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# Spontaneously released *Neisseria meningitidis* outer membrane vesicles as vaccine platform: production and purification

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ABSTRACT

Outer membrane vesicles (OMVs) are nanoparticles produced by Gram-negative bacteria that can be used as vaccines. The application of OMVs as vaccine component can be expanded by expressing heterologous antigens on OMVs, creating an OMV-based antigen presenting platform. This study aims to develop a production process for such OMV-based vaccines and studies a production method based on meningococcal OMVs that express heterologous antigens on their surface. As a proof of concept, the *Borrelia burgdorferi* antigens OspA and OspC were expressed on *Neisseria meningitidis* OMVs to create a concept anti-Lyme disease vaccine. Production of OMVs released in the culture supernatant was induced by high dissolved oxygen concentrations and purification was based on scalable unit operations. A crude recovery of 90 mg OMV protein could be obtained per liter culture. Expressing heterologous antigens on the OMVs did result in minor reduction of bacterial growth, while OMV production remained constant. The antigen expression did not alter the OMV characteristics. This study shows that production of well characterized OMVs containing heterologous antigens is possible with high yields by combining high oxygen concentrations with an optimized purification process. It is concluded that heterologous OMVs show potential as a vaccine platform.


## 1. Introduction

Vaccination is one of the most successful inventions as vaccines have saved many lives by preventing infectious diseases. Despite the successes, infectious diseases remain a major source of mortality worldwide. The development of new vaccines is continuously required as new infectious diseases emerge and there is an increasing demand for therapeutic vaccines. There is thus a need for new vaccine production technologies that enable more rapid production of vaccines. Vaccine platforms can provide enhanced safety, productivity and simplicity in vaccine development by thorough initial development of the platform. A vaccine platform for prophylactic vaccines aims at eliciting high and lasting antibody responses against specific antigens, while eliciting a standard supportive innate immune response. The design of the vaccine platform or antigen presenting platform should be such that protection against different infectious diseases can be induced by displaying different antigens. Bacterial outer membrane vesicles (OMVs) are suitable candidates for vaccine platforms. OMVs are nanoparticles derived from Gram-negative bacteria and are highly immunogenic while non-infective and non-replicating [7]. However, not all Gram-negative pathogens are suitable OMV vaccine producers because of their high pathogenicity, low growth rate, complex media requirements, lack of genetic accessibility, or low OMV productivity. Adding heterologous antigens to OMVs creates an OMV-based antigen presenting platform. Bacteria have been engineered so that they produce heterologous antigens in OMVs [14,20]. Alternatively, the antigen can be produced separately and added to the OMV [22,26,29]. Besides targeting infectious disease targets, OMV-based vaccine platforms can be designed as therapeutic vaccines [15,19,39].

Gram-negative bacteria produce spontaneously released OMVs (sOMVs) that are secreted in the culture medium. However, the amount of sOMVs produced per amount of biomass is generally low and therefore other methods of OMV production have been developed, like extraction of vesicles from the bacterial cells by detergents (dOMVs) or by EDTA or other detergent-free methods (eOMVs) [13]. Detergent extraction has been used in the production of OMVs since the detergent extraction reduces the

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lipopolysaccharide (LPS) content of the OMVs resulting in a safer to use vaccine composition. However, with the development of genetically detoxified LPS variants there remains no necessity of detergent extraction to reduce the LPS toxicity of OMVs [48].

OMVs from *Neisseria meningitidis* (Nm) are well studied, used to control outbreaks of serogroup B meningococcal disease, and Nm dOMVs are included in the 4CMenB/Bexsero vaccine [34]. Here we test the expression of heterologous antigens on both Nm eOMV and Nm sOMV. Outer surface protein A (OspA) and Outer surface protein C (OspC) of *Borrelia burgdorferi* were chosen as model antigens. *B. burgdorferi* causes Lyme disease and is the most common tick-borne illness in the Northern hemisphere [2,38]. Recombinant OspA has been shown safe and effective in randomize controlled trials and has been the basis of novel vaccine concepts [5,35,46]. These model antigens are surface expressed because we hypothesize that surface exposure of antigens is advantageous. However, the induction of antibody responses has also been shown for antigens expressed in the OMV lumen [3,8,27,32]. The heterologous antigens are surface expressed by fusion of the proteins to short N-terminal part of the Nm lipoprotein fHbp [31]. Spontaneous OMV production was triggered in batch cultures by cysteine depletion of the culture media [43], which is associated with oxidative stress responses. Additionally, high dissolved oxygen concentrations were used during cultivation to enhance OMV production [11].

This study aims to develop a production platform for Nm OMVs expressing heterologous antigens. First a purification process for sOMVs is designed on the available unit operations of the eOMV purification process. Optimization of the separation of sOMVs from the bacterial cells was required to achieve sufficient yields. Next, the impact of expressing heterologous antigens on the growth of the bacterial culture on the quality of OMVs expressing these heterologous antigens is assessed. Furthermore, the characteristics of eOMVs and sOMVs are compared.

## 2. Methods

### 2.1. Bacterial strains

The H44/76 isolate of *Neisseria meningitidis* serogroup B [17] was used as OMV production host. In this strain the *rmpM* gene was knocked out to induce vesicle formation [41]. Furthermore, the strain is non-encapsulated by *siaD* knockout, has reduced LPS toxicity by *lpxL1* knockout [44], has increased interaction with dendritic cells by *lgtB* knockout [37], and lacks the major outer membrane protein and immunodominant antigen *porA* [40]. Outer surface protein A (OspA) and Outer surface protein C (OspC) from *Borrelia burgdorferi* were expressed in Nm as heterologous antigens. The *ospA* and *ospC* genes were codon optimized for the codon usage of Nm and expressed by placement after a hybrid *porA-nadA* promotor [28]. To ensure surface exposure, OspA and OspC were linked to fHbp (fHbp-OspA, fHbp-OspC) as based on the method described previously [31] and placed in either *rmpM* or *lpxL1*. An overview of the strains used in this study is given in Table 1.

