

Chapter 10

Bacterial Membrane Vesicles and Their Applications as Vaccines and in Biotechnology



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Abstract Human society has coevolved with our invisible, microbial neighbors. From their use in the processing of food and drink to the manufacture of commercial products and therapeutics, bacteria, yeast, and fungi are invaluable tools to many aspects of our existence. Despite our long history together, researchers are only recently beginning to understand the complexities of these relationships and how the microbial world can fully be exploited. In the last 50 years alone, researchers have shown that bacteria can be used to manufacture drugs to aid in the treatment of medical disorders such as diabetes, that environmental microbes can be used to clean up chemical spills and disasters, and that the microbial communities that reside within our bodies are capable of influencing our mental and physical health. With this greater understanding comes new avenues of research to utilize these microbes to advance human society. Here we discuss efforts to utilize both native and engineered membrane vesicles shed by bacteria and their potential applications to several areas of biotechnology. We highlight the use of bacterial membrane vesicles in vaccine research and as emerging therapeutics as well as exploring their potential commercial applications and benefits.

10.1 Introduction

Society has a long and storied history with the microbial world. While most often thought of in the context of historical events of plague, disease, and infection, microbes such as bacteria, yeast, and fungi have benefited and advanced human society in countless ways. Microbes have become integral components in food and beverage production, in the manufacture of therapeutics, and as platforms for

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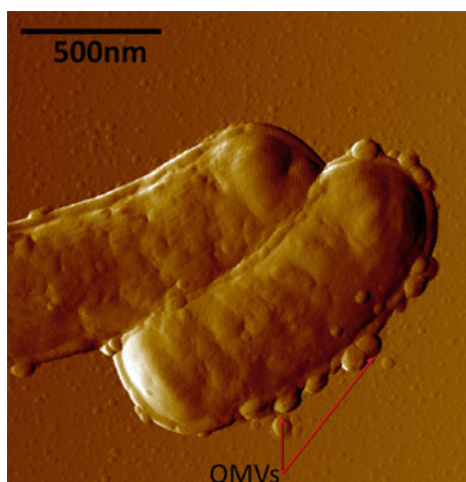
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synthesis of commercial enzymes and other products. In recent years, the ability to engineer biology has made exponential leaps forward allowing researchers to explore alternatives to using natural, “wild-type” bacteria. Advances in DNA sequencing, the accessibility of molecular engineering technologies, improvements in omics’ instrumentation and analysis tools, and many other factors have opened new avenues of biological engineering and synthetic biology.

Since they were first observed under scanning electron microscopy, the vesicles shed by microbes have gone by many names: membrane vesicles, exosomes, outer membrane vesicles, microvesicles, and others (Knox et al. 1967). Often the nomenclature follows the characteristics of the parental organisms such as membrane vesicle (MV) for those particles originating from Gram-positive bacteria and outer membrane vesicle (OMV) for the vesicles produced by Gram-negative bacteria, though this is not always the case. Regardless of the nomenclature and the parental organism, these vesicles are shed from the outermost membrane of their parental microorganism (Fig. 10.1). The biomolecules’ composition is highly variable, as are many of their morphological properties. Most Gram-negative OMVs typically range in size from 50 to 300 nm, though particles of 85–125 nm are by far the most prevalent (Deatherage et al. 2009; Schwechheimer et al. 2013). In comparison, the MVs of Gram-positive bacteria often exhibit a bimodal distribution of particle sizes with a significant number of vesicles in the 10–30 nm range (Dean et al. 2019; Grande et al. 2017). The exosomes of eukaryotic cells show a distribution similar to the OMVs, while microvesicles of mammalian cells have been reported from 100 nm to 1 μm in range (Raposo and Stoorvogel 2013; Tricarico et al. 2017; Zomer et al. 2010). Typically, vesicles are released from the parental bacteria into the surrounding environment, however, researchers have observed some elongated tubules extending from bacterial surfaces that appear as chains of vesicles as seen with some *Myxococcus* species (Berleman et al. 2014).

Fig. 10.1 Nascent MVs of *Lactobacillus reuteri*.

Atomic force microscopy was used to image the formation of MVs at the surface of *L. reuteri*. Cells were fixed at mid-log stage and immobilized to mica surfaces for imaging. Scale bar represents 500 nm



Beyond simple morphological differences, the composition of biological vesicles is highly variable, as would be expected. Proteomic analysis of several bacterial species of both Gram-positive and Gram-negative classification has shown that membrane proteins are often the most prevalent components of the structures (Kroniger et al. 2018; Lee et al. 2007). However, in these studies, researchers have also shown that in many instances the protein composition of the vesicles does not always mimic that of the parental microbe. Early studies of *Escherichia coli* OMVs by Lee et al. highlighted this phenomenon, reporting that highly abundant membrane and periplasmic proteins were often not found at detectable levels in OMV preparations (Lee et al. 2007, 2008). Similar observations have been made with regard to toxins, small molecules, and peptides that appear to be enriched in vesicles by some microbes, suggesting a cellular mechanism or signal that facilitates loading (Dean et al. 2019; Jan 2017). Cell membranes are very heterogeneous by nature with specific proteins and lipids distributed across the exterior of the cell (Barak and Muchova 2013). For most microbes, the outermost membrane is a dynamic surface that not only changes through the various stages of the cells' life cycle but also responds to environmental cues and conditions and changes in response to these stimuli. While there are many mechanisms proposed for vesicle biogenesis, evidence is mounting that formation and release may be a well-controlled cellular event in some bacteria (Elhenawy et al. 2016; Roier et al. 2016). The bacterial membrane vesicles therefore take on a variety of functions, serving as a defense mechanism from phages and antimicrobial peptides, facilitating toxin delivery in pathogenesis, and shuttling DNA and RNA between species allowing for horizontal gene transfer, among others.

In the subsequent sections, we will explore the potential biotechnology applications of both natural and engineered bacterial membrane vesicles. At present, membrane vesicles have seen their greatest acceptance in the area of vaccine development employing the OMVs of Gram-negative bacteria. Unlike many traditional strategies that require inactivation of the virus or toxin, OMV-based vaccines allow antigens to be maintained in their native state and delivered simultaneously with immunostimulatory epitopes that serve as the adjuvant. OMV-based vaccines offer an all-in-one vehicle that is relatively low cost and easy to manufacture. Beyond vaccines, the complexity of protein and other biomolecule composition opens numerous avenues of engineering bacterial membrane vesicles for specific applications that may have commercial value. Researchers have demonstrated that through careful design of molecular systems, specific proteins can be targeted to both the interior and exterior of these biological nanoparticles. These efforts have allowed for the development of OMV-based assays for antigen detection, enzyme-based systems for bioremediation, and tools for imaging cell–cell interactions. These early success in both health-related and commercial applications are foundational to ongoing efforts that may lead to new therapeutics, engineered probiotics, or tools for regulating complex microbial communities.

10.2 OMV Use in Vaccines

The design of vaccines is based on the concept that protective immunity against a given pathogen results from inducing the natural immune response against that pathogen without actually causing the associated disease. Therefore, the vaccine must resemble the pathogen enough to trigger the correct immune response, yet must not itself be infectious. Traditionally, this has been achieved through several strategies, including the use of attenuated pathogens (rendered nonpathogenic by various methods) or killed pathogens (inactivated by heat or chemical denaturation) (Zepp 2010). While numerous vaccines in use today are still produced by these strategies, there are drawbacks to both. Attenuated pathogens may potentially revert back to virulent forms or may pose a higher threat to individuals with compromised immune systems. On the other hand, killed pathogens may not stimulate the immune system enough to provide long-lasting protection, necessitating the use of adjuvants such as aluminum compounds that further stimulate the innate immune response (Coffman et al. 2010; Zepp 2010). Thus, modern vaccines are based on the use of carefully chosen antigens in combination with adjuvants to enhance the body's response to the antigen (Fig. 10.2).

OMVs possess a number of characteristics that make them ideal candidates as a vaccine platform. First, they are naturally produced by bacteria and have intrinsic immunostimulatory properties, as they contain species-specific antigens as well as pathogen-associated molecular patterns (PAMPs). Thus, OMVs induce both adaptive and innate immune responses, allowing them to function as antigen and adjuvant in one package (Ellis and Kuehn 2010; Kaparakis-Liaskos and Ferrero 2015). Second, OMVs are non-replicative and so do not need to be treated with inactivating agents, which preserves antigens and PAMPs in their native states. Third, while a number of nanoparticle-based vaccine delivery vehicles are in development today including OMVs, virus-like particles (VLPs), immunostimulatory complexes (ISCOMs), and inorganic nanoparticles (reviewed in Xiang et al. 2006), OMVs are uniquely suited to this purpose as their natural size range (20–250 nm) enables them to both drain freely into the lymph nodes to target immune cells residing there, as well as to be taken up by antigen-presenting cells such as dendritic cells (Fig. 10.3) (Gerritzen et al. 2017; Kulp and Kuehn 2010; Manolova et al. 2008). Finally, genetic engineering can be used in a number of ways to improve the production, immunostimulatory properties, and safety of OMV-based vaccines (discussed in detail below). As a result of their potential as a vaccine platform, a significant amount of research has been dedicated in recent decades to the engineering of OMV-based vaccines, including an FDA-approved vaccine against meningitis serogroup B.

