



Isolation and quantification of human urinary exosomes using a Tween-20 elution solvent from polyester, capillary-channeled polymer fiber columns

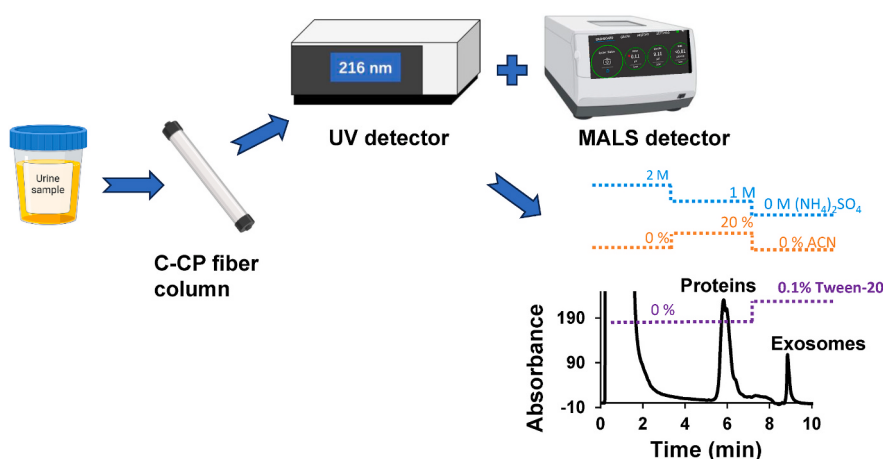
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HIGHLIGHTS

- Exosomes are isolated from human urine using an HIC protocol on C-CP fiber columns.
- Range of the exosome elution solvents has been expanded to include 0.1 % Tween-20.
- Use of this level of detergent poses no observable perturbations to the vesicular structures.
- Reductions of protein content of >99 % is realized for exosomes isolated from urine.
- Process throughput and repeatability bode well for scale-up to the preparative scale.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Exosomes, a subset of extracellular vesicles (EVs), are a type of membrane-secreted vesicle essential for intercellular communication. There is a great deal of interest in developing methods to isolate and quantify exosomes to study their role in intercellular processes and as potential therapeutic delivery systems. Polyester, capillary-channeled polymer fiber columns and spin-down tips are highly efficient, low-cost means of exosome isolation. As the methodology evolves, there remain questions as to the optimum elution solvent for specific end-uses of the recovered vesicles; fundamental biochemistry, clinical diagnostics, or therapeutic vectors.

Results: While both acetonitrile and glycerol have been proven highly successful in terms of EV recoveries in the hydrophobic interaction chromatography workflow, many biological studies entail the use of the non-ionic detergent, Tween-20, as a working solvent. Here we evaluate the use of Tween-20 as the elution solvent for the recovery of exosomes. A novel 10-min, two-step gradient elution method, employing 0.1 % v/v Tween-20, efficiently isolated EVs at a concentration of $\sim 10^{11}$ EV mL⁻¹ from a 100 μ L urine injection. Integration of absorbance and multi-angle light scattering detectors in standard HPLC instrumentation enables a comprehensive single-injection determination of eluted exosome concentration and sizes. Transmission electron microscopy

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verifies the retention of the vesicular structure of the exosomes. The micro-bicinchoninic acid protein quantification assay confirmed high-purity isolations of exosomes (~99 % removal of background proteins)

Significance: The effective use of Tween-20 as an elution solvent for exosome isolation/purification using capillary-channeled polymer fiber columns adds greater versatility to the portfolio of the approach. The proposed method holds promise for a wide range of fundamental biochemistry, clinical diagnostics, and therapeutic applications, marking a significant advancement in EV-based methodologies.

1. Introduction

Extracellular vesicles (EVs) are nanometer-sized, membrane-enclosed vesicles secreted by cells, containing a diverse cargo of bioactive molecules, including proteins, lipids, messenger RNAs, and DNA, all reflecting their cells of origin [1,2]. More specifically, exosomes, a subset of EVs having sizes ranging from 30 to 150 nm, are secreted by almost all cell types and are found in most body fluids. They serve as messengers relaying cellular information and function in several intricate physiological and pathological processes [3,4]. Exosomes also carry distinct cell-specific surface biomarkers, allowing their biological origins to be determined. Notably, exosomes are extremely stable and plentiful in biological fluids such as urine, making them ideal for non-invasive sampling, with their cargo and outer-vesicle protein composition providing diverse access as possible biomarkers [5]. Increased secretion of exosomes in numerous pathological states, such as cancer, indicates that exosomes may be useful for diagnostic purposes [6]. Moreover, exosomes actively engage in intercellular communication, effecting the transfer of cargo to target cells and therefore serving as vital disease mediators [7]. This same basic concept of targeted cargo delivery is the basis for the rapidly evolving use of exosomes as vectors for biotherapeutics delivery [8,9].

As the field moves towards using EVs as diagnostic biomarkers [10], drug delivery vehicles [9], and therapeutic agents in their own right [11], it is imperative to develop precise and efficient exosome isolation and quantification methods. As a wide variety of exosome isolation methods are available to affect fundamental biochemistry, clinical, and large-scale processing, it is essential to recognize that no universally applicable method exists. The selection of an isolation approach should be dictated by the subsequent characterization and intended use of the exosomes/EVs. A key consideration of exosome isolation protocol includes yield (microvesicles per unit volume of primary matrix), purity (EVs per mass of residual protein in the isolate), and the preservation of their physical and chemical integrity. Ultracentrifugation (UC), the most widely employed method, presents challenges in terms of cost and time, often requiring lengthy centrifugation procedures ranging from 2 h to overnight to remove contaminants and large vesicles, still yielding isolates with appreciable protein carryover [12,13]. Ultrafiltration, a centrifugation-based method, is prone to co-purifying protein aggregates, lacking time efficiency, and may result in the significant loss of exosomes, particularly when isolating from small fluid volumes due to the absorption of vesicles to the membrane [14]. Alternative exosome isolation strategies, such as size exclusion chromatography [15], immunoaffinity-based capture [16], and polymeric precipitation [17], are associated with limitations including low throughput, higher costs and the potential to induce morphological changes.

