The successful development of an efficient OMV vaccine process with the potential for producing a broad protective immune response against Neisseria meningitidis

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Introduction

Outer membrane vesicles (OMV) are naturally produced by Neisseria meningitidis bacteria by vesiculation or 'blebbing' from the bacterial membrane. The composition of OMVs makes them significant activators of host innate and acquired immune response pathways. In addition to the potent immunomodulatory molecule LPS, vesicles contain porins and other important innate immune-activating ligands. Together, vesicle components appear to act synergistically to modulate the host response to either stimulate the clearance of the pathogen, enhance the virulence of the infection or both. OMV vaccines have been successfully used to control single strain epidemics of *N. meningitidis*. However, these vaccines have several drawbacks which prevent clinical acceptance including injection site reactogenicity, limited strain coverage and limited duration of protection necessitating booster doses. Much development is proceeding to address these disadvantages.





Current methods of OMV production involve the use of detergents to extract OMVs (dOMV), which gives a good yield but can modify the composition and immunogenicity from the "native" spontaneous OMV (sOMV). sOMVs are thought to be the most native form of manufactured OMV.

OMVs are released from bacteria during all phases of growth; however vesiculation levels increase during periods of bacterial stress, such as that experienced during the colonization of host tissues.

ImmBio has investigated the use of a non-detergent method of OMV production using a 1.25L scale fermentation process. The process was designed to yield sOMV, which are released into the culture supernatant during stationary phase and an EDTAextracted OMV (eOMV) from the pelleted harvested bacteria (Bas van de Waterbeemd, 2013). sOMVs are thought to be the most native form of manufactured OMV.

The yield and composition of OMV can be dependent on manufacturing conditions. Process conditions can be closely monitored in the fermenter vessel facilitating reproducibility and scale up. Fermentation cultures were either exposed to temperature elevation and oxygen depletion methods (otherwise known as a heat-shocked process), or grown to a stationary phase before harvesting. Samples were taken throughout the process and protein extracted from the pelleted bacteria. Heat shock protein (Hsp) expression, (GroEL and DnaK) was examined in response to increased stress. Heat shock proteins may increase immunogenicity and facilitate the delivery of immunogenic antigens to the host immune system leading to both MHC class I and II presentation.

Following downstream processing, the resultant eOMV and sOMV were assessed by SDS PAGE, Western blots, ELISAs and electron microscopy and compared with OMVs produced by deoxycholic acid (DOC) detergent extraction from strain 44/76-SL (dOMV).

The sOMV and eOMV were also used in an *in vivo* study to assess immunogenicity.. Following analysis it was found that there was a high yield of sOMV derived from the 44/76-SL strain compared with that derived from the M01-240149 strain and that the yield of eOMV was similar for both strains. Using SDS-PAGE and Western blot analysis both eOMV and sOMV were found to have similar protein profiles and *in* vivo, both the eOMV and sOMV were immunogenic and induced a good antibody response. This study shows that this 1.25L scale fermentation of N. meningitidis can successfully produce both spontaneously released and extracted OMV for biochemical evaluation and comparison in vivo.

Upstream Process

One ampoule of N. meningitidis WCB was removed from -80 C and thawed at room temperature. Two tryptic soy agar plates supplemented with Vitox were inoculated with 100µL of thawed material. The plates were incubated at 37 C and 5% CO₂ for 13 - 16 hours. The growth was harvested and resuspended in 11mL of shake flask medium (Enhanced Frantz + 100mM HEPES pH 7.0). The resuspension was used to inoculate 200mL of medium in a shake flask at an optical density (OD₆₀₀) 0.3 and incubated 37 C with shaking at 200rpm. When an OD₆₀₀ of 1.5 - 2.0 was achieved the culture was used to inoculate 950mL of fermenter medium (Enhanced Frantz –HEPES) in a fermenter vessel to give a starting volume of 1.25L at OD_{600} 0.3. The fermentation culture was controlled at a temperature of 37 C, pH of 6.95 (± 0.05) and dissolved oxygen saturation (dO₂) of 40%. Fermentation cultures were either harvested at 3 hours (after the onset of stationary phase) or heat shocked at 42 C with dO₂ depletion for 1 hour from the onset of stationary phase with subsequent harvest. The harvest was clarified by centrifugation. The supernatant was separated for sOMV manufacture. The cell paste was processed for eOMV manufacture.

sOMV Purification

A tangential flow filtration system with a 100kD hollow fibre filtration unit was used to concentrate the supernatant to a volume of 200mL before diafiltration into two volumes of a pH neutral buffer (20mM Tris; 1mM MgCl₂; pH 7.0). The material was passed through a depth filter (Cuno Zeta Plus 25cm² 0.5µm nominal) and a sterilization filter (Sartorius 2 XLG 210cm² 0.8-0.2µm absolute) in a series configuration. The filtrate was ultracentrifuged at 200,000 RCF for 1 hour. The supernatant was discarded and the pellet was resuspended in pH neutral buffer. All manipulations following sterile filtration were performed aseptically in a sterile environment.