There are various factors to consider in the design and production of OMV-based vaccines. OMVs may be isolated directly from the target pathogen, though this often requires them to be extracted using detergents that remove certain toxic components from the OMV surface such as lipopolysaccharide (LPS) found on Gram-negative bacteria (Gnop et al. 2017; van de Waterbeemd et al. 2010). Alternatively, mutant

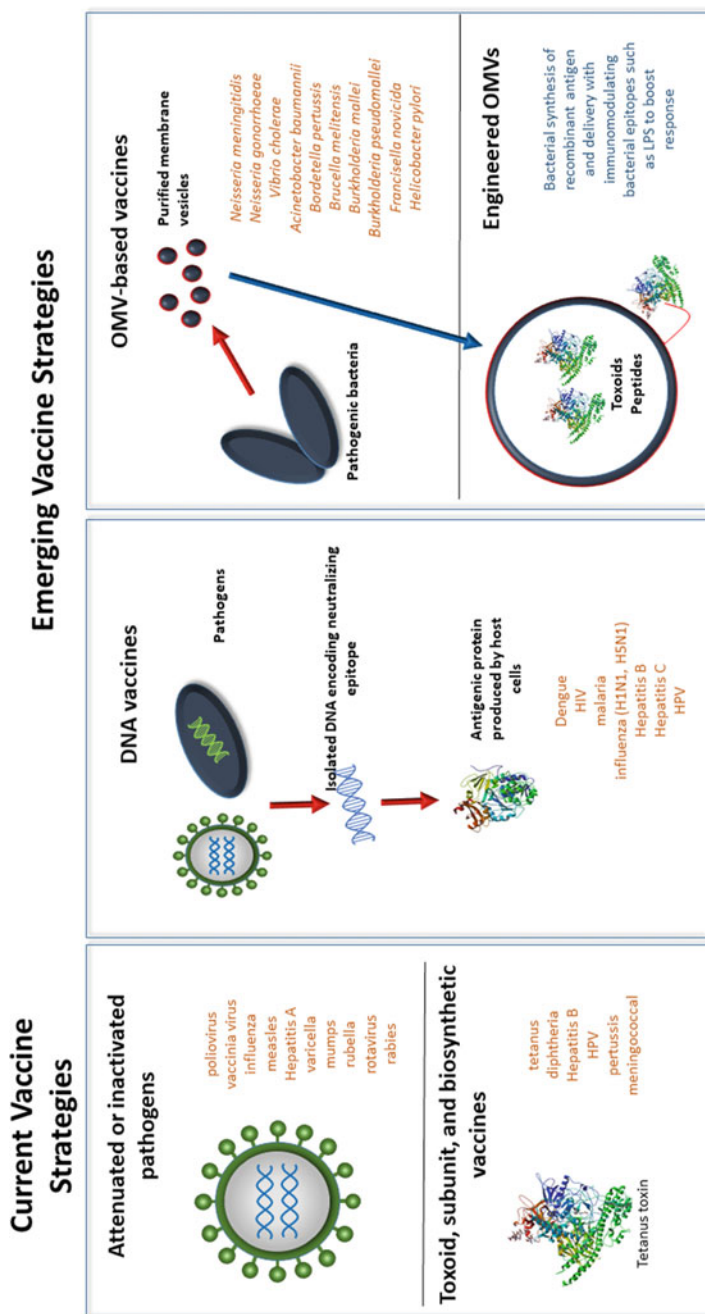


Fig. 10.2 Current and emerging vaccine strategies. Current vaccines employ live and/or attenuated pathogens (upper left panel) or inactivated biomolecules such as proteins, carbohydrates, or peptides (lower left panel). DNA vaccines are based on isolated genetic sequences that encode an antigenic, neutralizing protein or peptide for a target pathogen. DNA is taken up by antigen-presenting cells, then transcribed and translated into a protein sequence that is presented on the cell surface to stimulate an immune response (middle panel). Membrane vesicles can be used in their natural form as they mimic the cell surface of pathogenic bacteria, thereby allowing the host immune system to respond to a wide range of antigenic features of the bacteria (upper right panel). Membrane vesicles can be engineered to contain recombinant proteins and peptides thus allowing for the development of vaccines for any number of bacterial or viral targets determined solely by the cargo within or on the surface of the vesicle itself (lower right panel)

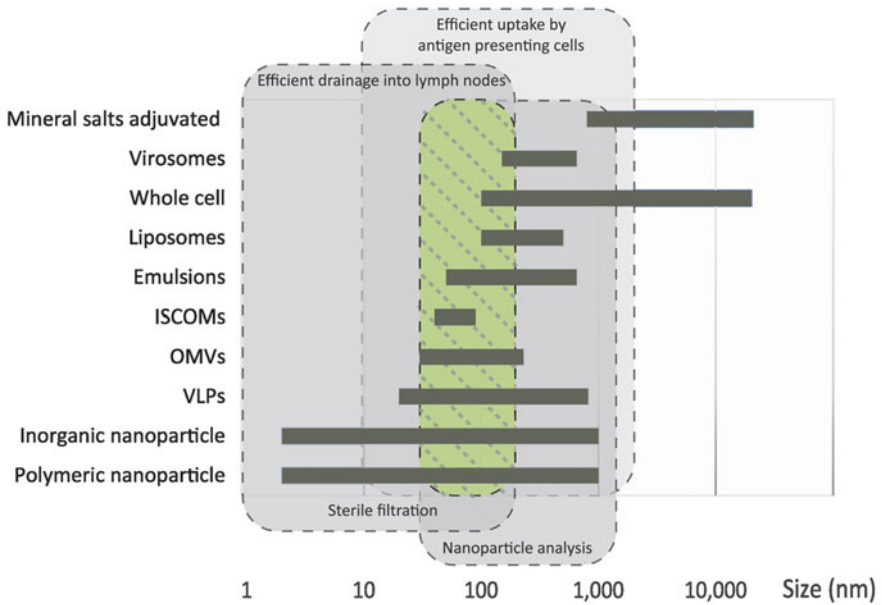


Fig. 10.3 Comparison of current vaccine platforms. Vaccine platforms vary in their size, methods of purification, and their mode of interaction with host immune cells. For each system, these properties have to be weighed to determine their feasibility in administration and commercialization. For example, the possibility of sterile filtrations simplifies the production design while the analysis of nanoparticle size distribution can increase the difficulty of standardizing the final product. Within this figure, the green box highlights the overall preferred size window for vaccine production. Used with permission from Gerritzen et al. *Bioengineering bacterial outer membrane vesicles as vaccine platform*. *Biotechnology Advances*, 2017. **35**(5): p. 565–574

pathogen strains producing modified, less-toxic LPS can be used as the OMV source, or antigens can be heterologously expressed in engineered host species with modified LPS. In either case, this can be done in strains carrying mutations that increase vesicle formation to increase the OMV yield. Genetic engineering can also be used to introduce multiple antigens into a single OMV platform, thus increasing the strength or broadening the scope of the immune response to protect against additional strains of the pathogen. These considerations will be discussed in detail below in the context of the meningitis B vaccine as a primary case study, followed by several other examples of OMV-based vaccines against various diseases. This topic has also been extensively reviewed by other researchers and their work may be referred to for additional information (Gerritzen et al. 2017; Gnopo et al. 2017; Tan et al. 2018; van der Pol et al. 2015).

10.2.1 *OMV Vaccines for Meningitis*

To date, the development of OMV vaccines has seen the most attention for Meningitis type B (MenB), which remains the only disease for which they have been approved for human use. *Neisseria meningitidis* strains are classified into serogroups based on their capsular type, and effective vaccines containing a capsular polysaccharide coupled to a carrier protein have been developed against several serogroups. However, the serogroup B capsule bears structural similarity to a neural cell adhesion molecule in the human brain, which means that it is poorly immunogenic and also that its use in a vaccine has the potential to induce an autoimmune response (Finne et al. 1983; Rosenstein et al. 2001). Consequently, vaccine development for MenB has focused on other antigens, particularly the immunodominant outer membrane protein porin A (PorA) (Holst et al. 2009, 2013). As PorA is abundant in the outer membrane, it is naturally also present at high levels in OMVs isolated from *N. meningitidis* strains, making them an ideal candidate for vaccine development.

OMVs isolated from local serogroup B meningococcal strains proved effective as vaccines in Cuba, Norway, Chile, Brazil, and New Zealand, with the significant limitation that the immune response was strain-specific due to the high sequence variability of PorA (reviewed in Holst et al. 2009). To combat this limitation, an OMV vaccine was developed at the Netherlands Vaccine Institute that is produced from two genetically modified *N. meningitidis* strains that each express three PorA subtypes (Claassen et al. 1996; van der Ley et al. 1995). This hexavalent vaccine “HexaMen” was safe and effective in clinical trials and has been further improved to include a third trivalent OMV to provide coverage against the nine most frequently occurring subtypes of MenB in industrialized countries (de Kleijn et al. 2001; van den Dobbelen et al. 2007).

