**TITLE:**

Directed Protein Packaging within Outer Membrane Vesicles (OMV) from *Escherichia coli*: Design, Production and Purification

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**SHORT ABSTRACT:**

A protocol for the production, purification, and use of enzyme packaged outer membrane vesicles (OMV) providing for enhanced enzyme stability for implementation across diverse applications is presented.

**LONG ABSTRACT:**

An increasing interest in applying synthetic biology techniques to program outer membrane vesicles (OMV) are leading to some very interesting and unique applications for OMV where traditional nanoparticles are proving too difficult to synthesize. To date, all Gram-negative bacteria have been shown to produce OMV demonstrating packaging of a variety of cargo that includes small molecules, peptides, proteins and genetic material. Based on their diverse cargo, OMV are implicated in many biological processes ranging from cell-cell communication to gene transfer and delivery of virulence factors depending upon which bacteria are producing the OMV. Only recently have bacterial OMV become accessible for use across a wide range of applications through the development of techniques to control and direct packaging of recombinant proteins into OMV. This protocol describes a method for the production, purification, and use of enzyme packaged OMV providing for improved overall production of recombinant enzyme, increased vesiculation, and enhanced enzyme stability. Successful utilization of this protocol will result in the creation of a bacterial strain that simultaneously produces a recombinant protein and directs it for OMV encapsulation through creating a synthetic linkage between the recombinant protein and an outer membrane anchor protein. This protocol also details methods for isolating OMV from bacterial cultures as well as proper handling techniques and things to consider when adapting this protocol for use for other unique applications such as: pharmaceutical drug delivery, medical diagnostics, and environmental remediation.

**INTRODUCTION:**
Presented here is a method for the design, production, and purification of enzyme-loaded bacterial outer membrane vesicles (OMV). OMV are small, primarily unilamellar, proteoliposomes that range in size from 30-200 nm[1,2]. All Gram-negative and Gram-positive bacteria that have been studied to date have demonstrated release of either OMV or extracellular vesicles (EV) from their surface[3,4]. The precise mechanism by which OMV are produced have yet to be fully elucidated due to the diverse bacterial populations that secrete them as well as the varying functions that they serve. OMV have been shown to transport a wide range of cargo from small molecules, peptides, and proteins to genetic material serving a variety of complex signaling, gene translocation, and virulence functions[5,6].

The exact mechanisms of OMV biogenesis are not well characterized, and appear to differ between bacterial species. Despite this fact, we have developed a method for enhancing the packaging efficiency of a recombinant protein into OMV by creating a synthetic linkage between a protein of interest and a highly abundant protein endogenous to the bacterial outer membrane and subsequent OMV. In the absence of a synthetic linkage, or artificially incorporated affinity, between the recombinantly expressed protein and the OMV the observed packaging efficiency is very low[7]. This result is to be expected as incorporation of the proteins within the OMV either occurs through random chance encapsulation at the precise moment of OMV formation at the bacterial surface or through directed packaging by mechanisms that are not well understood. Some success has been observed in packaging proteins simply through over expression within the periplasmic space which relies on random chance encapsulation but effective packaging is highly protein dependent with some proteins packaging at high efficiency compared to others that do not package at all[8-10]. By utilizing common synthetic biology techniques we sought to engineer *Escherichia coli* (*E. coli*) to simultaneously produce, package and secrete an active

enzyme of interest into OMV that circumvents current knowledge limitations regarding how OMV are formed and how cargo is selected by the bacteria for packaging.

For the purposes of this application a split protein bioconjugation system was selected as the synthetic linkage of choice to facilitate directional packaging into the OMV. As the name suggests a split protein bioconjugation system is comprised of two complementary subunit domains that interact with one another. The split protein domains selected for the purposes of this protocol are referred to as the SpyCatcher (SC) and SpyTag (ST) domain and are derived from the *Streptococcus pyogenes* fibronectin-binding protein (FbaB)[11]. This split protein system is unusual in that when the two subunits are within proximity an isopeptide bond spontaneously forms between the proximal aspartic acid and lysine amino acid residues creating a covalent linkage. Isopeptide bond formation does not require the addition of chaperone proteins, catalytic enzymes, or cofactors and can readily occur at room temperature (RT) and over a wide range of physiologically relevant conditions[12].

As a proof of concept, Phosphotriesterase (PTE) (EC 3.1.8.1) from *Brevundimonas diminuta* was selected to be packaged into *E. coli* derived OMV[13]. PTE contains a binuclear Zn/Zn active site and has the ability to break down organophosphates through a hydrolysis reaction converting aryldialkylphosphates into dialkylphosphates and aryl alcohols[14]. Exposure to organophosphates impairs proper neurotransmitter function through inhibiting the hydrolysis of acetylcholine by acetylcholinesterase at neuromuscular junctions making organophosphate derived compounds extremely dangerous[15]. Prolonged or significant exposure to organophosphates commonly results in uncontrollable convulsions and typically causes death *via* asphyxiation. While PTE exhibits the highest catalytic activity toward paraoxon, a very potent insecticide, it is also capable of hydrolyzing a broad range of other pesticides and V/G type chemical nerve agents[16]. To facilitate OMV packaging, a bacterial plasmid was designed that encodes a gene construct that contains an inducible promoter, a periplasmic localization sequence, and a short multiple cloning site upstream of the SC gene sequence. Insertion of the PTE gene between the leader and SC sequence allowed for creation of a genetic switch that targets the PTE-SC fusion protein to the periplasmic space for OMV packaging. While the efforts described here focus on PTE, the enzyme gene is interchangeable and could readily be replaced with another gene sequence to facilitate packaging of an alternate enzyme or protein.