With the goal of addressing the limitations of other exosome isolation methods, Marcus and coworkers have developed a novel cost-effective approach based on hydrophobic interaction chromatography (HIC) using capillary-channeled polymer (C-CP) fiber stationary phases [18–23]. The fibers, melt-extruded from commodity polymers (nylon 6, polypropylene, and poly(ethylene terephthalate) (PET)), feature a distinctive cross-sectional profile having "legs" that interdigitate when packed to form massive numbers of open (1–4 μm), parallel channels. This configuration, coupled with nonporous surfaces (on the size scale of proteins and EVs) that inhibits intraphase solute diffusion, allows for high-speed separations ($\sim 100 \text{ mm s}^{-1}$) without typical mass transfer

limitations of common porous-bead chromatographic phases [24]. These characteristics make the fiber-based columns promising alternatives for exosome isolation in comparison to those traditional methodologies. The method employs both high-performance liquid chromatography (HPLC) column and micropipette spin-down tip formats allowing for solid-phase extraction, with a specific focus on analytical-scale processing. This approach has been successfully employed to extract exosomes from a variety of complex biofluids, including urine, saliva, blood serum, cervical mucus, cell culture media, and plants [18,21,25]. Impressively, the technique yields high concentrations of EVs, reaching levels as high as $7 \times 10^{12} \text{ EV mL}^{-1}$, with purities that surpass community standards in all cases, and from sub-milliliter initial sample volumes [23,26,27]. Furthermore, the method demonstrates remarkable removal efficiency ($\sim 95 \%$) of contaminating proteins and lipoproteins from matrices as complex as human serum [20,28].

The core aspect driving exosome isolation in the C-CP platform relies on the use of an organic modifier-assisted HIC solvent system. Previously demonstrated organic solvents (e.g. acetonitrile (ACN) and glycerol) have been proven effective in yielding high concentrations of pure EVs from a variety of biofluids and cell culture media [23,26]. However, both solvents present unique challenges that limit the comprehensive characterization and utility of the isolated EVs. The utilization of high concentrations of ACN (50 %) could potentially impact the long-term stability of EVs, though the ability to simply remove excess ACN by ambient evaporation yields EVs in a virtually pristine PBS matrix for characterization by LC-MS, for example. Glycerol, on the other hand, possesses cryopreservative properties that support long-term storage of EVs, yet it may interfere with post-isolation characterization, particularly by blocking access to surface marker proteins that are crucial for immune and flow cytometry-based assays [29]. In this case, ultrafiltration is effective for solvent removal, but involves another processing step. In pursuit of an improved HIC-assisted solvent for long-term EV preservation and perhaps greater overall versatility, an alternative approach employing Tween-20, a non-ionic detergent, has been previously explored by this group [27]. That effort demonstrated the potential to isolate high-purity EVs from suspension-adapted human embryonic kidney (HEK-293) cells while remaining compatible with subsequent immuno-characterization methods. However, as reported in numerous studies, the use of high-concentrations of detergent may disrupt protein-protein and lipid-lipid interactions in EV membranes, and potentially compromising their structural integrity [30–32]. Moreover, elevated concentrations of Tween-20 may not be suitable for certain applications, such as mass spectrometry (MS) proteomic workflows [33]. Therefore, a more detailed evaluation of Tween-20 as an elution solvent was undertaken towards achieving high throughput isolations of high-purity exosomes while preserving their structural integrity.

This effort presents a low-cost, efficient, and gentle HPLC-based isolation protocol of exosomes from human urine utilizing a Tween-20 based HIC methodology on PET C-CP fiber columns. Human urine, a target sample biomatrix in various assays, is readily available and does not require prior concentration or centrifugation steps before injection onto the fiber column. Filtration with a 0.22 μm membrane effectively removes larger contaminants, such as cells, preparing the sample for injection. A step gradient, first to remove matrix proteins and then elution of the exosomes by Tween-20, was employed in the HIC

separation to affect recovery and purification. Exosome quantification relied on in-line optical absorbance detection and the generation of response curves prepared from exosome standards. The integration of standard HPLC absorbance and multi-angle light scattering (MALS) detectors enables quantification and size determination; offering an automated, high-throughput approach for purification and characterization. In contrast, most commonly used manual processing methods, such as centrifugation, require off-line characterization on completely separate platforms, potentially introducing operator-dependent variability and potentially impacting the purity of the collected fractions [34]. Verification of morphological integrity of the vesicles was conducted by transmission electron microscopy (TEM) imaging. The micro-bicinchoninic acid (micro-BCA) protein assay was performed to evaluate isolate purity. The Tween-20 elution flow rate was optimized to achieve high-throughput, high-purity separations. Finally, the method's reproducibility was assessed, incorporating an inter-column washing step to enhance EV purification processing. This methodology holds promise for advancing EV research, offering a reliable and reproducible approach to urine-derived exosome isolation.

2. Materials and methods

2.1. Sample preparation

Ultrapure grade ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$ was purchased from thermo scientific (Waltham, MA). Tween-20 was obtained from Anatrace (Maumee, OH). HPLC-grade acetonitrile (ACN) was purchased from VWR Chemicals (Radnor, PA). Deionized (DI) water was obtained from Elga PURELAB flex water purification system (18.2 M Ω -cm) (Veolia Water Technologies, High Wycombe, England). Gibco phosphate buffered saline (PBS) solution 10X pH 7.4 (ThermoScientific, Waltham, MA) was diluted in DI water to prepare a 1X solution which served as the stock PBS for further mobile phase solvent preparation. Lyophilized exosome standards derived from human urine of healthy donors were purchased from HansaBioMed Life Sciences (Tallinn, Estonia). These standards have a nominal as-constituted concentration of 1.4×10^{12} particles mL⁻¹. To prepare the exosome standards for further characterization and quantification studies, the lyophilized exosomes were reconstituted using 100 μ L of DI-H₂O, following the manufacturer's instructions. It is essential to note that the exosome stock used in this study was not designated as certified reference material, nor is an assay provided as to the absence of residual proteins (i.e., purity values) provided by the supplier. Fresh morning urine was collected from a healthy, consenting donor. In consideration of the anticipated sizes of the target analyte exosomes falling within the range of 30–150 nm, a preliminary filtration step employing a 0.22- μ m filter was implemented to eliminate macroscopic debris and larger impurities from the urine samples. While low-speed centrifugation could remove cellular debris, that method would entail an added manipulation of the sample and potentially result in losses of the target EV. The urine sample was filtered using 0.22 μ m PES (polyethersulfone) membrane syringe filters, without any additional manipulations, to obtain the sample required for this study.

2.2. Instrumentation

Chromatographic analysis was conducted using the Dionex Ultimate 3000 HPLC system, consisting of the LPG-3400SD quaternary pump and MWD-3000 UV–Vis absorbance detector from Thermo Fisher Scientific (Sunnyvale, CA, USA). The Chromeleon 7 software system was employed to control the HPLC system. A UV detection wavelength of 216 nm was used with the post-column UV–Vis detector during the analysis. A 1 mL HENKE-JECT Tuberculin syringe (Tuttlingen, Germany) and a 100 μ L loop were used for sample injection.

