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Quantitative Proteomics Reveals Distinct Differences in the Protein Content of Outer Membrane Vesicle Vaccines

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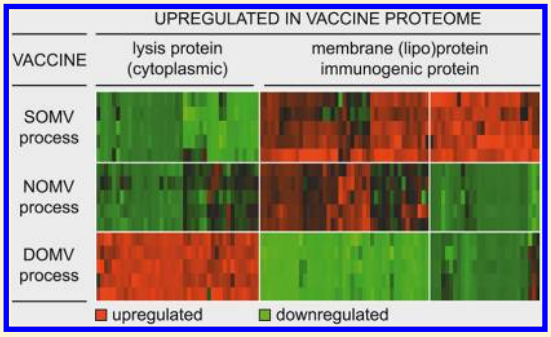
Supporting Information

ABSTRACT: At present, only vaccines containing outer membrane vesicles (OMV) have successfully stopped *Neisseria meningitidis* serogroup B epidemics. These vaccines however require detergent-extraction to remove endotoxin, which changes immunogenicity and causes production difficulties. To investigate this in more detail, the protein content of detergent-extracted OMV is compared with two detergent-free alternatives. A novel proteomics strategy has been developed that allows quantitative analysis of many biological replicates despite inherent multiplex restrictions of dimethyl labeling. This enables robust statistical analysis of relative protein abundance. The comparison with detergent-extracted OMV reveales that detergent-free OMV are enriched with membrane (lipo)proteins and contain less cytoplasmic proteins due to a milder purification process. These distinct protein profiles are substantiated with serum blot proteomics, confirming enrichment with immunogenic proteins in both detergent-free alternatives. Therefore, the immunogenic protein content of OMV vaccines depends at least partially on the purification process. This study demonstrates that detergent-free OMV have a preferred composition.



KEYWORDS: *quantitative proteomics, common reference, N-proteomics, PTAG, OMV, outer membrane vesicles, vaccine, immunogenicity*

INTRODUCTION

The use of outer membrane vesicles (OMV) is a promising approach for vaccine development against *Neisseria meningitidis* serogroup B, which causes acute and severe meningitis.[1−5] OMV consist of a phospholipid bilayer with outer membrane proteins, endotoxin and a lumen with periplasmic proteins.[6,7] Outer membrane porin A protein (PorA) was identified as the immunodominant antigen in OMV, but is antigenically variable between circulating strains.[8,9] To obtain a broadly protective vaccine, recombinant strains with multiple PorA subtypes were developed.[10,11] Recent studies demonstrated that conserved minor antigens, like factor H binding protein (fHbp, a lipoprotein) or iron-regulated membrane proteins, can complement PorA to further improve cross-protection.[12−14] In addition to well-described antigens the OMV proteome contains a considerable number of other proteins that may be relevant for immunogenicity.[15]

The first OMV vaccines were prepared with detergent-extraction (*detergent* OMV, DOMV) and have successfully stopped *N. meningitidis* serogroup B epidemics in several countries.[3−5,16−19] The detergent-extraction was required to remove endotoxin, but removed protective lipoproteins and caused partially intact and aggregated vesicles.[16,20] The *lpxL1* mutation successfully attenuated meningococcal endotoxin and allowed a detergent-free process for vaccine development.[21,22] A detergent-free process either uses extraction with a chelating agent to improve yield (*native* OMV; NOMV),[23−27] or no extraction at all (*spontaneous* OMV; SOMV).[28−30] It was confirmed that detergent-free OMV retain lipoproteins like fHbp, which improved cross-protection and functional immunogenicity in mice.[12,20,31,32] These immunological properties of OMV vaccines were measured with SBA (serum bactericidal activity), which is an established correlate of protection in humans, but does not provide in-depth information at the protein level.[33,34]

Proteomics has been used to assess the protein content of OMV in more detail. Initial studies on DOMV from *N. meningitidis* serogroup B used gel electrophoresis combined with LC−MS/MS peptide identification.[28,35−37] One study

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revealed that DOMV and SOMV vaccines have a different protein content.[31] More recently, quantitative 2D gel analysis with fluorescent labeling identified a limited number of differential proteins in DOMV after growth on two media.[38] Gel-based proteomics however is labor intensive and less compatible with hydrophobic membrane proteins from OMV than a gel-free approach.[39] It also has a strong bias toward highly abundant proteins.[40] To overcome these limitations, several gel-free quantitative proteomics methods have been developed.[41−43] Compared to other quantitative methods, multiplex dimethyl labeling of amino groups on N-termini and lysine residues is fast, robust and inexpensive.[41,44] When dimethyl labeling is performed at the protein level, the N-terminal part of the protein (blocked α-amino group) can be selectively purified from the internal peptides after proteolytic cleavage (free α-amino groups).[45−48] Such a positional proteomics strategy strongly reduces sample complexity and uncovers low-abundant peptides, while preserving the original proteome fingerprint.

Positional proteomics successfully addressed sample complexity issues observed in proteomes that are dominated by a few proteins, like human plasma or OMV.[49,50] Recently the PTAG strategy (phospho-tag) was developed for this purpose, using glyceraldehyde-3-phosphate reagent to derivatize free amino groups of internal peptides after proteolytic cleavage.[51] PTAG-modified internal peptides were depleted with efficient titanium dioxide (TiO_2) affinity chromatography, which purified protein N-terminal peptides for consecutive LC−MS/MS analysis. This PTAG strategy identified 572 unique proteins of *S. cerevisiae* and 428 unique proteins of *N. meningitidis* by their N-terminal peptides, representing one of largest N-proteome data sets available for these organisms. The study included 170 unique proteins from *N. meningitidis* NOMV vaccine, but dimethyl quantification was not implemented.

This work describes the first quantitative proteome comparison of detergent-extracted OMV vaccines with detergent-free alternatives. A novel quantification method was developed, based on dimethylation with stable isotopes and selective purification of N-terminal peptides. Distinct differences in protein content were observed, including several immunogenic proteins. These findings demonstrate that purification processes can change the protein content of OMV vaccines and support previously observed differences in functional immunogenicity.

MATERIALS AND METHODS

Strain, Growth Conditions and OMV Purification

The *N. meningitidis* vaccine strain that was used is a recombinant variant of isolate H44/76 (serogroup B), combining one wild type and two recombinant PorA antigens (trivalent PorA; subtypes P1.7,16; P1.5−1,2−2 and P1.19,15−1) with a non-functional *porB* gene.[10,52] The *cps* locus was deleted, resulting in a nonencapsulated phenotype with *galE*-truncated LPS. Additional deletions in *lpxL1* and *rmpM* genes were made to attenuate LPS toxicity and improve OMV yield, respectively.[20] Cultures were grown in chemically defined, iron-rich medium.[53] Erlenmeyer shake flasks with 150 mL medium were inoculated with 10 mL working seedlot (cells at OD_{590} = 1.5 ± 0.1; stored at −135 °C with glycerol). Preculture shake flasks were incubated at 35 °C, 200 rpm and 10 mL portions (OD_{590} of 1.5 ± 0.3) were used to inoculate secondary shake flasks. OD_{590} of the secondary flasks was monitored and bacteria were harvested for OMV purification after 5 h of stationary growth. OMV vaccines were purified as described previously.[20] For OMV quality control, total protein concentration (>1.0 mg/mL; Lowry with Peterson's modification), PorA content (>50% of total protein) and vesicle size distribution (average size 70−110 nm; polydispersity index <0.20) were performed.[23,54] As reported before, detergent-extraction caused aggregation of DOMV samples which resulted in high polydispersity. NOMV and SOMV vaccines passed all quality criteria.[20]

Dimethyl Labeling and N-Proteome Enrichment

Protein was extracted from OMV vaccines with Trizol reagent (Invitrogen, The Netherlands). An OMV amount corresponding to 500 μg of total protein was used for each extraction. OMV sample volume was first reduced to 20−50 μL by centrifugation at room temperature in a vacuum dryer (Eppendorf, The Netherlands) before adding 500 μL Trizol. Protein was extracted according to manufacturer's protocol and the resulting pellets were stored at −80 °C. Thawed pellets were dissolved in 50 μL buffer (100 mM KH_2PO_4, pH 7.5) containing 4 M Guanidine-HCl. Total protein concentration was measured using the Lowry assay with Peterson's modification (sample was diluted at least 100-fold to prevent interference of guanidine with the assay). SDS-PAGE analysis confirmed comparable protein compositions before and after Trizol extraction (data not shown). Protein labeling, digestion and purification protocols (including the PTAG strategy) were described previously.[51] The dimethyl labeling strategy was adapted for relative quantification purposes. The common reference (equal amounts of total protein from all experimental protein samples) and the individual experimental samples were incubated with either light formaldehyde label (CH_2O) or heavy label (CD_2O; Sigma Aldrich), respectively, with addition of sodium cyanoborohydride (Sigma Aldrich) for 24 h at room temperature. Each heavy labeled sample was pooled individually with light labeled common reference in a 1:1 (w/w) ratio and digested by trypsin under previously described conditions.[51] A sample aliquot was diluted 100 times in formic acid/DMSO in water (5/5/90, v/v) and stored at −20 °C until further analysis. Peptide mixtures after trypsin digestion were PTAG derivatized and the N-terminal peptides were recovered/enriched in flow-through fraction of TiO_2 affinity chromatography. Samples were dried and reconstituted in formic acid/DMSO in water (5/5/90, v/v) and stored at −20 °C until analysis.