**Table 1**
Overview of bacterial strains used in this study. The heterologous antigens OspA and OspC from B. *burgdorferi* were expressed in *N. meningitidis*.

| Strain | Knockout mutations | Heterologous antigen | |
|---|---|---|---|
| | | OspA | OspC |
| Nm | *rmpM*, *siaD*, *lgtB*, *lpxL1* | – | – |
| Nm OspA | *rmpM*, *siaD*, *lgtB*, *lpxL1* | in *rmpM* | – |
| Nm OspC | *rmpM*, *siaD*, *lgtB*, *lpxL1* | – | in *rmpM* |
| Nm OspA + OspC | *rrmpM*, *siaD*, *lgtB*, *lpxL1* | in *lpxL1* | in *rmpM* |

### 2.2. Batch cultivations

Pre-cultures were started by adding 10 mL of frozen working seedlot to a shaker flask containing 155 mL chemically defined growth medium [1]. The shaker flask was incubated in a shaker incubator at 35 °C, 200 rpm and after growth it was used to inoculate a second shaker flask. At $OD_{590\ nm}$ = 1.5 ± 0.5 this was transferred to a bioreactor containing 3 L medium to start the batch culture. The bioreactor (Pierre Guerin Tryton$^{i}$) controlled the temperature at 35 °C, pH at 7.2. The dissolved oxygen tension was controlled at 30% air saturation by increased agitation rate (300–1000 rpm) followed by addition of oxygen in the overlay aeration. The growth medium was designed such that cysteine depletion causes growth arrest and triggers sOMV release [43]. sOMV release can be further induced by oxidative stress from high dissolved oxygen concentrations in the culture. These cultures were controlled at 100% air saturation, further referred to as high dissolved oxygen. Filtered off-gas (0.22 μm cut-off) was measured by a Thermo Prima δb mass spectrometer to monitor oxygen consumption and carbon dioxide production.

### 2.3. Downstream processing of sOMVs

The harvested culture broth was cooled to 20 °C and transferred to a tangential flow microfiltration setup (Spectrum Mini-Kros pilot or Spectrum KR2i) with a 20 cm mPES hollow fiber module. The hollow fiber was used according to the manufacturer's instructions (Spectrum Labs). Transmembrane pressure scouting (TMP-scouting) and flow excursion were performed using the harvest of two 3 L batch cultures grown at 100% DOT as described above. For the TMP-scouting, 3 L harvest was cooled to 20 °C and transferred to a tangential flow filtration (TFF) setup using the Spectrum KR2i and Spectrum 85 $cm^2$, 0.65 μm pore size hollow fiber module with 0.7 mm lumen diameter. The permeate flowed back into the feed. TMP was controlled by the automatic backpressure valve. For the flow excursion, a similar setup was used with a new culture harvest, using a new 85 $cm^2$ 0.65 μm pore size membrane module without backpressure valve. The permeate flow was controlled by the KRJ pump of the KR2i. To produce sOMVs, a 520 $cm^2$ 0.65 μm pore size mPES hollow fiber module was used on a KroFlo Mini-Kros Pilot TFF system (Spectrum). The cooled harvest was concentrated 6-fold by processing with a shear rate of 16.000 $s^{-1}$ and a constant flux rate of 15 L per $m^2$ surface area per hour (LMH). Next, the remaining sOMVs were washed out by 2 volumes diafiltration by constant volume diafiltration performed at the same operating settings, using a 10 mM Tris-HCl 3% sucrose buffer of pH 7.2. Next, sOMVs were purified starting with the addition of 300 U/L Benzonase (Merck) to the crude sOMVs to digest DNA. The sOMVs were concentrated using a 790 $cm^2$, 100 kDa cut-off mPES membrane, followed by 3 volume diafiltration with buffer (10 mM Tris-HCl 3% sucrose buffer of pH 7.2). The concentrated OMVs were clarified by dead-end microfiltration (1.2 μm–0.5 μm cut off) before group separation by size-exclusion chromatography on a Sepharose 6 Fast Flow column (GE Life Sciences). Lastly, sOMVs were sterile filtered using a 0.2 μm cut-off dead-end filter (Pall).

### 2.4. Downstream processing of eOMVs

Nm eOMVs were produced based on the process described by Van de Waterbeemd et al. [42]. In brief, the biomass of the cooled culture harvest was concentrated 5-fold using a 790 $cm^2$ mPES hollow fiber module with 0.2 μm pore size and 0.5 mm lumen diameter (Spectrum Labs), followed by 2 volumes diafiltration in a buffer suited for OMV extraction (100 mM Tris-HCl pH 8.6). eOMVs were

separated from residual bacterial cells by 10 volumes diafiltration. Next, purification of eOMVs was identical to the purification of sOMVs described above.

### 2.5. Analytical methods

OMV samples were quantified by Nanoparticle Tracking Analysis (NTA) [24]. Culture samples were filtered (0.22 μm pore size) before measurement. NTA was performed on a calibrated NanoSight NS500 by capturing 10 captures of 30 s at 25 °C. Purified water was used to prepare correct sample dilutions. Automated flow-measurements were obtained as described previously [12]. Captures were analyzed using the NTA 3.2 software build 3.2.16. Total protein content of purified OMV samples was assessed using Lowry's protein assay with Peterson's modification and Bovine Serum Albumin as protein standard. The assay was performed according to manufacturer's protocol (Sigma-Aldrich). LPS content was measured by a modified gas chromatography method [18,45]. In brief, the LPS was quantified based on the peak area of C14:0-3OH using C12:0-2OH as internal standard [1]. Protein composition of OMVs was assessed by SDS-PAGE by loading OMVs with a total protein content of 4 μg on a precast polyacrylamide gel (Lonza) to perform SDS-gel electrophoresis. The gel was stained with InstantBlue protein stain (Expedeon).

Western blot analysis was performed as described [31] using α-OspC Rabbit Polyclonal Antibody (Rockland) as a primary antibody after separating the proteins on a Novex 16% Tricine Protein Gel (ThermoFisher Scientific). As a molecular weight marker the Precision Plus Protein Standard (BioRad) was used.

