Journal of Visualized Experiments

Scalable isolation and purification of extracellular vesicles from Escherichia coli and other bacteria

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63155R2
Full Title:	Scalable isolation and purification of extracellular vesicles from Escherichia coli and other bacteria
Corresponding Author:	Mohammed Dwidar, PhD Cleveland Clinic Lerner Research Institute Cleveland, OH UNITED STATES
Corresponding Author's Institution:	Cleveland Clinic Lerner Research Institute
Corresponding Author E-Mail:	DWIDARM@ccf.org
Order of Authors:	Dionysios Watson
	Sadie Johnson
	Akeem Santos
	Mei Yin
	Defne Bayik
	Justin Lathia
	Mohammed Dwidar, PhD
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biology
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Cleveland, Ohio, USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	

1 TITLE:

Scalable Isolation and Purification of Extracellular Vesicles from *Escherichia coli* and Other Bacteria

AUTHORS AND AFFILIATIONS:

Dionysios C. Watson[1,2,3], Sadie Johnson[1], Akeem Santos[1,4], Mei Yin[5], Defne Bayik[1], Justin D. Lathia[1,3], Mohammed Dwidar[1,3,4]

[1]Department of Cardiovascular & Metabolic Sciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA
[2]University Hospitals Cleveland Medical Center, Cleveland, OH, USA
[3]Case Western Reserve University, Cleveland, OH, USA
[4]Center for Microbiome & Human Health, Cleveland Clinic, Cleveland, OH, USA
[5]Electron Microscopy Core, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA

Email addresses of co-authors:

Sadie Johnson	(johnsos21@ccf.org)
Akeem Santos	(santosa4@ccf.org)
Mei Yin	(yinm@ccf.org)
Defne Bayik	(watsond3@ccf.org)
Justin D. Lathia	(lathiaj@ccf.org)

Corresponding authors:

Dionysios C. Watson	(dionysios.watson@uhhospitals.org)
Mohammed Dwidar	(dwidarm@ccf.org)

SUMMARY:

Bacteria secrete nanometer-sized extracellular vesicles (EVs) carrying bioactive biological molecules. EV research focuses on understanding their biogenesis, role in microbe-microbe and host-microbe interactions and disease, as well as their potential therapeutic applications. A workflow for scalable isolation of EVs from various bacteria is presented to facilitate standardization of EV research.

ABSTRACT:

Diverse bacterial species secrete ~20–300 nm extracellular vesicles (EVs), comprised of lipids, proteins, nucleic acids, glycans, and other molecules derived from the parental cells. EVs function as intra- and inter-species communication vectors while also contributing to the interaction between bacteria and host organisms in the context of infection and colonization. Given the multitude of functions attributed to EVs in health and disease, there is a growing interest in isolating EVs for *in vitro* and *in vivo* studies. It was hypothesized that the separation of EVs based on physical properties, namely size, would facilitate the isolation of vesicles from diverse bacterial cultures.

The isolation workflow consists of centrifugation, filtration, ultrafiltration, and size-exclusion chromatography (SEC) for the isolation of EVs from bacterial cultures. A pump-driven tangential flow filtration (TFF) step was incorporated to enhance scalability, enabling the isolation of material from liters of starting cell culture. *Escherichia coli* was used as a model system expressing EV-associated nanoluciferase and non-EV-associated mCherry as reporter proteins. The nanoluciferase was targeted to the EVs by fusing its N-terminus with cytolysin A. Early chromatography fractions containing 20–100 nm EVs with associated cytolysin A – nanoLuc were distinct from the later fractions containing the free proteins. The presence of EV-associated nanoluciferase was confirmed by immunogold labeling and transmission electron microscopy. This EV isolation workflow is applicable to other human gut-associated gram-negative and gram-positive bacterial species. In conclusion, combining centrifugation, filtration, ultrafiltration/TFF, and SEC enables scalable isolation of EVs from diverse bacterial species. Employing a standardized isolation workflow will facilitate comparative studies of microbial EVs across species.

INTRODUCTION:

Extracellular vesicles (EVs) are nanometer-sized, liposome-like structures comprised of lipids, proteins, glycans, and nucleic acids, secreted by both prokaryotic and eukaryotic cells[1]. Since the early studies visualizing the release of EVs from gram-negative bacteria[2], the number of biological functions attributed to bacterial EVs (20–300 nm in diameter) has constantly been growing in the past decades. Their functions include transferring antibiotic resistance[3], biofilm formation[4], quorum sensing[5], and toxin delivery[6]. There is also growing interest in the use of bacterial EVs as therapeutics, especially in vaccinology[7] and cancer therapy[8].

Despite the growing interest in EV research, there are still technical challenges regarding methods of isolation. Specifically, there is a need for isolation methods that are reproducible, scalable, and compatible with diverse EV-producing organisms. To create a unified set of principles for planning and reporting EV isolation and research methods, the International Society for Extracellular Vesicles publishes and updates the MISEV position paper[9]. Moreover, the EV-TRACK consortium provides an open platform for reporting detailed methodologies for EV isolation used in published manuscripts to enhance transparency[10].

In this protocol, previous methodologies used for the isolation of EVs from mammalian cell culture were adapted[11,12] to enable the isolation of EVs from bacterial cell culture [*Insert* ***Figure 1*** *here*]. We sought to employ methods that enable EV isolation from a variety of microbes, which can be scalable, and balance EV purity and yield (as discussed in the MISEV position paper[9]). After removing bacterial cells and debris by centrifugation and filtration, the culture medium is concentrated either by centrifugal device ultrafiltration (for a volume of up to ~100 mL) or pump-driven TFF (for larger volumes). EVs are then isolated by SEC using columns optimized for the purification of small EVs.

A mouse-commensal strain of *Escherichia co*li (*i.e., E. coli* MP1[13]) was used as a model organism and modified to express EV-associated nanoluciferase by fusion to cytolysin A, as previously reported[14]. The methods used here can process at least up to several liters of bacterial cultures

and effectively separate EV-associated from non-EV-associated proteins. Finally, this method can also be used for other gram-positive and gram-negative bacterial species. All relevant data of the reported experiments were submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV210211)[10].

PROTOCOL:

NOTE: Ensure that all work involving bacteria and recombinant DNA follows best practices for biosafety containment appropriate for the biosafety hazard level of each strain. Work should be done in accordance with local, national, and international biosafety regulations.

1. Bacterial strains and culturing conditions

NOTE: Bacterial strains used in this study were *Escherichia coli* MP1[13], *Akkermansia mucinophila*, *Bacteroides thetaiotaomicron*, *Bifidobacterium breve*, and *Bifidobacterium dentium.*

1.1. For *E. coli,* use a sterile loop to inoculate single colonies into 250 to 1,000 mL of Luria-Bertani (LB) broth and incubate aerobically in a shaking incubator at 300 rpm and 37 °C for 48 h before processing the culture. For recombinant *E. coli* MP1 strain harboring p114-mCherry-Clyluc (**Supplemental Method** and **Supplemental Figure S1**), add chloramphenicol to the LB agar and broth at a final concentration of 17 µg/mL.

1.2. For *A. mucinophila*, *B. thetaiotaomicron*, *B. breve,* and *B. dentium,* streak on Brain heart infusion (BHI) agar plates and incubate anaerobically inside a vinyl anaerobic chamber. Inoculate single colonies into 100 mL of pre-reduced BHI broth and incubate for 48 h anaerobically.

2. EV isolation

2.1. Clarifying bacterial culture medium by centrifugation and filtration

2.1.1. Transfer the bacterial cell cultures inoculated in step 1 to clean 250 mL or 500 mL polypropylene centrifuge bottles by pouring. Centrifuge the bottles in a large-capacity, fixed-angle rotor at 4 °C and 5,000 × *g* for 15 min. Transfer the supernatant to clean centrifuge bottles by careful pouring, and centrifuge again at 10,000 × *g* for 15 min.

NOTE: Reuse the bottles after biosafety-appropriate cleaning and decontamination.

2.1.1.1. If large pellets of bacterial cells are present after the second centrifugation, repeat the centrifugation in a clean bottle to further remove cells.

2.1.2. Transfer the supernatant to a 0.2 µm polyethersulfone vacuum-driven filter device of appropriate size by pouring. Filter by connecting the filtration device to a vacuum wall supply. If the filtration rate drops significantly, simply move any unfiltered material to a new device. Store the filtered medium at 4 °C overnight, and continue the protocol the following day if desired.

NOTE: The centrifugations above typically allow processing of ~2x the indicated volume of cell culture through each device. For example, a single 500 mL filter device could filter ~1,000 mL of pre-centrifuged culture. These devices are not typically reused. Using syringe filters at this step is not recommended without optimization, as significant losses were noted with the tested models. This is a potential stopping point.

2.1.3 Check for the complete removal of the viable cells at this point by spreading an aliquot of the filtered supernatant on suitable agar plates and ensure the absence of any colonies after incubation at optimum conditions for the bacterial strain. If bacteria are detected, further optimize the procedure above by performing additional centrifugations and/or filtrations.

2.2. Concentration of the filtered medium

2.2.1. **If working with volumes significantly >100 mL, proceed to step 2.2.2.** If working with volumes of ~100 mL, load 90 mL of filtered culture medium onto the reservoir of a respective capacity 100 kDa molecular weight cutoff (MWCO) centrifugal ultrafiltration device using serological pipettes. Always balance with a matching ultrafiltration device, and centrifuge in swinging bucket rotor at 4 °C and 2,000 × *g* for 15–30 min intervals, until the volume of the medium in the top reservoir has been concentrated to <0.5 mL.

2.2.1.1. Top up the reservoir with any remaining filtered culture medium. If "topping up," remove the flow-through in the bottom of the device and re-balance any devices.

NOTE: It was observed that the maximum volume of filtered culture medium that can be concentrated using these devices is <2-fold the recommended volume.

2.2.1.2. If the viscosity of the concentrated medium in the reservoir is visibly increased (dark, viscous material), dilute with phosphate-buffered saline (PBS) and re-concentrate by centrifugation to dilute any non-EV proteins smaller than the MWCO of 100 kDa.

NOTE: This is a potential stopping point.

2.2.1.3. Transfer the concentrated medium to a low-protein-binding tube, store at 4 °C overnight, and continue the protocol the following day if desired.

2.2.2. **If working with volumes significantly >100 mL,** select an appropriately sized TFF device (100 kDa MWCO) to accommodate the volume to be processed.

NOTE: This information is provided by manufacturers. Filtration devices for processing 100 mL to >1,000 mL are commercially available. Up to 2 L of culture medium were processed with the device indicated in the **Table of Materials** before needing to clean the filter (see step 2.3 below for the cleaning protocol).

2.2.2.1. Assemble a filtration circuit with #16 low-binding/low-leaching tubing, 1/8 inch hose-barb to Luer adapters, the TFF device, and a peristaltic pump, as indicated in **Supplemental Figure S2**.

NOTE: Perform TFF within a biosafety cabinet to minimize the risk of contaminating the EV preparation with environmental bacteria.

2.2.2.2. At room temperature, begin circulating the filtered, conditioned medium at approximately 200 mL/min (minimum 100 mL/min). Determine the appropriate RPM corresponding to the desired flow rate by pumping 200 mL of PBS into a graduated vessel. When circulating filtered, conditioned medium, collect the molecules <100 kDa crossing the ultrafiltration membrane as waste in a separate vessel.

NOTE: The example below will be assuming a starting volume of 2 L of culture.