While OMV vaccines are safe and effective, their primary dependence on the PorA antigen necessitates that even the multivalent vaccines would still require periodic reformulation as the dominant subtypes of MenB change over time. Thus, work continued toward the identification of new, conserved antigens that would allow for the development of a “universal” vaccine for serogroup B meningococcal strains. The availability of whole genome sequencing technology gave rise to a new strategy of vaccine development termed “reverse vaccinology,” in which candidate antigens identified *in silico* are individually expressed in *E. coli* and tested for immunogenicity (Rappuoli 2000). This approach allowed researchers to identify a number of novel antigens that are conserved across multiple *N. meningitidis* strains (Pizza et al. 2000). A new vaccine was then developed that includes five of these novel antigens in the form of three recombinant proteins: a fragment of NadA, plus two fusion proteins consisting of NHBA–GNA1030 and GNA2091–fHbp (Giuliani et al. 2006). NadA is an adhesin, NHBA is a heparin binding protein, and fHbp is a lipoprotein that binds to human complement factor H; the functions of GNA1030 and GNA2091 remain unknown (Comanducci et al. 2002; Madico et al. 2006; Serruto et al. 2010). The final formulation of this vaccine, registered as Bexsero by Novartis Vaccines, includes the five recombinant antigens plus OMVs prepared

from the New Zealand epidemic strain of *N. meningitidis*, which contribute both the PorA antigen to increase strain coverage and additional adjuvant activity (Gorringe and Pajón 2012).

In addition to the choice of antigenic component(s), other important factors to be considered in the development of OMV-based vaccines include the source organism and method of isolation for the OMVs. In the case of MenB vaccines, OMVs have traditionally been isolated from wild-type or recombinant strains of *N. meningitidis*, which requires them to be prepared by detergent extraction due to the highly toxic nature of lipopolysaccharide (LPS) found on the OMV surface (Holst et al. 2009). LPS (also known as endotoxin) is a potent adjuvant that stimulates the innate immune system through Toll-like receptor 4 (TLR4) found on macrophages, however, high levels of LPS can lead to fever, inflammation, and septic shock (Copeland et al. 2005; Raetz and Whitfield 2002). While detergent extraction of OMVs removes most of the LPS and can also increase OMV yield, there are also drawbacks to this process. Detergent use can result in aggregation of OMVs due to removal of negatively charged LPS and phospholipid molecules, which can increase size variability and decrease vaccine stability and shelf life (Holst et al. 2009; van de Waterbeemd et al. 2010). It also alters the proteomic profile of OMVs, through both contamination of OMVs with cytoplasmic proteins as a result of bacterial cell lysis and removal of components that contribute to immunogenicity and adjuvanticity (van de Waterbeemd et al. 2013, 2010; Zariri et al. 2016a). For example, detergent treatment removes the fHbp antigen, a surface-exposed lipoprotein that is highly immunogenic against various *N. meningitidis* strains and is naturally present on native OMVs (Masignani et al. 2003). The absence of antigens and PAMPs such as lipoproteins and phospholipids can reduce the immunogenic response to OMVs, requiring the use of additional adjuvants (Gnopo et al. 2017; Zariri et al. 2016a).

Recent efforts toward a second-generation OMV vaccine for MenB have focused on genetic engineering of *N. meningitidis* strains to reduce LPS toxicity and eliminate the need for detergent extraction of OMVs. The *lpxL1* and *lpxL2* deletion mutants produce modified lipid A, which is the major component of LPS responsible for its toxicity (Fig. 10.4) (van der Ley et al. 2001; Zariri et al. 2016b). The resulting lipid A is penta-acylated rather than hexa-acylated and is no longer toxic as it shows little to no stimulation of human TLR4 (Steeghs et al. 2008; van der Ley et al. 2001). Thus, OMVs isolated from *lpxL1* and *lpxL2* mutant strains do not require detergent treatment and are still immunogenic, though they may require additional adjuvants in order to be effective vaccines in humans due to their lack of stimulation of innate immunity through TLR4 (Fisseha et al. 2005; Koeberling et al. 2008; van de Waterbeemd et al. 2010). Further bioengineering of LPS biosynthesis or modification through the use of the *lptA* or *lgtB* mutations and heterologous expression of the *pagL* gene encoding lipid A 3-O-deacylase from *Bordetella bronchiseptica* has shown that the potential exists to produce OMVs that display a broad range of TLR4 activation, which show promise to be used as effective stand-alone vaccines (Geurtsen et al. 2006; Pupo et al. 2014; Zariri et al. 2016b).

Finally, there are additional genetic mutations available that can increase overall OMV yield. A mutation in the RmpM protein, which links the outer membrane of

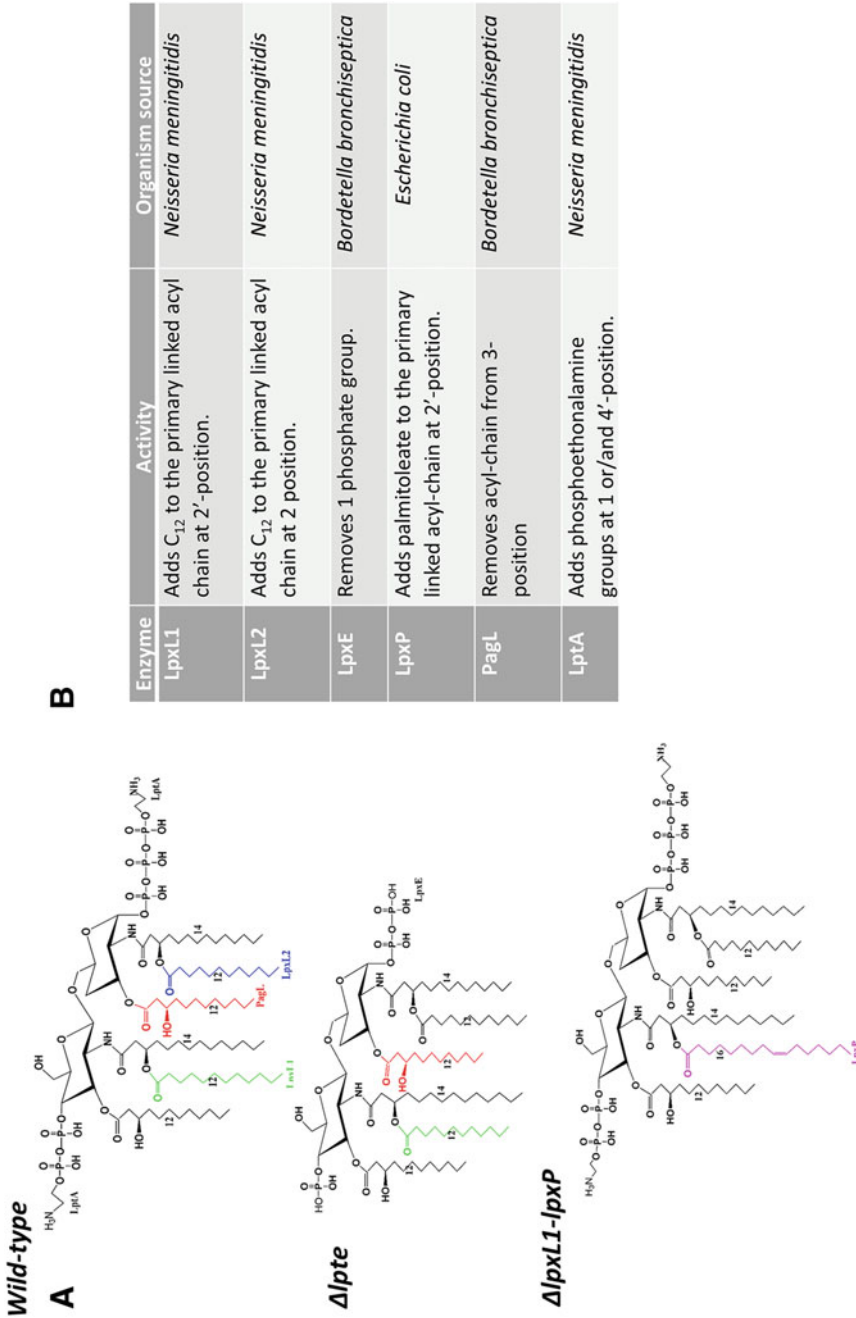


Fig. 10.4 Structural changes in Lipid A. Lipid A can be enzymatically modified by a number of enzymes as shown in the color panel (a). LpxL1 (green), LpxL2 (blue), LpxP (pink) and LptA (brown) all add the corresponding group to the molecule, whereas PagL (red) and LpxE (orange) remove the group. The $\Delta lptA$ and $\Delta lpxL1-lpxP$ mutants produce modified LPS that is less toxic than LPS from wild-type *E. coli*. The abbreviation of the enzymes, organism source, and activity are presented (b). Used with permission from Zairri et al. *Modulating endotoxin activity by combinatorial bioengineering of meningococcal lipopolysaccharide*. Scientific Reports, 2016. 6: p. 36575

the cell to the peptidoglycan layer, leads to a more loosely attached outer membrane and increased OMV release, but does not affect bacterial growth or OMV immunogenicity (Arigita et al. 2004; Klugman et al. 1989; van de Waterbeemd et al. 2010).

