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Size Exclusion Chromatography to Analyze Bacterial Outer Membrane Vesicle Heterogeneity

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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 peptidoglycans[1,2]. In particular, the role of OMVs in bacterial pathogenesis

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Disclosures

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has been extensively studied due to their enrichment in certain virulence factors and toxins[3, 4, 5, 6, 7, 8, 9, 10, 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, 13, 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, 17, 18, 19, 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, 13, 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. 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.
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.

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

1. Grow *A. actinomycetemcomitans* cells to the late exponential phase (optical density at 600 nm of 0.7). Pellet the cells by centrifuging twice at 10,000 *x g* at 4 °C for 10 min. Filter the supernatant through a 0.45 μm filter.
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.
3. Resuspend the pellet in PBS and ultracentrifuge again (105,000 *x g* at 4 °C for 30 min.) Resuspend the pellet in 2 mL of PBS.

3. Packing the S-1000 column

1. Mix the stock bottle of gel filtration medium with a glass stir rod and pour out into a glass bottle 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.
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. 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

1. Degas the elution buffer under vacuum. Wash the column with two column-volumes (180 mL) of elution buffer.
2. Allow the remaining buffer to fully enter the column. Once the buffer has reached the top of the gel layer, carefully pipette a 2-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.

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. 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.

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.

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

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.

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.

   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.

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

   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.

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

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

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

   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.

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

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.

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 samples 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.

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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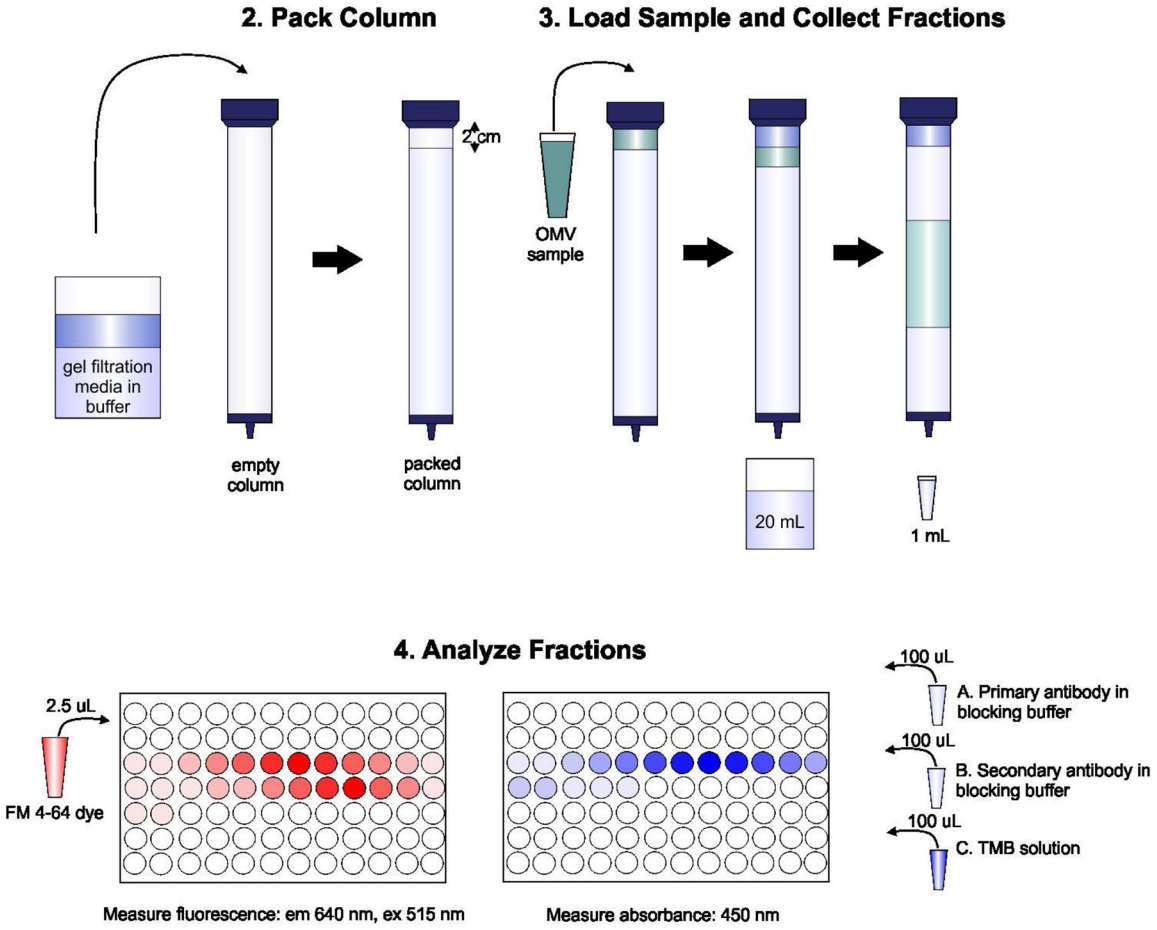

Figure 1: Schematic of SEC procedure.

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.

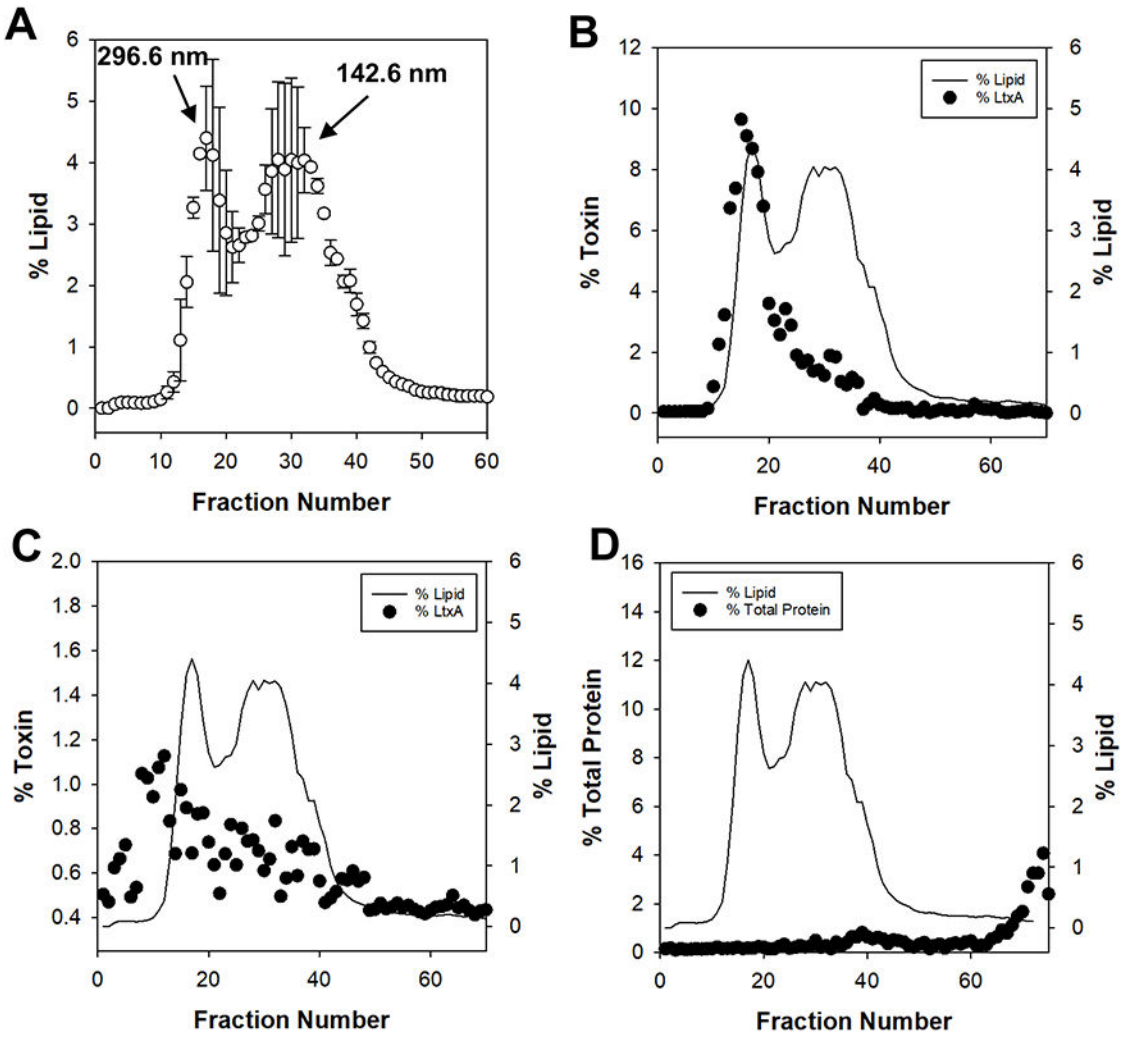

Figure 2: Representative results.

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 ± 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.

Materials

Name	Company	Catalog Number	Comments
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	