In-line with the HPLC flow, following the absorbance detector, size determination of isolated exosomes was carried out via multi-angle light scattering (MALS) using a DAWN (Wyatt Technology, Goleta, CA)

detection system controlled by ASTRA software. In a very recent communication [35], Wysor and Marcus have provided a very detailed description of this particular coupling, factors affecting exosome sizing and number density determinations, and the excellent quantitative agreement between absorbance detection and MALS-determined quantification. Particle eluates from the HPLC HIC elution passed through the MALS detector to determine the RMS radii, with the radii multiplied by 2 to get the approximate diameter of the eluted vesicles. For the MALS measurements, the parameters were configured as follows: the sphere real refractive index (RI) was set to 1.51 (per the instrument manufacturer's guidance for EV determinations), the refractive index of the solvent was adjusted to 1.336, and the specific refractive index increment (dn/dc) was set to 0.1850, a value commonly employed for proteins. Particle sizing was determined using the Zimm approximation, consistent with other applications involving EVs [36,37].

2.3. Construction of C-CP fiber columns

The assembly of tri-lobal, polyester (PET) C-CP fiber columns was accomplished using melt-extruded fibers obtained from Universal Fibers (Bristol, VA), and has been described previously [38]. PET C-CP fibers can function as the stationary phase, either integrated into HPLC columns [39] or as short segments, approximately 1 cm in length, mounted on the end of micropipette tips for solid-phase extraction (SPE) [40]. For this study, the PET C-CP fiber microbore column format was prepared by pulling 8 rotations of fibers (equating 448 single fibers) through a 30 cm long polyether-ether-ketone (PEEK) tube having an inner diameter of 0.76 mm (IDEX Health & Science LLC, Oak Harbor, WA). This number of fibers is used to achieve an interstitial fraction of ~ 0.6 [41]. Columns with such dimensions can accommodate dynamic loads of $\sim 5 \times 10^{12}$ exosomes [23], achievable within less than 10 min. After packing, the fiber column was flushed with DI-H₂O, ACN, and then DI-H₂O again at a flow rate of 0.5 mL min⁻¹ until a stable absorbance baseline was achieved with the UV–Vis detection at 216 nm. Once the columns were cleaned, they were stored at ambient conditions for further use. Microextraction tips are assembled in a similar manner, but using a more pliable 0.8 mm i.d. fluorinated ethylene propylene tubing [40]. Segments of 1.5 cm length, having a fiber bed of 1 cm, are affixed to the end of commercial 200 μ L low-retention micropipette tips with a drop of household adhesive. The primary C-CP fiber tip is placed inside of a 1 mL micropipette tip and secured within a 15-mL conical tube using an adapter-modified cap, facilitating SPE in a spin-down mode with a tabletop centrifuge.

2.4. Exosome isolation and quantification methods

The standard HIC elution strategy for the isolation of exosomes on PET C-CP fiber columns is very well established, to date using ACN and glycerol as the organic-modifier elution solvents [18,23]. The basic elements of the HIC method include sample injection (20–100 μ L) into the loading solvent of 2 M $(\text{NH}_4)_2\text{SO}_4$, wherein high polarity and ionic species pass through the column and proteins and exosomes are retained on-fiber. This loading step is performed for 5 min though could be reduced in time if desired. Following column loading, a step gradient is affected, where the salt content is reduced to 1 M $(\text{NH}_4)_2\text{SO}_4$, also containing 20–30 % of the organic modifier in 1X PBS for a period of 3–4 min, with the choice in modifier based on the ultimate exosome elution solvent; glycerol or ACN to this point. This solvent composition is sufficient to release free (matrix) proteins from the fiber surface, leaving the immobilized exosomes. The most readily implemented exosome elution sequence involves a single step where the salt content is reduced to zero, and the organic modifier solution concentration is increased to 40–50 % in PBS for a period of 3–5 min. Following the exosome elution, a column washing/regeneration cycle of 90 % ACN solvent (in PBS) is applied for 5 min between subsequent analyses. The exosome isolation and column cleaning processes employ a mobile

phase volume flow rate of 0.5 mL min⁻¹.

Key in the development of the applied step gradient methods has been the use of standard (linear) gradient elution protocols to establish the necessary solvent strengths for the sequential removal of the background proteins and the target EVs. In the evaluation of Tween-20 as an elution solvent, a 20 % ACN in PBS protein elution step is employed in all cases prior to the ultimate exosome elution gradient. The ACN organic modifier is chosen here as it presents fewer potential problems with regards to solvent mixing than glycerol. Following assessment of gradient elution characteristics, step gradients were then evaluated and used as the default process.

Very early studies of the HIC isolation strategy demonstrated the efficacy of absorbance detection and quantification through application of simple Beer's Law based on serial dilutions of EV standards [18,19,27, 42]. Eluting species were monitored at 216 nm for UV-Vis absorbance (actually microvesicle scattering). The gradient baseline absorbance was obtained from a chromatogram taken while injecting a blank PBS injection and subtracted from the subsequent gradient exosome isolation chromatograms. The integrated area of the exosome elution peak calculated from Chromleon 7 software represents the quantity of eluted species. To quantify the Tween-20 eluted exosomes, the same isolation procedures were applied to the human urinary exosome standards at five different concentrations (4.3×10^{10} – 7×10^{11} exosomes mL⁻¹) to generate a post-column response curve. A 100 μ L injection volume was used for each sampling, and the response curve was constructed from triplicate injections/separations of each. Based on the detector response of elution bands, analytical fractions were collected following exit from the MALS cell and stored for further characterization (e.g., TEM, protein purity assay).

2.5. Transmission electron microscopy (TEM)

The physical identification and structural integrity of isolated exosomes was characterized using TEM imaging using a Hitachi HR7830. The TEM sample preparation included 10 μ L of each recovery from the human urine sample isolate being carefully deposited onto EM-grade 200 mesh formvar/silicon monoxide coated copper grids and allowed to incubate at room temperature for 20 min. Following incubation, the excess sample liquid was removed from the grids by wicking using a paper towel. The immobilized vesicles were immediately subjected to fixation using a 2 % paraformaldehyde solution at room temperature for 5 min. Surplus paraformaldehyde was removed from the grids using a paper towel. The grids were then gently washed with DI-H₂O for 5 min, ensuring the elimination of any residual reagents. Staining of the exosomes immobilized on the grids was achieved by applying a filtered 1 % uranyl acetate solution at room temperature for a period of 1 min. Following the staining process, excess staining solution was thoroughly removed, and the prepared grids were once again washed to guarantee the removal of any excess staining reagent. Lastly, the prepared TEM grids were allowed to dry overnight within a sterile cell culture dish. To ensure the absence of moisture during the drying process, a desiccator was employed, with the procedure carried out at room temperature. This detailed protocol provides a standardized and reproducible method for the TEM imaging of EVs, ensuring the preservation of their structural integrity and enabling accurate morphological analysis.

