Chapter 16

Quantitative and Qualitative Preparations of Bacterial Outer Membrane Vesicles

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Abstract

Gram-negative bacterial outer membrane vesicle production and function have been studied using a variety of quantitative and qualitative methods. These types of analyses can be hampered by the use of impure vesicle preparations. Here we describe a set of techniques that are useful for the quantitative analysis of vesicle production and for preparative yields of highly purified vesicles for studies of vesicle function or composition. Procedures and advice are also included for the purification of vesicles from encapsulated and low-yield strains.

Key words: Gram-negative bacteria, Outer membrane, Vesicle, Bleb, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*

1. Introduction

Outer membrane vesicles are spherical structures that bud naturally from all Gram-negative bacteria studied to date (Fig. 1) (1–4). Vesicles were first identified in the 1960s in cultures of *Escherichia coli* grown under lysine-limiting conditions but were thereafter observed in cultures of normally growing bacteria (5). Vesicle production, or vesiculation, is a natural process and is not a result of membrane instability (6). Vesiculation has been shown to occur in planktonic cultures, biofilms, and *in vivo* (7–9). Although the exact mechanism of vesicle formation is unknown, vesicles from a variety of species have been shown to play roles in survival and host interactions.

Understanding the composition and function and determining the quantity of vesicles require the isolation of vesicles from bacterial culture supernatants. Proper functional characterization as well as quantitative assessments of vesicle content and biogenesis rely

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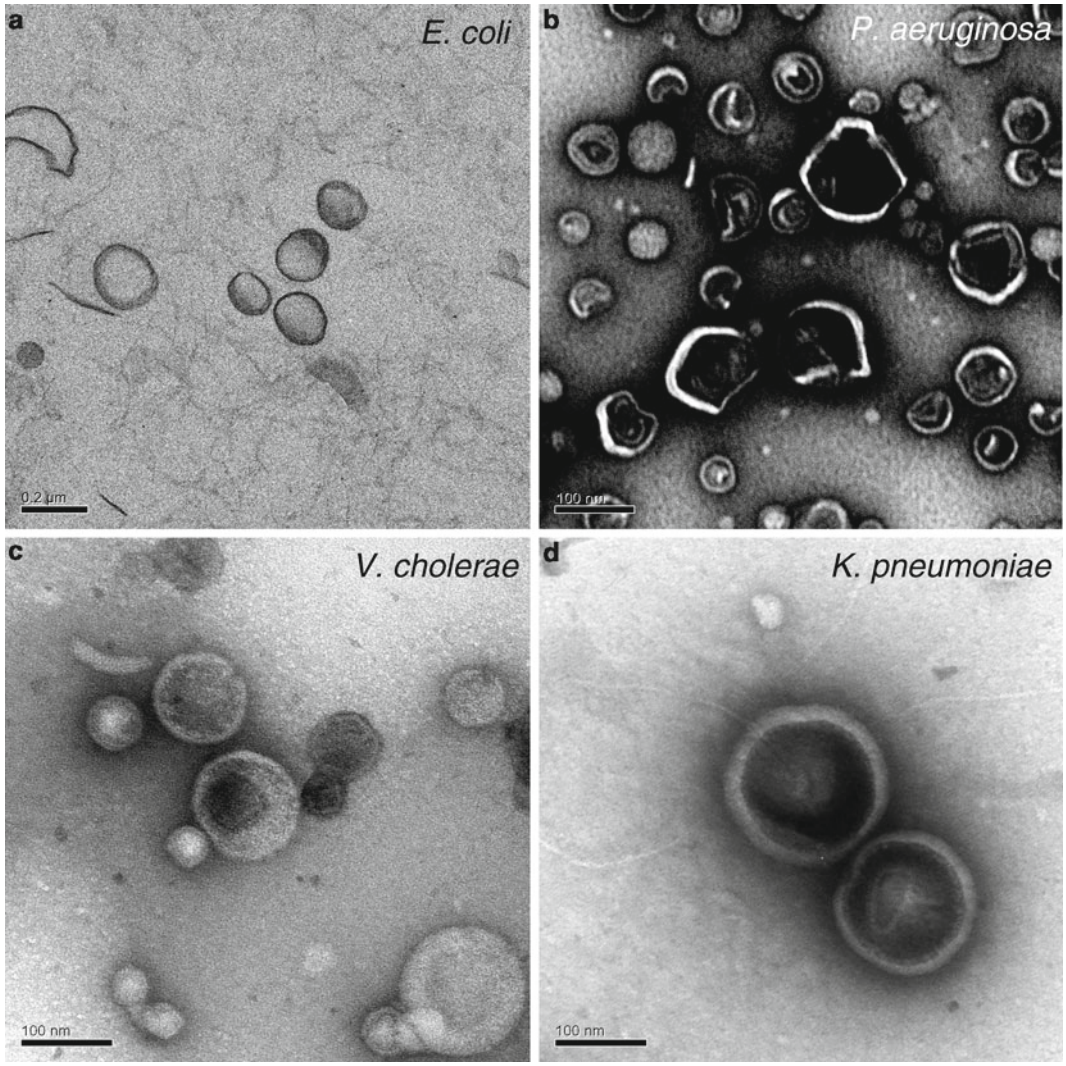