Research to date on MenB vaccines highlights both the advantages and challenges of using OMVs as a vaccine platform. Recent developments in bioengineering have done much to expand the potential in this area, and it is likely that the next generation MenB vaccine will make use of the various options in antigen choice and display, LPS detoxification, and OMV yield to result in improved effectiveness, safety, and ease of production.

10.2.2 *OMV Vaccines for Gonorrhea*

Gonorrhea is one of the most frequently reported communicable diseases in the USA, with a worldwide incidence estimated at 78 million new cases per year (Bolan et al. 2012; Newman et al. 2015). Despite more than a century of research, efforts to develop a vaccine against gonorrhea have been unsuccessful, while the need for such a vaccine has only increased as a number of antibiotic-resistant strains of gonorrhea have emerged (Bolan et al. 2012; Edwards et al. 2016). Interestingly, ecological data suggest a decline in gonorrhea during the time period immediately after the use of the OMV-based MenB vaccines in Cuba and Norway, indicating that the OMV vaccine may afford some protection against gonorrhea (Pérez et al. 2009; Whelan et al. 2016). Furthermore, a retrospective case-control study done in New Zealand estimated that the OMV vaccine used in that country, MeNZB, was 31% effective in preventing gonorrhea (Petousis-Harris et al. 2017). This landmark finding represents the first example of any vaccine being associated with protection against gonorrhea in humans.

Despite causing significantly different diseases, the causal pathogens of gonorrhea and meningitis, *Neisseria gonorrhoeae* and *N.meningitidis*, are closely related. The two are estimated to share 80–90% homology in DNA sequence, and several of the antigens found in the Bexsero MenB vaccine are also present in various *N. gonorrhoeae* strains at approximately 60–90% amino acid sequence identity to the *N. meningitidis* reference strain (Hadad et al. 2012; Semchenko et al. 2018; Tinsley and Nassif 1996). It was recently shown that both the OMV and recombinant protein antigen components of the Bexsero vaccine (which contains the same OMV component as the MeNZB vaccine) could elicit antibodies against *N. gonorrhoeae* in rabbits and humans (Semchenko et al. 2018). This provides some explanation of the cross-protection afforded by the MenB vaccines and suggests that development of a similar OMV-based strategy might be the key to an effective vaccine against gonorrhea.

The promising findings of Petousis-Harris et al. and Semchenko et al. with regard to vaccine cross-protection are very recent, and as such there have not yet been reports on OMV-based vaccines designed specifically against gonorrhea. However, some candidate antigens have been identified. For example, *N. gonorrhoeae* MetQ, a

subunit of the methionine binding ABC transporter, was recently shown to be highly conserved, localize to the bacterial and OMV surface, and to play a role in adherence to cervical epithelial cells. Most importantly, antibodies against MetQ are bactericidal and can block adherence, indicating its potential as a candidate vaccine antigen (Semchenko et al. 2017). Additionally, it was recently demonstrated that a vaccine consisting of formalin-inactivated whole bacterial cells encapsulated in microparticles was effective in a mouse model (Gala et al. 2018), further supporting the hypothesis that a vaccine presenting surface-exposed antigens in their native state may be the most effective avenue for a successful gonorrhea vaccine.

10.2.3 OMV Vaccines for Influenza

While the above examples demonstrate the use of pathogen-derived OMVs as vaccines, other species of bacteria can be engineered to heterologously express and display antigens on their outer membrane to produce antibacterial or antiviral OMV vaccines. Despite the availability of seasonal vaccines, influenza infection remains an ongoing threat, particularly with the ability of influenza A viruses to form pandemic strains. Current influenza vaccines generally produce an immune response against the immunodominant glycoproteins hemagglutinin and neuraminidase, however, these proteins are extremely variable and thus strain-specific vaccines must be redeveloped annually (Sato et al. 2001; Treanor 2015). Variations in these surface epitopes and others also contribute to a low efficacy for influenza vaccinations (Osterholm et al. 2012). Therefore, there is significant interest in the development of a universal influenza vaccine that could reduce the need for annual redesign and revaccination and would provide protection should a new pandemic strain arise.

One of the most promising target antigens for a universal vaccine is M2e, an integral membrane protein of influenza A virus. Unlike hemagglutinin and neuraminidase, the M2e sequence is highly conserved across strains, however, it is not as immunogenic as the aforementioned antigens and requires adjuvants to be effective (Lamb et al. 1985). To produce a self-adjuvant, OMV-based vaccine, the probiotic *E. coli* strain Nissle 1917 was engineered to express a fusion protein consisting of M2e4xHet, a multimeric construct containing four M2e variants, as a C-terminal fusion to ClyA, an *E. coli* transmembrane protein that is known to be enriched in OMVs (Rappazzo et al. 2016). Mice vaccinated with the resulting recombinant OMVs showed a 100% survival rate after challenge with a lethal dose of a mouse-adapted H1N1 strain (Rappazzo et al. 2016). The OMV-based vaccine was further improved by production in ClearColi, an *E. coli* strain engineered to contain only the LPS precursor lipid IVa instead of full LPS, and these OMVs provided equal protection against influenza in mice and ferrets without LPS-based endotoxicity, which would otherwise hamper translation of this therapy to use in humans (Watkins et al. 2017).

10.2.4 *OMV Vaccines for Cholera*

Cholera, a secretory diarrheal disease caused by the Gram-negative bacterium *Vibrio cholerae*, is a major cause of mortality in developing countries, particularly for infants and young children. While cholera vaccines are currently available, they still suffer from drawbacks of high cost, short shelf life, and the need for cold storage, all of which limit their implementation in developing countries and highlight the need for new candidate vaccines (Bishop and Camilli 2011). *V. cholerae* OMVs could induce a specific, high-titer, and long-lasting immune response in mice, and immunization of female mice also resulted in protection of their neonatal offspring via the transfer of IgG and IgA antibodies in the mother's milk (Schild et al. 2009, 2008). As was the case for the *N. meningitidis* and *E. coli* OMVs, genetic modification of lipid A resulted in reduced endotoxicity without diminishing the immunogenic potential of the vaccine (Leitner et al. 2013).

It has already been shown that there is a protective effect of breastfeeding against cholera due to the presence of IgA antibodies directed against *V. cholerae* surface structures and cholera toxin (Clemens et al. 1990; Glass et al. 1983; Hanson et al. 1985; Qureshi et al. 2006). The major protective antigen of the OMV vaccine is the O-antigen, which is present in high amounts on the OMV surface, and cholera toxin is known to be packaged into OMVs (Chatterjee and Chaudhuri 2011; Leitner et al. 2013). Thus, an OMV vaccine may be the ideal candidate to deliver effective antigens that can induce immunity via high IgA titers, and immunization of adult women would hopefully lead to significant protection of newborns and young children, often the population most affected during cholera epidemics (Leitner et al. 2013).

In addition to its effectiveness, the OMV vaccine is also a promising candidate to overcome the cost, stability, and transport limitations of the other available vaccines. Large-scale production of OMVs at reasonable cost has already been demonstrated for the meningitis vaccines. OMVs purified from *V. cholerae* were stable after 1 month at 37 °C, and could be easily administered for immunizations without accessory buffer solutions, indicating that cold storage and trained medical professionals may not be required for vaccine distribution (Leitner et al. 2013; Schild et al. 2009).

10.2.5 *OMV and EV Vaccines for Other Diseases*

In addition to the above examples, OMVs from various other pathogenic species have been tested as a vaccine platform against their associated diseases. These include *Acinetobacter baumannii*, *Bordetella pertussis* and *B. parapertussis*, *Bruceella melitensis*, *Burkholderia mallei* and *B. pseudomallei*, *Francisella novicida*, *Heliobacter pylori* and *H. felis*, *Klebsiella pneumoniae*, *Porphyromonas gingivalis*, *Salmonella enterica ssp. enterica ser. Typhimurium*, and *Shigella* spp. (Alaniz et al.

2007; Asensio et al. 2011; Avila-Calderón et al. 2012; Bottero et al. 2013; Keenan et al. 1998; Kesavalu et al. 1992; Lee et al. 2012; McConnell et al. 2011; Mitra et al. 2013; Nieves et al. 2014; Pierson et al. 2011; Roberts et al. 2008).

While the above examples all pertain to the use of OMVs from Gram-negative organisms, the relatively recent discovery that Gram-positive bacteria also produce membrane vesicles has led several groups to test whether Gram-positive MVs might also be effectively used as vaccines (Brown et al. 2015; Lee et al. 2009; Liu et al. 2018b) (See Chap. 2). Promising results from vaccinations with MVs have been demonstrated for *Bacillus anthracis*, *Clostridium perfringens*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (Choi et al. 2015; Jiang et al. 2014; Olaya-Abril et al. 2014; Prados-Rosales et al. 2014; Rivera et al. 2010). One potential advantage of using MVs from Gram-positive bacteria is that they do not contain LPS and thus are unlikely to require detoxification; for example, Choi et al. reported that unmodified MVs from *S. aureus* were self-adjuncting and could stimulate effective immunity against *S. aureus* with no observable toxic effects (Choi et al. 2015).