2.2.2.3. Continue to circulate the conditioned medium until its volume has been reduced to ~ 100–200 mL. Move to smaller vessels as needed. Dilute 2-fold with PBS, and continue to circulate with the pump, concentrating down to 75–100 mL. Dilute 2-fold with PBS, and continue to circulate to a final volume of 25 mL. Dilute 2-fold with PBS and continue to circulate until <10 mL.

2.2.2.4. Lift the feed tubing out of the sample reservoir, and continue to pump to purge the filter and recover the maximum amount of sample.

NOTE: This is a potential stopping point.

2.2.2.5. Transfer the concentrated sample to a conical tube and store overnight at 4 °C if desired. Alternatively, continue with the protocol.

2.2.2.6. Move the concentrated sample to a 15 mL capacity 100 kDa MWCO centrifugal ultrafiltration device. Centrifuge in a swinging bucket rotor at 4 °C and 2,000 × *g* for 15–30 min intervals until the volume of the medium in the top reservoir has been concentrated to <2 mL.

NOTE: This is a potential stopping point.

2.2.2.7. Transfer the concentrated medium to a low-protein-binding tube, and store at 4 °C overnight, continuing the protocol the following day if desired.

2.3. Cleaning the TFF device (optional)

NOTE: The filtration rate decreases as the TFF device begins to "clog" during the process (fouling). If necessary, the filter device can be cleaned to facilitate filtration of additional samples in the same purification run. Though theoretically possible, a cleaned TFF filter has not been used for a different purification run to avoid cross-contamination.

2.3.1. To clean, remove all tubing and caps from the TFF device and drain any residual liquid.

2.3.2. Use the peristaltic pump and tubing to flood both the inner and outer compartments of the TFF device (i.e., via the parallel and perpendicular ports in the model listed in the **Table of Materials**) with ~100 mL of distilled water. Remove all tubing/caps and drain the TFF device.

2.3.3. Cap the outer (perpendicular, filtrate) ports and circulate 250 mL of 20% ethanol in distilled water at >200 mL/min for 10 min through the inner compartment. Drain, flood with distilled water, and drain again as above.

2.3.4. Circulate 250 mL of 0.5 N fresh NaOH solution for 30 min through the inner compartment and drain again.

2.3.5. Reconnect all tubing and caps to the inlet, outlet, and filtrate ports, as in **Supplemental Figure S2**, and circulate 0.5 N NaOH solution again until a volume of NaOH > 1 mL/cm^2 filter surface area permeates through the filter membrane and is collected as filtrate/waste.

2.3.6. Rinse the TFF device with distilled water as above. Use the TFF device immediately or flood the device with ~100 mL of 20% ethanol and store overnight at 4 °C.

NOTE. If stored in ethanol, be sure to drain, rinse with water, drain, and circulate 250 mL of PBS through the device until a volume of >1 mL/cm^2 filter surface area permeates through the filter membrane and is collected as filtrate/waste to remove residual ethanol prior to sample processing.

2.4. Size exclusion chromatography (SEC)

NOTE: SEC is used to increase the purity of EVs and remove non-vesicular protein.

2.4.1. Use a small SEC column (10 mL bed volume) for the isolation of EVs from <100 mL of starting material and a larger column (47 mL bed volume) for the isolation of EVs from >100 mL of starting material.

NOTE: The example below will list volumes for the larger column, with volumes for the smaller column in parentheses.

2.4.2. Bring the SEC column and PBS to room temperature over several hours. Stabilize the SEC column in a vertical position using a standard laboratory stand and holder. Alternatively, use commercial chromatography column stands.

2.4.3. Before connecting to the SEC column, hydrate the sample reservoir by allowing 5 mL of PBS to flow through the frit and into a waste container. Unscrew the inlet cap of the SEC column, add 2 mL of PBS to the sample reservoir, and carefully connect the reservoir to the column as the PBS is dripping out through the frit (not applicable for small SEC columns).

NOTE: This previous step prevents any air bubbles from getting trapped at the top of the SEC column. If air is trapped, remove the reservoir, tap the column to get the air bubble out, and repeat the connection procedure. For the smaller column, simply uncap the top of the SEC column, and attach the sample hopper.

2.4.4. Add 47 mL (10 mL) of PBS to the sample reservoir and uncap the bottom of the SEC column. Allow all the loaded sample buffer to flow through the column for equilibration. Discard the flow-through.

2.4.5. Load a maximum of 2 mL (0.5 mL) of sample onto the sample reservoir, immediately begin collecting the flow-through, and allow the sample to enter the column completely.

2.4.6. Immediately add PBS to the sample reservoir or hopper at a volume of 14.25 mL minus the sample volume (3 mL minus the sample volume, for the small column). Allow the solution to flow through the column and discard this amount equal to the column void volume.

NOTE: For a typical 2 mL sample, the amount of PBS to be added to the sample reservoir or hopper will be 12.25 mL.

2.4.7. Position a 2 mL low-binding microtube directly below the SEC column. Immediately add 2 mL (0.5 mL) of PBS to the sample reservoir and allow it to enter the column. Label this first 2 mL (0.5 mL) of flow-through as **Fraction 1**. Continue to add 2 mL (0.5 mL) at a time to the sample reservoir to collect each subsequent fraction.

NOTE: Most bacterial EVs elute in the first 5 fractions. During optimization, the first 12 fractions were collected.

2.4.8. Store the fractions at 4 °C for short-term storage (days) or -80 °C for long-term storage.

2.4.9. Cleaning and storage of the reusable SEC columns

NOTE: The SEC columns described in this protocol can be reused up to 5 times according to the manufacturer.

2.4.9.1. To clean, add 2 mL (0.5 mL) of 0.5 M NaOH and allow it to enter the column completely. Run 100 mL (20 mL) of 20% ethanol through the column and store it at 4 °C until the next use. Before the next use, equilibrate the ethanol to room temperature as above, and exchange it with PBS buffer by running another 150 mL (30 mL) of PBS through the column.

3. EV preparation quality control

3.1. Sterility testing

NOTE: As these EVs come from bacterial cultures, it is critical to ensure sterility prior to downstream use.

3.1.1. Obtain 100 µL (20 µL) of the fractions to be used in assays and inoculate 3 mL of the medium used to grow the source bacteria. Culture under the respective optimal conditions for at least 3 days and observe for turbidity. Alternatively, apply the fraction samples to agar plates containing the medium used to grow the producing bacteria and look for colony formation.

NOTE: If bacterial contamination is detected, it is not recommended to use the EV preparation for experimentation. Instead, repeat the isolation, taking care to minimize the risk of bacterial contamination by (a) performing sufficient centrifugation/filtration of conditioned bacterial cell culture medium, (b) using clean bottles, tubing, filters, and chromatography columns, and (c) employing appropriate aseptic techniques.

3.2. Protein quantification

NOTE: A high-sensitivity, fluorescence-based protein quantification kit was used (see the **Table of Materials**). The kit works with a matching proprietary fluorimeter at excitation/emission wavelengths of 485/590 nm.

3.2.1. Bring all reagents, standards, and samples to room temperature.

3.2.2. Prepare a master mix of protein reagent and buffer by adding 1 µL of the reagent to 199 µL of buffer for each sample and standard to be assayed. Using thin-walled 0.5 mL PCR tubes, add 10 µL standard + 190 µL of master mix to each standard tube.

NOTE: To be within the range of the assay, the amount of each fraction to be added to each sample tube depends on the expected protein yield of the purification. Typically, 5 µL of each fraction + 195 µL of master mix were used. The final volume of sample + master mix must be 200 µL.

3.2.3. Vortex the assay tubes, and incubate for at least 15 min at room temperature in the dark.

3.2.4. Measure the standards on the appropriate proprietary fluorimeter (see the **Table of Materials**) by selecting the **Protein assay** option using the arrow buttons and pressing the **GO** button to confirm. Follow the on-screen instructions, inserting each standard tube and pressing **GO**.

3.2.5. Insert the experimental sample tube; press **GO** to read; and note the result displayed, which is the actual protein concentration in the assay buffer/sample mixture. To obtain the protein concentration in the sample, use the arrow keys to select the **Calculate sample concentration** option, press **GO**, and use the arrow keys to select the **sample volume** added to the assay buffer for the given sample. Press **GO** and record the sample protein concentration. Repeat this step for each sample to be analyzed.

3.3. Particle counting and size distribution

NOTE: Microfluidics resistive pulse sensing (MRPS) was used to quantify EV concentration and size distribution.

3.3.1. Dilute the samples in PBS supplemented with 1% Tween-20 that has been filtered through a 0.2 µm syringe filter to a protein concentration of approximately 0.1 µg/mL.

NOTE: The goal of dilution is to reach an expected particle concentration in the range of 10^{10} particles/mL in EV-containing fractions. The optimal dilution may need to be determined empirically. Few EVs are expected for later fractions (beyond Fraction 6). Thus, the particle concentration will likely be $<10^{10}$ particles/mL despite analyzing at low dilutions.

3.3.2. Load 3 µL of each sample into the disposable microfluidics cartridge with a micropipette, insert the cartridge into the MRPS instrument, and push the metal button with a blue illuminated rim.

3.3.3. Click **Go!** on the acquisition software and wait for the sample to be analyzed by the instrument. Acquire 1,000 to 10,000 particle events to minimize the technical statistical error of analysis. At this point, click **Stop** and **End Run** to complete the sample acquisition.

NOTE: Together with the raw data files, the instrument outputs a summary spreadsheet listing the particle concentration in the sample. Correct this value according to the sample dilution made.

3.3.4. Using analysis software, load the raw data and generate customized graphs of size distribution.

4. EV storage

4.1. Aliquot individual or pooled fractions to 25–50% of the individual fraction size (depending on the size of column used) in low-protein-binding tubes and store at -80 °C to avoid freeze-thaw cycles.

NOTE: Different applications may require smaller or larger aliquots depending on the expected amount utilized in each experiment. This will need to be determined empirically. The non-EV-containing fractions can be discarded if not applicable to the research objectives.

5. Transmission electron microscopy

5.1. Negative staining

5.1.1. Add 5 µL of the EV sample to the carbon-coated copper 400 mesh grid and incubate at room temperature for 10 min. Wash the specimen side with 5 drops of 5 mM Tris buffer (pH 7.1) and then with 5 drops of distilled water.

5.1.2. Stain a specimen side with 5 drops of 2% uranyl acetate. Blot away any extra amount of stain with filter paper, and allow the slide to dry completely for several hours or overnight. Visualize the specimens with an electron microscope operated at 80 kV.

5.2. Immunogold labeling

5.2.1. Apply 10 µL of the EV suspension to a formvar/carbon 400 mesh grid and incubate at room temperature for 1 h. Wash the grid in PBS three times, and then apply 4% paraformaldehyde for 10 min to fix the sample. Wash the grids five times with PBS.

5.2.2. Block the grid with three washes of PBS containing 0.1% bovine serum albumin (BSA). Then, apply 10 µL of a primary antibody for 40 min at room temperature (here, 1 µg/mL of nluc antibody). Wash three times again with PBS containing 0.1% BSA.

5.2.3. Add 10 µL of secondary gold-labeled antibody to the grid and incubate for 40 min at room temperature. Wash the grids three times with PBS.

NOTE: Here, a goat anti-mouse antibody conjugated with 10 nm gold nanoparticles was used after diluting 1:10 in blocking buffer.

