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Optimized Production and Analysis of Recombinant Protein-Filled Vesicles from *E. coli*

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Abstract

This innovative system, using a short peptide tag, that exports multiple recombinant proteins in membrane bound vesicles from *E. coli*, provides an effective solution to a range of problems associated with bacterial recombinant protein expression. These recombinant vesicles compartmentalise proteins within a micro-environment that facilitates the production of otherwise challenging, toxic, insoluble, or disulfide-bond containing proteins from bacteria. Protein yield is increased considerably when compared to typical bacterial expression in the absence of the vesicle-nucleating peptide tag. The release of vesicle-packaged proteins supports isolation from the culture medium and permits long-term active protein storage. This technology gives rise to increased yields of vesicle-packaged, functional proteins for simplified downstream processing for a diverse range of applications from applied biotechnology to discovery science and medicine. In the present article and the associated video, a detailed protocol of the method is provided, which highlights key steps in the methodology to maximize recombinant protein-filled vesicle production.

Introduction

The Gram-negative bacteria *E. coli* is an attractive system for recombinant protein production on both industrial and academic scale. It is not only cost-effective and straightforward to culture in batches to high densities, but a broad-spectrum of reagents, strains, tools, and promoters have been established to promote the generation of functional proteins in *E. coli*¹. Additionally, synthetic biology techniques are now overcoming obstacles typically related to the application of post-translational modifications and folding of complex proteins². The ability to target the secretion

of recombinant proteins into culture media is attractive for improving yield and reducing manufacturing costs. Controlled packaging of user-defined proteins into membrane vesicles assists the development of products and technologies within the applied biotechnology and medical industries. Until now, there has been a lack of widely applicable methods for secreting recombinant proteins from *E. coli*³.

Eastwood et al. have recently developed a peptide tagging-based method for producing and isolating recombinant protein-containing vesicles from *E. coli*¹. This Vesicle

Nucleating peptide (VNp) allows the production of extracellular bacterial membrane vesicles, into which the recombinant protein of choice can be targeted to simplify purification and storage of the target protein, and allows significantly higher yields than normally allowed from shaking flask cultures. Yields of close to 3 g of recombinant protein per liter of flask culture have been reported, with >100x higher yields than those obtained with equivalent proteins lacking the VNp tag. These recombinant protein-enriched vesicles can be rapidly purified and concentrated from the culture media and provide a stable environment for storage. This technology represents a major breakthrough in *E. coli* recombinant protein production. The vesicles compartmentalize toxic and disulfide-bond containing proteins in a soluble and functional form, and support the simple, efficient, and rapid purification of vesicle-packaged, functional proteins for long-term storage or direct processing¹.

The major advantages this technology presents over current techniques are: (1) the applicability to a range of sizes (1 kDa to >100 kDa) and types of protein; (2) facilitating inter- and intra- protein disulfide-bond formation; (3) applicable to multiprotein complexes; (4) can be used with a range of promoters and standard lab *E. coli* strains; (5) the generation of yields of proteins from shaking flasks normally only seen with fermentation cultures; proteins are exported and packaged into membrane bound vesicles that (6) provide a stable environment for storage of the active soluble protein; and (7) simplifies downstream processing and protein purification. This simple and cost-effective recombinant protein tool is likely to have a positive impact on the biotechnology and medical industries, as well as discovery science.

Here, a detailed protocol, developed over several years, describes the optimal conditions to produce recombinant protein-filled vesicles from bacteria with the VNp technology. Example images of this system in practice are shown, with a fluorescent protein being expressed, allowing the presence of vesicles during different stages of the production, purification, and concentration to be visualized. Finally, guidance is provided on how to use live cell imaging to validate the production of VNp fusion-containing vesicles from the bacteria.

Protocol

The bacterial work undertaken follows the local, national, and international biosafety containment regulations befitting the particular biosafety hazard level of each strain.