In each of the abovementioned cases, the administered OMVs or MVs induced an immune response in cell lines or animal models, and usually promoted survival or prolonged the time to death after challenge with the associated pathogen. As antibiotic-resistant strains are a serious problem for some of these pathogens, the potential for the development of OMV/MV vaccines that protect against these diseases is highly attractive.

10.2.6 OMV Vaccines Based on Recombinant Antigens

There are numerous reports of OMV vaccines based on heterologous expression of antigens. *E. coli* is often used as the host species for OMV production, as is the case for vaccine concepts against *A. baumannii*, *Francisella tularensis*, *S. pneumoniae*, *Campylobacter jejuni*, *Plasmodium sp.*, *Chlamydia sp.*, and *Leishmania sp.* (Bartolini et al. 2013; Chen et al. 2016; Fantappiè et al. 2014; Huang et al. 2016; Pritsch et al. 2016; Schroeder and Aebischer 2009).

Other host species have also been used for recombinant OMV production, particularly those whose OMVs have already been studied as vaccine candidates including *N. meningitidis* expressing antigens against Lyme disease or genital herpes, *V. cholerae* expressing enterotoxigenic *E. coli* antigens and *S. enterica ssp. enterica ser. Typhimurium* expressing protective antigens to prevent pneumococcal disease, tuberculosis, and chlamydia infections (Daleke-Schermerhorn et al. 2014; Del Campo et al. 2010; Kuipers et al. 2015; Leitner et al. 2015; Muralinath et al. 2011; Salverda et al. 2016).

As in the influenza vaccine described above, the target protein-based antigens are generally expressed as fusions with native membrane-localized proteins such as ClyA or OmpA in *E. coli* or fHbp in *N. meningitidis*. The *E. coli* autotransporter Hemoglobin protease (Hbp) was also recently engineered as a platform that can be

used for simultaneous display of multiple heterologous antigens on a single, stable scaffold that localizes to the OMV surface at high densities (Daleke-Schermerhorn et al. 2014; Kuipers et al. 2015). Similarly, a system has been designed in which multiple proteins can be produced separately from OMVs, then assembled onto a scaffold located on the OMV surface (see the below section on biomass conversion for more detail) (Park et al. 2014). These developments open up the exciting possibility of creating multivalent OMV vaccines that present several antigens from the same pathogen or even antigens from several different pathogens.

10.2.7 OMV Vaccines Based on Bacterial Glycans

In some cases, the target vaccine antigens are not proteins but rather glycans. Many successful current vaccines, such as those for non-serogroup B meningitis, are based on the use of glycoconjugates that consist of glycans coupled to T cell-dependent protein antigens, as glycans alone usually elicit T cell-independent responses which are weaker and short-lived. However, a major drawback to this glycan-based vaccine strategy is that current production techniques are technically demanding, costly, and unreliable (Price et al. 2016). As an alternative strategy, *E. coli* can be engineered to produce and display pathogen-specific polysaccharides on OMVs. For example, Chen et al. introduced the gene cluster required for synthesis of *F. tularensis* O-Polysaccharide (O-PS), a subunit of LPS, into a hypervesiculating laboratory *E. coli* strain which is O-PS-deficient but still produces the lipid A core to which O-PS attaches (Chen et al. 2016). In this model, pathogen-specific O-PS is synthesized on the cytoplasmic face of the inner membrane by plasmid-encoded enzymes, to which endogenous *E. coli* proteins complete the translocation of O-PS to the outer membrane and attach it to the lipid A core. The result of this process is that *F. tularensis*-specific O-PS is displayed on the *E. coli* outer membrane and consequently the OMVs as well (Fig. 10.5). In parallel, the *E. coli* host strain was also engineered to produce the less inflammatory, penta-acylated lipid A to circumvent LPS-based toxicity. Vaccination of mice using the subsequent glycoengineered OMVs significantly delayed time to death after lethal *F. tularensis* challenge. This is a promising result for the development of a vaccine against *F. tularensis*, which is a class A bioterrorism agent for which no licensed vaccine currently exists (Oyston et al. 2004). A similar strategy was employed by Price et al., who engineered *E. coli* to produce OMVs displaying the *S. pneumoniae* serotype 14 capsular polysaccharide, which elicited an immune response comparable to a commercial pneumococcal vaccine in mice (Price et al. 2016). They also designed glycoengineered OMVs that display the *C. jejuni* N-glycan, which resulted in an unprecedented level of protection against *C. jejuni* in chickens (Price et al. 2016). Collectively, these studies highlight the potential of glycoengineering in vaccine development for pathogens that have to date proven incompatible with other methods.

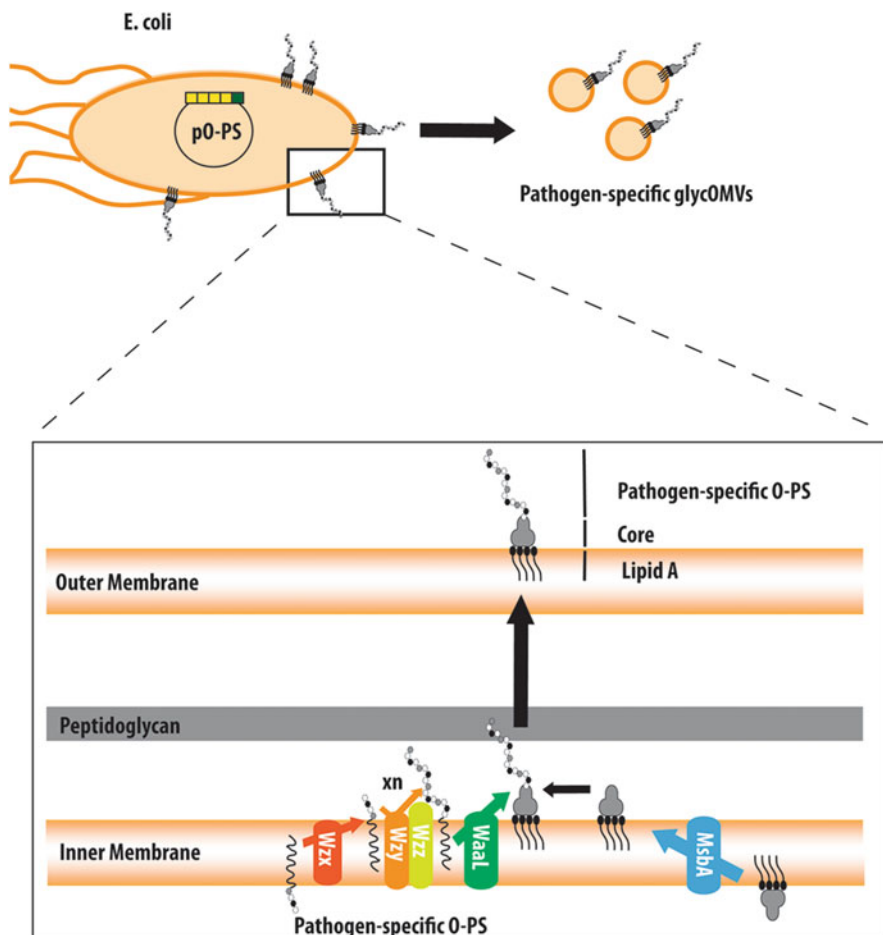


Fig. 10.5 Schematic of the assembly of pathogen-specific antigens on the surface of OMVs. Cellular machinery is responsible for synthesis and transport to the outer membrane where flippase (Wzx) is responsible for membrane translocation and surface expression. Membrane-bound antigen is released in budding vesicles. Reproduced with permission from Chen et al. *Outer membrane vesicles displaying engineered glycotopes elicit protection antibodies*. PNAS, 2016. **113**(26): p. E3609-E3618

10.2.8 OMV Vaccines for Host Glycans

Glycoengineering of OMVs also carries the exciting possibility for use in generating immune responses against clinically important host glycans such as those associated with certain types of cancer. For example, Valentine et al. engineered *E. coli* OMVs to display two clinically important human glycan structures, namely the tumor-specific carbohydrate antigens polysialic acid (PSA) and Thomsen-Friedenreich

antigen (T-antigen) (Valentine et al. 2016). Both of these glycans are highly expressed in several different cancers but not in normal cells and antibodies recognizing these antigens could have clinical benefits, however, on their own, they have low intrinsic immunogenicity (Heimburg-Molinari et al. 2011). OMVs displaying these antigens could elicit strong IgG antibody titers in mice, indicating that this may be an effective strategy for generating functional antibodies against clinically relevant carbohydrates. These results highlight the advantages of using OMVs to display glycan antigens: their production is less complicated and expensive than traditional glycoconjugate vaccines, the strategy can be easily tailored to various glycan antigens (provided the biosynthetic pathway for the target is known), and they provide the necessary adjuvanticity to activate long-lasting, T cell-dependent immunity.

