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Production of *Escherichia coli* outer membrane vesicles displaying anti-MUC1 single chain variable fragment via SpyTag/SpyCatcher system

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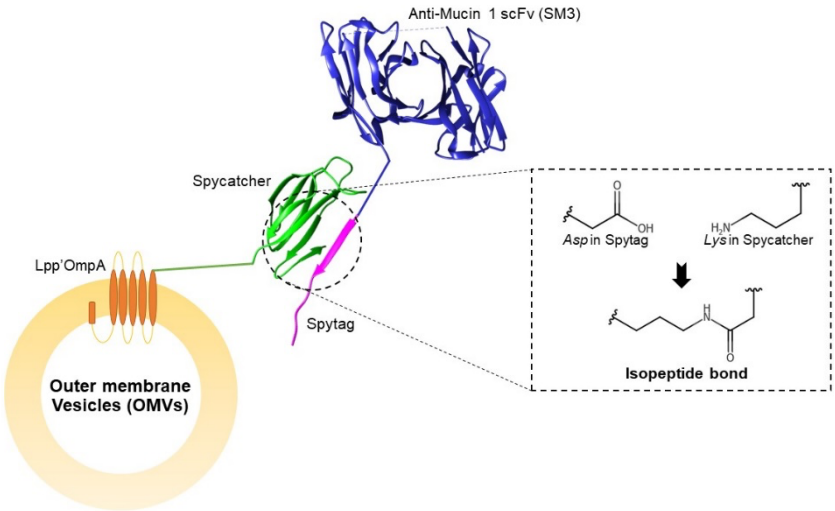
Abstract: Outer membrane vesicles (OMVs) are nanoparticles secreted from Gram-negative bacteria. Because of various properties of OMVs such as size, content, and simple production, bioengineered OMVs can be exploited as a vaccine/drug delivery system by developing OMVs to display desired proteins. Previous studies showed that proteins of interest must be genetically fused with outer membrane-localized proteins when inserting into OMVs. These proteins were produced in *Escherichia coli* hence they lack of correct post-translational modifications. To overcome this problem, we utilized the effective protein ligation system called SpyTag/SpyCatcher to attach foreign proteins from another expression system that retains their function on bacterial OMVs. SpyTag/SpyCatcher system enables coupling of two proteins through irreversible covalent bond resulting in extensive uses in surface decoration of various nanoparticles. In this study, we first produced SpyCatcher fused to Lpp'OmpA which is anchored on the surface of *E. coli* BL21(DE3)-derived OMVs. Then, SpyTag was fused to anti-Mucin 1 single-chain variable fragment and produced in ExpiCHO-S cells. After ligation of both components, Lpp'OmpA-SpyCatcher:SpyTag-SM3 complex was observed in Western blot analysis. It indicated that SM3 successfully displayed on OMVs. Thus, this ligation system will provide a robust and effective OMVs decoration.

Graphical abstract:



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Keywords: outer membrane vesicle; spytag/spycatcher system; surface display system; vaccine platform; drug delivery system; mucin 1; lipoprotein; outer membrane protein a

1. Introduction

Outer membrane vesicles (OMVs) are nano-sized spherical particles, ranging around 20 – 200 nm, budded from Gram-negative bacteria. Their functions are involved in bacteria communication leading to pathogenesis [1]. Importantly, because of antigens presenting in OMVs, they were served as a vaccine against their parent bacteria. For example, *Neisseria meningitidis* OMVs were purified and formulated in a vaccine to control serogroup B meningococcal disease, known as 4CMenB [2]. Apart from naturally secreted OMVs, non-pathogenic bacteria-derived OMVs can be developed to serve as a vaccine/drug delivery system. Using genetic engineering approach, OMVs was produced with desired proteins attached on their surface. This display system relies on a genetic fusion of target proteins with outer membrane (OM)-localized proteins such as Cytolysin A (ClyA) and Hemoglobin protease (Hbp) [3,4]. For instance, David J. Chen et al. illustrated that green-fluorescent protein (GFP) could be fused with ClyA and embedded in *E. coli*-derived OMVs. The recombinant OMVs could boost immunogenicity of GFP in mice without adjuvant [5]. Vipul Gukrati et al. also provided a finding to support the use of OMVs. They generated *E. coli* OMVs displaying antibody against human epidermal growth factor 2 (HER2). The resulting vesicles could deliver siRNA specifically to $HER2^+$ cancer cells and did not show nonspecific side effects [6]. Even though the display system using OM-localized protein fusion were intensively used, some proteins cannot be incorporated to OMVs via these systems. Since the proteins are expressed in bacteria along with production of OMVs, the proper post-translational modifications cannot be achieved resulting in loss of protein function [7]. Another decoration system in OMVs that retain biological activity of proteins is necessary.