1. Selection of different VNps

1. Identify the VNp sequences.

NOTE: For the present study, three VNp sequences have been identified¹ that result in maximal yield and vesicular export of the proteins examined to date: VNp2, VNp6, and VNp15. It is not currently clear why certain VNp variants perform more efficiently with some proteins than others; therefore, it is recommended that fusions are generated between a new protein of interest with each VNp variant (i.e., VNp2, 6, or 15).

VNp2: MDVFMKGLSKAKEGVVAAAEKTKQGVAEA
AGKTKEGVL

VNp6: MDVFKKGFSIADEGVVGAVEKTDQGVTEA
AEKTKEGVM

VNp15: MDVFKKGFSIADEGVVGAVE

Plasmids that allow expression of the protein of interest with different VNp amino terminal fusions have been made available commercially (see **Table of Materials**).

2. Design a cloning strategy to insert the gene of interest at the 3' end of the cDNA encoding for the VNp in one of these constructs, or adapt an existing plasmid by integrating synthesized VNp cDNA upstream of the first ATG codon of the gene encoding for the protein of interest. Use the methods as described in¹.
3. For toxic proteins, use a vector with a repressible expression promoter or a promoter with minimal uninduced expression noise.
4. Clone the VNp sequence tag at the amino-terminal of the fusion protein. Ensure that the affinity tags, protease cleavage sequences, etc., and the protein of interest are located on the carboxyl side of the VNp tag. It is recommended to separate the VNp from the downstream peptide with a flexible linker region, such as two or three repeats of a -G-G-S-G- polypeptide sequence (**Figure 1**).
NOTE: Use plasmids with an antibiotic selection that does not target peptidoglycan, which weakens the cell surface and reduces vesicle yield. Kanamycin and chloramphenicol (see **Table of Materials**) were the preferred antibiotics used for this study.

2. Bacterial cell culture and protein induction

NOTE: Bacterial strains typically used in this protocol are either *Escherichia coli* BL21 (DE3) or W3110. *E. coli* cells are cultured in lysogeny broth (LB) (10 g/L tryptone; 10 g/L NaCl; 5 g/L yeast extract) or terrific broth (TB) (12 g/L tryptone; 24 g/L yeast extract; 4 mL/L 10% glycerol; 17 mM KH₂PO₄; 72 mM K₂HPO₄, salts autoclaved separately) media (see **Table of Materials**). Example images showing each step of the protein induction and subsequent isolation and purification process are shown in **Figure 2**.

1. Culture 5 mL LB starters from fresh bacterial transformations at 37 °C to saturation and use them to inoculate 25 mL of TB in a 500 mL conical flask, all with appropriate antibiotic selection.
2. The surface area:volume ratio is an important factor in optimizing this system. Use as large a volume flask as possible (e.g., 5 L flask containing 1 L of culture; for optimization runs, use 25 mL of media in a 500 mL flask).
3. Incubate the larger shaking flask cultures in an incubator at 37 °C with shaking at 200 rpm (≥ 25 mm orbital throw) until a 600 nm optical density value (OD₆₀₀) of 0.8-1.0 is reached.
NOTE: Vesiculation is optimal when cells are grown at 37 °C. However, some recombinant proteins require expression in lower temperatures. If this is the case for the protein of interest, the VNp6 tag must be used, as this allows high-yield vesicle export at temperatures down to 25 °C.
4. To induce recombinant protein expression from the T7 promoter, add isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of up to 20 μ g/mL (84 μ M) (see **Table of Materials**). The induction of recombinant protein expression must occur at the late-log phase (i.e., typical OD₆₀₀ of 0.8-1.0) for the production of vesicles.

NOTE: The length of the induction period may differ between proteins, with some reaching maximum production at 4 h and others overnight (18 h). To date, maximum vesicle export has been obtained in overnight cultures.