2.6. Exosome isolate purity assessment via micro BCA protein assay

A standard micro BCA assay was employed to determine the residual protein concentration in the Tween-20 isolated exosome fractions. To be clear, while a value of zero residual protein would be ideal, the micro BCA determination will also register positively for the proteinaceous species incorporated in the exosome vesicular structure. The suggested practical target for describing exosome isolates as being of *high purity* is a value of 3×10^{10} EV μ g⁻¹ protein [43,44]. In the micro BCA assay, a 100- μ L aliquot of the sample was combined with 50 μ L of PBS and 150

μ L of the assay working reagent. Subsequently, the 96-well plates were covered and incubated at 37 °C for 2 h. The absorbance at 562 nm was measured utilizing a Synergy H1 Hybrid plate reader. For accurate protein quantification in unknown samples, a standard curve was generated using bovine serum albumin (BSA) standard solutions. The average absorbance reading of the blank (PBS) was subtracted from the standards and the EV samples. All measurements were conducted in triplicate, ensuring the reliability of the results.

3. Results and discussion

3.1. Chromatographic isolation of exosomes

Our prior investigation demonstrated the successful isolation of exosomes from the supernatant of an HEK-293 cell culture utilizing 1 % Tween-20 as the elution solvent in a PET C-CP fiber spin-down tip format [27]. The physical characteristics, encompassing shape and size distribution of the isolated exosomes, were confirmed using TEM and MALS, respectively. The method was particularly efficient as 10^{12} EVs could be obtained from only 100 μ L aliquots of milieu, with the exosome purity of the isolates exceeding the target values by 1–2 orders of magnitude in all cases. Despite this success, it is crucial to consider the fact that micelle-forming detergents can induce membrane solubilization [45, 46]. Ionic detergents at much lower concentrations (e.g., 0.01 % sodium dodecyl sulfate (SDS)) can lead to membrane disruption, impacting the exosomes' physical morphology and bioactivity [30]. While non-ionic detergents, such as Tween-20, are deemed milder in comparison to ionic detergents like SDS, higher concentrations may disrupt lipid-lipid and lipid-protein interactions, posing a risk to the phospholipid bilayer membrane of the isolated exosomes [32,47]. Therefore, a more thorough evaluation is necessary to utilize minimal concentrations of the detergent to affect maximum EV recovery from the biofluid samples while maintaining the morphology and physical integrity of the isolated vesicles.

In this study, the optimization of Tween-20 as an elution solvent for exosomes isolated from human urine is carried out using the PET C-CP fiber column format on an HPLC platform. This approach allows use of a conventional linear gradient for a controlled and gradual change in solvent composition following the initial release of the residual proteins using the 20 % ACN in PBS step. In this case, a linear gradient of 0–0.5 % Tween-20 was applied for the separation as this was anticipated based on the previous spin-down tip experiments and in consideration of potential vesicle disruption at higher Tween concentrations.

A representative chromatographic profile of the gradient elution is presented in Fig. 1a. The large absorbance transient occurring in the 0–2 min time frame reflects the injection of the pre-filtered (with a 0.22 μ m PES filter) urine sample, as small organic molecules and ionic species elute in the injection/loading volume [18]. As depicted, a step gradient dropping the ammonium sulfate concentration to 1 M, while adding the 20 % ACN in PBS is initiated at $t = 5$ min. The significant peak between ~6 and 9 min corresponds to the elution of proteinaceous materials from the fiber column. Finally, at $t = 10$ min, the salt and organic modifier concentrations are dropped to "0", and the linear Tween-20 solvent gradient (0–0.5 % v/v in PBS in 10 min) to elute the hydrophobic exosomes from the fiber column surfaces. The anticipated elution band between $t = \sim 11$ and 13 min corresponds to Tween-20 concentrations ranging from 0.08 to 0.16 % in PBS. Different from the protein elution band, which has an abrupt onset due to the step nature of the solvent change, the exosomes evolve over a range of solvent strengths which reflects a range of surface interaction strengths; reflecting the heterogeneity of exosome/EV sizes and/or surface protein composition.

Based on the elution solvent composition from the linear gradient in Fig. 1a, a step gradient program was investigated to shorten the elution time and simplify the elution procedures, as presented graphically in Fig. 1b. In this case, the step gradient times were shortened to minimize processing time and solvent consumption. As in all HIC-based EV

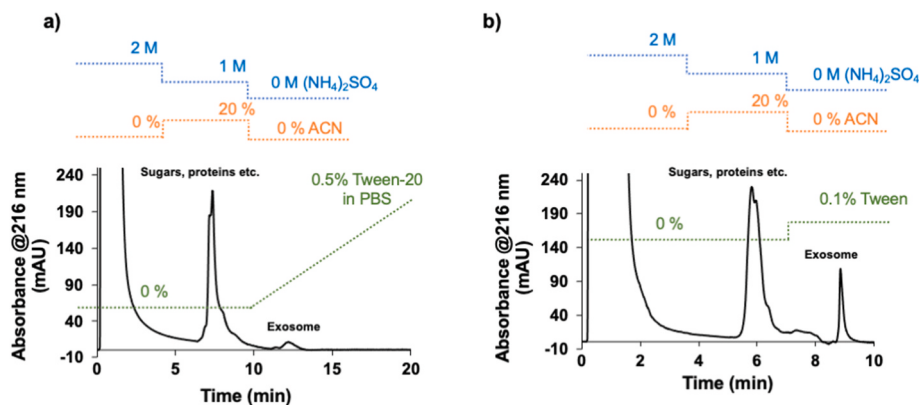


Fig. 1. HIC chromatograms of urinary exosome isolation using PET C-CP fibers employing different elution programs. **a)** 10 min linear gradient from 0 to 0.5 % (v/v) Tween-20 in PBS and **b)** step gradient of 0.1 % (v/v) Tween-20. A protein elution step (1 M $(\text{NH}_4)_2\text{SO}_4$ and 20 % ACN) was performed in each case before the isolation of EVs. 100 μL urine were injected with the flow rate applied 0.5 mL min^{-1} .