Fig. 1. Uranyl-acetate-stained electron micrographs of outer membrane vesicles purified from cultures of *E. coli*, *P. aeruginosa*, *Vibrio cholerae*, and *K. pneumoniae* (**a**–**d**, respectively). For electron microscopy procedure, *see* ref. 16. Size bar indicates 100 nm.

on the purity of the vesicles. If vesicles are simply sedimented by high-speed centrifugation from the culture supernatant they may be quite impure, depending on the propensity of other extracellular factors to co-sediment or co-precipitate with vesicles. These contaminants and other adherent extracellular material confuse studies of the vesicle proteome and host cell reactivity to vesicles. Differential density centrifugation of preparations generally allows the purification of vesicles away from such contaminants. However, as with any purification procedure, vesicle yield decreases as their purity increases. Therefore, other preparative and detection techniques must be used to acquire quantitative vesicle yield data.

Further complications in the preparation of vesicles arise from strains or species with low yields or which express capsule. In these cases, procedures can be modified to ensure sufficient vesicle yields.

For studies on the mechanism of vesicle formation or vesicle yield, a small-scale vesicle preparation is usually sufficient (6, 10). Vesicle quantity in these studies can be assessed by the specific analysis of outer membrane proteins and lipids in the pelleted supernatant and dividing by the amount of cells in the culture. These vesicle-specific probes would not detect contaminants, such as flagella, pili, and soluble factors, thus the vesicles produced from these small-scale preparations do not need to be further purified. Keeping the number of steps in quantitative vesicle analyses low reduces vesicle loss and error, increasing the accuracy and reproducibility of the data. By contrast, for studies on the interaction of vesicles with eukaryotic cells (e.g., toxicity or vesicle-mediated entry) or for vesicle content determination (e.g., proteomics or lipidomics), both a greater amount and a higher purity of vesicles are needed, and thus a large-scale vesicle preparation is required (11, 12). Density gradient centrifugation is commonly used to remove soluble and unassociated proteins from the vesicle fraction. The preparation of density-purified vesicles is particularly recommended for studies of vesicles from bacteria that secrete many other factors, such as *P. aeruginosa* (13). Although we generally recommend pooling the fractions which contain pure vesicles, it should also be considered that bacteria may produce vesicles of various densities with distinct functions. The nuances of the production and function of vesicles from the same organism, but with distinct densities, have yet to be explored.

Different protocols have also been used to isolate vesicles from different bacteria, including *Escherichia coli* (6), *Pseudomonas aeruginosa* (14), and *Neisseria meningitidis* (15). Here, we present protocols that have been used to efficiently isolate *E. coli* and *P. aeruginosa* vesicles for both quantitative (genetic) and qualitative (functional) studies (10, 11, 14). The *P. aeruginosa* protocol can be generally applied to bacteria that do not produce a lot of vesicles. Notes are included for capsulated strains (e.g., *Klebsiella pneumoniae*) since strongly associated cellular capsule can interfere with vesicle collection.