3. Recombinant vesicle isolation

1. Pellet the cells by centrifugation at 3,000 x g (4 °C) for 20 min.

- To sterilize vesicle-containing media for long-term storage, pass the cleared culture media through a sterile and detergent-free 0.45 μm polyethersulfone (PES) filter (see **Table of Materials**).

NOTE: To test the exclusion of viable cells from the vesicle-containing filtrate, plate onto LB agar and incubate overnight at 37 °C.

- To concentrate vesicles into a smaller volume, pass the sterile vesicle-containing media through a sterile and detergent-free 0.1 μm mixed cellulose esters (MCE) filter (see **Table of Materials**).

- Gently wash the membrane with 0.5-1 mL of sterile PBS using a cell scraper or plastic spreader to carefully remove vesicles from the membrane. Transfer to a fresh microfuge tube.

NOTE: Purified vesicles can be stored in sterile media or phosphate-buffered saline (PBS) at 4 °C. There are examples of recombinant proteins stored in these vesicles for 6 months, in this way, with no loss in enzymatic activity.

4. Soluble protein release from isolated vesicles

- Once protein-containing vesicles have been isolated into the sterile media/buffer, subject vesicular lipid membranes to sonication using an appropriate schedule for the apparatus (e.g., 6x 20 s on and off cycles) and centrifuge at 39,000 $\times g$ (4 °C) for 20 min to remove vesicle debris.

NOTE: Osmotic shock or detergent treatment can be used as an alternative to break open the vesicles, but consideration must be given to the impact upon protein functionality and/or downstream application.

- If VNp fusion remains cytosolic and does not release into the media, isolate the protein using standard protocols (e.g., resuspend the cell pellets in 5 mL of an appropriate extraction buffer (20 mM tris, 500 mM NaCl), sonicate, and remove the cell debris by centrifugation).

5. Protein concentration determination

- Determine the concentration of proteins by gel densitometry analysis of triplicate samples¹. Run alongside bovine serum albumin (BSA) loading standards on coomassie-stained sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Scan and analyze the gels using appropriate software (e.g., Image J; see **Table of Materials**).

6. Visualization of vesicle formation and isolated vesicles by fluorescence microscopy

NOTE: If the cells contain fluorescently labeled VNp fusion or membrane markers, live cell imaging can be used to follow vesicle formation. Alternatively, fluorescent lipid dyes can be used to visualize vesicles to confirm production and purification.

- Cell mounting
 - Induce VNp fusion expression for several hours before mounting onto the coverslip.
 - Agarose pad method (**Figure 3A-C**): pipette the cells onto a thin (<1 mm), circular LB-agarose (2%) pad that has been allowed to form and set on a clean glass slide. Allow the cells to settle and equilibrate and place a 50 mm x 25 mm coverslip onto the pad and cells. Hold the coverslip in place with spacers and adhesive tape.