Peptide Search List Compilation using Data-Dependent LC−MS/MS

A search list containing entries with unique combinations of peptide sequence, accurate mass and retention time window was obtained by data-dependent LC−MS/MS analysis.[55,56] Common reference sample aliquots were collected both before and after PTAG depletion and subsequently fractionated by strong cation exchange (SCX). The common reference samples were loaded onto a biphasic 200 μm ID trapping column packed with 20 mm 5 μm C18 resin (Reprosil Pur C18-AQ (Dr. Maisch, Ammerbuch-Entringen, Germany) and 20 mm 5 μm SCX resin (Polysulphoethyl A, PolyLC Inc., Colombia, MD) in 0.1 M HOAc at 5 μL/min. Following RP-to-SCX transfer using 50% acetonitrile in 0.1 M HOAc, peptide fractions were recovered by flushing the SCX bed stepwise with 2 μL plugs of potassium chloride in 0.1 mM acetic acid containing acetonitrile. Seven different acetonitrile concentrations were used in the plugs, ranging from 10 to 500 mM. Fractions were dried and reconstituted in formic acid/DMSO in water (5/5/90, v/v) and analyzed by LC−MS/MS. An LTQ-Orbitrap XL instrument (Thermo Fisher Scientific, The Netherlands) and Agilent 1100

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HPLC system (Agilent, The Netherlands) was modified for nanoflow LC separations as previously described.[57] The trap column was a 100-μm ID fritted microcapillary packed with 20 mm, 5 μm particle size Reprosil Pur C18-AQ particles (Dr. Maisch, Germany). The analytical column was a 50-μm ID fritted microcapillary packed with 31 cm 3 μm particle size Reprosil Pur C18-AQ. The column effluent was directly electrosprayed into the MS using an in-house prepared, gold and conductive carbon coated fused silica tapered tip of ~2 μm (typically at 2.0 kV).[57] Solvent A consisted of 0.1 M acetic acid in deionized water and solvent B of 0.1 M acetic acid in acetonitrile. Gradients were as follows: 100% solvent A during sample loading (0−10 min, flow rate 5 μL/min), 7% to 26% solvent B in 160 min followed by an increase to 60% solvent B in 20 min and reconditioning with solvent A for 10 min (total runtime 200 min). The mass spectrometer was set to acquire full MS spectra (m/z 350 to 1500) for mass analysis in the Orbitrap at 60000 resolution (fwhm) followed by data-dependent MS/MS analysis (LTQ) for the top 7 abundant precursor ions above a threshold value of 10^4 counts. The normalized collision energy was set to 35%, isolation width to 2.0 Da, activation Q to 0.250 and activation time to 30 ms. The maximum ion time (dwell time) for MS scans was set to 200 ms and for MS/MS scans to 2500 s. Charge state screening and preview mode were enabled. Precursor ions with unknown and +1 charge states were excluded for subsequent MS/MS analysis. Dynamic exclusion was enabled (exclusion size list with 500 entries) with repeat set to 1 and an exclusion duration of 180 s. The background ion at m/z 391.284280 was used as lock mass for internal mass calibration. Analysis of LC−MS/MS raw data was carried out with Proteome Discoverer 1.2 software (Thermo Fisher Scientific, Bremen, Germany) applying standard settings unless otherwise noted. MS/MS scans were searched against the *N. meningitidis* serogroup B database (Uniprot Knowledgebase, July 2012) using SEQUEST. Precursor ion and MS/MS tolerances were set to 10 ppm and 0.8 Da, respectively. Decoy database searches were performed with False Discovery Rate (FDR) tolerances set to 5% and 1% for modest and high confidence filtering settings, respectively. The data were searched with full trypsin cleavage specificity, allowing 5 miss-cleavages (lysine cleavage is prevented by dimethyl modification). Cysteine carbamidomethyl and lysine dimethylation were set as fixed modifications, while aspargine deamidation and methionine oxidation were set as variable modifications. An additional search was performed using C-terminal trypsin cleavage specificity and implementation a fixed N-terminal dimethyl modification. High confidence peptide sequence identifications (Xcorr values >2.2, false discovery rate <1%, rank No.1) were exported to an Excel data file (the peptide search list). Raw data files and protocols associated with this manuscript are available for the reader upon request.

High Resolution Peak Quantification using LC−MS

Quantification experiments of light (common reference) and heavy (experimental sample) labeled peptide mixtures were performed on the full MS level, relying on the high mass accuracy of the Orbitrap (data-dependent MS/MS disabled). All analyses were performed with the same nano LC column and identical gradient conditions as described above for maximal chromatographic reproducibility. Light/heavy peptide mixtures prior to PTAG-labeling and TiO_2 purification (15 samples) and N-terminally enriched peptide mixtures after TiO_2 purification (15 samples) were directly analyzed by LC−MS.

Data Processing for Analysis of Relative Protein Abundance

Relative peptide abundances were determined by using the high resolution LC−MS data in combination with the precompiled peptide search list (accurate mass and retention time approach).[55,56] Raw LC−MS data files were deconvoluted to monoisotopic masses to minimize isobaric interference of contaminants using Xcalibur software (Thermo Scientific; XtractAll plugin; MH+ mode; S/N threshold = 2).[58] The output was saved in NetCDF format (Thermo file converter) and imported in MS-Xelerator (MSMetrix, The Netherlands). The MSX-Quant plugin of this software traced and extracted the peak areas of all peptide entries of the precompiled Excel search list using accurate mass (±0.01 Da) and retention time (±10 min) information. Light/heavy peak area ratios were calculated from extracted ion chromatograms. Further data processing was performed in R (http://www.R-project.org). Individual MSX-Quant result files were compiled and stringent quality criteria were applied. A peptide search list entry was positively identified when both light and heavy labeled chromatographic peaks were extracted for at least 3 out of 5 independent biological replicates of at least 1 OMV vaccine type at an intensity threshold of $>10^4$ counts (above background noise). In addition, all chromatographic peaks were required to have an accurate mass deviation of <10 ppm and retention time window ±10 min compared to the search list entry. Starting with entries that were positively identified in any OMV vaccine type, it was determined whether or not these entries were identified in each of the three OMV vaccine types, by using the same quality criteria. The resulting lists of positively identified entries were mapped to corresponding peptides and proteins. The overlaps between the respective lists per OMV vaccine type were visualized as Venn diagrams.

Statistical Analysis of Relative Protein Abundance

For quantification of relative protein abundance, the light/heavy ratio values of positively identified peptide search list entries were ^{2}log transformed. For entries with a positive identification (found in ≥3 out of 5 replicates) for an OMV type, missing values (due to quality issues) were imputed as the mean of detected values. OMV types with a negative identification for a search list entry (found in ≤2 out 5 replicates) were imputed with background values. Background values were calculated from the mean of the minimal value for that entry across all experimental samples and the minimal value for that experimental sample across all search list entries. This ensured that both sample and entry-dependent background levels were taken into account. Next, log-transformed values of search list entries that belonged to the same peptide were averaged, followed by averaging of values from peptides that belonged to the same protein. The resulting protein expression data was analyzed across all OMV types with one-way ANOVA. This identified proteins that were differentially expressed between any of the OMV types (maximal Fold Ratio >2 and $p < 0.001$). This corresponded to a False Discovery Rate (FDR) of <1%. The expression values of differentially expressed proteins were visualized as a heat map using Genemaths XT (Applied Maths, Belgium). Functional annotations and keywords of identified proteins were adopted from Uniprot Knowledgebase, *N. meningitidis* strain MC58 (http://www.uniprot.org). A few entries were substituted with homologues from strain H44/76 or strain Alpha710, due to an outdated MC58 annotation. Predicted cellular locations were obtained with the PSORTb algorithm (http://www.psort.org/psortb).[59]

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Serum Blot Proteomics

Female BALB/c mice were immunized subcutaneously on day 0 and 28 with either DOMV, NOMV or SOMV vaccine and sera were collected on day 42, as previously described.[20] SDS gel electrophoresis was performed with 4 μg total protein of each OMV vaccine per lane.[20] Gels were either stained with Novex Colloidal Blue (Invitrogen, Carlsbad, CA) or blotted to nitrocellulose membranes and blocked with buffer containing 0.5% Protifar (Nutricia, The Netherlands). Blot membranes were incubated with pooled mice sera after immunization with the corresponding OMV vaccine (200× diluted), with monoclonal antibody against fHbp (NIBSC, United Kingdom) or with PorA P1.19 monoclonal antibody (RIVM, The Netherlands). Goat-antimouse antibody conjugated to alkaline phosphatase was used as a secondary antibody for staining. The protocol for direct surface digestion of blotted proteins was adapted from Luque-Garcia et al.[60] Differential serum bands were excised and incubated in 100 mM KH_2PO_4 (pH 7.5), 10% acetonitrille with trypsin at an enzyme/protein ratio of 1/20 (w/w) at 37 °C for 16 h. Peptide mixtures were purified with RP/SCX solid phase extraction as described above (peptide search list compilation paragraph). LC–MS/MS conditions and data analysis were also similar as above, however with 90 min. LC gradients (7–40% solvent B in 80 min). Cleavage specificity was set to full trypsin allowing 2 miss-cleavages. Aspargine deamidation and methionine oxidation were set as variable modifications. At least 2 unique, high-confidence peptides were required for protein identification and large deviation between theoretical and observed protein molecular weight on the immunoblot was not tolerated. Protein identifications that matched the immunoblot pattern were verified manually, to confirm presence of the corresponding tryptic peptides in the raw chromatographic data.