For LC-MS/MS analysis, OMVs were denaturated at 100 °C for 30 min in potassium phosphate buffer (100 mM, pH 7.8), also containing 100 mM Rapigest (Waters), at a protein concentration of 200 μg/mL. Reduction and alkylation of the proteins were performed by subsequent incubations with TCEP (1 h at 55 °C) and iodoacetamide (30 min at ambient temperature in the dark). Proteins were digested with LysC (0.4 μg, Roche) and trypsin (1 μg, Promega) by overnight incubation at 37 °C. Solid-phase extraction was performed to remove excess reagents using 1-mL C18 Sep-pack cartridges (Waters) according to the manufacturer's protocol. After drying, the peptide fraction was reconstituted in 1 mL of water containing 5% (v/v) DMSO and 0.1% (v/v) formic acid. Nanoscale reversed-phase liquid chromatography was used for peptide separation [25]. The peptides were measured in an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) utilizing data-dependent scanning: the MS-scan ($m/z$ 300–1500) with an orbitrap readout (120,000 FWHM). Precursor ions with +2 to +7 charge states (intensity threshold 25,000 counts) were selected for collision-induced dissociation (CID) with an ion-trap readout using default settings. Proteome Discoverer 2.1 software (Thermo Scientific) was used for peptide identification and quantification (based on the High-3 method). Identification of peptides was performed against the protein database of *N. meningitidis* H44/76 (NCBI 909420) and the fHbp-OspA/C constructs. Asparagine deamidation and methionine oxidation were set as dynamic modifications while cysteine carbamylation was set as static modification. The data were searched with full trypsin cleavage specificity, allowing 2 missed cleavages. Precursor ion and MS/MS tolerances were set to 5 ppm and 0.4 Da, respectively. Peptides were filtered using Perculator (1% FDR). Reported protein areas were multiplied with their respective molecular masses and used to calculate the percentage of the protein abundance for each individual protein relative to the total sum of all identified proteins. Protein localization was predicted using PSORTb v3.0 [47].

### 2.6. Stability of OspA expression during cultivation

A bioreactor culture was subcultured in shaker flasks from $OD_{590}$ = 0.1 to $OD_{590}$ = 3.0 ± 0.5 for over 20 generations after the end of a batch bioreactor culture. Culture samples from the shaker flasks as well as from the frozen seedlots, the preculture and the bioreactor culture were analyzed for the presence of OspA by Western blot analysis. As primary antibody a polyclonal rabbit anti-OspA (Rockland) was used with goat-anti-rabbit IgG-AP (Southern BioTech) as secondary antibody.

## 3. Results

### 3.1. Overall process design for sOMV production

A production process for Nm sOMVs was designed based on the scalable unit operations of the Nm detergent-free extracted OMV (eOMV) production process (Fig. 1). The production of biomass starts from a frozen working cell-bank, by growth in a shaker flask (1.1). After growth, this is sub-cultured to an inoculum culture (1.2) that is subsequently used to start a bioreactor production culture (1.3). The culture is cooled to reduce biological activity (1.4) during OMV purification. The biomass is concentrated (2.0) for eOMV production using tangential flow filtration (TFF). Next, the bacteria are diafiltrated to extraction buffer on the same hollow fiber module (2.1). eOMVs are extracted by incubation with EDTA (2.2) and separated from the residual bacterial biomass by collecting the filtrate (3.0). Next, eOMVs are purified by DNA degradation (4.0) and concentrated on a 100 kDa cut-off hollow fiber module (4.1). The concentrated OMVs are changed to a different buffer (4.2) and clarified (5.1) before separation from soluble proteins by size-exclusion chromatography (6.1). Lastly, the purified eOMVs are filtered to obtain sterile OMVs (7.1). For sOMVs, purification starts by separation of the sOMVs from the bacterial biomass by tangential flow filtration (2.0). Further purification of sOMVs is equivalent to the eOMV process, omitting the extraction process (2.1–3.0). In the following sections the sOMV separation process from the biomass is further discussed, followed by a proof of concept of OMV production of Nm OMVs containing heterologous antigens and analysis of their quality.

### 3.2. Optimizing tangential flow microfiltration based recovery of OMVs

Tangential flow microfiltration was used to separate sOMVs from the bacterial cells. A process was tested based on the process of biomass concentration used previously in eOMV production. Where for eOMV production tangential flow microfiltration is used for concentrating the biomass (step 2.0), the same step is used to remove bacterial cells in the case of sOMV production. A cooled Nm culture was processed by operating an 0.2 μm pore size hollow fiber filter in constant transmembrane pressure (TMP) mode. The production of Nm sOMVs yielded a total of $4 \times 10^{14}$ particles in the bacteria-free OMV fraction, which corresponds to a recovery of 77% of the $5 \times 10^{14}$ OMVs produced in a 3 L culture (Fig. 2A). The productivity of another culture was improved four-fold to $2.0 \times 10^{15}$ sOMVs in 3 L culture broth by increasing the dissolved oxygen concentration to 100% air saturation. Surprisingly, the recovery of sOMVs in step 2.0 (TFF microfiltration) from this culture was reduced to 1.0% (Fig. 2A).

OMV induction by high dissolved oxygen concentration showed severely reduced recovery of sOMVs using the 0.2 μm pore size hollow fiber filter. Since the size of the OMVs (100 nm) is close to the cutoff of the 0.2 μm pore size membrane, transmission can be affected by small increases in OMV size or slight fouling of the filter pores. Since we previously found that the size of OMVs