3. Polyethyleneimine (PEI) method (**Figure 3D-F**): spread 20 μL of 0.05% PEI (in dH_2O) onto a coverslip with a pipette tip and leave for 3-5 min to bind to glass without allowing to dry. Add 50 μL of cell culture and leave for 5-10 min to ensure bacteria have associated with the PEI-coated surface⁴. Wash the coverslip with 100 μL of media before placing it onto the slide and hold it in place with spacers and adhesive tape.
2. Mounting vesicles
 1. Pipette purified vesicles onto a thin (<1 mm), circular LB-agarose (2%) pad that has been allowed to form and set on a clean glass slide. Once the liquid has dried, place a 50 mm x 25 mm coverslip onto the pad and vesicles. Hold the coverslip in place with spacers and adhesive tape.
 2. The fluorescent lipid dye FM4-64 is able to stain membranes⁵, and therefore can be used to visualize vesicles. Add FM4-64 (see **Table of Materials**) to purified vesicles at a final concentration of 2 μM (from a 2 mM stock dissolved in dimethyl sulfoxide [DMSO]) and image after a 10 min incubation. This is especially useful for identifying vesicles containing non-fluorescently labeled cargoes⁵.
 3. Rinse the coverslips with the same media used to culture the observed cells.
NOTE: Some complex media (e.g., TB) can exhibit autofluorescence, which may result in excess background signal.
 3. Imaging vesicles
NOTE: Example microscopy images of VNp recombinant vesicles can be seen in **Figure 4**.
1. Mount the slide onto an inverted microscope (see **Table of Materials**) using an oil immersion objective and leave for 2-3 min to allow the sample to settle and the temperature to equilibrate.
NOTE: All live cell imaging for each sample must be completed within 30 min of mounting cells onto coverslips to minimize the impact of phototoxicity and anaerobic stress. For this reason, single-plane images are preferred to z-stacks.
 2. Use appropriate light sources (e.g., light emitting diode [LED] or halogen bulb; see **Table of Materials**) and filter combinations for the fluorescent protein(s)/dye(s) being used⁶.
 3. Use a high magnification (i.e., 100x or 150x) and high numerical aperture (i.e., $\text{NA} \geq 1.4$) lens for imaging the microbial cells and vesicles.
 4. Determine the minimal light intensity required to visualize fluorescence signals from cells and / or vesicles. This may require some adjustment of the exposure and gain settings for the camera in use.
NOTE: Typical exposure times from current complementary metal-oxide semiconductor (CMOS) cameras vary between 50-200 ms depending upon the imaging system.
 5. For single frame images, use three-image averaging to reduce hardware-dependent random background noise.
 6. For time-lapse imaging, allow 3-5 min between individual frames.
NOTE: Depending upon the microscope setup, the focus may need to be intermittently adjusted throughout longer time-lapse experiments.

Representative Results

BL21 DE3 *E. coli* containing the VNp6-mNeongreen expression construct were grown to late-log phase (**Figure 2A**). VNp6-mNeongreen expression was induced by the addition of IPTG to the culture (20 $\mu\text{g}/\text{mL}$ or 84 μM final concentration), which was subsequently left to grow overnight at 37 °C with vigorous shaking (200 rpm, ≥ 25 mm orbital throw). The following morning, the culture displayed mNeongreen fluorescence⁷ (**Figure 2B**), which remained visible in the media after the removal of bacterial cells by centrifugation (**Figure 2C**). The presence of VNp-mNeongreen within the culture and cleared culture media was confirmed by SDS-PAGE (**Figure 2D**). The mNeongreen-

containing vesicles were isolated onto a 0.1 μm MCE filter (**Figure 2E**) and resuspended in PBS (**Figure 2F**). The purified vesicles were subsequently mounted on an agarose pad (**Figure 3A-C**) and imaged using widefield fluorescence microscopy (**Figure 4A**). The presence of vesicle membranes was confirmed using the lipophilic fluorescent dye FM4-64 (**Figure 4B**). The *E. coli* cells expressing the inner membrane protein CydB fused to mNeongreen (green) and VNp6-mCherry2 (magenta)⁸ show vesicle production and cargo insertion in live bacterial cells (**Figure 4C**). **Figure 4A,B** were captured using a widefield fluorescence microscope, while **Figure 4C** was acquired using structured illumination microscopy (SIM), using methods described previously^{9,10}.

