

Review

Outer membrane vesicles as platform vaccine technology

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Outer membrane vesicles (OMVs) are released spontaneously during growth by many Gram-negative bacteria. They present a range of surface antigens in a native conformation and have natural properties like immunogenicity, self-adjuvation and uptake by immune cells which make them attractive for application as vaccines against pathogenic bacteria. In particular with *Neisseria meningitidis*, they have been investigated extensively and an OMV-containing meningococcal vaccine has recently been approved by regulatory agencies. Genetic engineering of the OMV-producing bacteria can be used to improve and expand their usefulness as vaccines. Recent work on meningitis B vaccines shows that OMVs can be modified, such as for lipopolysaccharide reactogenicity, to yield an OMV product that is safe and effective. The overexpression of crucial antigens or simultaneous expression of multiple antigenic variants as well as the expression of heterologous antigens enable expansion of their range of applications. In addition, modifications may increase the yield of OMV production and can be combined with specific production processes to obtain high amounts of well-defined, stable and uniform OMV particle vaccine products. Further improvement can facilitate the development of OMVs as platform vaccine product for multiple applications.

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1 Introduction

1.1 General

The release of various types of membrane vesicles is widespread, from prokaryotes to more complex eukary-

otic cells [1]. It has been known for decades that Gram-negative bacteria shed outer membrane vesicles (OMVs), but they are also released by other groups such as Gram-positive bacteria [2–4], mycobacteria [5], and archaea [6]. There is increasing interest in vesicles and recruitment of the immune system for medical applications which could be linked to the finding that 60% of the available pharmaceutical drugs exert their effect through interaction with membrane proteins [7]. A few years after their discovery, research started to explore OMVs as vaccine product, in particular for the application against meningitis B disease. In 2013 a meningitis serogroup B vaccine, Bexsero, was approved by the EMA, which contains a bacterial OMV component [8]. This review will focus on bacterial outer membrane vesicles as platform technology for vaccines, giving an overview of current possibilities and limitations

1.2 OMV as vaccine

A classical human vaccine is a pharmaceutical product that stimulates the immune system to prevent pathogens from causing disease. To evoke a broad and long-lasting

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Abbreviations: **D-OMV**, OMV extracted from cells with detergent; **EMA**, European Medicine Agency; **FDA**, Food and Drugs Administration (US); **fetA**, iron-regulated OM protein of *Neisseria*; **fHbp**, factor H binding protein; **FliC**, flagellin; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **IM**, inner (or cytoplasmic) membrane; **LOS**, lipo-oligosaccharide; **Lpp**, Braun lipoprotein; **LPS**, lipopolysaccharide; **MenB**, Meningitis type B; **MS**, mass spectroscopy; **NadA**, *Neisseria* adhesine A; **NHBA**, neisserial heparin binding antigen; **N-OMV**, OMV extracted from cells with detergent-free methods; **OM**, outer membrane; **OmpA**, outer membrane protein A; **OMV**, outer membrane vesicle; **opC**, opacity-associated protein C; **PAMPs**, pathogen-associated molecular patterns; **PE**, phosphatidylethanolamine; **PG**, peptidoglycan; **PL**, phospholipid; **porA**, porine A; **PQS**, *Pseudomonas* quinolone signal; **S-OMV**, OMV spontaneously released by cells

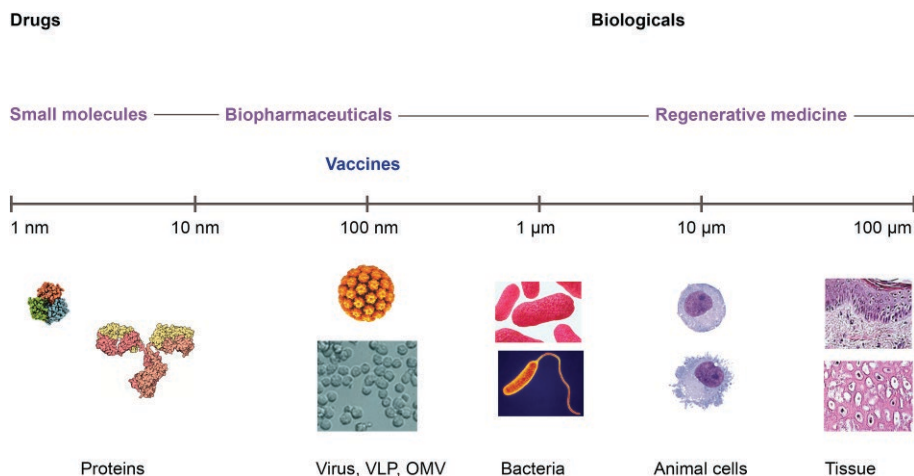


Figure 1. Vaccines as therapeutic products, positioned in complexity and size between well-characterized recombinant proteins and less-defined tissue products. For recombinant protein products, as monoclonal antibodies, the detailed molecular structure is usually known and there is scientific understanding on how active components of the product exert their therapeutic effect in humans (structure-function relationship). Such detailed knowledge on a molecular level is not available for the regenerative medicine products, such as modified cartilage or skin tissue. Presently, vaccines seem to possess an in-between position which affects the development strategy.

immune response, involving both innate and adaptive immune systems, a vaccine product should resemble a pathogen without causing the associated disease (Fig. 1) [9–12]. This implies that a vaccine product should have a proper size and should contain both PAMPs as well as pathogen specific antigens. In addition there are a number of properties the vaccine product should not have, because pathogens have developed a number of immune evasion strategies, such as creating enormous variety of certain surface components, mimicry with host components, production of proteases that degrade antibodies or developing biofilms.

OMVs have a proper size (20–200 nm) to enable their entry into lymph vessels and uptake by antigen presenting cells [9]. They naturally contain components that stimulate humoral and cell-mediated immune responses [13], since they resemble the bacterial antigenic surface of the pathogen.

The main challenges that may have to be addressed for OMV vaccine development include: (i) the high reactivity of PAMPs like LPS; (ii) low expression levels of relevant protective antigens; (iii) strain variation resulting in many subtypes of specific antigens, thus lower coverage; (iv) immuno-dominant antigens that misdirect the immune response; and (v) molecules which are immunosuppressive or otherwise interfere with a protective immune response. Genetic engineering of the OMV-producing strain can therefore be applied in many different ways to improve their vaccine application, by removing, adding, or altering OM proteins and other components.

2 Natural OMV

2.1 Composition of OMVs

Natural, or spontaneous, OMVs are spherical bi-layered membrane structures with a diameter in the range of 20–250 nm, that are pinched off from the outer mem-

brane of Gram-negative bacteria [14, 15]. In some cases the diameter range is increased to 10–500 nm and may include various irregular shapes, similar to the variation observed for many enveloped viruses [15]. The OMV membrane contains phospholipids (PL) on the inside and LPS and PL on the outside, mixed with membrane proteins in various positions, largely reflecting the structure of the outer membrane [14, 16]. The lumen of the vesicle may contain various compounds from the periplasm or cytoplasm, such as proteins, RNA/DNA, and peptidoglycan (PG) [17–20].

Emphasis of early research was on demonstrating that the OMVs indeed were formed from the outer membrane and not from IM or cytoplasmic components [16, 21], while current research is focusing more on which components from the cytoplasm, periplasm, or IM are directed towards the OMV as specific cargo and which OM proteins and lipids are included or excluded from the OMV [22, 23].

The used purification procedure for the OMVs as well as the use of specific mutant strains will affect the OMV composition. In addition the protein profile may change depending on the growth phase, media composition, or a specific stress signal that induces OMV formation [24, 25, 26].

2.1.1 Protein composition

The overall protein composition of purified OMVs was first analyzed with SDS-PAGE which can show the most abundant membrane proteins based on their molecular weight, while ELISA, flow cytometry, and Western blot or mass spectrometry (MS) in combination with 1D or 2D-gel electrophoreses can support identification of specific proteins. In proteomic studies of OMVs total numbers of identified proteins are in the range of 50–338, dependent on the technique and bacterium species, of which 40–80% was predicted to be OM protein [22–24, 27–32].

The identified proteins can be grouped in three categories: the basic constituents of the outer membrane,

the specific cargo proteins in the lumen, and unknown or contaminating proteins.

To the first category belong the major OM proteins such as porines, OM protein parts of larger transport systems, adhesins, enzymes such as phospholipases and proteases, and flagellum or pilus proteins. Adhesins mediate binding of OMVs to specific target cells [18, 33].

In OMVs of flagella-containing bacteria, usually flagellin (FliC) is found, even when elaborate purification techniques are used that should exclude flagella as contaminants of the OMV preparation [34]. For *Pseudomonas aeruginosa* the most abundant OM proteins identified in the OMVs were porines OprF and OprH/OprG, and flagellin, each representing >20% of the total protein content [29]. Flagellin was identified as the major discriminating protein, highly present in OMVs but hardly at all in other fractions. In natural OMVs of *Neisseria meningitidis* a total of 155 proteins were identified, including the membrane antigens used for MenB vaccine development such as porine A (PorA), factor H binding protein (fHbp), neisserial adhesine A (NadA) and opacity-associated protein C (Opc) [23]. The content of porines and anchor proteins, connecting OM to PG and IM, were reduced in OMVs compared to the OM fraction, while complement regulatory proteins, auto-transporters, proteins involved in iron and zinc acquisition, and a two-partner secretion system were enriched in the vesicles.

The second category of specific cargo proteins can include a number of toxins, such as cytolysin A of enterotoxigenic *Escherichia coli* [35, 36], cholera toxin of *Vibrio cholerae* [37], vacuolating cytotoxin (VacA) of *Helicobacter pylori* [38], and cytolethal distending toxin (CDT) from *Campylobacter jejuni* [39], and enzymes such as proteases and ureases. The last category of proteins consists of proteins from other cellular compartments, which are not expected to be normal OMV components, but this category is likely to become smaller with improved isolation methods. However, even in highly purified fractions of spontaneous natural OMVs, a number of cytoplasmic and IM proteins is usually found [40]. Common cytoplasmic proteins found in OMVs include elongation factors (EF-Tu), enzymes (enolase, GADPH), chaperones (GroEL), and heat shock protein (DnaK) [24, 41]. These proteins have also been identified as immunogenic surface proteins [35, 42]. Their presence may be an artefact resulting from cell fractionation, but some cytoplasmic proteins have been identified as “moonlighting” proteins, that seem to perform additional functions beyond their original cytoplasmic activity [43]. Examples are the enzyme enolase that functions as a plasminogen binding protein, and EF-Tu, that may also serve as adhesine for binding to host cells [43, 44]. However, surface associated cytoplasmic proteins were not found in recent proteomic studies of spontaneously released OMVs in *Porphyromonas gingivalis* [31], nor in an analysis of the OM proteasome in *E. coli* [45]. Though no true detergents were used, it can-

not be excluded that the applied purification methods and washing steps with surface active components caused the loss of associated proteins.

2.1.2 Lipid components (phospholipids and LPS)

The phospholipid composition of OMVs in *E. coli* was shown to be comparable to the OM, with phosphatidylethanolamine (PE) as most abundant phospholipid and elevated levels of phosphatidylglycerol and lyso-PE compared with the cytoplasmic membrane [46]. With alternative techniques a similar composition of OM and OMVs with respect to phospholipid content was confirmed for *H. pylori* [22]. The phospholipid content of OMVs has been found to differ for various Gram-negative bacteria. OMVs of *N. meningitidis* contained phosphatidylglycerol and PE as most abundant lipids [47]. In *P. aeruginosa* it was found that phosphatidylglycerol and stearic acid were present at higher levels in OMVs compared to the OM [26, 48], indicating a higher membrane rigidity of the vesicles. For *H. pylori*, PE, and to a lesser extent, cardiolipin were found as major lipid constituents, while elevated amounts of lyso-PE were found in OM and OMV compared to the IM [22, 49].

In *H. pylori*, one of the few bacteria containing cholesterol in the membrane, this lipid component was estimated to constitute 10% of the total lipids [22]. The OM of *Borrelia burgdorferi* also contains both free cholesterol and cholesterol glycolipids, which is contributing to the formation of lipid rafts [50]. While phospholipids are present in the inner leaflet of the OM for *E. coli* and other Enterobacteria, it can be a component of the outer leaflet in other bacterial species. LPS, however, is exclusively located at the outside surface of the OM. OMVs of *P. aeruginosa* were found to contain exclusively B-band LPS, while in the OM both A- and B-band LPS are present [51].

2.2 Function of OMVs

Several biological functions have been suggested for natural OMV release. One obvious possibility is that naturally occurring OMVs function as long distance delivery system of specific components [17, 41], protecting their cargo molecules from dilution and degradation. The transported components can be involved in acquiring nutrients, or help pathogens to create a suitable micro-environment for survival or growth in a host by supporting transfer of various virulence factors [18, 52]. They can also function as decoys which bind and remove antibodies and other bactericidal components in e.g. serum, as suggested for OM blebs of *Neisseria gonorrhoeae* [53]. For a number of bacteria it has been shown that OMVs participate in the formation of biofilms and thereby increase survival in hosts [54], or in soil [55]. Production of OMVs has also been reported to aid in the bacterial defense against antibiotics, since OMV production seems to dilute the damaging effect of the membrane perturbing agent gentamycin [51].

Protection against bacteriophages is possible through capture and inactivation of virus particles [56].

The possibility of gene transfer between bacteria by OMVs of various bacteria was recently summarized [19], including the example of the transfer of antibiotic resistance by DNA in OMVs. The OMVs can contain plasmids, but also chromosomal DNA fragments or bacteriophage DNA [57].

Finally, there remains the possibility that OMVs are sometimes released for strictly physiological reasons, i.e. as a consequence of an imbalance between cell growth and outer membrane synthesis, resulting in release of excess membrane material as OMVs.

2.3 OMV formation

The biogenesis of OMVs can be described as a budding process creating vesicles with the outside of the outer membrane at the surface of the OMV [18, 38, 58]. No universal mechanism for OMV formation can be given, only a number of events that are involved. These include: (i) the breaking of connections between OM and Peptidoglycan (PG); (ii) the accumulation of components in the periplasmic space; and (iii) the activity of specific signal and effector molecules such as PQS (*Pseudomonas* quinolone signal) [59].

A number of mutations, especially those involving Braun lipoprotein (Lpp) [60], the Tol-Pal system [61] of *E. coli*, and RmpM of *N. meningitidis* [62, 63], indicate the importance of losing anchoring structures for increased OMV production. In wild type *E. coli* the connections of OM with PG formed by Lpp are also in a dynamic equilibrium of breaking and binding [64]. Mutations affecting the enzymes involved in peptidoglycan degradation can also induce OMV production [65, 66]. A broad dissociation of connections between OM and PG layer does not seem to be an absolute requirement if accumulating components in the periplasm present an outward pressure [58, 67].

The initiation of the budding of an OMV by induction of membrane curvature has been associated with the lipid components of the membrane. Lysophospholipids, formed by one of the few enzymes present in the outer membrane, phospholipase-A [68], and CL seem to be involved in increasing membrane curvature required to form vesicles [22]. The formation of lysophospholipids has also been suggested as stress signal and associated with destabilization of the membrane in *H. pylori* [69]. For *E. coli*, however, mutations involved in synthesis of LPS or PG did affect vesicle formation, but no correlation was found for OMV production and instability of the membrane [70]. PQS in *P. aeruginosa* was not just reported as an inducing signal but also as a direct effector for OMV formation [59, 71]. PQS has a higher affinity for LPS than for phospholipids and could thus contribute to membrane curvature. For *P. aeruginosa* it was suggested that the presence of B-band type LPS in OMVs would add to the

curvature of the vesicle by charge repulsion, because it is longer and more negatively charged than the A-band LPS [72].

Current research does not support one particular mechanism for OMV formation but instead increases the number of possible mechanisms. During cell division, OMVs formed at septa are distinct from membrane vesicles formed along the cell body associated with local dissociation of the OM [73]. The septa OMVs were enriched with the TonB protein, while the cell body OMVs were enriched in flagellar proteins. The synthesis of FliC flagellum protein has been correlated with the formation of vesicles in *E. coli*, without yet presenting a mechanism for action [34]. The same holds true for outer membrane protein A (OmpA) that affects OMV formation in *Acinetobacter baumannii* [74] and *V. cholerae* [75, 76], although in that case the well-known anchor function of OmpA proteins to PG may play a role.

2.3.1 Induction of OMV formation

In general a number of signals that induce OMV formation are associated with stress [77], such as temperature stress [32, 77–79], amino acid limitation [80, 81], antibiotics [51], or the absorption of phages [82]. For *N. meningitidis* it was shown that cysteine depletion causes oxidative stress which triggered OMV release [81]. Also a number of media components can induce OMV production such as hexadecane for *Acinetobacter* [83], glucose for *Yersinia* [32, 84] and sodium carbonate for *V. cholerae* [85]. In *P. aeruginosa* the signal molecule PQS promotes OMV release [59], or under anoxic conditions, when no PQS can be synthesized, the bactericidal toxin pyocin [86].

Most early publications connect OMV formation with cell growth, since bleb formation was not observed in the stationary phase [87, 88], and cells growing at exponential phase produced more OMVs [79, 89]. The pattern is now mixed, with higher release of OMVs during late culture phase reported for *N. meningitidis* [90] and *Francisella* [91], while *Salmonella typhimurium* [73, 92], *Borrelia* [93], and *Gallibacterium* [94] seem to produce more vesicles during exponential growth. For *H. pylori* the release of OMVs is inversely related to the cell growth and is also correlated with shape transitions of the bacterium from spiral to coccoid [22]. *Legionella pneumophila* forms OMVs during intracellular and extracellular growth and in all phases of the cell growth cycle [95]. In general OMVs seem to be constitutively released by Gram-negative bacteria during all growth phases [96], also during normal stress-free growth conditions [21, 97]. Enhanced OMV production can be induced by specific signals, such as limitation at the end of the growth phase [38, 78, 81].

2.3.2 Cargo selection

Differences found in protein composition between OMV and OM are used as indication for the existence of a specific mechanism for exclusion of certain membrane

proteins during the formation of OMVs and the selection of specific cargo proteins to include in OMVs [41, 98]. A proteomic study analyzing differences in OM and OMV in *Bacteroides fragilis* identified 40 unique proteins in the OMVs, in particular glycosidases and proteases [99], illustrating that OMV formation is not only the non-specific entrapment of periplasmic components in the lumen of the OMV [29, 59, 100]. The exact mechanism by which specific proteins are sorted to OMVs is not yet known [15, 18], but some possibilities have been reported.

For *Lysobacter* a model was described for the excretion of proteases by OMVs [101]. The bacteriolytic enzymes are transported to the periplasmic space via the Sec transport system, and those enzymes having a high affinity for association with the inner leaflet of the OM have a higher probability to be included in OMVs. In *P. gingivalis* virulence factors and proteins containing a C-terminal secretion signal, that directs proteins to the cell surface via a new-type secretion system, are enriched in OMVs [31]. In the same organism it was found that LPS is involved in a mechanism responsible for the sorting of virulence factors towards OMVs, while major OM proteins were excluded [102]. For *V. cholerae* the periplasmic protein DegP, which has both a chaperone and protease function, was shown to be involved in the selection of nine cargo proteins as OMV content [85]. The presence of cholesterol and lipid rafts in *B. burgdorferi* could indicate involvement of these microdomain membrane structures in biological sorting processes, as has been reported for eukaryotic systems [15, 50]. Finally, the PG anchor proteins may not be randomly distributed over the cell surface but instead concentrated in specific micro-domains, which are then less likely to be released as OMVs [23]. Electron microscopy studies also point to the existence of substantial heterogeneity in OM structure within a single cell [103].

Besides proteins, OMVs can also transport PG, RNA and DNA. OMVs from *N. gonorrhoeae*, *H. pylori*, *P. aeruginosa*, and *V. cholerae* were shown to contain PG, which contributed to the innate immune response in host cells [104–106]. For *P. aeruginosa* and a number of other bacteria, DNA has been investigated as content of OMVs for transformation to other bacteria [18, 55, 78, 107]. For *Haemophilus influenzae* OMVs it was shown that 94% of the DNA was inside regular sized (20–200 nm) OMVs, while 6% was on the surface which has been associated with biofilm formation [108]. Presence of RNA in OMVs was indicated more than 25 years ago [109] and recently analyzed for *E. coli* [110].

3 OMVs vaccine development

3.1 Development of specific types of OMV vaccines

The development and use of OMVs as vaccine has received most attention for Meningitis type B (MenB).

Thus far only MenB OMV vaccine products have been tested in clinical studies (Table 1), but the performance of OMVs for various other diseases has been tested in rodents (Table 2). For meningococcal OMV vaccines a number of basic variants have been developed. First, there are non-recombinant, wild-type OMVs, derived from a particular strain and therefore monovalent in specific antigen content, mainly produced for the control of MenB outbreaks caused by a single clone [111]. Various forms of recombinant and/or multivalent OMV vaccines have also been used and will be discussed below.

Spontaneous – or natural – S-OMVs are purified and concentrated from culture supernatant, by separating intact cells from the already formed OMVs. The detergent D-OMVs, are extracted from cells with detergent which also reduces the content of reactogenic LPS. The term native N-OMV is used for intact spontaneous OMVs from cell supernatant [40], as well as for OMVs that are generated from concentrated dead cells with non-detergent cell disruption techniques [90, 112]. Here, native N-OMV will be used for vesicles extracted from cells with detergent-free methods, in accordance with previous nomenclature, and to be able to clearly distinguish them from the wild-type spontaneous OMVs and the detergent-extracted OMVs.

3.1.1 OMV (MenB) historical overview

For several serogroups of *N. meningitidis* effective conjugate vaccines consisting of capsular polysaccharide coupled to a carrier protein, are on the market. However, for serogroup B this approach was not feasible because this polysaccharide has homology to molecular structures in the human brain, generating the risk of autoimmunity [113]. Therefore, MenB vaccine development has mostly focused on other approaches, and OMVs have been in the picture for quite a few years [111, 114, 115].

An antigen which has received considerable attention is the immuno-dominant OM protein porin A (PorA), which is one of the major proteins in the meningococcal OM and a target for effective bactericidal antibodies, both in animal studies and in human sera [111]. A major challenge for PorA-based vaccines is the high sequence variability, which necessitates the inclusion of a high number of PorA subtypes to generate sufficient coverage [115]. However, by judiciously combining PorA with another antigen like FetA a limited number could still provide adequate coverage [116].

The availability of meningococcal genome sequences has enabled the search for novel antigens which might be used for the „holy grail“ of a universal MenB vaccine. With this so-called reverse vaccinology concept, genes encoding potential surface components were expressed in *E. coli* and the proteins tested for immunogenicity [117]. This omics approach initially yielded five suitable OM antigens, which are formulated as three recombinant proteins: a fragment of NadA, a fusion protein containing

Table 1. Overview *Neisseria meningitidis* type B or *Neisseria lactamica* OMV vaccines tested in clinical trials

Type OMV	Active component	Dose	Adjuvants	Response	Additional info	Reference
D-OMV/ proteoliposome	Multiple, OMPs (PorA), Men C polysaccharide, LPS	2 × IM 50 µg OMP 50 µg polysaccharide. 1% LPS	AlOH	Efficacy 83%	no severe or long lasting side effects VA-Mengoc-BC	[120]
D-OMV	multiple	2 × 25 µg	AlOH	Efficacy estimated at 57%	MenBvac Includes capsular B polysaccharide	[115, 119]
D-OMV	Multiple	2 × 25 or 50 µg IM	AlOH	Efficacy 73–85%	MeNZB	[111, 170]
D-OMV	6 PorA subtypes	3 × 45 or 90 µg PorA IM; 3 × 90 µg PorA	AlPO4	Induction of bactericidal antibodies (SBA)	Hexamen, phase I and phase II study	[171, 172, 173]
N-OMV	OpcA (+ multiple)	3 × 25 or 50 µg IM	+/- AlOH	Induction of SBA	Modified LPS, Lpx L2	[174]
N-OMV	fHbp, LOS, OpcA, PorA	3 × 10, 25, 50 or 75 µg IM	AlOH	Induction of SBA	Modified LPS, Lpx L1	[175]
D-OMV	NHBA, fHbp, NadA, PorA	1–4 doses, IM 50 µg NhbA, 50 µg fHbp, 50 µg NadA, 25 µg OMV/PorA	AlOH	Induction (SBA)	Bexero, multiple clinical studies	[8]
<i>Neisseria lactamica</i> , D-OMV	multiple	3–4 doses 25 µg IM,	AlOH	High specific antibody response against <i>N. lactamica</i>	Weak broad humoral response towards <i>N. meningitidis</i>	[124, 176]

IM intramuscular

NHBA, and a fusion protein containing fHbp. D-OMVs, as used for the outbreak in New Zealand, are added to the MenB vaccine product as adjuvant but might also contribute to the protective effect through the presence of PorA. This vaccine, Bexsero, has been approved for human use by the EMA and the FDA.

Traditionally, meningococcal OMV vaccines have been prepared with the use of DOC detergent to release the vesicles [115, 118]. This approach gives high yields of OMV material with reduced LPS content, but has some disadvantages such as loss of important protective lipoprotein antigens which are more loosely associated with the OM, compromised vesicle integrity, and increase of contaminating IM and cytoplasmic components. D-OMV vaccines have been used successfully to combat outbreaks of meningitis in Cuba, Norway and New Zealand where monovalent detergent-extracted OMVs were prepared from a manufacturing strain, matching the circulating strains causing the epidemic [111]. The efficacy of these wild-type OMV vaccines was estimated at 83% in Cuba, 57% to 87% in Norway and minimally 70% in New Zealand [111, 115, 119–121]. For the three outbreaks, summarizing findings for the application of 60 million doses of wild type OMVs have been published [111], showing a

consistent pattern of moderate reactogenicity and safety. In the last large outbreak in New Zealand three doses of 25 µg OMV were required to reach a sufficient level of protection. The immune response to wild-type OMV vaccines is, in particular in infants, largely directed towards PorA [115].

This limits the OMV approach to epidemic outbreaks caused by a single clone, and in the more common endemic situations where multiple strains are circulating it will be inadequate. In the Netherlands, a multivalent PorA-based OMV vaccine has been developed based on genetically engineered strains in which multiple *porA* genes were inserted. A single dose of 7.5 or 15 µg of D-OMVs containing six subtypes of PorA evoked an at least four-fold increase in bactericidal antibodies directed against most of the included PorA types in approximately half the number of volunteers in a clinical phase I study [122, 123]. This Hexamen vaccine, given with alum as adjuvant, showed a good safety profile. In a study to evaluate the feasibility of OMVs from *N. lactamica* as protective agent against *N. meningitidis* B based upon cross protection, demonstrated that *N. lactamica* derived OMVs did cause opsonophagocytosis of *N. meningitidis* but did not evoke a specific cross-protective humoral response

[124]. An overview of clinical studies with meningococcal OMV vaccines is given in Table 1.

3.1.2 *MenB OMV vaccines, recent development and current status*

As mentioned before, D-OMVs are included in the first generation vaccine product – Bexsero (Novartis) – that has been approved for the market. Recent developments towards a second generation MenB OMV product rather aim at the use of recombinant N-OMVs or S-OMVs. These S-OMVs contain genetically detoxified, and therefore, less reactogenic LPS, thus avoiding the use of detergent and preventing the loss of surface associated antigens [125–128]. The used *Neisseria* strains are deficient in capsular polysaccharide and make penta-acylated LpxL1 LPS which does not need detergent extraction because of its low endotoxic activity. The strongly reduced induction of IL-6 in human monocytes by OMVs containing this mutant form of LPS is a good indication for low in vivo reactogenicity [125], as this so-called MAT assay has already been accepted by the EMA as alternative indicator for in vivo pyrogenicity.

Whereas the immunodominant antigen in D-OMVs is PorA, N-OMVs give a broader response and increased coverage because additional antigens are present, such as the surface-exposed lipoprotein factor H-binding protein [30, 129]. In an analysis of the bactericidal antibody response to N-OMVs in rabbits it was found that the strongest response was against PorA, NadA and LOS, and to a lesser extend to fHbp [130]. An N-OMV vaccine containing modified LpxL1 LPS with PorA, fHbp and the conserved outer membrane protein OpcA as prime antigens, has been tested in a phase I clinical study, generating appropriate serum bactericidal assay (SBA) activity, while causing acceptable reactogenicity [126]. In Africa serogroup A is the main cause of meningococcal meningitis, though type W and X are increasing subtypes. Spontaneous S-OMVs from a serogroup W strain with overexpressed fHbp have been suggested as a novel approach, since they were also effective against serogroup A and X strains [128, 131].

3.2 Other human OMV vaccines; engineered homologous

3.2.1 *Bordetella pertussis*

The pertussis vaccine is one of the classical vaccines that was introduced as an whole-cell inactivated product. During the 1990's it was largely replaced by a purified subunit vaccine, in the developed world market. This acellular vaccine contains inactivated pertussis toxin, filamentous haemagglutinine and pertactin, and in some cases fimbriae. While acellular vaccines are less reactogenic than the classical whole cell vaccines, they have failed to effectively reduce pertussis infections; indeed, an increase has been seen in many countries which

switched to acellular vaccines. Reasons may include selection of strain variation and waning immunity due to a lower memory response. It has prompted the search for novel, more effective approaches to pertussis vaccination. One option is the use of OMVs from *B. pertussis*, because many of the known virulence factors are OM components or are at least associated with the OM. An OMV preparation, prepared by sonication of cells and treatment with DOC, combined with alum adjuvant provided protection against pertussis challenge in a mouse model [132], which was comparable to the effect of a whole-cell vaccine. Another version of OMVs containing a PagL-deacylated modified LPS showed both protection and a lower reactogenicity, the latter determined in vivo by both weight gain and cytokine induction [133]. Another interesting finding with *B. parapertussis* OMVs was their cross-protection against both pertussis and parapertussis [134]. The findings of OMVs of *B. pertussis* in mouse studies are summarized in Table 2.

3.2.2 *Other bacterial species*

For a number of human pathogens OMVs have been investigated in animal models, but none has yet progressed to the stage of clinical trials. The findings of these OMV vaccines in tests in mice or rabbits are presented in Table 2 (containing additional references [177–183]). The OMVs are usually S-OMVs from selected strains without specific increased or modified antigenic determinants. Most OMVs evoke a diverse antibody response, including different IgG subtypes and IgM [135] or IgA after mucosal administration [136, 137], as well as a cell-mediated immune response [92, 138]. The antibody response can be directed towards proteins as well as towards the LPS, as determined for *H. pylori* [139]. The active dose applied that provides protection is in the range of 1 to 500 µg, in most cases without the addition of adjuvants. As mentioned before, for some strains the LPS is already of low toxicity and does not need to be modified or extracted. Different routes of administration have been explored, which is also connected to the specific disease to be prevented (as e.g. intragastric for *H. pylori*). Intranasal immunization with OMVs did in this case not provide a protective effect [140], but did work for *V. cholerae* [136], *P. aeruginosa* [138], and *Francisella* [141].

4 Heterologous vaccine OMV

Genetic engineering can be used to introduce additional antigens into OMVs. First, antigens which are normally released from the cell surface by proteolytic processing can be retained by making mutants in which this is prevented. Second, novel antigens can be introduced in OMVs by targeting them to the outer membrane. Autotransporters have been used most often for such surface display, e.g. the Hbp protein from *E. coli* which was

Table 2. Overview OMV vaccines tested in mice (or other rodents)

Pathogen	disease	Type OMV	Active component	Dose	Adjuvants	Response	Additional info	Reference
<i>Acinetobacter baumannii</i>	Pneumonia, meningitis, sepsis	S-OMV	Multiple	2 × 50 µg IM	ALPhos	Humoral response (specific IgG and IgM) and protection in challenge study	Specific mouse model for sepsis	[177]
<i>Bordetella pertussis</i>	Whooping cough	D-OMV (EDTA, sonication + DOC)	Multiple	2 × 5 µg protein IP	ALOH IP; no IN	Protection against IN challenge (comparable to whole inactivated pathogen vaccine); <i>Also innate</i>	Reactogenicity: some loss in weight gain test (mice)	[132]
id	id	D-OMV (sonication + DOC)	Multiple	2 × 3 µg, 20 µg IN	No	Protection against IN challenge (both 3 and 20 µg)	LPS PagL modified; no weight loss; proper IL-6 respons	[133]
<i>Bordetella pertussis</i> and <i>B. parapertussis</i>	id	D-OMV	Multiple	2 × 3 µg IN	ALOH	Protection against IN challenge	Formalin inactivated; LPS of parapertussis less reactogenic	[134]
<i>Borrelia burgdorferi</i>	Lyme	N-OMV (vortexed, sucrose gradient)	Multiple, including DpbA, OspA and OspC	4 × 60 µl OMV from 10 ⁹ spirochetes	No	Protection against challenge; various antibodies measured	Tested in rabbits, no LPS modification required	[168]
<i>Brucella melitensis</i>	Brucellosis	S-OMV	Multiple	2 × 5 µg IM	no	Protection against challenge (smooth 2 log; rough 3 log units)	cytokine expression of APC (DC)	[178]
<i>Burkholderia mallei</i> & <i>pseudomallei</i>	Melioidosis	S-OMV	Multiple	3 × 2.5 µg SC & IN	no	Protection against nasal challenge for SC; reduction of pathogen no elimination; cellular & humoral response	no LPS removal required IN not effective	[140]
<i>Burkholderia pseudomallei</i>	Melioidosis	S-OMV (precipitation)	Multiple	3 × 5 µg SC	no	Protection against challenge	Diverse bactericidal antibody response	[179]
<i>Chlamydia muridarum</i> antigen in <i>E. coli</i>	Chlamydia	S-OMV	Serine protease HtrA (DegP)	3 × 50 µg IM (total protein)	ALOH	Induction of neutralizing antibodies	Antigen coupled to <i>E. coli</i> OmpA; No modified LPS	[147]
<i>Francisella</i> (strains)	Tularemia	S-OMV	multiple	1 × 20 µg IN	no	Protection against challenge	No LPS reduction required	[141]
<i>Helicobacter pylori</i> and <i>H. felis</i>	Gastritis, ulcer, gastric cancer	S-OMV	multiple	4 × 50 µg IG	Cholera toxin 10 µg	Protection against challenge	Mucosal IgA ; no cross protection	[137, 180]

Table 2. Overview OMV vaccines tested in mice (or other rodents) (continue)

Pathogen	disease	Type OMV	Active component	Dose	Adjuvants	Response	Additional info	Reference
<i>Klebsiella pneumoniae</i>	Lung infections	S-OMV	Multiple	20 µg IT	NA	Cytokine induction; innate response. OMVs induce lung pathology in mouse pneumonia model	No damage in vitro cell culture	[181]
<i>Neisseria meningitidis</i> type B	Meningitis	N-OMV	Multiple (iron limited culture)	2 × 0.1 – 20 µg IN or IP	No.	Bactericidal antibodies (SBA)	IN requires 8–10 times higher dose than IP; bacteria warmed and sheared	[112]
id	id	S-OMV/ N-OMV TFF	fHbp, (+ multiple)	2 × 2.5 µg IP	ALOH	High antibody titers with bactericidal effect (SBA)	Bacteria killed with phenol; modified LPS	[129]
id	id	S-OMV	Multiple (increased fHbp)	3 × 0.2, 2, or 5 µg	ALOH	Induction bactericidal antibodies; cross protection	Engineered GMMA, modified LpxL1	[128]
<i>Neisseria lactamica</i>		D-OMV	Multiple	2 × 10 µg IP	ALOH	Strain specific Bactericidal antibodies (SBA)	No Cross-reactive SBA, but antisera did mediate opsonophagocytosis	[182]
<i>Porphyromonas gingivalis</i>	Chronic periodontitis	S-OMV (ammonium sulph precipitat)	Multiple	2 × 100 µg SC	IFA	Protection against induction of lesions; IgG and IgM antibody response	Comparison with LPS, OMF	[135]
<i>Pseudomonas aeruginosa</i>	Lung infections	S-OMV TFF	Multiple	6 × 500 µg IN	No LPS)	Protection against challenge	potent innate immune response; induction of pro-inflammatory cytokines; TLR-4	[138]
<i>Salmonella typhimurium</i>	Typhoid fever, gastroenteritis	S-OMV (AmSulph precipitation)	Multiple	100 µg IP, repeated	No (NA)	Stimulation of macrophages and dendritic cells; pro-inflammatory cytokines	Innate response, involving but not only relying on TLR-4	[92]
<i>Shigella</i>	Shigellosis		Multi serotype	4 × 50 µg oral	No (ref to LPS)		Transfer of passive immunity to offspring	[183]
<i>Vibrio cholerae</i>	Cholera	S-OMV	Multiple	3 × 25 µg IN, IG, 1 × 1 µg + 2 × 0.25 µg IP	No	Elevated Ig titer against OMV antigens; IgA by mucosal route (IN best); Protection against challenge in offspring (prevent colonization)	Long lasting (3 months) response	[136]
id	id	S-OMV	Multiple	3 × 25 µg	no	Protection against challenge in offspring (prevent colonization)	oral response less robust than intranasal	[139]

IP, intraperitoneal; IM, intramuscular; ID, intradermal; IN, intranasal; IG, intragastric; IT, intratracheal; SC, subcutaneous; IFA, incomplete Freund's adjuvants ; APC, antigen presenting cell; DC, dendritic cell

successfully applied for surface expression of *Mycobacterium tuberculosis* antigens in Salmonella [142]. While effective, this approach seems to be limited to relatively small fragments of foreign antigens. Surface display of larger domains is more challenging and may require other approaches, e.g. based on lipoprotein export systems [143]. Kesty and Kuehn tested *E. coli* OMVs as delivery system for heterologous expressed proteins [100]. First, the Ail protein from *Y. enterocolitica* was used to produce OMVs with a heterologous adhesin in the membrane, thereby stimulating their uptake by human colon cells. In addition, as an example of a heterologously expressed protein, GFP could be directed into the lumen of these OMVs. Foreign antigens may be targeted to the periplasm and when retained in OMVs, provide an alternative for surface display [144]. Remarkably, there are very little published data on the need for surface exposure in OMVs versus only the presence within the vesicles; this aspect deserves more attention. Finally, there is the possibility to add external antigens to OMVs in such a form that binding to specific outer membrane components is facilitated, circumventing the problems inherent in heterologous expression [145]. Several examples have been published where OMVs carrying heterologous antigens confer protective immunity against the target pathogen. A surface protein of the Gram-positive pathogen *Streptococcus pneumoniae*, PspA, could be expressed in *Salmonella enterica* and directed to the periplasm, and collected in OMVs [146]. The OMVs containing PspA in the lumen of the vesicles protected intranasally immunized mice against a challenge with *S. pneumoniae*, while control mice receiving purified PspA or normal *S. enterica* OMVs were not protected. To generate an OMV-based vaccine against *Chlamydia* the serine protease HtrA (degP) was coupled to the OmpA leader sequence of a high-blebbing tolR deletion mutant *E. coli* [147]. The resulting recombinant heterologous *Chlamydia* HtrA-*E. coli* OMVs were able to induce neutralizing antibodies in an in vitro infectivity assay, while recombinant purified HtrA did not generate such a response.

5 OMV and innate response

As bacteria-derived products, OMVs will naturally contain a variety of molecules which can interact with the innate immune system. In the first place, LPS will be present as a major component of the outer leaflet of the outer membrane. It is a very potent activator of immune cells such as monocytes/macrophages, through specific recognition by the TLR4/MD2 receptor which results in triggering of NF- κ B and IRF3 mediated gene expression. The resulting activation is important in activating and directing the adaptive immune response, which is highly advantageous for vaccine applications of OMVs [148]. On the other hand, this inflammatory activation can also

result in high vaccine reactogenicity. The use of mutants with defects in lipid-A biosynthesis seems to provide a balanced response, generating sufficient adjuvant activity but preventing harsh side effects [63]. Inactivation of *msbA* in *E. coli*, or *lpxL1* in *N. meningitidis*, results in loss of a secondary acyl chain in lipid A which leads to strongly reduced capacity to activate TLR4/MD2 [125, 149], enabling the development of a detergent-free production process. Further fine-tuning of the lipid-A structure is possible by introducing lipid A modifying enzymes such as LpxE or PagL, leading to dephosphorylation or deacylation, respectively. In this way, LPS forms can be obtained with an optimal ratio between adjuvant activity and reactogenicity, e.g. by a bias towards the TRIF signaling pathway [150, 151]. In addition to LPS, OMVs always contain lipoproteins which are also activators of innate immunity through their recognition by TLR2 [152, 153]. This is more difficult to modify than LPS-mediated activation because many different lipoproteins are present in OMVs, and their contribution to adjuvant activity and reactogenicity is therefore less well characterized. Also, downstream signaling pathways differ for TLR4 and TLR2 which may result in different immune bias. Nevertheless, TLR2 does also undoubtedly play an important role in OMV-mediated activation of the immune response [154, 155]. Finally, recognition of various receptors on antigen-presenting cells by surface components of OMVs can interfere positively or negatively with the immune response, and this can vary depending on the particulars of the pathogen used for OMV preparation. For instance, Opa proteins in the outer membrane of pathogenic *Neisseria* have been reported to lead to immunosuppression by their interaction with CEACAM receptors in human immune cells, and in vivo experiments with transgenic mice showed that this led to a reduced Opa-specific antibody response [156]. Some LPS glycoforms can bind to receptors on dendritic cells, e.g. LgtB LPS from *N. meningitidis* to DC-SIGN, leading to a Th1-biased immune response [157]. Clearly, because many surface molecules of pathogenic bacteria have evolved to interact with the host immune system in a particular way, many further possibilities for fine-tuning the use of OMVs as vaccines, are possible.

6 OMV process development

The design of a process to obtain a biopharmaceutical product starts with defining the target product profile (TPP), and the methods to measure this. Based on the TPP a suitable expression system can be determined to produce an OMV vaccine. For most bacterial vaccines this will include cultivation of a pathogenic bacterium, either genetically modified or not.

From the selected strains a master and a working cell bank (MCB, WCB) is generated that serves as a stable

source for a manufacturing process. The standard vaccine production starts with the thawing of a vial from the WCB for an expansion culture, followed by the production fermentation, the down-stream purification process (DSP) and the final filling of a – often combination or multivalent – vaccine product. During all steps of the manufacturing, in-process controls will be taken to assess that the intermediate product meets pre-set quality criteria. General objectives include avoiding animal-derived components, chemically defined culture medium, and feasible sterile filtration of vaccine product. For the process development it makes a considerable difference whether an S-OMV has to be produced from the supernatant of the fermentation harvest, or that a native N-OMV has to be extracted from cells with detergent-free or detergent containing techniques.

6.1 Strain development

For some applications wild-type bacterial strains can be used directly to produce an OMV vaccine (Table 2), but in most cases, genetic engineering of the OMV-generating bacteria is required to improve the vaccine product. Such modifications can include: (i) modifying the LPS biosynthesis pathway in order to obtain less endotoxic and reactogenic variants; (ii) overexpression of crucial antigens; (iii) simultaneous expression of multiple antigenic variants; (iv) outer membrane retention of normally secreted antigens; (v) increased OMV production by removing outer membrane anchor proteins; (vi) removal of immune-modulating components which may trigger the wrong type of immune response; and (vii) expression of heterologous antigens from other pathogens than the host OMV producing strain.

An often used genetic modification of production strains is to delete genes encoding anchor proteins between OM and PG in order to increase vesicle formation. This can be done by inactivating the OmpA homologues which are commonly found in Gram-negative bacteria, e.g. the RmpM protein in *Neisseria* [62, 63]. Other mutations which can have the same effect target the tol-pal system [61], PG biosynthesis [66], or the cell envelope stress response [77]. However, while there are many reports on low versus high “blebbing” strains and conditions, very little comparative data have been published on OMV quantities produced by specific bacteria. Another crucial modification for most species is the reduction of LPS reactogenicity, for instance by inactivation of *lpxL1* in *Neisseria* [125] or *msbB* in *E. coli* or *Salmonella* [158]. A third standard genetic modification is the introduction of additional antigens or the upregulation of crucial protective antigens. Examples for *Neisseria* are PorA and fHbp. Since PorA is a highly variable antigen, multiple antigenic variants are required to confer a sufficiently broad protection, and this has been achieved by constructing strains with multiple chromosomal copies [125].

For fHbp, it has been shown that induction of bactericidal antibodies is directly related to its expression level, therefore up-regulation of expression by altering the promoter has been used [129].

Bacterial strains without capsular polysaccharide are generally preferred since these structures can interfere with the vesicle purification, as found for *Klebsiella* [159]. Since capsular polysaccharide is an essential virulence factor for many pathogenic bacteria, its removal has the additional benefit of rendering the production strains safer to handle. Many outer membrane antigens function by binding to some host receptor or ligand, and it has recently been found that in such cases their immunogenicity is reduced by this interaction [127, 156, 160]. By using mutants, which no longer bind their natural human ligand through small alterations in the binding site, one can increase their protective capacity.

6.2 Techniques USP

The start of the upstream process (USP), the expansion pre-culture of bacteria, is generally performed in shaker flasks. For the MCB it could be chosen to prepare a lyophilized seed lot, since secured storage and initial slow growth in agar plate culture is more important than fast expansion. When sufficient inoculum has been generated in expansion cultures, a production fermentation can be started. The standard upstream process for bacterial vaccines contains a fermentation in a stirred tank reactor to yield a crude product [161]. For larger scale processes a first reactor vessel can be used to inoculate the larger production reactor.

Medium composition can be of influence on formation of OMVs by bacteria, as mentioned by the signals that can induce OMV formation (section 2.3.1), which is also illustrated by the difference of OMV yield in different media for *Francisella tularensis* [91]. Cell densities reached during production fermentations to produce OMV vaccines have been reported as an OD of 6 [162] and 9 [163] at 60 to 1200 L scale for *N. meningitidis* B strains, and an OD of 30–45 was reported for a high blebbing mutant of *Shigella sonnei* in a 5 L stirred bioreactor [164]. The yield of OMV corresponding to these fermentations were 40 mg/L for *N. meningitidis* strains [165] and 100 mg/L for the *S. sonnei* strain [164]. A challenge for the scale-up of the fermentation process is to find alternatives for the use of antifoam, which is the standard method to prevent excessive foaming that is the result of the required aeration at higher cell densities. Most antifoams are not compatible with an OMV production process, because the surfactants can affect OMV integrity or will interfere with the OMV purification. Therefore, alternative techniques for mechanical foam breaking have been introduced as part of the scale-up of the fermentation process [162].

The harvest of S-OMVs of *P. aeruginosa* should be scheduled during the exponential phase, because pro-

tease synthesis can damage the S-OMV composition during stationary phase [159], and the appearance of irregular membrane structures has been reported for *H. pylori* during stationary phase [38]. For *N. meningitidis* it seems beneficial for D-OMVs and N-OMVs yield to maintain the cell culture to end of the exponential phase [90, 163], though this has to be optimized versus the increasing level of contaminants such as DNA.

6.3 Techniques DSP OMV

6.3.1 Techniques DSP for S-OMV

For the primary recovery of the S-OMV product from cell suspension, the standard technique to separate bacteria from vesicles, is to apply low speed centrifugation (2000–10 000 *xg*). In some cases the separation of OMVs from intact bacteria or from smaller soluble components is performed by tangential flow microfiltration, using 10 kDa [50] or 50–100 kDa filters [138, 166] or a combination of 0.2 and 0.1 μm filters [163]. The separation of S-OMVs from contaminants is combined with a filtration step through a 0.22 or 0.45 μm dead-end filter to remove larger debris fragments. The S-OMVs are collected with an ultracentrifugation (UC) step at 50 000–200 000 *xg*, which can be combined with density gradient UC [158, 159]. As alternative concentration step precipitation with ammonium sulfate can be performed [93, 158], followed by differential centrifugation.

Common contaminants during purification of S-OMVs include pili, flagella and soluble components, and as for all DSP processes the steps to yield a higher purified OMV fraction will reduce the overall yield [159]. Many small scale purification steps for S-OMVs are executed at 4°C, or with the addition of protease inhibitors, but for large-scale applications this is usually avoided because of costs.

Purified OMVs are mostly resuspended in PBS and frozen at -20 to -80°C. The storage of S-OMVs from *H. pylori* at -20°C did not change the structure of the vesicles [28].

6.3.2 Techniques DSP for N-OMV and D-OMV

For extracted vesicles the supernatant of the fermentation harvest containing spontaneous vesicles is discarded, and usually the cells are concentrated with centrifugation or tangential flow microfiltration. Subsequently, extraction of OMVs can be performed by detergent, with DOC as most frequently applied component to generate D-OMVs [63, 115]. As mild chemical alternative to detergents EDTA [94, 163] has been tested, and sonication as a mechanical vesicle releasing technique [167] and vortexing [168] has been used to prepare an N-OMV fraction. For *Neisseria* a combination of detergent-free techniques [112, 130] has been performed to obtain N-OMVs.

The further DSP to purify D-OMVs and N-OMVs make use of the same UC and TFF techniques as applied for the S-OMVs. Long term stability in liquid solution at 4°C

has been demonstrated for D-OMVs and N-OMVs [115, 133, 165].

7 Outlook

The development of vaccine products is slowly advancing from undefined whole inactivated or attenuated pathogens towards better characterized subunit vaccine products, resembling pathogen-like structures. OMVs contain a number of features necessary for an effective vaccine product: a native configuration for membrane surface antigens to elicit a humoral response [13], the potential to evoke a T-cell-mediated immune response, the presence of several PAMPs to trigger the innate immune response, and a size that is appropriate for efficient processing by antigen-presenting cells [9–12]. The successful application of OMVs as platform structure for vaccines includes the optimization of the appropriate innate and adaptive immune responses by either removing or including specific components, which has to be individually evaluated for each disease application. A crucial but still unresolved issue is whether the antigens have to be surface exposed or merely present in the OMV. The example of MenB shows that an OMV can be modified, such as for LPS reactogenicity, to yield a ready to use OMV product that is safe and effective as demonstrated in SBA scores. Development of a target manufacturing process shows that high yield of a stable, sterile product of constant quality can be produced, according to state-of-the-art conditions [164, 165].

With the given advantages, and the expected further decrease of the blind-spots with respect to unknown host-pathogen effects, the development of OMVs as platform vaccine is expected to enable a well-controlled development for new vaccine products based upon scientific understanding of product and process, in line with a structural vaccinology approach [169].

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