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Size exclusion chromatography to analyze bacterial outer membrane vesicle heterogeneity

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TITLE:

Size Exclusion Chromatography to Analyze Bacterial Outer Membrane Vesicle Heterogeneity

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SUMMARY:

Bacterial vesicles play important roles in pathogenesis and have promising biotechnological applications. The heterogeneity of vesicles complicates analysis and use; therefore, a simple, reproducible method to separate varying sizes of vesicles is necessary. Here, we demonstrate the use of size exclusion chromatography to separate heterogeneous vesicles produced by *Aggregatibacter actinomycetemcomitans*.

ABSTRACT:

The cell wall of Gram-negative bacteria consists of an inner (cytoplasmic) and outer membrane (OM), separated by a thin peptidoglycan layer. Throughout growth, the outer membrane, can bleb to form spherical outer membrane vesicles (OMVs). These OMVs are involved in numerous cellular functions including cargo delivery to host cells and communication with bacterial cells. Recently, the therapeutic potential of OMVs has begun to be explored, including their use as vaccines and drug delivery vehicles. Although OMVs are derived from the OM, it has long been appreciated that the lipid and protein cargo of the OMV differs, often significantly, from that of the OM. More recently, evidence that bacteria can release multiple types of OMVs has been discovered, and evidence exists that size can impact the mechanism of their uptake by host cells. However, studies in this area are limited by difficulties in efficiently separating the heterogeneously sized OMVs. Density gradient centrifugation (DGC) has traditionally been used for this purpose; however, this technique is time-consuming and difficult to scale-up. Size exclusion chromatography (SEC), on the other hand, is less cumbersome and lends itself to the necessary future scale-up for therapeutic use of OMVs. Here, we describe a SEC approach that enables reproducible separation of heterogeneously sized vesicles, using as a test case, OMVs produced by *Aggregatibacter actinomycetemcomitans*, which range in diameter from less than 150 nm to greater than 350 nm. We demonstrate separation of “large” (350 nm) OMVs and “small” (<150 nm) OMVs, verified by dynamic light scattering (DLS). We recommend SEC-based techniques over DGC-based techniques for separation of heterogeneously sized vesicles due to its ease of use, reproducibility (including user-to-user), and possibility for scale-up.

INTRODUCTION:

Gram-negative bacteria release vesicles derived from their outer membrane, so-called outer membrane vesicles (OMVs), throughout growth. These OMVs play important roles in cell-to-cell communication, both between bacteria and host as well as between bacterial cells, by carrying a number of important biomolecules, including DNA/RNA, proteins, lipids, and nucleic acids[1,2]. In particular, the role of OMVs in bacterial pathogenesis has been extensively studied due to their enrichment in certain virulence factors and toxins[3-11].

OMVs have been reported to range in size from 20 to 450 nm, depending on the parent bacteria and the growth stage, with several types of bacteria releasing heterogeneously sized OMVs[8,12-14], which also differ in their protein composition and mechanism of host cell entry[12]. *H. pylori* released OMVs ranging in diameter from 20 to 450 nm, with the smaller OMVs containing a more homogeneous protein composition than the larger OMVs. Importantly, the two populations of OMVs were observed to be internalized by host cells via different mechanisms[12]. In addition, we have demonstrated that *Aggregatibacter actinomycetemcomitans* releases a population of small (<150 nm) OMVs along with a population of large (>350 nm) OMVs, with the OMVs containing a significant amount of a secreted protein toxin, leukotoxin (LtxA)[15]. While the role of OMV heterogeneity in cellular processes is clearly important, technical difficulties in separating and analyzing distinct populations of vesicles has limited these studies.

In addition to their importance in bacterial pathogenesis, OMVs have been proposed for use in a number of biotechnological applications, including as vaccines and drug delivery vehicles[16-20]. For their translational use in such approaches, a clean and monodisperse preparation of vesicles is required. Thus, effective, and efficient methods of separation are necessary.

Most commonly, density gradient centrifugation (DGC) is used to separate heterogeneously sized vesicle populations from cellular debris, including flagellae and secreted proteins[21]; the method has also been reported as an approach to separate heterogeneously sized OMV subpopulations[12-14]. However, DGC is time-consuming, inefficient, and highly variable user-to-user[22] and is, therefore, not ideal for scale-up. In contrast, size exclusion chromatography (SEC) represents a scalable, efficient, and consistent approach to purify OMVs[21,23,24]. We have found that a long (50-cm), gravity-flow, SEC column, filled with gel filtration medium is sufficient for efficiently purifying and separating subpopulations of OMVs. Specifically, we used this approach to separate *A. actinomycetemcomitans* OMVs into “large” and “small” subpopulations, as well as to remove protein and DNA contamination. Purification was completed in less than 4 h, and complete separation of the OMV subpopulations and removal of debris was accomplished.

PROTOCOL:

1. Preparation of buffers

1.1. To prepare the ELISA wash buffer, add 3.94 g Tris-base, 8.77 g NaCl, and 1 g bovine serum albumin (BSA) to 1 L of deionized (DI) water. Add 500 µL polysorbate-20. Adjust the pH to 7.2

using HCl or NaOH.

1.2. To prepare the blocking buffer, add 3.94 g Tris-base, 8.77 g NaCl, and 10 g BSA. Add 500 µL polysorbate-20 to 1 L of DI water. Adjust the pH to 7.2 using HCl or NaOH.

1.3. To prepare the elution buffer (PBS), add 8.01 g NaCl, 2.7 g KCl, 1.42 g Na_2HPO_4, and 0.24 g KH_2PO_4 to 1 L DI water. Adjust the pH to 7.4 using HCl or NaOH.

NOTE: A 10x solution of this buffer can be made and diluted with DI water as needed.