SpyTag/SpyCatcher system employs powerful bio-ligation to link two protein components. SpyTag, a short peptide of 13 amino acids and its protein partner, SpyCatcher (15 kDa), perform spontaneously irreversible isopeptide bond that remain stable in various conditions (pH, temperature, buffer) [8]. SpyTag and SpyCatcher can be individually fused in N-terminus, C-terminus, or internal site of target protein then expressed in a number of expression systems [9]. Thus, SpyTag/SpyCatcher system was widely used to couple two heterologous proteins in many applications [10,11]. To apply the use of SpyTag/SpyCatcher system in OMVs decoration process, we aimed to produce OMVs displaying foreign proteins from another expression system via the ligation between OMVs having SpyCatcher and SpyTag-fused with protein of interest. SpyCatcher was constructed to embed in OMVs using genetic fusion of Lpp'OmpA known as a well-studied bacterial surface display system [12]. Lpp'OmpA display system consists of Lipoprotein signal sequence and Outer membrane protein A (residue 46 - 159) and can transport its C-terminus partner to anchor on outer membrane [13]. To validate this proof-of-concept, we chose anti-Mucin 1 single chain variable fragment (scFv; SM3) [14] to enhance functionality of OMVs as a cell-specific drug delivery. Thus, SpyTag was fused to anti-Mucin 1 scfv and expressed in mammalian cell expression system. After that, the fusion protein which includes histidine tag was purified by affinity column chromatography. Consequently, the components were ligated and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and dynamic light scattering (DLS) to confirm a presence of conjugated OMVs.

2. Materials and Methods

2.1. Expression plasmids

The lpp'ompa-GS linker-SpyCatcher-6xhistidine gene cassette was synthesized and subcloned into pET-21a+ via NdeI/EcoRI resuting in pLpp'OmpA-SpyCatcher. To construct pSpyTag-SM3, codon-optimized SpyTag and anti-muc1 scFv (SM3) were assembled with N-terminus 6xhistidine at EcoRI/NheI in pFUSE-hIgG1-Fc2 using NEBuilder HiFi

DNA Assembly Master Mix (New England Biolabs, USA). All insert sequences were verified by Sanger DNA sequencing.

2.2. Isolation of outer membrane vesicles

ECOS 21™ Competent Cells *E. coli* BL21(DE3) (YB Biotech, Taiwan) harboring pLpp'OmpA-SpyCatcher were cultured in TB broth with ampicillin at 37°C. Once OD_{600} reached 0.6, protein expression was induced using Isopropyl β-d-1-thiogalactopyranoside (IPTG) at final concentration of 0.5 mM and the cells were grown at 30°C for 16-18 h. After that, intact bacteria were removed by centrifugation of 7000 rpm at 4°C for 30 min followed by filtration using Steritop Millipore Express PLUS 0.22 μm (Merck, Germany). Then, crude OMVs were pelleted by ultracentrifugation at 150000 x g 4°C for 3 h and resuspended in phosphate buffer saline (PBS). Purified OMVs were collected from supernatant after an additional of centrifugation (15000 rpm, 10 min, 4°C).

