Biotechnology Advances 35 (2017) 565–574

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Bioengineering bacterial outer membrane vesicles as vaccine platform

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ARTICLE INFO

Keywords:
Outer membrane vesicles
Extracellular vesicles
Vaccine platform
Bioengineering
Nanobiotechnology

ABSTRACT

Outer membrane vesicles (OMVs) are naturally non-replicating, highly immunogenic spherical nanoparticles derived from Gram-negative bacteria. OMVs from pathogenic bacteria have been successfully used as vaccines against bacterial meningitis and sepsis among others and the composition of the vesicles can easily be engineered. OMVs can be used as a vaccine platform by engineering heterologous antigens to the vesicles. The major advantages of adding heterologous proteins to the OMV are that the antigens retain their native conformation, the ability of targeting specific immune responses, and a single production process suffices for many vaccines. Several promising vaccine platform concepts have been engineered based on decorating OMVs with heterologous antigens. This review discusses these vaccine concepts and reviews design considerations as the antigen location, the adjuvant function, physiochemical properties, and the immune response.

1. Vaccine platforms

Many vaccines are developed based on Pasteur's principle: “isolate, inactivate and inject” or by the selection of attenuated strains (Rappuoli 2004). Although many very effective vaccines have been developed by these methods, they have often led to adverse effects. Nowadays, vaccines must combine the lowest possible adverse effects with a high efficacy. Vaccine platforms can standardize the provoked immune response with high safety and low adverse effects, while the design allows for easy switching of displayed antigens leading to high efficacy. Protection against different diseases can be addressed by presenting different antigens.

The development of a highly safe and effective vaccine requires a lot of resources and time. At the same time the upcoming post-antibiotic era may require the development of more vaccines that also need to be developed in a short period (WHO 2014). The development trajectory can be shortened by vaccine platforms (Kushnir et al. 2012), since these platforms provide a blueprint for development of many different vaccines instead of a specific development trajectory for each separate vaccine with all its uncertainties. The safety of a vaccine platform can be established by thorough development of the platform itself. Once the safety and efficacy of a platform has been established, development time can be reduced for new vaccines, since less testing will be required and unexpected failures will occur less. This advantage of vaccine platforms will reduce significantly the time to market, which is notoriously long for new vaccines.

A vaccine platform should provoke a strong specific immune response. This response is triggered by conformationally correct antigen presentation, **PAMPs** (see glossary) to activate antigen presenting cells, and a nanosized particulate nature. Furthermore, it should be possible to easily add antigens onto the vaccine platform. Platform nanovaccines can be based on many components, for example **VLPs**, **ISCOMs**, polymeric nanoparticles, inorganic nanoparticles, liposomes, and emulsions. A component that is often overlooked are OMVs (Zhao et al. 2014). While many nanoparticles are capable of transferring heterologous antigens to antigen presenting cells, the ability to properly stimulate the immune system is often not natively present (Singh et al. 2007). OMVs, however, combine antigen presentation with proper adjuvant properties, making them highly suitable as a vaccine platform.

OMVs are non-replicative vesicles that are naturally produced by Gram-negative bacteria and contain excellent intrinsic immunostimulatory properties based on their particulate nature and composition (Ellis and Kuehn 2010). The vesicles consist of phospholipids, LPS, outer membrane proteins and entrapped periplasmic components (Kulp and Kuehn 2010). OMVs are ascribed many biological functions such as cell to cell communication, surface modifications and the expulsion of components (Kulp and Kuehn 2010). Overall, OMVs have been shown to be highly stable even upon elevated temperatures and several chemical treatments (Arigita et al. 2004). This review addresses the latest state of research with respect to the development of an OMV

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http://dx.doi.org/10.1016/j.biotechadv.2017.05.003
Received 15 February 2017; Received in revised form 24 April 2017; Accepted 12 May 2017
Available online 15 May 2017

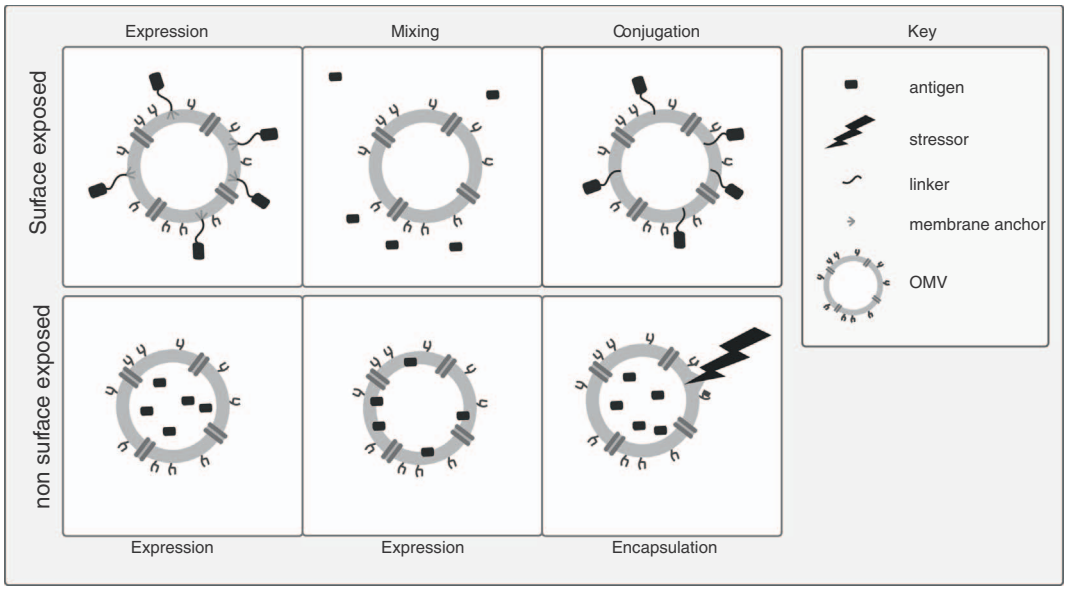

Fig. 1. Methods of antigen decoration on OMVs. Top row shows surface exposed antigens on the vesicles, bottom row shows the antigens as luminal cargo of OMVs. Antigens can be produced by the OMV production bacterium (left), while antigen addition to purified vesicles can be divided in mixing, conjugation and encapsulation (middle and right).