RESULTS

Development of Quantitative Proteomics Method

A novel quantitative proteomics method was developed based on duplex dimethyl labeling, combined with unbiased selection of N-terminal peptides (PTAG).[51] A detailed workflow is shown in Figure 1. Relative quantification was based on comparison of 15 experimental samples (3 different OMV vaccines; 5 biological replicates per vaccine) against a common reference (CR). The CR contained equal amounts of protein from all experimental samples. Samples for high-throughput quantification were collected after proteolytic digestion (all dimethylated peptides) and after PTAG depletion (N-terminal peptides). Quantification was performed with a two-step approach, using stringent quality criteria (Figure 2).[55] The CR allowed an unrestricted number of biological replicates despite inherent multiplex restrictions of the dimethyl labeling (either duplex or triplex[41,44]). As a result, each experimental group (OMV vaccine) had 3–5 independent measurements per peptide, allowing robust statistical analysis. Prior to OMV proteome analysis, the quantification method was successfully validated with a standard protein mixture containing known ratios (S-Table 1, Supporting Information).

OMV Protein Identification and Evaluation of the PTAG Procedure

The proteome analysis was performed on OMV vaccines from three different purification processes.[20] A total of 618 unique peptides were positively identified, 400 peptides in DOMV (detergent extraction), 509 peptides in NOMV (detergent-free extraction) and 497 peptides in SOMV (no extraction), with high

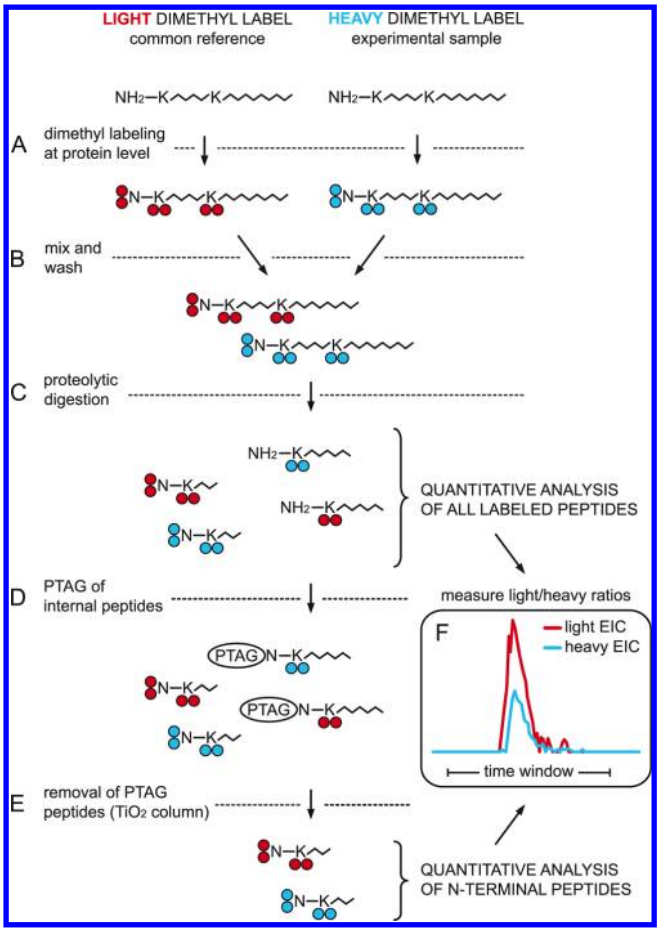

Figure 1. Workflow for purification of dimethylated N-terminal peptides with PTAG. (A) First, the common reference is mixed from equal amounts of protein from all experimental samples. Free N-terminal and lysine amino groups of the common reference are dimethylated at the protein level with formaldehyde (CH_2O, light label). In parallel, experimental samples are dimethylated with heavy formaldehyde (CD_2O isotope, heavy label). (B) Light and heavy labeled samples are mixed and washed with acetone precipitation. (C) Proteolytic digestion with trypsin generates internal peptides with free N-terminal amino groups. (D) Internal peptides are derivatized on the N-termini with PTAG reagent. (E) PTAG peptides are captured with TiO_2 columns for selective purification of dimethylated N-terminal peptides. (F) Samples are collected after step C (all dimethylated peptides) and after step E (N-terminal peptides) for quantification of light/heavy peak ratios with nano LC–MS (Figure 2).

reproducibility but moderate overlap between the three vaccines (282 peptides; Figure 3A). Merging of peptides with identical accession numbers yielded 185 unique proteins, of which 76 proteins were shared between the three OMV vaccines (Figure 3B). These results were obtained after merging of data sets from internal peptides and N-terminal peptides (S-Table 2B, Supporting Information). Added value of the PTAG strategy was evaluated by comparing the contribution of each data set to the total number of detected peptides (Figure 3C) and proteins (Figure 3D). At the peptide level, the PTAG strategy resulted in detection of 86 unique N-terminal peptides, of which 53 were not detected before the procedure. These additional N-terminal peptides accounted for 7 additional proteins, which indicates that most of the N-termini found with the PTAG strategy belonged to proteins that were already detected by their internal peptides (before PTAG). Cellular location prediction (PSORTb

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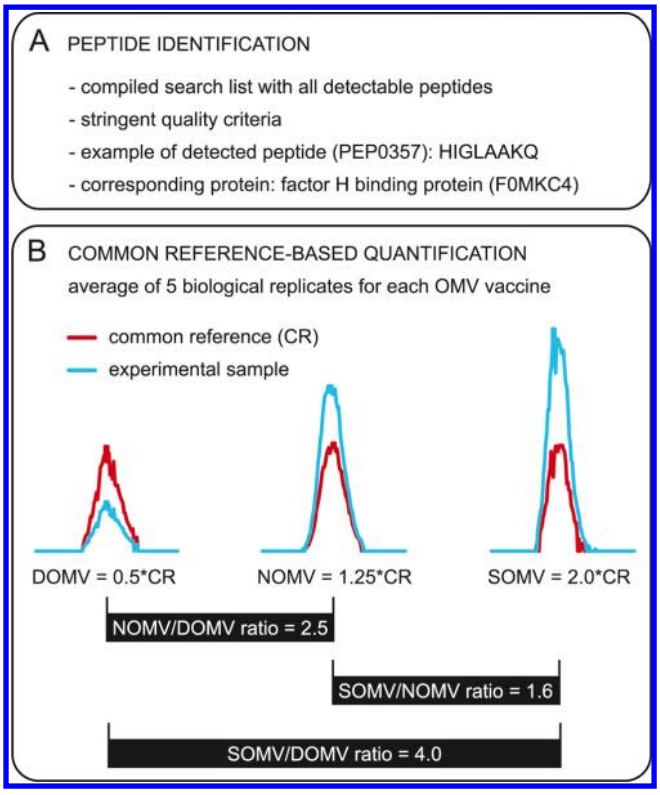

Figure 2. Common reference-based peptide quantification using stringent quality criteria. (A) High-throughput peptide identification was done with a two-step approach. First, the common reference (CR) was analyzed to compile a search list with accurate mass, retention time window and sequence ID of all detectable peptides (S-Table 2B, Supporting Information).[55,56] Second, full LC–MS chromatograms were acquired for all light/heavy mixtures. The light (CR) peptides were traced with the peptide search list. Corresponding heavy (experimental) peptides were matched based on calculated mass and retention time. Stringent criteria were required for a positive identification (see Material and Methods section). Search list entries that complied to these criteria nearly always provided a unique hit. (B) Peak areas were extracted for quantification of light/heavy ratios. The CR was used as an internal standard to calculate ratios between experimental samples, as illustrated for search list entry PEP0357, a peptide from factor H binding protein. After data processing, this protein had a low expression in DOMV compared to NOMV (2.5-fold higher) and SOMV (4.0-fold higher). Each detectable peptide in the search list was quantified with 3–5 independent biological replicates, allowing robust statistical analysis. See S-Table 2 (Supporting Information) for details.