As the second part of the synthetic linkage, an abundant outer membrane protein (OmpA) is chosen to present the ST peptide sequence. While the choice of anchor protein can vary, it is essential that the protein has a permissive domain that presents within the periplasmic space, tolerates the fusion construct without inducing cytotoxicity, is known to be present in OMV, and does not aggregate when it is recombinantly produced. OmpA is a 37.2 kDa transmembrane porin protein that is known to be highly expressed in the bacterial outer membrane and subsequent OMV[17]. It is implicated in the transport of small molecules, < 2 nm in size, across the bacterial membrane[18]. Native OmpA has two structurally unique domains, a transmembrane beta barrel motif and a periplasmically soluble C-terminal portion known to interact with the peptidoglycan[19]. In the mutant OmpA-ST fusion designed here the C-terminal portion of OmpA was deleted and the ST was fused to the periplasmically facing N- or C-termini. Deleting the

periplasmic portion of OmpA decreases the number of interactions between the outer membrane and the peptidoglycan resulting in membrane destabilization leading to hyper-vesiculation[7]. Genomic OmpA was maintained in addition to the recombinantly expressed OmpA-ST construct to mitigate gross membrane destabilization.

**PROTOCOL:**

**1. Preparation of Plasmids**

1.1) Prepare a plasmid (e.g. pET22) containing the anchor protein (OmpA) fused to a biorthogonal linkage domain (referred to in this protocol as anchor-ST), epitope tag (such as 6xHis, myc or FLAG tags for purification and identification), periplasmic localization tag, antibiotic resistance, and appropriate origin of replication based on the selected bacterial strain[7].

1.1.1) Extract plasmid DNA from overnight cultures using a commercially available DNA isolation kit following the manufacturer's protocol.

1.2) Prepare a plasmid (e.g. pACYC184) containing the enzyme/protein (PTE) to be packaged within the OMV fused to the complementary bioorthogonal linkage domain to that of the anchor protein (referred to in this protocol as enzyme-SC), epitope tag, periplasmic localization tag, antibiotic resistance that is different than the anchor protein plasmid, and appropriate origin of replication based on the selected bacterial strain[7].

1.2.1) Extract plasmid DNA from overnight cultures using a commercially available DNA isolation kit following the manufacturer's protocol[7].

**2. Generation of an OMV packaging *E. coli* culture**

2.1) Plasmid isolation

2.1.1) Purify plasmids encoding the enzyme-SC construct and anchor-ST constructs from separate maintenance cell lines using a commercial plasmid isolation kit as per manufacturer's protocol[7]. Determine DNA concentration and purity by measuring absorbance at 260 nm and 280 nm.

2.2) Co-transformation

2.2.1) Select an *E. coli* strain that is commercially available to facilitate protein expression.

Note: BL21(DE3) cells were utilized here. The T7 lysogen is required for expression of the anchor-ST construct, which relies on the T7 promoter and T7 RNA polymerase for protein expression. Use of other bacterial strains requires either the presence of the T7 lysogen gene or the use of a plasmid other than pET22.[7]

2.2.2) Dilute purified plasmid DNA to equivalent molar concentrations then transform them into

competent bacterial cells via either heat shock transformation or electroporation following the manufacturer's protocol for the competent cells chosen.

2.2.3) Plate transformed cells onto antibiotic-containing Luria-Bertani (LB) agar plates with both ampicillin (pET22) and chloramphenicol (pACYC184); both present at final concentrations of 25 μg/ml.

2.2.4) Place the culture dish at 37 °C overnight to isolate only clones containing both plasmids.

Note: Colonies that survive antibiotic selection will be used for all future OMV preparations. Antibiotics (ampicillin and chloramphenicol) will be added to all liquid cultures to maintain selective pressure and ensure the presence of both plasmids.

## 3. OMV Production

3.1) Colony expansion

3.1.1) Inoculate 5 ml of culture media (typically Terrific Broth (TB) or LB) containing antibiotics at the aforementioned concentrations (step 2.2.3) with a single colony from the selection plates described in 2.2.4.

3.1.2) Perform a 1:100 fold dilution of the overnight starter culture into baffled culture flasks.

Note: Use a volume of media between 250-500 ml.

3.1.3) Maintain the culture at 37 °C shaking at 250 rpm to ensure that sufficient aeration of the culture is attained.

3.2) Induction of each plasmid

3.2.1) Allow the bacterial culture to grow to an $OD_{600}$ of 0.6-0.8, approximately 3 hr of growth after inoculation of the primary growth flask.

3.2.2) To improve the final yield of PTE-SC loaded vesicles, centrifuge the entire culture at 7,000 x g for 15 min. Resuspend the cell pellet in pre-warmed, fresh culture media.

Note: This optional step helps to remove any empty OMV that are present in the culture media prior to PTE-SC induction.

3.2.3) Make a 20% stock solution of L-arabinose in water and sterile filter using a 0.22 μm filter. Store L-arabinose solution at 4 °C for 2-4 weeks.

3.2.4) Add the L-arabinose solution to the culture flask to a final 0.2% concentration to initiate PTE-SC production.

Note: This helps to preload the periplasmic space with PTE-SC prior to promoting increased vesiculation through induction of the mutant OmpA-ST.

3.2.5) Make a 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) solution in water and sterile filter using a 0.22 μm filter.

Note: IPTG must be used immediately once hydrated. Aliquots of IPTG can be stored frozen for 3-6 months and can be thawed immediately prior to use.

3.2.6) Add IPTG after an additional 3 hr incubation period to a final concentration of 0.5 mM to initiate production of the OmpA-ST.

3.2.7) Allow the culture to grow for an additional 8-14 hr at 37 °C while shaking.

**4. OMV Purification**

4.1) Removal of intact bacteria

4.1.1) Centrifuge the bacterial culture media for 15 min at 7,000 x g at 4 °C to pellet intact bacteria.

4.1.2) Decant, and save, the media off the top of the cell pellet post centrifugation being careful to not disturb the cell pellet.

4.1.3) Repeat steps 4.1.1 and 4.1.2 for one additional centrifugation cycle to ensure all intact bacteria are removed from the culture media being sure to decant and save the media.

4.2) Removal of protein aggregates

4.2.1) Further purify the bacterial culture media using a 0.45 μm membrane filter to remove residual bacteria and undesired large cellular material.

