Stability of mono- and trivalent meningococcal outer membrane vesicle vaccines

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(submitted)
Abstract

The stability during storage of outer membrane vesicles (OMV) of \textit{Neisseria meningitidis} group B was studied. Three types of OMV were compared for their stability, containing either one (monovalent) or three different PorA subtypes (trivalent), the latter with and without class 4 outer membrane protein (RmpM).

Aqueous formulations were stored at -70°C, 4°C, 37°C and 56°C. Some of the observed, unexpected changes in fluorescence characteristics were studied in more detail with tryptophan and tyrosine derivatives incubated at 56°C. Furthermore, the possibility to freeze-dry OMV was investigated.

The conformation of PorA and the physicochemical properties of the OMV were monitored during one year by electron microscopy, dynamic light scattering, fluorescence spectroscopy, and electrophoretic methods. The antigenicity of the PorA subtype P1.7-2,4, present in all formulations, was studied by ELISA with a bactericidal anti-P1.4 monoclonal antibody. The immunogenicity of OMV (i.e. bactericidal response) was determined in Balb/c mice.

When stored at -70°C or 4°C, the structure and immunogenicity of OMV was preserved. In contrast, storage of OMV at higher temperatures (37°C or 56°C) induced destruction of the OMV structure and denaturation of PorA, followed by chemical degradation, as well as a decrease or complete loss of immunogenicity. Changes observed in the fluorescence spectra of degraded OMV were also seen in tryptophan and tyrosine derivatives incubated at 56°C, indicating the occurrence of chemical degradation of tryptophan and tyrosine residues in PorA. Trivalent OMV were slightly more stable at 37°C than monovalent OMV as assessed by \textit{in vitro} methods, but these observations did not result in differences in the measured immunogenicity. The stability of trivalent OMV was not affected by the presence of RmpM. Both trivalent and monovalent OMV could be freeze-dried with preservation of their immunogenicity.

In conclusion, OMV are sensitive to degradation at elevated temperatures, but are fairly stable in the frozen state (-70°C) or when stored at 4°C in the liquid or freeze-dried state.
CHAPTER 2

Introduction

*Neisseria meningitidis* is a human pathogen and one of the major causes of bacterial meningitis and sepsis [1]. Vaccines against meningococci of serogroups A, C, Y and W-135 are based on capsular polysaccharides. However, the polysaccharide of group B meningococci is poorly immunogenic and cross-reactive with human tissues [2]. Vaccines containing outer membrane proteins (OMP) have been developed and have shown to induce protective immune responses [3,4]. The vaccines developed at the Netherlands Vaccine Institute (NVI, formerly a division of the National Institute of Public Health and the Environment, RIVM) consist of outer membrane vesicles (OMV) purified from genetically modified meningococcal strains expressing either one (monovalent) or three (trivalent) subtypes of class 1 OMP (PorA). Two different trivalent OMV are mixed to form a hexavalent vaccine that represents the majority of circulating subtypes in Western Europe. This vaccine has been proven to be safe and immunogenic [5]. The monovalent OMV vaccine contains PorA P1.7-2,4, the most prevalent subtype in The Netherlands and the subtype causing an epidemic in New Zeeland [1,9]. This vaccine is also safe and immunogenic in toddlers [6]. The trivalent strains contain class 4 outer membrane protein (RmpM). RmpM is firmly associated to PorA in the bacterial outer membrane, which is probably essential for the localization and the stability of the PorA trimers in the outer membrane [15].

In previous studies, we have shown that both the conformation and the presentation form determine the quality of the immune response against PorA [7]. Thus, both characteristics have to be maintained upon storage in order to preserve the vaccine functionality.

The composition of OMV is complex. The main constituents are: PorA, phospholipids, lipopolysaccharide (LPS), residual DNA, RmpM, and residual detergent [12]. The interaction of these compounds with each other through various non-covalent forces determine the physicochemical properties and stability of OMV. Diverse degradation processes may occur simultaneously during storage of OMV vaccines. Not only can the protein undergo chemical and conformational changes, but the OMV structure may also change or even be destroyed. However, it is not clear if changes in OMV affect the potency and efficacy of meningococcal vaccines.
The bactericidal activity of sera from humans after vaccination with OMV is accepted as correlate for protection [8].

To our knowledge, no studies on long-term stability of protein-containing vesicular systems such as PorA-OMV have been reported. In this study, trivalent OMV containing PorA subtypes P1.7-2,4, P1.5-2,10 and P1.12,13 were compared to monovalent OMV containing PorA subtype P1.7-2,4. The possible stabilizing effect of RmpM on trivalent OMV was also investigated. OMV containing subtype P1.7-2,4 were chosen, since 43% of all strains causing bacterial meningitis in The Netherlands belongs to this subtype [9]. At the same time, P1.7-2,4 is one of the weakest immunogens in multivalent OMV vaccines [5].

The stability of liquid (stored 4°C, 37°C and 56°C) and frozen (-70°C) PorA-OMV has been assessed during 12 months of storage. The possibility to freeze-dry OMV preserving its physicochemical and immunogenic characteristics was also studied. The stability of the presentation form was assessed with electron microscopy and dynamic light scattering measurements. The physicochemical stability of PorA was monitored by fluorescence spectroscopy, SDS-PAGE and ELISA. The functional immunogenicity (i.e. bactericidal response) of the vaccines was evaluated in vivo. The storage conditions for functionally stable OMV were determined.

