Development of new strategies for the purification of *Burkholderia cenocepacia* outer membrane vesicles (OMVs)

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**Abstract**

Outer membrane vesicles (OMVs), secreted by Gram-negative bacteria, are spherical bilayered structures that range between 20-250 nm in diameter enriched with lipopolysaccharides (LPS), phospholipids and outer membrane proteins. The purified OMVs of *Burkholderia cenocepacia* have shown potential applications in vaccines and in antibiotic therapy in Cystic Fibrosis (CF). Currently, the downstream processing is based on differential centrifugation methods. These methods have low yields, high costs and they are difficult to scale-up. This study focused on the development of new strategies to separate OMVs from the main impurities: cable pili and flagella. OMVs from a low-yield strain of *Burkholderia cenocepacia* were produced, concentrated in the supernatant by ultrafiltration and pelleted by ultracentrifugation. In order to purify them, were tested several alternatives, starting from the screening of aqueous two-phase systems (ATPS) with PEG-Dextran, followed by the using of PEG and a salt (ammonium sulfate/potassium phosphate). The possibility of separate OMVs by their negative charge was investigated as an alternative method using a monolithic platform with quaternary amines. Tests were also performed using size exclusion chromatography (SEC) with different commercially available which revealed to be an effective method. The use of a matrix of dextran-polyacrylamide (Sephacryl) achieved the best purification results.

**Keywords:** outer membrane vesicles, downstream processing, aqueous two-phase systems, size exclusion chromatography, monolithic chromatography.

**Introduction**

Cystic Fibrosis is the most frequent autosomal recessive genetic disease in populations of Caucasian origin and it affects close to 70,000 people in the whole world, with a life expectancy mid-30s due to chronic lung infections that are associated with high mortality [1]. This disease is caused by mutations in a gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The CFTR protein is present on epithelial cells throughout the body and it is a chloride ion channel involved in maintaining the water and ion homeostasis on cell surfaces. As it is ubiquitously expressed, multiple organs are affected including the lungs, sinuses, pancreas, gastrointestinal tract, sweat glands and reproductive tract [2]. Despite the various complications linked to the disease, the main cause of morbidity and mortality in CF is lung disease which results of an exaggerated pro-inflammatory response following bacterial infection [3]. At least 90% of patients with CF died from respiratory failure.

The bacterial species most commonly associated with respiratory tract infection in CF include common human pathogens such as *Staphylococcus aureus*, *Haemophilus influenzae*, species from *Burkholderia cepacia* complex (Bcc) and the most important, *Pseudomonas aeruginosa* [4]. *Burkholderia cepacia* complex (Bcc) bacteria have emerged as the highly opportunistic pathogens in patients with Cystic Fibrosis (CF) and immunocompromised individuals. Some of these infections with Bcc bacteria are inherently difficult to treat due to the resistance of these bacteria to multiple antibiotics, the ability to form biofilms, and the establishment of intracellular and chronic infection stages in the host [5]. A protective vaccine and new therapeutics against Bcc species would considerably prevent infection and increase life expectancy and quality of CF patients.

**Burkholderia cepacia complex (Bcc)**

The *Burkholderia* genus contains a group of versatile Gram-negative bacteria that are naturally found in moist soil, fresh water and
plant rhizopheres in relatively high population. Within this genus there is the *Burkholderia* cepacia complex (Bcc), a group of 18 closely related species of human opportunistic pathogens, capable of causing chronic and severe infection especially in cystic fibrosis and immunocompromised patients [6], [7]. In figure 1 are represented 17 species but a novel one was recently introduced: *Burkholderia pseudomultivoran* [8]. *Burkholderia cenocepacia* colonization, as well as patient-to-patient transmission of the organism, has been associated with the expression of filamentous adherence organelles known as cable pili [9]. These structures are expressed on the bacterial cell surface of *Burkholderia* species [10]. The cells are also constituted by flagella, important virulence determinants since the motility phenotype imparted by these organelles often correlates with the ability of an organism to cause disease [11].