5.2.4. Post-fix the grid with 10 µL of 2.5% glutaraldehyde for 10 min at room temperature. Wash three times in PBS. Perform negative staining with 2% uranyl acetate (10 µL) for 15 min. Embed the samples in 10 µL of 0.5% uranyl acetate and 0.13% methyl cellulose solution for 10 min.

5.2.5. Allow the sample grids to dry overnight at room temperature before imaging on the electron microscope.

5.2.6. On the microscope acquisition software, determine the exposure empirically to obtain the optimal quality of the image (e.g., 0.80851 s in this particular setup) and adjust it by typing this value into the **exposure time** option box. Select the **80 kV** option, and click **Start Acquisition** to capture the image.

REPRESENTATIVE RESULTS:

To assess which SEC chromatography fractions were enriched for EVs, the SEC column was loaded with 2 mL of *E. coli* MP1-conditioned culture medium that had been concentrated 1,000-fold by TFF, and sequential fractions were collected. Using MRPS, it was found that Fractions 1–6 contained the most EVs (**Figure 2A**). Subsequent fractions contained very few EVs, comprising instead of EV-free proteins (**Figure 2B**). EVs were primarily <100 nm in diameter (**Figure 2C**). TEM confirmed EV-enrichment and size, particularly in Fractions 2–6 (**Figure 2D**).

To further ensure that the methods were able to separate EVs from non-EV-associated proteins, a recombinant strain of *E. coli* MP1 expressing a cytolysin A–nanoluciferase fusion protein and free (non-fused) mCherry was generated (schematized in **Figure 3A**). Cytolysin A fusion proteins were previously shown to associate with *E. coli* EVs[14]. To monitor mCherry fluorescence, 100 µL aliquots from each chromatography fraction were transferred to the wells of a 96-well plate. Their fluorescence was measured in a microplate reader using 580 nm and 620 nm as absorption and emission wavelengths, respectively.

Similarly, for luminescence measurement, a 20 µL aliquot of each fraction was mixed with an equal volume of the Luciferase assay solution in a 384-well plate, incubated for 15 min, and visible light luminescence was measured. It was observed that EV-enriched fractions (Fractions 2–7) had high nanoluciferase activity comparable to that of later fractions but only a very low fluorescent signal from non-EV-associated mCherry (**Figure 3B**). Background signal from an equal amount of negative control EVs (isolated from a matched bacterial strain lacking nanoluciferase expression) was >1,000-fold lower in EV fractions. The signal from the positive control (a nanoluciferase-expressing bacterial cell pellet) was approximately 1,000-fold higher than that from the EV fractions (not shown). The latter was normalized to the initial volume of cell culture material yielding the analyzed cells or EVs, respectively. The EV-association of nanoluciferase was

confirmed in Fractions 2–5 by immunogold labeling (**Figure 3C**), observing the labeling within the small ~20 nm EVs isolated.

Finally, to assess the applicability of this protocol to other bacterial species, EVs were isolated from ~100 mL cultures of the following diverse anaerobic bacteria: *A. mucinophila, B. thetaiotaomicron, B. breve,* and *B. dentium* prepared in BHI culture medium. As a control, EVs were isolated from the fresh BHI culture medium. While EV yield varied by species, it was again observed that early chromatography Fractions 1–4 were enriched for EVs (**Figure 4A**). The complex BHI medium also contained EV-sized particles, albeit at <25% of the total EV yield in these preparations (**Figure 4A**, black bars). EVs of these bacteria were also found to be primarily <100 nm in size (**Figure 4B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Bacterial EV isolation workflow schematic overview. Abbreviations: EV = extracellular vesicle; TFF = tangential flow filtration; SEC = size exclusion chromatography; MWCO = molecular weight cut-off.

Figure 2: Representative *E. coli* MP1 EV elution in early chromatography fractions. (**A**) Particle count by MRPS in sequential 2 mL chromatography fractions. SEC input was 2 L of bacterial culture supernatant concentrated to 2 mL. Solid line represents mean; shaded area denotes SEM. (**B**) Protein concentration in each fraction. (**C**) Size distribution of Fraction 3 EVs measured by MRPS. Solid line represents mean; shaded area represents 95% CI. Note that the instrument cannot quantify particles <50 nm. (**D**) Transmission electron micrographs of sequential, pooled chromatography fractions and fresh culture medium (control). All images were taken with the same scale (100 nm). Wide-field TEM images are shown in **Supplemental Figure S3**. Abbreviations: EV = extracellular vesicle; MRPS = microfluidics resistive pulse sensing; SEC = size exclusion chromatography; SEM = standard error of the mean; CI = confidence interval.

Figure 3: Separation of *E. coli* MP1 EVs from EV-free proteins by SEC. (**A**) Schematic of *E. coli* MP1 expressing recombinant mCherry (red) in the cytoplasm and periplasm-trafficking cytolysin A–nanoLuciferase fusion protein (yellow diamonds). (**B**) nanoLuciferase bioluminescence activity and mCherry fluorescence were monitored in sequentially eluted chromatography fractions. Vertical dotted line represents the limit of EV-enriched fractions based on analyses in **Figure 2**. (**C**) EV fractions (F2–5) were immunogold labeled following staining with anti-nanoLuciferase antibody. Cyan arrowheads point to gold-conjugated secondary antibody colocalizing with small EVs. Control EVs from wildtype *E. coli* MP1 had negligible non-specific staining. Both images were taken with the same scale (100 nm). Wide-field TEM images are shown in **Supplemental Figure S4**. Abbreviations: EV = extracellular vesicle; SEC = size exclusion chromatography; F = fraction; TEM = transmission electron microscopy; ClyA–nLuc = cytolysin A–nanoLuciferase fusion protein.

Figure 4: Isolation of EVs from diverse bacterial species. Indicated species were cultured for 48 h in BHI medium under anaerobic conditions. EVs were isolated by ultrafiltration + SEC. (**A**) EV concentration in the first 4 SEC fractions, measured by MRPS. Mean ± SEM. Black bars represent

particles detected in a batch of fresh BHI medium (control). (**B**) Size distribution of EVs in Fraction 2. Abbreviations: EV = extracellular vesicle; MRPS = microfluidics resistive pulse sensing; SEC = size exclusion chromatography; BHI = brain heart infusion.

Supplemental Figure S1: p114-mCherry-Clyluc plasmid. (**A**) Map of p114-mCherry-Clyluc plasmid. (**B**) Sequence of the J23114-mCherry-clyA-nluc region. Violet, J23114 Promoter; Pink, *mCherry* gene; Grey, *clyA* gene; Green, Linker sequence; Orange, *nLuc* gene.

Supplemental Figure S2: Tangential flow filtration (TFF) setup schematic. The tubing with barb-hoses was connected to the TFF device, as shown. Feed flow tubing begins submerged in the filtered, conditioned culture medium vessel, continues through the peristaltic pump, and connects to the TFF device inlet port. The return flow begins at the TFF device outlet port and ends above the surface of the filtered, conditioned culture medium. Optionally, a backpressure clamp (e.g., a screw nut + bolt or simple paper clamp) can be used to increase the rate of filtration. As the pump circulates the conditioned medium, developed pressure within the TFF device leads to ultrafiltration and removal of components <100 kDa through the filtrate/waste flow tubing, which can be collected in a separate vessel for disposal (magenta).

Supplemental Figure S3: Widefield transmission electron micrographs. TEM images of sequential, pooled chromatography fractions and fresh culture medium (control) shown in **Figure 2D**. The images show that the *E. coli* MP1 extracellular vesicles elute in early chromatography fractions.

Supplemental Figure S4: Widefield TEM images for Figure 3C. The EV fractions (F2–5) were immunogold-labeled following staining with anti-nano-Luciferase antibody. Cyan arrowheads point to gold-conjugated secondary antibody colocalizing with small EVs.

DISCUSSION:

In the protocol above, a method is described that is scalable and reliably isolates EVs from various gram-negative/positive and aerobic/anaerobic bacteria. It has several potential stopping points throughout the procedure, although it is better to avoid taking longer than 48 h to isolate EVs from conditioned bacterial culture media.

First, it consists of culturing bacteria to generate conditioned bacterial culture medium. It was found that increasing the culture time to at least 48 h and using the optimal growth medium helps to maximize the EV yield. It is likely that each bacterial species will need to be optimized with regard to these two parameters. The volume of the bacterial culture is also important to ensure sufficient EVs are isolated for the desired application. For *in vitro* studies, the EVs are typically isolated from a minimum of 100 mL, while for *in vivo* studies, EVs are typically isolated from >1 L of culture medium. Again, the EV production characteristics of each bacterial strain and the required EV amount for downstream assays will dictate the minimum starting culture volume.

Once conditioned culture medium is available, cells and large non-EV debris must be removed. It was found that centrifugation is a critical step in this process. As noted in the protocol above,

two increasing *g*-force centrifugations were performed. Occasionally, an additional 10,000 × *g* centrifugation is performed if it was noted that the pellet of the second spin is not compact. Subsequently, sterile filtration of this supernatant is performed through a 0.2 µm filter. Insufficient centrifugation leads to clogging and poor performance of this filtration step. It was noted that continuing to filter the supernatant after the filtration rate has significantly slowed can lead to filter malfunction and contamination of the EV preparation with the parental bacteria. The solution to persistent clogging of the filter is to re-centrifuge and/or re-filter the supernatant, ensuring sterility.

Two variations of the protocol are described, depending on the starting volume of the bacterial culture. For volumes <100 mL, use centrifugal ultrafiltration devices to concentrate the culture media. The MWCO is critical in these steps. For mammalian EVs, >300 kDa MWCO were previously used[11,12]. However, this resulted in very poor EV yields from bacteria, presumably because of the smaller size distribution. Thus, it is recommended to use 100 kDa MWCO. A smaller MWCO can also be used but is associated with longer centrifugation times and less removal of small molecular weight contaminants, increasing sample viscosity. It is also helpful to have various sizes of ultrafiltration devices at 100 kDa MWCO to help concentrate different starting volumes of sample throughout the protocol.

Alternatively, for sample sizes significantly >100 mL, use pump-driven TFF to concentrate the sample; again, using a 100 kDa MWCO is critical. This method allows for processing large volumes of culture medium in a semi-automated fashion. It is important to obtain an appropriately-sized TFF device for the starting culture volume. The device used is rated at processing up to 200 mL of material by the manufacturer. It was possible to process up to about 2 L. However, a severe drop in the filtration rate was observed when trying to process larger volumes, requiring the process to be stopped and the device cleaned before additional processing. Thus, the characteristics of each bacterial culture and the amount of starting material will dictate the required size of the TFF device. Furthermore, the attainable pump speed is another important parameter for TFF. At low rates of ~100 mL/min, it was necessary to increase the backpressure in the TFF device using a clamp, as indicated in **Supplemental Figure S2**, to facilitate filtration, which increases the fouling rate of the filter. The tubing was reused up to 2 times after appropriate decontamination and autoclaving.

Once the sample is concentrated, it can then be loaded onto an SEC column to isolate the EVs. Commercial columns optimized for small EV isolation were used. For small starting samples, use columns with 0.5 mL loading volume, and use the columns with 2 mL loading volume for larger starting samples up to 2 L. It is likely that the processing of starting cultures >2 L will require larger columns. Manufacturers of EV-optimized columns currently offer SEC columns capable of accepting >100 mL of concentrated material.