**Materials and methods**

**Bacterial strains and growth conditions**

*N. meningitidis* strain F91 (P1.7-2,4, PorB⁻, RmpM⁻, low expression of Opa/Opc) was used for the production of monovalent PorA-OMV. Strains JB10124 (P1.5-2,10, P1.12,13, P1.7-2,4, PorB⁻, RmpM⁻, low expression Opa/Opc) and HP10124 (P1.5-2,10, P1.12,13, P1.7-2,4, PorB⁻, RmpM⁺, low expression Opa/Opc) were used for the production of trivalent PorA-OMV. Bacteria were grown in a defined medium, with as main components L-glutamic acid, glucose, L-cysteine and ammonium chloride, at 35°C for 15 h in a 40 L stirred fermentor under pH and pO2 control.

**Preparation of OMV formulations**

PorA-OMV were prepared as previously described [7]. Briefly, PorA-OMV were extracted from bacteria with EDTA. DNA was partially removed by DNase
treatment. Lipopolysaccharide (LPS) was partly depleted by gel permeation chromatography (GPC) in the presence of deoxycholate (DOC). In order to remove DOC, a second GPC step was performed, which also served to formulate the OMV in 10 mM Tris-HCl, 3% sucrose pH 7.4.

OMV derived from the three strains were stored at –70, 4, 37 and 56ºC in 2 ml plastic tubes with screw caps (Greiner, Alphen a/d Rijn, The Netherlands). For storage at 37ºC and 56ºC, 0.01% sodium azide was added. For fast freezing of OMV, 2 ml samples were submerged in an ethanol/dry ice mixture and immediately stored at –70ºC.

Freshly prepared OMV were freeze-dried in aliquots of 2 ml in 5-ml glass vials. The rubber stoppers were dried at 85ºC for 2 days prior to use. The vials containing the OMV formulations were placed directly on the shelf of a Leybold GT 4/6 freeze-dryer with a temperature of -35ºC. In the first step of the freeze-drying process, the temperature of the shelf was maintained at –35ºC and the chamber pressure set at 20 Pa. After 1-3 hours, the shelf temperature was raised to –25ºC and the pressure set to 5-6 Pa. These conditions were maintained for 65 hours, followed by additional secondary drying steps at plate temperatures of –20ºC, -5ºC, 10ºC, 25ºC and 35ºC, each for at least 5 hours at a pressure of 4-5 Pa. At the end of the freeze-drying process the vials were closed under vacuum with the dried rubber stoppers and capped with aluminium seals.

Freeze-dried OMV were stored at 4ºC until reconstitution. Analysis of frozen and liquid formulations was performed before storage (t=0) and after 3, 6 and 12 months. Freeze dried OMV were reconstituted and analyzed after 3 months storage.

Composition of OMV

The protein content of the OMV was measured according to Peterson [10] with BSA as standard. The PorA content (relative to total protein content) was determined by SDS-PAGE (see below). The presence of LPS was analyzed by gas chromatography (GC). In short, LPS was hydrolyzed in 3.45 M NaOH in 50% (v/v) methanol. Then, the fatty acids were methylated in 5.5 M HCl in 27% (v/v) methanol and extracted with a hexane/methyl tert-butyl ether mixture (1/1, v/v). Methylated fatty acids were separated and quantified by GC on an HP Ultra column (25 m x 0.2 mm, Agilent). The different fatty acids were identified based on their retention time using a calibration mixture of known fatty acids (Calmix, Midi, Newark, DE). The
amount of C14:3OH fatty acids was calculated with a calibration curve of *Serratia marcescens* LPS. As internal standard, C14:2OH was used. DNA content was determined by an ethidium bromide assay [11] with salmon sperm DNA (ICN, Rotterdam, The Netherlands) as standard.

The water content of freeze-dried OMV was measured with the Karl-Fisher method using a Mitubushi Moisture analyzer. Differential scanning calorimetry was performed to determine the glass-transition temperature of the freeze-dried OMV directly after drying and after 12 months of storage at 4°C.

*Physical characteristics of OMV*

To check the OMV structure after freeze-drying and storage, electron microscopy (EM) was performed as described by Claassen et al. [12]. The particle size of monovalent and trivalent OMV was measured by dynamic light scattering (DLS) at 25°C with a Malvern 4700 system equipped with a 75 mW Argon ion laser (488 nm, Uniphase, San José, CA, USA), a remote interface controller and PCS software, version 1.35 (Malvern Ltd., Malvern, UK). For refractive index and viscosity the values of water were used. The particle size distribution was reflected in the polydispersity index (PD), which ranges from 0.0 for a monodisperse to 1.0 for a heterodisperse dispersion. The zeta potential of the OMV was measured with a Malvern Zetasizer 2000 with an aqueous dip-in cell and a computer with PCS software (version 1.35, Malvern).

*Fluorescence measurements and guanidine denaturation assay*

The local environment of tryptophan (Trp) in PorA was monitored with fluorescence spectroscopy. Steady-state fluorescence measurements were performed with an LS-50B Luminescence spectrophotometer (Perkin Elmer, Norwalk, CT, USA).

Fluorescence emission spectra after excitation at 295 nm were recorded from 300-450 nm at 150 nm/min, with excitation and emission band widths of 2.5 and 5 nm, respectively. Samples contained either 25 µg/ml protein (F91, JB10124) or 20 µg/ml (HP10124). Sets of 5 spectra were averaged and corrected for buffer background.