**Outer Membrane Vesicles: OMVs**

Gram-negative bacteria, both pathogenic and nonpathogenic, constitutively release lipid bilayer vesicles derived from the outer membrane into the extracellular milieu [12]. These outer membrane vesicles (OMVs) are generally described as spherical proteoliposomes with an average diameter ranging from 10 to 500 nm that, like the outer membrane from which they are generated, are composed of lipopolysaccharide (LPS), phospholipids, and outer membrane proteins. The vesicle lumen contains mainly periplasmic components being enriched in many virulence factors [13]. Therefore, these structures were called membrane vesicles, extracellular vesicles, outer membrane fragments or blebs [14] although the historical term outer membrane vesicles is now generally accepted for Gram-negative bacteria. Gram-positive bacteria, fungi and archaea are also reported to exhibit the presence of membrane vesicles produced from cell surfaces [17].

OMVs are secreted by bacteria both in liquid and on solid media, as well as *in vivo*. OMVs have been observed in laboratory grown cultures and in natural environments such as freshwater, soil and biofilms [15], [16]. OMVs were found to be released in all growth phases of the bacterial culture [17] although their amount and composition may be dependent upon the growth conditions and the size distribution is characteristic from the strain [18].

**OMVs as Vaccine Carriers**

OMVs have become a promising target for vaccine development, as they carry many immunogenic components as the pathogen but are acellular. OMVs contain immune stimulators (e.g. LPS, proteins, and DNA) and antigenic molecules that can be delivered to immune competent cells of the immune system to trigger maturation as well as activation signals [19]. Therefore, they have an intrinsic adjuvant effect, but also multiple antigens in native conformation that are combined in a single vaccine formulation. Beside that they are stable after long-term storage at 5°C, which is an essential aspect for commercial viability [20].

At the time only meningococcal OMVs vaccines are commercially available and have shown particular utility in countering epidemics of *Neisseria meningitidis* serogroup B (MenB) [21].

**Materials and methods**

**Bacterial strains and culture conditions**

The bacterial strain of *Burkholderia cenocepacia* K56-2, kindly provided by Professor John Lipuma from the University of Michigan, was initially grown on a petri plate supplemented with Luria-Bertani broth (LB) at 37°C before experiment. The strain was pre-inoculated over-night in a flask with LB medium, at 37°C and 250 rpm orbital agitation. Two liters of LB liquid medium were inoculated with *Burkholderia cenocepacia*, initially with an OD640 nm of 0,1, and were grown to an OD640 nm of 1,5, in about 5/6 hours of incubation at 37°C and 250 rpm. This protocol was based on several experiments procedures previously described [22].

**OMVs Centrifugation**

The bacterial culture was centrifuged to remove cells at 9500 rpm, at 4°C for 10 minutes in a Beckman J2-MC centrifuge using 6 sterilized Beckman centrifuge bottles. OMVs
were gathered from the supernatant after removing residual cells by filtration through a 0.22µm membrane (Stericup&Steritop, Vacuum-driven filtration systems, Millipore Corporation, Billerica, USA) in order to maintain sterility. To the vacuum sterile filtration, it was used a vacuum pump from KIF Laboport. The 2 L filtered solution containing OMVs was stored at 4ºC in Schott flasks. A portion of filtrate was plated on LB agar and incubated at 37ºC overnight to verify that the suspensions were free of bacteria.

**Supernatant Ultrafiltration/Diafiltration**

Two liters of supernatant containing OMVs were concentrated by ultrafiltration with a 100 KDa (NMWC) hollow fibers cartridge with a membrane area of 110 cm². (QuixStand Benchtop System, GE Healthcare). After that, the supernatant was di-filtered with a buffer of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7 and 10 mM [23]. Last experiments were concentrated with a 500 kDa hollow fibers cartridge with 650 cm² of membrane area.

**Ultracentrifugation**

The concentrate was ultracentrifuged at 30000 rpm for 3 h at 4ºC in a Beckman XL-90 ultracentrifuge. The supernatant was removed and the OMVs pellet is re-suspended to obtain an enriched-OMVs pellet in a 10 mM HEPES buffer at pH 7. The OMVs solution obtained was storage at 4ºC.

**OMVs Purification**

**Aqueous Two-Phase Systems**

**PEG-Dextran Systems**

Systems of 2.5 g were prepared from stock solutions of 50% (w/w) PEG 3350 (Sigma-Aldrich,St. Louis, MO, USA), and 800 with 25% (w/w) Dextran 500 KDa (Fluka, Buchs, Switzerland). Several system combinations with concentrations of PEG and Dextran ranging from 6 to 8% were prepared by weighting the appropriate amounts of each component. The mass of OMVs feedstock solution used in all the systems was 40%.