based vaccine platform. First we discuss the location of the antigen, which is either inside the OMV or displayed on the OMV surface. Location is important for the provoked type of immune response. Two approaches of location specific antigen addition are discussed, namely the endogenous addition based on antigen production by the bacterium itself and the exogenous methods that introduce the antigen in a separate process step. The bioengineering of the provoked immune response and the endotoxicity is discussed. Additionally, we discuss the bioengineering of the physiochemical properties of the OMV and the potential of outer membrane vesicles as vaccine platforms. Lastly we propose a uniform naming of different vesicles based on the origin of the OMVs.

2. Designing the OMV: antigen location

Heterologous antigens on OMVs can be presented with or without surface exposure, attached to the vesicle or non-attached and directly produced by the bacterium or combined in a later production stage. Various possibilities of antigen locations and their production method are schematically shown in Fig. 1. At this moment it is unclear what the most preferred setup for an OMV based vaccine platform is. This section describes the impact of the heterologous antigen location, the endogenous loading of antigens to the vesicle lumen and the vesicle surface, and the exogenous loading of antigens to the vesicle lumen and the vesicle surface.

Surface exposed antigens are accessible for antigen-specific B cell binding, while the inside of the vesicle is shielded from these cells. Luminal antigens may be skewed towards cytotoxic T-cell responses (Galen and Curtiss, 2014), hence the desired immune response determines the design of the OMV. Many groups have expressed antigens in the lumen of OMVs to develop OMV vaccines (Table 1) (Bartolini et al. 2013; Fantappie et al. 2014; Kesty and Kuehn 2004; Kim et al. 2009; Muralinath et al. 2011; Schild et al. 2009). Surprisingly, these studies also find antibody-mediated immune responses against the luminal heterologous antigen. Muralinath et al. studied the luminal expression of Pneumococcal PspA in *Salmonella enterica* serovar Typhimurium OMVs (Muralinath et al. 2011). These vesicles triggered minor antibody responses against OMVs and PspA in immunized mice and provided protection in a challenge experiment. OMVs without PspA or purified PspA alone did neither evoke an antibody response nor provide protection. Schild et al. showed minor specific antibody responses against periplasmic alkaline phosphatase (PhoA) from *Escherichia coli* expressed in *Vibrio cholerae* OMVs (Schild et al. 2009). In addition to the minor antibody titers found in the previous two studies, it was shown by Fantappie et al. that also high functional antibody titers can be obtained by expressing heterologous antigens in their native conformation in the lumen of *E. coli* OMVs (Fantappie et al. 2014). This systematic study characterized the vesicles by showing incorporation of the antigens and antigen localization. Because of the findings in a previous study that the Chlamydial HtrA protein expressed in OMVs was partially surface exposed, the authors checked the antigen localization (Box 1) by proteinase K treatment (Bartolini et al. 2013). After all, contamination with surface exposed antigen could be a cause for the observed response. The heterologous antigens in OMVs were found not to be surface exposed. Further analysis of these proteins showed their native conformation in the vesicle lumen that remarkably appears to be sufficient to trigger antibody mediated responses.

Antigens can be presented on the surface of the OMV with exposure to the exterior side of the vesicle. We recently studied the expression of the Borrelial surface-exposed lipoprotein **OspA** in *Neisseria meningitidis* OMVs (Salverda et al. 2016). Expression of the protein in meningococci did not result in surface exposure on OMVs. To obtain surface exposure, OspA was fused to a Neisserial lipoprotein. The immunogenicity of this surface exposed fusion construct was compared to that of a luminal expressed OspA in mice. Results showed that only the surface-exposed OspA was able to elicit an OspA-specific antibody response. In a study on Salmonella OMVs by Muralinath et al., higher immune responses against outer membrane proteins and LPS were found than against the heterologous expressed antigen present in the vesicle lumen (Muralinath et al. 2011).

It remains unclear whether antigens in the lumen of OMVs provide sufficient antibody responses. The observed antibody responses of some studies may be biased by extracellular antigen or surface attached antigen. On the contrary the lack of an antigen specific antibody response against non-surface exposed OspA may not be predictive for other antigens (Salverda et al. 2016). Antibody responses have been observed for all surface exposed antigens, while for luminal antigens the provoked responses remain ambiguous. Altogether more research is required on the exact effect of the antigen location on efficacy of an OMV vaccine platform.

Table 1
Overview of heterologous antigens expressed on OMVs as vaccine concept.