10.3 OMV-Based Therapeutics

Biologically derived nanoparticles isolated from bacteria or mammalian cells have received substantial attention in recent years for their potential as biodegradable carriers that can specifically deliver cargo to targeted sites (Yoo et al. 2011). While many drugs face limitations in application due to toxicity, poor stability, and inability to cross cell membranes, engineered OMVs/MVs can circumvent many of these drawbacks as they can protect their cargo from degradation, target it to specific cells, and deliver it into those cells efficiently.

The capacity for engineered OMVs to target a specific cell population and deliver cargo was effectively demonstrated by Gujrati et al., who developed *E. coli* OMVs for use in cancer therapy (Gujrati et al. 2014). They engineered *E. coli* to express an affibody specific to HER2, a transmembrane receptor overexpressed in many cancers, as a fusion protein with the C-terminus of the native ClyA protein, which targets the resulting protein to the OMV surface (Fig. 10.6). Isolated OMVs were loaded via electroporation with a therapeutic siRNA that targets the kinesin spindle protein (KSP), which is overexpressed in rapidly proliferating cells such as those found in tumor tissue. Silencing of KSP blocks the formation of mitotic spindles, leading to cell-cycle arrest and apoptosis. When injected into mice, the resulting OMVs targeted HER2-overexpressing cells with high affinity and were rapidly internalized, leading to significant inhibition of tumor growth and reduction of tumor size that could be attributed to knockdown of KSP expression. As these OMVs were isolated from an *E. coli* strain with modified LPS, they have low endotoxicity and did not cause significant side effects, which is a major limiting factor of many drug therapies. This work highlights the potential of vesicle-based nanoparticles as cell-specific delivery vehicles that can overcome many of the drawbacks and limitations that hamper the development and release of new cancer therapies.

Various other health benefits have been observed for membrane vesicles, particularly those produced by probiotic bacteria. These include bacterial species that

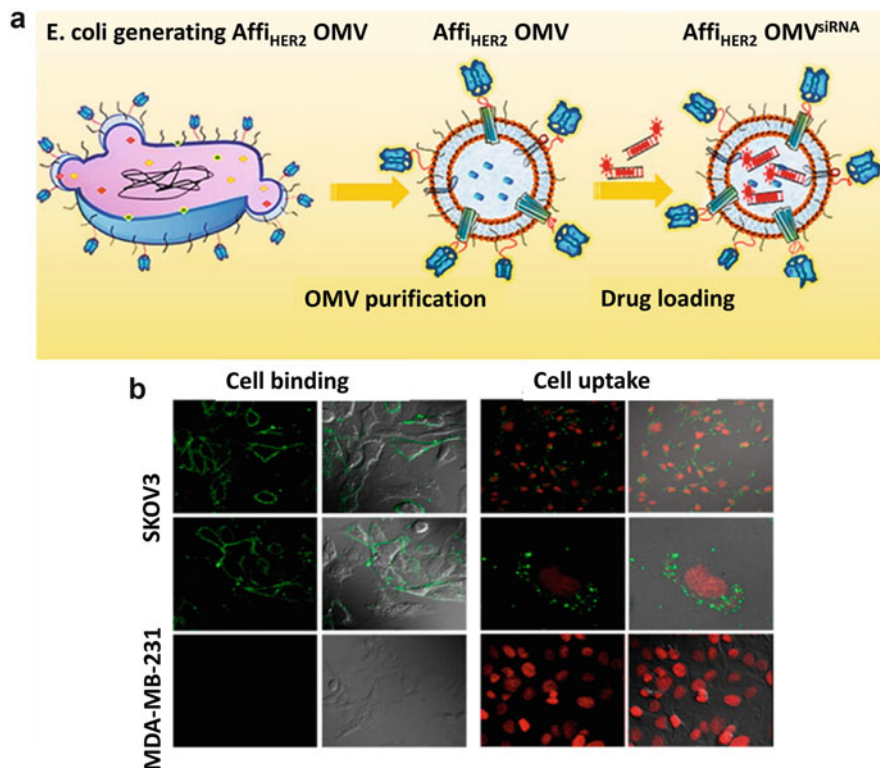


Fig. 10.6 OMV as therapeutic agents for cancer. **(a)** *E. coli* OMVs were labeled with anti-HER2 affibodies then loaded with cytotoxic siRNA labeled with a fluorophore for visualization. **(b)** For cell binding and uptake studies, HER2-overexpressing SKOV3 cells and HER2-negative MDA-MB-231 cells were co-incubated with Affi_{HER2} OMV and stained with an anti-affibody antibody (green). Receptor-specific cell binding and uptake were seen only with HER2-overexpressing SKOV3 cells. Reproduced with permission Gujrati et al. (2014) *Bioengineered Bacterial Outer Membrane Vesicles as Cell-Specific Drug-Delivery Vehicles for Cancer Therapy*. ACS Nano 8(2): pp. 1525–1537

colonize the gastrointestinal tract and confer benefits to the host through either direct communication with host cells or through interaction with other probiotic or pathogenic bacteria (Bron et al. 2011). Often, the beneficial effects result from the interaction of probiotic bacteria (and their associated membrane vesicles) with the host immune system. For example, recent studies have suggested that probiotic bacteria can suppress inflammatory and allergic responses through modulation of immune responses. MVs from *Bifidobacterium longum* could alleviate a food allergy response in a mouse model by penetrating through intestinal epithelial cells and selectively targeting and inducing apoptosis of mast cells (Kim et al. 2016). The commensal species *Bacteroides fragilis* delivers the immunomodulatory molecule Polysaccharide A to dendritic cells via OMVs, thereby suppressing immune responses that drive inflammation (Shen et al. 2012). A similar result was also

shown for *Lactobacillus rhamnosus*, for which MVs could recapitulate the previously demonstrated immunoregulatory and neuronal effects of whole bacteria (Al-Nedawi et al. 2015). Finally, MVs of Kefir-derived *Lactobacillus* strains reduced inflammatory cytokine production and alleviated symptoms in a mouse model of inflammatory bowel disease (Seo et al. 2018).

The potential modulation of the host immune system by probiotic bacteria can also have a protective role against various pathogens. While it has not been definitely shown that these benefits are conveyed solely by membrane vesicles, researchers have performed studies using culture media and other preparations devoid of cells and seen similar results as discussed above. As specific examples of MV-mediated protection, Li et al. showed that MVs derived from *L. plantarum* provided protection to *C. elegans* against vancomycin-resistant *Enterococcus faecium* through upregulation of multiple host defense genes (Li et al. 2017). The authors also performed these experiments with the common human intestinal epithelial cell line Caco-2 and observed similar changes in gene expression. Further exploration into the mechanisms of this and other protective effects induced by probiotic MVs might allow for the development of new treatments for antibiotic-resistant pathogens.

Probiotic bacteria have also been suggested to have cancer prevention properties, particularly against colon cancer (Commane et al. 2005; dos Reis et al. 2017; Paolillo et al. 2009). While the exact mechanisms of this are unknown, it has been reported that many probiotic species exert this effect through the secretion of factors that induce apoptosis, and it is likely that these factors are delivered to host cells via bacterial membrane vesicles (Oelschlaeger 2010). Indeed, it was recently shown that purified MVs from *L. rhamnosus* have significant cytotoxic effects on hepatic cancer cells (Behzadi et al. 2017). As membrane vesicles derived from the microbiota in the gastrointestinal tract can travel to the liver and other nearby organs through the bloodstream, this provides further evidence for the potent anticancer properties of probiotic bacteria and their membrane vesicles (Salminen et al. 2004). Furthermore, as probiotic bacteria generally do not have deleterious effects on the host, exploitation of these properties may make possible the development of new cancer treatments that do not also cause the damaging side effects of traditional chemotherapeutic agents (Behzadi et al. 2017).

10.3.1 Emerging Therapeutic Applications

In addition to the potential anticancer and other uses discussed above, there are a number of other therapeutics that could benefit from delivery via OMVs/MVs. Compounds such as antimicrobial peptides which would be susceptible to degradation in their free form, or antibiotics that would otherwise not be able to cross the cell membrane, could be packaged into bacterial membrane vesicles for protection and delivery into target cells (Liu et al. 2018b). This may be particularly important for treatment of pathogens that can ordinarily resist antibiotics through mechanisms such as outer membranes with low permeability, or biofilms that delay penetration of

the antibiotic or directly inactivate it via secreted enzymes such as β -lactamases (Messiaen et al. 2013). Unlike free molecules, vesicles may be transported through the biofilm and taken up into cells, bypassing these defense mechanisms. For example, packaging of the antibiotic tobramycin into artificial liposomes greatly increased its effectiveness against multiple pathogens including *Burkholderia cepacia*, *Pseudomonas aeruginosa*, and *S. aureus* (Beaulac et al. 1998). Several liposome-based drugs are currently in clinical trials; however OMV/MV-based vesicles may offer additional benefits related to ease of production or the ability to incorporate specific cell targeting motifs through genetic engineering.

