6	Pool 3 (BGA)
7	Pool 1 (SCL2B)
8	Pool 2 (SCL2B)
9	Pool 3 (SCL2B)

Fig. 6. Stain-Free technology SDS-PAGE analysis of fraction pools from SEC workflows with BGA and SCL2B columns. Criterion TGX Stain-Free Precast Gels, 4–20% gradient loaded with total protein of 8–10 µg/lane (lanes 1–6) and 2–3 µg/lane (lanes 7–8). Lanes 7–8 image contrast adjusted for comparison. ◀, ~60 kD protein absent in pool 1 sample; ◁, ~100 kD protein present in pool 2 sample with BGA column and absent with SCL2B column. BGA, Bio-Gel A-1.5m Resin; CCM, concentrated conditioned medium; SCL2B, Sepharose CL-2B; SEC, size exclusion chromatography.

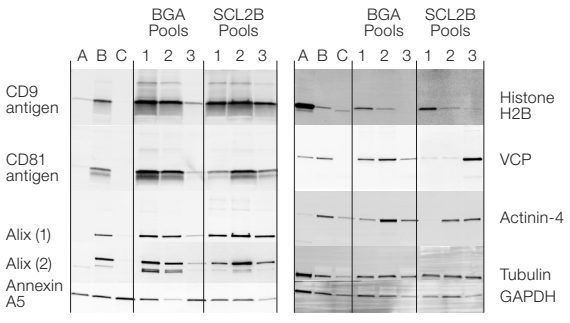
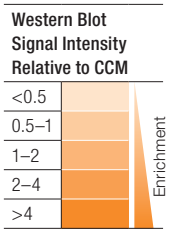


Fig. 7. Multiplex fluorescent western blotting. Stain-Free technology SDS-PAGE gels shown in Figure 6 were subjected to multiplex fluorescent western blotting with various primary antibody combinations from Table 2 as shown. Alix (1) is polyclonal, and Alix (2) is monoclonal. The fluorescent signals were recorded with the ChemiDoc MP Imaging System at different excitation wavelengths and filter combinations. Lane A, MCF-7 cell lysate; B, CCM sample; C, CCM sample flowthrough; BGA pools 1–3 (see Figure 6 lanes 4–6); SCL2B pools 1–3 (see Figure 6, lanes 7–9). Alix, programmed cell death 6-interacting protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VCP, transitional endoplasmic reticulum ATPase.

Table 4. Semi-quantitative data analysis of multiplex fluorescent western blotting signals from Figure 7 for pools 1–3 obtained after SEC of CCM sample with BGA and SCL2B columns. Pool 1 represents the main EV elution zone for both columns. Stain-Free technology signals were used as a loading control, and fluorescent western blot signals of various targets (see Table 1) were further normalized against the CCM sample. The fold-change analysis is presented as a color-coded range. All relevant EV-specific targets of ISEV categories 1 and 2a (Table 5) are increased, indicating successful EV enrichment for both columns. Signal changes of all other targets are discussed in section Multiplex Western Blot Analysis. Alix (1) is polyclonal, and Alix (2) is monoclonal.

		BGA			SCL2B		
Target	Category*	Pool 1	Pool 2	Pool 3	Pool 1	Pool 2	Pool 3
CD9	1						
CD81	1						
Alix (1)	2a						
Alix (2)	2a						
Anax5	2a						
Histone H2B	4a						
VCP	4c						
Actinin-4	4d						
Tubulin	2b						
GAPDH	2b						



* For information on ISEV categories see Table 5.

Alix, programmed cell death 6-interacting protein; BGA, Bio-Gel A-1.5m Resin; CCM, concentrated conditioned medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SCL2B, Sepharose CL-2B; VCP, transitional endoplasmic reticulum ATPase.

elevated levels in pool 1 of both resins and is almost absent in pools 2 and 3. Actinin-4 is not present in pool 1 of the SCL2B column and is clearly enriched/present in pool 2 of the BGA column. The protein VCP assembles as a homohexamer (540 kD) and is here regarded as a high molecular contamination marker of endoplasmic reticulum origin. VCP is detected in sample pools 1–3 of the BGA column and appears only in pool 3 of the SCL2B column. As indicated earlier, the BGA column is less capable of removing protein contaminants with a molecular weight higher than 500 kD. In addition, the visual inspection of the Criterion TGX Stain-Free Precast Gel lanes of fraction pool 3 shows that the SCL2B lane has a higher proportion of protein species above 150 kD.