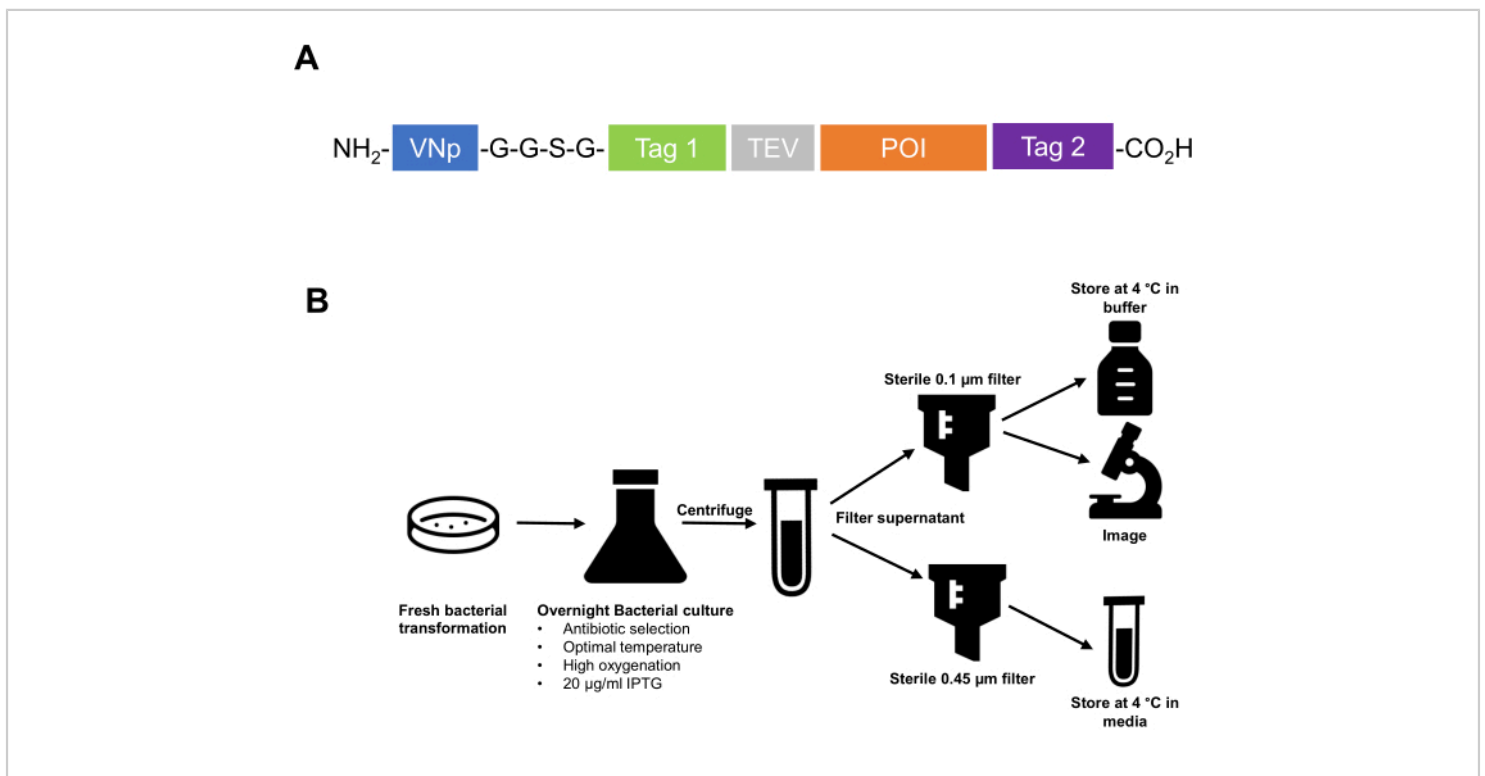


Figure 1: Summary of VNp technology from designing a cloning strategy to the purification and storage of extracellular vesicles. (A) Schematic of a typical VNp fusion protein. VNp at the NH₂ terminus, followed by a flexible linker and an appropriate combination of affinity and fluorescence tags (Tag1, Tag 2, protease cleavage site [e.g., TEV])

and protein of interest. **(B)** Schematic diagram summarising the protocol for the expression and purification of recombinant protein-filled membrane vesicles from *E. coli*. [Please click here to view a larger version of this figure.](#)

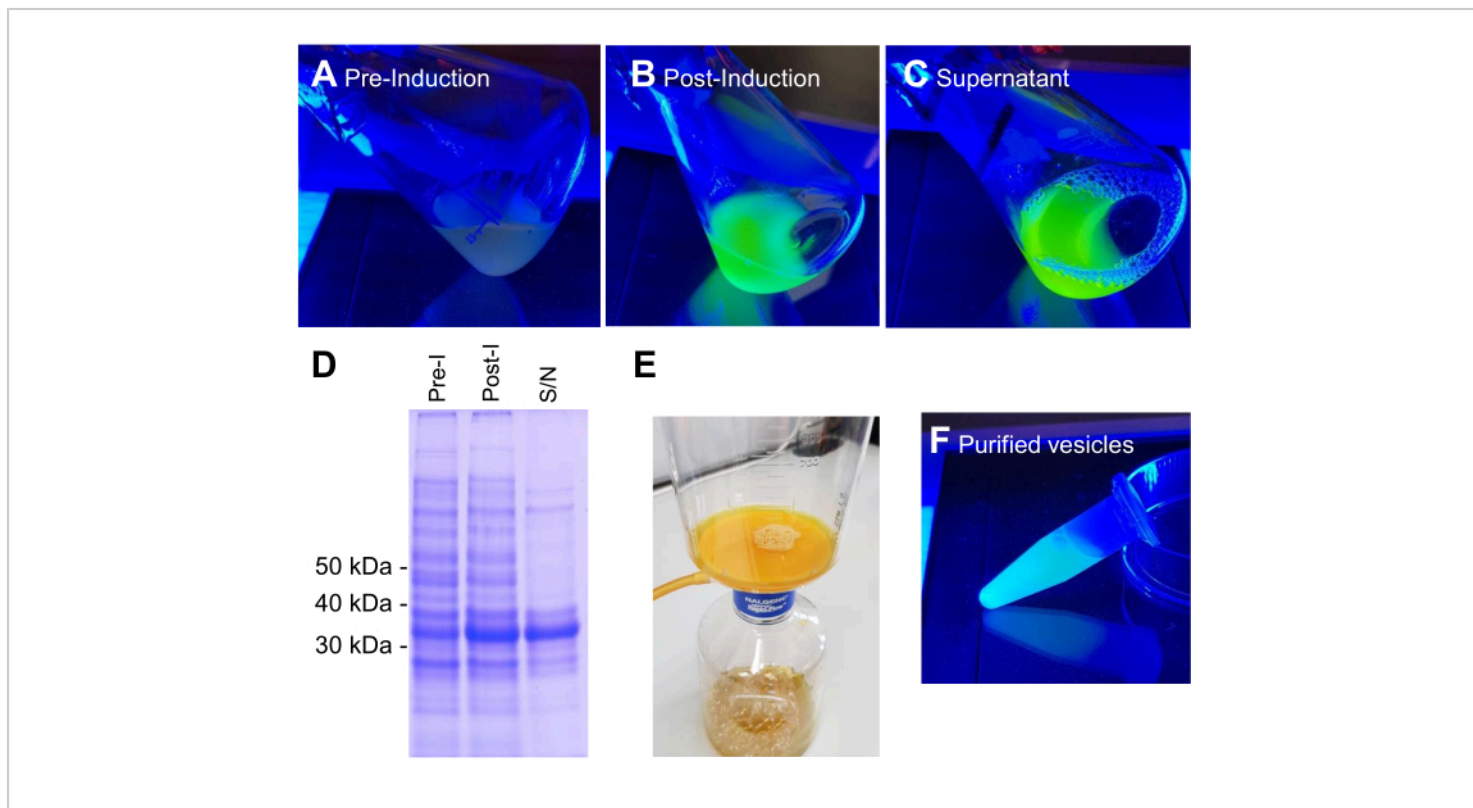


Figure 2: Stages of production and purification of VNp6-mNg vesicles. Cultures of *E. coli* cells containing the VNp-mNeogreen expression construct in blue light either before **(A)** or after **(B)** IPTG-induced expression of the fusion protein. The cells from **(B)** were removed by centrifugation, leaving VNp-mNeogreen-filled vesicles in the media **(C)**. **(D)** Equivalent samples from **(A)**, **(B)**, and **(C)** were analyzed by SDS-PAGE and coomassie staining. The vesicles were isolated onto a 0.1 μm filter **(E)** and subsequently washed off into an appropriate volume of buffer **(F)**. [Please click here to view a larger version of this figure.](#)