Various methods are used to characterize the isolated EVs, most of which are widely available. Normalization was based on the protein concentration for most assays because this is not affected by the inability of other quantification methods (namely, particle quantification by technologies such as MRPS) to detect very small EVs <50 nm. MRPS and other nanoparticle

quantification technologies remain useful in the relative quantification of EVs among the different fractions.

One critical aspect of MRPS quantification is the level of dilution. When diluted appropriately, the frequency of detected EVs should continue to increase to the limit of detection in most cases, as the instrument cannot quantify particles <50 nm. Insufficient dilution will lead to high instrument noise, which will generate an artifactual bell-shaped curve with a peak >65 nm (when using the recommended C-300 microfluidics cartridge). During size frequency distribution data analysis, an artefactual peak between 50 nm (the absolute limit of detection of the instrument) and 60 nm is still sometimes observed, despite adequate dilution. This is likely due to the presence of significant numbers of very small bacterial EVs (as visualized in TEM, **Figure 2D**) that are below the limit of accurate detection by MRPS and again lead to instrument noise. In this case, exclude data points smaller than the observed "peak," which becomes the *de facto* lower limit of quantitation of the given sample.

As described in this protocol, the quantification of EV abundance, total protein concentration, and abundance of non-EV proteins in the eluted chromatography fractions can help users decide which fractions to use for downstream assays. For example, small EVs were detected in pooled Fractions 7–8 (**Figure 2D**); however, their abundance was lower than that in the immediately preceding fractions, while the total protein concentration (**Figure 2B**) was higher. This may suggest that Fractions 7–8 contain higher amounts of non-EV-associated proteins and may thus not be desirable for certain downstream applications.

In summary, a versatile EV isolation protocol that relies on commercially available materials is described here. The significance of this methodology compared to widely used ultracentrifugation-based methods is that it comprises steps that can be easily reproduced by different users and is highly scalable. This is especially important to facilitate the generation of sufficient material for *in vivo* studies. It was used to isolate EVs from cultures of 100 mL to 2 L. Given the wide range of available TFF devices, it is possible that this protocol could be adapted to larger-scale purifications with some modification. The isolation protocol described is primarily based on the physical properties of EVs, namely their size, and is likely applicable to bacterial species beyond those described in this study.

One limitation of the protocol described is that it favors the isolation of small EVs, particularly <100 nm, as seen in the representative results. Prior reports also describe the presence of larger bacterial EVs[15,16]. Isolation of larger bacterial EVs may require modifications of the protocol above, for example, by using SEC columns optimized for larger EVs. Such SEC columns are also commercially available. Moreover, other protocols can likely attain higher EV purity (for example, density gradient ultracentrifugation or immuno-isolation). However, these methods lack the throughput and scalability of the methods described in this study. Modification of this protocol with additional purification steps in the future may further increase the yield and purity of preparations, which could be important for experimental and therapeutic applications.

ACKNOWLEDGMENTS:

The research described above was supported by NIH TL1 TR002549-03 training grant. We thank Drs. John C. Tilton and Zachary Troyer (Case Western Reserve University) for facilitating access to the particle size analyzer instrument; Lew Brown (Spectradyne) for technical assistance with analysis of the particle size distribution data; Dr. David Putnam at Cornell University for providing pClyA-GFP plasmid[14]; and Dr. Mark Goulian at the University of Pennsylvania for providing us with the *E. coli* MP1[13].

DISCLOSURES:

The authors have no conflicts of interest to declare.

REFERENCES:

1 Yanez-Mo, M. et al. Biological properties of extracellular vesicles and their physiological functions. *Journal of Extracellular Vesicles.* **4**, 27066 (2015).

2 Chatterjee, S. N., Das, J. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. *Journal of General Microbiology.* **49** (1), 1–11 (1967).

3 Ciofu, O., Beveridge, T. J., Kadurugamuwa, J., Walther-Rasmussen, J., Hoiby, N. Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy.* **45** (1), 9–13 (2000).

4 Yonezawa, H. *et al.* Outer membrane vesicles of *Helicobacter pylori* TK1402 are involved in biofilm formation. *BMC Microbiology.* **9**, 197 (2009).

5 Mashburn, L. M., Whiteley, M. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature.* **437** (7057), 422–425 (2005).

6 Kato, S., Kowashi, Y., Demuth, D. R. Outer membrane-like vesicles secreted by A*ctinobacillus actinomycetemcomitans* are enriched in leukotoxin. *Microbial Pathogenesis.* **32** (1), 1–13 (2002).

7 Petousis-Harris, H. et al. Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. *Lancet.* **390** (10102), 1603–1610 (2017).

8 Kim, O. Y. et al. Bacterial outer membrane vesicles suppress tumor by interferon-gamma-mediated antitumor response. *Nature Communications.* **8** (1), 626 (2017).

9 Thery, C. et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles.* **7** (1), 1535750 (2018).

10 Consortium, E.-T. et al. EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. *Nature Methods.* **14** (3), 228–232 (2017).

11 Watson, D. C. et al. Efficient production and enhanced tumor delivery of engineered extracellular vesicles. *Biomaterials.* **105**, 195–205 (2016).

12 Watson, D. C. et al. Scalable, cGMP-compatible purification of extracellular vesicles carrying bioactive human heterodimeric IL-15/lactadherin complexes. *Journal of Extracellular Vesicles.* **7** (1), 1442088 (2018).

13 Lasaro, M. et al. *Escherichia coli* isolate for studying colonization of the mouse intestine and its application to two-component signaling knockouts. *Journal of Bacteriology.* **196** (9), 1723–1732 (2014).

14 Kim, J. Y. et al. Engineered bacterial outer membrane vesicles with enhanced

functionality. *Journal of Molecular Biology.* **380** (1), 51–66 (2008).
15 Beveridge, T. J. Structures of gram-negative cell walls and their derived membrane vesicles. *Journal of Bacteriology.* **181** (16), 4725–4733 (1999).
16 Reimer, S. L. et al. Comparative analysis of outer membrane vesicle isolation methods with an *Escherichia coli* tolA mutant reveals a hypervesiculating phenotype with outer-inner membrane vesicle content. *Frontiers in Microbiology.* **12**, 628801 (2021).

Figure 1

Bacterial culture
48 hours at strain-optimal conditions

Remove cells and debris

Culture supernatant

~100 mL

>100 mL

Concentrate Large volumes (100 KDa MWCO)

Ultra-filtration

Tangential Flow Filtration (TFF)

Optional: Additional concentration

Isolate EVs

Size-exclusion Chromatography (SEC)