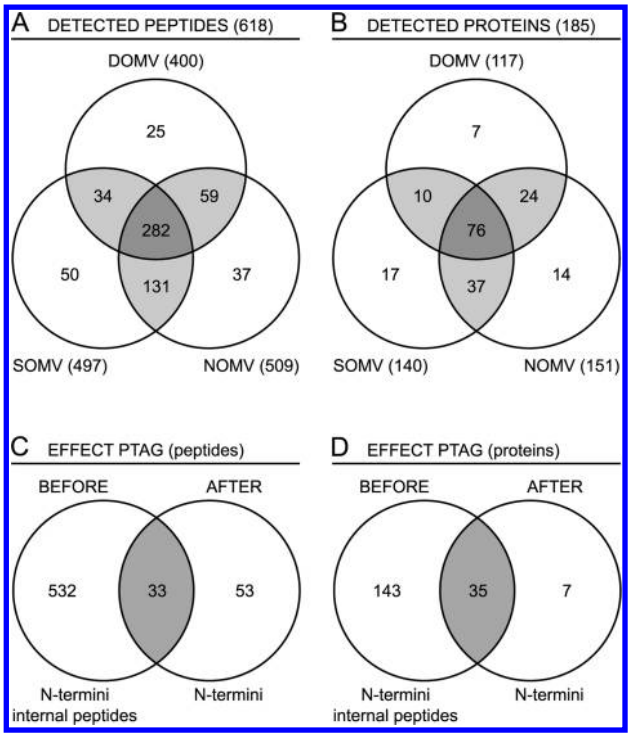

Figure 3. Venn diagrams of identified peptides and proteins. (A) Peptide overlap between DOMV vaccine (detergent-extraction), NOMV vaccine (detergent-free extraction) and SOMV vaccine (no extraction). A moderate overlap of 282 out of 618 unique peptides was observed. (B) Peptides corresponded to 185 unique proteins, of which 76 were shared. (C) Assessment of the added value of PTAG depletion at the peptide level. A total of 53 N-terminal peptides out of 86 (62%) were were uncovered by the PTAG procedure. (D) Additional N-terminal peptides represented only 7 additional proteins, since most proteins were already detected by their internal peptides. This indicates that OMV samples may not have sufficient complexity to challenge the PTAG procedure. S-Table 2 (Supporting Information) links the data in the Venn diagrams to corresponding peptides and proteins.

algorithm[59]) did not reveal enrichment in protein groups that were either detected before (143 proteins) or after PTAG (7 proteins; Figure 3D). The 35 proteins that belonged to both groups were enriched with a membrane location ($p < 0.012$). Since this group includes PorA, OpcA, PilQ and other major proteins of OMV, the observed enrichment is most likely related to protein abundance rather than a membrane location. A total of 42 proteins were identified by N-terminal peptides, of which 36 were positively verified. These N-termini were positioned at the first, second or SignalP predicted residue of the full protein sequence, or were cleaved after an AxA motif.[61] To account for unknown signal peptides, N-termini within the first 70 residues of the full protein sequence were also considered valid. The remaining 6 proteins were identified with degradation peptides from abundant proteins (e.g., PorA), which are also dimethylated during the PTAG procedure (neo N-termini). N-terminal peptides of lipoproteins were absent, due to a lipid modification that prevents dimethylation.[62] Details of the verification are provided in S-Table 2E (Supporting Information).

DOMV Vaccines Contain Cytoplasmic Proteins as a Result of Lysis

Relative protein abundance of all 185 detected proteins (Figure 3B) was quantified to identify differences between the OMV vaccines. A good overall correlation between biological replicates was observed ($R = 0.96$, 0.90, and 0.90 for DOMV, NOMV and SOMV, respectively). Proteins were clustered in 7 groups based on their expression profile (Figure 4). Group VII contained 39 proteins that were detected without significant differences in relative protein abundance (therefore not shown in Figure 4). This group included several well-known membrane proteins (PorA, OpcA, FetA).[63,64] Groups I to VI each had a specific expression pattern (e.g., downregulated in DOMV). Functional annotation revealed interesting differences. The 146 differentially expressed proteins in group I to VI together had 33 ribonucleoprotein annotations (from Uniprot keywords) of which 17 were found in group II (upregulated in DOMV; p-value <0.05). Group II was also significantly enriched in RNA-binding (14/23 annotations), nucleotide binding (9/17), cytoplasm (9/16) and transferase functions (7/12). These proteins originate from the cytoplasm and may be present exclusively in DOMV as

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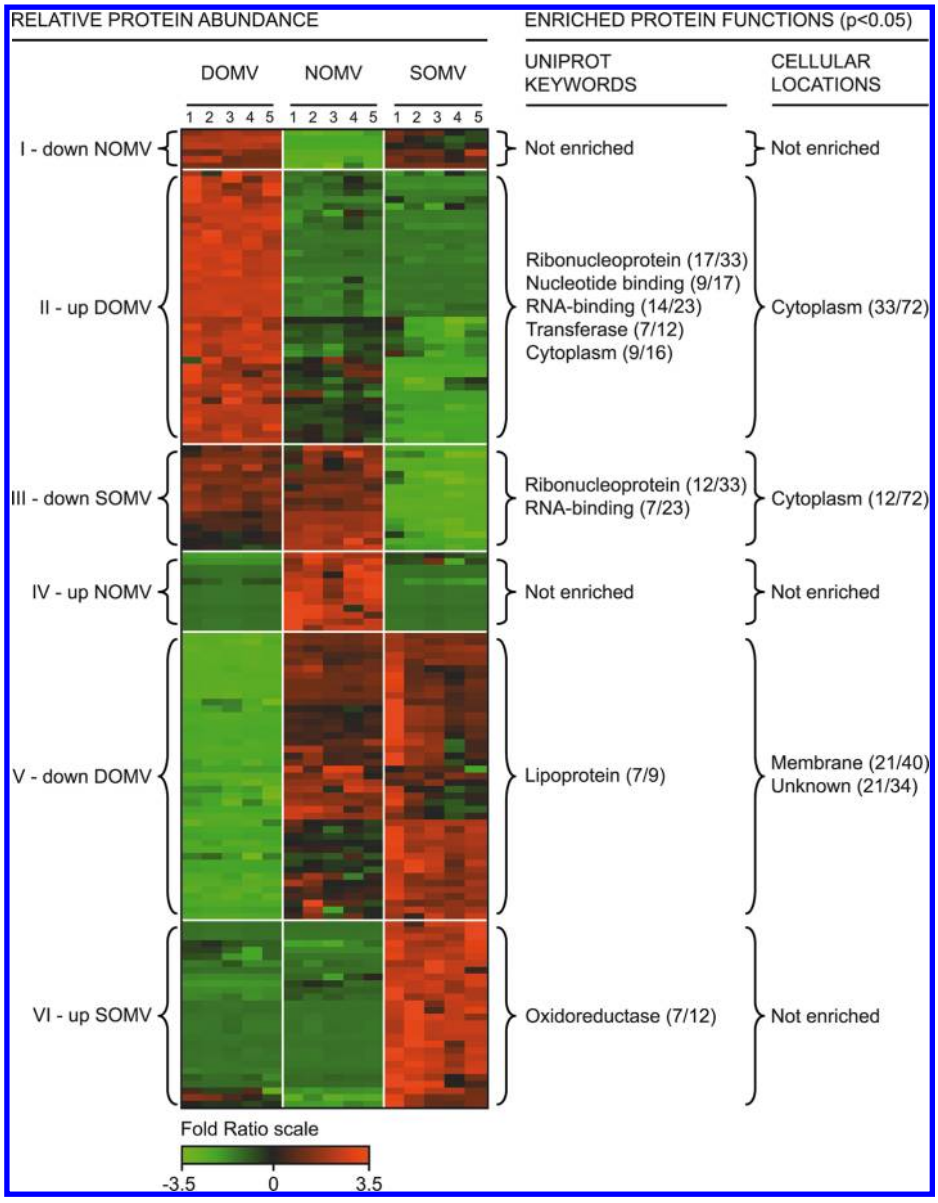

Figure 4. Differentially expressed OMV proteins. Quantitative proteome analysis was performed on OMV vaccines from 3 different purification processes, using 5 biological replicates per vaccine. Proteins were clustered in expression pattern groups, for example, group V (downregulated in DOMV). The color scale covers 3.5-fold downregulation (green), via no regulation (black) to 3.5-fold upregulation (red). Functional annotation was based on Uniprot keywords and predicted cellular locations (PSORTb algorithm). Enriched protein functions are depicted for each expression group ($p < 0.05$). The results demonstrate that OMV vaccines from biomass extraction processes (especially DOMV and to a lesser extent NOMV) are enriched with lysis-derived cytoplasmic proteins, including proteins with ribonucleoprotein, RNA-binding or nucleotide binding function. NOMV and SOMV vaccines (detergent-free processes) are enriched with membrane proteins and proteins with unknown location, including lipoproteins like fHbp. Therefore detergent-free OMV vaccines, SOMV in particular, have a preferred protein composition.

a result of lysis, an undesired side-effect of detergent extraction. Group III (upregulated in DOMV and NOMV) confirmed this hypothesis because it was also significantly enriched in ribonucleoprotein (12/33) and RNA-binding functions (7/23). This group however contained less proteins since NOMV purification (mild, detergent-free extraction) indeed caused less lysis than DOMV, but still more than SOMV (no extraction).

Detergent-free OMV Vaccines Are Significantly Enriched with Membrane (Lipo)proteins

The lysis-related results were further substantiated with cellular location predictions (PSORTb algoritm[59]), which confirmed that groups II and III indeed were enriched with cytoplasmic proteins. Group V (upregulated in detergent-free NOMV and SOMV vaccines) however was enriched with proteins that had a membrane location (21/40 annotations) or unknown location (21/34 annotations). Also, this group contained 6 out of 8 proteins with a lipoprotein annotation (all enrichment p-values <0.05). Proteins with such locations or functions are more likely to be relevant for the immunogenicity of OMV vaccines than lysis-derived cytoplasmic proteins. Notably, several oxidoreductases were found specifically in SOMV vaccines (group VI; 7/12 annotations). In addition to these redox-related proteins, SOMV vaccines contained proteins with nonenriched functions that are involved in iron uptake (e.g., heme utilization protein and bacterioferritin BfrA, which is regulated in response to iron availability[65,66]). Since SOMV are spontaneously released and

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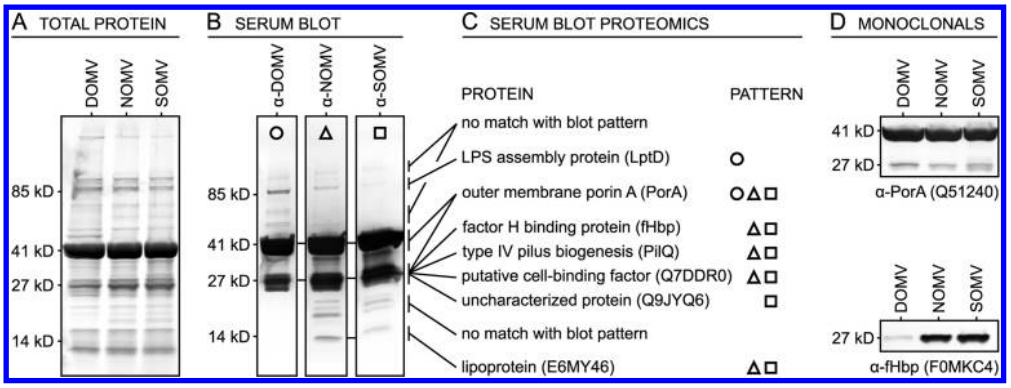

Figure 5. Serum blot proteomics reveals differences in immunogenic protein content. (A) Protein composition of OMV from three purification processes after SDS gel electrophoresis. (B) Sera of immunized mice were blotted against corresponding OMV vaccines. Despite a comparable SDS page pattern, the serum blots reveal differential immunogenicity (several variable bands at 50−85 kD and 14−27 kD) and confirm immunodominance of PorA (41 kD). (C) Serum blot proteomics identified proteins that matched the differential pattern (○, found in DOMV; △, NOMV; □, SOMV). This demonstrates that NOMV and SOMV vaccines (detergent-free process) are enriched with immunogenic proteins. (D) Results were verified with monoclonal antibodies against two protective antigens, PorA and fHbp. This confirms that PorA immunogenicity is not dependent on the purification process, while fHbp lipoprotein is largely removed after detergent-extraction (DOMV process).

most similar to natural OMV, these virulence-related proteins may be relevant for the pathogenicity of *N. meningitidis*.[67] Detailed protein information with expression data and functional annotation is provided in S-Table 2 (Supporting Information). An overview of the PSORTb location distribution per OMV vaccine is shown in S-Figure 1 (Supporting Information).

Serum Blot Proteomics Supports the Quantitative Proteomics Results for Several Immunogenic Proteins

Serum blot proteomics was performed to identify immunogenic proteins in the different OMV vaccines. Figure 5A shows the overall protein composition of the OMV vaccines after 1D gel electrophoresis, visualizing the major contributions of PorA (41 kD), Omp85 (85 kD) and several proteins at 27 kD (e.g., OpcA protein). As mentioned above, these proteins were found in all OMV types without significant expression differences. Next, mice sera after two vaccinations were used for immunoblotting against corresponding OMV vaccines. Despite a heavy immunodominance of PorA at 41 kD the serum blots revealed clear differences in immunogenicity, especially for DOMV compared to NOMV and SOMV (Figure 5B). This indicates that the different OMV processes retain or remove specific immunogenic proteins. These immunogenic proteins were not visible with 1D gel electrophoresis, therefore they represent a small but potentially important portion of the total protein content.

Immunoblot bands with a differential pattern between OMV sera were excised and digested separately with trypsin for qualitative LC−MS/MS analysis (serum blot proteomics). The analysis identified several proteins that matched the blot pattern (Figure 5C). At 41 kD, PorA was found abundantly on all blots. Lipoprotein E6MY46 (14 kD) was found on NOMV and SOMV blots, while LptD (LPS assembly protein; 85 kD) was unique for DOMV. In other sections no matching proteins were detected (100 kD, 50−70 kD and 15−20 kD) despite a differential serum blot pattern. Around 27 kD all serum blots had a visible band, but the bands for NOMV and SOMV were clearly more pronounced. Serum blot proteomics of this 27 kD band provided several interesting matches like PilQ, a protein involved in type IV pilus biogenesis and only found in NOMV and SOMV. Proteins Q7DDR0 (putative cell-binding factor) and Q9JYQ6 (uncharacterized protein) were found in NOMV and SOMV, and only in SOMV, respectively, but have not been described in literature. Two important identifications on the 27 kD blot section were fHbp and a C-terminal PorA fragment cleaved-off at the Pro_{268} position. These two immunogenic proteins were verified with monoclonal antibodies (Figure 5D). Detergent-extraction during DOMV purification is known to remove lipoproteins like fHbp.[12,29] The anti-fHbp immunoblot confirmed that fHbp indeed was absent in DOMV. The blot with anti-PorA monoclonal confirmed an overall strong band at 41 kD and confirmed the C-terminal PorA fragment. This PorA fragment explains the weaker 27 kD band on the DOMV serum blot, while the presence of fHbp explains the stronger 27 kD bands on NOMV and SOMV serum blots. Notably, the serum blot identifications were in full agreement with the quantitative proteomics results. Only LPS assembly protein LptD was somewhat ambiguous. LptD expression was 5-fold higher in DOMV, which may be induced by the detergent treatment and matches the serum blot results. The upregulation however was not significant (*p*-value just above threshold).

DISCUSSION

To investigate previously observed changes in functional immunogenicity, the proteomes of OMV vaccines from different purification processes have been compared and quantified.[20] Dimethyl labeling of free amines at the protein level is followed by selective purification of N-terminal peptides with the PTAG strategy.[51] This novel protocol combines the analysis of peptides before PTAG and N-terminal peptides after the PTAG procedure, using a straightforward workflow (Figure 1). Dimethylated peptides are quantified relative to a common reference, which represents an internal standard between all experimental samples (Figure 2). Strong cation exchange (SCX) fractionation followed by LC−MS/MS analysis is only required to compile a peptide search list, linking accurate mass and retention time to peptide and protein identifications.[55,56,68] Once the search list is available, a single LC−MS run for each experimental sample is sufficient to identify and quantify all detectable peptides in the OMV proteome. This accurate mass and retention time approach reduces total acquisition time and allowes a high number of independent biological replicates, resulting in robust statistical analysis of relative protein abundance. These advantages are not available with other

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quantitative proteomics methods, which mainly rely on weighed peptide fold-changes in a single biological sample for statistics.[42]

The PTAG strategy effectively reduces sample complexity and uncovers the majority of N-terminal peptides in this study. The uncovered N-terminal peptides correspond to only 7 new proteins, since most proteins are already detected by their internal peptides. A limitation of positional proteomics approaches like PTAG is undersampling of the N-proteome, because these methods rely on detection of a single N-terminal peptide.[51] As a result, the N-termini of lipoproteins are absent due to a lipid modification that prevents dimethylation. To our knowledge PTAG does not introduce any other experimental bias in this study. In addition, the strategy is likely to be beneficial for highly complex protein samples like cell lysates, because complexity reduction will improve specificity of the quantification. The present results comprise a data set of 618 peptides from 185 unique OMV proteins (Figure 3). Other studies identify between 25 and 166 OMV proteins with qualitative methods, like 1D or 2D gel electrophoresis and one gel-free approach.[31,35,36,39] A quantitative 2D electrophoresis study identifies 74 OMV proteins, of which 10 are differentially expressed.[38] Overlap of these studies with the current results is moderate to high (S-Table 3, Supporting Information), therefore this study is representative and comprises the largest quantitative OMV data set available to date.

Quantitative analysis of relative protein abundance has revealed distinct differences between OMV vaccines (Figure 4). Several proteins are absent in one or two vaccine types, resulting in large fold ratios (>10). The data set also contains a variety of subtle changes that remain unnoticed with a qualitative approach. Reproducibility of biological replicates is sufficient for reliable protein quantification, but the use of less than five independent replicates may reduce the statistical power to detect subtle differences. DOMV vaccines contain substantially more cytoplasmic proteins than NOMV or SOMV vaccines. These proteins may be released during detergent-extraction, which removes endotoxin from DOMV but apparently causes lysis and contamination with cytoplasmic proteins. A smaller but distinct set of cytoplasmic proteins is shared between DOMV and NOMV vaccines, while SOMV remains largely free of cytoplasmic contamination. This supports the extraction hypothesis, since NOMV are purified with a milder, detergent-free extraction and SOMV purification does not require any extraction at all. Other studies also found cytoplasmic proteins in DOMV, including one qualitative comparison of DOMV and SOMV from the New-Zealand vaccine strain.[31,36−39] The present study adds an intermediate vaccine (NOMV) to the comparison, which demonstrates that cytoplasmic protein contamination of OMV is directly related to the purification process that is used.

In addition to less cytoplasmic contamination, detergent-free OMV vaccines are significantly enriched with membrane proteins and proteins with an unknown cellular location. For SOMV vaccines, the difference is most pronounced (S-Figure 1, Supporting Information). Such proteins are more likely to be important for the immunogenicity of OMV vaccines than cytoplasmic proteins, which predominantly represent well-characterized aspects of microbial metabolism. The importance of unknown proteins is illustrated by the observation that 6 out of 9 lipoproteins in the data set have an unknown predicted location rather than a membrane location. The data also shows that most lipoproteins are selectively removed by detergent extraction. This includes factor H binding protein (fHbp), which contributes to functional immunogenicity and can be used as a purified protein vaccine.[32,69,70] SOMV vaccines contain an additional set of proteins with virulence-related functions. Therefore detergent-free OMV, in particular SOMV, have a preferred protein composition compared to DOMV.

The detergent-free OMV vaccines in this study previously had improved cross-protection and functional immunogenicity in mice,[20] which was confirmed by others.[12,31,32] Functional immunogenicity is measured with SBA, which correlates to protection in humans but does not provide in-depth information on the protein content of vaccines. This study now supplements SBA results with quantitative proteomics data. The results are refined with serum blot proteomics to visualize and assess differences in immunogenic protein content. Differential serum blot bands have been excised, digested with trypsin and analyzed with qualitative LC−MS/MS. Several antigens match the immunogenicity pattern on the serum blot, including PorA (constant pattern) and proteins that are found exclusively in detergent-free OMV like fHbp, type IV pilus assembly protein PilQ or putative cell-binding factor (Figure 5). Notably, the pattern of serum blot identifications is in full agreement with the quantitative proteomics results, indicating that both novel methods produce reproducible data. Serum blot proteomics demonstrates that detergent-free OMV are enriched with immunogenic proteins, but this does not necessarily translate to functional immunogenicity.[71] Differential endotoxin content of OMV vaccines should also be taken into account, since attenuated *lpxL1* endotoxin in detergent-free OMV vaccines adjuvates the immune response against proteins in the vaccine.[21,72] Also the endotoxin itself can contribute to functional immunogenicity.[24] Even though these aspects are not covered with proteomics, the current results provided valuable insight in the immunogenic protein content of different OMV vaccines. Such an approach can support functional assays like SBA.

■ CONCLUSIONS

A novel proteomics method has been developed that provides a quantitative fingerprint of complex protein products. Quantification is based on a common reference sample, which enables a high number of independent biological replicates for robust statistics. The method has broad potential applications in the field of biotechnology, like time course analysis of relative protein abundance in whole-cells, or batch-to-batch comparison of product consistency. This study compares the proteomes of OMV vaccines from different purification processes and reveals distinct protein profiles. Serum blot proteomics substantiates these results by identifying differential immunogenic proteins. The results indicate that the (immunogenic) protein content of OMV vaccines is at least partially determined by the purification process. This supports previously observed functional differences between OMV vaccines and illustrates that detergent-free OMV have a preferred protein composition.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figure and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

OMV, outer membrane vesicles; DOMV, *detergent* OMV (extraction with deoxycholate); NOMV, *native* OMV (detergent-free extraction with chelating agent); SOMV, *spontaneous* OMV (no extraction); PorA, outer membrane porin A protein; fHbp, factor H binding protein; SBA, serum bactericidal activity; PTAG, phospho tag reagent (glyceraldehyde-3-phosphate).

REFERENCES

(1) Girard, M. P.; Preziosi, M. P.; Aguado, M. T.; Kieny, M. P. A review of vaccine research and development: meningococcal disease. *Vaccine* **2006**, *24* (22), 4692−700.

(2) Jodar, L.; Feavers, I. M.; Salisbury, D.; Granoff, D. M. Development of vaccines against meningococcal disease. *Lancet* **2002**, *359* (9316), 1499−508.

(3) Bjune, G.; Hoiby, E. A.; Gronnesby, J. K.; Arnesen, O.; Fredriksen, J. H.; Halstensen, A.; Holten, E.; Lindbak, A. K.; Nokleby, H.; Rosenqvist, E.; et al. Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. *Lancet* **1991**, *338* (8775), 1093−6.

(4) Sierra, G. V.; Campa, H. C.; Varcacel, N. M.; Garcia, I. L.; Izquierdo, P. L.; Sotolongo, P. F.; Casanueva, G. V.; Rico, C. O.; Rodriguez, C. R.; Terry, M. H. Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. *NIPH Ann.* **1991**, *14* (2), 195−207. Discussion: 208−10.

(5) Thornton, V.; Lennon, D.; Rasanathan, K.; O'Hallahan, J.; Oster, P.; Stewart, J.; Tilman, S.; Aaberge, I.; Feiring, B.; Nokleby, H.; Rosenqvist, E.; White, K.; Reid, S.; Mulholland, K.; Wakefield, M. J.; Martin, D. Safety and immunogenicity of New Zealand strain meningococcal serogroup B OMV vaccine in healthy adults: beginning of epidemic control. *Vaccine* **2006**, *24* (9), 1395−400.

(6) Ellis, T. N.; Kuehn, M. J. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* **2010**, *74* (1), 81−94.

(7) Deatherage, B. L.; Lara, J. C.; Bergsbaken, T.; Barrett, S. L.; Lara, S.; Cookson, B. T. Biogenesis of Bacterial Membrane Vesicles. *Mol. Microbiol.* **2009**.

(8) van der Ley, P.; Heckels, J. E.; Virji, M.; Hoogerhout, P.; Poolman, J. T. Topology of outer membrane porins in pathogenic Neisseria spp. *Infect. Immun.* **1991**, *59* (9), 2963−71.

(9) Martin, D. R.; Ruijne, N.; McCallum, L.; O'Hallahan, J.; Oster, P. The VR2 epitope on the PorA P1.7−2,4 protein is the major target for the immune response elicited by the strain-specific group B meningococcal vaccine MeNZB. *Clin. Vaccine Immunol.* **2006**, *13* (4), 486−91.

(10) van der Ley, P.; van der Biezen, J.; Poolman, J. T. Construction of *Neisseria meningitidis* strains carrying multiple chromosomal copies of the porA gene for use in the production of a multivalent outer membrane vesicle vaccine. *Vaccine* **1995**, *13* (4), 401−7.

(11) van den Dobbelsteen, G. P.; van Dijken, H. H.; Pillai, S.; van Alphen, L. Immunogenicity of a combination vaccine containing pneumococcal conjugates and meningococcal PorA OMVs. *Vaccine* **2007**, *25* (13), 2491−6.

(12) Zollinger, W. D.; Donets, M. A.; Schmiel, D. H.; Pinto, V. B.; Labrie, J.; Moran, E. E.; Brandt, B. L.; Ionin, B.; Marques, R.; Wu, M.; Chen, P.; Stoddard, M. B.; Keiser, P. B. Design and evaluation in mice of a broadly protective meningococcal group B native outer membrane vesicle vaccine. *Vaccine* **2010**, *28* (31), 5057−67.

(13) Urwin, R.; Russell, J. E.; Thompson, E. A.; Holmes, E. C.; Feavers, I. M.; Maiden, M. C. Distribution of surface protein variants among hyperinvasive meningococci: implications for vaccine design. *Infect. Immun.* **2004**, *72* (10), 5955−62.

(14) Zollinger, W. D.; Poolman, J. T.; Maiden, M. C. Meningococcal serogroup B vaccines: will they live up to expectations? *Expert Rev. Vaccines* **2011**, *10* (5), 559−61.

(15) Wheeler, J. X.; Vipond, C.; Feavers, I. M. Exploring the proteome of meningococcal outer membrane vesicle vaccines. *Proteomics Clin. Appl.* **2007**, *1* (9), 1198−210.

(16) Claassen, I.; Meylis, J.; van der Ley, P.; Peeters, C.; Brons, H.; Robert, J.; Borsboom, D.; van der Ark, A.; van Straaten, I.; Roholl, P.; Kuipers, B.; Poolman, J. Production, characterization and control of a *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicle vaccine. *Vaccine* **1996**, *14* (10), 1001−8.

(17) Fredriksen, J. H.; Rosenqvist, E.; Wedege, E.; Bryn, K.; Bjune, G.; Froholm, L. O.; Lindbak, A. K.; Mogster, B.; Namork, E.; Rye, U.; Stabbetorp, G.; Winsnes, R.; Aase, B.; Closs, O. Production, characterization and control of MenB-vaccine "Folkehelsa": an outer membrane vesicle vaccine against group B meningococcal disease. *NIPH Ann.* **1991**, *14* (2), 67−79 discussion 79−80..

(18) de Moraes, J. C.; Perkins, B. A.; Camargo, M. C.; Hidalgo, N. T.; Barbosa, H. A.; Sacchi, C. T.; Landgraf, I. M.; Gattas, V. L.; Vasconcelos Hde, G.; et al. Protective efficacy of a serogroup B meningococcal vaccine in Sao Paulo, Brazil. *Lancet* **1992**, *340* (8827), 1074−8.

(19) Boslego, J.; Garcia, J.; Cruz, C.; Zollinger, W.; Brandt, B.; Ruiz, S.; Martinez, M.; Arthur, J.; Underwood, P.; Silva, W.; et al. Efficacy, safety, and immunogenicity of a meningococcal group B (15:P1.3) outer membrane protein vaccine in Iquique, Chile. Chilean National Committee for Meningococcal Disease. *Vaccine* **1995**, *13* (9), 821−9.

(20) van de Waterbeemd, B.; Streefland, M.; van der Ley, P.; Zomer, B.; van Dijken, H.; Martens, D.; Wijffels, R.; van der Pol, L. Improved OMV vaccine against *Neisseria meningitidis* using genetically engineered strains and a detergent-free purification process. *Vaccine* **2010**, *28* (30), 4810−6.

(21) van der Ley, P.; Steeghs, L.; Hamstra, H. J.; ten Hove, J.; Zomer, B.; van Alphen, L. Modification of lipid A biosynthesis in *Neisseria meningitidis* lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. *Infect. Immun.* **2001**, *69* (10), 5981−90.

(22) van der Ley, P.; van den Dobbelsteen, G. Next-generation outer membrane vesicle vaccines against Neisseria meningitidis based on nontoxic LPS mutants. *Hum. Vaccin.* **2011**, *7* (8), 886−90.

(23) van de Waterbeemd, B.; Streefland, M.; van Keulen, L.; van den IJssel, J.; de Haan, A.; Eppink, M. H.; van der Pol, L. A. Identification and optimization of critical process parameters for the production of NOMV vaccine against Neisseria meningitidis. *Vaccine* **2012**, *30* (24), 3683−90.

(24) Keiser, P. B.; Biggs-Cicatelli, S.; Moran, E. E.; Schmiel, D. H.; Pinto, V. B.; Burden, R. E.; Miller, L. B.; Moon, J. E.; Bowden, R. A.; Cummings, J. F.; Zollinger, W. D. A phase 1 study of a meningococcal native outer membrane vesicle vaccine made from a group B strain with deleted lpxL1 and synX, over-expressed factor H binding protein, two PorAs and stabilized OpcA expression. *Vaccine* **2010**, *29* (7), 1413−20.

(25) Pinto, V. B.; Moran, E. E.; Cruz, F.; Wang, X. M.; Fridman, A.; Zollinger, W. D.; Przysiecki, C. T.; Burden, R. An experimental outer membrane vesicle vaccine from N. meningitidis serogroup B strains that

induces serum bactericidal activity to multiple serogroups. *Vaccine* **2011**, *29* (44), 7752−8.
(26) Prachayasittikul, V.; Isarankura-Na-Ayudhya, C.; Tantimongcolwat, T.; Nantasenamat, C.; Galla, H. J. EDTA-induced membrane fluidization and destabilization: biophysical studies on artificial lipid membranes. *Acta Biochim. Biophys. Sin. (Shanghai)* **2007**, *39* (11), 901−13.
(27) Haque, H.; Russell, A. D. Effect of chelating agents on the susceptibility of some strains of gram-negative bacteria to some antibacterial agents. *Antimicrob. Agents Chemother.* **1974**, *6* (2), 200−6.
(28) Post, D. M.; Zhang, D.; Eastvold, J. S.; Teghanemt, A.; Gibson, B. W.; Weiss, J. P. Biochemical and functional characterization of membrane blebs purified from *Neisseria meningitidis* serogroup B. *J. Biol. Chem.* **2005**, *280* (46), 38383−94.
(29) Koeberling, O.; Seubert, A.; Granoff, D. M. Bactericidal antibody responses elicited by a meningococcal outer membrane vesicle vaccine with overexpressed factor H-binding protein and genetically attenuated endotoxin. *J. Infect. Dis.* **2008**, *198* (2), 262−70.
(30) Koeberling, O.; Seubert, A.; Santos, G.; Colaprico, A.; Ugozzoli, M.; Donnelly, J.; Granoff, D. M. Immunogenicity of a meningococcal native outer membrane vesicle vaccine with attenuated endotoxin and over-expressed factor H binding protein in infant rhesus monkeys. *Vaccine* **2011**, *29* (29−30), 4728−34.
(31) Ferrari, G.; Garaguso, I.; Adu-Bobie, J.; Doro, F.; Taddei, A. R.; Biolchi, A.; Brunelli, B.; Giuliani, M. M.; Pizza, M.; Norais, N.; Grandi, G. Outer membrane vesicles from group B *Neisseria meningitidis* delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. *Proteomics* **2006**, *6* (6), 1856−66.
(32) Koeberling, O.; Delany, I.; Granoff, D. M. A critical threshold of meningococcal factor h binding protein expression is required for increased breadth of protective antibodies elicited by native outer membrane vesicle vaccines. *Clin. Vaccine Immunol.* **2011**, *18* (5), 736−42.
(33) Borrow, R.; Balmer, P.; Miller, E. Meningococcal surrogates of protection−serum bactericidal antibody activity. *Vaccine* **2005**, *23* (17−18), 2222−7.
(34) Holst, J.; Feiring, B.; Fuglesang, J. E.; Hoiby, E. A.; Nokleby, H.; Aaberge, I. S.; Rosenqvist, E. Serum bactericidal activity correlates with the vaccine efficacy of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B disease. *Vaccine* **2003**, *21* (7−8), 734−7.
(35) Vipond, C.; Suker, J.; Jones, C.; Tang, C.; Feavers, I. M.; Wheeler, J. X. Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. *Proteomics* **2006**, *6* (11), 3400−13.
(36) Vipond, C.; Wheeler, J. X.; Jones, C.; Feavers, I. M.; Suker, J. Characterization of the protein content of a meningococcal outer membrane vesicle vaccine by polyacrylamide gel electrophoresis and mass spectrometry. *Hum. Vaccin.* **2005**, *1* (2), 80−4.
(37) Williams, J. N.; Skipp, P. J.; Humphries, H. E.; Christodoulides, M.; O'Connor, C. D.; Heckels, J. E. Proteomic analysis of outer membranes and vesicles from wild-type serogroup B Neisseria meningitidis and a lipopolysaccharide-deficient mutant. *Infect. Immun.* **2007**, *75* (3), 1364−72.
(38) Tsolakos, N.; Lie, K.; Bolstad, K.; Maslen, S.; Kristiansen, P. A.; Hoiby, E. A.; Wallington, A.; Vipond, C.; Skehel, M.; Tang, C. M.; Feavers, I. M.; Wedege, E.; Wheeler, J. X. Characterization of meningococcal serogroup B outer membrane vesicle vaccines from strain 44/76 after growth in different media. *Vaccine* **2010**, *28* (18), 3211−8.
(39) Gil, J.; Betancourt, L. H.; Sardinas, G.; Yero, D.; Niebla, O.; Delgado, M.; Garcia, D.; Pajon, R.; Sanchez, A.; Gonzalez, L. J.; Padron, G.; Campa, C.; Sotolongo, F.; Barbera, R.; Guillen, G.; Herrera, L.; Besada, V. Proteomic study via a non-gel based approach of meningococcal outer membrane vesicle vaccine obtained from strain CU385: A road map for discovery new antigens. *Hum. Vaccin.* **2009**, *5* (5), 347−56.
(40) Gevaert, K.; Van Damme, P.; Ghesquiere, B.; Impens, F.; Martens, L.; Helsens, K.; Vandekerckhove, J. A la carte proteomics with an emphasis on gel-free techniques. *Proteomics* **2007**, *7* (16), 2698−718.
(41) Boersema, P. J.; Raijmakers, R.; Lemeer, S.; Mohammed, S.; Heck, A. J. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat. Protoc.* **2009**, *4* (4), 484−94.
(42) Bantscheff, M.; Schirle, M.; Sweetman, G.; Rick, J.; Kuster, B. Quantitative mass spectrometry in proteomics: a critical review. *Anal. Bioanal. Chem.* **2007**, *389* (4), 1017−31.
(43) Cox, J.; Mann, M. Quantitative, high-resolution proteomics for data-driven systems biology. *Annu. Rev. Biochem.* **2011**, *80*, 273−99.
(44) Boersema, P. J.; Aye, T. T.; van Veen, T. A.; Heck, A. J.; Mohammed, S. Triplex protein quantification based on stable isotope labeling by peptide dimethylation applied to cell and tissue lysates. *Proteomics* **2008**, *8* (22), 4624−32.
(45) Gevaert, K.; Goethals, M.; Martens, L.; Van Damme, J.; Staes, A.; Thomas, G. R.; Vandekerckhove, J. Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides. *Nat. Biotechnol.* **2003**, *21* (5), 566−9.
(46) McDonald, L.; Beynon, R. J. Positional proteomics: preparation of amino-terminal peptides as a strategy for proteome simplification and characterization. *Nat. Protoc.* **2006**, *1* (4), 1790−8.
(47) McDonald, L.; Robertson, D. H.; Hurst, J. L.; Beynon, R. J. Positional proteomics: selective recovery and analysis of N-terminal proteolytic peptides. *Nat. Methods* **2005**, *2* (12), 955−7.
(48) Kleifeld, O.; Doucet, A.; auf dem Keller, U.; Prudova, A.; Schilling, O.; Kainthan, R. K.; Starr, A. E.; Foster, L. J.; Kizhakkedathu, J. N.; Overall, C. M. Isotopic labeling of terminal amines in complex samples identifies protein N-termini and protease cleavage products. *Nat. Biotechnol.* **2010**, *28* (3), 281−8.
(49) Guryca, V.; Lamerz, J.; Ducret, A.; Cutler, P. Qualitative improvement and quantitative assessment of N-terminomics. *Proteomics* **2012**, *12* (8), 1207−16.
(50) Wildes, D.; Wells, J. A. Sampling the N-terminal proteome of human blood. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107* (10), 4561−6.
(51) Mommen, G. P.; van de Waterbeemd, B.; Meiring, H. D.; Kersten, G.; Heck, A. J.; de Jong, A. P. Unbiased Selective Isolation of Protein N-terminal Peptides from Complex Proteome Samples Using Phospho Tagging (PTAG) and TiO2-based Depletion. *Mol. Cell. Proteomics* **2012**, *11* (9), 832−42.
(52) Holten, E. Serotypes of *Neisseria meningitidis* isolated from patients in Norway during the first six months of 1978. *J. Clin. Microbiol.* **1979**, *9* (2), 186−8.
(53) Baart, G. J.; de Jong, G.; Philippi, M.; Van't Riet, K.; van der Pol, L. A.; Beuvery, E. C.; Tramper, J.; Martens, D. E. Scale-up for bulk production of vaccine against meningococcal disease. *Vaccine* **2007**, *25* (34), 6399−408.
(54) Baart, G. J.; Willemsen, M.; Khatami, E.; de Haan, A.; Zomer, B.; Beuvery, E. C.; Tramper, J.; Martens, D. E. Modeling *Neisseria meningitidis* B metabolism at different specific growth rates. *Biotechnol. Bioeng.* **2008**, *101* (5), 1022−35.
(55) Smith, R. D.; Anderson, G. A.; Lipton, M. S.; Pasa-Tolic, L.; Shen, Y.; Conrads, T. P.; Veenstra, T. D.; Udseth, H. R. An accurate mass tag strategy for quantitative and high-throughput proteome measurements. *Proteomics* **2002**, *2* (5), 513−23.
(56) Conrads, T. P.; Anderson, G. A.; Veenstra, T. D.; Pasa-Tolic, L.; Smith, R. D. Utility of accurate mass tags for proteome-wide protein identification. *Anal. Chem.* **2000**, *72* (14), 3349−54.
(57) Meiring, H. D.; van der Heeft, E.; ten Hove, G. J.; de Jong, A. P. J. M. Nanoscale LC−MS(n): technical design and applications to peptide and protein analysis. *J. Sep. Sci.* **2002**, *25* (9), 557−68.
(58) Cappadona, S.; Baker, P. R.; Cutillas, P. R.; Heck, A. J.; van Breukelen, B. Current challenges in software solutions for mass spectrometry-based quantitative proteomics. *Amino Acids* **2012**, *43* (3), 1087−108.
(59) Yu, N. Y.; Wagner, J. R.; Laird, M. R.; Melli, G.; Rey, S.; Lo, R.; Dao, P.; Sahinalp, S. C.; Ester, M.; Foster, L. J.; Brinkman, F. S. PSORTb 3.0: improved protein subcellular localization prediction with refined

localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* **2010**, *26* (13), 1608−15.
(60) Luque-Garcia, J. L.; Zhou, G.; Spellman, D. S.; Sun, T. T.; Neubert, T. A. Analysis of electroblotted proteins by mass spectrometry: protein identification after Western blotting. *Mol. Cell. Proteomics* **2008**, *7* (2), 308−14.
(61) Petersen, T. N.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **2011**, *8* (10), 785−6.
(62) Kovacs-Simon, A.; Titball, R. W.; Michell, S. L. Lipoproteins of bacterial pathogens. *Infect. Immun.* **2010**, *79* (2), 548−61.
(63) Tsai, C. M.; Frasch, C. E.; Mocca, L. F. Five structural classes of major outer membrane proteins in Neisseria meningitidis. *J. Bacteriol.* **1981**, *146* (1), 69−78.
(64) Frasch, C. E. Vaccines for prevention of meningococcal disease. *Clin. Microbiol. Rev.* **1989**, *2* (Suppl), S134−8.
(65) van Ulsen, P.; Kuhn, K.; Prinz, T.; Legner, H.; Schmid, P.; Baumann, C.; Tommassen, J. Identification of proteins of Neisseria meningitidis induced under iron-limiting conditions using the isobaric tandem mass tag (TMT) labeling approach. *Proteomics* **2009**, *9* (7), 1771−81.
(66) Grifantini, R.; Sebastian, S.; Frigimelica, E.; Draghi, M.; Bartolini, E.; Muzzi, A.; Rappuoli, R.; Grandi, G.; Genco, C. A. Identification of iron-activated and -repressed Fur-dependent genes by transcriptome analysis of Neisseria meningitidis group B. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (16), 9542−7.
(67) Kulp, A.; Kuehn, M. J. Biological functions and biogenesis of secreted bacterial Outer Membrane Vesicles. *Annu. Rev. Microbiol.* **2010**, *64*, 163−84.
(68) Andreev, V. P.; Petyuk, V. A.; Brewer, H. M.; Karpievitch, Y. V.; Xie, F.; Clarke, J.; Camp, D.; Smith, R. D.; Lieberman, A. P.; Albin, R. L.; Nawaz, Z.; El Hokayem, J.; Myers, A. J. Label-free quantitative LC-MS proteomics of Alzheimer's Disease and normally aged human brains. *J. Proteome Res.* **2012**, *11* (6), 3053−67.
(69) Jiang, H. Q.; Hoiseth, S. K.; Harris, S. L.; McNeil, L. K.; Zhu, D.; Tan, C.; Scott, A. A.; Alexander, K.; Mason, K.; Miller, L.; DaSilva, I.; Mack, M.; Zhao, X. J.; Pride, M. W.; Andrew, L.; Murphy, E.; Hagen, M.; French, R.; Arora, A.; Jones, T. R.; Jansen, K. U.; Zlotnick, G. W.; Anderson, A. S. Broad vaccine coverage predicted for a bivalent recombinant factor H binding protein based vaccine to prevent serogroup B meningococcal disease. *Vaccine* **2010**, *28* (37), 6086−93.
(70) Snape, M. D.; Dawson, T.; Oster, P.; Evans, A.; John, T. M.; Ohene-Kena, B.; Findlow, J.; Yu, L. M.; Borrow, R.; Ypma, E.; Toneatto, D.; Pollard, A. J. Immunogenicity of 2 investigational serogroup B meningococcal vaccines in the first year of life: a randomized comparative trial. *Pediatr. Infect. Dis. J.* **2010**, *29* (11), ee71−9.
(71) Arigita, C.; Kersten, G. F.; Hazendonk, T.; Hennink, W. E.; Crommelin, D. J.; Jiskoot, W. Restored functional immunogenicity of purified meningococcal PorA by incorporation into liposomes. *Vaccine* **2003**, *21* (9−10), 950−60.
(72) Steeghs, L.; Tommassen, J.; Leusen, J. H.; van de Winkel, J. G.; van der Ley, P. Teasing apart structural determinants of 'toxicity' and 'adjuvanticity': implications for meningococcal vaccine development. *J. Endotoxin Res.* **2004**, *10* (2), 113−9.