separations, salts and ionic small molecules are manifested in the unretained injection peak ($\sim 0\text{--}2$ min), while proteins and other non-polar molecules are eluted with the initial gradient step (1 M ammonium sulfate: 20 % ACN) initiated at $t = 4$ min. While the previous spin-down tip method employed 1 % Tween-20, clearly that concentration is not required based on the linear gradient results. Indeed, it is reasonable to use as low of a value as practical, to alleviate potential problems. Therefore, exosomes were eluted employing a 0.1 % v/v Tween-20 gradient step at $t = 7$ min, appearing in the chromatogram as a sharp band from ~ 8.5 to 9.5 min. The application of a total 10-min gradient, demonstrates superior efficiency in comparison to the linear gradient (Fig. 1a), resulting in shortened processing time, sharper elution bands, improved resolution between the protein and EV eluates, and higher exosome yields. Specifically, the integrated absorbance for EV elution in the linear gradient was $9.1 \text{ mAU}\cdot\text{min}$, while the value for the step gradient was $18.7 \text{ mAU}\cdot\text{min}$, a two-fold improvement in recovery. Consequently, the 0.1 % Tween-20 step gradient was adopted to advance the development of the Tween-20-based HIC exosome isolation strategy.

3.2. Characterization of isolated exosomes

After assessing basic chromatographic conditions which affect the elution of exosomes, the next question becomes whether or not the particles retain their vesicular structure and some assessment as to the obtained particle size distribution. Transmission electron microscopy (TEM) plays a critical role in the characterization of the isolated EVs by providing high-resolution images of morphological attributes, allowing inferences of EV health and likely retention of important cargo [48,49]. Fig. 2 presents representative TEM micrographs of exosomes collected

post-column following elution with 0.1 % Tween-20 solvent. Fig. 2 presents representative TEM micrographs of multiple EVs collected post-column following elution with 0.1 % Tween-20 solvent. Fig. 2a offers a broader view at lower magnification ($20,000\times$ vs $100,000\times$ in Fig. 2b), revealing multiple discrete yet structurally intact vesicles within the characteristic size range of exosomes. Fig. 2b showcases one of many vesicles, consistent with the typical size range of exosomes (30–200 nm). It is important to note that the membrane structure suggests that the 0.1 % Tween-20-based method does not compromise the structural integrity of exosomes and that the captured and eluted exosomes are preserved intact. The membrane structure suggests that the 0.1 % Tween-based method does not compromise the structural integrity of exosomes and that the captured and eluted exosomes are preserved intact. Indeed, ELISA assays for the CD9 and CD81 tetraspanin proteins in the previous spin-down processing with 1 % Tween-20 showed that those proteins were retained in the vesicular membranes [27]. It must be reiterated, though, that full verification of vesicle integrity requires some assessment of the cargo (e.g., miRNA) loading; those studies are planned for the near future.

While TEM provides crucial morphological information, its ability to verify EV sizing is limited by simple counting statistics. To address this, size verification of isolated fractions was performed using the multi-angle light scattering (MALS) detector, which inherently offers particle/vesicle sizing and number density. A more commonly used method, nano particle tracking analysis (NTA) is susceptible to providing operator- and environmental-dependent results with inconsistencies in precision and accuracy [50]. MALS on the other hand can provide high levels of precision and has been employed in many studies of EV size determinations [27,51,52]. Moreover, the integrated approach of combining absorbance and MALS detectors on a standard HPLC platform

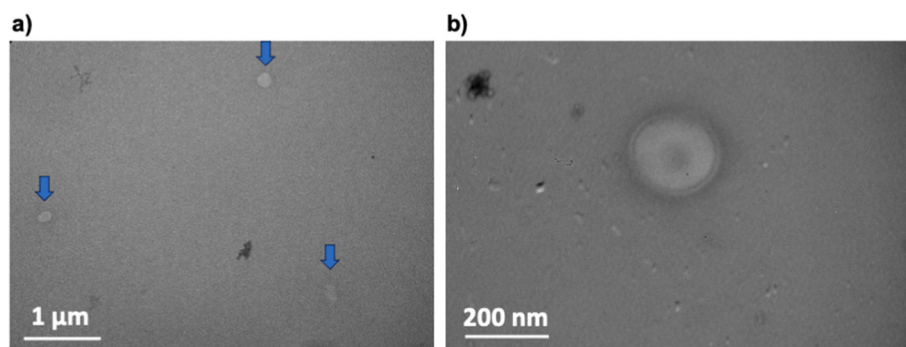


Fig. 2. Transmission electron microscopy (TEM) analysis of urinary exosomes eluted from 0.1 % (v/v) Tween-20 **a)** image at low magnification ($20,000\times$) of multiple EVs (scale bar, 1 μm) and **b)** image at higher magnification ($100,000\times$) of a singular EV (scale bar, 200 nm).

allows for a comprehensive one-injection determination of the concentration and sizes of eluted exosomes [35]. As illustrated in Fig. 3, MALS-determined size distributions of urine-derived EVs show that 50 % of the population falls in the range of ~ 100 and 380 nm in diameter. This aligns well with the previously determined size distribution of C-CP tip isolated EVs using Tween-20 from cell culture media, where the average diameter of EVs found to be 249 nm across the cell culture incubation time period [27]. Clearly, there is a discrepancy between the MALS-derived sizing and the TEM results of the individual vesicles. It is to be noted that the average size of urinary EVs from Tween-20 isolation and MALS determination are higher than the sizes determined with NTA and organic solvent (ACN)-based isolation in previous studies of this group [42]. There are questions as to the absolute values produced for the Tween-20 isolation as one variable in the MALS calculation has to do with the refractive index of the “particle” which here also includes a likely layer of detergent coverage. That finite layer itself may effect scattering responses.

There are indeed many variables in the MALS sizing of EVs. Another important factor to consider is the selection of instrumentation calibration bead materials and sizes. Indeed, the decision of reference particles and the assigned refractive index is a topic of much discussion. While polystyrene beads (RI = 1.59) are commonly employed for EV detection, silica nanospheres (RI = 1.51) were utilized in this study based on recommendations by the instrument manufacturer. This choice has also been suggested in other EV scattering studies as having a value closer to those of the bionanoparticles [53,54]. In fact, Gardiner and co-workers [54] have presented a method to determine the RI of EVs based NTA scattering measurement, presenting values ranging from 1.38 to 1.59, varying with host cell type. The impact of different calibration particles and elution solvents, such as acetonitrile (ACN), glycerol, and Tween-20, on the apparent particle sizes of EVs measured by MALS warrants investigation in future studies.