Phage therapy has also received significant attention in recent years as an alternative treatment for antibiotic-resistant bacteria (Lin et al. 2017). One challenge to the implementation of phage therapies is that many phages have narrow host ranges, limiting their applicability. As membrane vesicles naturally play a role in broadening phage host ranges through the transfer of phage receptors between bacterial hosts, this capability could be harnessed for clinical use (Tzipilevich et al. 2017). For example, vesicles derived from phage-sensitive bacteria could be given to a patient prior to administration of the phage itself. If those vesicles are taken up by bacteria already infecting the patient, the phage receptor could be transferred and could potentially enhance targeting of the phage to the infectious bacteria (Liu et al. 2018b).

It has also been proposed that vesicles could be used for delivery of genome editing tools, such as the Cas9-guide RNA ribonucleoprotein complex required for CRISPR-based genome editing, to correct genetic disorders or to combat pathogens (Knott and Doudna 2018; Liu et al. 2019).

Membrane vesicles could also be used as vehicles for delivery of nutritional compounds to the host gastrointestinal tract. For example, vitamin K2 (menaquinone) is a cofactor required for the production of blood coagulation factors and osteocalcin (a bone-forming protein) and may also prevent osteoporosis, coronary heart disease, and liver cancer, but recent data indicate that subclinical vitamin K deficiency is not uncommon (DiNicolantonio et al. 2015). Vitamin K cannot be synthesized by humans, but rather is mainly produced by bacteria in the intestine such as *B. subtilis* and certain strains of lactic acid bacteria (Liu et al. 2018a). In particular, a strain of *B. subtilis* isolated from the traditional Japanese fermented soybean product natto could be engineered to produce very high amounts of vitamin K2 (Sato et al. 2001). As menaquinones are hydrophobic compounds that accumulate in the bacterial cell membrane, the potential exists for vitamin K-containing MVs isolated from *B. subtilis* or lactic acid bacteria to be administered as dietary supplements (Liu et al. 2018a). A similar strategy could be used for other important nutritional or medically relevant compounds that are not naturally produced in high amounts by these bacteria, if they could be genetically engineered to produce and package such compounds in vesicles.

10.3.2 *Commercial Application of EVs*

The protein composition of bacterial membrane vesicles is highly variable dependent upon growth conditions, culture age, and many other factors. Despite the variability, abundant membrane proteins such as porins, membrane channels, and others consistently appear in proteomic analysis of bacterial membrane vesicles and offer potential anchors for their functionalization (Dean et al. 2019; Kroniger et al. 2018; Kwon et al. 2009; Lee et al. 2007; Schwechheimer et al. 2013; Yun et al. 2017). With the targeted loading of bacterial membrane vesicles, researchers can begin to exploit the inherent natural advantages afforded by these vesicles, such as protection from environmental conditions, as well as others of specific design such as enzyme localization and assembly. This engineering of OMVs has the potential for designer probiotics, biological catalysts, and even development for new therapeutic platforms. In the subsequent section, we highlight some of the areas where engineered OMVs and MVs are already making inroads as new tools for a range of commercial, environmental, and health-related applications.

10.3.3 *OMVs for Biomass Conversion*

The successful engineering of *E. coli* and other bacteria to express target proteins and display them on the OMV surface makes possible their use not just in biomedical applications, but for other purposes in which nanoparticles are needed. Many biological processes, such as the Krebs TCA cycle in mitochondria or cellulose hydrolysis by cellulosomes on the surface of anaerobic bacteria, are carried out through complex multienzyme cascades that achieve specificity and efficiency through compartmentalization and precise spatial organization of individual proteins. In an effort to mimic this organization for biotechnological applications, several studies have demonstrated synthetic assembly of multiple enzymes onto liposomes or polymersomes; however, the process is complex, costly, and the resulting liposomes are often fragile, making this approach impractical for large-scale applications (Fischer et al. 2002; van Dongen et al. 2009; Vriezema et al. 2007). Conversely, OMVs could provide the ideal backbone for synthetic nanoreactors if some or all of the enzyme production and assembly could be driven by genetic engineering of the *E. coli* host strain.

This approach was successfully demonstrated by Park et al., who engineered *E. coli* OMVs to display a functional multienzyme complex similar to a cellulosome (Park et al. 2014). Natural cellulosomes are composed of a structural scaffold containing repeating cohesin domains that are bound individually to cellulases via corresponding dockerin domains (Fontes and Gilbert 2010). To mimic this structure on the surface of OMVs, Park and colleagues engineered *E. coli* to express a scaffold consisting of three different cohesin domains and a cellulose binding domain, attached to the outer membrane by the ice nucleation protein anchor (Fig. 10.7).

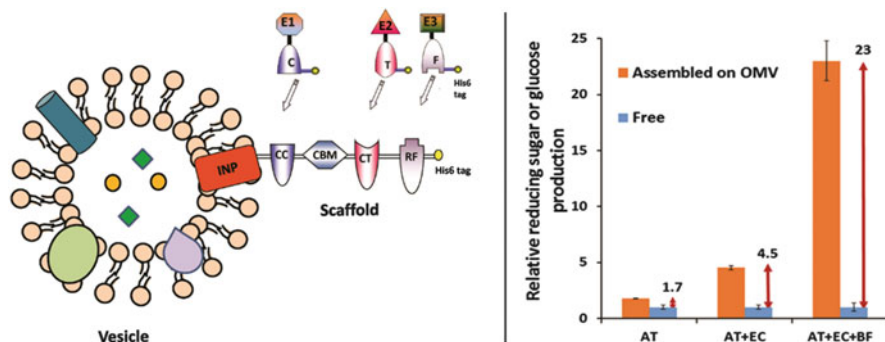


Fig. 10.7 Multienzyme assembly on engineered OMVs. *Left panel:* A trivalent scaffold was developed that contained three orthogonal cohesion domains that enabled the assembly of cellulose enzymes tagged with complementary dockerin domains. *Right panel:* Assembly of the enzyme system on OMV surfaces significantly improved enzyme activity (orange bars) compared to the free enzyme controls (blue bars). Reproduced with permission from Park et al. (2014) *Positional Assembly of Enzymes on Bacterial Outer Membrane Vesicles for Cascade Reactions*. PLoS ONE 9(5): e97103

Three different cellulases, each possessing a dockerin domain corresponding to one of the cohesins, were produced separately in *E. coli* and subsequently incubated with the OMVs to allow cohesin–dockerin interactions to assemble the full multienzyme complex. The resulting OMVs showed 23-fold enhancement of cellulose hydrolysis as compared to free enzymes. This result is very promising for the potential of OMVs as nanobioreactors in biotechnology, with the added benefit that the same strategy could be used for various other enzymatic cascades by replacing the dockerin-bound cellulases with other enzymes engineered to contain the dockerin domain.

10.3.4 OMVs for Bioremediation

Fortunately for mankind, microbial populations are able to exploit ancestral, bi-functional, or newly evolved cellular processes to degrade and consume many of the chemical contaminants we have produced or inadvertently released into the environment. Take for example, the rapid degradation of the oil plume released following the Deep Sea Horizon disaster (Atlas and Hazen 2011; Scoma et al. 2016) or the more recent identification of marine bacteria capable of degrading some of the plastics released into the ocean (Dash et al. 2013; Urbanek et al. 2018). Observations such as these and others stimulate the continued efforts of researchers and government agencies to develop biological tools for environmental remediation. Natural bioremediation, where an indigenous organism degrades the environmental contaminant is, of course, the ideal scenario. Unfortunately, this is often not achievable as spills and targeted release of toxic compounds can occur in locations where such

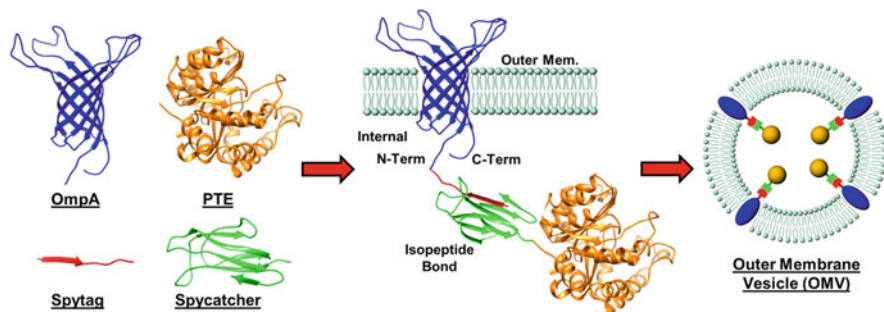


Fig. 10.8 Directed packaging of the OMV lumen. A protein–protein ligation system (SpyCatcher/SpyTag) was used to anchor a recombinant phosphotriesterase (PTE) enzyme to the bacterial outer membrane enabling the directed loading of nascent OMVs. Reproduced with permission from Alves, N.J., et al., *Bacterial Nanobioreactors—Directing Enzyme Packaging into Bacterial Outer Membrane Vesicles*. ACS Appl Mater Interfaces, 2015. 7(44): p. 24963–72

microbes are not native. As an alternative, the enzymatic systems from these organisms can be isolated, produced recombinantly, and deployed at the point of concern to facilitate decontamination. Enzymes, however, have their own limitations such as stability and cost of manufacture.