Note: Use a biologically compatible membrane material for higher recoveries and lower protein adsorption such as cellulose acetate (CA) or polyvinylidene difluoride (PVDF). Syringe filter volumes less than 100 ml and vacuum filter volumes greater than 100 ml.

4.2.1.1) To syringe filter, draw culture medium into sterile 10 – 50 ml syringe. Attach 0.45 μm filter to Luer end of syringe. Depress plunger and collect flow-through in a new vessel.

4.2.1.2) To vacuum filter, transfer culture medium to a sterile filtration apparatus. Attach unit to vacuum pump or sink aspirator to generate suction. Transfer filtered medium to new vessel.

4.3) Isolation of OMV via ultracentrifugation

4.3.1) Add the filtered culture media into centrifugation tubes being careful to balance opposing tubes in the centrifuge.

4.3.2) Pellet at ~150,000 x g for 3 hr at 4 °C in an ultracentrifuge using the corresponding rotor matching both the instrument and tubes being utilized.

4.3.3) Decant the OMV depleted culture media from the OMV pellet, being careful not to disturb the OMV pellet, immediately at the completion of centrifugation as allowing the OMV pellet to remain in the media will soften the pellet reducing OMV recovery.

Note: This pellet will be slightly brown and depending on the starting amount of bacterial culture may or may not be visible by eye. Aspirating the media directly from the top of the centrifugation tube is also an option keeping in mind that the more media removed from the OMV pellet the more pure the final OMV sample will be.

4.3.4) Save the centrifuged supernatant for later dynamic light scattering (DLS) testing to verify that the OMV particles were fully depleted from the media.

4.3.5) Add 0.5-1 ml of sterile filtered Phosphate Buffered Saline (PBS) pH 7.4 or 100 mM *N*-Cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer at pH 8.0 to the OMV pellet allowing it to incubate for 30 min at RT. Ensure that the pellet does not dry.

Note: Extremes in solution ionic strength, pH, and temperature may result in reduced OMV solubility and promote aggregation.

4.3.6) Carefully pipet the purified OMV solution from the centrifuge tube, mixing gently to ensure the entire pellet has been solubilized.

**5. OMV characterization**

5.1) Use any commercially available DLS or nanoparticle tracking software following the provided manufacturer's protocols unique to each instrument to determine OMV size distribution and particle concentration.

Note: Here, OMV size distribution and concentration was determined utilizing NanoSight LM10 Nanoparticle Tracking and NTA 2.3 Analysis software.

5.1.1) Open the software.

5.1.2) Turn on microscope and clean all surfaces on the microscope and nanoparticle tracking unit.

5.1.3) Add ~0.5 ml of OMV sample directly to the viewing window of the particle tracking device

through the Luer fitting with a 1 ml sterile syringe being sure to cover the entire viewing window without injecting any air bubbles into the system.

5.1.4) Place the nanoparticle tracking unit on the microscope stage and turn on the laser.

5.1.5) Click "Capture" in the software.

5.1.6) Adjust the microscope focus and stage to visualize the particles on the preview screen present in the software window.

5.1.7) Adjust the "Camera Shutter" and "Camera Gain" until bright particles are clearly visualized on a dark background.

5.1.8)  Adjust capture duration to 60 sec.

5.1.9) Click "Record".

5.1.10) When prompted, input the sample/device temperature, label the sample, and save the video data at the completion of the each run into a user designated folder.

5.1.11) Keep all analysis parameters (Detection Threshold, Blur, Min Track Length, Min Expected Particle Size) on auto detect mode and click "Process Sequence."

Note: Consult the manual for how each parameter can be adjusted independently to fine tune analysis results.

5.1.12) Record particle size distribution and particles/ml concentration as determined by the software.

Note: A typical OMV hydrodynamic size range is between 30-200 nm with a particle/ml concentration of ~$10x10^8$.

5.2) Determining OMV protein content

5.2.1) Use standard SDS-PAGE and Western blot protocols to assess OMV purity, enzyme production, and the SC-ST crosslinking efficiency[7].

Note: Crosslinking efficiency will not be 100%[7].

**6. Verification of enzyme packaging**

6.1) PTE activity assay

6.1.1) Perform all enzymatic assays in a suitable buffer such as 100 mM CHES buffer (pH 8) at 25

°C in either a cuvette or multiplexed in a 96/384 well plate.

6.1.2) Add 5 μL of a 1:1,000 dilution of paraoxon in CHES buffer to 90 μL of CHES and 5 μL of purified OMV (~36-fold concentrated compared to the OMV concentration present in the culture media).

Caution: Paraoxon is a toxic pesticide and must be handled per manufacturer and institutional guidelines.

6.1.3) Take absorbance readings at regular intervals (every 20 sec for ~2 hr total reaction time) at 405 nm ($\varepsilon$ = 18.1 mM$^{-1}$cm$^{-1}$) and at 348 nm (isosbestic point $\varepsilon$ = 5.4 mM$^{-1}$cm$^{-1}$) to monitor the reaction progression of the chromogenic paraoxon breakdown product, *p*-nitrophenol.

Note: At pH values above 8 the dominant deprotonated form of *p*-nitrophenol is present allowing for accurate monitoring at 405 nm. In all other complex or lower pH solutions it is necessary to utilize the isosbestic point of 348 nm as multiple forms of the *p*-nitrophenol will be present.

6.1.4) Calculate initial reaction velocities by determining the slope of the linear portion of the progress curves from step 6.1.3 to compare the relative quantity of PTE in each sample, packaging efficiency, and total PTE production.

6.1.5) Perform standard enzymatic assay protocols to determine $K_M$, $V_{max}$ and $k_{cat}$ for the OMV encapsulated PTE.

Note: A Lineweaver-Burk analysis can be utilized for determination of these kinetic parameters where the y-intercept = $1/V_{max}$ and slope = $K_M/V_{max}$[20].

6.2) Transmembrane substrate/product diffusion.