3.3. Quantification of the isolated exosomes

Prior investigations have established a direct correlation between the concentration of exosomes in solution and the observed amount of light scattering (exosomes are nanometer-sized vesicles which are expected to more efficiently scatter than to absorb 216 nm photons) as manifest in a standard absorbance measurement [18,19,42]. This relationship is

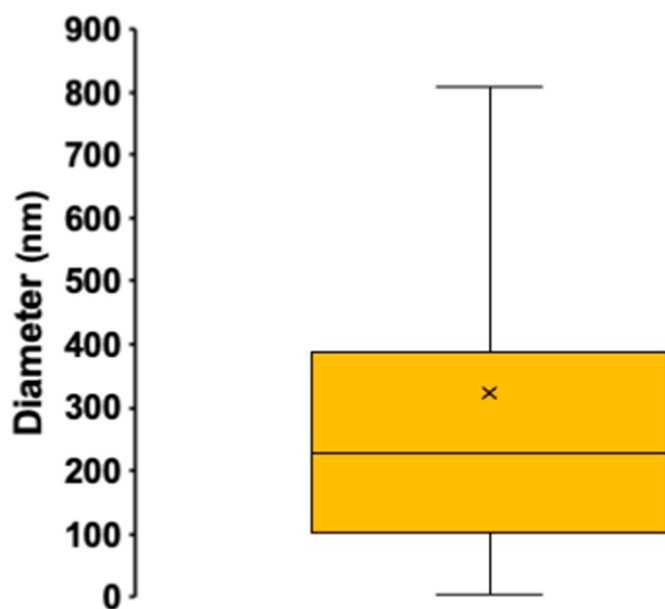


Fig. 3. Post column size determination of the isolated EVs using multi-angle light scattering (MALS) detection.

quantitatively expressed through Beer's law, wherein the quantity of exosomes is proportional to the absorbance peak area. It is essential to note that the exosome standard stock utilized in this study does not represent a thoroughly characterized or standardized reference material. Instead, it consists of EVs with known concentrations, with the concentration determined by nanoparticle tracking analysis (NTA) disclosed by the manufacturer. Consequently, the characterization of EV concentration in this study is limited to general approximations. To obtain a quantitative relationship between exosome concentration and the integrated elution absorbance peak area, six different concentrations of the commercial human urine derived exosome standards were applied to generate a standard response curve. Different concentrations of commercial exosome standards (in a range of 4.3×10^{10} - 7×10^{11} exosomes mL^{-1}) were obtained through serial dilution of the primary stock solution, with 100 μL injection volumes loaded onto the column and eluted using the step gradient elution program with 0.1 % Tween-20. The data with regression statistics are presented in Fig. 4, in terms of the injected exosome concentrations and the integrated absorbance values. The agreement aligns well ($R^2 = 0.9892$) with the linear response function, with the range of triplicate values in most cases falling within the plotted symbols. Subsequently, a 100 μL human urine sample was injected onto the column and eluted using the same gradient program protocol to determine the concentration of exosomes in the sample. Using the HIC protocol, the urine injection yields an average integrated absorbance of 22.50 ± 0.02 $\text{mAU} \cdot \text{min}$ for triplicate measurements, representing an EV recovery concentration of 1.07×10^{11} EV mL^{-1} .

3.4. Purity assessment of recovered exosomes via micro BCA assay

A major challenge in all forms of exosome isolation, regardless of the end application, is achieving high purity exosome isolates; i.e., minimizing protein carryover [43,55]. Previous efforts in this laboratory have shown excellent performance in the case of spin-down tip isolations, regardless of the elution solvent [21,25,27]. Indeed, direct comparison to more established methods has proven the efficacy of the approach [26]. What has not been established is the purity levels obtainable via the HPLC column methodology and so the purity in terms of protein content of the recovered EV fractions from the human urine samples was determined. The micro BCA protein assay employs a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein content [44].

Fig. 5 represents the micro BCA assay-determined protein concentrations in the raw urine sample, the eluted EV fraction from the raw urine, the reconstituted commercial exosome standard, and the eluted

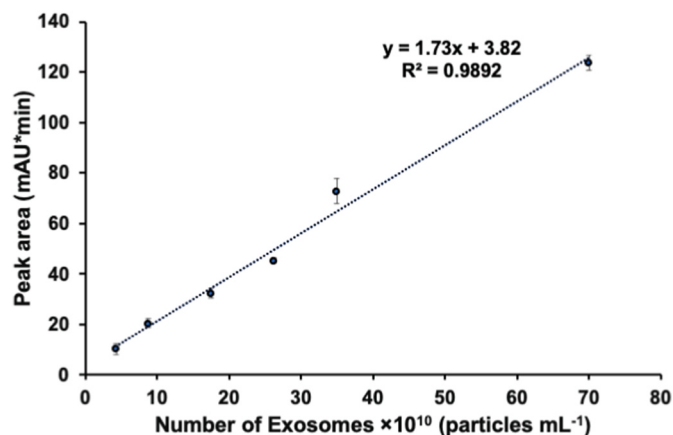


Fig. 4. Response curve generated for the dilution of a human urine-derived exosome standard prepared with a 100 μL injection volume. Triplicate injections were performed for each sample volume.

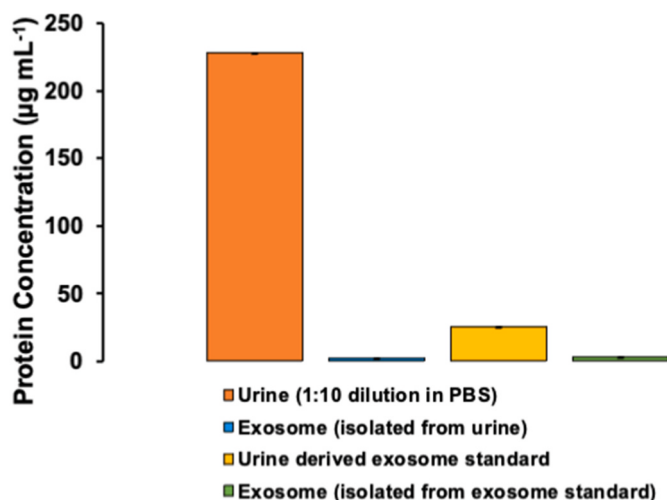


Fig. 5. Micro BCA assay-determined protein concentrations in the raw urine sample, EV fraction eluted from the raw urine, the commercial exosome standard, and the EV fraction eluted from the standard, respectively.