2. Preparation of OMV sample

2.1. Grow *A. actinomycetemcomitans* cells to the late exponential phase (optical density at 600 nm of 0.7). Pellet the cells by centrifuging at 10,000 *x g* at 4 °C for 10 min. Filter the supernatant through a 0.45 µm filter.

2.2. Concentrate the bacteria-free supernatant using 50 kDa-molecular weight cut-off filters. Ultracentrifuge the concentrated solution at 105,000 *x g* at 4 °C for 30 min.

2.3. Resuspend the pellet in PBS and ultracentrifuge again (105,000 *x g* at 4 °C for 30 min.) Resuspend the pellet in 1 mL of PBS.

3. Packing the S-1000 column

3.1. Mix the stock bottle of gel filtration medium with a glass stir rod and pour out into a beaker the volume necessary to fill the column, plus approximately 50% excess (about 135 mL). Let these beads sit until they have settled, and then decant off the excess liquid. Resuspend the beads in elution buffer, so that the final solution is approximately 70% (by volume) gel, 30% buffer. Degas the solution under vacuum.

3.2. Mount the glass column vertically using a ring stand and fill with elution buffer to wet the walls of the column. Drain the buffer until there is only about 1 cm of buffer remaining in the column.

3.3. Without creating bubbles, carefully pipette beads into the column, filling the column to the top. Continue to drain excess buffer throughout this process. Be sure to not let the beads settle completely before adding additional beads to the top of the column. The column should be packed to a height of about 2 cm below the bottom of the column reservoir.

4. Loading the sample and collecting fractions

4.1. Degas the elution buffer under vacuum. Wash the column with two column-volumes (180 mL) of elution buffer.

4.2. Allow the remaining buffer to fully enter the column. Once the buffer has reached the top of the gel layer, carefully pipette a 1-mL sample containing OMVs (at a lipid concentration of approximately 100 – 200 nmol/L) onto the surface of the beads, being careful not to disturb any of the beads at the top of the column. Allow the sample to fully enter the gel, that is, when no liquid remains above the gel layer. Then stop the column.

4.3. Carefully and slowly add elution buffer on top of the gel column. Do not disturb the top layer of the gel, as this will cause sample dilution.

4.4. Place a single 50-mL tube under the column and open the column. Collect the first 20 mL of the eluent. Add additional elution buffer to the top of the column, carefully, as needed to ensure the column is never dry. Stop the column.

4.5. Place a series of 1.5 mL tubes under the column. Start the column and collect a series of 1-mL samples in each tube. As the samples are being collected, continue to add elution buffer to the top of the column, as necessary. Repeat until 96 fractions have been collected. Stop the column.

NOTE: The samples should be stored at -20 °C for long-term storage or 4 °C for short-term storage until further analysis.

4.6. To clean the column, run one column volume (90 mL) of 0.1 M NaOH through the column. Run two column volumes (180 mL) of elution buffer through the column.

5. Sample analysis

5.1. To measure the lipid concentration in each fraction, pipette 50 µL of each fraction into a single well of a 96-well plate. To each well, add 2.5 µL of lipophilic dye. Incubate for 15 s. Measure the fluorescence intensity on a plate reader with an excitation wavelength of 515 nm and an emission wavelength of 640 nm. To calculate the fraction of all lipid in each sample, sum all of the emission intensities and divide each individual intensity by the total.

5.2. To measure the concentration of a particular protein, pipette 100 µL of each fraction into a single well of an ELISA immuno-plate. Incubate at 25 °C for 3 h.

5.2.1. Decant the samples. Add 200 µL of ELISA wash buffer to each well and decant. Repeat four times for a total of five washes.

5.2.2. Add 200 µL of blocking buffer to each well and incubate for 1 h at 25 °C. Decant.

5.2.3. Incubate plates with 100 µL blocking buffer plus primary antibody (1:10,000 for purified antibody; 1:10 for unpurified antibody) overnight at 4 °C. Decant.

5.2.4. Add 200 µL of ELISA wash buffer to each well and decant. Repeat four times for a total of

five washes.

5.2.5. Add 100 µL of ELISA wash buffer plus secondary antibody (1:30,000) to each well. Incubate for 1 h at 25 °C.

5.2.6. Add 200 µL of ELISA wash buffer to each well and decant. Repeat four times for a total of five washes.

5.2.7. Add 100 µL of the 3,3',5,5'-tetramethylbenzidine (TMB) one-step solution and incubate for 15-30 min or until a blue color develops. Stop the TMB reaction with 50 µL of the stopping solution.

5.2.8. On a plate reader, read the absorbance of each well at a wavelength of 450 nm.

5.3. To measure the total protein concentration, record the absorbance at a wavelength of 280 nm (A_{280}) of each fraction, using a UV-vis spectrophotometer.

A schematic of the protocol is shown in **Figure 1**.

[Place **Figure 1** here]

REPRESENTATIVE RESULTS:

Figure 2 shows representative results from this method. OMVs produced by *A. actinomycetemcomitans* strain JP2 were first purified from the culture supernatant using ultracentrifugation[15]. We previously found that this strain produces two populations of OMVs, one with diameters of about 300 nm and one with diameters of about 100 nm[15]. To separate these OMV populations, we purified the sample using the SEC protocol described above. Each fraction was analyzed for lipid content using the lipophilic dye and for toxin (LtxA) content using enzyme-linked immunosorbent assay (ELISA) or an immunoblot. The lipid and toxin concentrations are reported as percentages, where "%lipid" indicates what percentage of the total lipid content of the sample is in each fraction and "%toxin" indicates what percentage of the total toxin content of the sample is in each fraction.

Figure 2A shows the averaged lipophilic dye results with standard deviations from three separate purifications, each performed by a different user, demonstrating the reproducibility of this technique. Two distinct lipid peaks are observed, corresponding to "large" OMVs (fraction number 13) and "small" OMVs (fraction number 25). We confirmed the size of the OMVs in these peaks using dynamic light scattering (DLS) and found the mean diameters of the OMVs in fractions 13 and 25 to be 296.6 nm and 142.6 nm, respectively, as shown in Fig. 2A. In comparison, the mean diameter of the OMV sample after ultracentrifugation but before SEC purification was previously found to be 161.0 nm[15].