Similar to their function in the natural world, engineered bacterial membrane vesicles afford protection to encapsulated biomolecules from harsh environmental conditions. The controlled loading of enzymes into bacterial OMVs/MVs provides for a method of producing reagents that can easily be isolated from bacterial cultures, lyophilized for storage and distribution, then rehydrated to facilitate environmental cleanup. In a series of publications, Alves et al. showed that a protein–protein ligation system could be used to direct the packaging of a phosphotriesterase (PTE) enzyme capable of degrading an organophosphate compound into *E. coli* OMVs (Alves et al. 2015a, 2016). The authors employed a recombinant version of a native porin protein (ompA) presenting a small peptide (SpyTag) and a PTE fusion with its counterpart (SpyCatcher) (Fig. 10.8). As described by Zakeri and colleagues, the SpyCatcher/SpyTag system allows for the spontaneous formation of an isopeptide bond between the two components which in this system, facilitated the anchoring of PTE to the outer membrane and subsequent packaging of the enzyme into the OMVs (Zakeri et al. 2012). The authors demonstrated that not only did the enzyme maintain activity but also survived a number of storage and environmental conditions better than the free enzyme. In subsequent studies, Alves et al. also showed that these materials had relevance outside the laboratory by testing their materials in environmental water samples spiked with substrate and on a number of materials chosen to mimic military vehicle paint and surfaces (Alves et al. 2018). In these studies, the authors also examined enzyme activity under nonideal conditions such as variable pH, high salinity, and in environmental water samples with varying microbial populations and debris composition. Given the aversion of many societies to employ genetically modified organisms or release nonindigenous species into the

wild, OMV-based reagents offer an alternative approach to the development of Green reagents for environmental remediation.

10.3.5 OMVs for Imaging and Biosensing

Today, synthetically manufactured liposomes are routinely used for the delivery of therapeutics and in many diagnostic assays (Akbarzadeh et al. 2013; Alavi et al. 2017; Xing et al. 2016). Despite their many successes, liposomes can be difficult to manufacture, load, and store for prolonged periods of time (Alves et al. 2015b). In contrast, engineered OMVs offer a potential path to a simple manufacture platform that allows for controlled packaging and a final product that exhibits biophysical properties making them ideal reagents for drug delivery and the building of biological sensors. As an example, Chen et al. demonstrated how the ability to modify both the exterior proteins and interior cargo could be used in combination to develop reagents that have both assay and imaging capabilities (Fig. 10.9) (Chen et al. 2017). Here the authors expanded upon a previous construct for OMV modification that presents a cohesion–dockerin scaffold on the OMV exterior, adding a terminal domain (Z-domain) that allows for attachment of an antibody (Chen et al. 2010; Park et al. 2014). Interior packaging is accomplished using a modified bacterial lipoprotein (SlyB) fusion which is known to localize to the outer membrane of *E. coli* (Tokuda and Matsuyama 2004). This system allows for versatility in assay and imaging development as any number of antibodies could be added to the exterior and used in combination with a wide array of reporter proteins, from fluorescent proteins to luminescent proteins.

10.3.6 Future Commercial Applications of Bacterial Membrane Vesicles

While therapeutic applications of OMVs/MVs are at the forefront of research efforts, the encapsulation of biomolecules within bacterial membrane vesicles has great potential for commercial applications. The ease of production and the protection afforded to encapsulated proteins make this a highly versatile system that could easily be adapted to benefit many applications that are not currently explored.

Recombinant production of proteins, enzymes, and other biomolecules is a critical component of both the commercial and medical industries (Adrio and Demain 2010; Demain and Vaishnav 2009; Ferrer-Miralles and Villaverde 2013; Sanchez-Garcia et al. 2016). While successes such as the *E. coli*-produced insulin serve as banners for this technology, there are numerous other biomolecules that are never able to come to market due to complications associated with biomanufacturing. Often, large-scale production of biomolecules is hindered by

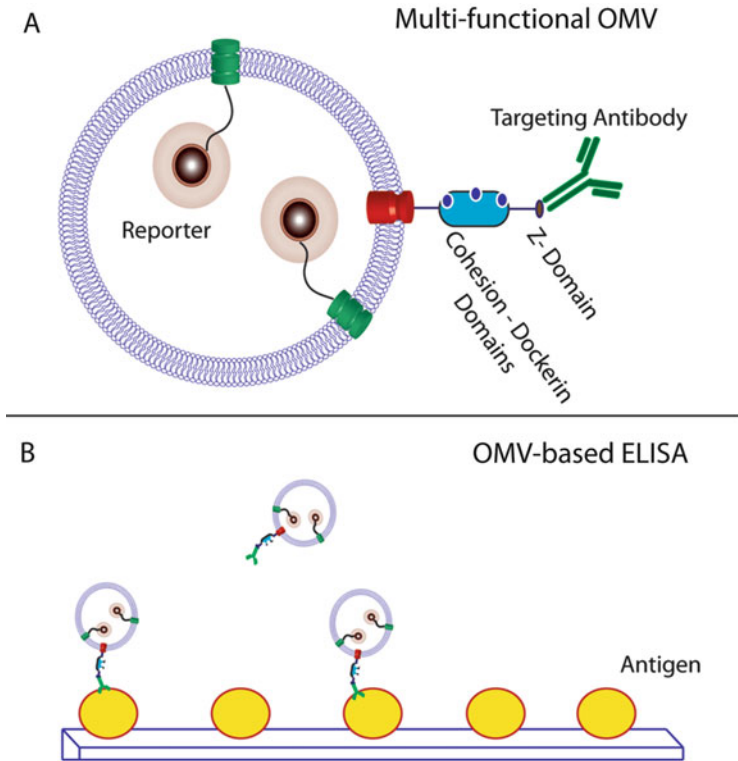


Fig. 10.9 OMV-based reagents for imaging and biosensing. OMVs from Gram-negative bacteria offer numerous opportunities for modification by targeting both the exterior and interior domains. (a) Researchers have shown that exterior domains of OMVs can be modified to add targeting moieties while the interior cavity can be loaded with a range of reporter molecules using membrane anchors, fusions, and various other methods. (b) Multifunctional OMVs produced in this manner can be used as a one-pot material for bioassays such as the traditional ELISA or used in cell targeting and imaging

low yields that can be associated with toxicity, insolubility, or any number of other issues. Membrane vesicles could potentially serve as a method of “off-loading” recombinant products before they could accumulate in the engineered organism leading to toxicity. Enclosed within OMVs, these biomolecules could be isolated directly from batch cultures through engineered epitopes on the OMV surface as shown by Alves et al. (2017).

Researchers have shown that OMVs function in many microbial community interactions including interspecies communication and regulation of microbial populations. Additionally, antimicrobial peptides and other cytotoxic compounds are readily packaged within vesicles and purified OMVs alone can display bactericidal activity (Dean et al. 2019; Park 2018; Schulz et al. 2018). While this has obvious therapeutic benefits, these properties of OMVs and MVs could also be harnessed for nonmedical purposes. Microbial communities and the biofilms they

form also plague many industrial, military, and environmental systems. In addition to obvious examples such as food production facilities (Glass et al. 1983; Marchand et al. 2012), damaging biofilms can also be found in fuel storage containers (Bücker et al. 2014), oil and gas pipelines (Tingyue Gu 2015), on the hulls of ships (Schultz et al. 2011), and countless other locations. The use of biological reagents that are easily resorbed into the environment to eliminate or reduce biofilms could prove beneficial in developing technologies that can readily be implemented without concern for additional harmful environmental effects.

10.4 Conclusion

Scientists continue to gain a greater understanding of the microbial world and the valuable tools it provides to society. We have long exploited them for food processing, as a pipeline for drug discovery, and as miniature factories for the production of target biomolecules; however, as shown here, bacteria can begin to aid the development of new vaccines or even as reagents for environmental cleanup. With the rapid emergence of antimicrobial resistance, OMV/MV derived vaccines may serve as mankind's next line of defense in the battle against pathogenic microbes, particularly those pathogens that can be weaponized such as *B. anthracis* or intracellular pathogens such as *F. tularensis* that are difficult to treat with conventional methods. Beyond vaccines, the observations and theories that support the presence of OMVs in the bloodstream and passing through the blood-brain barrier suggest that with advances in synthetic biology and a greater understanding of the human microbiome, scientists may be able to harness microbial communities to effect changes and treat disorders. Well beyond traditional probiotics, advances in these areas of research will dramatically alter the way we think about the foods we eat as well as how medical therapies are administered. Finally, by mimicking the bacteria themselves and loading enzyme systems into OMVs, we may be able to develop cell-free catalytic reagents that can be used to remediate environmental disasters, treat waste water, or even synthesize novel materials. Both natural and engineered OMVs and MVs have great potential in many areas of human society and will be an area of scientific investigation for years to come.

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