Upon incubation with increasing concentrations of guanidinium hydrochloride (Gnd-HCl), PorA in OMV denatures, causing increased exposure of fluorescent
groups (Trp), which results in a shift of the fluorescence maximum towards higher wavelengths [13]. Samples were diluted with Gnd-HCl to final Gnd-HCl concentrations ranging from 1 M to 5 M, and incubated for 2 hours at room temperature before measurement. Fluorescence spectra of these samples were measured as described above. Sets of 5 spectra were averaged and corrected for buffer/Gnd-HCl background. The emission maximum was plotted against the Gnd-HCl concentration. The curves obtained were fitted, assuming a two-state equilibrium between the native and the denatured protein [14]. The concentration of Gnd-HCl necessary to obtain 50% denaturation of PorA was determined based on the midpoint of the denaturation curve.

**Analysis of the denaturation of model compounds**

N-acetyl tryptophan amide (NATrA), N-acetyl tyrosine amide (NATyA) were dissolved in 10 mM Tris-HCl, 3% sucrose pH 7.4 plus 0.01% sodium azide and incubated at 56ºC. Spectral properties of the samples were monitored during 6 months. UV spectra (200-400 nm) of samples were taken with a Perkin-Elmer spectrophotometer and normalized (Abs_{280} = 1). Samples used for fluorescence measurements had an absorbance maximum \( \leq 0.1 \). Fluorescence emission spectra after excitation at 295 (for tryptophan) and 280 nm (tyrosine) were recorded as described above.

**SDS-PAGE and Western blotting**

The trimeric folding of PorA was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in ‘native’ gels as described by Jansen et al. [15]. Prior to electrophoresis, samples were incubated for 10 min at room temperature or 100ºC in sample buffer [16] containing 0.05% SDS. Polyacrylamide gels were prepared as described [16], except that SDS was omitted from the stacking and running gels. The acrylamide concentration of the running gel was 12%. Gels were run at 20 mA in a temperature-controlled room (4ºC) to prevent denaturation of the formulations containing folded PorA. Gels were stained with Coomassie Brilliant Blue.

Relative PorA content was measured by SDS-PAGE under denaturing conditions [16]. Gels were scanned after running and staining in an Agfa Fotolook 3.5
scanner and analyzed with Phoretix 1D Quantifier software (version 5.10, Biozym, Landgraaf, The Netherlands) for determination of the relative PorA content.

Western blotting was performed as described [17] after SDS-PAGE under denaturing conditions. The monoclonal antibodies (mAb) used to detect the different PorA subtypes were MN20B9.34 (anti-P1.4); MN20F4.17 (anti-P1.10); MN20A7.10 (anti-P1.12) and MN3B9F (anti-RmpM).

PorA inhibition ELISA

Antigenicity of the P1.4 epitope of PorA P1.7-2,4 in OMV was tested by an inhibition ELISA as previously described using the mAb MN20B9.34 [7]. Briefly, a dilution series of the sample was incubated with a fixed concentration of mAb. Free mAb was quantified with an OMV-coated ELISA plate. The coating OMV batch contained at least 90% PorA, as determined by SDS-PAGE. The results are shown as the ratio between the PorA concentration determined by ELISA and the protein concentration determined by Peterson (total protein) corrected for protein impurities. As a reference, the antigenicity of freshly produced monovalent OMV was arbitrarily set at 1.

Immunization experiments

Balb/c mice, 6 to 8 weeks old (10 animals in each group) were immunized subcutaneously on day 0, 14 and 28. PorA (1.5 µg/subtype) in OMV were administered adjuvated with 1.5 mg AlPO₄ (NVI) (0.25 ml/mouse) (see Table 3). The mice were bled on day 42, and sera were collected and stored at -20ºC until analysis.

Anti-PorA whole-cell ELISA

The antibody titer (total IgG) of each individual mouse serum was determined by whole cell ELISA as described [18]. Neisseria meningitidis isogenic H44/76-based strains (B:15P1.7,16:L3,7,9) expressing P1.7-2,4 (H44/76 7-2,4), P1.12,13 (H44/76 12,13) or P1.5-2,10 (H44/76 5-2,10), and the H44/76-derived mutant strain HI5 (lacking PorA) were used. Titers were determined with goat anti-mouse (GAM) total IgG conjugates, labeled with horseradish peroxidase (HRP) (Southern Technology Associates; dilution 1/5000). The titer is defined as the dilution of the serum where 50% of the ODₘₐₓ in the assay is reached.
Serum bactericidal assay

The serum bactericidal activity was measured as previously described [19] against the four *N. meningitidis* strains: H44/76 7-2,4; H44/76 12,13; H44/76 5-2,10 and Hi5 (PorA'). From each group of 10 mice, 2 pools were made (5 mice/pool) and every pool was tested at least twice. Pooled sera were heat inactivated for 30 min at 56°C prior to use. Serum samples and bacteria were incubated for 10 to 15 min at room temperature before the addition of complement. A final concentration of 80% (v/v) rabbit serum was used as complement source. As positive controls, the bactericidal anti-P1.4 (MN20B9.34) and anti-LPS (MN15A17F12) monoclonal antibodies were used. Also, test sera were incubated without complement as a negative control. The serum bactericidal titer was measured as the reciprocal serum dilution showing more than 90% killing of the number of bacteria used.

Statistical methods

Before statistical analysis, antibody and bactericidal titers were log_{10} converted. IgG titers are expressed as the mean log_{10} titer of ten independent observations. Bactericidal titers are expressed as the mean log_{10} titer of at least four independent observations. Analysis of variance was used for statistical evaluation of the data. The significance of the differences between the mean values of the antibody and bactericidal titers was determined by the least-significant-difference (LSD) test at a confidence level of 95%.