In **Figure 2B**, the amount of LtxA in each fraction, obtained using ELISA with a monoclonal antibody against LtxA[25], is shown overlaid on the lipid concentration from panel A. This technique

demonstrates that the toxin is associated primarily with one subpopulation of OMVs. **Figure 2C** shows the amount of LtxA in each fraction, measured using an immunoblot technique with the same anti-LtxA monoclonal antibody[25], overlaid on the lipid concentration from panel A. While the overall trend is similar to what is observed in **Figure 2B**, the immunoblot approach is much less sensitive than the ELISA technique, resulting in noisier profiles. **Figure 2D** shows the percentage of the total protein concentration in each fraction, measured using the A_{280}, overlaid on the lipid concentration profile. This panel demonstrates that SEC is able to remove significant amounts of free proteins from the OMV preparations, as evidenced by the high A_{280} values in fractions greater than 60. (In fact, most of the protein is found in these fractions, which do not contain OMVs, demonstrating that a large amount of protein co-purifies with the OMVs.) In addition, this result shows that the total protein concentration does not necessarily correlate with the concentration of specific proteins. In the case of *A. actinomycetemcomtians* OMVs, LtxA associates primarily with the population of larger OMVs, while more of the total protein associates with the smaller OMVs.

Together, these representative results demonstrate a number of important features of the SEC protocol for OMV purification. First, the technique is highly reproducible, even between users. Second, the use of a lipophilic dye to detect OMVs in each fraction is a simple and reliable method. Third, to detect specific protein concentrations, ELISA is more robust than an immunoblot. Fourth, SEC is able to remove large amounts of impurities, including proteins and nucleic acids.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of SEC procedure. The column is packed with degassed gel filtration medium carefully to avoid bubbles and discontinuities, then washed with two column volumes of elution buffer. Next, the sample is carefully pipetted onto the top of the gel, without disrupting gel packing. The column is opened and run until the sample completely enters the gel. At this point, buffer is placed on the top of the column, and the first 20 mL of eluate is collected. Next, a series of 1-mL fractions is collected. These fractions are then placed in a 96-well plate or 96-well immuno-plate for analysis of lipid and protein content.

Figure 2: Representative results. *A. actinomycetemcomitans* JP2 OMVs were run through the SEC column and each fraction was analyzed for lipid content using a lipophilic dye and toxin (LtxA) content using a monoclonal antibody. (**A**) The average lipid content of each fraction reported as a percentage of the total lipid content, from three trials. Each data point represents the mean $\pm$ standard deviation. (**B**) The LtxA content of each fraction, reported as a percentage of the total LtxA content, as measured by ELISA with a monoclonal anti-LtxA antibody. (**C**) The LtxA content of each fraction, reported as a percentage of the total LtxA content, as measured by an immunoblot with a monoclonal anti-LtxA antibody. (**D**) The total protein content of each fraction, reported as a percentage of the total protein content, as measured by A_{280}. Some of the data are reproduced from Chang et al.[26] with permission from John Wiley and Sons Ltd.

DISCUSSION:

Here, we have provided a protocol for the simple, fast, and reproducible separation of bacterial

OMV subpopulations. Although the technique is relatively straight-forward, there are some steps that must be performed extremely carefully to ensure that efficient separation occurs in the column. First, it is essential that the gel be loaded into the column carefully and slowly to avoid air bubbles. We have observed that leaving the gel at room temperature for several hours before loading the column allows the gel to equilibrate and minimizes bubble formation within the column. When the gel is pipetted into the column, it should be carefully pipetted along the side of the column to minimize turbulence. At all times during loading, excess buffer should be maintained in the column to avoid discontinuities in the settled gel. If a disjunction should occur, add more buffer and pipette up and down to resuspend the gel.

Similarly, loading the column with sample is critically important. Because the sample will become diluted as it passes through the column, the loaded sample should be sufficiently concentrated before separation by SEC. For the *A. actinomycetemcomitans* OMVs, we have found that a 1-mL sample containing approximately 100-200 nmol/L of lipids is ideal. After the sample is loaded carefully at the top of the column without disrupting the gel layer, the column should be run just until the sample fully enters the gel column. At this point, the column should be stopped so that a layer of buffer can be carefully added to the top of the gel. It is helpful to load only a small volume (~ 1 mL) of buffer, ensuring that the gel layer is not disrupted. Once the sample has been run further into the gel, buffer can be added in larger volumes and the concern with disrupting the gel layer is less of an issue. The column can be reused multiple times, as long as it is maintained in a fully hydrated state and cleaned well (Step 4.6) between runs.

All OMV purification procedures follow the same initial steps that include bacterial growth, removal of bacterial cells, and OMV isolation[27]. Although this “crude” preparation has commonly been used in OMV studies[28], it is becoming increasingly apparent that a subsequent purification step is necessary to remove co-precipitating proteins and other contaminants, as well as to separate OMV subpopulations. In OMV studies, this purification step is commonly completed using density gradient centrifugation. In the eukaryotic extracellular vesicle field, the use of SEC to separate vesicle populations and to remove contaminants is increasing in importance, as it is simpler, faster, and less expensive than DGC[29]. In addition, SEC has the advantage of being possible to automate, unlike DGC[29]. Thus, while DGC remains the “gold standard” of vesicle isolation in the bacterial OMV field, we propose that the numerous advantages of SEC make it an extremely useful, if not better, method of OMV purification than DGC. In this work, we have demonstrated that a 1.5 x 50 cm column of Sephacryl S-1000 is capable of separating two subpopulations of OMVs. We have also observed that the approach is capable of removing nucleic acids and free proteins from the OMV solution. Previous reports have found SEC to be able to remove free LPS from OMV preparations, as well[28].