**PEG - Ammonium Sulfate Systems**

The PEG-Ammonium sulfate systems used for ATPS had a total weight of 3 g with loads of 14 and 15% of ammonium sulfate with PEG 1000 (Fluka, Buchs, Switzerland) and 3350 (Sigma-Aldrich,St. Louis, MO, USA) in concentrations of 15 and 13% (w/w), respectively. The systems were prepared by weighting the correct amount of each component, adding 34% of OMVs solution and reaching the final weight by adding milli-Q water. The experiments were conducted with pH 7.

**PEG- Potassium Phosphate Systems**

Two 3g systems with different combinations of PEG and potassium phosphate were tested including i) 10% of PEG 3350, 10% phosphate at pH 7 and 34% of OMVs solution, and ii) 10% PEG 3350, 13% of phosphate buffer at pH 7 and 34% of OMVs solution. The systems were prepared by weighting the appropriate amounts of each component, adding 1g of OMVs solution and reaching the final weight of 3 g by adding milli-Q water.

**Size Exclusion Chromatography (SEC)**

Several runs using two different gel filtration media were performed. The OMVs feedstock solution obtaining in the diafiltration step was loaded either onto a column pre-packed with Superose 6 (10/300 GL, GE Healthcare Life Sciences) or in a Tricorn 5/100 column packed with Sephacryl S-1000 Superfine (GE Healthcare Life Sciences). In the case of Sephacryl S-1000 gel filtration, the column was equilibrated with 2 column volumes (CV) of 10 mM HEPES buffer pH 7, prior to injection at 1 mL/min. A load of 1.5 mL of OMVs sample without any pre-conditioning step was injected at 0.5 mL/min. The chromatographic runs performed using the Superose 6 column, followed the same protocol but 2 mL of OMVs sample was injected at 0.5 ml/min. All chromatographic experiments will be carried out on an AKTA™ Purifier 10 system (GE Healthcare, Uppsala, Sweden).
Ion-Exchange Chromatography (IEX)

IEX was evaluated using a CIM® disk monolithic column (BIA Separations, Ljubljana, Slovenia) containing quaternary amines (QA). The volume of OMVs sample applied to the monolithic column was 2.5 mL. Equilibration of the disk monolithic column was carried out with 2 CVs of the adsorption buffer, 10 mM HEPES, at different pH values of 7, 6, 5, and 4 (depending on the runs). Unbound substances were washed out using 5 CV of adsorption buffer at a flow rate of 0.5 mL/min. Linear gradient elution was undertaken with 5 CV using 10 mM HEPES at pH 7 with 1 M NaCl.

Particle size distribution and measurement of potential zeta

The size and zeta-potential parameters were evaluated in the samples, before and after purification using chromatography, in an electrophoretic light scattering apparatus (Zetasizer Nano ZS, Malvern Instruments, United Kingdom). The data was analyzed with a software to obtain the average hydrodynamic diameter of the particles in solution. The measurements were conducted at 22°C with three replicated runs of 5 min each, for each sample, and the average intensity weighted diameter was calculated. To measure these parameters, the samples were diluted with 10mM HEPES buffer, pH 7, with a refractive index of 1.59 and a viscosity of 0.89 [70].

Protein gel electrophoresis

ATPS samples were first diluted in milli-Q water and then diluted in a loading buffer containing 62.5 mM Tris-HCl, pH 6.2, 2% SDS, 0.01% bromophenol blue and 10% glycerol, and denatured in reducing conditions (0.1 M dithiothreitol (DTT), Sigma), at 100 °C for 10 minutes. For a final volume of 50 µL, 25 µL of loading buffer, 20 µL of sample, and 5 µL of 1 M DTT were used. A volume of 20 µL of these samples was applied in a 12% acrylamide gel, prepared from a 40% acrylamide/bisacrylamide (Hercules, CA, USA) stock solution (29:1), and run at 90-120 mV using a running buffer containing 25 mM Tris-HCl, 192 mM glycerine and 0.1% (w/v) SDS at pH 8.3. The molecular marker used in all gels was the Precision Plus Protein™ Dual Color Standards, from Bio-Rad. Gels were stained in an aqueous solution of 0.1% Coomassie Brilliant Blue R-250 (Hercules, CA, USA), in 30% ethanol and 10% acetic acid for 1 hour. Distaining was achieved by successively washing the gels with 30% (v/v) ethanol and 10% (v/v) acetic acid. Gels were further stained with silver nitrate from Bio-Rad (Hercules, CA, USA).