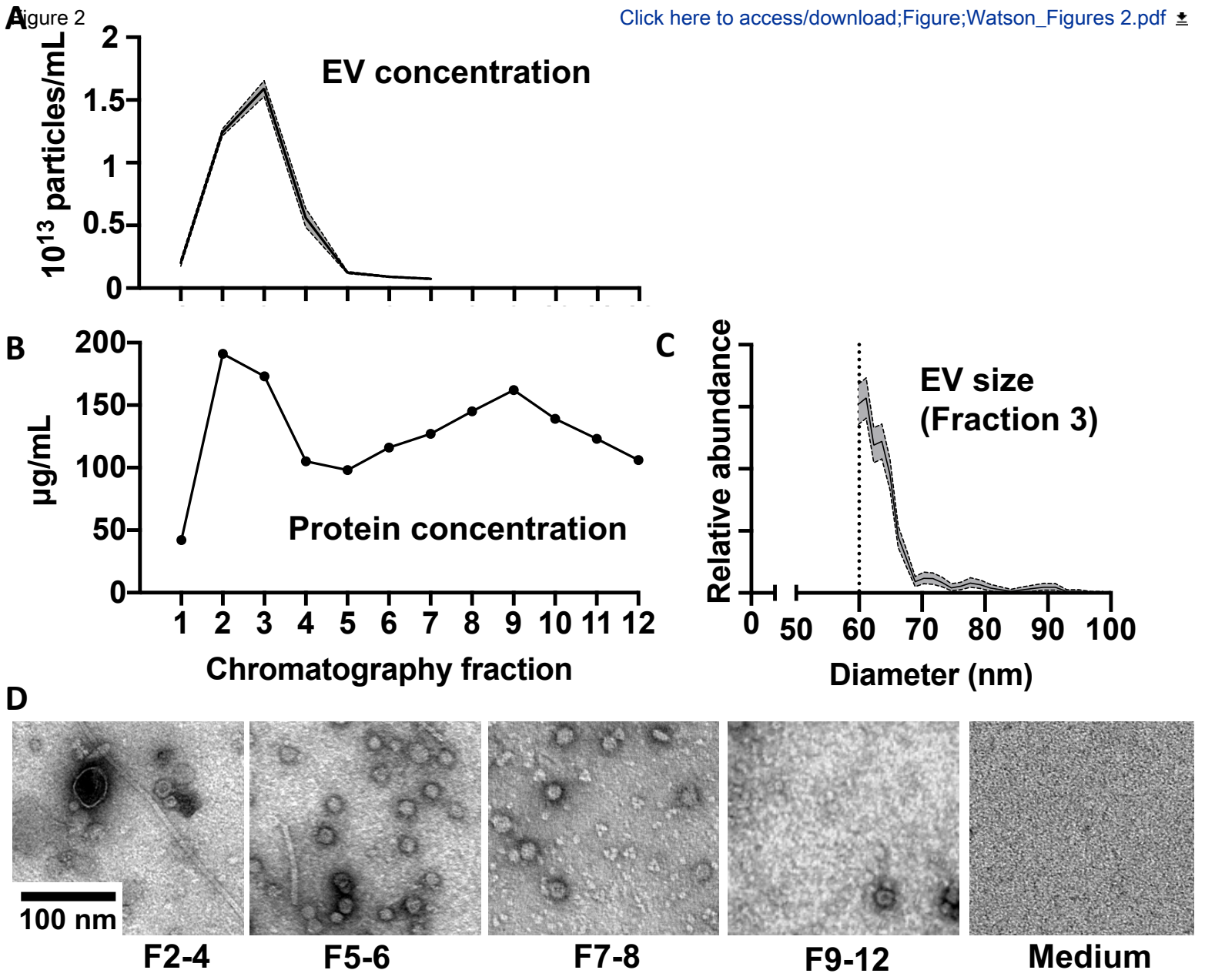


Figure 3

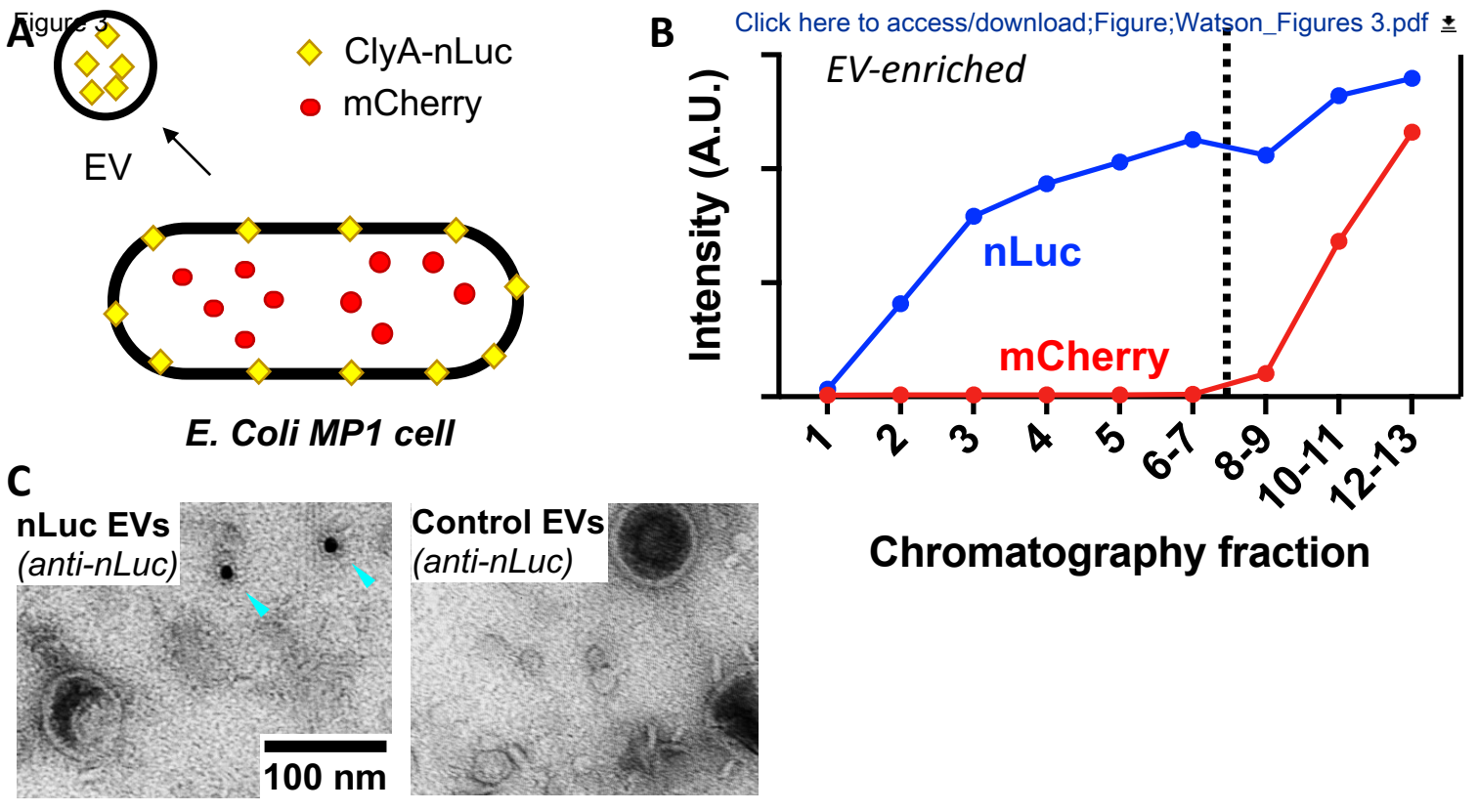
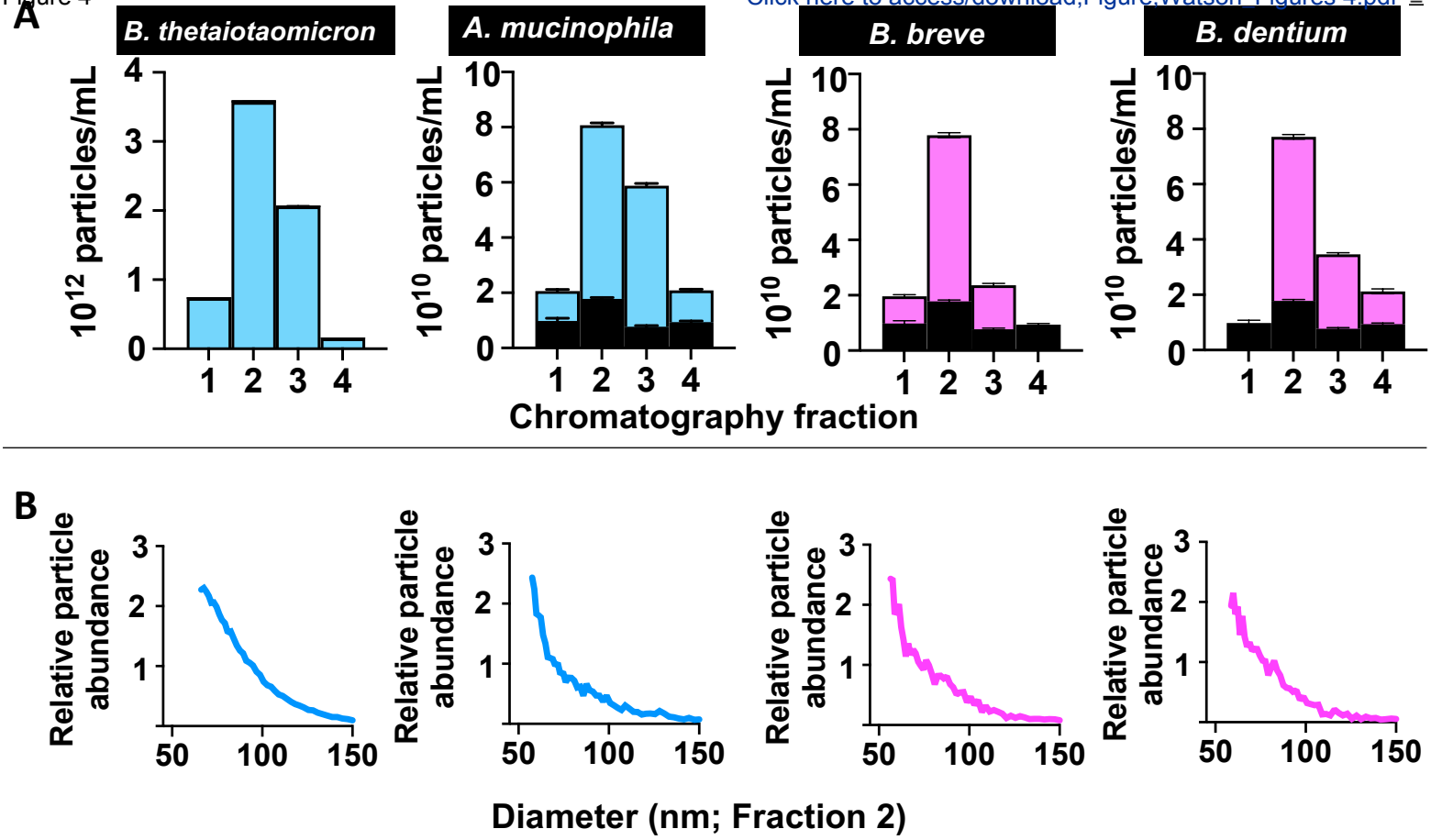

A
ClyA-nLuc
mCherry
EV
E. Coli MP1 cell
B
Click here to access/download;Figure;Watson_Figures 3.pdf
EV-enriched
Intensity (A.U.)
nLuc
mCherry
1
2
3
4
5
6-7
8-9
10-11
12-13
Chromatography fraction
C
nLuc EVs
(anti-nLuc)
Control EVs
(anti-nLuc)
100 nm

Figure 4



Click here to access/download

Table of Materials

Watson_EV table of materials (1).xls

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. Please define all abbreviations upon first use.

The text was revised as suggested.

2. Your Abstract is over the 150-300 word limit. Please rephrase it to be within 300 words.

It was shortened as suggested. Thank you

3. JoVE cannot publish manuscripts containing commercial language. 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.
For example: Akkermansia mucinophila ATCC BAA-835, Bifidobacterium dentium ATCC 27678, nCS1 Viewer, Tecnai G2 SpiritBT, Nano-Glo Luciferase etc.

The commercial products were deleted from the main manuscript as suggested

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The text was revised as suggested.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

The protocol was revised as suggested.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

The protocol was revised as suggested.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

It was corrected

8. Please use the symbol µ instead of u throughout the manuscript.

It was corrected

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Step 2: How was plasmid linearized and how was the promoter and gene sequence deleted? How were the promoter and reported inserted? How was the gfp replaced and the linker inserted? How were the cloning, verification of cloning, extraction, and transformation done? If all this has been described in a publication, please cite that publication.

More details were added to this paragraph, and the whole paragraph was moved to the supplementary information to avoid distracting from the main protocol as it describes only cloning steps which are not relevant to EV isolation technique.

Step 3.1.1: Please specify the cell cultures used here, are they from Step 1 or 2 or both.

We clarified that the bacterial culture was generated by inoculation of bacteria from Step 1.

Step 3.1.2: How was the filtration done?

We added the sentence, "Filter by connecting the filtration device to a vacuum wall supply set to the open position".

Step 3.2.1.2.3: Please specify volume of ethanol, NaOH, water, and PBS used.

Volumes were added to protocol

Step 4.2: How were the fluorimeter-based measurements done: wavelengths etc

Information was added, "The kit works with a matching proprietary fluorimeter, at excitation/emission wavelengths of 485/590 nm."

Step 4.3: How was the value corrected? What was the particle concentration obtained here and how was it diluted to obtain the desired concentration?

Information has been clarified:

"Dilute samples in PBS supplemented with 1 % Tween-20 that has been filtered through 0.02 µm syringe filter to a protein concentration of approximately 0.1 µg per mL.

Note. The goal of dilution is to reach an expected particle concentration in the range of 10^{10} particles/mL. The optimal dilution may need to be determined empirically. For later fractions (beyond Fraction 6) few EVs are expected. Thus, despite analyzing at low dilutions, particle concentration will likely be $<10^{10}$ particles/mL."

Step 4.3, 6.1.2, 6.2.4: Please include button clicks for software usage, parameters for image acquisitions, etc.

Information has been added:

"3.3.2 Load 3 µL of each sample into the disposable microfluidics cartridge with a micropipette, insert the cartridge into the MRPS instrument, and push the metal button that has a blue light rim.

3.3.3 Click "Go!" on the acquisition software and wait for the sample to be analyzed by the instrument. Acquire 1,000 to 10,000 particle events to minimize the technical statistical error of analysis. At this point, click "Stop," and "End Run" to complete sample acquisition.

3.3.4 Together with the raw data files, the instrument outputs a summary spreadsheet listing the particle concentration in the sample. Correct this value according to the sample dilution made"

AND

"**On the microscope acquisition software, adjust exposure to obtain optimal quality of image. This may need to be determined empirically, for example 0.80851 seconds was used in this particular setup, by typing this value into the "exposure time" option box. Select the 80 kV option, then click "Start Acquisition" to capture the image. "**

Step 5: What was the aliquot volume? Please mention.

Information was added.

"**Aliquot individual or pooled fractions to 25-50% of individual fraction size (depending on the size of column used) in low protein binding tubes and store at -80 °C to avoid freeze-thaw cycles. Different applications may require smaller or larger aliquots depending on the expected amount to be utilized in each experiment. This will need to be determined empirically. Also, you may choose to discard non-EV containing fractions if not applicable to your research. "**

10. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.
Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Please do not highlight any steps describing euthanasia.

The layout was corrected as suggested.

11. As we are a methods journal, please add any limitations and the significance of your technique with respect to existing methods to the Discussion.

Updated wording to clearly reflect significance and limitations:

"**The significance of this methodology compared to widely used ultracentrifugation-based methods are that it is comprised of steps that can be easily reproduced by different users, and is highly scalable. This is especially important to facilitate the generation of sufficient material for *in vivo* studies. It was used to isolate EVs from cultures of 100 mL to 2 L. Given the wide range of available TFF devices, it is possible that this protocol could be adapted to larger-scale purifications with some modification. The isolation protocol described is primarily based on the physical properties of EVs, namely their size, and is likely applicable to bacterial species beyond those described in this study. Regarding limitations, there are other protocols that can likely attain higher EV purity (for example density gradient ultracentrifugation or immuno-isolation). However, these methods lack the throughput and scalability of the methods described in this study. Modification of this protocol with additional purification steps in the future may further increase the yield and purity of preparations, which could be important for experimental and therapeutic applications**."

12. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

A) We included a sentence in the legends of figures 3, and 4 to indicate that all images were taken with the same scale.

13. Figure 5A: Please provide y-axis values and y-axis titles for B. breve and B. dentium.

Figure has been updated

14. Figure 5B: Please provide y-axis values.

Figure has been updated

15. Supplementary Figure 3 and 4: Please include a short description of each figure. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

A) The descriptions were added as suggested to supplementary figures 3 and 4.

16. Please move information about sources of plasmids (e.g., lines 129-130) to the acknowledgment section.

A) It was moved as suggested

17. Please do not abbreviate journal names in references.

Journal names have been updated

18. Please ensure the inclusion of all items (tubes, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

A) The table was sorted alphabetically

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript the authors propose a combination of ultrafiltration and SEC separation to isolate bacterial EVs from conditioned medium.

Major Concerns:

This method seems to enrich for very small EVs less than 100 nm. However, many bacteria produce EVs that are more heterogeneous in size 60-300 nm and an average of ~100-150.

Many factors can affect the size distribution, including the producing species, growth conditions, and enrichment protocol. In our protocol, most of the isolated EVs are <100 nm. As the reviewer suggests, it is possible that there exist other sub-populations of EVs that are larger. We added the sentence below to acknowledge this limitation:

"One limitation of the protocol described is that it favors isolation of small EVs, particularly <100 nm, as seen in the representative results. Prior reports also describe the presence of larger bacterial EVs[15,16]. Isolation of larger bacterial EVs may require modifications of the protocol above, for example by using SEC columns optimized for larger EVs. Such SEC columns are also commercially available."

Minor Concerns:
Temperature for large volume concentration (TFF) is not mentioned. Was this step done at 4 degrees or Room temperature?
TFF was done at room temperature. The manuscript text has been updated:

"2.2.2.2 At room temperature, begin circulating the filtered conditioned medium at approximately 200 mL/min (minimum 100 mL/min)."

Luciferase assay (How long is the incubation). Positive control for luciferase activity should be used in the assay.

information on incubation time was added to the manunscript:

"Similarly, for luminescence measurement, 20 µL aliquot of each fraction was mixed with equal volume of the Luciferase assay solution in 384-well plate, incubated for 15 minutes, and visible light luminescence was measured."

We also added the information regarding positive/negative controls to the text:

"Background signal from equal amount of negative control EVs (isolated from matched bacterial strain lacking nanoluciferase expression) was >1,000-fold lower in EV fractions; signal from positive control (nanoluciferase-expressing bacterial cell pellet) was approximately 1,000-fold higher than the EV fractions (not shown). The latter is normalized to the initial volume of cell culture material yielding the analyzed cells or EVs, respectively."

Is MCherry secreted into the medium where it can "contaminate EVs" if it is not secreted I do not see the rational of this control.

Thank you for this comment. Our data suggest that some mCherry was indeed released into the culture medium (Figure 3B). Excretion of cytoplasmic proteins through cell lysis or other mechanisms, has been previously reported (reviewed for example in PMID 25596889).

Fractionation of the culture medium by SEC led to elution of mCherry in fractions that lacked EVs (as assessed by both MRPS and transmission electron microscopy), which indicates that the detected mCherry fluorescence was independent of EVs. Taken together, the data presented in Figure 3B provides evidence in favor of our bacterial EV isolation protocol's ability to separate EVs from non-EV-associated secreted proteins.

For animal experiment - A Cy5.5 (alone) in PBS control is more appropriate than PBS alone.

This is a great suggestion. Unfortunately, the quenching reagent with this particular kit turned out to be non-compatible with our filtration devices. This became evident when we repeated the staining reaction with Cy5.5 alone (no EVs). We thus decided to omit the biodistribution figure in the absence of the most appropriate control.

Reviewer #2:
Manuscript Summary: The manuscript describes two methods of purifying bacterial membrane vesicles adaptable to several bacterial species. Choice of protocol is largely determined by volume with the TFF method described being applicable to large-scale purification of bacterial vesicles. Protocol is well written and easy to follow. Would benefit from a better description of materials and equipment (though I may have missed these in the version I downloaded)

Thank you for this comment. Per the journal editor's instructions, detailed description of materials/equipment with commercial names is not allowed in the main manuscript text. This information is provided in the Materials Table spreadsheet.

Major Concerns:
- in section 4.1 the authors suggest checking for contamination but do not suggest a course of action if bacterial growth is observed. While most can infer that you should move back a step there should be some language here to explain or describe contingencies.

We added language clarifying that TFF should be performed in a biosafety cabinet:

Perform TFF within a Biosafety Cabinet to minimize risk of contaminating the EV preparation with environmental bacteria.

Furthermore, we added language describing contingency to bacterial contamination:

After the initial centrifugation/filtration:

"If bacteria are detected, it is recommended to further optimize the procedure above by performing additional centrifugations and/or filtrations."

And at the final quality control of the EV preparation:

“If bacterial contamination is detected, it not recommended to use the EV preparation for experimentation. Instead, it is recommended to repeat the isolation, taking care to minimize the risk of bacterial contamination by: (a) performing sufficient centrifugation/filtration of conditioned bacterial cell culture medium, (b) using clean bottles, tubing, filters, and chromatography columns, and (c) performing appropriate aseptic techniques.”

- in section 4.2 the authors indicate that EVs have low concentration of protein but this is not always the case. protein concentration can be affected by a number of conditions and often OMVs/MVs can be quite rich in proteins dependent on parental organisms, growth conditions, etc. Authors should provide references or data to support this statement.

The reviewer is correct. We have deleted this phrase, as it is not universally applicable.

- also in section 2 the authors talk about a highly sensitive protein quantification kit but give no details as to the manufacturer or product name.

As mentioned above, the journal does not allow providing commercial details in the main manuscript text. This information is provided in the Materials Table. In this instance, we are referring to the Qubit protein assay kit (ThermoFisher).

Minor Concerns:
- there is some discussion on whether or not bacterial vesicles should be defined as EVs. The EV nomenclature is more commonly used with vesicles from eukaryotic cells. OMVs for gram negative bacterial vesicles or MVs for gram positive vesicles are more commonly used.

Indeed, there is discussion on the appropriate nomenclature. The latest nomenclature recommendation from the International Society of Extracellular Vesicles (ISEV; PMID 30637094) recommends using the general term “extracellular vesicles” in their “nomenclature section”. The same consensus paper uses the term “EVs” to describe prokaryote-derived vesicles in Table 3. Thus, we decided to also uses the more general term “EVs” and “bacterial EVs” in this protocol, as it encompasses vesicles produced broadly by a variety of species.

- be consistent with the use of "mL" versus "ml" and "ul" versus 'uL" throughout there are sections in one format and others in the other format.

This has been corrected.

-section 6.1.1 the sentence describing the buffer is in an unusual format. suggest "5mM Tris (pH #.#)"

This has been corrected:

"**5 mM Tris (pH 7.1)**"

Reviewer #3:
Manuscript Summary:
In this paper, Watson et al describe a method to isolate bacterial membrane vesicles from monocultures using a combined approach with ultrafiltration and SEC. This method can be used for small volumes, but also larger volumes >1L. Several techniques have been used to validate the purity and the yield. Also recombinant bacteria have been used to validate the method. These recombinant vesicles have also been used to study the in vivo distribution in mice, showing that most vesicles end up in the liver.

Major Concerns:
Methods are described in great detail, with an extensive list of consumables/equipment needed. This will make is possible for most researches interested in this field to repeat the experiments. With respect to the results, however, I do have some concerns. In one of the first papers showing that SEC is very useful to separate EVs from (lipo-)proteins (Boing et al 2014) is was shown that the vesicles appear starting from fraction 5-6. This has been confirmed by many others in the meanwhile (depending a little on the samples size (0.5 vs 1.0 ml/fraction). In the current paper the authors claim that vesicles already appear in high numbers in fraction 2 (fig 2A) and they seem to have disappeared from fraction 5 eben though they also collect 0.5ml fractions. This is quite contradictory to the results by others. TEM pictures on the other hand suggest that the highest density of vesicles seems to be in fractions 5-8, which is more in accordance to findings of other groups.

Thank you for these comments. Boing et al 2014 (PMID: 25279113) used an in-house Sepharose CL-2B column to isolate EVs. We used a commercial SEC column from Izon, with undisclosed column bed composition. Thus, direct comparison may not be accurate. Nonetheless, we noted that there is a difference in the way fractions are numbered between our protocol and the work published by Boing et al. Specifically, we do not count the eluted column void volume in our fraction numbering. This was done to avoid confusion with fraction numbering of columns with different void volumes, and has also been adopted by Izon in their instruction manuals as they have expanded their column repertoire to many more sizes (ranging from 0.1 – 100 mL of sample loading capacity) in addition to their "original" column which accepts up to 0.5 mL sample.

Keeping in mind the potential for an imperfect comparison between columns with potentially different composition, our Fraction 1 of the smaller SEC column corresponds to the 3.0 – 3.5 mL elution, if we also consider the void volume. Using 0.5 mL fraction volumes, this would correspond to Fraction 7 (using the Boing numbering scheme). Our peak EV elution by resistive pulse sensing was observed at Fraction 3 (or Fraction 9, using the Boing numbering). This is very similar to the observed EV peak in the Boing paper (Fraction 10).

Regarding the differences observed in EV abundance between MRPS and TEM analyses, this can be explained by the significant secretion of EVs <50 nm in size by the bacteria we tested.

These are below the limit of detection of MRPS, and can be more accurately assessed with electron microscopy.

The text was updated to clarify this observation:
"**This is likely due to the presence of significant numbers of very small bacterial EVs (as visualized in TEM, Figure 2D) that are below the limit of accurate detection by MRPS and again lead to instrument noise.**"

Finally, we did find EVs in pooled Fractions 7-8, however these were less abundant than preceding fractions (Figure 2D). Together with the rising total protein concentration in Fractions 7/8 (as compared to the more EV-rich Fractions 5/6), this suggests that Fractions 7/8 are of lower purity, containing also non-EV-associated proteins. Thus, for our experiments, we do not use Fractions ≥7 for EV studies. Others may choose to also include these fractions in their studies.

The text was updated to discuss these nuances:

"**As described in this protocol, quantification of EV abundance, total protein concentration, and abundance of non-EV proteins in the eluted chromatography fractions can help users decide which fractions to use for downstream assays. For example, small EVs were detected in pooled Fractions 7-8 (Figure 2D); however, their abundance was lower than immediately preceding fractions, while the total protein concentration (Figure 2B) was higher. This may suggest that Fractions 7-8 contain higher amounts of non-EV-associated proteins and may thus not be desirable for certain downstream applications**."

Likewise, although the authors claim that there's only low amount of protein in the EV fractions, fig 2b tell a different story. It shows that the protein concentration in the fractions containing the vesicles is even higher than those fractions which are supposed to contain the highest concentration.

Thank you also for this comment. In our manuscript we specifically propose that TFF + size-exclusion chromatography is useful in separating EVs from non-EV associated proteins, resulting in a low amount (specifically) of non-EV proteins in the EV-enriched fractions. We used a combination of EV quantification by 2 different methods, total protein quantification, and a recombinant non-EV-associated protein (mCherry) to make this argument.