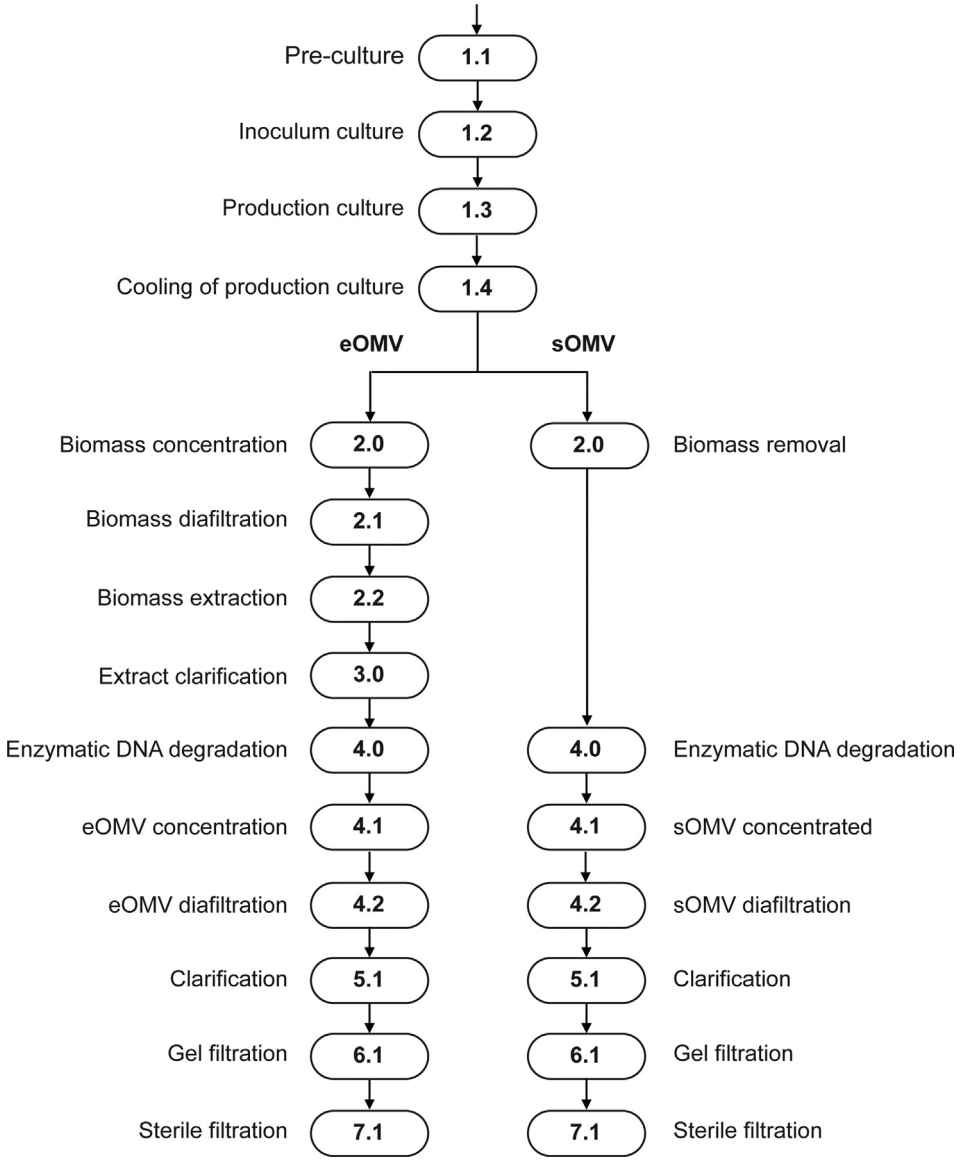


**Fig. 1.** Schematic production process of eOMVs and sOMVs. Production of both Nm eOMV as Nm sOMV starts by batch cultivation of *N. meningitidis* on chemically defined growth medium. After bacterial growth, sOMVs can be purified from the culture supernatant directly, while the production of eOMVs requires an extraction process of the concentrated biomass under specific conditions. Further down-stream processing of both types of OMVs consists of the same process steps: DNA removal, vesicle concentration, buffer exchange, removal of residual proteins, and sterile filtration. The unit operations are explained in the main text.

was not affected by the increased oxygen levels, we hypothesized that the mild stress may have caused a decrease in pore size due to DNA or other cell debris. We assessed the performance of a hollow fiber membrane filter with increased pore size to restore the recovery of sOMVs. A pore size of 0.65 μm resulted in complete retention of bacteria, which are 0.6–1.0 μm in diameter, and OMV recovery increased to 29% (Fig. 2B). Additionally, it was tested whether DNA degradation by DNAse before filtration would improve the transmission, but the transmission was similar (31%) to processing without DNA degradation (Fig. 2B).

Further analysis of the filtration performance showed that initial transmission of OMVs over the 0.65 μm pore size membrane was high, but reduced quickly, indicating fouling of the filter membrane. To explore the possibilities of improving the recovery of sOMVs induced by the high dissolved oxygen concentration, TMP scouting was performed. By measuring the flux at different transmembrane pressures, an optimum flux can in principle be found at increased shear rates and increased transmembrane pressures (Fig. 3A). Interestingly, we observed that the passage of OMVs through the filter was highly reduced at increasing transmembrane pressures (Fig. 3B), while the transmission of total protein remained unaffected for all samples (data not shown). Especially at a moderate shear rate of 5000 $s^{-1}$, the cross flow along the membrane was apparently not strong enough to clean the surface. This shear rate (5000 $s^{-}1$) was initially chosen due to the non-fouling feed flow observed in the eOMV process. If only a minor amount of fouling occurs, it reduces the possibility of the relatively large OMVs to pass the pores of the membrane. Operating the membrane at higher TMPs will thus cause a reduced recovery of OMVs.

Minimization of membrane fouling by this highly fouling culture harvest could be achieved by operating under constant permeate flow instead of at constant TMP conditions. This constant flux method ensures the lowest fouling conditions since the flow through the pores of the membranes is limited by the permeate pump, while the cleaning action of the flow along the membrane is maintained. To determine the operating conditions for this