Type[1]	Antigen location	Antigen display	Antigen	Target	Backbone	Reference	Year
sOMV	Surface exposed	ClyA fusion	Omp22	*Acinetobacter baumannii*	*Escherichia coli*	(Huang et al. 2016)	2016
sOMV	Surface exposed	ClyA fusion	M2e	Influenza A	*Escherichia coli*	(Rappazzo et al. 2016)	2016
eOMV & sOMV	Surface exposed	fHbp fusion	OspA	Lyme's disease	*Neisseria meningitidis*	(Salverda et al. 2016) (Intravacc)	2016
glycOMV	Surface exposed	Lipid A glycan	*F. tularensis* O-PS	*Francisella tularensis*	*Escherichia coli*	(Chen et al. 2016)	2016
geOMV	Surface exposed	Lipid A glycan	CPS14	Pneumococcal disease	*Escherichia coli*	(Feldman et al. 2013; Price et al. 2016)	2016
sOMV	Mixing	Mixing	AnAPN1, Pfs48/45	Malaria	*Escherichia coli*	(Pritsch et al., 2016)	2016
sOMV	Not determined	Hybrid flagellin FlaA	FliC	ETEC	*Vibrio cholerae*	(Leitner et al. 2015)	2015
sOMV	Surface exposed	Hbp fusion	PspA or Ply fragments	Pneumococcal disease	*Salmonella typhimurium*	(Kuipers et al. 2015) (Abera Biosciences)	2015
sOMV	Surface exposed	Hbp fusion	ESAT6, Ag85B fragments, and Rv2660c	Tubercolosis	*Salmonella typhimurium/Escherichia coli*	(Daleke-Schermerhorn et al. 2014) (Abera Biosciences)	2014
sOMV	Surface exposed	Hbp fusion	MOMP fragments	Chlamydia	*Salmonella typhimurium*	(Daleke-Schermerhorn et al. 2014) (Abera Biosciences)	2014
sOMV	OMV lumen	OmpA fusion	SpyCEP, Streptolysin O, Spy0269	Group A Streptococcus disease	*Escherichia coli*	(Fantappie et al. 2014) (Novartis)	2014
sOMV	OMV lumen	OmpA fusion	SAM_1372	Group B Streptococcus disease	*Escherichia coli*	(Fantappie et al. 2014) (Novartis)	2014
sOMV	Mixing	Mixing	O-antigen	Shigellosis	*Shigella spp.*	(Koley et al. 2014; Mitra et al. 2013)	2013
sOMV	OMV lumen	OmpA fusion	HtrA	Chlamydia	*Escherichia coli*	(Bartolini et al. 2012; Bartolini et al. 2013) (Novartis)	2012
sOMV	Outer membrane	OprI fusion (*Pseudomonas aeruginosa*)	A104R	African swine fever	*Escherichia coli*	(Basto et al. 2012)	2012
sOMV	OMV lumen	Type II secretion signal from β-lactamase	PspA	Pneumococcal disease	*Salmonella enterica (serovar Typhimurium*	(Muralinath et al. 2011)	2011
dOMV	Mixing	Mixing	glycoprotein D	Genital herpes	*Neisseria meningitidis*	(Del Campo et al. 2010; Romeu et al. 2014)	2010
sOMV	OMV lumen	OmpA fusion	FLAG	Proof of concept	*Escherichia coli*	(Kim et al. 2009)	2009
sOMV	Surface exposed	AIDA fusion	KMP-11	Leishmaniosis	*Escherichia coli*	(Schroeder and Aebischer 2009)	2009
sOMV	Vesicle lumen	Inherent	PhoA	Proof of concept	*Vibrio cholerae*	(Schild et al. 2009)	2009
sOMV	Surface exposed	ClyA fusion	GFP	Proof of concept	*Escherichia coli*	(Chen et al. 2010; Kim et al. 2008)	2008
dOMV	Mixing	mixing	*Dermatophagoides siboney* allergens	Dust mite allergy	*Neisseria meningitidis*	(Lastre et al. 2006)	2006
sOMV	Surface exposed	Inherent	NspA	*Neisseria meningitidis*	*Neisseria flavescens*	(O'Dwyer et al., 2004)	2004
sOMV	Outer membrane	Inherent	Ail	Proof of concept	*Escherichia coli*	(Kesty and Kuehn 2004)	2004
sOMV	OMV lumen	Tat signal	GFP	Proof of concept	*Escherichia coli*	(Kesty and Kuehn 2004)	2004

[1] See Box 2 for an explanation of the OMV types.

Box 1
Analyzing the surface exposure of antigens on OMVs.

Analysis of the surface exposed protein expression is important, but challenging. Expression levels of the antigen can be assessed by immunostaining. The heterologous antigen expressed on the bacteria and on the OMV can be stained by a labeled antibody and detected by fluorescent microscopy (Salverda et al. 2016). Alternative options for the detection of immunostained bacteria or vesicles can be flow cytometry (Rioux et al. 2001), ELISA or nanoparticle tracking analysis. Besides immunostaining, proteins associated to the outside of the outer membrane can be determined by proteinase K digestion of surface exposed proteins followed by SDS-PAGE analysis. Western Blotting can be used to test the proteinase K susceptibility of a specific antigen. This method has been used since the early 1980s for the detection of surface exposed proteins (Freudl et al. 1986). The protein profile of proteinase K treated vesicles can be compared to the protein profile of non-treated OMVs to detect proteinase K susceptibility of certain proteins. As a control, SDS can be added to the treatment to check the digestion of previously shielded proteins.

