Published in final edited form as:

Anal Chem. 2016 November 1; 88(21): 10390–10394. doi:10.1021/acs.analchem.6b03348.

Quantification of small extracellular vesicles by size exclusion chromatography with fluorescence detection

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Abstract

Chemical analysis of small extracellular vesicles (sEVs) circulating in body fluids holds potentials in non-invasive diagnosis of diseases and evaluation of therapeutic treatments. However, quantification of sEVs remains a challenge due to lacking of cost-effective analytical protocols. Herein we report a facile method based on size exclusion chromatography with fluorescence detection (SEC-FD) for sEVs quantification. After removal of cells and cell debris, a 0.50-mL sample (e.g. cell culture medium) is incubated with CM-Dil dye to fluorescently label sEVs. The incubation solution is then separated on a SEC column packed with Sepharose CL-4B. The eluent is monitored fluorescently at Ex553nm/Em570nm by using a fluorometer equipped with a 50-μL flow through cuvette. Separation efficiency of the proposed SEC-FD method was evaluated by analyzing 100-nm liposomes and albumin-FITC conjugate. Liposomes were eluted out in less than 6 min, about 10 min before albumin-FITC. A separation repeatability (RSD in retention time) of 1.4% (n=5) was obtained for liposomes. In analysis of cell culture media, linear calibration curves based on SEC-FD peak height versus sEVs concentration were obtained with r^2 value of 0.996. Intra-day quantification repeatability (RSD in peak height) was 3.2% (n=5). Detection limit was estimated to be 2.9×10^7 exosome particles/mL. The proposed assay was applied to the first study of sEVs secretion from TK6 cells cultured in serum-free medium for a culturing period from 1 to 48 hours.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: DSL measurements of the liposome preparation; identification of SEC-FD peaks by DSL, TEM, and protein assay; calibration curve for exosome quantification (PDF)

Notes

The authors declare no competing financial interest.

Keywords

Small extracellular vesicles; Exosomes; Quantification; Size exclusion chromatography; Fluorescence detection; TK6 cells

Cells shed vesicles into extracellular environments. Small extracellular vesicles (sEVs) are classified by size into micro vesicles (100–300 nm) and exosomes (40–100 nm).^{1–3} Current protocols for sEVs isolation/purification are most widely based on differential ultracentrifugation (UC), immune-magnetic isolation, or size exclusion chromatography (SEC).^{4–8} Since these techniques do not discriminate microvesicles from exosomes, most relevant studies include both. Molecular analysis of sEVs circulating in body fluids opens up a new avenue to achieve non-invasive disease diagnosis and treatment evaluation (so called “liquid biopsy”).^{9–12} sEVs in body fluids, including blood, saliva, urine, and breast milk have been detected. Quantification of sEVs has been achieved by methods based on nanoparticles tracking analysis (NTA), tunable resistive pulse sensing (tRPS), ELISA, and high-resolution flow cytometry (hFC).^{13–16} These assays do not only require access to expensive instrumentation and necessitate technical skills, but also involve tedious procedures for sample pretreatments.

The aim of this study was to develop a cost effective protocol for quantification of sEVs. Separation of sEVs from other endogenous components in sample matrix such as lipoproteins and amino acids by size exclusion chromatography (SEC) was investigated. Columns packed with different SEC matrices were evaluated to obtain a fast separation of sEVs with a high recovery. In order to achieve high assay sensitivity, fluorescence detection (FD) was deployed for monitoring SEC eluent. The sEVs peak from SEC separation was characterized by using various spectroscopic and electrochemical methods to confirm its vesicular identity. The SEC-FD method was applied to monitoring sEVs secretion from TK6 cells cultured in serum free medium.

EXPERIMENTAL SECTION

Cell Culture

The human B-lymphoblastoid cell line TK6 was obtained from ATCC. The cells were maintained in RPMI 1640 medium supplemented with 10% horse serum, 2mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO₂. Medium was replaced every 3 days after initial plating. When confluence was reached 80%, cells were washed 3 times with PBS and then incubated in horse serum free medium (i.e. the conditioned culture medium, CCM) before sEVs assay.