2. Materials

2.1. Small-Scale (Quantitative) Vesicle Preparation

2.1.1. General Preparation

1. 500 mL glass culture flask.
2. CFA (colonization factor antigen) broth: to approximately 3 L of water in a 4 L graduated cylinder or beaker, add 40 g casamino acids, 6 g yeast extract, 0.2 g $MgSO_4$, and 0.02 g $MnCl_2$. Adjust the pH to 7.4 using NaOH and then Q.S. (quantity

sufficient) to 4 L with water. Aliquot into culture flasks or bottles as necessary, autoclave, and store at room temperature.

3. LB broth: to 1 L of dH_2O in a 2 L culture flask, add 10 g of Bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl. Autoclave and allow media to cool before inoculating.
4. DPBSS (Dulbecco's phosphate buffered saline supplemented with salt): to approximately 750 mL of water in a 2 L graduated cylinder or beaker, add 0.2 g KCl, 0.2 g KH_2PO_4, 11.70 g NaCl, 1.15 g Na_2HPO_4, 0.1 g $MgCl_2 \cdot 6H_2O$, and 0.1 g $CaCl_2$; Q.S. to 1 L with water.
5. Centrifuge, rotor, and 250 mL centrifuge bottles capable of spinning at 38,000 × *g*.
6. Vacuum filtration apparatus: glass filter holder and ground joint flask.
7. 0.45 μm PVDF Millipore filters, 90 mm.
8. 0.45 μm centrifugal filters.
9. LB agar plates: to approximately 750 mL of water in a 2 L beaker, add approximately 10 g of Bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl and 15 g of agarose, and Q.S. to 1 L with water. Autoclave the solution and pour into plastic Petri plates. Allow the agar to set overnight. Store wrapped in plastic at 4°C.
10. Table top ultracentrifuge, rotor, and 1.7 mL tubes capable of spinning at 100,000 × *g*.
11. Protein concentration determination assay.
12. SDS-PAGE apparatus and gel, with Ruby or Coomassie stain.
13. FM4-64 fluorescent dye.
14. Spectrofluorometer.

2.1.2. Additional Material for Encapsulated Strains

1. Low-speed centrifuge with 250 and 500 mL polycarbonate centrifuge bottles.
2. 0.8 μm Cellulosic Acetate membrane filters, 90 mm.

2.2. Large-Scale Vesicle Preparation Using a Tangential Flow Concentrator

1. CFA or LB broth: see items 2 and 3, respectively, Subheading 2.1.1.
2. 3 L Fernbach culture flasks.
3. Low-speed centrifuge.
4. Tangential flow concentrator with a 100-kDa filter.
5. Concentrator wash buffer (1–2% Alconox): to approximately 2 L of water in a 4 L graduated cylinder or beaker, add approximately 45 g of Alconox, and Q.S. to 3 L with water.
6. Concentrator storage buffer (0.1 M NaOH): to approximately 2 L of water in a 4 L graduated cylinder or beaker, add 12 g of NaOH, mix, and Q.S. to 3 L with water.

7. 0.45-μm PVDF Millipore filter.
8. Glass vacuum filtration apparatus.
9. DPBSS: see item 4, Subheading 2.1.1.
10. LB agar: see item 9, Subheading 2.1.1.

2.3. Large-Scale Vesicle Preparation Without Concentrator

1. CFA or LB broth: see items 2 and 3, respectively, Subheading 2.1.1.
2. 3 L Fernbach culture flasks.
3. Low-speed centrifuge.
4. 0.45-μm PVDF Millipore filter.
5. Glass vacuum filtration apparatus.
6. DPBSS: see item 4, Subheading 2.1.1.

2.4. Small-Scale P. aeruginosa Vesicle Preparation

1. LB broth: see item 3, Subheading 2.1.1.
2. Amicon Ultra 10,000 MWCO centrifugation filters.
3. Slide-A-Lyzer® Dialysis Cassette 10,000 MWCO 3–12 m.
4. Low-speed centrifuge, rotor, 250 and 500 mL polycarbonate centrifuge bottles.
5. 50 mM Hepes, pH 7.5: to approximately 750 mL of water in a beaker add 11.9 g Hepes and adjust pH to 7.5 using NaOH. Q.S. to 1 L with water and filter-sterilize.
6. 0.45 μm PVDF Millipore filters.
7. Glass vacuum filtration apparatus.
8. 10 mL plastic syringe and needle.
9. Ammonium sulfate.

2.5. Purification of Vesicles by Density Gradient

1. OptiPrep diluent buffer: 0.85% NaCl in 10 mM Hepes, pH 7.4. Add 8.5 g NaCl and 2.38 g Hepes to approximately 750 mL of water in a graduated cylinder or beaker. Bring the pH to 7.4 with NaOH. Q.S. to 1 L with water and filter-sterilize.
2. OptiPrep solutions: dilute OptiPrep 60% stock solution in OptiPrep diluent buffer. The series of concentrations in the gradient steps depends on the strain of bacteria used, but a standard, recommended range of dilutions for the gradient is 40, 35, 30, 25, and 20% (final % Optiprep, v:v).
3. SDS-PAGE apparatus.
4. SDS-PAGE running buffer: to approximately 750 mL water in a graduated cylinder or beaker, add 14.4 g glycine, 3 g Tris base, and 1 g SDS. Q.S. to 1 L with water.
5. 15% SDS polyacrylamide gel: for the separating gel, mix 10.2 mL water with 7.5 mL 1.5 M Tris–HCl, pH 8.8, 0.15 mL 20% (w:v) SDS, and 12.0 mL Acrylamide/Bis-acrylamide

(30%/0.8% w:v). Add 0.15 mL 10% w:v ammonium persulfate (APS) and 0.02 mL tetramethylethylenediamine (TEMED).

6. Stacking gel (4% acrylamide): mix 3.075 mL water, 1.25 mL 0.5 M Tris–HCl, pH 6.8, 0.025 mL 20% w:v SDS, and 0.67 mL acrylamide/bis-acrylamide (30%:0.8%, w:v.) Add 0.025 mL 10% (w:v) APS and 0.005 mL TEMED.
7. 5× sample loading buffer: mix 0.1 M Tris–HCl, pH 6.8, 5 mL 100% glycerol, 0.5 g SDS, 1 mL β-mercaptoethanol, and 0.5 mL 2% bromophenol blue. Q.S. to 10 mL with dH_2O. Store aliquots at −20°C.
8. Ruby wash/fix solution: 7% acetic acid and 10% methanol in water. Add 70 mL acetic acid and 100 mL methanol to 830 mL dH_2O, and mix.
9. LB agar plates see item 9 Subheading 2.1.1.
10. 12.5-mL Ultraclear centrifuge tube.
11. Ultracentrifuge.
12. 0.45-μm PVDF Millipore centrifuge filters.
13. Tube rocker.

3. Methods

Perform all procedures at room temperature unless otherwise specified.

3.1. Small-Scale (Quantitative) Vesicle Preparation

1. Start an overnight culture in 5 mL of CFA or LB.
2. Inoculate 250 μL–2 mL of the overnight starter culture in each of up to six 500 mL-flasks containing 250 mL of CFA or LB (see Note 1).
3. Grow cultures at the desired temperature for the desired time and agitation (see Note 2).
4. Collect 1 mL, dilute 1,000- to 10,000-fold in sterile medium, and plate a tenfold dilution series to determine the amount of colony-forming units (CFU) in the culture (see Note 3).
5. To obtain vesicle-containing, cell-free supernatant for non-encapsulated strains, pour 240 mL of the growth culture into 250 mL bottles using a graduated cylinder and centrifuge at 10,000 × *g* for 10 min.
6. To obtain vesicle-containing, cell-free supernatant for encapsulated strains, follow these steps:
   (a) Centrifuge the cultures at 11,000 × *g* for 13 min in 500 mL polycarbonate bottles and collect the low-speed supernatant (see Note 4).

(b) Pour the low-speed supernatant into 250 mL polycarbonate bottles and centrifuge at 13,000 × *g* for 15 min and collect the mid-speed supernatant (see Note 5).

(c) Filter the mid-speed supernatant through a 0.8 μm filter, one sample at a time, and collect the filtrate that contains the vesicles. After each sample, remove the filter, wash the apparatus with 500 mL of dH_2O, replace with a new filter, and run the next sample through the apparatus.