2.1. Endogenous loading of surface exposed antigens

Recombinant expression of proteins in bacteria is a large and ever-growing field (Baneyx and Mujacic 2004; Rosano and Ceccarelli 2014). Several approaches have been described to express proteins on the outer membrane of bacteria (van Bloois et al. 2011). However, many of these approaches have a low yield or are only suited for small proteins or parts thereof. One method relies on antigenic proteins that are normally exported beyond the cell surface by proteolytic processing. These antigens can be retained by preventing the proteolysis (Georgiou and Segatori 2005). Another method relies on targeting the protein to the outer membrane and thus to the OMVs (Kesty and Kuehn 2004). One of the first concepts of outer membrane targeting for surface exposed antigen expression was based on autotransporters. Autotransporters are transported as unfolded protein over the inner membrane via the Sec pathway. Next, the autotransporter is transported over the outer membrane by a so far unknown mechanism that most likely requires direct involvement of the BamA protein (Sauri et al. 2009). The rightly folded autotransporter anchors to the outer membrane via a C-terminal β domain. Fusion to the autotransporter hemoglobin protease (Hbp) of *E. coli* was used to express recombinant proteins in an attenuated strain of *Salmonella typhimurium* (Jong et al. 2014). The authors engineered Hbp to facilitate the surface expression of *Mycobacterium tuberculosis* proteins and epitopes of the major outer membrane protein MOMP from *Chlamydia thrachomatis* (Daleke-Schermerhorn et al. 2014). This method of antigen display is highly efficient, however it seems limited to smaller protein fragments (Luirink and Jong 2016).

To express larger proteins on the surface of the outer membrane, Kim et al. fused several heterologous protein including GFP with a five-residue glycine linker to the C-terminus of the pore-forming cytotoxin ClyA (Kim et al. 2008). These fusion constructs were efficiently transported across the inner membrane to the outer membrane of *E. coli*. Recently it was shown by their group that surface expressed **M2e** antigens on *E. coli* OMVs provided protection against influenza A infection in a mice study (Rappazzo et al. 2016). Besides single M2e-ClyA fusion constructs, the authors also successfully constructed a nearly 50 kDa multimeric variant containing the serine analogs of human, swine and two avian M2e variants. Apart from incorporation of a single protein on the OMV it was shown in a different study that a three-enzyme cascade could also be successfully engineered on *E. coli* OMV (Park et al. 2014). The enzyme scaffold was expressed in the bacterium and anchored to the outer membrane by the truncated ice nucleation protein anchor (Shimazu et al. 2003). The enzymes were produced separately from the OMV, which would allow the expression of different enzymes on the OMV by simple replacement of these enzymes for different proteins. The enzymes were attached to the protein anchor by using three cohesion-dockerin pairs. Cohesin domains interact with dockerin domains of enzymes to form multiprotein complexes (Carvalho et al. 2003). To decorate the OMV with a three-enzyme cascade, the authors used the unique interaction between each of the three cohesion-dockerin pairs for the sequential attachment of the enzyme complex. The OMVs with the enzyme scaffold had a 23-fold higher conversion rate when compared to the individual enzymes free in solution (Park et al. 2014). Although this example is not related to vaccines it shows the potential of attaching proteins to the outside of the OMV.

Surface exposed expression of heterologous lipoproteins has been achieved by fusion of the heterologous lipoprotein to a membrane anchoring second lipoprotein. In this way, the heterologous lipoprotein signaling is not required to match the hosts signaling to achieve surface exposure. The borrelial lipoprotein OspA was fused via a tether to the N-terminal part of factor H binding protein that acts as a membrane anchor (Salverda et al. 2016). The extracted OMV (eOMV) contained surface exposed OspA. With this approach it was possible to purify OMVs with surface exposed heterologous lipoprotein antigens.

2.2. Endogenous loading of antigens to the OMV lumen

Differences in protein composition of OMVs and the outer membrane have been described (Haurat et al. 2011; Lappann et al. 2013; Veith et al. 2014). This difference hints towards a regulated protein sorting to OMVs or a regulated OMV release but the mechanism of protein enrichment remains unclear. Accumulation of misfolded and aggregated proteins in the periplasm is suggested as one of the possible mechanisms of vesicle release (Kulp and Kuehn 2010). An increase in OMV formation was found in strains lacking the chaperone DegP, resulting in enrichment of misfolded DegP substrates in OMVs (Schwechheimer and Kuehn 2013). McBroom and Kuehn showed that it was possible to selectively enrich a protein in the OMV lumen by adding a misfolded outer membrane protein sequence to the periplasmic cytochrome b_{562} (McBroom and Kuehn 2007). A 12-fold enrichment of cytochrome b_{562} in OMVs was observed based on SDS-PAGE analysis. Although this method provides valuable insights in the method of protein sorting in OMVs, it may not be beneficial for the folding of the antigen. Thus this method might not be preferred for the addition of antigens that require proper folding in the lumen of OMVs.

Antigens have been bioengineered to be targeted to the lumen of OMVs. Kesty and Kuehn fused GFP to the twin-arginine (Tat) signal sequence to produce *E. coli* OMVs with GFP in their lumen. This Tat pathway transfers folded proteins over the cytoplasmic membrane. To transfer unfolded proteins over the cytoplasmic membrane the Sec pathway could be used. However, GFP and some other proteins are unable to fold correctly in the periplasm. The OMVs, containing GFP in their lumen, showed to shield GFP from proteinases, which indicates the stability of proteins inside the vesicles (Kesty and Kuehn 2004). Another method for luminal protein expression in *E. coli* OMVs is based on the fusion of proteins to the periplasmic side of the abundant outer membrane protein OmpA (Kim et al. 2009). A FLAG-tag was attached to the truncated C-terminus of the OmpA protein, which is located at the periplasmic side of the outer membrane (Kim et al. 2009). OmpA truncations or deletions are known to result in a blebbing phenotype.

This approach combines stimulation of OMV production by the host with a site for antigen addition. This method was successfully used to express fusions of OmpA to several different antigens against Group A Streptococcus, Group B Streptococcus and Chlamydia (Bartolini et al. 2013; Fantappie et al. 2014).