Liposome preparation

A liposome kit purchased from Sigma-Aldrich (Catalog Number L4395, St. Louis, MO) was used. The user manual was followed to prepare multilamellar liposomes. Briefly, the lipid mixture was dissolved in $\text{CHCl}_3/\text{CH}_3\text{OH}$. The solution was dried under a stream of nitrogen. The vial containing the lipid film was placed under vacuum for 6 hrs to remove the traces of organic solvent. The dry lipid film was hydrated by adding PBS and vigorously shaking with a vortex mixer for >1 min. The MLV suspension were frozen and thawed for 6 cycles. A single freeze-thaw cycle consisted of freezing for 5 min at liquid nitrogen temperature (-196°C) and thawing for 5 min in a water bath at 65°C . Extrusion of the liposome suspension obtained was carried out with a filtration device (LiposoFast Liposome Factory, Sigma-Aldrich Chemicals) through a porous polycarbonate membrane with a pore size of 100 nm to obtain liposomes of uniform size. Size distribution of the liposomes prepared was measured by dynamic light scattering (DLS). Results are shown in Figure S1.

SEC-FD Set-up

SEC columns of 10 mm $\times$ 10 cm packed with Sepharose CL-4B (Sigma-Aldrich, St. Louis, MO) were in-house prepared. Glass tubes equipped with an on-off valve near the column outlet were used for preparing columns. The outlet of a column was connected with a 50- μL quartz flow-through cuvette (Hellma Analytics) which was placed in a fluorescence spectrometer (Fluoromax-4, Horiba Scientific). The effluent flow rate was controlled by adjusting the valve in column.

SEC-FD Assay

In analysis of cell culture medium, the sample was centrifuged at 300 rpm at 4°C for 10 minutes to remove cells, and then at 10,000 rpm for 30 minutes to remove cell debris (Centrifuge 5810 R, Eppendorf). A portion of the supernatant (0.500 mL) was transferred into a vial and incubated with 2 μL CM-Dil dye (Molecular probes, Eugene, OR) for 30 minutes at 37°C on a rotator (Tube Revolver, ThermoFisher scientific). In analysis of liposome preparations, the sample was diluted and incubated with the dye directly without centrifugation. The incubation solution was loaded onto the SEC column and eluted with PBS/0.2 M NaCl (pH 7.4, 0.22 μm filtered) at a flow rate of 0.5 mL/min. The eluent was fluorescently monitored at 553nm/570nm.

Transmission Electron Microscopy^{17, 18}

A portion of a SEC fraction was incubated with 4% osmium tetroxide for 30 min at 4°C , and then applied onto a 400 mesh carbon coated copper grid (Electron Microscopy Sciences). The grid was stained with 10 μL 1% phosphotungstic acid and imaged under a JEM-1011 computer-controlled high-contrast 100 kV transmission electron microscope.

Protein Analysis

SEC fractions were lysed in RIPA buffer (Santa Cruz, Dallas, TX). The protein contents were determined by using microBCA assay (Pierce, Rockford, IL).

Exosome Quantification by ELISA

An ELISA kit (CD 63 ExoELISA™, System Biosciences, Mountain View, CA) was used to quantify exosomes in a pooled sample of cell culture medium. The procedure described in the manufacturer's instructions was followed. The sample (5.0 mL) was incubated with ExoQuick-TC (1 mL) at 4 °C for 10 hours after removal of cells and cell debris by centrifugation. Number of exosome particles was obtained using the CD 63 protein standard curve calibrated by NanoSight instrument provided by the ELISA kit.