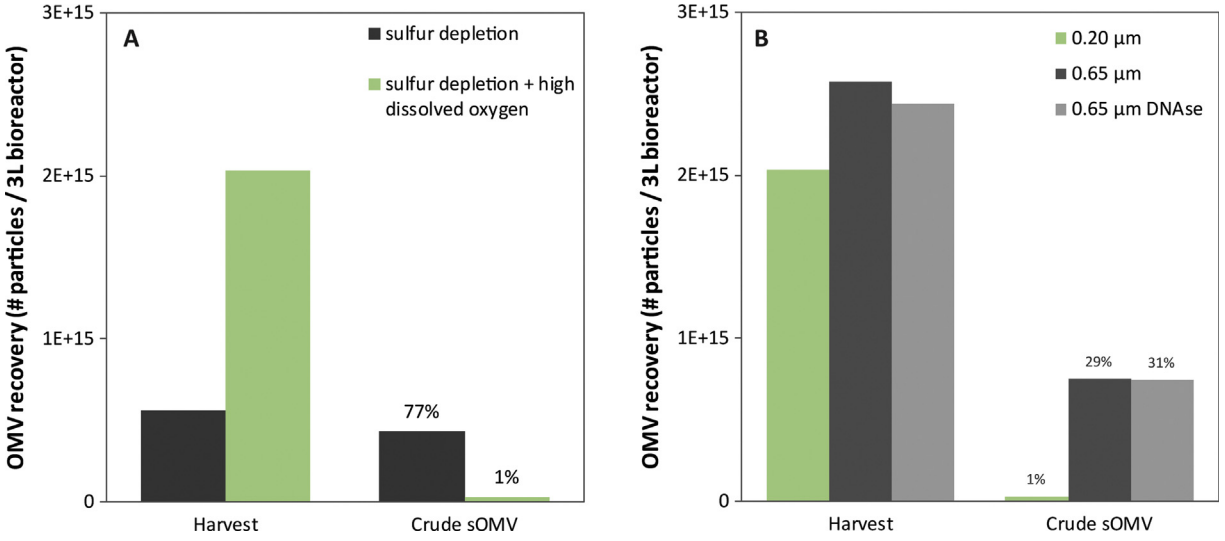


**Fig. 2.** *N. meningitidis* biomass removal and sOMV recovery by tangential flow filtration. Recovery of sOMVs by tangential flow filtration at constant TMP from a bioreactor culture (harvest) (A). Nm sOMVs produced in a sulfur-source (cysteine) depleted culture (black) showed 77% recovery, while a sulfur-source depleted culture combined with a high dissolved oxygen concentration (green) showed 1% recovery. Recovery of sOMVs from a culture with high oxygen concentration by tangential flow filtration at constant TMP is optimized using an increased pore size membrane and DNAse treatment (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

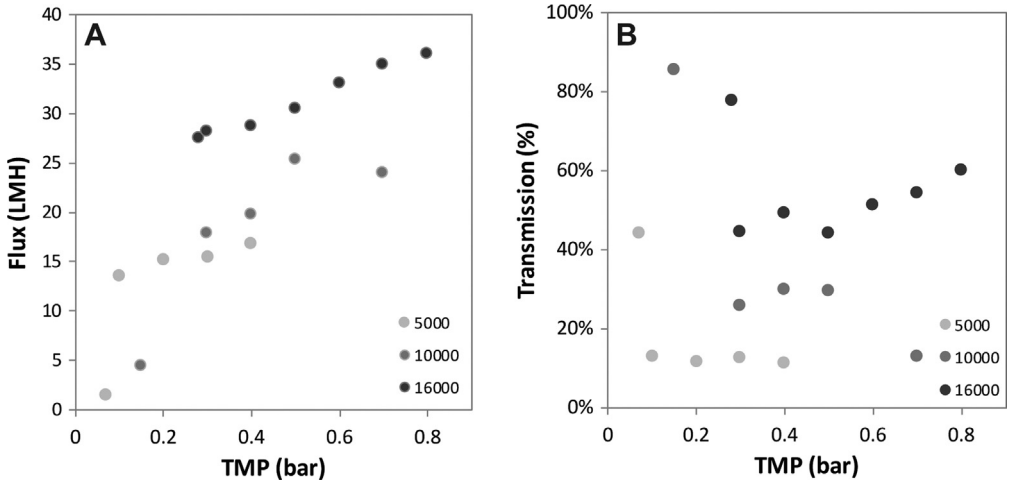


**Fig. 3.** TMP scouting of the TFF of a dissolved oxygen induced sOMV harvest. Filter performance at varying shear rates and increasing transmembrane pressures using a *N. meningitidis* high dissolved oxygen induced sOMV harvest. The resulting permeate flux in liter per $m^2$ surface area per hour (LMH) is shown at increasing transmembrane pressures (A). The transmission of sOMVs at increasing transmembrane pressures is given for crossflow rates at 5000 $s^{-1}$ shear, 10,000 $s^{-1}$ shear, and 16,000 $s^{-1}$ shear (B).

mode, the critical flux (maximum flux at which the membrane system can operate without accumulation of foulants) was determined at three different shear rates (Fig. 4). The shear rate of 5,000 $s^{-1}$ showed the lowest critical flux (10 LMH), while the shear rates 10,000 $s^{-1}$ and 16,000 $s^{-1}$ showed higher critical fluxes (22 LMH). Transmission of the OMVs through the membrane was constant up to the critical flux. For the higher shear rates transmission was constant at 70%. Processing the culture harvest at high shear rates and a flux below the critical flux should allow the processing of the entire harvest volume with 70% transmission. By processing 5/6th of the volume, followed by 2 volumes diafiltration, a theoretically OMV recovery of 93% could be reached.

### 3.3. Production of meningococcal sOMVs containing heterologous antigens

The process with optimized microfiltration (step 2.0) was used to produce Nm sOMVs, and Nm sOMVs containing heterologous antigens. Heterologous antigens were added to sOMVs by genetical linkage to an outer membrane linking protein of *N. meningitidis* to ensure surface exposure [31]. Fusion constructs of fHbp to Outer Surface Protein A (OspA) and Outer Surface Protein C (OspC) of *Borrelia burgdorferi* were expressed individually in Nm (Nm OspA, Nm OspC) as well as combined (Nm OspA + OspC). The stability of the heterologous expression of antigens was analyzed for the OspA expressing Nm strain. Western blot analysis showed that OspA was expressed for at least 20 generations after the end of the batch production culture (Supplemental Fig. 1). All three strains, and the control strain without OspA and OspC, were grown in a batch culture with cysteine depletion in combination with high dissolved oxygen concentrations to trigger sOMV release. The growth of the four cultures was similar during the first 7 h of cultivation (Fig. 5). Then, a reduction in growth rate for the strains expressing heterologous antigens was observed resulting in a shift of 4 h (Nm OspA, Nm OspC) and 7 h (Nm OspA + C) before reaching the maximum carbon dioxide evolution rate. Cultures were harvested