6.2.1) Perform the same kinetic assay analysis as described in step 6.1 utilizing increasing concentrations of Triton X-100 in CHES buffer from 0-3% by volume.

Note: The addition of Triton X-100 to the solubilized OMV functions to disrupt the lipid bilayer allowing for the contents of the OMV to be freely accessible to the reaction solution.

6.2.2) Calculate and compare the initial velocities at each Triton X-100 level to verify transmembrane passage of the substrate/product indicated by minimal change in enzyme activity (initial velocities) compared to enzyme activity in the absence of Triton X-100. If a substantial increase in enzyme activity is observed in the presence of Triton X-100 it is likely that the substrate does not freely diffuse into the OMV.

6.2.3) Assay free enzyme activity (as described above in section 6.1) in the presence of Triton X-100 to verify that enzyme activity is not inhibited by the surfactant itself.

Note: If activity is greatly impacted by Triton X-100, alternative additives, such as saponins or various polysorbate/tween surfactants may be more suitable to rupture the OMV membrane.

**7. OMV storage**

7.1) Freezing

7.1.1) Place ~100 µL aliquots of purified OMV directly into liquid nitrogen to snap freeze the aliquots being sure not to fully submerge the vials or contact any liquid nitrogen with the sample itself.

Note: Purified OMV can be snap frozen directly in the buffer that was selected for solubilizing the OMV pellet during the purification process with no additional cryoprotectants necessary[7].

7.1.2) Store the snap frozen aliquots at -80 °C.

7.1.3) Thaw frozen aliquots from step 7.1.1 by placing them at RT being sure not to heat the samples.

7.1.4) Prior to preparing all samples for storage in this manner perform the enzyme assay described in step 6.1 comparing initial velocities before and after freezing to verify that there is no significant loss in enzyme activity post freeze-thaw.

Note: Store purified OMV at 4 °C if a reduction in enzyme activity is observed due to freezing.

7.2) Lyophilization

7.2.1) Lyophilize the snap frozen purified OMV aliquots utilizing commercially available lyophilization equipment[21].

7.2.2) Store lyophilized aliquots at RT for weeks or at -80 °C for many months.

7.2.3) Add the same volume of purified water to the lyophilized OMV as prior to lyophilization and let stand at RT for 30 min to rehydrate the sample. Mix gently when necessary being sure not to vortex or sonicate the OMV.

7.2.4) Add lyophilized OMV powder directly to point-of-care for applications where prior dilution/solubilization is not necessary.

**REPRESENTATIVE RESULTS:**
Simultaneous expression of two recombinant proteins, as is required for the OMV packaging strategy detailed in this protocol, can be accomplished through a number of different avenues. Here, a two vector system was utilized with compatible origins of replication and separate inducible gene cassettes. For the expression of the PTE-SC construct a commercial plasmid

backbone (pACY184) was engineered to include an arabinose inducible gene cassette and a twin arginine periplasmic localization leader sequence followed by a series of unique restriction sites to facilitate cloning of the enzyme gene as a fusion to a C-terminal SC protein. The plasmid construct encoding the outer membrane anchor protein is the commercial vector pET22 which utilizes the *lac* operon for control of protein expression and the *pelB* periplasmic localization sequence. The short ST sequence was cloned into the truncated OmpA protein prior to insertion into the expression plasmid. In both plasmids, the C-terminal hexahistidine sequence was maintained to allow for simultaneous identification of the fusion proteins in OMV samples. Construction and characterization of these gene constructs, including both the N- and C- terminal ST fusion constructs to OmpA, are fully described in Alves *et al*[7]. The gene constructs described herein may not be conducive to the packaging of all enzymes. In some instances it may be necessary to change the periplasmic localization signal, the regulatory elements, or the epitope tags. Additionally, some proteins may be prohibitivley large and unable to transverse the inner membrane altogether. Enzymatic activity and OMV packaging efficiency cannot be determeined *a priori* and will need to be emperically determined for each target protein.

Below are results that one would typically expect after successfully carrying out this protocol. Included is a schematic of the two ST/SC split protein domains, OmpA-ST and PTE-SC fusion constructs as well as an example of isopeptide formation at the bacterial outer membrane and subsequent encapsulation of PTE-SC within an OMV (Figure 1). Also included are representative results of the purified OMV morphology via SEM as well as size distribution and absolute OMV concentration determined via particle tracking software (Figure 2). Further characterization of the OMV protein content and recombinant protein expression are seen via SDS-PAGE and Western blot (Figure 3). Molecular weights for proteins of interest specific to the protocol provided here are as follows: native OmpA (37.2 kDa), OmpA-ST (23 kDa), PTE-SC (51 kDa), OmpA-ST/PTE-SC (74 kDa). Purified OMV should have a dark band at approximately 37 kDa which is indicative of the highly abundant native OmpA. Depending on the gel resolution there may be multiple bands present in this region of the gel as OmpA, OmpF and OmpC are all relatively abundant and share a similar molecular weight. An additional band that is 2.2 kDa larger than OmpA-ST may also be observed due to improper cleavage of the leader sequence as a result of overwhelming the *E. co*li expression machinery. It is important to note that a successfully purified OMV sample will not have many other highly expressed proteins. If other bands are present either the purification was carried out improperly or the bacteria strain being utilized may be packaging other proteins not observed in this *E. coli* BL21(DE3) strain.

Furthermore, activity assays and vesicle rupture experiments have been provided to demonstrate representative results that would be expected if the enzymatic substrate can freely enter the OMV through transmembrane porin proteins (Figure 4). Paraoxon relatively freely enters the OMV through endogenous transmembrane porin proteins to react with the packaged PTE and the reaction product, *p*-nitrophenol, also passes relatively freely through the OMV membrane. This phenomenon will not be ubiquitous among all product, substrate, and enzyme sets and must be experimentally determined. Though successful OMV rupture and enzyme release has been seen with low concentrations of detergent and PTE (herein Triton X-100), such additions may affect the activity of other enzymes.