Another study has directed the pneumococcal protein PspA to the lumen of *Salmonella typhimurium* OMVs by fusion of PspA to the N-terminal β-lactamase signal sequence (Muralinath et al. 2011). This signal sequence allows the transport of β-lactamase to the periplasm by the type II secretion system, which yields OMVs with PspA in their lumen.

Taken together, heterologous antigens can be expressed in the lumen of OMVs by fusion of the antigen to secretion signals or periplasmic proteins. These fusion based methods are relatively straightforward and have been successfully used to express full size antigens in a conformational correct manner and antibody have been found against these antigens. While it is unclear how the antigens are processed to eventually elicit antibody mediated immune responses, heterologous expression of antigens in the lumen of OMVs may be a feasible approach for an OMV vaccine platform.

2.3. Exogenous loading of surface exposed antigens

Antigens can be added afterwards by introducing affinity to the antigen on the OMV. This method of exogenous loading was used by adding a **SpyTag** to OmpA (Alves et al. 2015). The SpyTag peptide is designed to form a covalent bond with the SpyCatcher protein to ease conjugation of proteins (Zakeri et al. 2012). The SpyTag-OmpA fusion was expressed on OMVs and can be coupled to SpyCatcher fused to any protein. By this method, OMVs can be produced in mass production followed by adding antigens on the OMV. Similarly, this SpyTag based approach has been used to decorate VLPs with antigens as vaccine platform (Brune et al. 2016).

2.4. Exogenous loading of antigens to the OMV lumen

Loading of antigens to the vesicle lumen, after the vesicles and antigens have separately been produced in bulk, can be an attractive approach. The concentration of the active components may be better controllable when compared to endogenous loading. To load antigens in their native conformation to the OMV lumen in vitro, the vesicle should be opened and closed again without permanent damage. The exogenous loading of proteins to the vesicle lumen has not been described although several methods of loading smaller molecules into extracellular vesicles **(EVs)** are explored. EVs can be loaded by simple incubation (passive loading) or by active treatment of the EVs (active loading). Methods of loading EVs by electroporation, saponin-treatment, extrusion or dialysis are well described for EVs and even compared in a side by side study (Fuhrmann et al. 2015). In this study different porphyrins were loaded in endothelial, stem cell, and cancer derived EVs. Active encapsulation techniques and especially saponin-treatment showed higher porphyrin loading than passive techniques. Active encapsulation by electroporation has been described to load small-molecule drugs and siRNA in to EVs (reviewed in Lamichhane et al. 2015). Active encapsulation of siRNA in to *E. coli* OMVs by electroporation was applied by Gujrati et al. (2014). This siRNA targets kinesin spindle protein, which is upregulated in tumor and rapidly growing cells. The siRNA was electroporated to the lumen of the OMV resulting in intact siRNA-loaded vesicles. The loaded vesicle was targeted to HER2+ tumor cells by a HER2 antibody fused to ClyA. The authors were successful in reducing tumor growth in mice by treatment with these OMVs. For the loading of vesicles derived from the fungus *Neurospora crassa*, a single freeze-thaw method has been shown to be very effective (Mayer et al. 1995). Loading efficiencies of over 80% were reported and the authors claim the method is suitable for any protein. Interestingly, this method has to our knowledge not been tested for bacterial OMVs.

2.5. Mixing OMVs

It is currently unknown whether antigens require attachment to the vesicles. It has been shown that for Adjuvant System 03, a marketed oil in water adjuvant, it is sufficient to inject the antigen at the same location as the adjuvant to obtain an adjuvating effect (Morel et al. 2011). In this case, simple mixing of the adjuvant and antigen is sufficient. The mixing of the weak antigen GFP with *E. coli* OMVs showed however, that immunized mice did not elicit anti-GFP IgG responses (Chen et al. 2010). Attachment of GFP to the OMVs did show enhanced responses, comparable to GFP absorbed to the commercial standard adjuvant alum. Alternatively, antigens can be conjugated to the OMV if attachment is desired. This has been successfully accomplished for Brucella LPS that was conjugated to *N. meningitidis* OMVs (Siadat et al. 2015). Along the same line capsular polysaccharides from serogroup C meningococci were conjugated to a *N. meningitidis* serogroup B OMV (Fukasawa et al. 2006). Using conjugation, mixing, heterologous expression, or a combination thereof, also multivalent vaccines can be designed using OMVs as a main component. A combination of mixing and recombinant antigen expression has been applied for the eOMV based *N. meningitidis* serogroup B concept vaccines Hexamen and Nonamen (Claassen et al. 1996; Kaaijk et al. 2013). Mixing dOMVs from two strains has been applied to produce a combined *N. meningitidis* serogroup A and W concept vaccine (Tunheim et al. 2013). Mixing OMVs has been applied by a recently proposed sOMV based Shigellosis concept vaccine (Mitra et al. 2013). The authors of this latter study produced sOMV from 6 serogroups of Shigella that were mixed to form a hexavalent vaccine. This composition was named MOMV (not to be mistaken with modified OMVs or the previous naming of mitochondrial EVs, see Box 2 for naming of different types of OMVs) because of the multi-serotype OMV composition (Mitra et al. 2013). Roier et al. created OMV mixtures of nontypeable *H. influenzae* strains to obtain a broad covering immunization mixture (Roier et al. 2012). The OMV mixture showed a broadly covering protective immune response, although it should be noted that OMVs derived from a single strain also showed cross-protection against other nontypeable *H. influenzae* strains. Mixing of OMVs from enterotoxigenic *E. coli* and *V. cholerae* was described by Leitner and coworkers (Leitner et al. 2015). The authors showed an improved protection for the mixed OMVs when compared to the provoked immune response from the individual OMVs alone. Mixing of OMVs can thus be a simple yet very effective method of improving protection.