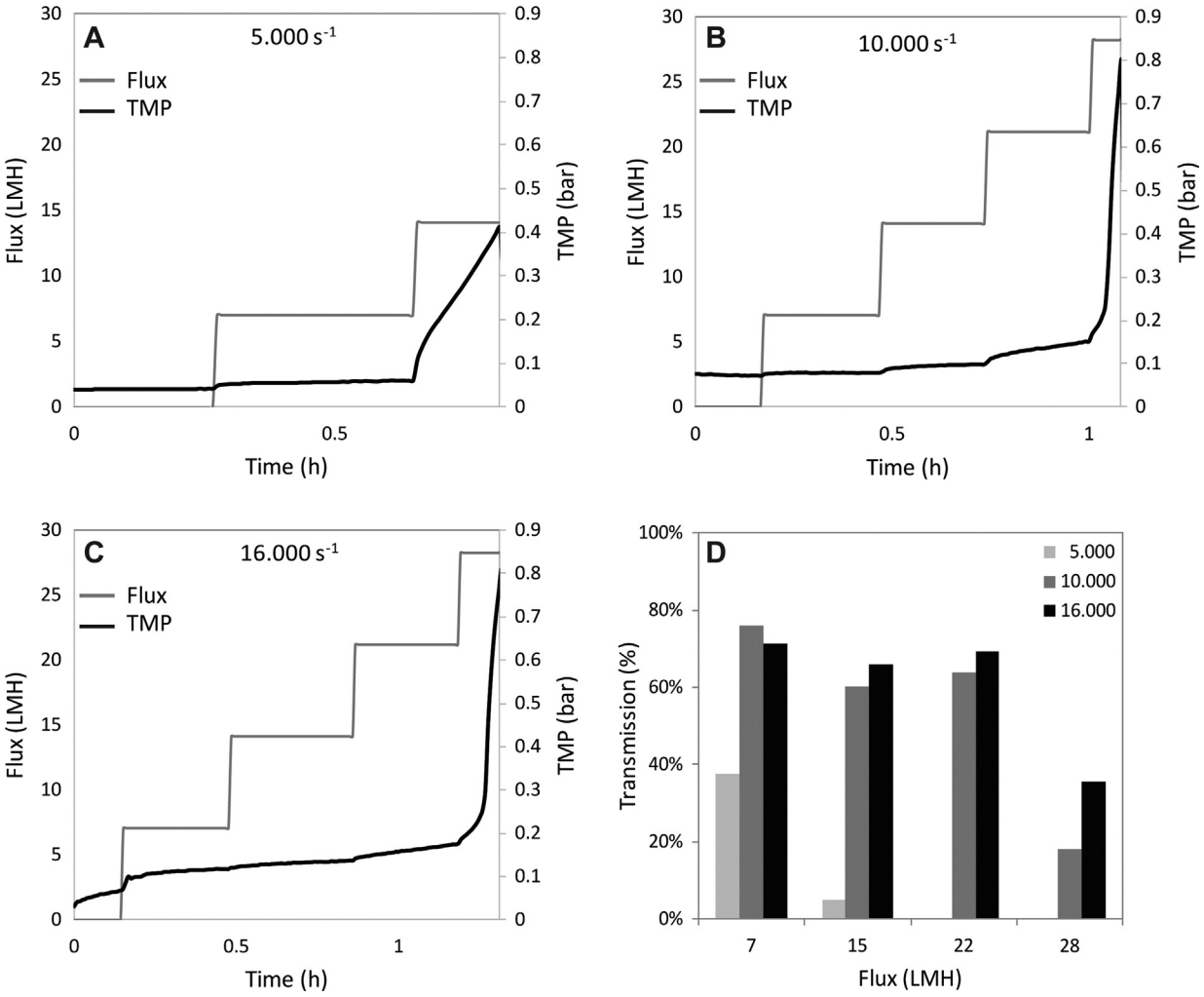


**Fig. 4.** Flux excursion of the TFF of a dissolved oxygen induced OMV harvest. The performance of constant flux-mode processing of a high dissolved oxygen induced sOMV harvest was tested. The permeate flux (grey line) at increasing transmembrane pressures (black line) was monitored at shear rates of 5000 $s^{-1}$ (A), 10,000 $s^{-1}$ (B), and 16,000 $s^{-1}$ (C). The resulting sOMV transmission is shown (D).

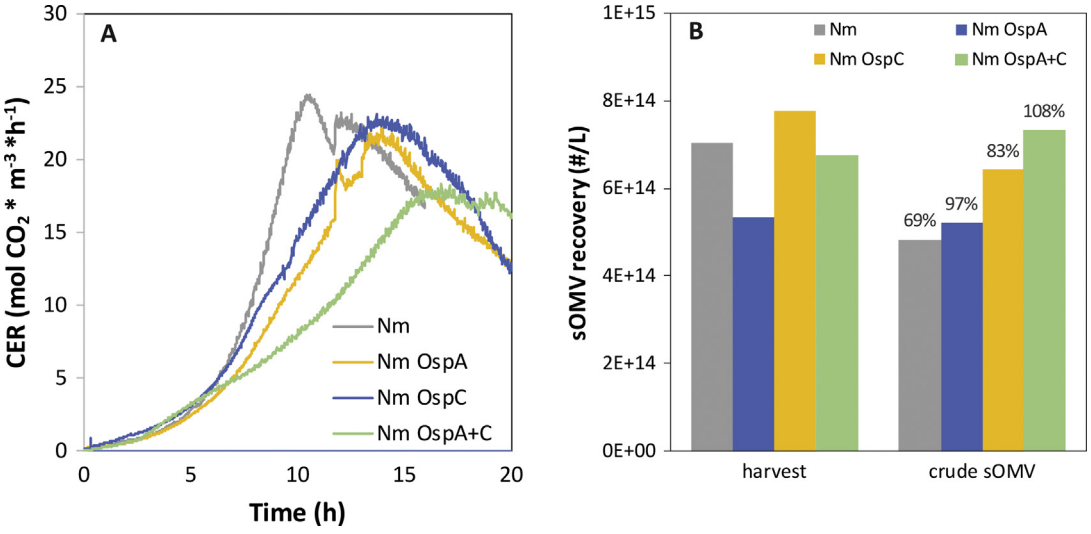


**Fig. 5.** Bacterial growth and sOMV production of *N. meningitidis* expressing *B. burgdorferi* antigens. Bacterial growth of Nm strains expressing heterologous antigens in batch cultures at high oxygen concentrations is plotted (A) by the carbon dioxide evolution rate (CER). The respiratory quotient remained 1.0 ± 0.05 throughout the cultivation for all cultures (data not shown). The resulting amounts of sOMVs in the harvested culture (harvest) and after removal of bacterial cells (crude sOMV) are shown in panel B.

6 ± 1 h after reaching the maximum carbon dioxide evolution rate, resulting in an average harvest of $2.0 \times 10^{15}$ sOMVs in 3 L culture. The optimized microfiltration process results in an average recovery of 90 ± 17% of sOMVs. Next, the sOMVs were further purified using the same unit operations as the detergent-free purification process (data not shown). In brief, sOMVs were treated with DNAse, concentrated and diafiltrated. After clarification, the sOMVs were further purified using size-exclusion chromatography and filter sterilized.