The reviewer correctly points out that the early EV-enriched Fractions 2-3 have a higher protein concentration than the later EV-enriched fractions 4-6. This likely reflects a different amount of protein loading in the larger vs. smaller EVs that elute in the early and later fractions, respectively. We speculate that this could be due to different biosynthetic pathways and/or biophysical constraints of the smaller EVs that might limit protein loading, but this falls beyond the scope of this manuscript.

I think it is really important to clarify these issues, which cannot simply be related to the difference between eukaryotic and bacterial vesicles. Kameli et al. (2021) recently demonstrated

by using a similar methodology that bacterial vesicles are predominantly found in fractions 7-11. Since it is of great importance to know in which fraction the vesicles end up after SEC, this issue needs to be addressed carefully.

Kameli et al. 2021 (PMID 34026664) references the Boing 2014 paper discussed above as the method of SEC. As discussed above, our Fractions 1-5 correspond to Fractions 7-11 using the Boing 2014 numbering scheme.

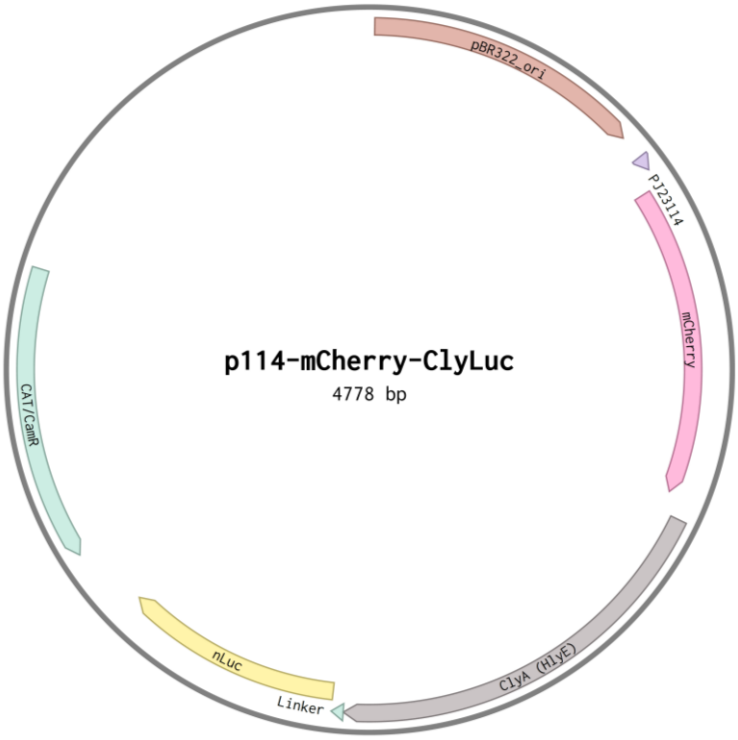
Minor Concerns:
none

Supplemental method:

1. **Generation of the p114-mCherry-ClyLuc plasmid**

   Plasmid pClyA-GFP expressing Cytolysin A-Green fluorescent protein fusion was a generous gift from Dr. David Putnam (Cornell University, New York). This plasmid was linearized through a PCR reaction to amplify a 5.7 Kb fragment spanning the region from the *clyA* gene to the pBR322 origin. The medium strength constitutive promotor J23114 (http://parts.igem.org/Part:BBa_J23114), together with *mCherry* gene was then PCR-amplified and inserted upstream of the *ClyA-GFP* fusion gene to generate the p114-mCherry-ClyGFP plasmid. Subsequently, to generate the p114-mCherry-ClyLuc plasmid, the *gfp* gene was replaced with the nanoluciferase (*nluc,* Promega) gene, and a linker peptide "GGGGSGGGG" sequence was inserted in-between the *clyA* gene and the *nluc*. The map and sequence of p114-mCherry-ClyLuc are shown in **Supplemental Figure S1**. The PCR-amplified inserts were cloned into the linearized vectors using ligation-independent cloning (In-Fusion cloning). The ligated plasmids were transformed into chemically competent commercial *E. coli*, verified through Sanger sequencing, and then extracted and subsequently transformed into *E. coli* MP1 through electroporation.

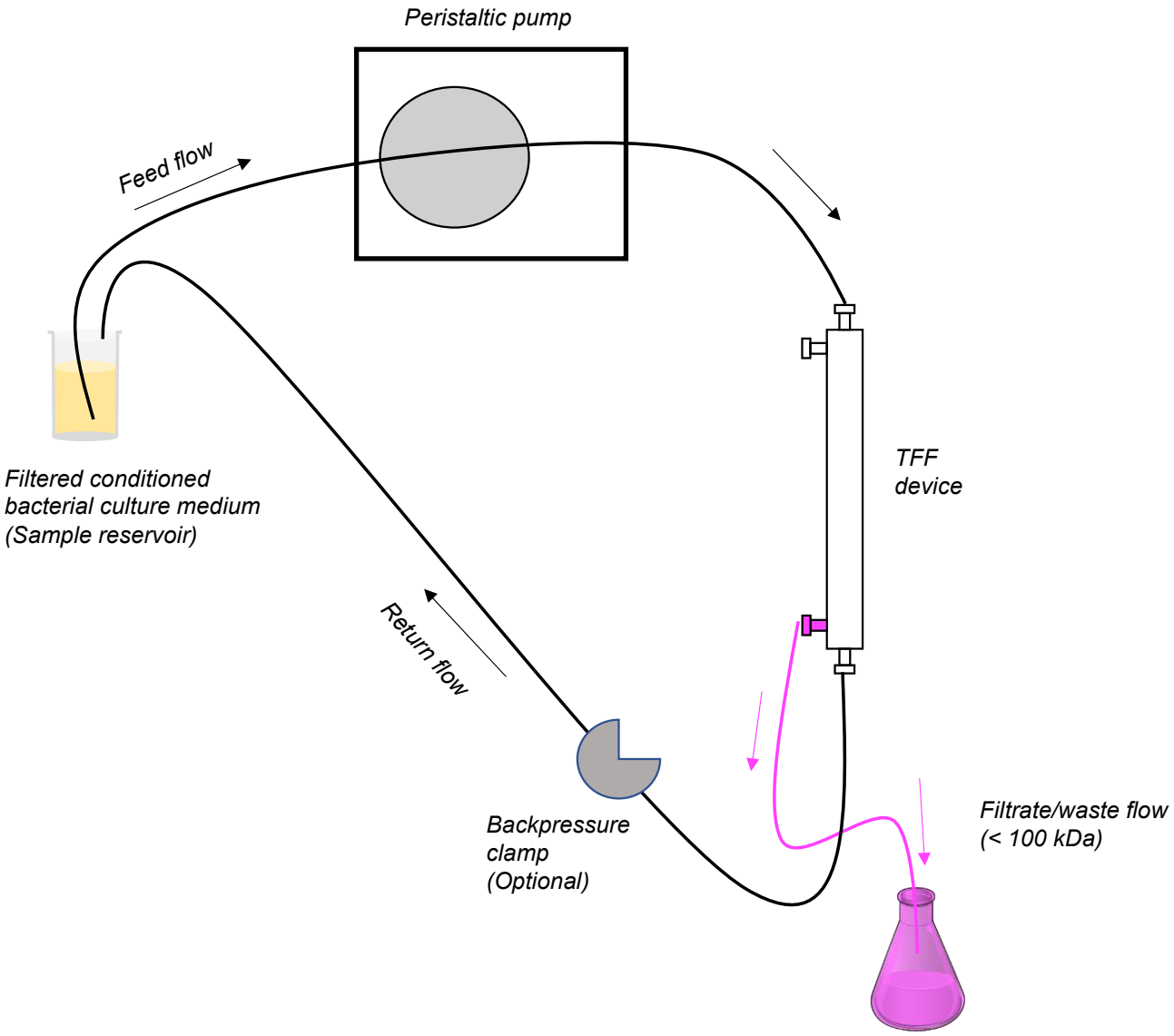
A)



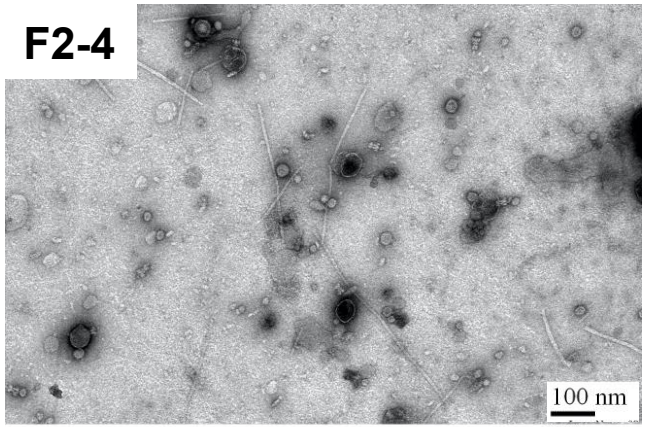
B)