In conclusion, we propose that SEC holds much promise in the purification of bacterial vesicles. While we have demonstrated the ability of the technique to separate subpopulations of OMVs produced by a specific bacterium (*A. actinomycetemcomitans*), we anticipate that the technique will be found to be extremely valuable in analyzing other bacterial vesicle samples, as it sees additional use. In particular, as the biotechnological applications of OMVs increase, the need for consistent and pure vesicle preparations will also increase; SEC is a promising method for these

applications.

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DISCLOSURES:

The authors have no conflicts of interest to report.

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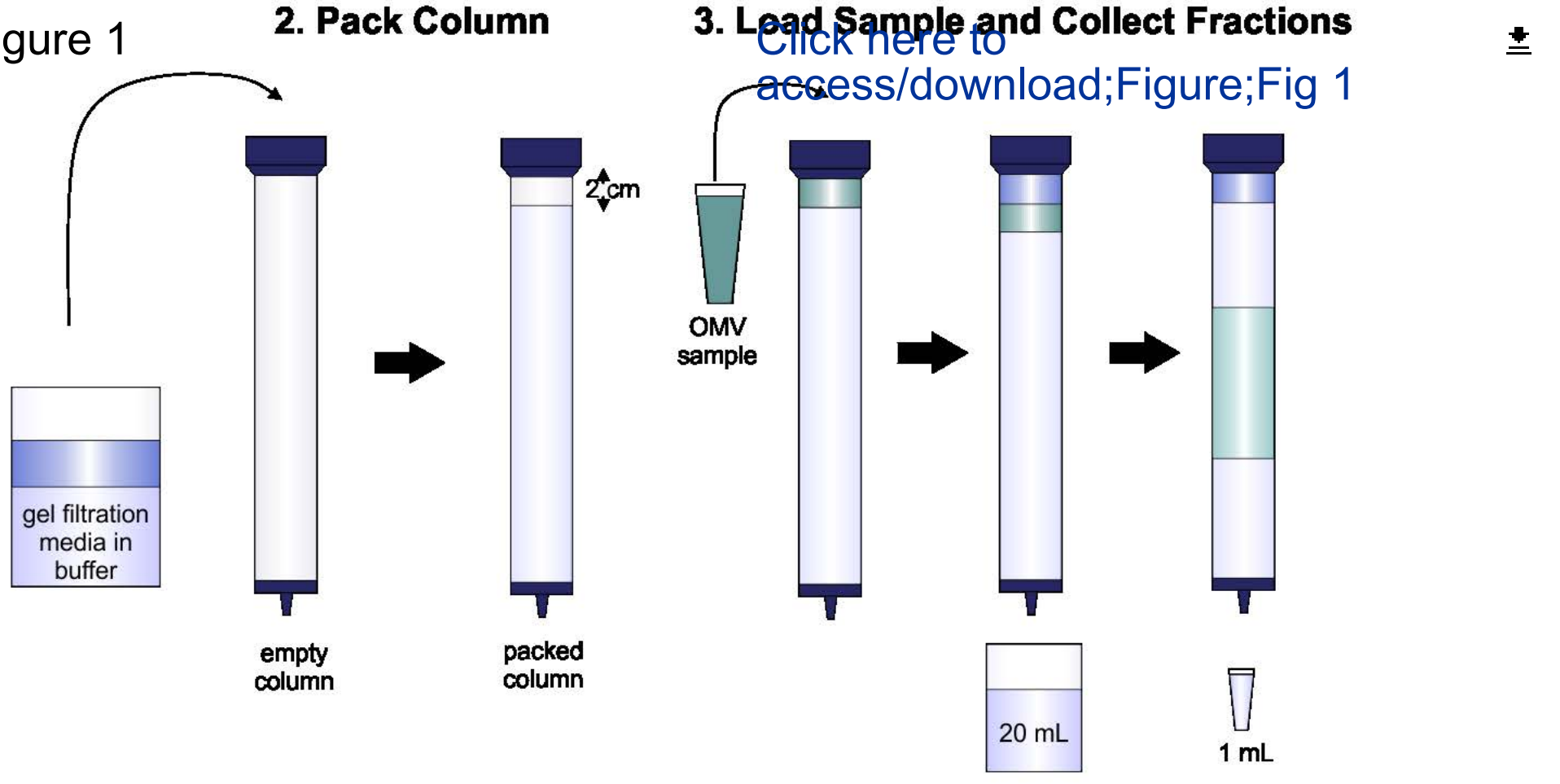
Figure 1