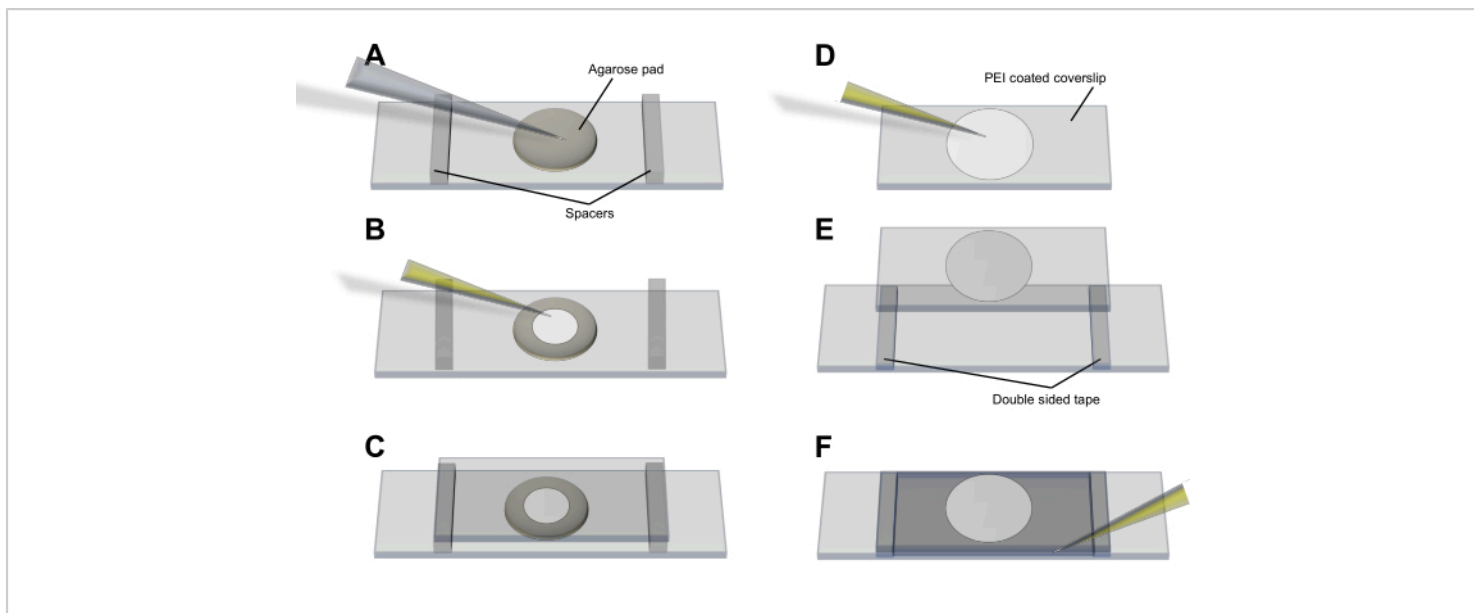


Figure 3: Cell mounting procedure for imaging vesicles and vesicle production. (A-C) The agarose pad method and **(D-F)** the PEI method for mounting *E. coli* cells onto the coverslip. [Please click here to view a larger version of this figure.](#)