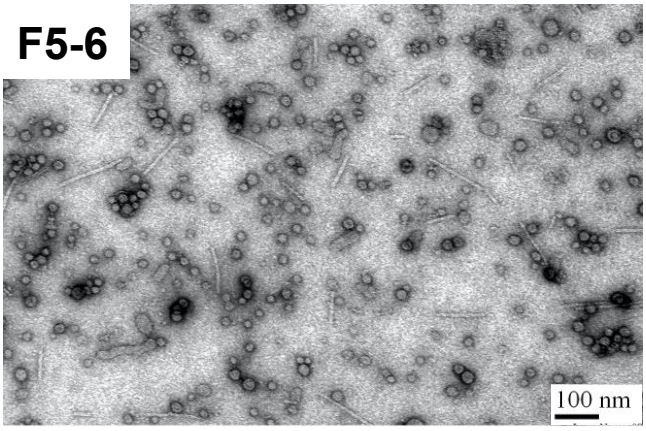
TTTATGGCTAGCTCAGTCCTAGGTACAATGCTAGCGGGCCCACTCACATACGAGATATATAGAAGGAGGTAATAAATGCAAGTCGACCTGCTGGATCCAAACATGGCAATCATTAAGGAGTTCATGAGATTCAA
AGTTCACATGGAAGGTTCTGTAAATGGACATGAATTTGAAATAGAAGGTGAAGGAGAAGGAAGGCCTTATGAAGGAACCCAAACCGCGAAGCTAAAAGTTACTAAGGGTGGCCCATTACCATTTGCATGGGAT
ATCCTTAGCCCTCAATTCATGTATGGGTCAAAGGCTTATGTCAAGCACCCCGCCGACATTCCAGACTATCTAAAGTTATCTTTTCCCGAAGGGTTTAAGTGGGAGCGTGTGATGAACTTCGAAGACGGTGGCGTGG
TAACAGTGACTCAGGATTCGTCCCTGCAAGATGGTGAATTTATCTACAAAGTCAAATTAAGAGGAACTAACTTTCCATCTGACGGCCCGGTTATGCAAAAAAAGACAATGGGCTGGGAGGCCTCCTCAGAACGA
ATGTACCCTGAAGATGGTGCCTTGAAGGGTGAGATTAAACAAAGATTGAAATTGAAAGATGGTGGACATTATGACGCTGAGGTTAAAACGACATACAAAGCTAAGAAACCTGTCCAGCTCCCAGGTGCTTACAA
TGTAAATATAAAACTTGATATTACATCACATAATGAAGATTATACGATAGTTGAACAATACGAAAGGGCTGAGGGGAGACATAGTACTGGTGGCATGGATGAACTATACAAAGGTTCTGGTACCGCATAAACCC
GTTTTTTTGGGCTAGCGAATTCGAGCTCTTAAAGAGGAGAAAGGTCATGACTGAAATCGTTGCAGATAAAACGGTAGAAGTAGTTAAAAACGCAATCGAAACCGCAGATGGAGCATTAGATCTTTATAATAAAT
ATCTCGATCAGGTCATCCCCTGGCAGACCTTTGATGAAACCATAAAAGAGTTAAGTCGCTTTAAACAGGAGTATTCACAGGCAGCCTCCGTTTTAGTCGGCGATATTAAAACCTTACTTATGGATAGCCAGGATAA
GTATTTTGAAGCAACCCAAACAGTGTATGAATGGTGTGGTGTTGCGACGCAATTGCTCGCAGCGTATATTTTGCTATTTGATGAGTACAATGAGAAGAAAGCATCCGCCCAGAAAGACATTCTCATTAAGGTACT
GGATGACGGCATCACGAAGCTGAATGAAGCGCAAAAATCCCTGCTGGTAAGCTCACAAAGTTTCAACAACGCTTCCGGGAAACTGCTGGCGTTAGATAGCCAGTTAACCAATGATTTTTCAGAAAAAAGCAGCT
ATTTCCAGTCACAGGTAGATAAAATCAGGAAGGAAGCATATGCCGGTGCCGCAGCCGGTGTCGTCGCCGGTCCATTTGGATTAATCATTTCCTATTCTATTGCTGCGGGCGTAGTTGAAGGAAAACTGATTCCAG
AATTGAAGAACAAGTTAAAATCTGTGCAGAATTTCTTTACCACCCTGTCTAACACGGTTAAACAAGCGAATAAAGATATCGATGCCGCCAAATTGAAATTAACCACCGAAATAGCCGCCATCGGTGAGATAAAAA
CGGAAACTGAAACAACCAGATTCTACGTTGATTATGATGATTTAATGCTTTCTTTGCTAAAAGAAGCGGCCAAAAAAATGATTAACACCTGTAATGAGTATCAGAAAAGACACGGTAAAAAGACACTCTTTGAG
GTACCTGAAGTCGGCGGCGGCGGCAGCGGCGGCGGCGGCATGGTCTTCACACTCGAAGATTTCGTTGGGGACTGGCGACAGACAGCCGGCTACAACCTGGACCAAGTCCTTGAACAGGGAGGTGTGTCCAGTT
TGTTTCAGAATCTCGGGGTGTCCGTAACTCCGATCCAAAGGATTGTCCTGAGCGGTGAAAATGGGCTGAAGATCGACATCCATGTCATCATCCCGTATGAAGGTCTGAGCGGCGACCAAATGGGCCAGATCGAA
AAAATTTTTAAGGTGGTGTACCCTGTGGATGATCATCACTTTAAGGTGATCCTGCACTATGGCACACTGGTAATCGACGGGGTTACGCCGAACATGATCGACTATTTCGGACGGCCGTATGAAGGCATCGCCGTG
TTCGACGGCAAAAAGATCACTGTAACAGGGACCCTGTGGAACGGCAACAAAATTATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTGTTCCGAGTAACCATCAACGGAGTGACCGGCTGGCGGCTGTG
CGAACGCATTCTGGCGTAA

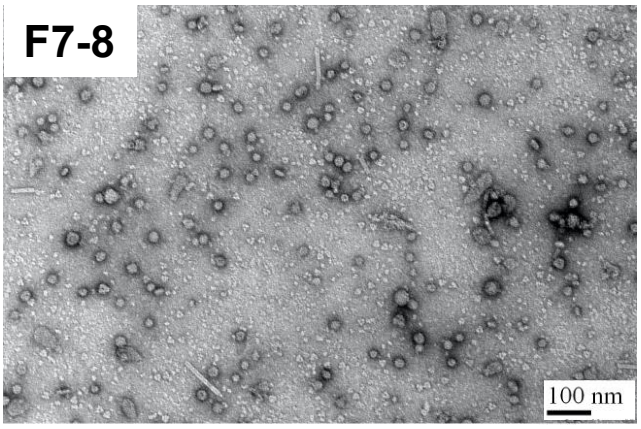
Supplemental Figure S1: p114-mCherry-Clyluc plasmid. (**A**) Map of p114-mCherry-Clyluc plasmid. (**B**) Sequence of the J23114-mCherry-clyA-nluc region. Violet, J23114 Promoter; Pink, *mCherry* gene; Grey, *clyA* gene; Green, Linker sequence; Orange, *nLuc* gene.

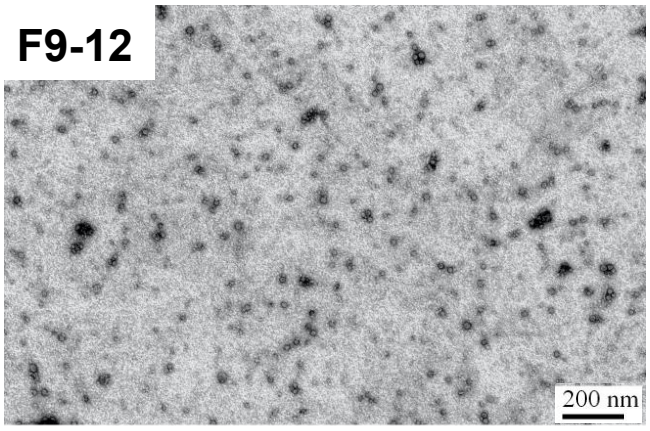


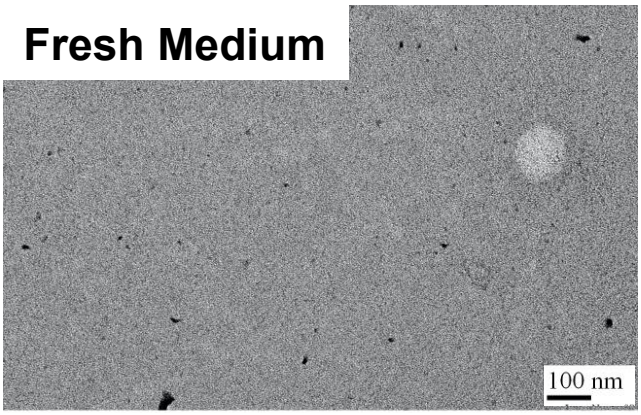
Supplemental Figure S2: Tangential flow filtration (TFF) setup schematic. The tubing with barb-hoses was connected to the TFF device as shown. Feed flow tubing begins submerged in the filtered conditioned culture medium vessel, continues through the peristaltic pump, and connects to the TFF device inlet port. The return flow begins at the TFF device outlet port and ends above the surface of the filtered conditioned culture medium. Optionally, a backpressure clamp
(e.g., a screw nut + bolt or simple paper clamp) can be used to increase the rate of filtration. As the conditioned medium is circulated by the pump, developed pressure within the TFF device leads to ultrafiltration and removal of components <100 kDa through the filtrate/waste flow tubing, which can be collected in a separate vessel for disposal (magenta).



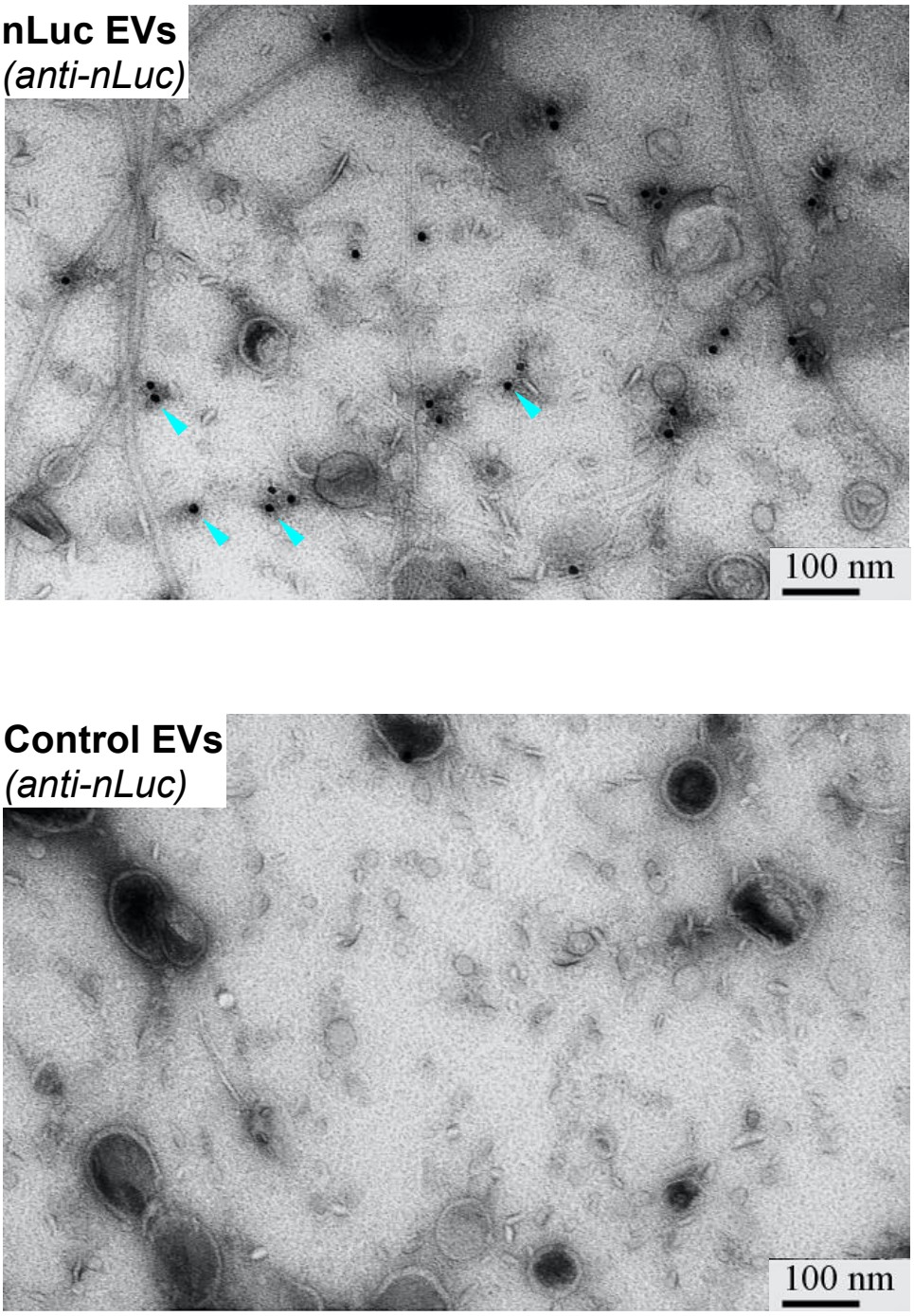








Supplemental Figure S3: Widefield transmission electron micrographs. TEM images of sequential, pooled chromatography fractions and fresh culture medium (control) shown in **Figure 2D**. The images show that the *E. coli* MP1 extracellular vesicles elute in early chromatography fractions.



Supplemental Figure S4: Widefield TEM images for Figure 3C. The EV fractions (F2-5) were immunogold-labeled following staining with anti-nano-Luciferase antibody. Cyan arrowheads point to gold-conjugated secondary antibody co-localizing with small EVs.