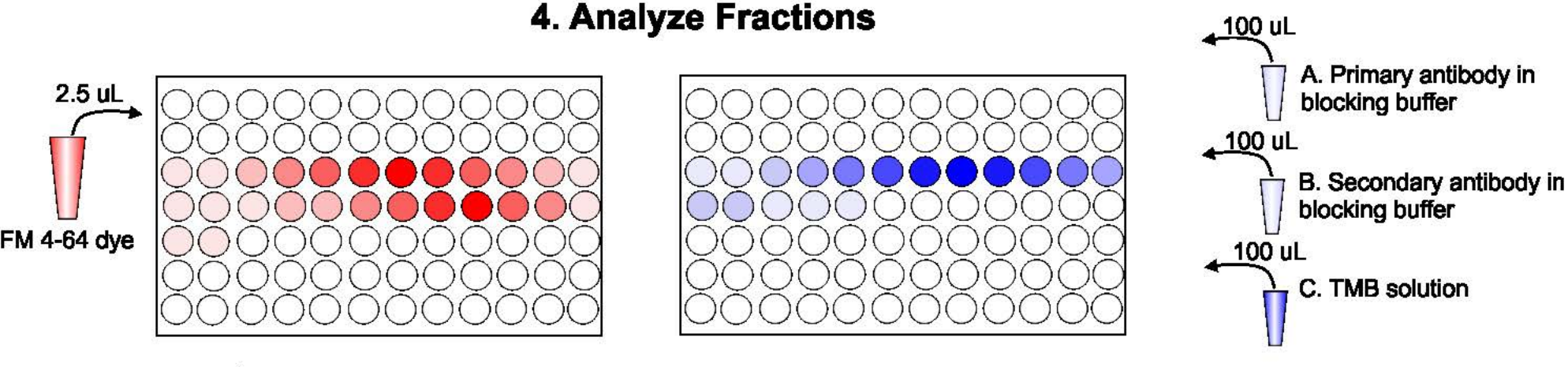
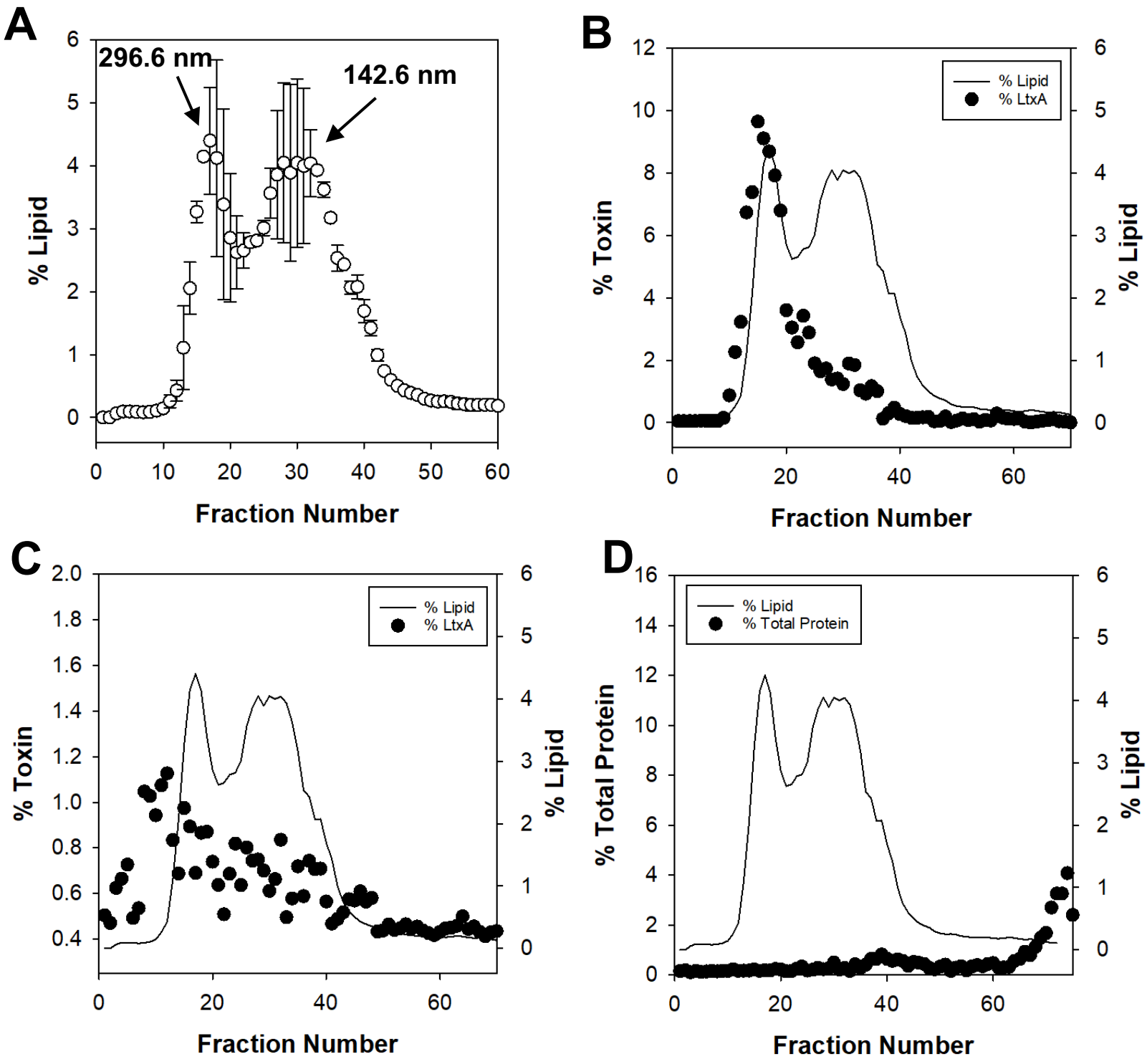


Figure 2



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1-Step Ultra TMB-ELISA	Thermo Scientific	34028	
Amicon 50 kDa filters	Millipore Sigma	UFC905024	
Bovine Serum Albumin (BSA)	Fisher Scientific	BP9704-100	
ELISA Immuno Plates	Thermo Scientific	442404	
FM 4-64	Thermo Scientific	T13320	1.5 x 50 cm
Glass Econo-Column	BioRad	7371552	
Infinite 200 Pro Plate Reader	Tecan		
Potassium Chloride (KCl)	Amresco (VWR)	0395-500G	
Potassium Phosphate Monobasic Anhydrous (KH_2PO_4)	Amresco (VWR)	0781-500G	
Sephacryl S-1000 Superfine	GE Healthcare	17-0476-01	
Sodium Chloride (NaCl)	Fisher Chemical	S271-3	
Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4)	Amresco (VWR)	0404-500G	
Tris Base	VWR	0497-1KG	
Tween$^{(R)}$ 20	Acros Organics	23336-2500	

We thank the editors and reviewers for the constructive and comprehensive feedback. We have carefully analyzed and addressed the comments below, including all modifications made to the manuscript as appropriate. The editor/reviewer comments are listed in **bold** below, followed by our responses and revisions in each case. Blue text is used to note the revised text from the manuscript. In addition, the revised manuscript and supplementary information with new changes being highlighted have been resubmitted together with the revised versions of these files without highlights.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript.