BCA Assay

Total protein concentration was determined with a BCA reagent kit from Pierce (Rockford, IL, USA) following the procedure using Bovine serum albumin (BSA) as protein standard calibration.

Transmission electron microscopy (TEM)

Ten microliters of the fractions were negatively stained with freshly prepared uranyl acetate 2% on carbon-coated copper grids. The pool of fractions obtained in the chromatography had to be ultracentrifuged in order to increase the concentration.

Results

Total Protein Quantification

After fermentation, the clarified supernatant had a protein content of around 600 µg/mL which was considerably reduced to 50-100 µg/mL, despite the concentration of the initial feedstock in the UF/DF step. The concentration ranges obtained in the last primary isolation step of OMVs isolation (ultracentrifugation) comprises the values between 70 and 300 µg/mL, which reveals a low production of outer membrane vesicles by the Burkholderia cenocepacia K56-2.

<table>
<thead>
<tr>
<th>Operation</th>
<th>Total Protein µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1. Centrifugation</td>
<td>500-700</td>
</tr>
<tr>
<td>Step 2. Ultrafiltration / Diafiltration</td>
<td>50-100</td>
</tr>
<tr>
<td>Step 3. Ultracentrifugation</td>
<td>70-300</td>
</tr>
</tbody>
</table>

Table 1- Total protein in µg/mL present after each operation method.
Particle size distribution and potential zeta

It was obtained a Z-average about 200-250 nm and there were observed three types of relevant population, represented by the first, second and third peak. The third peak had a huge deviation comparing to others, which indicates the presence of particle aggregates in solution. It was obtained a PdI of 0.62, revealing the low homogeneity of the solution. Table 4 with the results obtained on Zetasizer and the graphic of the second run (figure 1) are shown above. The results of zeta potential shown that for three runs using the same sample, the average potential estimative varied between -25 and -30 mV at neutral pH.

<table>
<thead>
<tr>
<th>Run</th>
<th>PdI</th>
<th>Z-average (nm)</th>
<th>1st Peak</th>
<th>2nd Peak</th>
<th>3rd Peak</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>d (nm)</td>
<td>Area (%)</td>
<td>d (nm)</td>
</tr>
<tr>
<td>1</td>
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<td>238.5</td>
<td>131.5</td>
<td>38.8</td>
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</tr>
<tr>
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</tr>
<tr>
<td>3</td>
<td>0.663</td>
<td>230.1</td>
<td>27.71</td>
<td>1</td>
<td>518.3</td>
</tr>
</tbody>
</table>

Table 2-Size distribution of OMVs particles analyses in triplicate in a Zetasizer.

Feed characterization

The sample analyzed by SDS-PAGE (figure 3) confirm the presence of one band with high molecular weight between 250 and 150 KDa and one intensive band between 50 and 37 KDa. There are other two bands between 50 and 75 KDa that are also present in all the batches. Other outer membrane proteins are also present in the gel with different sizes, mainly between 37 and 25 KDa. Among so many proteins in solution, there are two important ones: the adhesin with 22 KDa and the 16 KDa basic protein from cable pili [35]. In general, it can be found a similar protein pattern in the various samples from different batches. By observing the feedstock with a transmission electron microscope (Fig. 4), it is possible to see the various filaments corresponding to flagella, cable pili and spherical vesicles in different sizes.

Figure 1- Size distribution intensity of the solution analyzed by Zetasizer.

Figure 2- Size distribution volume of the solution analyzed by Zetasizer.

Figure 3- Silver-stained SDS-PAGE illustrating the proteins in the feedstock (lane 1) containing OMVs through SDS-PAGE. M: Protein molecular weight marker.
Figure 4- Feedstock imaged with a Transmission Electron Microscope (TEM). Arrows point to outer membrane vesicles (OMV), cable pili (cb), flagella (f).