2.3. Transient expression and purification of SpyTag-SM3

SpyTag-SM3 was transiently expressed in ExpiCHO-S™ using PEI MAX-mediated transfection method. In brief, ExpiCHO-S™ cells were maintained in ExpiCHO™ expression medium (Gibco, USA) 37°C, 8% CO_2, and 125 rpm. Then, ExpiCHO-S™ cells were seeded at 0.5 x 10^6 cells/ml for overnight. At the day of transfection, 1 μg/ml of pSpyTag-SM3 plasmid DNA and 3 μg/ml of PEI MAX (MW 40,000; Polyscience, USA) was separately diluted in 1/20 of starting culture volume of fresh ExpiCHO™ expression medium and PEI MAX solution was subsequentially dropped to plasmid DNA solution. The plasmid DNA/PEI MAX complex was incubated at room temperature for 10 min and slowly transferred to cell suspension. At 5 days after transfection, the cells were removed by centrifugation at 5000 x g 4°C for 30 min and the supernatant was clarified by filtration using Acrodisc syringe filter 0.22 um (Pall, China). SpyTag-SM3 was purified from the filtered supernatant by Histrap FF 1 mL column (Cytiva, USA) using ÄKTA Start (Cytiva, USA). The column was previously equilibrated with binding buffer (50 mM phosphate buffer, 300 mM NaCl, 20 mM Imidazole pH 8.0) and then loaded with the supernatant. Unbound proteins were washed with binding buffer. SpyTag-SM3 was eluted using elution buffer (50 mM phosphate buffer, 300 mM NaCl, 250 mM Imidazole pH 8.0) followed by buffer exchange to dilute buffer (50 mM phosphate buffer, 150 mM NaCl pH 8.0) and concentrated using Amicon® Ultra-4 centrifugal filter unit (Merck, USA). Protein concentration of purified SpyTag-SM3 was measured by Nanodrop™ One Microvolume Spectrophotometer (Thermo Scientific, USA).

2.4. SDS-PAGE and Western blot analysis

Protein samples were diluted in 5X SDS-PAGE Protein Loading Buffer (Enzmart Biotech, Thailand) and heated at 95°C for 5 min. After that, the samples were resolved in 12% SDS-PAGE at 50 mA for 50 min and then transferred to Immobilon-NC transfer membrane (Millipore, USA) using Trans-Blot Semi-Dry Transfer Cell (Bio-Rad, USA). Gels were stained with Coomassie Blue, while the membrane was briefly washed with tris-buffer saline with 0.1% Tween-20 (TBST) and blocked with 5% skim milk (Hardy Diagnostics, USA) in TBST at room temperature for 3 h. The blot was probed by 1:20000 diluted mouse anti-histag antibody (Invitrogen, USA) in 5% skim milk in TBST with gently rocking at room temperature for 60 min. Excess antibodies were removed by several washes using TBST. After that, 1:200000 diluted of horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (BioLegend, USA) in 5% skim milk in TBST was incubated with the membrane at room temperature for 30 min and unbound antibodies were then washed by TBST. HRP enzymatic reaction was carried out by Immobilon Forte Western HRP substrate (Merck, USA). Subsequently, histidine-tagged proteins were detected using Chemiluminescent ImageQuant LAS4000 (Cytiva, USA).

2.5. Conjugation of OMVs:Lpp'OmpA-SpyCatcher and SpyTag-SM3

Protein ligation was conducted at a ratio of 1:60 w/w (OMVs and the protein) in 15 μL of dilute buffer and the ligated sample was incubated in 4°C for 21 h. The reaction was stopped by adding 5X SDS-PAGE Protein Loading Buffer. After that, conjugated product (Lpp'OmpA-SpyCatcher:SpyTag-SM3) was visualized by Western blot as described above.

2.6. Dynamic light scattering (DLS)

Size distribution profile of the particles were observed by Zetasizer Nano ZS instrument (Malvern Panalytical, UK) using a standard protocol (Run duration = 60 s, number of runs = 5, number of measurements = 3). The conjugated OMVs was prepared at final concentration of ~0.1 μg/μL in 1 mL of dilute buffer then loaded in a 12mm square polystyrene cuvette. Data was collected by measuring 173° backscatter at 25°C. After that, the result was analyzed with Zetasizer Software (version 8.01).

3. Results

*3.1. Lpp'OmpA-*SpyCatcher *was expressed in E. coli BL21(DE3) and localized in OMVs*

SpyCatcher is aimed to present on *E. coli* BL21(DE3) OMVs through a genetic fusion of Lpp'OmpA which is previously described in another report [15]. After 16 – 18 hours of the protein expression under IPTG induction, whole cell lysates at pre-induction and post-induction were collected. After that, OMVs were isolated from cell-free supernatant by ultracentrifugation and purified by additional centrifugation. The resulting vesicle was analyzed with SDS-PAGE. As shown in Figure 1a, we found that Coomassie blue-stained gel did not provide distinctive band of Lpp'OmpA-SpyCatcher owing to intensive protein contents. As a result, the protein fusion which contains 6xhistidine was visualized in Western blot using anti-histag antibody. As expected, a visible band of 29.9 kDa was presented in both post-induction whole cell lysate and OMVs corresponding to its calculated size (Figure 1b). This result demonstrated that Lpp'OmpA-SpyCatcher was expressed and incorporated into *E. coli* BL21(DE3) OMVs.