2. Please ensure that the Summary is within the 50-word limit.

We have revised the Summary to be within the 50-word limit.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Tween®, FMTM 4-64 etc.

We have replaced all commercial product names in the text.

4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have added additional details to our protocol, as requested.

5. 3.2: What is the sample in your experiment? what is the sample volume? How do you ensure that the sample has fully entered the gel?

We have revised this step to read:

Run the buffer to the top of the gel, and stop the column. Carefully pipet a 1-mL sample containing OMVs onto the surface of the beads, being careful not to disturb any of the beads at the top of the column. Run the column until the sample has fully entered the gel, that is, when no liquid remains above the gel layer, then stop the column.

6. Please include single line space and highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted sections 3-5 as the most essential steps of the protocol.

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Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have updated the legend of Figure 2 and have uploaded the editorial policy for the referenced work.

8. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have updated our Discussion to include paragraphs describing the critical steps, limitations, and trouble-shooting. Additionally, we have added a paragraph to better explain why this technique is superior to the other most commonly used technique and to describe future applications of the technique.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This study demonstrates the utility of a faster and easier method (Sephacryl-based SEC) to isolate two distinguishable OMV subpopulations from *A. actinomycetemcomitans* strain JP2, one carrying a toxin.

Major Concerns:

no major concerns are raised.

Minor Concerns:

The biggest limitation of this study is the lack of testing in OMVs from other species, to consolidate Sephacryl-based SEC method to be useful across many research areas. Also feels like the results are focused on showing antibody optimisation instead of a full characterisation of OMV fractions obtained by SEC (at least presenting Electron microscopy data and DLS data).

We have updated Fig. 2 based on this and other reviewer comments to now focus on the reproducibility of the approach (panel A) and the ability to separate vesicles with different protein compositions (panel B). In our first submission, we included DLS data from the two populations, but it was somewhat hidden in the text. To improve the accessibility of that data, we have now also included it in Fig. 2. We have also included a reference to our published work in which we measured the DLS of the ultracentrifuged OMVs before SEC. We hope that these changes highlight the advantages of the SEC approach with less focus on antibody optimization.

The protocol is well described, with plenty practical tips for researchers to avoid unnecessary mistakes in the future. The method is very straightforward and with potential to up-scale procedures to isolate specific OMVs for biotechnological applications.

Few minor comments are below:

Line 104. Loading the sample and collecting fractions

3.2 Run the buffer to the top of the gel, and stop the column. Carefully pipet the sample onto the surface of the beads, being careful not to disturb any of the beads at the top of the column. Run the column until the sample has fully entered the gel, then stop the column. The volume of loaded OMV sample is missing. This is a very crucial step in SEC protocols, because it directly relates back to the column volume/area and resulting fraction volumes.

We have revised this step to read:

Run the buffer to the top of the gel, and stop the column. Carefully pipet a 1-mL sample containing OMVs onto the surface of the beads, being careful not to disturb any of the beads at the top of the column. Run the column until the sample has fully entered the gel, that is, when no liquid remains above the gel layer, then stop the column.

Line 124. Sample analysis

4.2.3 Incubate plates with 100 µL blocking buffer plus primary antibody overnight at 4 °C. NOTE: The necessary volume of primary antibody will vary depending on the concentration and purity of the antibody. Decant.
4.2.5 Add 100 µL of ELISA wash buffer plus secondary antibody to each well. Incubate for 1 hr at 25 °C.
I think an approximate value or range of the concentration of primary and secondary antibodies used in ELISA is necessary.

We have added the necessary details, as shown below. (Note that the numbering of the protocol has changed slightly in the revised manuscript.)

5.2.3 Incubate plates with 100 µL blocking buffer plus primary antibody (1:10,000 for purified antibody; 1:10 for unpurified antibody) overnight at 4 °C. NOTE: Decant.

5.2.5 Add 100 µL of ELISA wash buffer plus secondary antibody (1:30,000) to each well. Incubate for 1 hr at 25 °C.

Line 187, The sample should be sufficiently concentrated before it is loaded on the column to avoid the need to pool samples for analysis.

What is the OMV sample load (by protein and volume) that was optimised for separation by this column? Since it is one of the main conclusions of this manuscript, it is useful for other researchers to know the range of OMV load successfully fractionated with the described protocol, i.e. above XX µg OMV the column may block or below XX µg OMV the column's recovery of fractions is significantly decreased, etc. What is the absolute value of "sufficiently concentrated"?

Unfortunately, we do not have this information. However, we consistently load a one-mL sample, with a lipid concentration of 100-200 nmol/L. We have added this information to the manuscript.

4.2 Allow the remaining buffer to fully enter the column. Once the buffer has reached the top of the gel layer, carefully pipet a 1-mL sample containing OMVs (at a lipid concentration of approximately 100 – 200 nmol/L) onto the surface of the beads, being careful not to disturb any of the beads at the top of the column. Allow the sample to fully enter the gel, that is, when no liquid remains above the gel layer. Then stop the column.

Line 163. Each panel of this figure includes the averaged FM™ 4-64 results from three separate runs, each collected by a different user, demonstrating the extreme reproducibility of this technique. Line

186. Together, these representative results demonstrate that separation of OMV subpopulations by SEC is highly reproducible, even between users.

For this claim to be true, readers would need to see the variability of data obtained from each user, which is hidden in averaged plotted values shown in Figure 2. I recommend to add SD or SEM to all graphs in Figure 2 to justify the 'reproducibility' statement.