EV fraction from that specimen, respectively. Not surprisingly, the raw human urine (undiluted) sample yielded a concentration value that was beyond the extent of the linear response of the assay; therefore, a dilution of the sample was required (1 : 10 in 1X PBS). Clearly seen, the protein content in the diluted raw urine sample is reduced a significant amount in the eluted EV fraction from $228 \mu\text{g mL}^{-1}$ to $2.1 \mu\text{g mL}^{-1}$, with the lower value in fact equating to the limit of detection (LOD) for the micro BCA assay ($\text{LOD} = 2 \mu\text{g mL}^{-1}$). The extent of the residual protein removal was found to be the same in the case of commercial exosome standard, where the isolation reduces the protein concentration from $25 \mu\text{g mL}^{-1}$ to $3 \mu\text{g mL}^{-1}$. It is to be noted that a high level of precision was observed for the protein assay, with the variability in each triplicate measurement set being $<3\%$ RSD. The determined protein concentrations in the EV eluates occurring in the vicinity of the LOD underscores both the efficacy of the C-CP fiber processing method and limitations in accurately quantifying the obtained EV purities. That said, the change in total protein content from the diluted raw sample to the eluted fraction corresponds to a $>99\%$ reduction in background proteins. As mentioned earlier, the commercial exosome standard is not a certified reference material and its starting material purity value is not provided by the manufacturer. Even so, the reduction of residual protein content of $>10\text{X}$ is further support of the efficiency of the C-CP fiber processing. It is noted that the most abundant protein in urine, Tamm-Horsfall protein (THP) can present challenges in the isolation of EVs from urine samples [56]. THP tends to form high molecular weight filamentous aggregates, which can entrap EVs, resulting in lower yields and thereby reducing their availability for analysis [57,58]. Although the specific types of proteins removed here are not explicitly identified, it may be that the organic modifier-assisted protein elution step in the C-CP HIC method may disrupt potential aggregates. Ultimately, the elution protocol effectively removes the vast majority ($>97\%$) proteinaceous materials, including THP, providing EVs with superior yield and purity as presented previously [21,26]. That said, a comprehensive assay of the protein eluate content would be very informative.

In assessing the overall efficacy of any EV isolation method, the crucial metric of interest is the ratio of number of recovered exosomes per unit volume to the protein content within the same volume. In the case of the 0.1 % Tween-20 step gradient HIC method, the purity value obtained for the introduction of raw human urine was $\sim 5 \times 10^{10}$ EVs per μg of total protein recovered. As noted above, based on limitations to the micro BCA methodology, it is reasonable to say that the obtained purity is likely far greater than that value. It is important to note that the purity value obtained from Tween-20 based C-CP column isolation is within a

factor-of-2 of that obtained from C-CP tip isolation employing ACN and glycerol solvents ($\sim 1 \times 10^{11}$ EV μg^{-1} protein) [26], with the differences lying in either the actual urine starting materials or the fact that the column method involves $>10\text{x}$ greater elution volumes (dilution) than the tip processing. In any case, the obtained values exceed the recognized "high purity" standard of $>3 \times 10^{10}$ EVs per μg of protein [26,44].

3.5. Effect of the mobile phase flow rate

Looking towards the practical implementation of the fiber column exosome isolation methodology to exosome purification, the concept of process throughput will be an important feature. Consistent throughout all previous applications of C-CP fiber stationary phases to the separation of protein molecules is the fact that higher mobile phase linear velocities provide higher throughput while also delivering higher resolving powers [59–61]. This phenomena is related to the highly efficient solute mass transport through the open parallel channels formed in between the non-porous polymer fibers [24]. To that end, separations at linear velocities up to $\sim 100 \text{ mm s}^{-1}$ have been applied. To the same end, dynamic binding capacities are not sacrificed at high linear velocities [61]. Therefore, it is reasonable to question the efficacy of using high flow rates in the processing of exosomes via the Tween-20 elution methodology. In three previous studies, while not as pronounced as the benefits in protein separations, it has been consistently true that while lower loading velocities tend to improve the EV binding capacities, higher flow rates yield greater exosome process throughput and recoveries from the fiber columns [62–64].

The primary assessment of the impact of flow rates on the isolation of urinary EVs by the 0.1 % Tween-20 HIC program, variations of the EV elution flow rates (0.3, 0.5, 0.7, and 0.9 mL min^{-1}) were evaluated, as illustrated in Fig. 6 and quantified in Table 1. These flow rates equate to linear velocities of $\sim 24\text{--}72 \text{ mm s}^{-1}$. In this case, the loading flow rate was held constant at 0.5 mL min^{-1} for the $100\text{-}\mu\text{L}$ human urine injections. The precision of the loading process is reflected in part, in the overlap of the bands in the protein elution step. As presented in the table, each of the characteristic quantities is measured with high precision for the triplicate measurements. Qualitatively, the figure illustrates that higher flow rates lead to shorter EV elution times following the onset of the solvent step, indicative of the swifter elution process after release, with the elution peak widths decreasing at the same time. This response is completely consistent with prior investigations utilizing the HIC isolation method for proteins and EVs on C-CP fiber phases [41, 42]. The observed trends in elution characteristics are ascribed to a decrease in longitudinal diffusion (van Deemter B-term) coupled with the absence of solution-solid mass transfer limitations (van Deemter

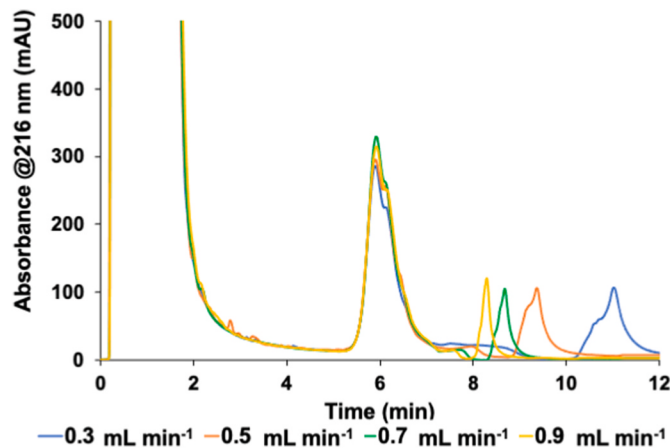


Fig. 6. Characteristic chromatograms for the isolation/recovery of EVs from human urine as a function of elution flow rate. Flow rate varied at 0.3, 0.5, 0.7, and 0.9 mL min^{-1} , respectively. $100 \mu\text{L}$ urine was injected in each case.

Table 1

EV elution peak characteristics from human urine isolation at different elution flow rates. Averages from triplicate measurements with standard deviation (SD) values are presented. 100 μL urine was loaded at a flow rate of 0.5 mL min^{-1} for each injection.