3. Designing the OMV: bioengineering the immune response

The interaction of the OMV with the immune system can be considerably tuned. Outer membrane vesicles can be directly used as a vaccine, since the vesicles are non-replicating and contain antigens as well as PAMPs in a particle configuration. Furthermore, OMVs can induce both humoral and cellular immune responses (Ellis and Kuehn 2010). Recently it was shown by Rosenthal et al. that *E. coli* OMVs can trigger a Th1-biased immune response (Rosenthal et al. 2014). The authors were able to induce both protective humoral and Th1-biased cellular immune responses against the heterologous antigen by using the probiotic *E. coli* Nissle 1917 bacteria as OMV production host. This probiotic bacterium itself is highly immunosuppressive, and thus not an obvious vaccine candidate, nevertheless its OMVs lack this capacity (Kim et al. 2013). In contrast to the OMVs from *E. coli* K12, the probiotic derived OMVs elicit Th1 cellular immune responses, however these responses were not necessarily directed against the heterologous expressed antigens (Rosenthal et al. 2014). To improve the immune response against an antigen of interest, the antigen amount per OMV can be increased or multiple antigens can be included. Zhang and co-workers improved the immunogenicity of a trivalent *N. meningitidis*

Box 2
Naming of OMVs.

With the production of extracted OMVs, many prefixes have been used to clarify the origin of the OMVs (van der Pol et al. 2015). The naming of the different types of vesicles has been based on the method of extraction, for example, the detergent deoxycholate (DOC) has been used to extract OMVs from bacteria to develop the first OMV-based vaccines (Sierra et al. 1991). This harsh extraction method was used to decrease the toxic LPS in the vesicles and the produced vesicles were referred to as detergent OMVs (dOMVs). The use of prefixes in OMV naming starts to become confusing due to the development of more and more methods of extracting vesicles and methods of increased "spontaneous" release of vesicles in the culture supernatant. The term nOMV has been used for both natural OMVs and native OMVs, where native OMVs were extracted by EDTA. It was shown that EDTA-extracted OMVs were similar to natural OMVs and hence the term nOMVs or N-OMVs was used (van de Waterbeemd et al. 2010). To distinguish between spontaneous and extracted vesicles we favor to refer to EDTA extracted vesicles as extracted OMV: eOMV. Moreover, spontaneously released OMV in vivo should be distinguished from OMV released in vitro. The in vitro production of spontaneous released OMV during cultivations is known to be induced by many mutations or stressors. The possible difference between these stress released vesicles and vesicles released spontaneously by the bacterium are more difficult to observe and researchers should be careful when comparing differently produced OMV. We suggest to refer to in vitro produced OMVs as supernatant OMVs: sOMVs. Overall it has become challenging to apply a uniform naming system of OMVs and thus it should be cared for that the method of producing OMVs is accurately described. We propose to uniform the naming of OMVs as listed in Table 2.

Table 2
Overview of the proposed naming of OMVs.

Abbreviation	Type of OMV
dOMV	Detergent extracted OMVs from in vitro cultured bacteria
eOMV	All non-detergent extracted OMV from in vitro cultured bacteria
mOMV	Preferably not to be used due to its ambiguity (modified OMV, multivalent OMV, mitochondrial OMV)
nOMV	Preferably not to be used due to its ambiguity (native OMV or natural OMV)
sOMV	Spontaneous released OMV, typically obtained from the supernatant of in vitro cultured bacteria

vaccine by genetic engineering a second fHbp protein in the *porB* gene, which increased fHbp yields in the OMV and increased antibody responses against fHbp (Zhang et al. 2016).

OMVs can be engineered such that the vesicles contain proteins altered in their interaction with human receptors. Opacity (Opa) proteins of *Neisseria* sp. are known to interact with human CEACAM receptors (Billker et al. 2000). This interaction causes a reduced Opa-specific immune response, however the immune response against the other OMV antigens was not significantly affected (Zariri et al. 2013). Another hypothesis is that binding of OMV proteins by human complement inhibitors could interfere with the development of the immune response. A reduction in antibody responses was shown due to binding of the antigen with a human protein (Costa et al. 2014). Human factor H could be bound by Neisserial fHbp, NspA and Porin B2. Especially the interaction with fHbp is studied widely due to the recent licensing of two fHbp based *N. meningitidis* serogroup B vaccines (Marshall et al. 2013; Serruto et al. 2012). It was shown that partial inhibition of fH binding did not enhance the immunity in a mouse model (Daniels-Treffandier et al. 2016). Human Factor H transgenic mice were immunized with a low fH binding mutant of fHbp and showed to elicit higher serum bactericidal antibody responses than when immunized with fHbp capable of binding fH (Rossi et al. 2013). Furthermore, immunizations with a low fH binding recombinant fHbp mutant in an infant rhesus macaques model showed antibody responses directed to the region of fHbp that binds human factor H (Granoff et al. 2016). Antibody responses against these host protein binding antigens can be improved by low-binding mutants, although these show no alteration in the antibody responses against other proteins present on the OMV.