We have updated this figure to show the standard deviations from the different runs to better demonstrate the reproducibility. This information can be found in the revised Fig. 2A.

Line 189. Finally, the selection of an appropriate antibody, with sufficient specificity and affinity for the target protein(s), is an essential aspect of this approach.

Would total protein concentration of OMV fractions follow the two subpopulations trend as well? Just wondering if a more affordable and applicable-to-all analysis approach would be of utility to characterise OMVs from other bacterial species, when antibodies are not available.

We have modified Fig. 2 to include the absorbance at 280 nm (A_{280}), as a measure of protein concentration, of each fraction. We found that for our *A. actinomycetemcomitans* OMVs, this measurement did not accurately predict which fractions contained OMVs. We agree with the reviewer that if it had, it could be a more versatile approach to measure protein content in each fraction. However, we propose that using the lipid concentration of each fraction is a versatile approach in itself to detect fractions containing OMVs, in cases where specific antibodies are not available. Additionally, we believe the A_{280} results shown in the modified Fig. 2 further support the value of this SEC approach, as it is clear that a significant amount of "free" protein can be separated from the OMV populations.

Figure 2. The LtxA content of each fraction represents results from three separate runs, each quantified in a different way. What does it mean each 3 separate runs, each quantified in a different way? Line 163 says each run was performed by different users. Please clarify.

We have revised this figure substantially based on the reviewer feedback. In the new figure, we only show LtxA content quantified in one way, so we believe this clarity issue has been resolved.

Please clarify how percentages of Toxin and Lipid were calculated in Panel A and B in the figure legend.

We have added the following explanation of these calculations:

The lipid and toxin concentrations are reported as percentages, where "%lipid" indicates what percentage of the total lipid content of the sample is in each fraction and "%toxin" indicates what percentage of the total toxin content of the sample is in each fraction.

Please clarify the units of the Toxin Intensity in Panel C and D.

We have updated this figure, based on reviewer suggestions, and we no longer show the results included in the original Panels C and D.

Line 234. For these reasons, we propose that SEC is a more promising method than DGC for future biotechnological applications of OMVs.

In order to conclude that SEC is more promising than DGG (which I agree), more Sephacryl-based SEC assays were required to be tested in OMVs from few different species. Bacteria MVs quickly respond to environmental cues (culture conditions), so there is a vast diversity in MV molecular or population-wide composition. Sephacryl-SEC may be useful to distinguish subpopulations is some cases and some others may not. Perhaps it's worth to narrow down the biotechnological applications

potential to the tested strain in the discussion section, while also adding some discussion of what's the current state of SEC and bacterial vesicles.

We appreciate these suggestions and we have incorporated them into two new paragraphs in the Discussion section. In these paragraphs, we more directly compare SEC and DGC. We expect that as the technique becomes more common in the bacterial OMV field, it will be found to be superior to DGC in many applications; however, we agree with the reviewer that with our current data, we cannot make such a broad statement. We have toned down our claims in these new paragraphs to instead propose that SEC might become increasingly important in the OMV field.

All OMV purification procedures follow the same initial steps that include bacterial growth, removal of bacterial cells, and OMV isolation.[27] Although this "crude" preparation has commonly been used in OMV studies[28], it is becoming increasingly apparent that a subsequent purification step is necessary to remove co-precipitating proteins and other contaminants, as well as to separate OMV subpopulations. In OMV studies, this purification step is commonly completed using density gradient centrifugation. In the eukaryotic extracellular vesicle field, the use of SEC to separate vesicle populations and to remove contaminants is increasing in importance, as it is simpler, faster, and less expensive than DGC[29]. In addition, SEC has the advantage of being possible to automate, unlike DGC[29]. Thus, while DGC remains the "gold standard" of vesicle isolation in the bacterial OMV field, we propose that the numerous advantages of SEC make it an extremely useful, if not better, method of OMV purification than DGC. In this work, we have demonstrated that a 1.5 x 50 cm column of Sephacryl S-1000 is capable of separating two subpopulations of OMVs. We have also observed that the approach is capable of removing nucleic acids and free proteins from the OMV solution. Previous reports have found SEC to be able to remove free LPS from OMV preparations, as well.[28]

In conclusion, we propose that SEC holds much promise in the purification of bacterial vesicles. While we have demonstrated the ability of the technique to separate subpopulations of OMVs produced by a specific bacterium (*A. actinomycetemcomitans*), we anticipate that the technique will be found to be extremely valuable in analyzing other bacterial vesicle samples, as it sees additional use.

Are there other non-MV contaminants that can be effectively removed/cleaned with Sephacryl-based SEC as compared to just having the ultracentrifuged OMVs?

We have found that SEC is able to remove nucleic acids and proteins that co-pelleted with the OMVs during ultracentrifugation. We have included mention of this in the text.

Representative Results section:

Fig. 2D shows the percentage of the total protein concentration in each fraction, measured using the A_{280}, overlaid on the lipid concentration profile. This panel demonstrates that SEC is able to remove significant amounts of free proteins from the OMV preparations, as evidenced by the high A_{280} values in fractions greater than 60. (In fact, most of the protein is found in these fractions, which do not contain OMVs, demonstrating that a large amount of protein co-purifies with the OMVs.)

Discussion section:

We have also observed that the approach is capable of removing nucleic acids and free proteins from the OMV solution.

Reviewer #2:

The manuscript by Dr. Collins et al, described a size exclusion chromatography (SEC) based method to purify bacterial derived outer membrane vesicles (OMVs). Using size exclusion column to purified extracellular vesicles from body fluid or culture media is a well established method; however, except a few prior descriptions, applying SEC on OMV purification is relatively new.

The protocol described in the manuscript lacks some details.

1. The authors need to provide some more information of the column since the length of column will affect the particle separation in SEC. In addition, the authors also need to provide a range of sample volumes that can be applied.