OMV products were loaded (2.5µg /lane Total protein) onto 4-12% Bis-Tris SDS-PAGE gels and stained with Coomassie. Similar protein profiles were observed for both sOMV and eOMV samples. Bands can be observed in the PorA and PorB positions for the sOMV and the eOMV products. Duplicate gels were used for Western blotting (JAR 4 anti-fhbp and anti PorA 1.7)



<u>Western Blot Analysis :</u> Comparison with dOMV eOMV 44/76-SL contain both GroEL and DnaK Hsp proteins and increased fhbp as compared to 44/76-SL dOMV. sOMV contain GroEL Hsp protein and increased fhbp as compared to 44/76-SL dOMV. PorA is present in all OMV products analysed. Lane 2 sOMV 44/76-SL, Lane 3 eOMV 44/76-SL, Lane 4 dOMV.

Electron Microscopy





Fermentation

Online and offline data trace of N. meningitidis strain M01-240149 heat-shocked fermentation process

Culture viability was monitored by measuring numbers of colony forming units by serially diluting in 96 well plates and inoculating angled TSA + Vitox plates using a multi-channel pipette. Viability at harvest may affect conformity and yield of OMV. Differences in growth profile and resistance to stress conditions were observed between the two strains.



eOMV Purification

The cell paste was resuspended in extraction buffer (20mM Tris; 1mM MgCl2; pH 8.4) at a ratio of 7.5mL/g of cell paste. The resuspension was supplemented with EDTA at a final concentration of 10mM and incubated at room temperature for 1 hour with agitation. The material was diafiltered into two volumes of a pH neutral buffer (20mM Tris; 1mM MgCl₂; pH 7.0) before an overnight incubation at +4 C with nuclease (BaseMuncher, Expedeon). The material was diafiltered again on the following day into two volumes of pH neutral buffer before depth and sterile filtration as described in **sOMV Purification**. The filtrate was ultracentrifuged and the pellet was resuspended as described in **sOMV Purification**.

Total Protein Assessment



Yield was estimated by total protein. A bicinchoninic acid assay (BCA) was used to assess the total protein yield of each OMV product. sOMV derived from the substrate strain 44/76 non heat-shocked (non H/S) process yielded a significantly high content of total protein.

Hsp Assessment

Heat-shocked and non heat-Hsp60 - GroEL shocked native sOMV and eOMV on Western blots were probed with anti-GroEL and anti-DnaK primary antibodies. Densitometry was performed to analyse the relative levels of Hsps. B12 sOMV B12 eOMV B13 sOMV B13 eOMV B11 eOM/ Western blots were loaded

Electron microscopy images of sOMV and dOMV were taken at a direct magnification of x14,500. The observed differences may be strain or process related.

In vivo Immunogenicity

IgG Detection ELISA

An ELISA was used to detect levels of IgG and other isotypes in raised sera from the CD1 mice. ELISA plates were coated with lysates from strain 44/76-SL to capture antibodies specific to the antigens present in the sera. The plates were blocked and sera samples were added and serially diluted. After incubation with a secondary antibody and HRP substrate the optical density was measured at 450nm (OD_{450nm}). Data was plotted to obtain dilution curves. Three points within the linear region of the dilution curve were selected and linear regression values were obtained to generate an end point titre (y-axis). This value represents the maximum dilution which can still produce a valid positive reading.





Increased levels of total IgG, IgG1, IgG2a, IgG2b and IgG3 were detected in serum

H. Contraction

with 1µg of total protein. Hsp70 - dnaK Increased levels of Hsp60 were observed in heat Jg shocked (H/S) sOMV deriving from strain 240149. Levels of Hsp70 were not detectable in sOMV B11 sOMV B11 eOMV B12 sOMV B12 eOMV B13 sOMV B13 eOMV products, however, increased levels were detected in heat 44 / 76 240149 240149 shocked eOMV. non H/S H/S non H/S

raised from 44/76-SL OMV (4,5) compared with the antibody levels raised against M01-240149 OMV (2,3,6,7) when tested against 44/76-SL lysate by ELISA. Detected levels of these isotypes were higher in serum from heat shocked M01-240149 OMV (6,7) compared with those that were not heat shocked (2,3). The presence of complement fixing isotypes in these sera against more than one strain of *N. meningitidis* indicates a heterologous immune response that may have relevance for vaccine development. Future work will involve the assessment of the sera in a serum bactericidal assay (SBA).

Conclusion

- sOMV and eOMV were successfully manufactured and were found to be immunogenic in an IgG isotype detection ELISA.
- A significantly high estimated yield of 44/76-SL sOMV was achieved.

• Proteins thought to be important in an immunological response were detected in sOMV and eOMV. The heat shocked process increased levels of Hsp60 in M01-240149 sOMV and Hsp70 in M01-240149 eOMV.

- 44/76-SL sOMV and eOMV had increased levels of fHbp compared with 44/76-SL dOMV. PorA was detected in all products.
- Heat shocked M01-240149 sOMV and eOMV demonstrated increased levels of IgG compared with non heat shocked M01-240140 sOMV and eOMV against the heterologous 44/76-SL strain.

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