The purity of EV preparations after enrichment by SEC can be demonstrated individually, for example, by western blotting, or in a more generic way by comparing the total number of EVs to the total protein amount, which can be obtained by protein assay or spectrometry at 280 nm during SEC. Here, fraction pools 1 of both columns show similar ratios of particle count to the respective 280 nm peak area (see Table 3), while pool 2 obtained after BGA purification can be regarded as less “pure” compared to pool 1. A question remains: How pure should an EV preparation be, for example, after SEC? An adequate generic EV purity, as obtained with pool 1 from the BGA column and pools 1 and 2 from the SCL2B column, should be complemented by additional individual purity assays for specific contaminating proteins with known effects on planned functional assays. In general, the most suited resin and fractions will depend on the sample of interest and downstream application since there will be a tradeoff between EV yield and purity.

Conclusions

Several techniques exist for the isolation of EVs. Ultracentrifugation and polymer-based precipitation methods are widely used; however, these are difficult to standardize in terms of reproducibility and data acquisition and are not considered scalable solutions to meet the demands of the field developing EVs for numerous therapeutic applications. The use of SEC has increased significantly over the years because it offers a wide range of analytical potential, especially when performed in an instrument-controlled manner. The advantages of automated SEC over other methods are multifaceted and include scalability, reproducibility, purity, and gentleness (Sidhom et al. 2020). Here, we compared two SEC resins, Bio-Gel A-1.5m and Sepharose CL-2B, in a single-step chromatography approach to separate EVs from serum-free, conditioned cell culture media. Self-packed columns connected to an NGC Chromatography System enabled reproducible flow over the columns, allowed real-time separation monitoring at multiple wavelengths, and enabled accurate collection of fractions. The results demonstrate the usefulness of Bio-Gel A-1.5m Resin for the separation of EVs. EV separations using Bio-Gel A-1.5m Resin were characterized by a bulk-like elution of EVs at a high particle yield, while with Sepharose CL-2B, which has a larger pore size, a wider EV elution profile is obtained. In addition to the stand-alone usefulness of Bio-Gel A-1.5m Resin, the narrower elution profile may make it beneficial for automated multistep chromatography (for example, SEC combined with ion exchange chromatography), in cases where a higher EV preparation purity is essential. Overall, it can be anticipated that there will be a growing importance of automated SEC in purifying EVs from diverse sources and it will contribute significantly to method standardization in EV research.

Table 5. ISEV Categories* and subcategories for monitoring EV separations and purity.

Category and Description	1. Transmembrane or GPI-anchored proteins associated to plasma membrane and/or endosomes	2. Cytosolic proteins recovered in EVs	3. Major components of non-EV co-isolated structures	4. Transmembrane, lipid-bound and soluble proteins associated with intracellular compartments other than PM/endosomes	5. Secreted proteins recovered with EVs
Subcategory, Description, and Example Targets	1a. Non-tissue-specific: CD63, CD81	2a. Lipid or membrane protein-binding ability: Alix, annexins, TGS101	3a. Abundant in serum/plasma: apolipoproteins, albumin	4a. Nucleus: histones	5a. Cytokines, growth factors: TGF-β1/2, IFNG, VEGFA, FGF1/2, EGF
	1b. Cell/tissue-specific: CD9	2b. Promiscuous incorporation in EVs and possibly exomers: GAPDH, tubulin, actin	3b. Protein and protein/nucleic acid aggregates: Tamm-Horsfall protein, ribosomal proteins	4b. Mitochondria: cytochrome C, VDAC	5b. Adhesion and extracellular matrix proteins: fibronectin (FN1), MFGE8; galectin3-binding protein (LGALS3BP), CD5L
				4c. Secretory pathway including ER, Golgi apparatus: VCP, BiP, calnexin	
				4d. Others such as cytoskeleton: actinins 1–4	

* See Théry et al. (2018).

Alix, programmed cell death 6-interacting protein; BiP, binding immunoglobulin protein; EGF, epidermal growth factor; ER, endoplasmic reticulum; EV, extracellular vesicle; FGF, fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPI, glycosylphosphatidylinositol; IFNG, interferon-gamma; ISEV, International Society for Extracellular Vesicles; PM, plasma membrane; TGF-β, transforming growth factor beta; TSG101, tumor susceptibility gene 101 protein; VCP, transitional endoplasmic reticulum ATPase; VDAC, voltage-dependent anion channel protein; VEGFA, vascular endothelial growth factor A.

Best Practices for EV Isolation Using Size Exclusion Chromatography (SEC)

The study of EVs mainly comprises four experimental areas: 1) collection and preprocessing, 2) contaminant separation, 3) characterization, and 4) functional studies. To improve the reliability and reproducibility of experimental results, the International Society for Extracellular Vesicles (ISEV) has published a position editorial with valuable guidelines (Théry et al. 2018). In the following sections, we will briefly discuss a few of those ISEV recommendations (see Table 5) in conjunction with the application of SEC, NTA, and fluorescent multiplex western blotting for the separation and characterization of EVs obtained from conditioned cell culture medium.

Sample Collection and Preprocessing

Cell Culture

Stable, well-defined cell culture, harvesting, and storage conditions are crucial for reproducible EV recovery of similar quality and quantity. In upstream processing, the number of experimental parameters that can affect the composition of EVs is enormous and they need to be carefully controlled (Ludwig et al. 2019). Cell culture supplements, such as growth factors, can affect EV production and their constitution. It is also important to mention that EVs are best obtained from FBS-free cell culture media to avoid sample contamination with foreign EVs and other soluble proteins found in serum, which complicate downstream sample processing. If exosome/EV-depleted serum or other supplements are used, it is advisable to verify the impact of such supplements on cell growth and EV release (Théry et al. 2018). Cell culture conditions and time of harvest should be optimized to keep cell apoptosis to a minimum. Dying cells release their biochemical content, including membranous particles, into the medium and decrease the purity of the released EVs.

After harvest, cells and debris are removed from the media by centrifugation at 2,000 x g. At this point, media can be stored at –80°C for at least one month (Sivanantham and Jin 2022). After thawing on ice, the media is centrifuged again at 10,000 x g to reduce the concentration of larger extracellular particles, such as apoptotic bodies.

EV Sample Preparation

One should keep in mind that highly concentrated samples are required for purification by SEC. For optimal performance, it is recommended to apply sample volumes in the range of 1–5% of the total column bed volume. Very dilute samples must be concentrated before column loading, especially cell culture media and urine. Ultrafiltration spin columns with a 100 kD cutoff can achieve a 100-fold concentration of serum-free, conditioned media from cell cultures. In most cases, a 100-fold concentration step yields particle concentrations of about 1 x 10^{11}/ml, which is a good starting point for downstream processing. If it is not possible to reach a desired particle concentration for SEC by ultrafiltration, the sample can be further concentrated by polymer-based (for example, PEG 6000) precipitation techniques. The success of precipitation protocols is often sample dependent and should be carefully evaluated for particle yield with NTA. The downstream application of the precipitated EVs should be considered. Some low levels of precipitation agent may remain in the EV sample and may interfere with downstream analysis methods such as mass spectrometry. Thus, EV precipitation is best combined with SEC to remove excess precipitation agents. In addition, it should be considered that precipitation could diminish the biological activity of EVs, as reported by Paolini et al. (2016). EV precipitation techniques are more commonly utilized for smaller-scale EV preparations for research or diagnostic purposes. For larger-scale EV preparations where the ultimate intent is to use EVs in therapeutic applications, a very effective way to concentrate large volumes of conditioned media is the application of tangential flow filtration (Visan et al. 2022), or anion-exchange chromatography (AEX) since

EVs have a net negative charge. Heath et al. (2018) isolated 2.4 x 10^{11} EVs from 1 liter of cell culture media using AEX within 3 hr and removed multiple contaminating proteins in parallel.

Contaminant Separation

Separation Principle of SEC

SEC, or gel filtration, separates molecules based on their size, and molecules are eluted in order of decreasing molecular weight. The SEC resins consist of spherical beads containing pores of a specific size distribution. Separation occurs when molecules of different sizes are included or excluded from the pores within the resin matrix. In the case of resins used for EV isolation, smaller molecules, such as contaminating proteins or protein complexes, diffuse into the pores, and their flow through the column is retarded. In contrast, very large molecules such as EVs do not enter the pores and are eluted in the column's void volume. Generally, column performance is influenced by flow rate, column dimension, and particle size. A widely used chromatography buffer for the isolation of EVs is phosphate buffered saline, and the highest resolution is obtained at low flow rates (10–15 cm/hr).