As reported in our Materials List, we have used a 1.5 x 50 cm column. For clarity, we have included this information in the text (Introduction) as well. We have also added information to the Method about the volume of sample that can be applied to this size column (1 mL).

2. How many times the column can be re-used? What is the washing condition between samples?

In our experience, the column can be reused indefinitely, as long as it is consistently hydrated with buffer and has not become contaminated. We have run a single column up to fifteen times. We have added this information to the manuscript (Discussion section).

The column can be reused multiple times, as long as it is maintained in a fully hydrated state and cleaned well (Step 4.6) between runs.

3. It is not necessary to provide results from 4 different measurement methods for LtxA. Instead, it would be helpful if the authors can provide a direct comparison between the ultracentrifugation and SEC based OMV purification results. Actually, in the "Representative Results" section, the authors did mention ultracentrifugation results are shown in Figure 2 (Lines 157-158)

We agree with the reviewer and have altered the representative results show in Fig. 2. Also, in the text, we have compared the DLS results of an ultracentrifuged OMV sample and the SEC-purified samples.

We confirmed the size of the OMVs in these peaks using dynamic light scattering (DLS) and found the mean diameters of the OMVs in fractions 13 and 25 to be 296.6 nm and 142.6 nm, respectively, as shown in Fig. 2A. In comparison, the mean diameter of the OMV sample after ultracentrifugation but before SEC purification was previously found to be 161.0 nm.

Reviewer #3:

Manuscript Summary:

The authors describe a method of purifying the membrane vesicles from a Gram negative bacteria using SEC. Vesicles isolated from *Aggregatibacter actinomycetemcomitans*, which the authors state, produces two discrete populations of OMVS. While there are numerous methods of isolating OMVs, the authors speak to the simplicity and low cost of this method compared to other methods such as ultra-centrifugation. The methods provided are informative but I believe there are a lot of components that are missing that would make this a more comprehensive protocol. OMV purification has multiple steps which are not even discussed in this protocol including bacterial growth, cell removal, and final OMV/sample analysis to confirm results.

Major Concerns:

1) The authors do not discuss the preparation of OMVs from bacterial culture which makes the starting point for this protocol quite confusing. For several reasons

a. Is this straight culture media that is being purified here? Obviously there would be a centrifugation step to pellet bacteria, however, most methods have multiple stages of centrifugation followed by

filtration to ensure that cells, cellular debris, and large aggregates are removed. The authors should provide some information here or at least provide some citations to other published methods.

We apologize for the omission of those important details. We first pellet the bacteria and collect the supernatant. We then ultracentrifuge the supernatant to concentrate the OMVs. It is those purified OMVs that we then separate by SEC to obtain more homogeneous samples. We did not originally include the specific details of this process in the revised manuscript because we do not want the method to focus solely on *A. actinomycetemcomitans.* However, we have added an explanation of this point in the Protocol section of the manuscript.

2. Preparation of OMV Sample

2.1 Grow *A. actinomycetemcomitans* cells to the late exponential phase (optical density at 600 nm of 0.7). Pellet the cells by centrifuging at 10,000 *x g* at 4 °C for 10 min. Filter the supernatant through a 0.45 µm filter.

2.2 Concentrate the bacteria-free supernatant using 50 kDa-molecular weight cut-off filters. Ultracentrifuge the concentrated solution at 105,000 *x g* at 4 °C for 30 min.

2.3 Resuspend the pellet in PBS and ultracentrifuge again (105,000 *x g* at 4 °C for 30 min.) Resuspend the pellet in 1 mL of PBS.

b. Is there a concentration step (following 1 above) such as a high speed centrifugation step achievable with most table top centrifuges to reduce the amount of sample loaded to the column?

Please see our response above.

2) In section 3.2, the authors us the word "run" but do not define this term. Are they suggesting that column has been attached to a pump and is being "run" at a specific flow rate? Without this information one would have to assume that the column is being used as a gravity flow column which should be indicated and might not be the best approach if a long column is being used.

We apologize for our confusing word choice. The column is a gravity flow column. We have updated this section to read (note that the protocol numbering has changed slightly):

4.2 Allow the buffer to fully enter the column. Once the buffer has reached the top of the gel layer, carefully pipet a 1-mL sample containing OMVs onto the surface of the beads, being careful not to disturb any of the beads at the top of the column. Allow the sample to fully enter the gel, that is, when no liquid remains above the gel layer, then stop the column.

We have also added “gravity-flow” to the Introduction:

We have found that a long, gravity-flow, SEC column, filled with gel filtration medium is sufficient for efficiently purifying and separating subpopulations of OMVs.

3) The authors indicate that this method is ideal for separating population of OMVs but they provide no evidence to indicate that discrete populations of OMVs have been isolated. An analytical method such as Laser Particle Tracking, Dynamic Light Scattering, or similar method should be used to verify that the subpopulations have been separated.

We have included DLS results in the Representative Results section of the text. For clarity, we have added those results to Fig. 2 as well.

4) The information in figure 2 does not show separation of OMV populations. There is too much overlap in samples, both lipids and toxin, to suggest that these are discreet population. The data may be clearer in a different format such as a line ran than spheres, however, as presented it is confusing.

We have modified Fig. 2 significantly based on this and other reviewer comments. We hope these changes make the data clearer.

5) I do not see why the authors include Figure 2B and 2C which both show failed detection assays. These do not convey any useful information.

We originally included these panels as an example of "suboptimal" results, as requested in the Guide for Authors. However, based on this and other reviewer comments, we have significantly updated Fig. 2 in the revised manuscript.

6) The authors use chemical formulas in the protocol section and then list full chemical names at the end in the Material/ Equipment section. It may be beneficial to some readers to include the name and formula in the Material/Equipment section.

We have added this information to the Material/Equipment Section.

Minor Concerns:

No minor comments. Manuscript was well written and the information that was provided was detailed and thorough.

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