7. Collect the supernatant in a sterile container, and filter it through a 0.45 μm Durapore PVDF Millipore filter using the glass vacuum filtration apparatus.
8. Centrifuge the filtered supernatant at 38,400 × *g* for 1–2 h (see Note 6).
9. Decant the supernatant (see Note 7).
10. Resuspend the vesicle-containing pellet in the residual supernatant remaining in the centrifuge bottle after decanting (approximately 1 mL) or resuspend in 1 mL fresh DPBSS. Transfer to 1.7 mL ultracentrifuge tube and centrifuge in tabletop ultracentrifuge at 100,000 × *g* for 1 h.
11. Decant the supernatant and resuspend the pellet in 100 μL DPBSS (see Note 8). The resuspended pellet is the vesicle preparation.
12. Filter-sterilize the vesicles using a 0.45 μm centrifugal filter. Check for sterility by plating on LB agar. Re-sterilize using 0.45 μm filters if needed.
13. To quantitate the vesicle yield in terms of mg vesicle protein/CFU, use a protein concentration determination assay to determine the total protein concentration in the vesicle preparation and divide the number by the CFU obtained from dilution plating of the culture at the time of harvest. In some cases, flagella and other non-vesicle proteins contaminate the preparation; however, further purification steps (e.g., using density gradient purification) prevent quantitative recovery. Therefore, vesicle yields often can be best compared using the quantity of vesicle-specific protein or lipid in the pelleted cell-free supernatant preparations. Here, an aliquot of the vesicle preparation is run on SDS-PAGE, stained for protein using Ruby or Coomassie, and either the total protein in each sample or the major outer membrane proteins (e.g., Omps F/C and A for *E. coli*) in each sample are determined by densitometry. Subsequently, the densitometry value is divided by the CFU and this vesicle yield compared between strains or treatments. Finally, vesicle yield can also be determined based on lipid content using FM4-64, a lipophilic fluorescent dye. For FM4-64-based measurements, 20 μL vesicle preparation is diluted in 560 μL of DPBSS, and 20 μL FM4-64 is added. Fluorescence

(RFU) is measured with an excitation of 506 nm and emission of 750 nm using a spectrofluorometer. Subsequently, the RFU value is divided by the CFU and this vesicle yield value compared between strains or treatments.

3.2. Large-Scale Vesicle Preparation Using a Tangential Flow Concentrator

1. Start an overnight culture in 10 mL of CFA or LB.
2. Autoclave 3 L culture flasks with 1.5 L of medium in each (12 L total).
3. Inoculate each flask with 750 μL of culture and grow at 37°C overnight.
4. Centrifuge the cultures at 15,000 × *g* for 15 min. Collect and pool the supernatants (see Note 9).
5. Drain the concentrator reservoir of concentrator storage solution and rinse with dH_2O.
6. Add the supernatants to the concentrator and turn on the flow through the filter. Take care to ensure that the volume in the reservoir does not go below 250 mL. Add the supernatant to the reservoir until all of the supernatant has been concentrated.
7. Once all of the supernatant has been concentrated down to 250 mL, backwash with 250 mL of medium through the filter for a total of 500 mL.
8. Drain the supernatant from the reservoir into centrifuge bottles, bypassing the filter, and centrifuge at 6,000 × *g* for 6 min.
9. Filter the supernatant through a 0.45-μm PVDF Millipore filter using the glass vacuum filtration apparatus.
10. Centrifuge the supernatant at 38,400 × *g* for 1–2 h and resuspend the pellet in a small volume of DPBSS (see Note 8).
11. Streak out a small amount of the resuspended vesicle-containing pellet on an LB plate to verify sample sterility. Re-filter if necessary.
12. Wash the concentrator filter with 2 L of concentrator wash buffer. Backwash approximately 500 mL of concentrator wash buffer through the filter into the reservoir. Bypassing the filter, drain the reservoir, and rinse with dH_2O until the reservoir is no longer sudsy. Drain 2.5 L dH_2O through the filter. Rinse the filter with 500 mL of concentrator storage buffer and store the reservoir with 2 L of concentrator storage buffer.