### 3.4. sOMV characteristics

The optimized sOMV production process was used to produce sOMVs and the effect of introducing different antigens on the characteristics of sOMVs was assessed. Purified sOMVs were highly similar in size distribution with a mean particle size of 105 nm (Fig. 6A). The average composition of purified OMVs also showed high similarities for the sOMVs containing heterologous antigens. The ratio of protein per vesicle and the ratio of LPS per vesicle was determined by combining concentration measurements of protein and LPS to the concentration of particles (Fig. 6B). The protein pattern on an SDS-PAGE gel is also very similar (Fig. 6C). The most abundant protein in all sOMVs is PorB (33.8 kDa). A minor amount of OspA (29.1 kDa) can be observed in the Nm OspA and Nm OspA + OspC OMVs. The fusion construct of OspC to fHbp (10.8 kDa) was not observed by SDS-PAGE, however it is visible by Western blot (Fig. 6D) at the expected size in the Nm OspC sOMVs and the Nm OspA & OspC sOMVs. Based on LC-MS/MS, an estimated relative protein content of 0.04% (w/w) and 0.07% (w/w) OspC was retrieved for the Nm OspC and Nm OspA + OspC OMVs. The estimated relative content of OspA in Nm OspA and Nm OspA + OspC OMVs was 2.2% (w/w) and 0.31% (w/w). Since repetitive epitope presentation is required in proper vaccine function [16], we estimated the average number of OspA and OspC antigens per vesicle based on the particle and protein concentration and the relative abundance of the antigens. The strains expressing single antigens contain 55 and 2 molecules of OspA and OspC per OMV, respectively. Simultaneous expression yields 10 molecules of OspA and 4 molecules of OspC per OMV.

Besides OspA and OspC, the overall protein composition of OMVs produced by the different production methods (eOMV, sOMV and sOMV by increased oxygen concentration) were compared. Based on the LC-MS/MS data the subcellular protein location of the OMV proteome was predicted by PSORTb. The sOMVs produced at high dissolved oxygen concentrations using the improved purification process are mainly composed of outer membrane proteins (55%) and cytoplasmic proteins (25%). The sOMVs produced at standard oxygen concentration show a comparable composition (Fig. 6E). However, eOMVs contain more cytoplasmic proteins (39%) than outer membrane proteins (37%) and are also enriched in cytoplasmic membrane associated proteins. Overall, eOMVs show a different protein composition to sOMVs. Expressing OspA or OspC on the surface of sOMVs does not impair the tested characteristics of the sOMVs. Furthermore, the results show that sOMVs formed at high dissolved oxygen concentrations have similar protein compositions to sOMVs produced at standard oxygen concentration.

## 4. Discussion

In this study, Nm sOMVs containing *B. burgdorferi* antigens were produced in a batch process by a purification process with scalable unit-operations. The heterologous antigens were expressed on the outer surface of the bacterium and were found in spontaneously

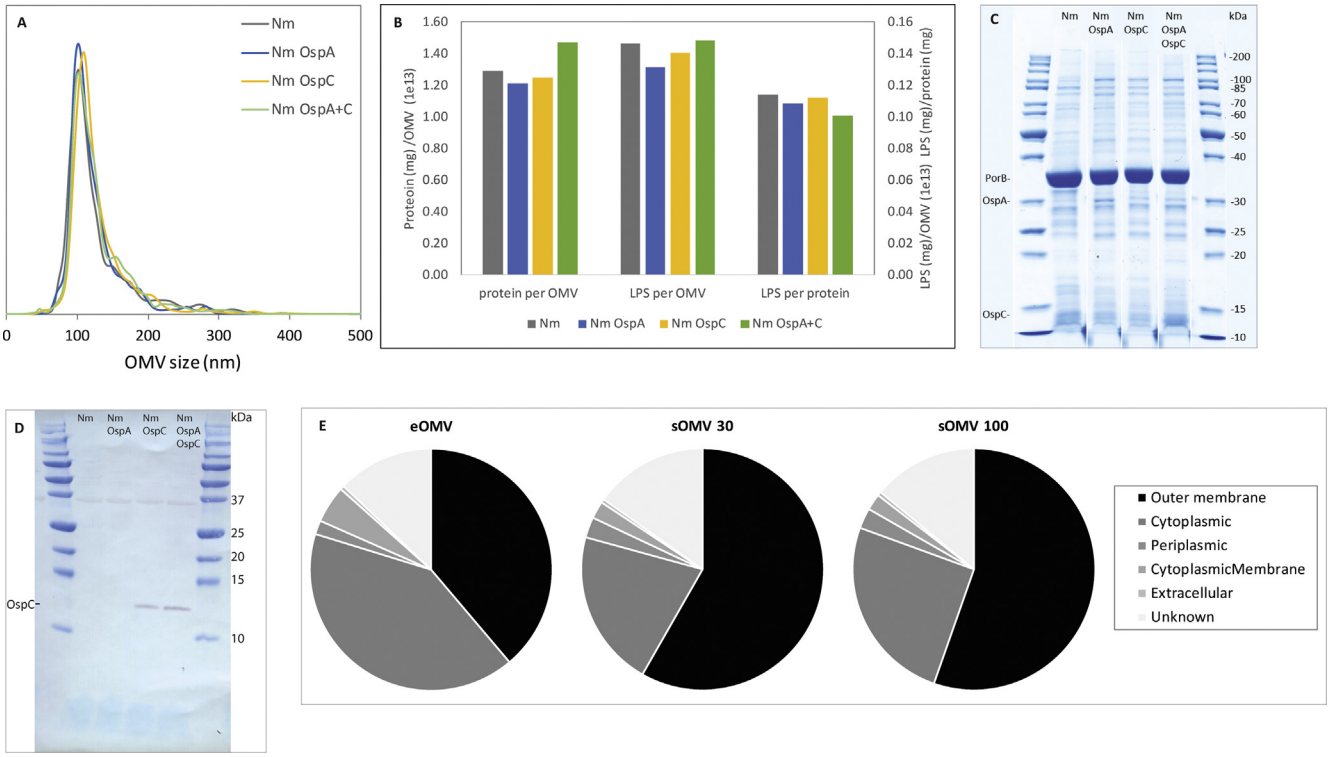


**Fig. 6.** *Characteristics of the OMVs.* Nm sOMVs produced at high oxygen concentrations using the improved purification method were characterized. The size distribution of Nm sOMVs containing heterologous antigens shows a mode size of 105 nm (A). The composition of the sOMVs is shown in amount of protein per OMV, amount of LPS per OMV and amount of LPS per protein (B). Protein composition of the sOMVs is analyzed by SDS-PAGE (C). OspC is visualized by Western blot (D). Proteins from OMVs with different production methods (eOMV, sOMV by cysteine depletion (sOMV 30), sOMV by high dissolved oxygen (sOMV 100) were measured by LC-MS/MS and their subcellular location was assessed by PSORTb (E).

