HARNESSING *E. COLI* TO IDENTIFY POLYSTYRENE MICROPLASTICS

by

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ABSTRACT

Microplastics (MPs) are broadly defined as plastic materials that are less than 5 mm in size. Exposure to MPs can be traced back to synthetic products used every day, yet the potential health consequences of MP exposure are unknown. The detection of the ultra-small MPs in environmental or biological samples pushes the limitations of our current technologies, and they cannot determine MP chemical composition at high throughputs. A recent study demonstrated the ability to quantify algae that bind micro-polystyrene (micro-PS) on their cell surface. Several peptides have been identified to potentially have specific protein-plastic interactions; however, they have yet to be used in an application for MP detection. In this thesis, the cell surface of bacteria was genetically engineered to express peptides previously shown to bind bulk PS materials in an effort to develop a novel identification method for the chemical composition of micro-PS. Genes of PS-binding peptides (PBPs) were inserted into plasmid DNA that could express the PBPs on the surface of *Escherichia coli*. A combination of Western blots and flow cytometry were used to validate the expression of PBPs on the cell surface, and the ability of these cells to bind micro-PS was assessed using flow cytometry.

INTRODUCTION

Microplastics (MPs) are broadly defined as plastic materials that are less than 5 mm in size.[1] MPs can be traced back to bulk plastic products used every day; for example, a single plastic tea bag can release billions of MPs in a cup of tea.[2] Polystyrene (PS) is one of the most used plastics worldwide due to its industrial versatility and it is commonly used in food packaging. Unfortunately, PS, especially in its foam form as Styrofoam, accumulates in the environment and is not considered biodegradable due to its high covalent nature of materials.[3] Moreover, evidence suggests MPs can absorb persistent, bioaccumulative, and toxic (PBT) compounds from seawater.[4]

While it has been established that plastic pollution is a global environmental health concern, much remains unknown about the potential effects of MP exposure. Despite this uncertainty, MPs that are less than 20 μm in size are particularly of interest because of the increasing evidence that they can be absorbed and accumulated in the gut and other organs.[5] However, the detection of the ultra-small MPs in environmental or biological samples pushes the limitations of current technologies (Raman and IR spectroscopy), and they cannot determine the molecular composition of MPs at high throughputs.[6] A technique with the ability to track and quantify MPs at high throughputs could bring new horizons to this field. Could there be another way to identify the molecular composition of MPs?

Synthetic biology is a rapidly growing interdisciplinary field that aims to engineer biological systems to have functions that are not found in nature. Practical applications of synthetic biology include developing biosensors, revolutionizing agriculture, bioproduction, and more.[7] For example, the use of microbes designed to display proteins

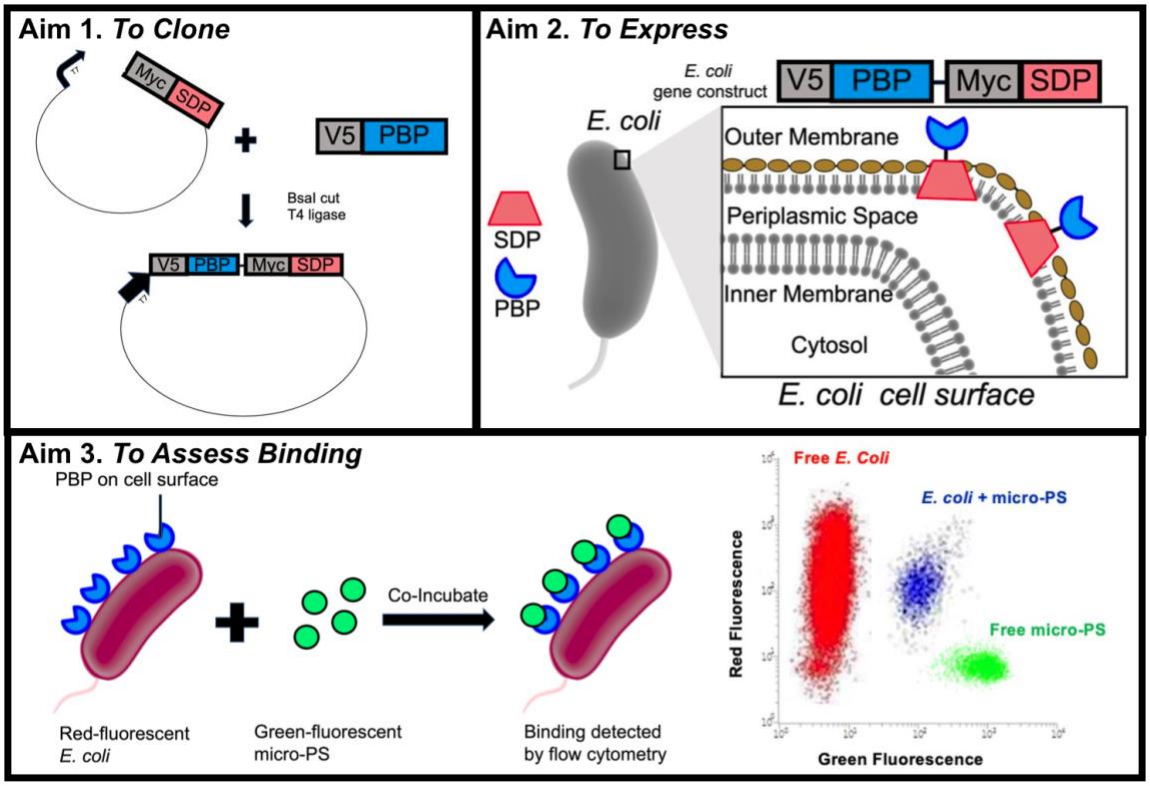
on their surface has become a useful system for the directed evolution of target proteins.[8] To do this in gram-negative bacteria like *E. coli*, well-studied autotransporter proteins, which can traverse the inner and outer cell membranes, are exploited to display proteins on the cell surface.[9]

Recently, Soudant and colleagues developed a novel flow cytometry approach to observe the interaction of fluorescent micro-polystyrene (micro-PS) with the cell surface of *Chaetoceros neogracile* algae.[10] Flow cytometry, typically used to study cells, is a tool that allows for the quantification and characterization of particle surfaces by passing a stream of the sample through lasers to measure light scattering and/or fluorescence. In this study, the red-fluorescent chlorophyll in *C. neogracile* was contrasted with green-fluorescent micro-PS to characterize unique populations of free cells, free micro-PS, and the hetero-aggregates of the two. The findings from this study suggest flow cytometry may be a suitable MP detection strategy.

Approximately 50 plastic-binding peptides have been identified to have the potential for specific protein-plastic interactions; however, they have yet to be used in an application for MP detection.[11] Of these peptides, the majority demonstrated a relatively high affinity to PS substrates determined by screenings of random peptide libraries using phage display.[12,13,14] This widely used technique involves the display of random peptides on the surface of bacteriophage, a type of virus, for the selection of peptides with high affinity to a specific substrate. Although some studies have reported common PS-binding motifs in some peptides, such as WXXWXXXW, not all peptides have clear motifs.[13] Additionally, the most frequently appearing amino acids in PS-binding peptides (PBPs)

are Trp, Phe, Pro, and Val, which form strong hydrophobic interactions with the hydrophobic nature of the benzene ring in PS.[14]

It is our hypothesis that the cell surface of genetically engineered *E. coli* can express selected peptides previously shown to bind bulk plastic materials, and these engineered cells can be used to identify the chemical composition of micro-PS. To test our hypothesis, the work in this thesis aimed (i) to clone plasmid DNA that can express PBPs on the surface of *E. coli*, (ii) to verify the expression of the gene constructs using a combination of Western blot and flow cytometry analysis, and (iii) to assess the ability of these cells to bind micro-PS using flow cytometry (Schematic 1). In this thesis, protein expression conditions were optimized by investigating two cell strains of *E. coli* and two auto-transporter proteins, hereafter referred to as surface display proteins (SDPs). The first SDP is a protease with an amino acid substitution D1120N derived from enterohemorrhagic *E. coli* O157:H7 (EspP), and the second SDP is an esterase from *Pseudomonas putida* (EstA).[15,16] Finally, preliminary flow cytometry experiments have been designed to investigate interactions of the engineered cells with micro-PS and future work is proposed.



Schematic 1. Aim 1. Plasmid DNA cloning strategy designed to place polystyrene-binding peptides (PBPs) on the surface of *E. coli*. Aim 2. Fusion protein (PBP-SDP) expression in the outer membrane of *E. coli*. Aim 3. Microbe-MP binding strategy with theoretical flow cytometry findings.

MATERIALS AND METHODS

DNA Recombination and Cloning

Four PBPs were selected for investigation in this thesis and will be referred to as "PBPs 1 – 4" from this point on (Table S1). A gene block for each PBP was purchased from Twist Bioscience as two or three tandem repeats. Additionally, the V5-tag and internal ALFA-tag were linked to the 5' end of the PBP gene alongside restriction sites for two endonucleases. This gene block was inserted into the pET28a expression vector under the control of the *lac* operon at a locus that would link the peptide to one of two SDPs, EstA or EspP. Additionally, the insertion of a gene at this locus would disrupt the DNA sequence that encodes a red cassette protein, which was leveraged as a marker for successful cloning. The 5' end of the SDP gene was also linked to a Myc-tag. The gene

insertion was performed using a Golden Gate Assembly Kit, which contains an optimized mixture of BsaI-HFv2 restriction endonuclease and T4 DNA Ligase (New England Biolabs). The DNA recombination reaction was performed in a C1000 Touch Thermal Cycler (BioRad) set to cycle 30 times between 5 min at 37 °C and 16 °C, followed by 5 min at 60 °C.

Competent cells of *E. coli* DH5α were transformed with the recombinant plasmid DNA and recovered in 200 µL of LB broth at 37 °C and shaken at 300 rpm for 1 h. The transformed DH5α culture was inoculated onto LB growth plates with the appropriate antibiotic (kanamycin, chloramphenicol, or both) for no more than 20 h at 37 °C. A single colony was selected to make a liquid culture in LB with the appropriate antibiotic at an optical density at 600 nm (OD_{600}) of 1.0 – 1.75 from a 2-fold dilution. The culture was diluted to an OD_{600} of 2.5 for plasmid DNA extraction using the centrifugation protocol in the ZymoPURE Plasmid Miniprep Kit (Zymo Research). Plasmid DNA concentrations above 50 ng/µL were considered acceptable.

Restriction Digests of Plasmid DNA

A restriction digest of the plasmid DNA was performed as a diagnostic for successful DNA recombination. Prior to experimental validation, the restriction digest was simulated virtually using SnapGene. Restriction endonucleases that simulated a distinguishable plasmid DNA digest were selected to experimentally determine whether the plasmid was successfully cloned. Using 200 ng plasmid DNA, the restriction digest was performed using 20 units of NdeI and 10 units of BsmBI endonucleases in Buffer 3.1 to a final volume of 130 µL (New England Biolabs). The reaction occurred at 37 °C and

then 55 °C for 1 h at each temperature. Notably, the plasmid DNA was not standardized to the same amount, which ideally should be done in the future. The restriction digest product was visualized using ethidium bromide in a 1 % agarose gel made with TAE buffer (40 mM Tris base, 1 M acetic acid, and 1 mM EDTA). A 1 kb Plus DNA Ladder (New England Biolabs) was compared to the restriction digest products. Plasmid DNA was stored at -20 °C to be used later for expression.

Making Chemically Competent Cells

Strains of *E. coli* with a plasmid DNA that encoded constitutively expressed mkate2 or GFP were made competent to uptake an additional plasmid that encodes PBPs 1 – 4. Liquid cultures were grown to an OD_{600} of 0.4, then incubated on ice for 20 min. The cooled cultures were then centrifuged at 4000 rpm and 4 °C for 10 min and the supernatant was discarded. The cell pellets were resuspended in 3 mL of cold 0.1 M calcium chloride that was filtered through a 0.2 µm syringe filter. The resuspended pellets were centrifuged again at 4000 rpm and 4 °C for 10 min and the supernatant was discarded. The cell pellets were then resuspended in 750 µL of cold, filtered 0.1 M calcium chloride, 15 % glycerol. The glycerol stocks were stored at -80 °C or directly proceeded to transformation.

Protein Expression

Several conditions were tested to optimize protein expression. For example, protein expression was performed using the *E. coli* strains BL21 (DE3) and Top10 (DE3), the latter of which are Top10 cells with the DE3 T7 RNA polymerase operon

obtained from the Thomas Ward lab.[17] Strains of *E. coli* expressing each SDP were investigated, and the dual expression in chemically competent strains constitutively expressing mkate2 or GFP was also investigated. Strains of *E. coli* with an empty plasmid DNA vector (EV) were used as a negative control. Taking from 50 % glycerol -80 °C freezer stocks, strains of *E. coli* were streaked on LB plates with the appropriate antibiotic. A single colony would be inoculated into 2 mL of LB with the antibiotic and incubated overnight at 37 °C and shaken at 220 rpm. The following morning, a working culture was made by inoculating a 1:50 dilution of the starting culture, which was incubated at 37 °C and shaken at 220 rpm until an OD_{600} of 0.6 – 0.8. Once at this OD_{600}, 1 mL samples were collected for negative controls, then the cultures were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). After inducing, the cultures were incubated at 16 °C and shaken at 160 rpm. Expression time was investigated at 1 – 4 h and 16 h.

Cells were harvested in 1.0 OD_{600} fractions by centrifugation at 16000 xg for 30 s. Cell lysis was performed by resuspending the cell pellet in 100 µL of a lysis mix for each OD_{600} unit. The lysis mixture consisted of 1 mL of lysis buffer (50 mM sodium phosphate pH 7.5, 300 mM NaCl, 0.5 % DM), 10 µL of 100 mg/mL lysozyme (GoldBio), 10 µL of 10 mg/mL RNase (VWR Life Science), 5 µL of 500 mM magnesium sulfate, 0.5 µL benzonase (MilliporeSigma), and 10 µL of protease inhibitor cocktail in DMSO and ethanol (Sigma Aldrich). Resuspended pellets were placed on a nutator for 15 min at room temperature, then 10 % N-lauroylsarcosine (MP Biomedicals) was added to 1 % and returned to the nutator with gentle shaking for an additional 5 – 10 min. Finally, LDS

sample buffer (Thermo Scientific) was added to the cell lysate, which was either stored at -20 °C or prepared for polyacrylamide gel electrophoresis (PAGE).

SDS PAGE and Coomassie Staining

The whole-cell lysates were heated at 90 °C for 5 min and loaded into a 12 % Tris-glycine NuPAGE gel in sodium dodecyl sulfate (SDS) buffer. For gels intended to be stained, 5 µL of Color Prestained Protein Standard (New England Biolabs) was added along with 10 µL of each cell lysate. Gels intended for Western blotting were run with 2 µL of a 1:5 dilution of protein standard and 5 µL of each cell lysate. Single gels were run in a Mini Gel Tank (Thermo Scientific) set to 150 V for 1 h or until the tracking dye ran off the bottom of the gel.

Gels intended to be stained were rinsed with enough deionized water to cover the gel for 5 min, and this process was repeated two more times with fresh deionized water. The gel was then covered with Coomassie stain (0.2 % [w/v] Brilliant Blue R-250, 30 % [w/w] ethanol, 10 % [v/v] glacial acetic acid) for 1 h on the nutator with gentle shaking. The gel was de-stained using a de-stain solution (30 % [v/v] ethanol, 10 % [v/v] glacial acetic acid), which was replaced with a fresh solution once saturated. De-staining was stopped by leaving the gel in water, then it was imaged using a BioRad ChemiDoc MP Imaging System.

Western Blots

Gels intended for Western blotting were assembled onto a nitrocellulose membrane in transfer buffer (25 mM tris base, 192 mM glycine, 20 % [v/v] methanol) following the

Mini Blot Module (Thermo Scientific). Electroblotting was set to 22 V and 0.03 A for 12 h at 4 °C. After transferring protein onto a nitrocellulose membrane, the membrane was blocked with 5 % (w/v) milk, which was prepared by dissolving dry non-fat milk in tris-buffered saline with 0.1 % Tween 20 (TBST). The membrane in milk was placed on a nutator with gentle shaking for at least 1 h. Then, the milk was discarded and replaced with a 1:5000 dilution of the mouse antibody Myc (9E10; Thermo Scientific) in fresh milk and returned to the nutator for 1 h. The membrane was washed by immersing it in TBST for 5 min on the nutator and then discarding the solution. This wash step was repeated two more times with fresh solution. The goat antibody anti-mouse Alexa fluor 647 or Fluorescein (FITC) was used based on availability and prepared by making a 1:5000 dilution in fresh milk (Thermo Scientific). Once the secondary antibody was added to the membrane, it was placed on the nutator in the dark for 30 min. Finally, the membrane was washed using the same protocol and imaged using the BioRad ChemiDoc MP Imaging System (FITC 532/28 nm; Alexa fluor 647 700/50 nm).

Flow Cytometry

Cell surface expression of PBPs was tested using flow cytometry and a green fluorescent protein fused to an ALFA nanobody (GFP-nbALFA) for ALFA tag labeling.[18] The GFP-nbALFA fusion protein also contains a 6x-histidine tag, which was used to purify it from *E. coli* Shuffle T7 using an NGC chromatography system with an immobilized metal affinity column (IMAC) nickel-charged resin (BioRad). Nonfluorescent strains of *E. coli* with PBPs 1 – 4 were tested and compared to *E. coli* with cytoplasmic GFP and nonfluorescent *E. coli* with an EV. The cultures were

harvested as 1.0 OD_{600} cell pellets after protein expression and resuspended in 200 µL of phosphate-buffered saline (PBS), then kept on ice and placed on a nutator to shake for 30 min. The cells were washed by centrifugation at 5000 xg and 4 °C for 30 s and the supernatant was discarded. The strains with PBPs 1 – 4 and the EV strain were resuspended in 200 µL of GFP-nbALFA in PBS. Another cell pellet of these strains, and the strain with cytoplasmic GFP, were resuspended in 200 µL of PBS as a negative control. The resuspended cell pellets were placed on ice and the nutator for 30 min, then an additional wash step was performed as before.

E. coli cells were counted using a CytoFLEX S flow cytometer (Beckman Coulter) equipped with a blue laser (excitation 488 nm). Each sample was diluted in PBS so that it could be recorded at approximately 500 events/s for a total of 10,000 events. All particles were characterized by their forward scatter (FSC), side scatter (SSC), and green fluorescence intensity (GFI; green emission filter band pass, 525/40 nm). Aggregates of cells with GFP-nbALFA were identified by the high FSC and GFI.

Micro-PS Exposure

The BL21 (DE3) cells expressing the PBP-EspP fusion proteins 1 – 4 were harvested as 1.0 OD_{600} cell pellets after protein expression and resuspended in 200 µL of 0.22 µm filtered PBS. The resuspended pellets were then centrifuged at 5000 xg for 30 s at 4 °C and the supernatant was discarded. This PBS wash was repeated two more times before the resuspended pellets went through 4 10-fold serial dilutions in PBS to a total volume of 200 µL. Fifty µL of 0.5 µm Dragon Green PS beads (Bangs Laboratory Inc; Fishers, IN) working solution ($1/3000^{th}$ dilution in PBS) was added to each dilution of

cells and incubated on ice for 30 min. These samples, along with a pET28a strain and the EV, were observed with and without the addition of the micro-PS beads on the flow cytometer.

RESULTS

Restriction Digests of Plasmid DNA

A good practice adopted was to virtually simulate a restriction digest for each plasmid using SnapGene Software (Insightful Science) prior to conducting the experiment (Figure 1).

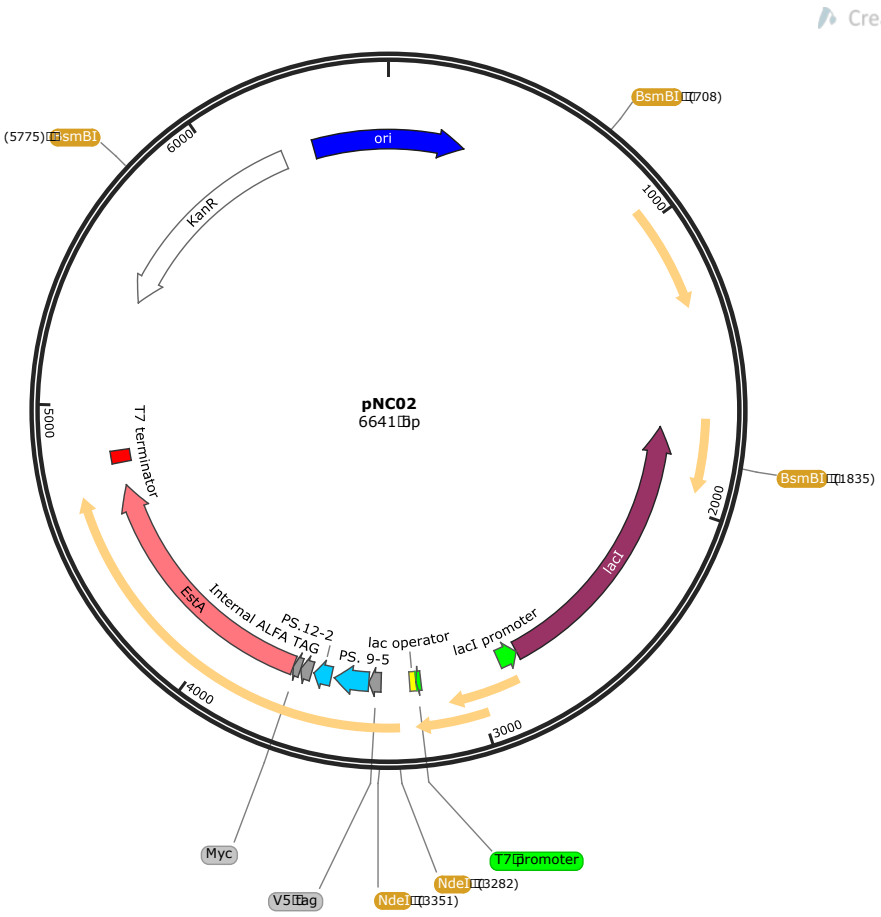


Figure 1. Virtually cloned plasmid DNA with PBP2 ligated to EstA (pNC02). The origin of plasmid DNA replication is represented by the dark blue arrow. The restriction sites for BsmBI and NdeI are highlighted in

gold along with the base number. In the *lac* operon, the EstA gene (coral) is ligated to the PBP2 gene (light blue) along with the Myc-, V5-, and ALFA-tags (gray). Transcription of these genes begins at the T7 promoter (green) and ends at the T7 terminator (red). Transcription is controlled by the *lac* operator (yellow) and the *lacI* gene (plum) encodes the *lac* repressor protein, which binds to the *lac* operator. A kanamycin resistance gene (white) is constitutively expressed away from the *lac* operon. This plasmid DNA map was created using SnapGene.

On SnapGene, it was suggested the combination of BsmBI and NdeI restriction endonucleases would provide sufficient band separation that could distinguish the proper construction of the plasmid DNA and used to experimentally validate them. For the PBP-EstA plasmid DNA, the anticipated size of bands upon restriction digest can be seen for plasmids 1, 2, and 3. However, a high molecular weight band can be seen near approximately 5 kb for plasmid 2, and plasmid 4 only has one band near 6 kb (Figure 2). For the PBP-EspP plasmid DNA, bands appear nearly identical to the simulation, although they are very faint for plasmids 3 and 4 (Figure 3).

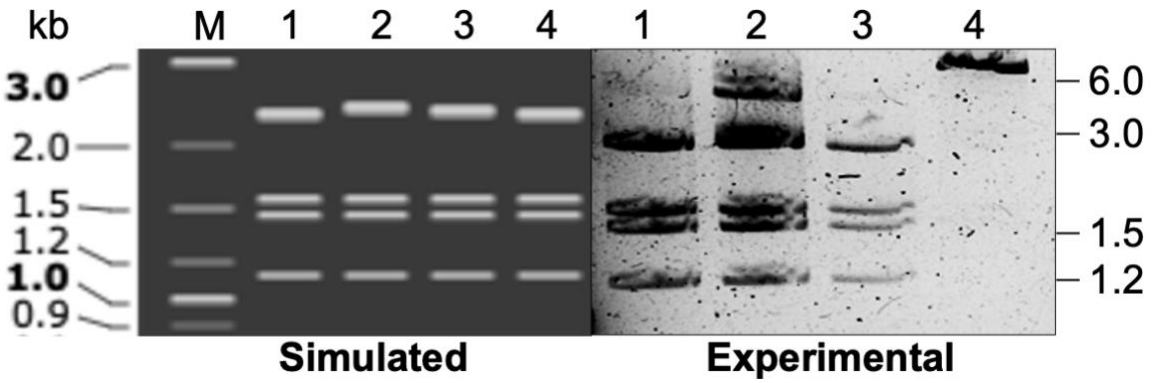


Figure 2. Restriction digest of plasmid DNA with PBPs 1 – 4 ligated to EstA. A 1 kb Plus DNA Ladder (M) is compared to the plasmids containing each PBP (1 – 4) on a 1 % agarose gel. Left. Simulated digest using BsmBI and NdeI restriction endonucleases using SnapGene. Right. Experimental restriction digest using BsmBI and NdeI restriction endonucleases. The highest band can be seen at 2.9 kb, followed by two bands centered at 1.6 kb, and the lowest band at 1.2 kb.

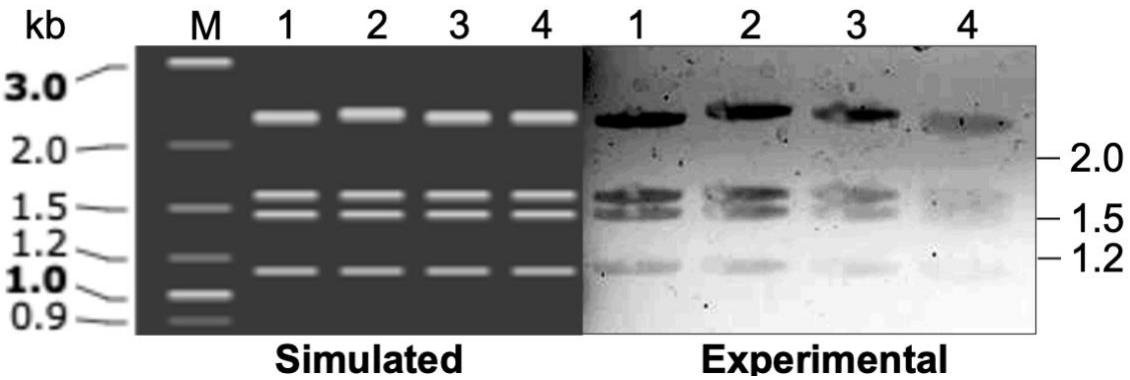


Figure 3. Restriction digest of plasmid DNA with PBPs 1 – 4 ligated to EspP. A 1 kb Plus DNA Ladder "M" is compared to the plasmids containing each PBP "1 – 4" on a 1 % agarose gel. Left. Simulated digest using BsaI and NdeI restriction endonucleases using SnapGene. Right. Experimental restriction digest using BsaI and NdeI restriction endonucleases. The highest band can be seen near 2.5 kb, followed by two bands centered at 1.5 kb, and the lowest band at 1.1 kb.

Protein Expression

Having validated the plasmid DNA, the ability of the plasmids to optimally express the PBP-SDP fusion protein in *E. coli* was investigated. Note, these strains did not contain an additional plasmid for a cytoplasmic fluorescent protein. Protein expression was compared with plasmids containing the EstA or EspP SDP. The expression time was initially investigated at 16 h (Figure 4). The SDP for the control can be seen at the expected molecular weight and strains 1 – 4 show the SDP at slightly offset migration distances. This pattern likely corresponds to the fusion of each PBP to the SDP. The band intensity corresponds to the amount of protein isolated, which is noticeably greater in the EstA constructs. Some strains appear to have doublet bands associated with the PBP-SDP, and there are several lower molecular weight bands present.

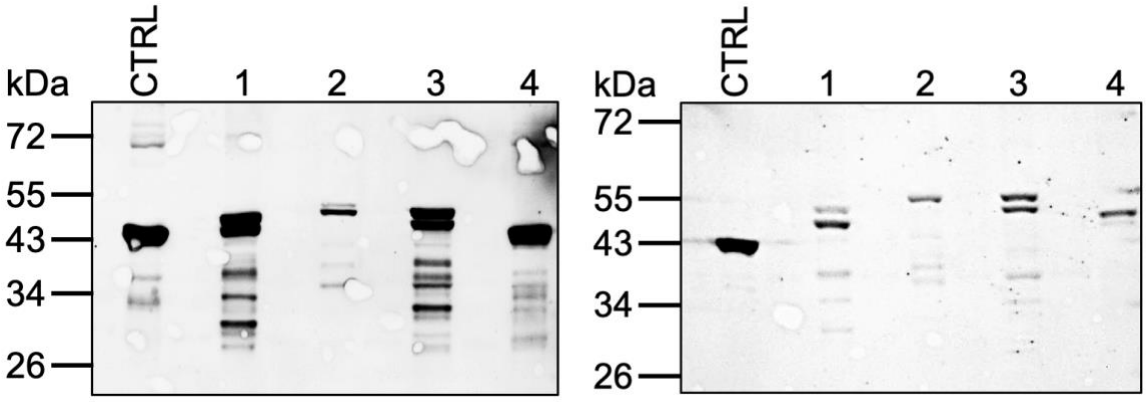


Figure 4. 16-hour protein expression of PBPs 1 – 4 fused to EstA (left) and EspP (right) from whole-cell lysates of Top10 (DE3) cells visualized via Western blot. The Color Prestained Protein Standard "kDa" is compared to the EV "CTRL" and each PBP-SDP fusion protein "1 – 4" on a nitrocellulose membrane. Anti-Myc primary antibodies were visualized using the Alexa Fluor 647 secondary antibody.

The abundance of lower molecular weight bands was hypothesized to be degradation of the SDP, so a shorter expression time of 4 h was investigated (Figure 5). The shorter expression time arguably reduced the presence of lower molecular weight bands but did not eliminate them. The bands associated with the SDPs did maintain an appreciable amount of protein.

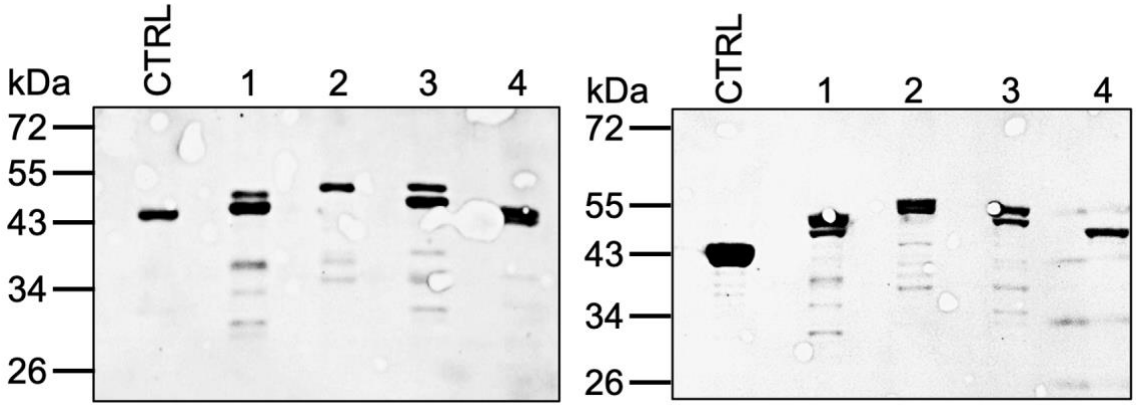


Figure 5. 4-hour protein expression of PBPs 1 – 4 fused to EstA (left) and EspP (right) from whole-cell lysates of Top10 (DE3) cells visualized via Western blot. The Color Prestained Protein Standard "kDa" is compared to the EV "CTRL" and each PBP-SDP fusion protein "1 – 4" on a nitrocellulose membrane. Anti-Myc primary antibodies were visualized using the Alexa Fluor 647 secondary antibody.

In a continued effort to eliminate the lower molecular weight bands, a 16-hour protein expression of PBPs fused to EspP was performed in protease-deficient BL21 (DE3) cells, although no change was observed (Figure S1). Similarly, using Top10 (DE3) strains that also expressed cytoplasmic mkate2 fluorescent protein did not noticeably influence the expression of the PBP-EspP fusion proteins (Figure S2). After several rounds of protein expression at 4 h, it was wondered whether sufficient protein could be generated at a shorter expression time (Figure 6). A comparable amount of protein could be seen between 3 and 4 h for the strain that was investigated. Ultimately, the presence of lower molecular weight bands could not be eliminated, yet the potential consequence of this was not known so the strains proceeded to be tested in PS binding assays.

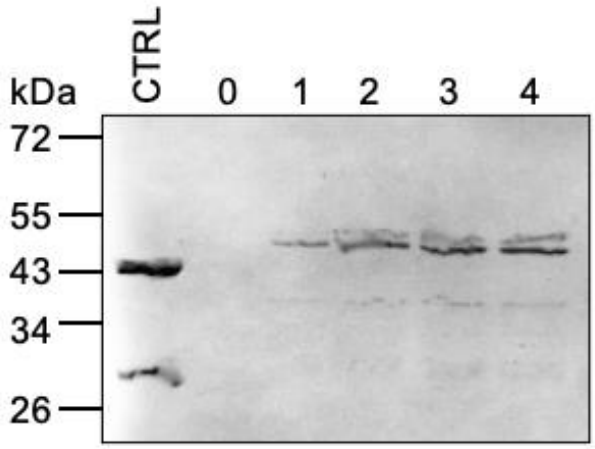


Figure 6. 4-hour time-course protein expression of PBP1 fused to EspP from whole-cell lysates of Top10 (DE3) cells visualized via Western blot. The Color Prestained Protein Standard "kDa" is compared to 1 mL of the EV "CTRL" culture at 4 h and 1 mL of the culture expressing PBP1-EspP fusion protein from 0 – 4 h. Anti-Myc primary antibodies were visualized using the Fluorescein secondary antibody.

An inherent limitation of this protocol for Western blot analysis of protein expression is that it cannot characterize where the protein is being expressed in the cell. Thus, flow cytometry was used to observe the surface of cells interacting with GFP-

nbALFA that would bind to the ALFA-tag fused to the SDP (Figure 7). This experiment was performed in BL21 (DE3) strains that express each PBP-EspP fusion protein. Notably, at least 65 % of cells for each strain have more than a 20-fold increase in GFI when mixed with the GFP-nbALFA fusion protein.

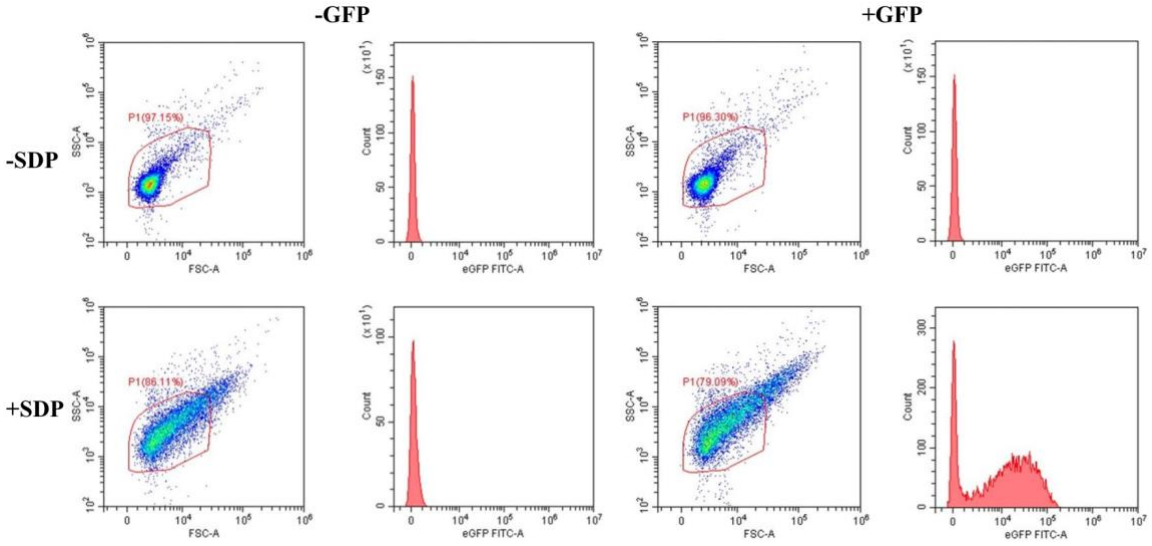


Figure 7. Flow cytometry observations of *E. coli* cells expressing the PBP1-EspP fusion protein labeled with GFP-nbALFA. Top. The side-scatter (SSC-A) versus forward scatter (FSC-A) and cell count versus fluorescence intensity (eGFP FITC-A) plots for *E. coli* BL21 (DE3) that do not express the ALFA-tagged EspP SDP are compared with (right) and without (left) the addition of the GFP-nbALFA fusion protein. A gate was added to the SSC-A/FSC-A plot to characterize *E. coli* cells. Bottom. The same plots are used for cells that express the ALFA-tagged PBP1-EspP fusion protein with (right) and without (left) the addition of GFP-nbALFA.

Micro-PS Binding Experiments

In an experiment nearly identical to the GFP-nbALFA experiment, the BL21 (DE3) cells expressing the PBP-EspP fusion proteins 1 – 4 were incubated with 0.5 µm Dragon Green PS beads instead of the nanobody and run on the flow cytometer. All samples showed a small fraction of cells shift in GFI that appeared similar to the EV control.

DISCUSSION

The work in this thesis has successfully engineered *E. coli* to display PBPs on the cell surface via a surface display protein scaffold. This is a seminal step in the process of developing the proposed *in vivo* MP detection system.

By replacing the gene for a red cassette protein with the gene for a PBP in the plasmid DNA vector, colorless colonies of *E. coli* could be selected for plasmid DNA extraction and verification via restriction digest. With the PBP gene inserted adjacent to the gene for a SDP, the PBP may be transported to the outer membrane. To observe protein expression, anti-Myc Western blots were performed. Ideally, supplemental anti-V5 Western blots should be used to specifically observe the expression of PBPs because the V5 epitope is inserted with the PBP gene into the plasmid DNA. Nonetheless, the conserved migration distance pattern for each band compared to the EV reflects the size of each PBP, which supports that it was successfully fused to the SDP.

Combining Western blot and flow cytometry findings, we conclude the PBP-SDP fusion protein is being expressed on the surface of *E. coli.* The optimal protein expression conditions determined are incubating Top10 (DE3) or BL21 (DE3) cells at 16 °C for 4 h. Notably, the time-course experiment suggests as little as 3 h is sufficient. The two SDPs investigated, EstA and EspP, showed similar expression levels via Western blot analysis. This protocol was also tested with cells designed to express both PBP-SDP and cytoplasmic fluorescent protein mkate2, although the higher metabolic demands may warrant a slightly longer protein expression time (Figure S2). Additionally, by inoculating cultures onto growth plates after centrifugation it was determined that harvesting cultures by centrifuging at 16000 xg was killing the cells that expressed the

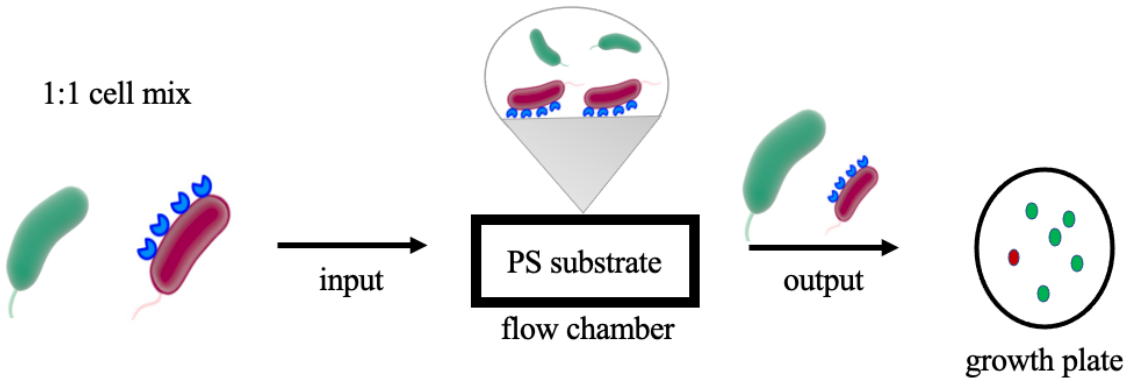
SDPs, which is not the case for cells that do not express the SDPs. Potentially compromising cell viability is a common obstacle in the application of surface display systems, which is especially true in gram-negative bacteria like *E. coli* that have an inner and outer cell membrane and periplasmic space in between.[9,16] The GFP-nbALFA specifically labels the ALFA-tag on SDPs and cannot penetrate the outer membrane, thus it was used to confirm the surface-level protein expression on the flow cytometer.

There was no condition tested that could eliminate the persistent lower molecular weight bands seen for each control on the Western blots. Initially, these bands were hypothesized to be degraded protein, but the use of the protease-deficient cell line BL21 (DE3) and a protease inhibitor suggest the bands are not due to protein degradation. However, the unintentional cell lysis when harvesting cultures by centrifugation at high speeds may account for protein degradation prior to the addition of the protease inhibitor. Another unexpected result is the occasional appearance of doublet bands for the PBP-SDPs fusion proteins in all controls on the Western blots. Considering there are no cysteine residues in the PBP-SDP fusion protein that could be reduced, it is thought the doublets may be due to post-translational modifications common in *E. coli* like phosphorylation or glycosylation. These findings have been reported in similar studies, so the project was advanced to assess how the cells interact with micro-PS.[16]

Using the flow cytometer to observe cells expressing the PBP-EspP fusion protein incubated with 0.5 µm Dragon Green PS beads showed nonspecific interactions compared to the EV control. The leading hypothesis for the difference in results compared to the GFP-nbALFA fusion protein is that the interactions of PBPs on cells with micro-PS are not strong enough to resist the shear stress experienced in the channels

of the flow cytometer (approximately 30 m/s). The high-affinity nanobody nbALFA has demonstrated low picomolar affinity to the rationally designed ALFA-tag epitope, which is fused to EspP.[19]

Future work should consider testing the hypothesis of shear stress on the cells expressing the PBP-SDP fusion proteins and its potential influence on the interaction with micro-PS. For example, a proposed flow cell experiment, where the flow rate can be controlled and the flow-through can be quantified, is described (Schematic 2). The results from such an experiment could elucidate any specific binding of cells with PBPs to micro-PS and any effect the flow rate may have on these cells. Further, fluorescent microscopy of the microbe-MP interactions should be pursued as qualitative supporting evidence. Finally, future directions for this project should aim to ultimately use yeast as the microbial display system. Yeast like *Saccharomyces cerevisiae*, commonly known as "Baker's yeast", is typically the most robust and well-established microbial display system for directed protein evolution, which can be exploited for the discovery of novel peptides with higher affinity to PS or other plastic substrates.[8,9]



Schematic 2: Proposed flow cell experiment. A 1:1 mixture of green-fluorescent cells without PBPs and red-fluorescent cells with PBPs are added to a flow chamber containing a PS substrate. Theoretically, cells with PBPs have a higher affinity to the PS substrate and will be retained in the flow chamber more than cells without PBPs. A flow-through (output) sample can then be inoculated onto a growth plate to generate colonies that can be counted to determine percent recovery.

SUPPLEMENTARY INFORMATION

Table S1. Amino acid sequence of polystyrene-binding peptides (PBPs)

Code	Name	Sequence	Reference
PBP1	0-1	ssssVDWVGWGASWggs	Gebhardt et al.[13]
PBP2	PS. 9-5 and PS. 12-2	ssssRLAYDHYFPSWRSYIFPGSNSSYYNNSWPTITMETNSRggs ssssPWWWVSWVDAGGGSLALPTQPSD	Adey et al.[12]
PBP3	PB-TUP	ssssVHWDFRQWWQPSggs	Qiang et al.[14]
PBP4	Fibrinogen	ssssDNGIIWATWKTRWYSMKKggs	Gebhardt et al.[13]

The one-letter amino acid sequences correspond to the name given by each reference, which have been coded "PBP1 – 4" in this thesis. The uppercase letters in the peptide sequences are the exact sequences identified from each reference. Note, two peptides were selected from Adey *et al.* and combined as one long peptide. The appropriate serine/glycine linkers (lowercase letters) were determined using Twist Bioscience's codon optimization tool.

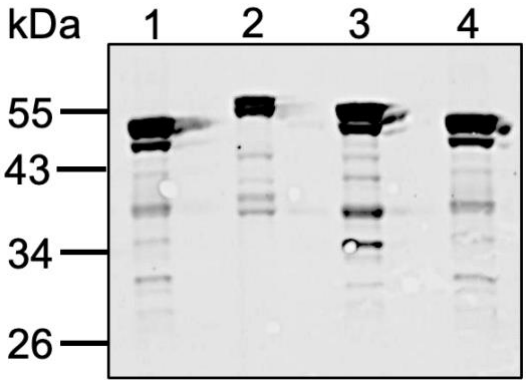


Figure S1. 4-hour protein expression of PBPs 1 – 4 fused to EspP from whole-cell lysates of BL21 (DE3) cells visualized via Western blot. The Color Prestained Protein Standard "kDa" is compared to each PBP-SDP fusion protein "1 – 4" on a nitrocellulose membrane. The EV control was not made at the time this blot was performed. Anti-Myc primary antibodies were visualized using the Alexa Fluor 647 secondary antibody.

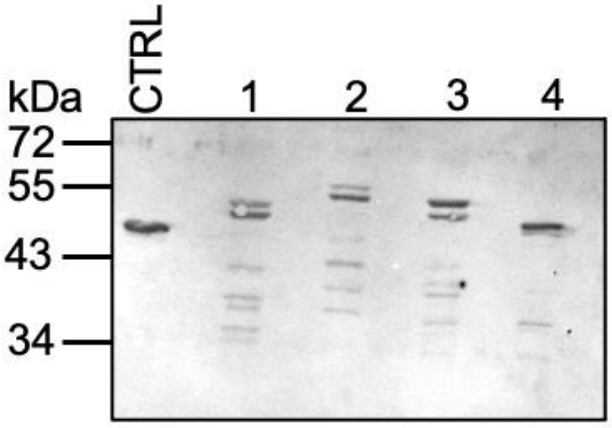


Figure S2. 4-hour dual protein expression of PBPs 1 – 4 fused to EspP and cytoplasmic mkate2 from whole-cell lysates of Top10 (DE3) cells visualized via Western blot. The Color Prestained Protein Standard "kDa" is compared to the EV "CTRL" and each PBP-EspP fusion protein "1 – 4" on a nitrocellulose membrane. Anti-Myc primary antibodies were visualized using the Fluorescein secondary antibody.

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