3.3. Large-Scale Vesicle Preparation Without Concentrator

1. Follow steps 1–4 of Subheading 3.2.
2. Filter each batch of supernatant through 0.45 μm PVDF Millipore filters using the glass vacuum filtration apparatus and store the cell-free supernatant at 4°C.
3. Once all of the cultures have been centrifuged and filtered, add the cell-free supernatant to six centrifuge bottles, and centrifuge at 38,400 × *g* for 1 h.

4. Decant the supernatant, add another aliquot of cell-free supernatant to the same bottles, and re-centrifuge until all of the cell-free supernatant has been pelleted.
5. Resuspend the vesicle pellets in five of the bottles and add the resuspended vesicles to the sixth bottle to pool all vesicle pellets.
6. Centrifuge the pooled vesicles at 38,000 × *g* for 1–2 h, decant the supernatant, and resuspend the vesicle pellet in the smallest possible volume of DPBSS.

3.4. Small-Scale P. aeruginosa Vesicle Preparation

1. Start an overnight culture in 5 mL of LB broth.
2. Inoculate a 1 L flask containing 250 mL of LB broth with 5 mL of culture and incubate at 37°C until an OD_{600} ~0.9–1.0 (see Note 10).
3. Pour the culture into a centrifuge bottle and centrifuge at 10,000 × *g* for 10 min. Collect the supernatant.
4. Filter the supernatant through a 0.45 μm PVDF Millipore filter using the glass vacuum filtration apparatus.
5. Pour the filtered supernatant into a 500 mL centrifuge bottle.
6. Add 129 g of ammonium sulfate to the filtered supernatant, add a stir bar, and dissolve at room temperature at moderate speed on a stir-plate.
7. Once dissolved, move to 4°C and continue stirring for at least 3 h to precipitate the vesicle-containing fraction from the supernatant.
8. Remove the stir bar and centrifuge the sample for 20 min at 10,000 × *g* (see Note 11).
9. Decant the supernatant carefully, without disrupting the pellet or the precipitate on the side of the bottle.
10. Resuspend the precipitate by gently rocking the bottle with 10 mL of 50 mM Hepes, pH 7.5 buffer.
11. Using a syringe and needle, inject the resuspended sample into 3–12 mL 10,000 molecular weight cut-off slide-a-lyzer dialysis cassettes. Remove the remaining air bubbles from the cassettes.
12. Dialyze the vesicle preparations in 50 mM Hepes, pH 7.5 buffer for at least 12 h at 4°C, with stirring. Use at least 100-fold the sample volume of buffer.
13. Collect, pool, and concentrate the samples to a final volume of 500 μL using 10,000 molecular weight cut-off Amicon Ultra centrifugation filters (see Note 12).

3.5. Purification of Vesicles by Density Gradient

1. Add OptiPrep (60% stock) to the resuspended vesicles at a ratio of 1:3 (by volume) to adjust the vesicle preparation to 45% OptiPrep (v:v) (see Note 13).