SEC as an Isolation Method in EV Research

The use of gravity-driven SEC columns for EV isolation contradicts efforts toward standardization and reproducibility made by the EV research community. To unlock the full potential of SEC, it is highly recommended to operate the respective pre- or self-packed columns with the help of a medium-pressure chromatography system such as the NGC Chromatography System with ChromLab Software from Bio-Rad. Automated sample application and multi-wavelength recording of the runs with a 10 mm analytical flow cell will increase inter-run reproducibility and enhance data analysis and interpretation. As previously mentioned, EVs can be labeled with various fluorescent probes, which can be detected using chromatography systems equipped with a multiwavelength detector such as NGC Chromatography Systems. Sample labeling is beneficial for specific tracking during SEC protocol development or quality control. In addition, small-volume fraction collectors such as the BioFrac Fraction Collector with a 25 µl microplate drop head can achieve EV subfractionation when combined with a high-resolution SEC column.

Resin, Column Packing, and Performance Testing

SEC resins are characterized by several parameters (see Table 1), such as fractionation range for globular proteins, particle size, maximum flow velocity, chemical stability, and recommendations for cleaning and storage. For example, the fractionation range of Bio-Gel A-1.5m Resin for globular proteins is 10,000–1,500,000 Da; the latter value is the resin's exclusion limit. Particles such as EVs are in the mega- to gigadalton range and are typically eluted within or just after the void volume. Most protein contaminants in cell culture media have molecular weights smaller than the exclusion limit of SEC resins used in EV isolation and elute later in the purification process.

Detailed packing instructions and necessary equipment are specified when purchasing a resin such as Bio-Gel A-1.5m Resin. It is beyond the scope of this work to explain this process in detail, but consider the following recommendations:

- In SEC, resolution increases with bed height
- Determine the desired final bed height to calculate the appropriate amount of resin. Include the compression factor in calculations if the resin is compressible
- The resin slurry and buffers used for column packing should be thoroughly degassed
- Packing speed is typically different from the flow rate applied during chromatography

After packing an SEC column, a simple performance test can be conducted. Column Performance Test Phase is available in ChromLab Software to easily add to a New Method and is located in the Phase Library. The determination of the peak asymmetry factor (PAF) is a measure of column packing quality. It is defined as the distance from the center line of the peak to the back slope divided by the distance from the center line of the peak to the front slope. All measurements are made at 10% of the maximum peak height. Extremely well-packed columns have a PAF close to 1, but a PAF between 0.8 and 1.8 is sufficient for SEC columns used in EV chromatography. A PAF below 1 results in peak fronting, while values above 1 lead to peak tailing. Peaks that show intense fronting are often caused by overpacking or column overload, while underpacking is often the reason for significant tailing (Figure 8). In those cases, it is recommended to repack the column using either lower or higher flow rates. A suitable sample for PAF calculations is 2.5% (v/v) acetone in water, and a typical performance run for a 15.5 ml BGA column is shown in Figure 9.

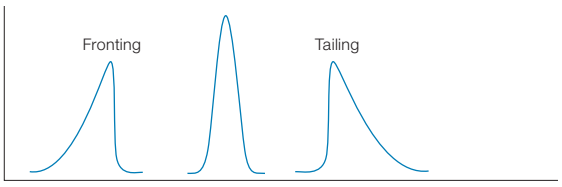


Fig. 8. Representation of various peak shapes. Peak anatomy is an important parameter to assess the quality of column packing. In an ideal case, peaks should be highly symmetrical. Fronting peaks are often caused by column overpacking or overloading with the sample. Tailing peaks are an indication of underpacking. Simple column performance tests can be conducted with sample injections of 2.5% acetone in water (see Figure 9).

Column Void Volume Determination

An important parameter for SEC columns used in EV isolation is the knowledge of the void volume (V_0) of the bed, which is the volume of interstitial fluid. Molecules such as EVs that are larger than the pore size of most SEC resins are completely excluded from the resin, and their elution volume is equal to or