OMVs Purification

Polymer-polymer systems

Figure 5- Qualitative analysis of the purity of both phases from PEG-dextran ATPS through SDS-PAGE. Top phases are illustrated in (A) and bottom phases in (B) composed by the following systems: 6% PEG 3350-6.14% dextran (2), 6.5% PEG 3350-6.5% dextran (3), 7% PEG 3350-7% dextran (4), 8% PEG 3350-8% dextran (5), 3.8% PEG 8000-5.2% dextran (6), 4.4% PEG 8000-6.2% dextran (7), 5% PEG 8000-7% dextran (8), 5.8% PEG 8000-8.4% dextran (9) at pH 7. Protein molecular weight marker (M). Feed (1). It was used a dextran with a molecular weight of 500 kDa. Proteins were separated by 12% SDS-PAGE and visualized by silver staining.

Figure 6- Qualitative analysis of the purity of both phases from PEG-ammonium sulfate and PEG-potassium phosphate ATPS. Top phases are illustrated in (A) and bottom phases in (B) composed by the following systems: 15% PEG 1000-14% (NH₄)₂SO₄ (2), 13% PEG 3350-15% (NH₄)₂SO₄ (3), 10% PEG 3350-10% KH₂PO₄ (4), 13% PEG 1000-15% KH₂PO₄ at pH 7. Protein molecular weight marker (M). Feed (1).
Chromatography on Superose 6 (SEC)

Figure 7 - SEC run with a Superose 6 column for the separation of OMVs from a 2.5 mL sample of the OMVs feedstock and 10 mM HEPES at pH 7 of preparation buffer.

Chromatography on Sephacryl-S1000 Superfine

Figure 9 - SEC run with a Sephacryl-S1000 Superfine column for the separation of OMVs from a 1.5 mL sample of the OMVs feedstock and 10 mM HEPES at pH 7 of preparation buffer.

The main goal of this purification is to separate the pili and flagella from the vesicles, which can be subsequently confirmed by visualization of the chromatographic fractions by transmission electron microscopy (TEM). According to TEM analysis, the first peak from the size exclusion chromatography with Sephacryl-S1000 contained no pili or flagella, only OMVs in a low amount and some aggregates (figure 11).

Chromatography with QA monolithic disk

Figure 10 - Silver stained reducing SDS-PAGE analysis of each chromatography fraction. Lane 1: Feedstock loaded; Lane 2: first elution peak; Lane 3: second elution peak; Lane 8,9: last fractions of SEC; M: protein molecular weight marker in kDa. Proteins were separated by 12% SDS-PAGE electrophorese.
Discussion and Conclusions

The results of the multicomponent protein mixture partitioning with PEG-dextran showed a higher affinity for the PEG-rich top phase for all the 8 systems performed. The increase in the molecular weight of PEG from 3350 to 8000 Da did not seem to have interfered with the partition profile (Fig.5). The best results were obtained for PEG-Dextran systems. The fact that the proteins present on top have only affinity for that phase and vice versa is advantageous to perform an efficient separation. However, it cannot be affirmed that these systems allowed the separation of the outer membrane proteins from the pili without performing a Western blotting. The results for PEG-sulfate and PEG-phosphate with molecular weights of 1000 and 3350 did not show many differences in the profile obtained for the 4 systems. The molecular bands with lower molecular weight were not detected in the top or bottom phase showing that they could probably have partitioned towards the interface. Aqueous two-phase systems were not efficient for the purification of the OMVs due to the fact that the systems cause a dilution of the already very diluted feedstock, and consequently this method would not be apppellative at an industrial scale. With the monolith, no effective purification was achieved. By observing the fractions with a transmission electron microscope, it was possible to analyze that in the first elution peak (fig.11A), flagella and pili are presented along with OMVs in aggregate form and in the second elution peak (fig.11B), the filaments of flagella are notable along OMVs, cable pili and biomolecules aggregates. The main goals were accomplish with a size exclusion chromatography with Sephacryl (Fig 10), but the concentrations of OMVs obtained are too low. It is necessary to optimize the OMVs production, using different growth media or testing the use of high yield strains. The immunogenicity of the purified OMVs should be investigated for a vaccine that can improve the quality of life of CF patients.

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References