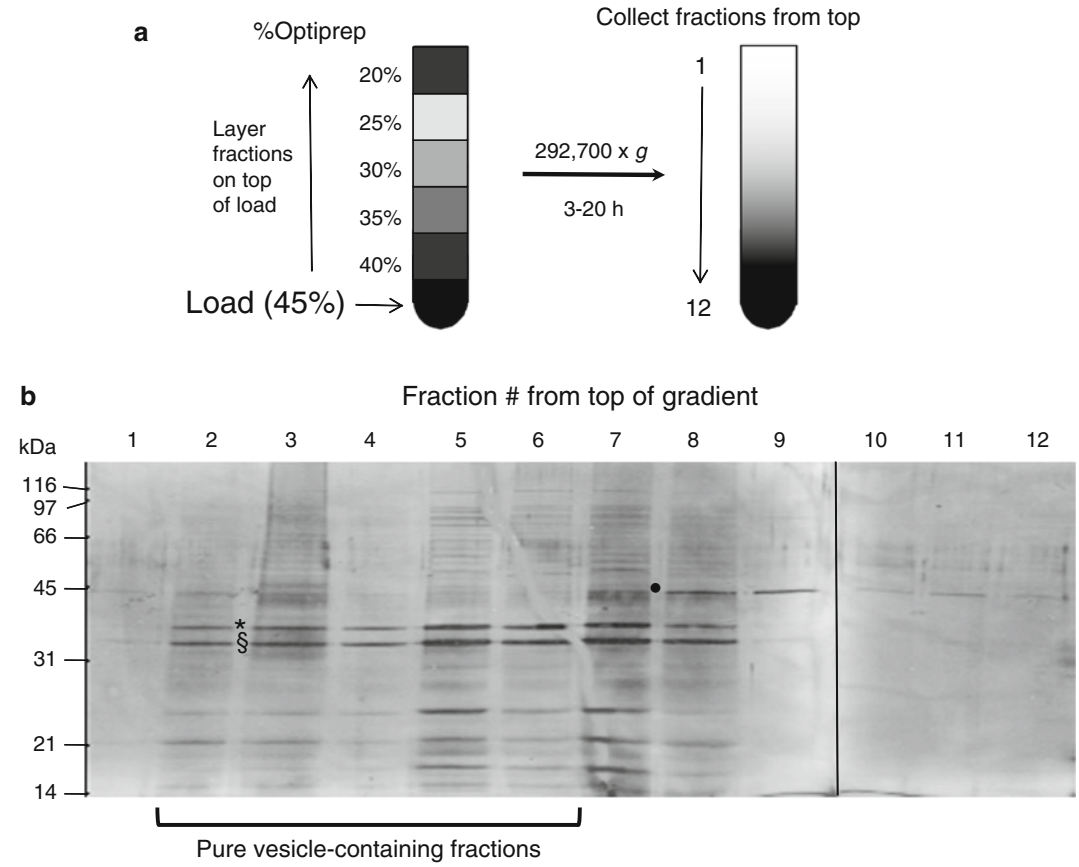

Fig. 2. (**a**) Schematic of ultracentrifuge tubes loaded with the vesicle preparation in the 45% Optiprep at the bottom of the tube, layered with decreasing density of Optiprep prior to and after centrifugation. After the gradient has reached equilibrium (3 h to overnight), fractions (typically 12 equal fractions of approximately 1 mL each) are removed sequentially from the top of the tube. (**b**) Optiprep gradient fractionation analysis by SDS-PAGE. Vesicles were collected from a culture of enterotoxigenic *E. coli* grown in LB, and the vesicle-containing pelleted supernatant was loaded on a 45–20% Optiprep gradient and ultracentrifuged. Twelve fractions (1 mL each) were collected and analyzed by Coomassie-stained 15% SDS-PAGE. The migration of molecular weight standards is indicated (kDa). Symbols * and § indicate the migration of OmpsF/C and OmpA, respectively, which are vesicle outer membrane proteins; • indicates the migration of a contaminating, non-vesicle protein that was present in the material originally pelleted at 38,400 × *g* from the culture supernatant. Pure vesicle-containing fractions are *bracketed*.

2. Pipette 2 mL of vesicles in 45% OptiPrep to the bottom of a 12.5-mL Ultraclear centrifuge tube.
3. Carefully layer 2 mL of each OptiPrep dilutions in descending order on the top of the preceding layer (see Note 14) (Fig. 2a).
4. Centrifuge the tube at 292,700 × *g* (120,000 × *g* for *P. aeruginosa*) in an ultracentrifuge for 3 h to overnight.
5. Collect sequential fractions by placing the tip of a 1 mL pipette at the top of the gradient and carefully removing 1 mL (see Note 15).

6. Analyze a portion (12 μL) of each fraction using 15% SDS-PAGE and visualize the proteins in the gel using Coomassie or Ruby protein staining. Identify the vesicle fractions by the presence of outer membrane proteins (OmpF/C and OmpA for *E. coli* and OprF/D and OprH for *P. aeruginosa*) (Fig. 2b).
7. Pool the selected vesicle-containing fractions, and add them to a centrifuge bottle with at least tenfold the sample volume using DPBSS.
8. Centrifuge at 38,400×*g* for at least 3 h to remove the OptiPrep.
9. Resuspend the vesicle pellet in the smallest volume of DPBSS, and filter-sterilize the vesicles using 0.45-μm PVDF Millipore centrifuge filters (see Note 16).
10. Confirm sterility by plating an aliquot of vesicles on LB agar plates. Filter again through 0.45-μm PVDF Millipore centrifuge filters, if necessary.