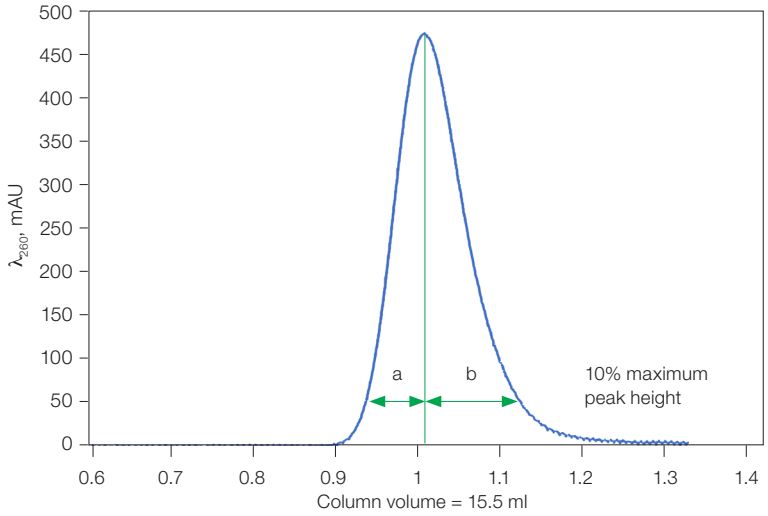


Fig. 9. Performance test of a 15.5 ml self-packed Bio-Gel A-1.5m column. Absorbance trace following sample injection with 250 µl of 2.5% (v/v) acetone in water. The calculated peak asymmetry (b/a) at 10% of maximum peak height is 1.65. For column packing information, see Table 1. AU, absorbance units.

close to V_0 of the column. Suitable test substances for V_0 must exhibit minimal interaction with the resin and be large enough to ensure elution in the true void volume. Blue Dextran 2000 or tobacco mosaic virus (TMV) with molecular weights of 2.0 x 10^6 and 39.0 x 10^6 Da respectively, are often used to determine the void volume of size exclusion columns. Unfortunately, Blue Dextran 2000 is not homogenous in size. It contains dextran species with molecular weights below 2,000 kD that can travel through the resin, not just the interstitial space, resulting in the appearance of peak tailing (see Figure 3B).

Confirmation of EV Elution Zone of SEC Columns

When using an SEC resin for EV isolation, it is recommended to verify the respective elution zone of EVs. As already outlined, void volume markers such as Blue Dextran 2000 or TMV are helpful to define a putative EV elution area but require confirmation by a biological sample. One possibility is direct fluorescent membrane labeling of an EV-enriched sample (Simonsen 2019), for example, with CMO dye, and recording the respective SEC run at a dye-specific wavelength of 556 nm compared to the unstained sample. For the characterization of a 15.5 ml BGA column, a PEG-precipitated CCM sample from an MCF-7 cell line can be used for labeling, and a specific peak at 556 nm can be recorded with a total elution volume of about 2 ml (Figure 10).

Another option is to equally split an EV-containing sample and apply ultracentrifugation (2 hr at 100,000 x g) to one half of the sample. The supernatant is recovered, and the SEC chromatograms of both samples are recorded at standard wavelengths and analyzed for qualitative differences (data not shown).

Characterization

Monitoring EV Quantity, Purity, and Composition

The quality of the purification process in terms of yield, size characteristics, and purity should be monitored by multiple complementary techniques. Nanoparticle tracking analysis (NTA) can be used to assess both total particle number and particle size distribution. Multiplex western blotting is

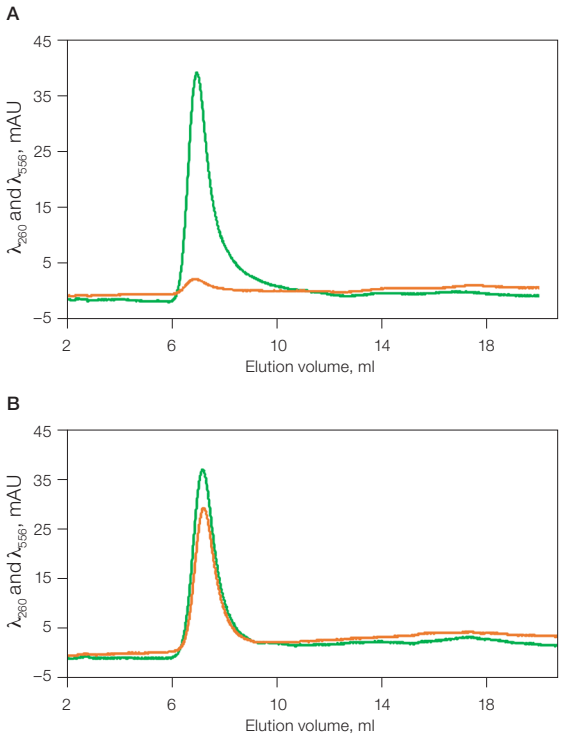


Fig. 10. Schematic elution profiles for identification of the EV elution zone of a 15.5 ml BGA column by direct fluorescent membrane labeling of EVs in CCM samples with CMO dye. **A**, precipitated CCM sample, unlabeled; **B**, precipitated CCM sample, CMO labeled. Chromatograms were recorded at wavelengths 280 nm (—) and 556 nm (—). Note that precipitated samples are less complex and mostly contain EVs and were chosen to highlight the difference between unlabeled and labeled cells. BGA, Bio-Gel A-1.5m Resin; CCM, concentrated conditioned medium; CMO, CellMask Orange Plasma Membrane Stain; EV, extracellular vesicle.

useful for characterizing EV-containing samples by their protein composition. Knowledge about sample purity is of importance when functional or biomarker studies are planned. It is also recommended to perform total protein assays whenever possible. Ratios of different quantitation methods (for example, total particle count/total protein) provide useful relative generic measures of sample purity during a purification scheme. Instrument-controlled SEC offers the advantage of monitoring EV isolation at multiple wavelengths, which can be used to calculate additional purity metrics of sample fractions obtained after the run, for example, total particle count/total peak area at 280 nm.

Western blotting is by far the most common technique for protein content–based EV characterization. Other popular methods are reviewed by Shao et al. (2018), including flow cytometry and mass spectrometry. ISEV recommends monitoring a few marker proteins of different categories; these are further subdivided (Table 5) to verify the presence of EVs and to assess their purity by assaying sample-specific contaminants.

Representatives of ISEV category 1 and subcategory 2a are used to confirm the presence of EVs, and category 3 lists highly abundant contaminating proteins, primarily present in biological fluids. The analysis of category 4 targets is recommended when isolating small EV subtypes (<200 nm). This category is not meant to be strictly black and white and leaves room for interpretation. Protein members of category 5 might interfere with functional assays, and their analysis is required to avoid any unwanted biological/assay side effects.

Nanoparticle Tracking Analysis Considerations

The principle of NTA is based on the characteristic movement of nanoparticles in solution according to Brownian motion and documented by a camera that measures the scattered light upon illumination. In conventional NTA, all particles are unlabeled and equally visible, and membrane fragments, protein aggregates, and other background particles are also detected with no differentiation. To enhance method specificity, fluorescence NTA (F-NTA) was introduced (Desgeorges et al. 2020). EV-containing samples are incubated with fluorescent dyes or antibody-dye conjugates, resulting in a more specific EV signal since only particles emitting fluorescence are detected. It is suggested to use fluorescent dyes that are resistant to photobleaching. A comprehensive protocol for the characterization of EVs with F-NTA is given by Midekessa et al. (2021).

Concentrating Samples Before Multiplex Western Blotting

Western blotting analysis of individual or pooled fractions after SEC of CCM samples usually requires a concentration step. Concentration methods can include protein precipitation with chemical agents (for example, acetone and trichloroacetic acid) and ultrafiltration. There is a wide selection of ultrafiltration devices that vary, for example, by capacity and membrane type and orientation. It is advisable to check the performance of ultrafiltration devices and observe for sample loss during method development before valuable samples are applied. Polyether sulfone (PES)–ultrafiltration containers with horizontally integrated membranes can be used to prepare western blotting samples after SEC. This allows the partial recovery of precipitated material on the membrane. After concentrate removal, the membrane is incubated with sufficient Laemmli Sample Buffer for 2–3 min, and the solution is recovered by inverse centrifugation at 3,000 x g.

Stain-Free Technology, Electrophoresis, and Multiplex Western Blotting

After sample concentration, electrophoresis using Criterion TGX Stain-Free Precast Gels in combination with multiplex fluorescent western blotting can be used to verify the presence of EVs in pooled SEC fractions and possibly their contamination level. Electrophoresis using Stain-Free technology is superior to other electrophoretic techniques since it allows the near-immediate visualization of proteins at any point during electrophoresis and western blotting (Gürtler et al. 2013). A large panel of antibodies is available for EV research, and multiplex analysis can maximize the data output from precious EV-containing samples. After protein transfer and membrane blocking, while typically not recommended for western blot experiments, the membrane can be cut horizontally into two pieces (at about 45 kD), which allows each membrane piece to be probed with multiple primary antibodies, for example, a pool of three primary antibodies per membrane piece for a total of six targets.

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