Volume flow rate (mL min^{-1})	Elution time (min) $\pm$ SD	Peak width (min) $\pm$ SD	Peak area ($\text{mAU} \cdot \text{min}$) $\pm$ SD
0.3	11.1 ± 0.05	0.67 ± 0.05	34.03 ± 1.04
0.5	9.4 ± 0.01	0.63 ± 0.08	23.91 ± 1.62
0.7	8.6 ± 0.04	0.43 ± 0.002	16.4 ± 0.52
0.9	8.2 ± 0.05	0.29 ± 0.002	13.3 ± 0.65

C-term). While the peak absorbance values for each of the elution bands are fairly equivalent, Table 1 presents a steady, proportional decline in the integrated absorbances at higher flow rates. In fact, the values are proportional to the corresponding dilution factors linked to the increasing volume flow rates, with the decline actual values ($34.03\text{--}13.3 \text{ mAU} \cdot \text{min}$) suggesting that the recoveries at the higher flow rates are $\sim 35\%$ higher than predicted based on the lowest flow rate. Thus, increased processing throughput at increased flow rates is anticipated in future preparative applications.

3.6. Reproducibility of chromatographic cycles

Another practical concern in all applications of the fiber column exosome purification technology is ensuring the consistent recyclability of columns following elution and washing procedures, though this is particularly true in the context of bioprocessing. Many bioprocessing procedures necessitate some form of clean-in-place (CIP) to eliminate contaminants during analytical separations [65]. Given the strong interaction between exosomes/EVs and the column surface, addressing potential carryover becomes a natural area of concern. Existing literature highlights the challenge of removing biological impurities from porous, silica-based phases, especially when utilizing pure organic solvents such as ACN or methanol [66,67]. The PET fiber column exhibits remarkable stability and reproducibility in the HIC mode, particularly when incorporating a thorough washing protocol. To maintain stability, an inter-injection wash buffer consisting of 90% ACN in PBS was introduced for 5 min . Fig. 7a illustrates seven successive chromatographic cycles of a freshly packed PET C-CP fiber column, each including a washing step, demonstrating the efficacy of the wash buffer in maintaining column performance across multiple cycles. The corresponding EV recoveries from each cycle are depicted in Fig. 7b, revealing that the recovery for the seventh cycle is 83.7% of that observed in the first cycle. The variability in EV elution peak area across these seven replicates is minimal, at 6.85% RSD. Perhaps most importantly, the drop in recoveries is practically observed for just the first three cycles, with the variability over the final four being only 1.31% RSD. Such a high level of repeatability is crucial for future endeavors, and will be an essential

parameter for further evaluation/improvement involving the realm of process analytics or bioprocessing.

4. Conclusions

This study presents a novel HIC-based HPLC method, utilizing Tween-20 as the elution solvent for the isolation of EVs from human urine employing PET C-CP fiber columns. The utilization of a step gradient program enabled rapid processing within 10 min for $100 \mu\text{L}$ samples, which would be typical of biochemical characterization studies and clinical analysis. Notably, the procedure integrates purification, quantification, and size characterization into a single operation, utilizing a HIC chromatographic method on a standard HPLC platform with in-line optical absorbance and MALS detection. TEM micrographs confirmed the structural integrity of the isolated exosomes, although further investigations are necessary to fully validate the vesicular integrity of the Tween-20 based isolations based on analysis of the genetic (e.g., miRNA) cargo. Processing of native human urine yields high-purity separations with remarkable removal ($>99\%$) of background proteins, with presumably high-purity commercial exosome isolates also seeing appreciable levels of latent protein reduction. The method demonstrates reproducibility across multiple chromatographic cycles, while the low-cost PET C-CP fiber ($<\$1$ per analytical column) offers significant benefits in terms of process throughput and materials cost in comparison to commercial HIC column bed materials ($<\$50$ vs. $\sim\$3000$ per liter bed of volume) [68]. The method offers distinct advantages in various automation aspects of commercial HPLC technologies, including auto-sampling, complete cycle programming, in-line detection and characterization (absorbance and MALS), and fraction collection.

While Tween-20 at this low concentration (0.1% v/v utilized in this study) shows promise in avoiding membrane disruption of exosomes, future efforts will be directed towards evaluating the Tween-20 elution strength to ensure the maintenance of both structural and biological integrity in high-throughput separations. A comprehensive characterization of the Tween-20 solvent regarding its suitability for subsequent genomic/proteomic analyses and vector applications is warranted, including comprehensive evaluation of surface (e.g., tetraspanin) proteins and miRNA content. For example, the presence of residual Tween-20 may impede immunochemical labelling, but perhaps not impede subsequent cargo loading processes. The isolation and quantification of EVs from a key biomatrix such as human urine holds significant promise for diverse clinical and biochemical applications, with extension to other matrices such as plasma, saliva, and breast milk (as has been demonstrated using ACN and glycerol solvents) required as well. As a mild detergent-based solvent, Tween-20 holds potential for process scale-up, serving as a valuable tool for harvesting large quantities of EVs for therapeutic applications. In this case, the emergence of Tween-20 as a protective storage solvent that enhances exosome preservation may be a key attribute [69].

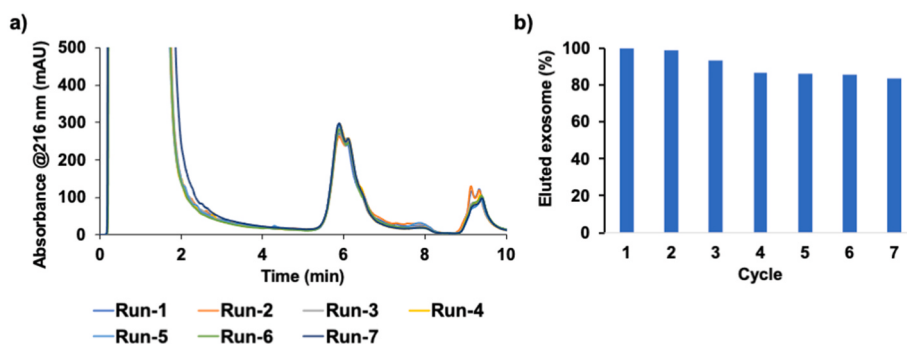


Fig. 7. Relative amounts of eluted EVs (based on absorbance peak areas) for successive chromatographic cycles. **a)** 7 consecutive chromatograms employing the column washing step and **b)** resulting recoveries of EVs as a function of the first injection response. $100 \mu\text{L}$ urine was injected with a loading and elution flow rate of 0.5 mL min^{-1} .

CRediT authorship contribution statement

Md Khalid Bin Islam: Writing – original draft, Visualization, Methodology, Data curation. **R. Kenneth Marcus:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

R. Kenneth Marcus reports financial support was provided by National Science Foundation. R. Kenneth Marcus reports financial support was provided by National Institutes of Health. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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