4. Notes

1. If the preparations will be compared for quantitative yield (e.g., amount of vesicles/CFU), the preparation of duplicate samples is suggested to improve accuracy. Consequently, given the capacity limit of the rotor, a typical run can be used to compare three strains in duplicate at a time.
2. For encapsulated strains, stop growth incubation once the culture reaches an OD_{600} of 1.0–1.6 and place the culture on ice. Growth is restricted for capsulated strains to partially alleviate the problems of pouring off supernatants of heavily capsulated bacteria. The more you allow the strains to grow the harder it is to separate the capsule from the supernatant.
3. Plating of samples of the culture diluted 10^{-5}-, 10^{-6}-, and 10^{-7}-fold is recommended for determining an accurate bacterial count.
4. Encapsulated strains will have a sloppy pellet, meaning that as the supernatant is decanted, a substantial amount of capsule will mix with the supernatant after the first spin. This is why two pelleting steps are recommended. It is, however, important that a different centrifuge bottle is used for each spin (or at the least, that they are washed between spins) as the capsule will not stick to the bottle as well if there is already a pellet there.
5. This spin step is used to capture all of the capsule in the pellet of each bottle. Pour cautiously, and attempt to not disturb the pellet so as to mix the capsule back into the supernatant. If the

pellet is coming off the bottle wall, attempt to collect as much supernatant as possible (and avoid pouring off the pellet), but retain some of the supernatant, supplement with uncentrifuged cell-free supernatant to balance the bottles, and centrifuge again before filtration step.

6. Because the vesicle pellet may not be visible after this step, it is easiest to use a lab marker to designate the area on the outside of the centrifuge bottle where the pellet will form.
7. Vesicle pellets may not be visible, use caution when pouring off your supernatant and watch your pellet the entire time. If the sample appears to have disappeared, stop pouring and spin again. To ensure that no excess liquid leads to sample dilution, it may help to vacuum aspirate all liquid from the lip and wall of each centrifuge flask.
8. Generally 100 μL will resuspend the pellet without a problem. If the pellet is too large, resuspend in a more appropriate volume but do not forget to account for that change when comparing yields for samples. However, if the vesicle pellet is not visible after centrifugation, focus the pipetting of the 100 μL to the spot that was marked on the centrifuge bottle to identify the location of the vesicle pellet. Resuspending the pellet in DPBSS may result in a frothy solution. To ensure that the entire vesicle fraction is collected, take care to collect all of the bubbles.
9. The cell pellets may be very loose. Take care not to pour out the pellet with the supernatant. If the pellet is too loose, leave some supernatant in the bottom of the tube and re-centrifuge the bottle with fresh culture.
10. Cultures of *P. aeruginosa* are only grown to late log phase because degradative enzymes that could degrade outer membrane vesicles are produced during stationary phase.
11. If the precipitate remains suspended in the supernatant, re-centrifuge as necessary.
12. During concentration, the sample will become compacted at the bottom of the concentrator. Between spins, resuspend the concentrated sample by gentle pipetting.
13. OptiPrep is very viscous. We recommend using disposable plastic pipettes near a flame for all OptiPrep manipulations. When pipetting the OptiPrep solution, draw in approximately 1 mL more OptiPrep than necessary, and then during dispensing, ensure that the liquid drains down to the appropriate measurement.
14. Place the tip of a plastic pipette to the inside of the tube below the rim and slowly add each OptiPrep solution in a slow, steady stream. There should be a distinct boundary between each layer.

15. Because OptiPrep is so viscous, we recommend using a new tip for each tube, even for the same fraction number.
16. Sometimes, the vesicle suspension will be too concentrated to pass through the filter and may be retained on the filter. In these cases, a visual pellet will be visible on the filter. Resuspend the pellet in sterile DPBSS and re-sterilize the vesicles by filtering the suspension using more centrifugal filters.

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