Results

Characterization of OMV

The composition of the OMV derived from the three strains (F91, JB10124 and HP10124) is shown in Table 1. The protein present in all OMV was mainly PorA. Most impurities were found in OMV from strain JB10124. OMV had a negative surface charge (zeta potential) ranging between -40 and -50 mV.

The average particle size of freshly prepared OMV ranged between 150-350 nm, with a rather high polydispersity around 0.4, typical of OMV (Table 1). Both particle size and polydispersity were smaller for OMV derived from the trivalent strain JB10124, indicating a more homogenous particle size distribution for these OMV. The presence of partly aggregated vesicles with a diameter around 80 nm for all
OMV was shown by electron microscopy (see Fig. 2 A for a representative picture). Both freezing and freeze-drying of OMV induced the formation of large aggregates, accompanied by an increase of the polydispersity index (see Fig. 1 A-D). The size of the aggregates was smaller in freeze-dried OMV than in freeze-thawed OMV. A representative picture of the aggregates present after freeze and thawing can be observed in Fig. 2 B.

**Table 1. Physicochemical characteristics of the OMV used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>% PorA</th>
<th>PorA subtypes</th>
<th>Class 4 protein</th>
<th>LPS content (%)</th>
<th>DNA content (%)</th>
<th>Particle size (nm)</th>
<th>PDd</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F91 (monovalent)</td>
<td>94</td>
<td>P1.7-2.4</td>
<td>-</td>
<td>9.4</td>
<td>7.7</td>
<td>362</td>
<td>0.42</td>
<td>-49.4</td>
</tr>
<tr>
<td>JB10124 (trivalent)</td>
<td>76</td>
<td>P1.7-2.4, P1.12,13, P1.5-2.10</td>
<td>-</td>
<td>10.6</td>
<td>19.2</td>
<td>138</td>
<td>0.22</td>
<td>-41.8</td>
</tr>
<tr>
<td>HP10124 (trivalent)</td>
<td>87</td>
<td>P1.7-2.4, P1.12,13, P1.5-2.10</td>
<td>+</td>
<td>3.7</td>
<td>8.2</td>
<td>304</td>
<td>0.45</td>
<td>-47.5</td>
</tr>
</tbody>
</table>

- Determined by scanning the SDS-PAGE profile.
- Relative to the total amount of protein present in the formulation.
- The presence of class 4 protein was demonstrated by Western blotting with monoclonal antibody MN3B9F.
- Polydispersity: indication of the size distribution of the OMV; ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion.

The water content of freeze-dried OMV was below 3% for all formulations. Freeze-dried OMV showed a glass transition temperature of ca. 50ºC. These characteristics were maintained over one year of storage at 4ºC.

The fluorescence emission spectrum of PorA was similar in OMV derived from the three different strains studied. The emission maximum was around 338 nm, indicating a relatively hydrophobic environment of the tryptophan residues [13]. Upon titration with Gnd-HCl, a shift in the fluorescence maximum was observed from ca. 338 nm to ca. 350 nm. This shift was similar in magnitude for OMV from the three strains. The denaturation midpoint was at 3.8 M Gnd-HCl for monovalent OMV and slightly higher (4.1 M) for both trivalent OMV.

The trimeric structure typical of native PorA was monitored by ‘native’ SDS-PAGE (Fig. 3) [15]. Freshly prepared PorA-containing OMV showed the presence of a high molecular weight band (~130 kDa) corresponding to PorA trimers (Fig. 3 A, lanes a); no monomers were visible. In samples incubated at 100ºC prior to electrophoresis the trimers were converted to monomers (41 kDa) (Fig. 3 A, lanes b).
Freeze-drying of OMV did not induce changes in the electrophoretic characteristics of PorA in the three OMV studied (not shown).

**Figure 1.** Panels A, B, C: effect of storage on the particle size of OMV of strains F91 (A), JB10124 (B) and HP10124 (C). Frozen samples were stored at -70ºC (squares); liquid samples at 4ºC (diamonds), 37ºC (triangles) or 56ºC (cross). Bars indicate SD (n=3). **Panel D:** effect of freeze drying on the particle size of OMV. The bars indicate the particle size ± SD (n=3) of OMV before (grey) and after (white) freeze-drying and reconstitution after 3 months storage at 4ºC. The numbers above the bars indicates the polydispersity of the samples.

**Figure 2.** Electron micrographs of OMV stored at 4ºC (A: strain JB10124), -70ºC (B: strain F91) and 56ºC (C: strain HP10124) during 6 months. The bar indicates 0.2 µm. Micrographs are representative for all three OMV stored at the indicated temperature.
Figure 3. SDS-PAGE under mild conditions (see materials and methods) of OMV after production (A), after 6 months storage (B) or after 12 months storage (C). In panel A is indicated the molecular weight of PorA monomers (43 kDa) and trimers (~130 kDa). The OMV strains and the storage temperature are indicated above the gels. Each sample was mixed with sample buffer containing 0.05% SDS and incubated either at room temperature (lanes a) or at 100°C (lanes b).

The interaction of PorA P1.7-2,4 with the bactericidal mAb MN10B9.34 (directed against loop 4 of this PorA subtype) was studied by ELISA (Fig. 4). When assuming that the ratio of the three PorA subtypes in trivalent OMV is 1:1:1, the antigenicity of PorA P1.7-2,4 in trivalent OMV is expected to be one third of that of monovalent OMV. However, the results show that the antigenicity of PorA P1.7-2,4 in trivalent OMV was only 1/8 and 1/4 for strains JB10124 and HP10124, respectively, as compared with monovalent OMV. Freeze-drying and reconstitution of OMV did not significantly reduce the antigenicity of PorA P1.7-2,4 in any of the OMV (Fig. 4 D).
Figure 4. Panels A, B, C: Effect of storage on the antigenicity of PorA P1.7-2,4 in OMV of the strains F91 (A), JB10124 (B) and HP10124 (C). Samples were stored at -70°C (squares), 4°C (diamonds), 37°C (triangles) or 56°C (cross). Panel D: effect of freeze drying and storage on the antigenicity of PorA P1.7-2,4 in OMV. The bars indicate the antigenicity of PorA P1.7-2,4 in OMV before (grey) and after (white) freeze-drying in samples stored for 3 months at 4°C. The results are shown as mean values (±SD) of at least 3 measurements.

Effect of storage on the physicochemical characteristics of OMV

During 12 months, the pH of the preparations increased from 7.4 to 8.1 for almost all the formulations studied, except for trivalent OMV incubated at 56°C, where the solution acidified, reaching pH values around 6. The zeta potential of OMV did not change upon storage under different conditions (not shown).

OMV stored at -70°C and thawed (at 37°C, 10 min) showed aggregation, which tended to increase with storage time (Fig. 1), parallel to an increase in the polydispersity index. The size of OMV in liquid formulations did not change during storage at 4°C. At higher temperatures (37°C, 56°C), the average particle size of OMV derived from the three strains gradually decreased (Fig. 1 A, B, C). After 6 months, the particle size and zeta potential of samples incubated at 56°C could not be measured anymore, as the scatter intensity had become too low. This indicates total destruction of the OMV, as confirmed by the presence of amorphous granular material in electron micrographs (Fig. 2 C).
The concentration of Gnd-HCl necessary to induce a shift in the fluorescence emission maximum decreased in liquid samples of all three strains incubated at 37°C, as compared to non-treated OMV. This indicates a decreased stability of these samples. The red shift of samples stored at 56°C could not be measured, as in the absence of Gnd-HCl they showed a broadened emission spectrum with a maximum above 350 nm after 3 months. After 6 and 12 months totally different fluorescence spectra were observed: the tryptophan emission peak disappeared and a new spectrum was formed instead with an emission maximum of ca 405 nm (Fig. 6). In these degraded samples, the fluorescence spectra were similar when measured at an excitation wavelength of 280 or 295 (not shown). The newly formed spectra, which cannot originate from natural aromatic amino acids, indicate the formation of (fluorescent) chemical degradation products due to storage at high temperatures. To study these observations in more detail, the fluorescent amino acids N-acetyl tryptophan (NATrA) and N-acetyl tyrosamine (NATyA) were used as model compounds. Solutions of these amino acids in the same buffer as OMV were stored for 6 months at 56°C, and their fluorescence (λexc 295 and 280 nm, respectively) and
Figure 7. Panels A and B. Fluorescence spectra of NATyA (A, $\lambda_{\text{exc}}$: 280 nm) and NATrA (B, $\lambda_{\text{exc}}$: 295 nm) freshly prepared (thick black line) and after 3 (thin black line) and 6 months (thick gray line) incubation at 56ºC. See materials and methods for details. Panels C and D: normalized UV spectra of NATyA (C) and NATrA (D) freshly prepared (thick black line) and after 3 (thin black line) and 6 months (thick gray line) incubation at 56ºC.

UV spectra were monitored (Fig. 7). The intensity of the maximum fluorescence signal decreased in all model compounds and a new fluorescence emission peak was formed at higher wavelengths (ca. 405 nm for NATyA, 375 nm for NATrA). As
expected (because NATyA does not absorb at 295 nm), when freshly prepared
NATyA was excited at 295 nm, no emission spectra could be observed. After 3 and 6
months of incubation at 56°C, however, the NATyA sample did absorb at 295 nm
(Fig. 7A, inset) and showed an emission spectrum with a maximum around 405 nm
upon excitation at 295 nm (not shown), indicating the formation of a fluorescent
degradation product.

The trimeric structure of PorA was still present in frozen and liquid OMV stored
at 4 and 37°C after 3 months (not shown). At this time point no protein bands were
observed for samples incubated at 56°C. Samples incubated at 56°C were not
studied further by SDS-PAGE. After 6 months, part of the trimeric PorA was
converted to monomers for all OMV stored at –70, 4 and 37°C (Fig. 3B). In trivalent
OMV (JB10124 and HP10124) incubated at 37°C, extra bands with lower molecular
weights appeared below the monomer band (43 kDa) and this band was separated in
two bands (Fig. 3 B, lanes b). The structural changes of PorA were clearer after 12
months of incubation at all temperatures (Fig. 3 C). In native samples (lanes a),
trimers and monomers were still observed, and an extra band appeared at ~ 80 kDa,
indicating the presence of PorA dimers. Degradation bands at lower molecular weight
were also observed. This degradation pattern was most apparent for samples
incubated at 37°C, especially for monovalent OMV, where the intensity of the trimer
band had strongly decreased (Fig. 3 C, F91). For all samples incubated at 37°C, a
smear could be observed. The presence of P1.4, P1.5-2 and the P1.12 epitopes in
degradation bands was checked by Western blotting. No degradation bands were
observed for PorA subtypes P1.7-2, 4 and P1.5-2,10. Only in the case of the
P1.12,13 subtype one minor degradation band in addition to the original band was
observed containing the P1.12 epitope (not shown).

Effect of storage on the immunochemical characteristics of OMV

The antigenicity of liquid samples stored at 4°C was preserved in time. Frozen
monovalent OMV showed a slight decrease in the antigenicity, which was not
observed for trivalent samples stored at –70°C. When OMV were incubated at 37°C
the antigenicity decreased to (almost) zero within 6 months of incubation. When
samples were incubated at 56°C the antigenicity disappeared within 3 months (Fig. 4
A, B, C).
**Table 2.** IgG titer induced by OMV incubated at different temperatures

<table>
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<tr>
<th>OMV strain</th>
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<th>t=12</th>
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<td>F91</td>
<td>-70°C</td>
<td>3.4</td>
<td>3.9</td>
<td>4.2</td>
<td>3.8</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<td>&lt;1</td>
<td>&lt;1</td>
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<tr>
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*The total IgG titer of each mouse serum was determined by whole-cell ELISA and is expressed as the mean log$_{10}$ titer. Plates were coated with Neisseria meningitidis strains: H4/476 7.2, H4/76 5.2,10, H4/76 12.13 and HI.5 (see materials and methods). The means were compared by the LSD test with a confidence level of 95%. LSD$_{0.05}$ (7-2,4-3) = 0.4; LSD$_{0.05}$ (7-2,4-3) = 0.4; LSD$_{0.05}$ (7-2,4-3) = 0.4; LSD$_{0.05}$ (7-2,4-3) = 0.4; LSD$_{0.05}$ (7-2,4-3) = 0.6; LSD$_{0.05}$ (5-2,10,4-3) = 0.3; LSD$_{0.05}$ (5-2,10,4-3) = 0.3; LSD$_{0.05}$ (5-2,10,4-3) = 0.3; LSD$_{0.05}$ (5-2,10,4-3) = 0.4; LSD$_{0.05}$ (12,13,4-3) = 0.4; LSD$_{0.05}$ (12,13,4-3) = 0.7; LSD$_{0.05}$ (12,13,4-3) = 0.3; LSD$_{0.05}$ (12,13,4-3) = 0.5; LSD$_{0.05}$ (HI.5,4-3) = 0.9; LSD$_{0.05}$ (HI.5,4-3) = 0.5; LSD$_{0.05}$ (HI.5,4-3) = 1.2; LSD$_{0.05}$ (HI.5,4-3) = 0.9.

*PorA subtype against which IgG titer is specific.

* IgG titers specific for other structures than PorA.

* months

* Not determined.
Table 3. Bactericidal titers induced by OMV incubated at different temperatures

<table>
<thead>
<tr>
<th>OMV strain</th>
<th>Storage</th>
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<th>5-2.10°C</th>
<th>12,13°C</th>
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<td>&lt;1</td>
</tr>
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<td>2.0</td>
<td>&lt;1</td>
<td>&lt;1</td>
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* The bactericidal titer was determined as the reciprocal value of the serum dilution that effectuates >90% killing of H44/70-based strains expressing the indicated PorA subtype (see material and methods) and is expressed as the mean log₂ titer. Serum of each group was pooled into two samples. Each pool was tested at least four times. The means were compared by the LSD test with a confidence level of 95%: LSD₀̂₉₅ (7-2,4°C) = 0.5; LSD₀̂₉₅ (7-2,4°C) = 0.4; LSD₀̂₉₅ (7-2,4°C) = 0.4; LSD₀̂₉₅ (5-2,10°C) = 0.3; LSD₀̂₉₅ (5-2,10°C) = 0.5; LSD₀̂₉₅ (5-2,10°C) = 0.4; LSD₀̂₉₅ (5-2,10°C) = 0.2; LSD₀̂₉₅ (5-2,10°C) = 0.1; LSD₀̂₉₅ (12,13°C) = 0.6; LSD₀̂₉₅ (12,13°C) = 0.6; LSD₀̂₉₅ (12,13°C) = 0.4; LSD₀̂₉₅ (12,13°C) = 0.2.

* Sera were tested for bactericidal activity against the PorB-containing N. meningitidis strain H15. None of the pools tested induced killing of cells.

* PorA subtype against which bactericidal titers are specific.

* months.

* -, not determined.
Table 4. Effect of freeze-drying on the immune response induced by OMV. Freeze-dried OMV were reconstituted after 3 months storage at 4°C and measured in parallel to liquid OMV stored under the same conditions.

<table>
<thead>
<tr>
<th>OMV strain</th>
<th>Storage conditions</th>
<th>IgG titers*</th>
<th>Bactericidal titers*</th>
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<td>7-2,4°C</td>
<td>5-2,10°C</td>
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<td>-</td>
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<td>F-D, 4°C</td>
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<tr>
<td></td>
<td>F-D, 4°C</td>
<td>3.6</td>
<td>4.0</td>
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</table>

* The total IgG titer of each mouse serum was determined by whole-cell ELISA and is expressed as the mean log_{10} titer. Plates were coated with *Neisseria meningitidis* strains: H44/76 7-2, H44/76 5-2,10, H44/76 12,13 and H1.5 (see materials and methods). The means were compared by the LSD test with a confidence level of 95%: LSD_{0.05} (7-2,4) = 0.3; LSD_{0.05} (5-2,10) = 0.4; LSD_{0.05} (12,13) = 0.3; LSD_{0.05} (H1.5°) = 0.9.

° The bactericidal titer was determined as the reciprocal value of the serum dilution that effectuates >90% killing of H44/76-based strains expressing the indicated PorA subtype (see material and methods) and is expressed as the mean log_{10} titer. Serum of each group was divided in two pools. Each pool was tested at least four times. The means of these tests were compared by the LSD test with a confidence level of 95%: LSD_{0.05} (7-2,4) = 0.5; LSD_{0.05} (5-2,10) = 0.4; LSD_{0.05} (12,13) = 0.8.

Subtype against which IgG/bactericidal titers are specific.

Subtype specific for other structures than PorA. Sera were tested for bactericidal activity against the strain H1.5. None of the pools tested induced killing of cells.

F-D: Freeze-dried.
Effect of storage on the immunogenicity of OMV

The ability of monovalent and trivalent OMV stored at –70°C, 4°C, and 37°C to induce high IgG titers against the different PorA subtypes did not decrease with time (Table 2). A pronounced decrease in the ability to elicit an IgG response was only observed with OMV stored at 56°C. Antibodies induced by monovalent OMV and trivalent OMV from strain HP10124 (containing RmpM) were mainly PorA-specific, as demonstrated by the low titers against the PorA-lacking strain HI.5. OMV from strain JB10124 induced the formation of antibodies against other structures than PorA, probably LPS or impurities (cf. Table 1). The antibody titers against other structures than PorA were fairly constant for samples stored during 12 months at –70°C and 4°C, but the titers were lower in sera of mice immunized with OMV stored at 37°C and 56°C.

Sera of animals were also tested for the presence of bactericidal antibodies (Table 3). Only antibodies directed against PorA were bactericidal, as determined by the lack of bactericidal serum activity against meningococci of strain HI.5 (not shown). The capability of monovalent OMV to induce a bactericidal response against the PorA P1.7-2,4 was unchanged in samples stored at –70 or 4°C, and significantly reduced in OMV stored at 37°C for 12 months. OMV of strain F91 stored for 3 months at 56°C did not induce a bactericidal response at all. The P1.7-2,4 specific bactericidal activity induced by OMV from strain JB10124 was weak or absent. OMV from strain HP10124 induced anti-P1.7-2,4 bactericidal antibodies only in samples stored at –70°C. OMV from this strain stored at 4°C did not induce bactericidal anti-P1.7-2,4 antibodies after 3 or 6 months storage. Surprisingly, after 12 months storage at 4°C this formulation induced again significant bactericidal antibodies. This is probably due to the variability in the test and the fact that the titers against this subtype are low [5]. Although samples incubated at 37°C induced detectable bactericidal titers after 6 and 12 months, these titers were not significantly above the background (LSD of 0.4 and 0.6, respectively). The PorA P1.5-2,10 induced the highest bactericidal titers of all three subtypes in both trivalent strains. Also, the anti-P1.5-2,10 bactericidal titers were maintained for OMV of both strains (JB10124 and HP10124) upon storage at all temperatures except 56°C, where bactericidal activity of these formulations dropped to undetectable levels after 3 months storage. A similar trend was observed for the P1.12,13-specific bactericidal response. The anti-P1.12,13 bactericidal titers induced by trivalent OMV after 12 months were relatively
high (i.e. ≥ 2.7); the OMV from strain HP10124 induced a bactericidal response that was even higher than the one induced by the same OMV freshly prepared.

Freeze-drying and reconstitution did not affect the immunogenicity of monovalent OMV and trivalent OMV of strain JB10124 (without RmpM) (Table 4). Surprisingly, in trivalent OMV containing RmpM, only PorA subtype P1.5-2,10 preserved the capacity to induce a bactericidal response after freeze-drying.

Discussion

The stability of three types of outer membrane vesicle vaccines from Neisseria meningitidis was studied, with a detailed in vitro and in vivo characterization of these OMV. The preservation of the native PorA conformation as well as the structure of the presentation form were monitored, as both are crucial for the quality of the immune response [7]. PorA is a porin, belonging to a class of membrane proteins that appear to be exceptionally stable [20]. The physical stability of purified recombinant PorA has been previously studied, showing its ability to maintain its trimeric membrane arrangement in the presence of low SDS concentrations [15].

As expected, the physicochemical characteristics of PorA-OMV were best preserved by storage at 4ºC. However, partial unfolding of PorA was observed in all three OMV formulations stored at this temperature after 12 months (Fig. 3). Although this unfolding did not measurably affect the antigenicity or the protective immunogenicity generated by OMV, it may be a first step in the degradation leading to decreased immunogenicity of PorA during storage.

Only the incubation of OMV at high temperatures (56ºC) resulted in the total destruction of both the OMV structure and the protein conformation (DLS, EM, SDS-PAGE, ELISA), accompanied by change of the pH. The modification of the PorA structure was accompanied by a dramatic change in the fluorescence emission maximum towards higher wavelengths (from 338 nm to ca. 405 nm), reflecting chemical modifications of PorA, with formation of new fluorescent degradation product(s). This was confirmed using NATrA and NATyA as model compounds. Similar changes in the fluorescence spectrum have been reported for tetanus toxoid incubated at pH 2.5 and 37ºC for 14-28 days [21] and tetanus and diphtheria toxoids stored at 60ºC for 21 days [22]. The modifications of Trp and Tyr described in literature that lead to fluorescence shift are oxidation of Trp or dimerization of Tyr
[23]. Other oxidation reactions (e.g. methionine, histidine) as well as other chemical degradation reactions (e.g. deamidation, chain scission) may also have occurred [24,25], but this was not investigated in this study.

Unfolding of PorA and degradation of the OMV structures was also observed at 37°C but obviously, much more slowly than at 56°C. At this temperature, the change in the conformation of PorA could also be observed by SDS-PAGE, with monomerization of PorA (Fig. 3). This was also translated in a drastic drop in antigenicity (Fig. 4). However, a decrease in functional immunogenicity could not be detected, indicating that the epitope investigated in our ELISA system is not the only epitope important for the development of a protective immune response. The unfolding of PorA was accompanied by a reduced stability of the OMV structure, as their particle size decreased (Fig. 1).

Freezing or freeze-drying of OMV resulted in aggregation (Fig. 1). Probably, the freezing step is responsible for this aggregation. During freezing, ice crystal formation may partially rupture the vesicle structure, resulting in formation of larger vesicles and/or aggregates after thawing or reconstitution. The presence of 3% sucrose that could act as cryoprotectant or lyoprotectant was not enough to prevent this aggregation. However, the conformation of PorA did not change upon freeze-thawing and freeze-drying/reconstitution, which may explain the fact that the immunogenicity of aggregated OMV was not decreased. Although the functional immunogenicity of monovalent OMV and trivalent OMV from strain JB10124 was maintained when freeze-dried, lyophilized OMV from strain HP10124 induced a decreased P1.7-2,4- and P1.12,13-specific bactericidal response upon reconstitution. This difference can be related to the strain or the batch. No attempts were made to improve the freeze-drying process by adding other lyoprotectants or cryoprotectants than sucrose.

The trimeric PorA association was more stable in trivalent OMV than in monovalent OMV. This is shown by the relatively higher amounts of trimeric PorA in trivalent OMV incubated at 37°C (Fig. 3 B, C) and the higher concentration of Gnd-HCl necessary to unfold PorA in trivalent OMV (Fig. 5). As the composition of both trivalent and monovalent OMV is similar, i.e. most of the protein is PorA, this increased physicochemical stability of trivalent OMV could be due to differences in the stability among PorA serosubtypes or differences among strains in the quality of the membrane association of PorA.
It is difficult to correlate physicochemical characteristics of trivalent and monovalent OMV with immunogenicity, as the PorA subtype present in monovalent OMV (P1.7-2,4) is the least immunogenic subtype in trivalent OMV [6]. Antigenicity could not help to predict the bactericidal response induced by OMV stored at high temperatures. Whereas the antigenicity of the P1.4 epitope in all OMV was decreased or completely absent in OMV stored at 37ºC, these OMV were still able to induce polyclonal bactericidal antibodies in mice. Possibly, the immune response was directed against other epitopes of PorA subtype P1.7-2,4 than the epitope recognized by the monoclonal antibody used for the antigenicity test. Also, as previously shown in man [27], the presence of IgG in mice serum was not always accompanied by a bactericidal activity.

The ELISA data (relative to epitope P1.4 of PorA P1.7-2,4) show that the antigenicity of this epitope was lower than expected in trivalent OMV than in monovalent OMV. This may be due to a lower P1.7-2,4 expression in trivalent OMV, or to a decreased accessibility of the P1.4 epitope in these OMV for the monoclonal antibodies used in the assay. Moreover, the P1.7-2,4-mediated bactericidal serum activity induced by trivalent OMV was reduced as compared to the bactericidal activity induced by monovalent OMV. It was previously shown that bactericidal activity induced by the P1.7-2,4 subtype in trivalent OMV and mixtures of three monovalent OMV was decreased in both cases [26]. This indicates that the exposure of this PorA subtype is similar in trivalent and monovalent OMV, but that P1.7-2,4 – when simultaneously administered with other subtypes– is less efficiently processed and/or that P1.7-2,4 epitopes generate antibodies with low affinity. From the other PorA subtypes in trivalent OMV, P1.5-2,10 induced the highest bactericidal serum activity. This subtype was also previously found to induce the highest bactericidal response in toddlers and school children [27]. Even after 3 months incubation at 56ºC, still bactericidal activity was detected against this subtype. This is very surprising, as no PorA could be seen by SDS-PAGE and samples incubated at 56ºC showed no antigenicity. This bactericidal activity could be due to the presence of a non-detectable fraction of intact, denatured or fragmented PorA in the formulations.

The low molecular weight bands observed by Western blotting, indicating the presence of degradation products containing epitopes located in the variable regions 1 and 2 (VR1 and VR2, respectively) [28], was observed for PorA subtype P1.12,13 in trivalent OMV stored for 12 months at –70ºC, 4ºC or 37ºC. The fragmentation of
PorA did not affect the immunogenicity of this PorA subtype in trivalent OMV, probably because the fragmentation was incomplete.

The presence of RmpM in the OMV from strain HP10124 did not improve their long-term stability. On the contrary, the immunogenicity induced by these OMV was decreased upon freeze-drying (Table 4). It was not expected that these OMV were less stable (after freeze-drying) than trivalent OMV that do not contain RmpM, as this protein has an important role in the localization and stability of PorA trimers in the outer membrane [15]. The yield (by means of the amount of total protein) of OMV purified from HP10124 strain (RmpM+), was also 3-fold lower than the yield in RmpM- strains (not shown), and the amount of RmpM present in OMV was very low, only detectable by Western blotting. A reason for these relatively low yields may be the strong effect of RmpM on the structure of the bacterial outer membrane. RmpM+ bacteria form a well fitted, tight outer membrane, whereas the outer membrane of RmpM- stains has an ‘over-sized’ appearance [29]. The RmpM seems to influence the attachment of the outer membrane to the underlying peptidoglycan layer. The result is that the OMV yield of the RmpM- strains is higher, at least in the case of detergent-free extraction.

In conclusion, trivalent OMV appeared slightly more stable than monovalent OMV, although these differences are not reflected in the in vivo activity. All OMV are stable for at least one year if stored at 4ºC or frozen. Freeze-drying provides a good alternative to storage in the liquid state and may be advantageous for long-term (> 1 year) storage.

Acknowledgements

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References