Plasmid maps have also been included to demonstrate the design of a two plasmid packaging strategy utilizing the SC/ST system (Figure 5).
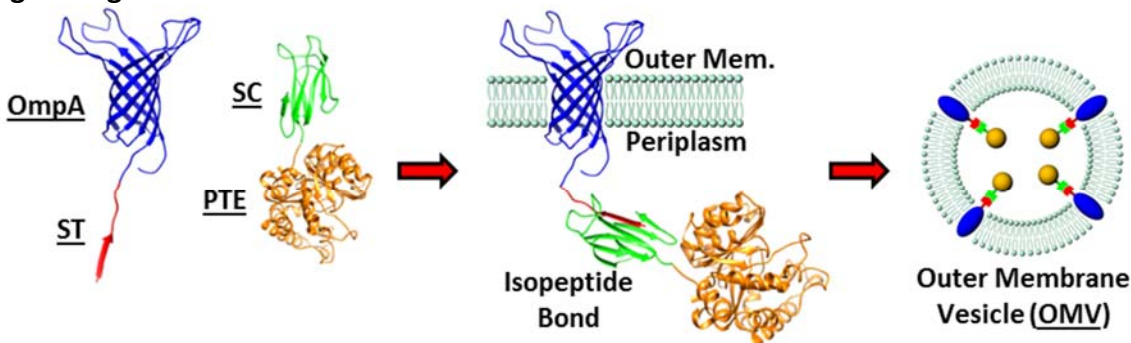
**Figure Legends:**



**Figure 1: Directed packaging of proteins into OMV.** Crystal structures for the proteins utilized in the described OMV packaging strategy: OmpA, PTE, SpyTag (ST) and SpyCatcher (SC); PDB: 2GE4, 1PTA, 4MLI, 4MLI, respectively. A schematic representation of the OmpA-ST and PTE-SC fusion constructs forming an isopeptide bond at the outer membrane of the bacteria is shown. This membrane fusion drives incorporation of the PTE within the forming OMV. Figure reproduced (adapted) from Alves et al. *ACS Appl Mat Interfaces* (2015), 7(44), pp 24963 – 24972 [7]. Copyright 2015 American Chemical Society.
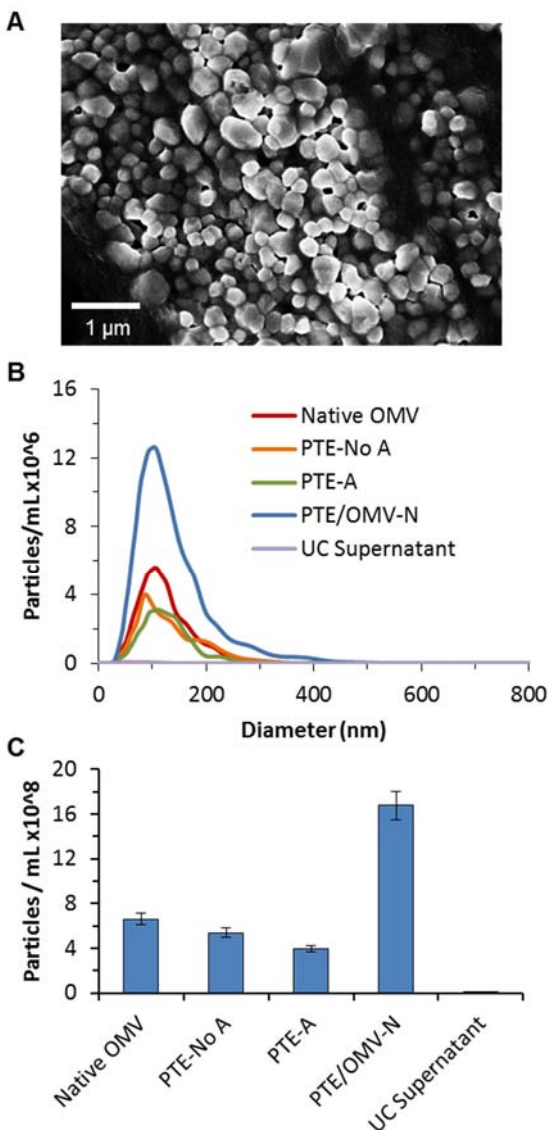
**Figure 2: OMV characterization.** A) SEM of ultracentrifuge purified OMV from native *E. coli* [BL21(DE3)]. B) Representative particle tracking size distributions and (C) total vesicle concentration averaged over 90 sec sample reads for Native OMV, PTE-SC in the absence of arabinose activation (PTE-No A), PTE-SC in the presence of arabinose activation (PTE-A), N-terminal OmpA-ST co-transformed with PTE-SC in the presence of arabinose and IPTG activation (PTE/OMV-N), and the ultracentrifuge supernatant (UC Supernatant). A significant increase in OMV production was observed in the PTE/OMV-N sample compared to the Native OMV and PTE-SC constructs alone. OMV were quantified in the UC supernatant to demonstrate a nearly complete recovery of OMV in the UC pellet leaving little to no OMV in the supernatant post ultracentrifugation. All data represents means (±SD) of triplicate experiments. Figure reproduced (adapted) from Alves et al. *ACS Appl Mat Interfaces* (2015), 7(44), pp 24963 – 24972 [7]. Copyright 2015 American Chemical Society.
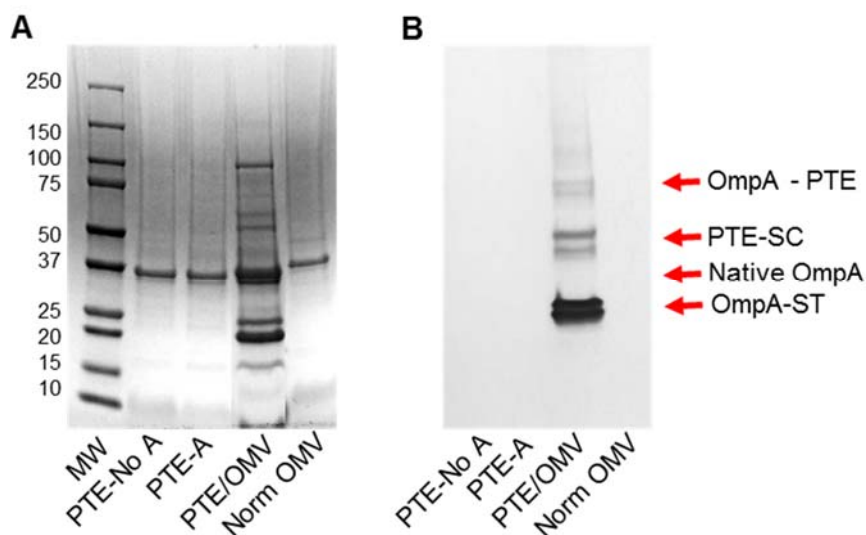
**Figure 3: Purified OMV protein content determination.** A) SDS-PAGE of the purified OMV UC pellet of the C-terminal OmpA-ST fusion coexpressed with PTE-SC (PTE/OMV) construct demonstrating representative abundance of OmpA-ST, Native OmpA, PTE-SC and the OmpA-ST/PTE-SC isopeptide fusion. B) Western blot analysis utilizing the included his-tag present in the OmpA and PTE constructs to facilitate visualization via an anti-6xhis antibody. It is important to note that PTE is known to dimerize, as evidenced on the blot by the presence of larger molecular weight his-tagged species. Even though there are very high OmpA-ST expression levels observed there is not a complete conversion of free PTE-SC to membrane bound OmpA-ST/PTE-SC. Despite this fact, the increase in overall PTE production and improved packaging efficiency suggest that while covalent bond formation is not ubiquitous the non-covalent association of the ST and SC domains is an important factor in directed packaging within the OMV.  Figure reproduced (adapted) from Alves et al. *ACS Appl Mat Interfaces* (2015), 7(44), pp 24963 – 24972 [7]. Copyright 2015 American Chemical Society.
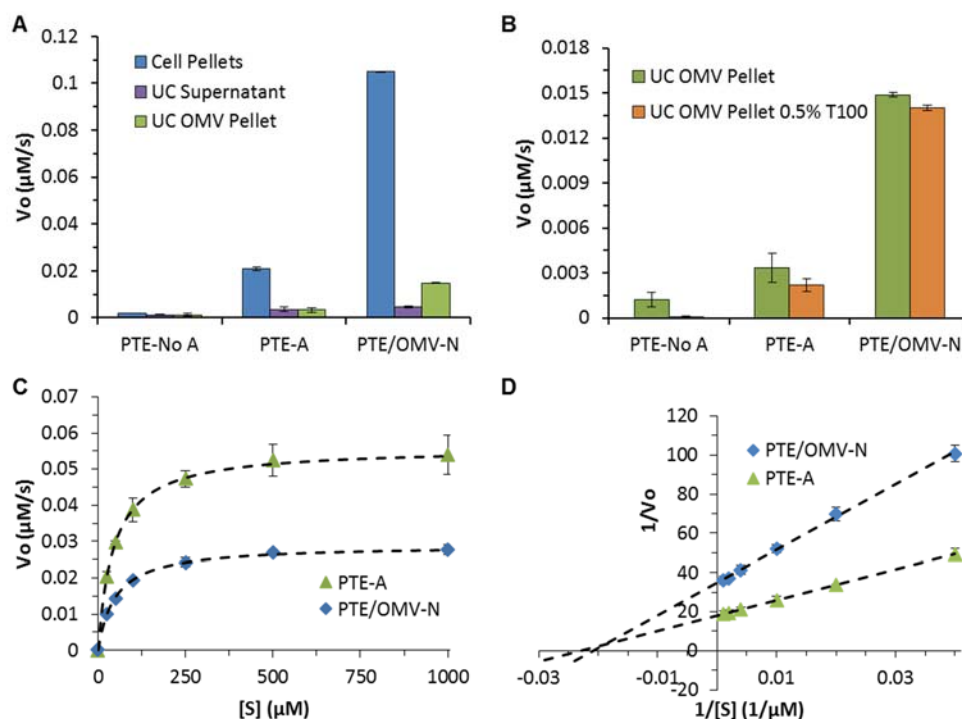
**Figure 4: PTE activity and packaging characterization.** A) Overall PTE expression levels and OMV packaging efficiency determined via initial velocity measurements utilizing paraoxon as a chromogenic substrate comparing PTE present in the cell pellets, the UC supernatant, and the purified UC OMV pellets. B) Representative data demonstrating the use of Triton X-100 (0.5% T100) to disrupt the OMV bilayer allowing unimpeded access of substrate to the OMV interior. Comparing this data to assays performed in the absence of T100 facilitates verification of translocation of substrate across intact OMV bilayers if enzymatic initial velocities are unchanged. In the case of paraoxon there was very little activity difference observed in the presence or absence of T100 indicating that paraoxon passes freely through the endogenous pores on the OMV. C) PTE-SC kinetic data fit to standard Michaelis-Menten enzyme kinetics equation for PTE/OMV-N and PTE-A. D) A Lineweaver-Burk analysis used for determining $K_M$ and $k_{cat}/K_M$ (48, $4.4 \times 10^7$; 44 µM, $4.9 \times 10^7$ $s^{-1}M^{-1}$, respectively) demonstrating similar PTE literature kinetic parameters as native enzyme (90 µM, $2.7 \times 10^7$ $s^{-1}M^{-1}$) with $R^2 \geq 0.999$ in all cases. All data represents means (±SD) of triplicate experiments. Figure reproduced (adapted) from Alves et al. *ACS Appl Mat Interfaces* (2015), 7(44), pp 24963 – 24972 [7]. Copyright 2015 American Chemical Society.

**Figure 5: Representative plasmid constructs for the example system described here.** SpyCatcher modified enzyme-SC (PTE-SC) plasmid targeted for encapsulation within the OMV (left). SpyTag modified membrane anchor-ST (OmpA-ST-N) plasmid (right).