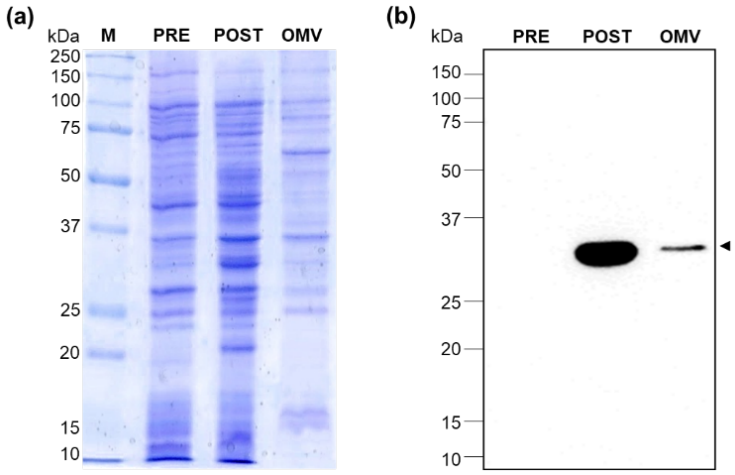

Figure 1. Expression of Lpp'OmpA-SpyCatcher in *E. coli* BL21(DE3) and OMVs. (**a**) SDS-PAGE of whole cell lysates and purified OMVs. Total cell lysates were collected from *E. coli* BL21(DE3) harboring pLpp'OmpA-SpyCatcher at pre-induction (PRE) and post-induction (POST) by IPTG as well as ~120 μg of purified OMVs (OMV). (**b**) Western blot analysis of OMVs:Lpp'OmpA-SpyCatcher was performed by using mouse anti-histag antibody followed by anti-mouse IgG antibody. An arrow indicates Lpp'OmpA-SpyCatcher at its theoretical size.

3.2. SpyTag-SM3 was successfully produced in mammalian expression system

We chose ExpiCHO-S™ cells as an expression host since this CHO-based system can produce recombinant protein with disulfide bond formation that scFv requires to maintain its native structure [16]. The expression plasmid (pSpyTag-SM3) was delivered to the cells by PEI MAX which is a transfection reagent extensively used in previous studies [17]. Then, the cells were maintained in a standard condition for five days where cell viability was dropped to 75% determined by Trypan blue exclusion method. SpyTag-SM3 which its gene cassette was constructed under IL2 signaling sequence was secreted into the supernatant, the cells and debris were thereby removed by centrifugation. Consequently, the fusion protein was purified from the supernatant by Histrap FF 1ml (Ni-NTA resin column). After increased volume of imidazole containing buffer (elution buffer) was loaded into the column, histidine-tagged SpyTag-SM3 was eluted in a single peak (Figure 2a). Excess imidazole was sequentially removed and SpyTag-SM3 was buffer-exchanged to dilute buffer. To examine SpyTag-SM3 expression and purity, fractions collected from purification process including crude (the supernatant before purification), flow-through, and eluted fraction were analyzed by 12% SDS-PAGE. The result revealed a homogenous band of SpyTag-SM3 at ~29.4 kDa which matches its theoretical size (Figure 2b), and the fusion protein was confirmed by immunoblotting analysis with anti-histag antibody (Figure 2c). These results indicated that purified SpyTag-SM3 was obtained in a homogeneity. The yield of SpyTag-SM3 production was achieved around ~3 mg/L of culture.