released OMVs. For the production of OMVs, sOMVs are advantageous over eOMVs, because no OMV-extraction step is required, resulting in fewer unit operations. Here we applied high dissolved oxygen concentrations to bacterial cultures to stimulate sOMV production. Applying high dissolved oxygen concentrations to trigger sOMV release resulted in increased productivity, but was lowering the recovery during microfiltration. Microfiltration optimization showed that at constant TMP, sOMV transmission, but not protein transmission, is reduced in slightly fouling conditions. Using a larger pore size membrane and operating the tangential flow filtration at a constant permeate flux of 50% of the critical flux, improved the recovery to 90%. sOMV quality attributes were highly consistent between the different antigen expressing strains, and highly comparable to sOMVs produced at standard oxygen concentrations. However, the Zeta potential was measured in one of the intermate samples and a lower potential was found in the OMVs purified from the cultivations with a high dissolved oxygen concentration as compared to a low dissolved oxygen concentration. This reduced potential could have aided to the initial fouling of the membrane before optimization.

The amount of Nm sOMVs recovered in this study reached $2 * 10^{15}$ OMVs for a 3 L culture, corresponding to approximately 90 mg of OMV associated protein per liter culture (mg/L). This is an improvement over *N. meningitidis* eOMV production, where 20 to 50 mg/L was reached [42], and *N. meningitidis* dOMV production where up to 22 mg/L was reached [4,9]. It should be noted that the strains used in the other studies harbored different mutations than this strain. The parental strain, however, is the same. Other sOMV production described in literature include the *Shigella sonnei* sOMV yields of 140 mg/L [33], and 68 to 80 mg/L [10]. These yields were obtained from high-cell density cultures ($OD_{600}$ 35 to $OD_{600}$ 45), while this study is based on cultivation of up to 4 g dry weight per liter culture (gdw/L). Interestingly a high cell density *N. meningitidis* perfusion cultivation has been described to reach a biomass concentration of 58 gdw/L [6], indicating that there is a possibility of process intensification if enhanced productivities are desired.

Expressing heterologous antigens in *N. meningitidis* resulted in cultures with a lowered growth rate, however, production of sOMVs was maintained. More research is needed to assess the repeatability, the effect of additional antigens, and possibly the screening of clones or the use of an inducible production system could further improve the productivity of the heterologous antigens.

The number of antigens present on OMVs reported here are between 2 and 55 antigenic proteins per vesicle. These values were based on semi-quantitative LC-MS/MS protein composition measurements combined with nanoparticle tracking analysis of the OMV concentration and should thus be interpreted with some caution. Furthermore, the level of expression could have been influenced by the genomic location of the heterologous antigen as *OspA* was expressed by insertion in either *rmpM* or *lpxL1*. Hypothetically more antigens per vesicle would increase the potency of the composition, but it is unknown what an optimum composition would be. Together, these design criteria are important aspects of future research.

In designing an OMV production process, sOMVs are advantageous over eOMVs from a process perspective. Here we show that the composition of eOMV differs from sOMV, but it is unknown what the effect of OMV type is on the immunogenicity. sOMVs are enriched in outer membrane proteins, which are usually immunogenic proteins. For a nonavalent PorA based Nm serogroup B vaccine, the cross-protection against PorA not included in the strain were assessed for dOMV, eOMV and sOMV [41]. dOMVs did not show bactericidal titers against strains not included in the vaccine, whereas eOMVs showed some cross-protection against strains expressing PorA not included in the vaccine. sOMVs did elicit broad cross protection against all tested strains and a minor response against a strain lacking PorA. sOMVs are thus advantageous over eOMV and dOMV for vaccine compositions providing protection against the OMV-producing strain. However, further research is required to elucidate the differences between the type of OMV on the efficacy of an OMV-based vaccine platform.

In this platform design we use a pathogenic bacterium as production host, in contrast to common production bacteria that are closely related to commensal bacteria. This approach ensures that the vaccine platform does not cause unwanted responses against commensal bacteria. Another concern of vaccine platforms is the possible response against the platform itself, possibly causing immune dominance of the carrier. Currently it is unknown if reduction of the antigenicity of the OMV-backbone is required to yield an effective vaccine platform. Here we aimed to reduce the antigenic content of the backbone by removing the capsular polysaccharide, LPS and the antigen outer membrane porin A (PorA). However, the OMVs showed to be enriched in another porin, PorB. Nm PorB is a TLR-2 ligand [36], that may be advantageous because of its adjuvating function [30]. The observation of a large amount of PorB in sOMVs is contrary to the proteome of Nm sOMVs described by Lappann et al., where sOMVs were found to be reduced in PorA and PorB [21], which bind to the periplasmic protein RmpM [23]. Here we used a Nm strain without RmpM to enhance OMV release, which could explain the increase of PorB, since linkage of PorB with RmpM and the peptidoglycan is not possible in this strain.

Overall, this study demonstrates the feasibility of the production of an OMV-based vaccine platform based on the expression of heterologous antigens on *N. meningitidis* sOMV. We showed that the addition of two Lyme disease antigens affected the growth, but yielded similar amounts of OMVs with similar characteristics as the Nm OMV control. Purification of sOMVs was possible by optimizing the purification process. By applying high oxygen concentrations to the bacteria, yields of 90 mg/L highly pure OMVs were obtained. With this process it becomes feasible to produce sufficient *N. meningitidis* OMVs with various heterologous antigens and study their potential as future vaccines.

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## Conflict of interest

M.J.H. Gerritzen, M.L.M. Salverda and M. Stork are employees of Intravacc, and M.J.H. Gerritzen and M. Stork are co-inventors of a patent application for the use of increased dissolved oxygen tension as trigger for sOMV production (patent application no. EP17205138).

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.01.076.

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