RESULTS AND DISCUSSION

SEC Column Evaluation

To achieve accurate quantification, sEVs have to be separated from other endogenous components in the sample matrix. These include inorganic salts, small organic molecules, and macromolecules such as DNAs and proteins. In this work the SEC technique was deployed for the separation not only because of its size-based separation mechanism, but also for keeping sEVs intact through the whole process. SEC columns packed with several commercially available SEC matrices, i.e. Sepharose CL-2B, Sepharose CL-4B, Sephacryl S-100, and their combinations at various ratios were evaluated by separating fluorescently labeled liposomes and albumin-FITC conjugate. After examining the separation results in terms of separation efficiency, speed, and SEC peak heights, Sepharose CL-4B SEC columns were chosen for further tests. Although Sepharose CL-2B was used as the SEC media to isolate sEVs in previous studies,⁵ we noted that tailing peaks were often obtained with this SEC media. It is likely because Sepharose CL-2B gel has a pore size of ~75 nm, bigger than that of Sepharose CL-4B. Figure 1 shows the SEC chromatograms obtained from separating solutions of a 100 nm liposome preparation after fluorescently labeled with CM-Dil and a solution of albumin-FITC conjugate on a Sepharose CL-4B column (0.5 mL sample loaded). In both cases SEC peaks obtained are symmetrical with an asymmetric factor of about 1.1. It is worth noting that liposomes of 100 nm in size were eluted out about 10 min before albumin-FITC conjugate. Herein albumin-FITC conjugate is used as a model solute of macromolecules such as DNAs and lipoproteins. It is well documented that DNAs and lipoproteins are co-isolated with sEVs in differential ultracentrifugation based preparation procedures.^{19, 20} Separation repeatability was assessed by repeatedly separating these samples and examining the variations in retention times of the solutes. For the peak of 100 nm liposomes, relative standard deviation (RSD) values in retention time were 1.4% (n=5) on the same column and 3.9% (n=3) on different columns. For the peak of albumin-FITC conjugate, RSD values were 3.1% (n=5) on the same column and 4.7% (n=3) on different columns. It should be pointed out that the RSD values in retention times on different columns depended largely upon how skillfully the columns were packed with the SEC separation media.

SEC Separation of sEVs and Peak Identification

sEVs samples enriched from cell culture media by differential ultracentrifugation were separated by the proposed SEC-FD method after tagging with CM-Dil dye. Separation of these samples all produced two SEC-FD peaks: a big peak at 6 min and a small one at 15 min. To characterize the sEVs peak, several detection techniques, including DLS, TEM, and

protein assay were used to analyze the fractions collected during elution (one fraction for each min). In these tests, large quantities of sEVs were loaded on to the SEC column so that good analytical signals could be obtained. DLS assay measures the average size of particles contained in the eluent. As shown in Figure S2a, the DLS data indicate that the average particle size of sEVs eluted at about 6min are in a range from 100 nm to 300 nm, and it decreases to a range from 50 to 100 nm between 7 and 9 min. After 10 min no particles bigger than 30 nm were detected in the eluent. To verify these DLS results, SEC fractions collected at 6, 8, and 10 min were examined by TEM after staining with phosphotungstic acid. The results are shown in Figure S2 b–d. From these TEM images, big particles are contained in the fraction collected at 6 min, particles of 40~100nm sizes are contained in the fraction at 8 min, and few particles are seen in the fraction collected at 10 min. Both DLS and TEM results indicate the SEC peak at ca. 6 min is from sEVs which have a size distribution ranging from 40 to 300 nm.

It is well documented that sEVs contain DNA, protein, etc. macromolecules.^{1~3} Therefore, measurement of protein contents in the SEC eluent was carried out to identify the sEVs peak. As shown in Fig. S3, increased protein contents are observed in eluent fractions collected at around 6 min, which suggests that sEVs may be present in these eluent fractions. This finding is in consistence with those from DLS and TEM assays. In fractions collected between 11 and 16 min, the protein level increases significantly, indicating that a large quantity of free proteins presented in this enriched sEVs sample prepared by differential ultracentrifugation. Separation of sEVs from macromolecules such as DNA and proteins is critical for accurate quantification of sEVs by fluorescence detection. It should be pointed out that although the proposed SEC-FD method separates sEVs from salt, small molecules, and many NDA/protein molecules, some macromolecules such as certain lipoproteins having molecular sizes of >30nm will be co-eluted with sEVs. This is because SEC is a molecular size-based separation technique, and the SEC medium used in this work (i.e. Sepharose CL-4B) has an average pore size of 30nm.²⁰

To establish the relationship between SEC-FD peak height and sEVs quantity, a pooled sample of TK6 cell culture was assayed by using an ELISA method.^{21, 22} The concentration of exosomes in this sample was determined to be $(2.3 \pm 0.41) \times 10^9$ exosome particles/mL (n=5) using the CD 63 protein standard curve calibrated by NanoSight instrument provided by the ELISA kit.

Using this sample as the stock exosome solution, a set of standard exosome solutions were prepared by sequential dilution. A calibration curve was obtained by SEC-FD assay of these solutions. As shown in Figure S4, SEC-FD peak height was proportional to sEVs quantity:

$$\text{Peak Height} = 0.328 X - 0.011 \quad r^2 = 0.996$$

where X is exosome concentration in 10^9 exosome particles/mL. The assay repeatability (RSD in peak height) was 3.2% (n=5) for a solution of 4.0×10^8 exosome particles/mL. The detection limit (S/N = 3) was estimated to be 2.9×10^7 exosome particles/mL. These results indicate that the present cost-effective SEC-FD method is reproducible and sensitive. It is

well suited for extracellular vesicle studies where changes in sEVs quantity need to be sensitively monitored.

Quantification of sEVs secreted from TK6 cells

The human B-lymphoblastoid cell line TK6 is widely used in cancer biology study. Monitoring sEVs secretion from these cells is highly desired in relevant studies. Since bovine (or horse) serum contains a large quantity of sEVs, the cells were cultured in serum free culture medium (conditioned culture medium, CCM) before tests. During the incubation, portions (0.50 mL each) of the CCM were pipetted out and transferred into vials at different times. After removing cells and cell debris, these CCM samples were fluorescently labeled with CM-Dil and then loaded on to the SEC-FD system. Neat CCM was assayed as a negative control to determine the fluorescence background with CM-Dil under the assay conditions. The SEC-FD chromatograms obtained are shown in Figure 2a. The sEVs peaks were seen at 6 min for all samples. From assaying neat CCM, a very small peak is observed at ~15 min, which indicates the fluorescence background from CM-Dil is very low and doesn't interfere with sEVs quantification. In all the CCM samples taken from TK6 cell culture at 1, 12, 24, 36 and 48 hours sEVs were detected at various concentrations ranging from 2.0×10^8 to 1.5×10^9 sEVs particles/mL. The trend of sEVs quantity versus cell culture time is shown in Figure 2b. It is worth noting that sEVs quantity in CMM doesn't increase linearly with cell culture time. The number of sEVs in the cell culture media is little changed in the first 12 hours of cell culture, suggesting that the cells do not secrete significantly sEVs during this time period. After 12 hours, the quantity of sEVs secreted from the cells increases enormously. These results prove that the present SEC-FD method is effective and easy to carry out for quantifying sEVs in cell culture media.

CONCLUSIONS

A facile and cost-effective protocol for quantifying small extracellular vesicles (sEVs) in cell culture media has been developed based on size exclusion chromatography with fluorescence detection (SEC-FD). The assay had a detection limit of 2.9×10^7 exosome particles/mL, and proved sensitive enough to measure sEVs quantity in small volumes (e.g. 0.50 mL) of culture media without enrichment. The quantification repeatability (RSD in SEC-FD peak height) was found 3.2% (n=5) for a sEVs solution of 4.0×10^8 particles/mL. The method was applied to the first study of monitoring sEVs secretion from TK6 cells cultured in serum free media for up to 48 hours. No significant secretion of sEVs from the cells was observed in the first 12 culture hours. However, sEVs quantity in the culture media increased drastically after that. To our knowledge, this is the first SEC method with fluorescence detection for sensitive quantification of sEVs in biological samples. Compared with other methods for sEVs quantification such as ELISA and NTA the proposed SEC-FD method is easy to carry out, cost-effective, highly sensitive, and of good repeatability.

Acknowledgments

Financial support from US National Institutes of Health (GM089557) is gratefully acknowledged.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SEC-FD chromatograms obtained from separating: (a) sequentially diluted solutions of CM-Dil labeled 100-nm liposomes, and (b) a solution of albumin-FITC conjugate on an in-house prepared Sepharose CL-4B column.

Figure 2. Quantification of sEVs in TK6 cell culture: a) SEC-FD chromatograms obtained from assaying the neat serum-free culture medium and media samples taken from cell culture at different times, and b) trend of sEVs quantity in the culture media versus culture time (three samples were analyzed for each time point).