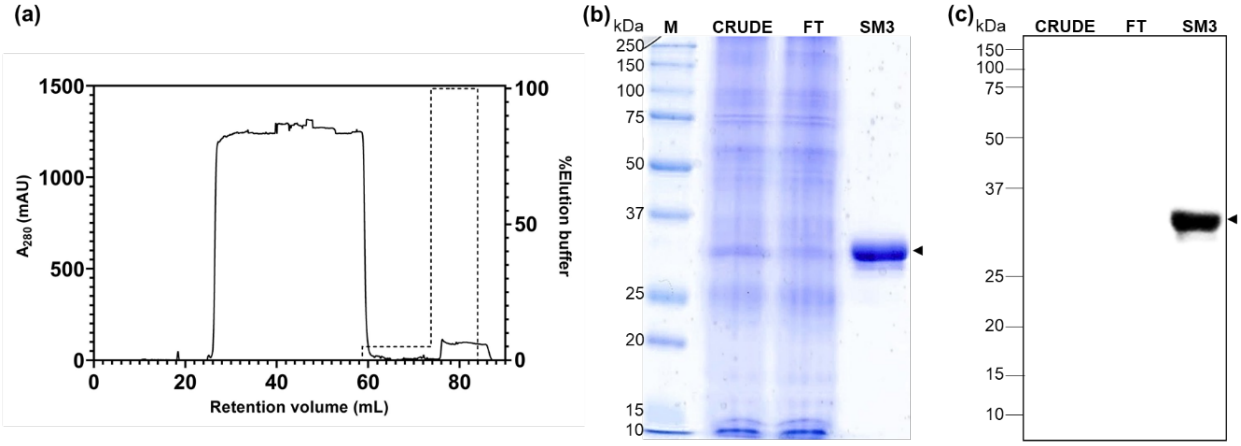

Figure 2. Purification of SpyTag-SM3. (**a**) Chromatogram of SpyTag-SM3 purified by Histrap FF from the supernatant of ExpiCHO-S cells. UV absorbance of 280 nm was plotted as a solid line, while percentage of elution buffer was displayed as a dash line. (**b**) Coomassie blue-stained gel of fractions from purification including the supernatant (CRUDE), flow-through (FT), and 3 ug of purified SpyTag-SM3 (SM3). (**c**) Western blot analysis of SpyTag-SM3 using anti-histag antibody to probe the protein. An arrow points SpyTag-SM3 in both Coomassie blue-stained gel and immunoblotting analysis.

3.3. Western blot analysis and DLS result revealed conjugation of OMVs:Lpp'OmpA-SpyCatcher and SpyTag-SM3

To validate isopeptide bond formation between OMVs:Lpp'OmpA-SpyCatcher and SpyTag-SM3, both components were excessively mixed in dilution buffer and incubated at 4 °C for 21 h. Then, Western blot analysis using anti-histag antibody was conducted to visualize the conjugated product. As seen in Figure 3a, a significant band of SpyTag-SM3 was shifted to higher molecular weight in consistent with calculated conjugated Lpp'OmpA-SpyCatcher:SpyTag-SM3 size (~57.5 kDa). The size of conjugated product is summary of both components (Lpp'OmpA-SpyCatcher, 29.9 kDa and SpyTag-SM3, 29.4 kDa) plus the removal of water (1.8 kDa). This result confirmed that OMVs:Lpp'OmpA-SpyCatcher and SpyTag-SM3 were conjugated. Even though band of the conjugated product was presented, we also observed unreacted substrates. Due to small different of size between Lpp'OmpA-SpyCatcher and SpyTag-SM3 (29.9 kDa and 29.4 kDa, respectively), we could not observe their individual bands. Next, size distribution of the

vesicle was assessed by dynamic light scattering (DLS). The result showed that conjugated OMVs displayed polydisperse profile (pDI of 0.465) with an averaged peak at 34.87 nm of diameter. Similar to typical *E. coli* BL21(DE3) OMVs, our conjugated OMVs formed nanosized particles as displayed in Figure 3b.

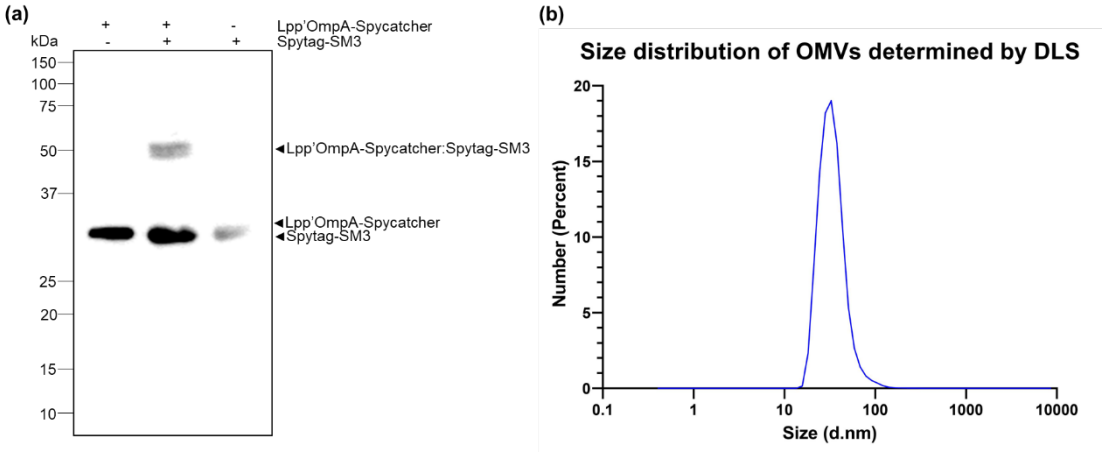

Figure 3. Characterization of conjugated OMVs. (**a**) Immunoblotting analysis of conjugated product (Lpp'OmpA-SpyCatcher:SpyTag-SM3) was conducted using anti-histag antibody. (**b**) Size distribution of OMVs determined by dynamic light scattering (DLS). The data collected from conjugated OMVs and presented in a blue line.