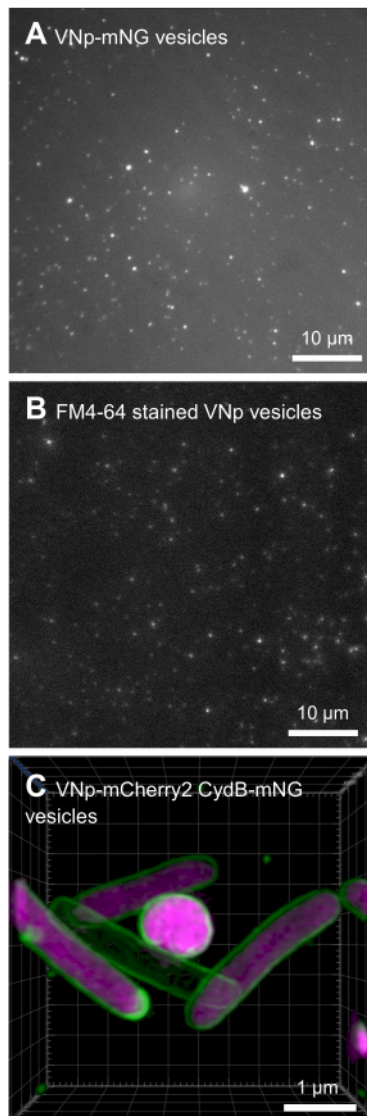


Figure 4: Microscopy images of VNP recombinant vesicles. Green (A) and red (B) emission images from different fields of FM4-64 labeled VNP6-mNeogreen-containing vesicles mounted on an agarose pad. (C) Imaging *E. coli* cells expressing the inner membrane protein CydB fused to mNeogreen (green) and VNP6-mCherry2 (magenta) shows vesicle production and cargo insertion in live bacterial cells. (A,B) were imaged using a widefield fluorescence microscope, while (C) was acquired using structured illumination microscopy (SIM). Scale bars: (A,B) = 10 μm; (C) = 1 μm. [Please click here to view a larger version of this figure.](#)

Discussion

The amino-terminal peptide-tagged method for the production of recombinant proteins described above is a simple process,

which consistently yields large amounts of protein that can be efficiently isolated and/or stored for months.

It is important to highlight the key steps in the protocol that are required for optimal use of this system. Firstly, the VNp tag¹ must be located at the N-terminus, followed by the protein of interest and any appropriate tags. It is also important to avoid using antibiotics that target the peptidoglycan layer, such as ampicillin.

In terms of growth conditions, rich media (e.g., LB or TB media) and a high surface area:volume ratio is necessary to maximize vesicle production. The optimal temperature for the production of extracellular vesicles is 37 °C, but the conditions typically required for expression of the protein of interest must be considered too. For lower induction temperatures, VNp6 must be used. Crucially, induction of the T7 promoter must be achieved using no greater than 20 µg/mL (84 µM) IPTG once the cells reach an OD₆₀₀ of 0.8-1.0. Proteins expressed using the system reach maximum vesicle production at either 4 h or after overnight induction.

Despite the simplicity of this protocol, it requires optimization. VNp variant fusion, expression temperatures, and induction time periods may differ depending on the protein of interest. Furthermore, there is a need to optimize the purification and subsequent concentration of extracellular vesicles from the media. The current procedure is not scalable and can be time-consuming. These are the limitations of this methodology.

The VNp technology has many advantages over traditional methods². It allows the vesicular export of diverse proteins, with the maximum size successfully expressed to date being 175 kDa for vesicles that remain internal and 85 kDa for those that are exported. Furthermore, this technology can significantly increase the yield of recombinant proteins with a range of physical properties and activities. Exported vesicles containing the protein of interest can be isolated by simple filtration from the precleared media and can subsequently be

stored, in sterile culture media or buffer, at 4 °C for several months.

The applications for this system are diverse, from discovery science to applied biotechnology and medicine (e.g., through the production of functional therapeutics)³. The ease of production, downstream processing, and high yield are all attractive qualities in these areas and especially in industry.

Disclosures

The authors declare no competing financial interests or other conflicts of interest.

Acknowledgments

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