OMVs contain by nature many immunogenic components that could cause a reaction against the OMV backbone. Currently it is unknown whether this reaction causes undesired effects. The possibility of this effect affects the choice of backbones (Table 1). The use of *E. coli* as backbone has the advantage of being extensively studied (Bartolini et al. 2012; Basto et al. 2012; Chen et al. 2016; Fantappie et al. 2014; Huang et al. 2016; Kesty and Kuehn 2004; Kim et al. 2008; Kim et al. 2009; Price et al. 2016; Pritsch et al., 2016; Rappazzo et al. 2016; Schroeder and Aebischer 2009), however the immune response directed against *E. coli* could negatively influence commensal populations. This negative effect could be prevented by the use of a pathogenic bacterium as backbone. Pathogenic backbones as *N. meningitidis*, *S. typhimurium* and *V. cholerae* have been described (Daleke-Schermerhorn et al. 2014; Salverda et al. 2016; Schild et al. 2009), although it is unknown what the effect is on the spread of the production pathogen itself. Another aspect is the possible response against the backbone during the repetitive use of the OMV platform against different diseases. Alternatively, a stealth backbone could be developed. Cross reactivity of the backbone with closely related species was described for *N. meningitidis* (Oliver et al. 2002; Sanchez et al. 2001) illustrating the possible broader effect of immune responses against the OMV backbone. Currently it is unknown to what extent minor cross-reactivity impacts a vaccine platform or if a complete stealth OMV backbone is required.

4. Designing the OMV: bioengineering toxicity and adverse effects

As a very potent activator of the immune system, lipopolysaccharids (LPS) can induce severe side effects and must be detoxified. Detoxification by reducing the amount of LPS has been performed by a detergent extraction process yielding dOMV (Zollinger et al. 1978). To use native or spontaneous OMVs, most vaccine concepts are based on genetically detoxified LPS, with potent activation of the innate immune system by TLR4. The TLR4 activation can be altered by bioengineering the LPS (Zariri et al., 2016b), since too potent TLR4 reactivity causes endotoxicity (Fitzgerald et al. 2004). The endotoxin activity of LPS was modified by *msbA* deletion in *E. coli* or *lpxL1* deletion in *N. meningitidis*, resulting in lipid A without the secondary acyl chain (Somerville et al. 1996; van der Ley et al. 2001). These modifications strongly alter the endotoxicity by reducing the TLR4/MD-2 activation by the LPS. Further bioengineering of *N. meningitidis* LPS showed that a broad range of TLR4 activation can be obtained (Zariri et al., 2016b). Furthermore, a broad range of differential cytokine inducing properties was observed by the different bioengineered LPS species. LPS bioengineering to reduce LPS reactivity has been applied on many different species (Zariri and van der Ley 2015). Besides OMVs from bioengineered *N. meningitidis*, vesicles with genetically detoxified LPS have also been produced from *E. coli* and *V. cholarae* (Leitner et al. 2013; Leitner et al. 2015). Recently, *E. coli* OMVs containing only the lipid IVa of LPS instead of the full LPS have been produced (Watkins et al. 2017). These OMVs retained sufficient capabilities of eliciting immune responses against the included heterologous antigens.

Besides targeted attempts to alter the endotoxicity of the OMV, it is important to take into account that the production method has severe impact on the endotoxicity. It was shown that different OMVs (sOMV, eOMV and dOMV, see Box 2) derived from the same strain have differences in TLR4 and TLR2 activation (Zariri et al., 2016a). To improve the OMV productivity of *H. pylori*, Tol-Pal knockouts were constructed (Turner et al. 2015). However, the downside of the increased vesicle production was the more potent induction of inflammation of these OMVs, by significantly higher IL-8 levels, in host cells than wildtype OMVs. This indicates once more the complexity involved in the design of an OMV based vaccine.

Recently a novel type of vaccine platform based on OMVs was described by two groups, based on heterologous expressed glycan antigens instead of antigenic proteins (Chen et al. 2016; Feldman et al. 2013; Price et al. 2016). These glyco-engineered OMVs (geOMVs) designed by Price et al. are based on the similarity between the polysaccharide translocation in the expression host and the vaccine target (Price et al. 2016). The capsular polysaccharide of *S. pneumoniae* was expressed in an *E. coli* strain lacking its own O-antigen yielding attachment of the *S. pneumoniae* capsular polysaccharides to the *E. coli* lipid A core. In another study, Chen et al. produced glycosylated OMVs that provided protection against *F. tularensis* challenge in mice, by expression of recombinant O-antigen polysaccharide on *E. coli* OMVs (Chen et al. 2016). Furthermore, several lipid A modifications were made resulting in lowered TLR4 activation similarly to the attempts of detoxifying OMV associated LPS. The ease of adding heterologous glycans on OMVs emphasizes the potential of an OMV based vaccine platform for polysaccharide vaccines.

5. Designing the OMV: physiochemical properties

The size of OMVs is an overlooked part of OMV vaccine research that is, however, very relevant since the size influences the uptake by antigen presenting cells (APCs). Macrophages preferentially take up larger particles (Fig. 2) where dendritic cells (DCs) take up particles in the size range of OMVs and VLPs (Xiang et al. 2006). OMVs range in size from 20 to 250 nm in diameter (Kulp and Kuehn 2010) and size is assumed to be homogeneous and similar between different bacterial species. However, minor differences are reported in the size distributions of sOMV, eOMV and dOMV from the same bacterium in similar production processes (van de Waterbeemd et al. 2010). The size of OMVs is important for the design of the production process (Klimentova and Stulik 2015). For example, sterile filtration is only possible for smaller sized OMVs. Moreover, a minimum size of OMVs is required for adequate analysis of OMV quality. Conventional flow cytometry, for example, is not suitable for the analysis of particles smaller than 300 nm (Steen 2004). Improvements on flow cytometry have been made improving the detection limit (van der Vlist et al. 2012) and methods focused on nanoparticles, such as Nanoparticle Tracking Analysis (NTA) (Malloy and Carr 2006) and Tunable Resistive Pulse Sensing (Vogel et al. 2011), have been developed. With these improved methods a size limit of around 20 nm remains for appropriate detection of nanoparticles.