4. Discussion

SpyTag/SpyCatcher system enables efficient surface decoration system in various nanoparticles [18,19]. Isopeptide bond linked by SpyTag and SpyCatcher that remains stable in various environment is a key benefit to those platforms. In this report, we emphasized the use of SpyTag/SpyCatcher system in protein decoration of OMVs. As protein display systems in OMVs are based on direct genetic fusion to OM-localized proteins, they are not suitable to disulfide-linked proteins and glycoproteins. Thus, we presented a novel OMVs decoration platform that foreign protein can be attached on the surface by *in vitro*. This platform consists of two components. First, SpyCatcher-displaying *E. coli* BL21(DE3) OMVs via Lpp'OmpA fusion purified by ultracentrifugation which is the most common method and can achieve good homogeneity [20,21]. Second, SpyTagged anti-Mucin 1 (SM3) was expressed in CHO-based system. Rather than conventional periplasmic expression of bacterial system, mammalian expression system is preferable as a protein production host because it can perform glycosylation processes. Also, scFv produced in prokaryotic system were found to be aggregated and less thermal stability leading to loss of protein yield [22,23].

Despite of successful ligation as shown in Figure 3, condition optimization is still needed to achieve better decorated OMVs. Since unreacted substrates were observed after the ligation, ratio of the components could be modified. Besides, even though SpyTag/SpyCatcher bonding occurs within minutes, decoration of OMVs that contain massive contents needs longer incubation time up to 24 hours similar to other reports suggesting that time of incubation could be optimized [24]. However, we assumed that fully decorated OMVs could not be approached since bulkiness of other outer membrane compositions may interfere the ligation [3,25]. In addition, SpyTag/SpyCatcher was previously utilized to decorate heterologous antigens in bacterial OMVs by fusion of SpyCatcher to other OM-localized proteins including Cytolysin A (ClyA) and Hemoglobin protease (Hbp) [21,22]. Here, we suggest that Lpp'OmpA could serve as a superior choice of protein carrier in OMVs. First, Lpp'OmpA is the smallest carrier compared to the others so we expected that density of Lpp'OmpA expression in OMVs would be the highest. In particular, complexity and bulkiness of Hbp are weakness resulting in reduced ligation efficacy. However, there would be additional study to confirm this hypothesis. Second, there is unknown whether ClyA which is bacterial hemolysin may display undesirable

side effects in clinical uses. Even though no related outcomes were observed in several studies, they suggested that its hemolytic activity should be depleted prior to be used [5,28]. These reasons strongly support the use of Lpp'OmpA as a favorable SpyCatcher carrier in OMVs.

In the future, we will continue to investigate whether SM3 is attached on the surface of OMVs to confirm with this result. Later, this MUC1-targeted OMVs will be functionalized by loading drugs or therapeutic siRNA and then deliver them to $MUC1^{+}$ cells which is successfully illustrated in other studies [6,29]. This vesicle will be applied as a powerful selective drug delivery in cancer therapy applications.

5. Conclusions

Here, we reported a novel decoration system on OMVs that can present protein from another expression system. SpyTag/SpyCatcher system was utilized and linked between *E. coli* BL21(DE3)-derived OMVs and anti-Mucin 1 scFv (SM3). The results showed that SpyCatcher could be incorporated in OMVs through genetic fusion with Lpp'OmpA, while SpyTag fused with SM3 was successfully expressed in ExpiCHO-S and purified with a homogeneity. Conjugated vesicles were ligated and verified as Lpp'OmpA-SpyCatcher:SpyTag-SM3 complex was observed in Western blot analysis. Besides, DLS revealed a nanosized particle size related to ordinary OMVs. To sum up, this study provided simple and robust process to display foreign protein on OMVs serving as an efficient drug/vaccine vehicle.

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Conflicts of Interest: The authors declare no conflict of interest.

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