**DISCUSSION:**
This protocol functions to demonstrate a representative directed packaging technique in which an enzyme of interest is produced and packaged into OMV by *E. coli*. As with many complex techniques there are multiple areas in which the protocol can be modified to accommodate for

use in different unique applications, some of which are detailed below. While the mechanism of OMV packaging and enzyme encapsulation can be adapted to specific needs there are several steps within this protocol which are critical to its success. The initial removal of intact bacterium and cellular debris is of significant importance and will affect all downstream attempts at quantitation or sample analysis. Two successive centrifugation steps followed by filtration is typically adequate to remove these contaminants. However, in all instances care should be taken when decanting solutions after centrifugation to minimize the transfer of contaminants. Similarly, the final OMV pellet obtained following ultracentrifugation is often only loosely adhered to the bottom of the centrifuge tube. In the final stages, care must be taken when removing the spent culture medium to ensure that the pellet remains undisturbed at the bottom of the vessel. In some instance it may be best to retain the final culture medium until after sample analysis using DLS or nanoparticle tracking device to ensure the OMV pellet was not lost in the final steps of the protocol. Below are some additional concerns that may arise with both this particular protocol and others that are tailored to specific needs of the researchers.

The use of PTE in this system provides for an excellent model enzyme encapsulation for the environmental remediation of organophosphate contaminated regions with PTE being readily replaceable by an alternate enzyme or protein of interest. As described here the coexpression of PTE-SC with OmpA-ST resulted in a large increase in overall PTE expression as well as higher vesicle production levels with more PTE being packaged within the vesicles compared to PTE and PTE-SC expressed alone. By reducing the number of interactions between the outer membrane and the peptidoglycan through the C-terminal deletion of OmpA hyper-vesiculation is attained. This provides an easy route for the bacteria to export the recombinant PTE which mitigates the toxic effects that are often observed in the overexpression of non-native proteins.

While PTE can readily be replaced in this model system it is important to note that not all enzyme substrates will freely pass through the OMV bilayer and it is therefore imperative that each unique enzyme and substrate pair be tested on a case by case basis. Even if a substrate does not pass through the membrane this technique can still be utilized to produce and package an enzyme into OMV and the addition of Triton X-100, or suitable alternative surfactant, can be added at sufficient levels to rupture the vesicles prior to use.

In the absence of recombinant protein expression and packaging this protocol can also serve as a basic method for OMV production and purification from diverse bacterial species. Alternate methods for OMV purification, such as density gradient fractionation,[22] membrane filtration,[23] and ultra-filtration,[24] also exist and may be more suitable techniques depending upon the intended application. These alternate purification techniques can also be readily supplemented into this protocol in place of the ultracentrifugation pelleting of the OMV for the directed packaging of a protein into an OMV through use of a biorthogonal synthetic linkage.

Other modifications can be made to the specific synthetic linkage system utilized in this protocol as well as the selected membrane tethering protein. There are various synthetic strategies for pairing two different proteins within a biological system that include: split proteins,[25] coiled coils,[26] and split inteins,[27] just to list a few. Other membrane bound or transmembrane proteins

such as OmpF and OmpC would likely provide for suitable ST modification sites as well[28]. In some instances it may also be necessary to swap the ST and SC domains placing the SC on the anchoring protein and the much smaller ST on the recombinant enzyme/protein. There are also a number of different cloning strategies that can be implemented here including the use of multiple plasmids under the same or different induction systems, a single plasmid with multiple proteins encoded, or even utilizing homologous recombination techniques to incorporate all or parts of the expression system into the bacterial genome.

The microenvironment of the OMV helps to stabilize the packaged enzyme reducing enzymatic inactivation that commonly occurs under less than ideal storage conditions, freeze-thaw, and lyophilization[29]. It is also anticipated that the OMV will provide greatly improved resistance to proteolytic cleavage as the OMV bilayer will function as a physical barrier between the active enzyme and the proteins present in the extra-OMV space. This protocol can further be expanded to include an exterior facing small molecule, peptide, or protein tag to facilitate targeted delivery of OMV encapsulated proteins and affinity purification. While PTE was selected for this unique application the results of this study, and contents of this protocol, can easily be utilized to design analogous protein packaging strategies for use in diverse pharmaceutical delivery, medical diagnostic, and environmental remediation applications[30].

**DISCLOSURES:**
The authors have nothing to disclose

**REFERENCES:**
1.      Avila-Calderon, E. D. *et al.* Roles of bacterial membrane vesicles. *Arch. Microbiol.* **197**, 1-10, doi:10.1007/s00203-014-1042-7 (2015).
2.      Kulp, A. & Kuehn, M. J. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu. Rev. Microbiol.* **64**, 163-184, doi:10.1146/annurev.micro.091208.073413 (2010).
3.      Beveridge, T. Structures of Gram negative cell walls and their derived membrane vesicles. *J. Bacteriol.* **181**, 4725-4733 (1999).
4.      Kulkarni, H. M. & Jagannadham, M. V. Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. *Microbiology* **160**, 2109-2121, doi:10.1099/mic.0.079400-0 (2014).
5.      Ellis, T. N. & Kuehn, M. J. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* **74**, 81-94, doi:10.1128/mmbr.00031-09 (2010).
6.      Berleman, J. & Auer, M. The role of bacterial outer membrane vesicles for intra- and interspecies delivery. *Environ. Microbiol.* **15**, 347-354, doi:10.1111/1462-2920.12048 (2013).
7.      Alves, N. J. *et al.* Bacterial nanobioreactors–directing enzyme packaging into bacterial outer membrane vesicles. *ACS Appl. Mater. Interfaces* **7**, 24963-24972, doi:10.1021/acsami.5b08811 (2015).

8.      Kim, J.-Y. *et al.* Engineered bacterial outer membrane vesicles with enhanced functionality.  *J. Mol. Biol.* **380**, 51-66, doi:10.1016/j.jmb.2008.03.076 (2008).

9.      Haurat, M. F. *et al.* Selective sorting of cargo proteins into bacterial membrane vesicles. *J. Biol. Chem.* **286**, 1269-1276, doi:10.1074/jbc.M110.185744 (2011).

10.     Kesty, N. C. & Kuehn, M. J. Incorporation of heterologous outer membrane and periplasmic proteins into *Escherichia coli* outer membrane vesicles. *J. Biol. Chem.* **279**, 2069-2076, doi:10.1074/jbc.M307628200 (2003).

11.     Zakeri, B. *et al.* Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesion. *Proc. Natl. Acad. Sci. U. S. A.* **109**, e690-697, doi:10.1073/pnas.1115485109/-/DCSupplemental (2012).

12.     Li, L., Fierer, J. O., Rapoport, T. A. & Howarth, M. Structural analysis and optimization of the covalent association between SpyCatcher and a peptide tag.  *J. Mol. Biol.* **426**, 309-317, doi:10.1016/j.jmb.2013.10.021 (2014).

13.     Dumas, D. P., Durst, H. D., Landis, W. G., Raushel, F. M. & Wild, J. R. Inactivation of organophosphorus nerve agents by the phophotriesterase from Pseudomonas-Diminuta. *Arch. Biochem. Biophys.* **277**, 155-159, doi:10.1016/0003-9861(90)90564-f (1990).

14.     Bigley, A. N. & Raushel, F. M. Catalytic mechanisms for phosphotriesterases. *Biochimica et Biochim. Biophys. Acta, Proteins Proteomics* **1834**, 443-453, doi:10.1016/j.bbapap.2012.04.004 (2013).

15.     Minton, N. A. & Murray, V. S. G. A review of organo-phosphate poisoning. *Med. Toxicol. Adverse Drug Exper.* **3**, 350-375, doi:10.1007/BF03259890 (1988).

16.     Bigley, A. N., Xu, C., Henderson, T. J., Harvey, S. P. & Raushel, F. M. Enzymatic neutralization of the chemical warfare agent VX: Evolution of phosphotriesterase for phosphorothiolate hydrolysis. *J. Am. Chem. Soc.* **135**, 10426-10432, doi:10.1021/ja402832z (2013).

17.     Chatterjee, S. N. & Chaudhuri, K. Gram-negative bacteria: the cell membranes. In: *Outer Membrane Vesicles of Bacteria*. SpringerBriefs in Microbiology. 15-34, doi:10.1007/978-3-642-30526-9_2 (2012).

18.     Wang, Y. The function of OmpA in Escherichia coli. *Biochem. Biophys. Res. Commun.* **292**, 396-401, doi:10.1006/bbrc.2002.6657 (2002).

19.     Danoff, E. J. & Fleming, K. G. The soluble, periplasmic domain of OmpA folds as an independent unit and displays chaperone activity by reducing the self-association propensity of the unfolded OmpA transmembrane beta-barrel. *Biophys. Chem.* **159**, 194-204, doi:10.1016/j.bpc.2011.06.013 (2011).

20.     Alves, N. J. & Kline, J. A. Comparative study on the inhibition of plasmin and delta-plasmin via benzamidine derivatives. *Biochem. Biophys. Res. Commun.* **457**, 358-362, doi:10.1016/j.bbrc.2014.12.117 (2015).

21.     Wang, W. Lyophilization and development of solid protein pharmaceuticals. *Int. J. Pharm*. **203**, 1-60, doi:10.1016/S0378-5173(00)00423-3 (2000)

22.     Goormaghtigh, E. & Scarborough, G. A. Density-based separation of liposomes by glycerol gradient centrifugation. *Anal. Biochem.* **159**, 122-131, doi:10.1016/0003-2697(86)90316-7 (1986).

23.     Alves, N. J. *et al.* Functionalized liposome purification via Liposome Extruder Purification (LEP). *Analyst* **138**, 4746-4751, doi:10.1039/c3an00680h (2013).

24.     Mayer, L. D. & StOnge, G. Determination of free and liposome-associated doxorubicin and vincristine levels in plasma under equilibrium conditions employing ultrafiltration techniques. *Anal. Biochem.* **232**, 149-157, doi:10.1006/abio.1995.0001 (1995).

25.     Blakeley, B. D., Chapman, A. M. & McNaughton, B. R. Split-superpositive GFP reassembly is a fast, efficient, and robust method for detecting protein–protein interactions in vivo. *Mol. BioSyst.* **8**, 2036, doi:10.1039/c2mb25130b (2012).

26.     De Crescenzo, G., Litowski, J. R., Hodges, R. S. & O'Connor-McCourt, M., D. Real-time monitoring of the interacation of two-stranded *de novo* designed coiled-coils: effect of chain length on the kinetic and thermodynamic constants of binding. *Biochemistry* **42**, 1754 – 1763, doi:10.1021/bi0268450 (2003).

27.     Charalambous, A., Antoniades, I., Christodoulou, N. & Skourides, P. A. Split-Intein for simultaneous site-specific conjugation of quantum dots to multiple protein targets in vivo. *J. Nanobiotechnol.* **9**, 1-14, doi:10.1186/1477-3155-9-37 (2011).

28.     Lee, E.-Y. *et al.* Global proteomic profiling of native outer membrane vesicles derived fromEscherichia coli. *Proteomics* **7**, 3143-3153, doi:10.1002/pmic.200700196 (2007).

29.      Alves, N. J., Turner, K. B., Medintz, I. L., & Walper, S. A. Protecting enzymatic function through directed packaging into bacterial outer membrane vesicles. *Sci. Rep.* **6**, 24866, doi:10.1038/srep24866 (2016).

30.     Alves, N. J., Turner, K. B., Medintz, I. L. & Walper, S. A. Emerging therapeutic delivery capabilities and challenges utilizing enzyme/protein packaged bacterial vesicles. *Ther. Delivery* **6**, 873-887, doi:10.4155/tde.15.40 (2015).