The size and shape are important parameters in the field of synthetic nanoparticles, and these physiochemical properties are highly tunable. Recently Gao and co-workers made an interesting combination of bacterial outer membrane vesicles and synthetic gold nanoparticles (AuNPs) (Gao et al. 2015). OMVs were coated with mechanical force on gold nanoparticles to produce 30 nm sized membrane-coated AuNPs. Mice vaccinated subcutaneously with these nanoparticles showed rapid activation of DCs in lymph nodes, while vaccination with 90 nm sized membrane coated nanoparticles showed reduced accumulation of DCs. The membrane coated AuNPs of 30 nm showed higher induction of specific antibody responses, IFN-gamma production, and IL-17 production when compared to OMVs alone. The IL-4 production was low for all groups indicating a strong Th1- and Th17-biased cellular response. Another study compared ovalbumin loaded styrene nanoparticles with different size and shape, concluding that the smallest (193 nm) spherical particles elicit the strongest Th1 and Th2 immune responses (Kumar et al. 2015). This smaller spherical particle showed a Th1-biased response while the larger (1530 nm) rod-shaped particle elicits a Th2-biased immune response. Smaller nanoparticles (10–200 nm) are able to drain freely to the lymph nodes, while larger nanoparticles (500 nm - 2000 nm) require dendritic cells (Manolova et al. 2008). The larger particles are thus unable to target cells residing in the lymph nodes. Size of the vesicles is thus an important parameter in navigating the immune response by the efficient uptake by antigen presenting cells and efficient entry in to lymph nodes.

6. Concluding remarks and future perspectives

OMVs have been proven to be a flexible vaccine production plat-

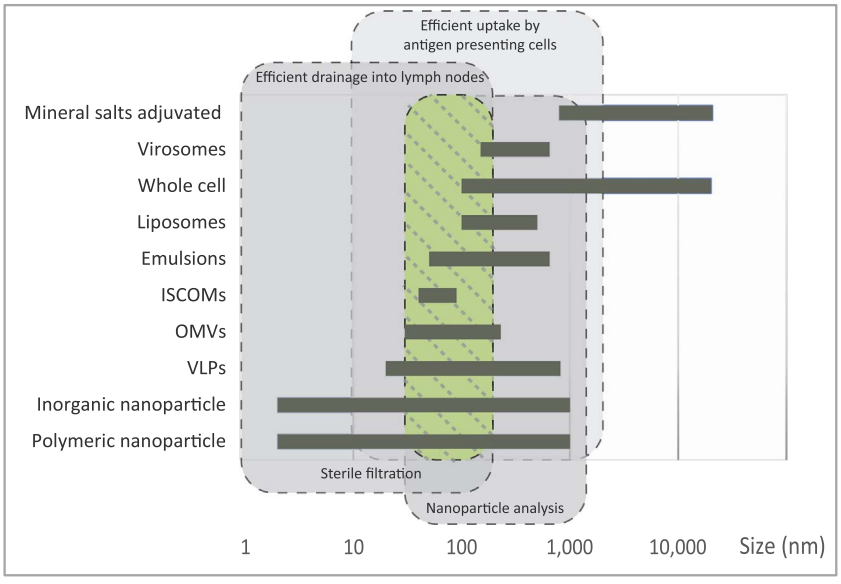

Fig. 2. The size of vaccine concepts and the required size range for accessible production compared to the interaction with the immune system. The possibility of sterile filtration simplifies the design of the production process, while the analysis of nanoparticle size and the number of nanoparticles is essential for quality control. The green box highlights the overall preferred size window for vaccine production. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

form. Heterologous proteins and glycan antigens can be easily added to the vesicle, the immune stimulating properties of the vesicle can be engineered, and the toxicity can be reduced. Future research should address the immune response against the platform backbone, the heterogeneity of the vesicle product and the relatively underexplored effects of luminal OMV components. Besides the use of OMVs as prophylactic vaccines, vast possibilities of OMVs as therapeutic vaccines exist. With the approval of human use of several OMV based meningococcal vaccines, the future of OMV vaccines is bright, paving the way to designer and tailor made OMV vaccines. The use of OMV based vaccine platforms will facilitate enhanced vaccine design and will speed up the introduction of new much needed vaccines.

Conflict of interest statement

The authors declare no financial or commercial conflict of interest.

Acknowledgements

This work was supported by the Dutch Ministry of Health, Welfare and Sport.

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Glossary

EVs: Extracellular vesicles are membrane structures derived from eukaryotic cells, involved in intercellular communication. EVs are widely studied because of their diagnostic and therapeutic potential.:

ISCOMs: Immune stimulating complexes, adjuvating spheres of approximately 40 nm that can be combined with membrane antigens.:

M2e: the extracellular domain of the Matrix 2 protein of influenza A. M2e is evolutionary conserved among influenza A viruses and widely studied as a possible universal vaccine antigen.:

OspA: Outer surface protein A of *Borrelia burgdorferi* is one of the major surface proteins. OspA has been the basis of LYMErix, a vaccine against Lyme's disease marketed until 2002.:

PAMPs: Pathogen associated molecular patterns that are recognized by pattern recognition receptors to activate innate immune responses. Examples of PAMPs are virus associated RNA, bacterial endotoxins, flagellin or LPS. The latter is specifically recognized by the innate immune system via Toll-like receptor 4 (TLR4).:

SpyTag: A peptide derived from *Streptococcus pyogenes* (Spy) designed such that it can form a covalent bond with SpyCatcher, a complementary domain. SpyTag/SpyCatcher pairs can be used for bioconjugations.:

VLPs: Virus-like particles are virus structures without viral genetic material, which makes them non-infectious. VLPs have been recently developed as new-generation of viral vaccines.: