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Outer Membrane Vesicles (OMVs) as a Platform for Vaccination and Targeted Drug Delivery

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Abstract

Outer membrane vesicles (OMVs) are nanoparticles secreted from bacterial cell surface. They range from 10 to 300 nm in size and possess similar surface characteristics to the cell from which they are derived. OMVs are a common phenomenon observed in Gram-negative bacteria. These spherical vesicles with their enclosed periplasmic content are flexible and pliable; therefore, can be engineered for different purposes. They have recently gained popularity owing to their unique characteristics and have been explored for use in diverse biotechnological applications like adjuvants, vaccine development, drug delivery, and fluorescence tracking. OMVs contain pathogen-associated molecular patterns (PAMPs) on their bacterial outer membranes which imparts immunomodulatory characteristics to the OMVs. Their ability to suppress or elicit an immune response makes them attractive for vaccine development. OMVs have several unique advantages over conventional nanoparticles and notably the ability to protect the cargo in their

lumen and heterologous protein presentation make them novel platforms for target drug delivery. In this chapter, we begin with biogenesis of OMVs and their functions in bacterial virulence and pathogenesis, we present an overview of medical applications to exploit OMVs for therapeutic approaches, we discuss the current discoveries of OMVs and engineering approaches for drug delivery, and we conclude with future perspectives.

Keywords: OMVs; vaccine development; OMV-based drug delivery; bacterial vesicles

List of Abbreviations:

APCs	Antigen presenting cells
COVID-19	Coronavirus disease 2019 pandemic
ClyA	Cytolysin A
DCs	Dendritic cells
DLS	Dynamic light scattering
DOX	Doxorubicin
ETEC	Enterotoxigenic <i>E. coli</i>
HER2	Human epidermal growth factor 2
HEK293T	Human epithelial cells
IM	Inner membrane
KSP	Kinesin spindle protein
LPS	Liposaccharides
MALS	Multiangle light scattering
MR	Mannose receptor

NPs	Nanoparticles
NTA	Nanoparticle tracking analysis
NSCLC	Non-small-cell lung cancer
OMPs	Outer membrane proteins
OmpA	Outer membrane protein A
OM	Outer membrane
OMVs	Outer membrane vesicles
PTX	Paclitaxel
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
PGN	Peptidoglycan
PTE	Phosphotriesterase
PLs	Phospholipids
PD-1	Programmed death 1
PD-L1	Programmed death 1 ligand 1
SEC	Size exclusion chromatography
TLR	Toll-like receptor
TAM	Tumor-associated macrophages
TME	Tumor microenvironment
siRNA	Small interfering RNA
NOVC OMVs	<i>Vibrio cholerae</i> -derived OMVs

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1. What are OMVs

Outer membrane vesicles (OMVs) are nanosized vesicular structures generated through a ‘budding out’ of the bacterial outer membrane surface. OMVs are spherical vesicles with

diameter ranging between 10-300 nm (1) and possess similar characteristics to the cell from which they are derived (2) (Figure 1). OMVs were initially reported by Chatterjee and Das (3) to be an artifact of bacterial growth from observation of *Vibrio cholera*.

Today they are well studied and known to be a common phenomenon observed in almost all Gram-negative bacteria purposefully secreted to serve as a mediator of bacterial communication and homeostasis and also harnessed for their potential in biotechnological applications. OMVs contain cargo derived from their parent bacterium such as; phospholipids (PLs), membrane proteins, periplasmic proteins, nucleic acids (DNA and RNA), ions, metabolites and signaling molecules, and pathogen-associated molecular patterns (PAMPs). Notably, PAMPs such as; liposaccharides (LPS) and peptidoglycans (PGN) are able to activate host innate immune response via activation of the pattern recognition receptors (PRRs). Their cargo transportation ability makes them ideal for bacterial communication to distant sites, transfer of virulence factors and maintenance of bacterial communities (4). Due to their small size, diverse cargo, transportation ability and immune-stimulatory properties they are pliable and gaining popularity in the scientific community. They are currently genetically engineered by protein expression for use in diverse biotechnological applications such as adjuvants, vaccine development, drug delivery, fluorescence tracking, cancer immune therapy, and anti-bacterial therapy. For the purpose of this chapter we focus on the vaccine development and target drug delivery (Figure 2).

1.1. Biogenesis of OMVs

Despite over 60 years of studying OMVs (3, 5), there is no unified mechanism for their formation. Several models have been developed over the years and it has been extensively

reviewed by Avila-Calderón et al. (6). However, three key mechanisms exist elucidating the role lipoproteins, LPS, flagellin and PGN respectively on the biogenesis of OMVs but it is possible that more than one molecule is involved in the biogenesis of OMVs. Overall, OMVs biogenesis seems to be a collective set of multi-factorial mechanisms that influence their production and cargo selection.

Mashburn and Whiteley showed that the accumulation of the outer membrane with LPS, PLs and other curvature inducing molecules causes curvature and OMV production (7). Conditions of stress also lead to the production of OMVs such as change in temperature in *Serratia marcescens* (8) and *E. coli* (9). Kadurugamuwa & Beveridge showed the presence of antibiotics caused *Pseudomonas aeruginosa* to produce OMVs (10).

A recent study described a highly conserved model for the biogenesis OMVs in Gram-negative bacteria. Briefly, Roier et al. (11) have identified genes which are involved in the biogenesis of OMVs. Their result showed that the deletion of the outer membrane lipoprotein *VacJ* and/or the inner membrane permease *Yrb* genes significantly increase the production of OMVs by *H. influenzae*, where *VacJ* and *Yrb* proteins play a role in maintaining the lipid asymmetry in the Gram-negative bacteria outer membrane by reverse trafficking of PLs from the outer membrane to the inner membrane. The authors conclude that the distribution of *VacJ* and *Yrb* proteins cause the accumulation of PLs in the outer leaflet of the OM, which initiated the outward bulging of the OM (1). Thus, accumulation of PLs in the outer leaflet of the OM lead to induce a positive and negative curvature which lead to the budding of the OM (2). And eventually, pinching of the OM (Figure 3) (11).

1.2. Structure, Function, and Composition of OMVs

1.2.1. Structure and Compositions of OMVs:

OMVs are produced by all Gram-negative bacteria in both planktonic culture and the native host environment (12). OMVs belong to a type of lipid bilayer nanostructure of spherical structures, and the compositions of OMVs include proteins, lipids and nucleic acids, which were analyzed by high-speed ($\geq 40,000\times g$) density gradient centrifugation and mass spectroscopy (13).

I. Proteins

The protein content of OMVs has been well studied by immunoblotting, Mass spectrometry, and other functional experiments. To date, over 3500 diverse proteins have been identified in OMVs that originate from the membranes of host bacteria such as OM proteins and periplasmic proteins. OM proteins are related to transport systems, adhesins, enzymes (e.g. phospholipases and proteases), and flagellum or pilus proteins. Of note, periplasmic proteins that are associated with the inner surface of OM, display increased incorporation within OMVs, in comparison to proteins firmly bound to the inner membrane. However, certain cytoplasmic and inner-membrane proteins were also identified in OMVs.

II. Lipids

Lipids are a crucial structural component of OMVs. According to Chowdhury and Jagannadham's paper, OMVs are primarily composed of phosphatidylglycerol and phosphatidylethanolamine (14). In addition, a high extent of saturated fatty acids builds a rigid structure for OMVs (15). Of note, despite the fact that virulence factors, such as LPS are present in the Gram-negative bacteria, only a small percentage of parent LPS is found in the OMVs (16).

According to the investigation of Kadurugamuwa and Beveridge, they found that charged B-band types of LPS in OMVs are of enrichment in *P. aeruginosa* (10). However, in *Porphyromonas gingivalis*, they are of high components of A-band LPS of OMVs (17).

III. Nucleic Acids

OMVs also contain nucleic acids, including DNA, RNA, plasmid, phage, and chromosomal DNA, in several studies. In addition, surface-associated and luminal DNA are both present in OMVs (18). The DNA fragments can be circular and linear (19). However, Zhou et al. discovered that some strains' OMVs didn't contain DNA. Zhou et al. found the presence of OMVs without DNA in *Porphyromonas gingivalis* strain 33277, *P. gingivalis* strains W50 and A7436 (20).

1.2.2. Functions of OMVs:

OMVs exhibit diverse functions in the interactions between bacteria and the host. For example, Lee et al. reported that OMVs provided a long-distance delivery system for several specific components, such as adhesion molecules and virulence factors (13), and certain toxins transported by OMVs have been demonstrated to modulate the physiology of host cells (1). Additionally, OMVs have been shown to be responsible for promoting homeostasis, formatting biofilm, and inducing the SOS response to repair DNA.

Furthermore, Mashburn-Warren & Whiteley found that OMVs signal between cells through encapsulated small molecules to control gene expression and confer favorable mutations in bacteria (21, 22). Of note, Li et al. reported that OMVs could be utilized by bacteria for horizontal gene transfer (23). In addition, OMVs-based communication is also associated with

the antibacterial activity due to the fact that the small size and endogenous membrane structure of OMVs facilitate the entry of antibacterial molecules into the bacteria (24).

The production of OMVs can be elevated in response to stress conditions (25). For instance, OMVs can help remove misfolded proteins when bacteria are subject to physical or chemical stresses (26). Additionally, OMVs play a role in scavenging or neutralizing antibiotics when bacteria are exposed to antibiotics (27). Of note, OMVs are also responsible for nutrient acquisition by carrying metal ions, degradative enzymes, and target-specific receptors (2, 28). For example, in *Myxococcus xanthus*, their OMVs equip with alkaline phosphatase to liberate phosphate which can provide nutrition to promote the improvement of a multicellular community (29). Pulido et al. demonstrated that *Acinetobacter baumannii* OMVs deficient in LPS synthesis, and that immunization with these OMVs elicits protective immunity against infection with *A. baumannii* (30). Interestingly, this strain of bacteria is resistant to antibiotics and are difficult to treat. Furthermore, Martora et al. confirmed the secretion of OMVs by *Klebsiella pneumoniae*, an important pathogen of nosocomial infections due of resistance to antibiotics, and the showed the presence of outer membrane proteins (OMPs) and porins (OmpA and OmpC), which are important innate immune-activating ligands that play a role in activation of host innate immune response pathways. The authors concluded the involvement of OMVs in the pathogenesis of *Klebsiella pneumoniae* and could be future targets for novel therapy and potential vaccine against *Klebsiella pneumoniae* (31). However, as existing studies of OMVs suggest more functions than we have previously thought, it warrants further investigation into the roles of OMVs for both fundamental and translational studies.

1.3. Isolation and Purification of OMVs

To accelerate OMV-based therapeutic applications, it is critical to report a detailed description method of isolation and purification of OMVs from pathogenic species. The majority of techniques for isolation and purification of OMVs, was described in the literature with very similar procedures, start with low speed centrifugation followed by sub-sequential filtration of the supernatants through the use of both 0.22 μm and 0.45 μm filters and this primarily depend on the size of the vesicles (32). After filtration, the filtrate was subjected to several steps including concentration, purification and have been listed in Table 1. Hence, ultracentrifugation alone is not sufficient for purification of OMVs. Thus, OMVs need to be purified by density gradient ultracentrifugation will be resulted in the purest fraction of OMVs (32-34).

Method	Advantage	Disadvantage	Reference(s)
Differential centrifugation	Low technical requirements; ease of execution	Laborious, low purity, generally needs to be combined with density gradient centrifugation for further purification	(35)
Size-exclusion chromatography	Rapid isolation process; high purity	High cost; unsuitable for large-scale production	(36)
Hydrostatic filtration dialysis	Low cost; suitable for large scale production	Limited data on purity of the isolated OMVs	(37, 38)
Affinity purification	Fast; specific isolation of targeted	Only available for OMVs carrying exposed tags; low	(39)

	OMV populations	recovery rate	
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1.4. Characterization of OMVs

In OMV vaccines development, it is essential important to check their consistency of production, size, quality and stability (40). For the last two decades, researchers have been interested in understanding the proteinaceous cargos of OMVs from different species of bacteria however the characteristics and functions of the OMVs is still lacking. Particle size, appearance, structure and stability need to be reported for complete characterization of OMV particles.

Mostly, the Gram-negative OMVs are spherical nanoparticles consisted of a single lipid bilayer encapsulating a proteinaceous lumen (41). Their lipid bilayer composed of an inner membrane (IM) and outer membrane (OM), separated by thin layer of peptidoglycan (42, 43) (Figure 4). OMVs carry nucleic acids (DNA, RNA), LPS, PLs, peptidoglycan hydrolases, periplasmic content, as well as proteins localized to the cytoplasm, IM, periplasm, and OM, and other insoluble components that are delivered to the environment to accomplish several functions. OMVs membrane-bound proteins, including protein channels, signaling molecules, transporters, antigens and receptors (1, 30, 44-47). The OMV's cargos depend on species of bacteria from which they originate, mechanism of biogenesis, and stress conditions (48-50). Numerous studies have demonstrated that the composition of OMSs differs from that the bacterial outer membrane by the enrichment or exclusion of specific OMPs and LPS modifications (10, 51).

There are multiple techniques to determine the OMV particle size including; Dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and multiangle light scattering (MALS)

coupled with high-performance liquid chromatography-size exclusion chromatography (SEC) (40).

1.5. Mechanisms of OMVs Entry into Cells

OMVs can transfer diverse range of cargo, including DNA, RNA, microRNA, protein, and lipids to proximal host cells. Recent study demonstrated the presence of DNA on the external and internal surfaces of OMVs (52). Moreover, they showed that most of the DNA is present externally and the authors concluded that external and internal OMV-associated DNA play a vital role in pathogen-host communication and may modulate host cell responses and highlighted their importance for their use as vaccines.

OMVs can interact with many host cell types including mucosal epithelial cells and with immune cell populations in host's submucosa, where they can directly interact with immune cell, including macrophages, neutrophils, and dendritic cells (DCs). Where the later suggest the involvement of OMVs as a modulator for immune response. Moreover, OMVs can interact with cells that are distal to the site of OMV entry (12).

Consistent with they are able to enter host cells and delivery their DNA, LPS, peptidoglycan, protein cargos (12, 53). Bielig et al. reported that *Vibrio cholerae*-derived OMVs (NOVC OMVs) deliver PGN to host cells and has intrinsic inflammatory potential. NOVC OMVs internalized by human epithelial cells (HEK293T) and induced inflammatory responses via activation of two cytosolic expressed members of the nucleotide-binding domain-containing proteins (NOD1, NOD2) (54).

Notably, OMVs express several physiologically relevant PAMPs on their surface that can be recognized by host PRRs and that may facilitate their entry into host cells.

OMVs enter host epithelial cells by the use of multiple endocytic mechanisms including, lipid raft-dependent and lipid raft-independent endocytosis, micropinocytosis, clathrin mediated endocytosis, caveolin mediated endocytosis, and dynamin-dependent entry (12, 55, 56). Moreover, OMVs present a range of surface-exposed antigens such as Toll-like receptor (TLR) activating components (flagellin, triacylated lipoproteins, diacylated lipoproteins) which allow them to be internalized by the host cells via activation of TLR. Kaparakis-Liaskos and Ferrero concluded that OMVs use multiple routes of entry into host cells and this may be regulated by the size and membrane composition of OMVs (12).

The most common reported mechanism of OMVs entry into host cells involves lipid rafts. Kesty et al. were the first group who identified the ability of OMVs to enter non-phagocytic host's epithelial cells via lipid raft fusion. They have demonstrated that enterotoxigenic *E. coli*-derived OMVs internalized by host cell via cholesterol-rich lipid rafts in a temperature- dependent manner (57). Following endocytosis into host cells, OMVs traffic into early endosomes and trigger signaling cascades where they interact with intracellular PRRs. In addition, OMVs degraded by autophagy and their degradation results in the generation of pro-inflammatory response and this mediated by the production of cytokines and chemokines. And this ultimately result in the recruitment and activation of DCs to facilitate the development of T cell immunity (57).

2. Cargo Loading and Surface Modification Using OMVs

OMVs have become well appreciated in recent times as nanodrug delivery system due to the fact that they could be utilized as delivery agents by encapsulating drugs and deliver them to target tissues. Hence, they have unique properties including biodegradable, biocompatible, non-toxic, easy to synthesize, cost effective, prolonged circulation, evading the immune system, selective targeting which are desirable for drug delivery system. The lipid bilayer of OMVs act as a barrier to protect the loading materials from decomposition in circulation system and prolong the half-life. Ligands and proteins can be functionalized on OMVs surface to achieve targeted delivery (58). There are two strategies through which cargos are loading into OMVs: passive and active loading approaches (Figure 5).

2.1 Active Cargo Loading

Active loading is commonly used for loading small molecules or hydrophilic drugs into the OMVs lumen. OMVs can be functionalized via different physical methods including electroporation, extrusion, sonication (59, 60). However, only few studies have been addressed the mechanism behind specific cargo loading into OMVs. Taking electroporation methods as an example.

2.1.1 Electroporation

Electroporation technique involves the application of short high-voltage pulses to OMV membrane to create a transient state of permeability which allows the entry of drugs and different cargos. The phospholipid membrane then recovers its structure once this process is completed. Ayed et al. showed for the first time the successful of encapsulating of metallic gold (Au) nanoparticles into bacteria-derived OMVs using electroporation. In this study, small gold

nanoparticles (AuNPs) were loaded into OMVs derived from *Pseudomonas aeruginosa* by applying an optimal voltage of 470 V and 1 pulse yielding and encapsulation efficiency of ~35%. and further validated the integrity of the vesicles after electroporation. They concluded that their approach could be adapted to include other nanomaterials or drugs for biomedical applications (59).

In another encapsulation approach, wherein OMVs derived from genetically engineered *E. coli* BL21 (Δ msbB) was used for the delivery of nucleic acid drugs and genetic medicine with less endotoxicity. They designed a pH-sensitive drug delivery system coloaded with therapeutic siRNA (Redd1 siRNA) and paclitaxel (PTX, a microtubule-stabilizing drug) (siRNA@M-/PTX-CA-OMVs) that sequentially targets different cells in tumor microenvironment (TME) and regulate the tumor metabolic and suppress tumor growth. The loading of Redd1 siRNA in this case was achieved through the electroporation technique leading to a high loading efficiency of siRNA in OMVs. The surface of OMVs was modified with mannose to target M2 macrophages via the mannose receptor (MR, CD206⁺). Upon arrival of siRNA@M-/PTX-CA-OMVs to tumor site where the Ph 6.8 and this trigger the release of PTX and the rest of the system is uptake by M2 macrophages to increase their level of glycolysis. Their system showed a promise potential for tumor-associated macrophages (TAM) repolarization, tumor suppression, tumor immune activation, and TME remodeling in the triple-negative breast cancer model (Figure 6).

2.2 Passive Cargo Loading

2.2.1 Simple Incubation

Passive loading is to load the drugs of interest OMVs that have been isolated via simple incubation with cargo. Passive diffusion is suitable for small-molecule drugs that are positively charged and hydrophobic, which interact with the negatively charged membranes and subsequently are retained in or cross the membranes. Kuerban et al. reported the successful encapsulating of doxorubicin (DOX) into OMVs. OMVs obtained from *Klebsiella pneumonia* to drive chemotherapeutic agents to non-small-cell lung cancer (NSCLC) A549 cells. OMVs were isolated from attenuated *Klebsiella pneumonia* and loaded with DOX (DOX-OMVs) through a gentle co-incubation approach. Further found that DOX-OMVs nanocarriers exhibited promising positive immune response with no obvious cytotoxicity in NSCLC mouse model. The efficacy of their approach was characterized by the accumulation of OMVs and DOX inside tumor cells (61).

3. OMVs in Vaccination

3.1 OMVs as Modulators of the Innate Immune Response

Naturally derived bacterial outer membrane vesicle (OMVs) contain a large number of PAMPs which can induce the activation of antigen-presenting cells of the host tissues, including the maturation of DCs and their production of cytokines, and drive the activation of the innate immune response and qualified as immunoadjuvants (12, 62). Moreover, OMVs can function as vaccine carriers. In fact, OMVs have been shown to be safe as immunotherapeutic agents and have been approved by the European Commission to be used as a meningococcal B vaccine against gonorrhoeae disease. Both OMV-based vaccines (MeNZB and Bexsero) induced anti-gonococcal antibodies which recognize gonococcal proteins (63, 64). Furthermore, OMVs can be genetically engineered and modified to express any chosen antigen and can manipulated to

reduce their endotoxicity. Intravenous injection of OMVs which produced by *E. coli* BL21 mutant showed a reduction in systemic cytokines release and low endotoxicity compared to wild type OMVs (65). Moreover, their system siRNA@M-/PTX-CA-OMVs induced a favorable synergistic antitumor effect at the tumor site. Upon reaching the tumor site where the pH is 6.8 and this cause the release PTX from the complex due to the pH-sensitive linker inserted in the OMVs and this cause a direct tumor killing and the rest of the system were uptake by M2 macrophages and downregulate and suppress Redd1 and increase the macrophages glycolysis and block tumor metabolic microenvironment and inhibit tumor growth and metastasis (Figure 7).

In a previous report, Li et al. developed a genetically engineered OMVs carrying on their surface the ectodomain of programmed death 1 (PD-1) which function as immune suppressive on active T cells and has been uses to normalize the immune system. Briefly, their data have showed a strong accumulation of OMV-PD1 at the tumor site which increased the infiltration of immune cells and activated the immune response. Simultaneously, PD1 ectodomain bind to its ligand, immune checkpoint programmed death 1 ligand 1 (PD-L1) on the surface of tumor cells and antigen presenting cells (APCs) and facilitate their internalization and reduction, thereby protecting CD8⁺ T cells from the PD1/PD-L1 immune inhibitory axis and lead to tumor growth reduction both in mouse melanoma and colorectal cancer models (Figure 8) (66).

3.2 Bioengineered OMVs for Vaccination

Over the past decades, OMVs have been used for as vaccine delivery vehicles by incorporating antigens of interest. Engineering efforts of OMVs for antigen delivery largely center around genetic fusion between antigens and anchor proteins such as Cytolysin A (ClyA). Furthermore,

some researchers revealed that engineered OMVs with some fusion proteins would dramatically enhance OMVs' functions (67).

3.2.1 Recombinant OMVs based on ClyA fusion protein

OMVs can present antigens on the outer layer of the vesicles through proteins, like a ClyA fusion protein. The ClyA protein is a transmembrane protein with a molecule weight of 34 kDa found in OMVs (68). By fusing green fluorescence protein (GFP) with ClyA protein, Cheng et al. successfully expressed GFP on the surface of OMVs (69) by producing OMVs in *E. coli*. Of note, while no additional adjuvant was applied in their immunization protocol, the GFP-OMVs elicited GFP-specific antibodies, suggesting the effect of self-adjvanticity. In addition, Kim et al. demonstrated that only the C terminal fusion of ClyA could produce desired functions of derived OMVs, while the N terminal fusion of ClyA is often problematic (67). To further prove the translational potential of recombinant OMVs, in a murine sepsis model, Omp22 antigens from *Acinetobacter baumannii* were fused into *E. coli* DH5 α -derived OMVs for treating *Acinetobacter baumannii* infection. The results showed that active immunization with Omp22-OMVs increased the survival rate of mice (70). Meanwhile, Rosenthal et al. demonstrated that recombinant OMVs could produce novel anti-viral vaccines (71).

3.2.2 Recombinant OMVs Based on Other Carrier Fusion Proteins

Currently, other carrier fusion proteins are also applied for OMV-mediated vaccine delivery (Table 2). Salverda et al. expressed the lipoprotein Outer Surface Protein A (OspA) in *Borrelia burgdorferi* on the surface of OMVs through fusion with fHbp in *Neisseria meningitidis* for

Lyme's disease (72), and their experimental results proved that OMVs in *Neisseria* can generate OspA-specific antibodies for *Borrelia* vaccines.

Additionally, Basto et al. explored Outer Membrane Protein I (OprI) from *E. coli* fusion can infuse A104R antigen into OMVs in *Pseudomonas aeruginosa* to defend against African swine fever (73). Also, researchers infused Outer membrane protein A (OmpA) protein to locate antigens of *S. pyogenes*, such as SpyCEP, Streptolysin O and Spy0269, in *E. coli* OMVs lumen to treat Streptococcus disease. And experiment results proved Slo-OMVs and SpyCEP-OMVs could help mice against *S. pyogenes* infection (74). However, according to several analyses, lumen fused OMVs just evoked minor specific antibody production and even decreased protection (75). Therefore, we can conclude that bioengineered OMVs which focus on the surface of OMVs would produce higher antibodies and could practice protection abilities. However, we need more research

to clarify the mechanism of the different results between lumen and surface of OMVs.

OMVs Source	Fusion protein	Antigen	Diseases application	Reference
<i>Escherichia coli</i>	ClyA	Omp22	<i>Acinetobacter baumannii</i>	(70)
<i>Escherichia coli</i>	ClyA	M2e	Influenza A	(76)
<i>Salmonella typhimurium</i>	fHbp	PspA or Ply	Pneumococcal disease	(77)
<i>Neisseria meningitidis</i>	fHbp	OspA	Lyme's disease	(72)

<i>Salmonella typhimurium</i>	fHbp	MOMP fragments	Chlamydia	(78)
<i>Salmonella typhimurium/Escherichia coli</i>	fHbp	ESAT6, Ag85B fragments, and Rv2660c	Tuberculosis	(78)

In summary, while we have discussed the potential of using OMVs as a novel platform for vaccines, there remain many limitations for clinic applications. As a result, innovative approaches must be developed in order to scale up the manufacturing of OMVs with high purity.

4. OMVs in Drug Delivery

Synthetic nano-carriers have been studied for many decades and optimized, as drug carriers however are unable to effectively replicate the trafficking pathways seen by OMVs. Cytotoxicity is another disadvantage of liposomes and other synthetic nano-carriers. OMV possess many attributes that make them beneficial for use in drug driver and have gained attention in the scientific community due to the aforementioned properties such as the diversity of proteins on its surface, small size and pliability to enable them carry diverse cargos. Target drug delivery can also be achieved by modification of the outer membrane surface composition as well as genetic manipulations (79).

The effective delivery of Enzymes has posed a problem for scientist but with OMV's native ability to carry enzymes in their cargo and deliver enzymes without degradation is another advantage of OMV in drug delivery. Alves et al. successfully packaged phosphatidylesterase (PTE) into OMV by conjugating it to Outer membrane protein A. the study showed that enzyme activity was unchanged (80). Koeppen et al. also showed that OMV protect nucleic acid cargo from degradation (50). several studies have also shown that genes encapsulated in OMVs were

protected from d DNase digestion (18, 81). Alves and colleges went on to further show that protein cargo of OMVs were remained protected in OMVs at different stability testing parameters such as varying temperature conditions and freeze drying (80). Making OMVs an effective vehicle for delivery of drugs in vivo furthermore the protection of cargo in storage and would enhance the drug formulation development and improve the efficacy of the drug as lower quantities would need to be delivered (82).

4.1. Cancer Therapy

Application of OMVs in cancer therapy is just in its infancy. However, OMVs have gained a great attention in the biotechnology fields as a novel cancer-targeting nanocarrier. OMVs have the added advantage of being non-replicating but possessing immunostimulatory molecules that enable recognition and uptake by immune cells thereby inducing an immune response. Their nano size allows for accumulation in at tumor site to induce local immunity through enhanced permeation and retention effect (83).

Gujrati et al. reported for the first time the use of bioengineered OMVs as targeted drug-delivery vehicles for tumor cells. In their study, they have co-deliver of anti-HER2 and therapeutic siRNA targeting kinesin spindle protein (KSP). OMVs were generated from ClyA-affibody-overexpressing *E. coli* to express human epidermal growth factor 2 (HER2) affibody on their outer membrane surface (Affi_{EGFR}-OMVs) (84). In addition, Sepahdar and colleagues showed that bioengineered Affi_{EGFR}-OMVs were more internalized into triple-negative breast cancer cells expressing EGF receptors (85).

Kim et al. were the first to successfully utilize OMVs capability to illicit an immune response to develop OMVs for use as cancer immunotherapeutic agents. In their study OMVs were engineered to reduce the endotoxic effects of LPS by using modified *E. coli* with inactivated gene for lipid A acyltransferase (*msbB*); which is the lipid component of LPS. Mice were subcutaneously transplanted with CT26 murine colon adenocarcinoma and treated with varying amounts of W3110 Δ *msbB* OMVs derived from *E. coli* (86). Their OMVs accumulated mostly in the tumor cells displaying highly specificity and also induced an immune response. The study showed that OMV treatments significantly reduced of the tumors in dose dependent manner and with IFN- γ antibody determined to be the major mechanism of antitumor response. Immune memory was also established as cured mice rejected second and third challenge of tumor cells. Whereas IFN- γ deficient mice were unable to reject the tumor cells after OMV treatment OMVs immunomodulatory effects have also be studied by several scientist (61, 87-89).

OMV are able to elicit an anti-tumor immune response, in order to completely eradicate tumors and prevent recurrence and metastasis. Combination therapy would be required to enhance the immune therapeutic effects of OMVs (89). Chen et al. combined OMVs and polymeric nanoparticles to boost the efficacy of cancer immunotherapy and prevent of cancer metastasis. In their study they have collected OMVs from the culture of attenuated *Salmonella* and then they fuse OMVs with DSPE-PEG-RGD through extrusion to generate OMV-DSPE-PEG-RGD (OR). Then, they have coated OR with Pluronic F127 micelles (ORFT). They have demonstrated that the functionalized OMV-coated polymeric micelles hold a great potential as immunotherapeutic and anti-metastatic delivery system.

Kuerban et al. went a step further to harness the immune stimulation function of OMV in combination with their role as effective nano-carriers for delivery of chemotherapeutics (61). OMVs derived from *Klebsiella pneumonia* were loaded with DOX (DOX-OMV) and treated against NSCLC cells A549. Their results showed that DOX-OMV had better uptake than DOX alone and DOX Liposomal formulation. DOX although is an FDA approved drug for treatment of neoplastic diseases including NSCLC its clinical application has been limited due to its side effects of cardiac toxicity such as cardiac myopathy (90). Kuerban and colleagues successfully showed that OMV-DOX enhanced the effects of DOX without any obvious toxicity in vivo (61). OMVs have also been used for the delivery of nucleic acids for cancer therapy (84). Gujrati and colleagues bioengineered *E. coli* to generate OMVs displaying anti-HER2 affibody as a targeting ligand for the delivery of small interfering RNA (siRNA) targeting kinesin spindle protein (KSP) which resulted in tumor regression and tolerability in animal models.

4.2. Antibacterial Therapy

One of the special properties of OMVs is their ability to be utilized to kill other bacteria to reduce competition in bacterial flora. Many NPs have been developed as anti-bacterial carriers, however one impediment remains which is the issue of bacterial resistance that inhibits the drugs from penetrating infected cells or the bacterial membrane. OMVs being derived from bacteria, possess the characteristic of the bacterial membrane therefore are advantageous over other synthetic NPs in terms of easier delivery of their cargo to gram-negative bacterial cells because they are recognized as biocompatible. Studies showed that *Shigella flexneri* exposed to gentamycin produce OMVs containing gentamycin. Interestingly when these OMVs were delivered to HeLa cells infected by *Shigella*, it caused a reduction in the growth of the pathogen

(91). Kudurugamuwa and colleagues also observed that OMVs play a role in micro biota interactions and have inherent bacteriolytic effect on other bacteria. The group also demonstrated that OMVs from *Pseudomonas aeruginosa* contain virulence factors, hydrolytic enzymes, DNA and endotoxin and the amount of OMVs produced increased in the presence of Gentamycin (10, 92). Li et al. observed the lytic effects of OMVs from several strains of bacteria and noted that they contained peptidoglycan hydrolases; which serve to dissolve the peptidoglycan layer of dissimilar cells to increase the nutrient load (47). Goes and colleagues explored the use of OMVs derived from myxobacterial strains *Cystobacter velatus* Cbv34 and *Cystobacter ferrugineus* Cbfe23 as natural antibacterials for the treatment of intracellular infections caused by *Sphylococcus Aureus* (93). Myxobacteria are found in soil and are known to produce potent antimicrobial compounds (94-96) that are non-pathogenic to humans. Their results showed that the OMVs displayed selective uptake by infected cells and displayed a bacteriostatic effect against *S. aureus* intercellular infection versus bacteriomimetic liposomes which were rapidly taken up by all cells. The OMVs from Cbv34 also showed storage stability at different storage conditions and maintained its dose-dependent antibacterial effect; which is an important criterion for successful formulation development for clinical use. Interestingly, scientists have combined NPs and OMVs for active targeting of bacteria infected cells. Gao et al. developed an actively targeted nanoparticle coated with OMV from *E. coli*. And showed that in vitro NP@OMV had higher uptake in *S. Aureus* infected macrophages compared to NP coated with polyethyleneglycol or NPs coated with EVs derived from *S. aureus* (NP@EV) (97). However, when loaded with antibiotics the NP@EV showed significantly higher uptake than the OMV this could be because the EVs used were secreted from *S. aureus* and would therefore have higher biocompatibility and uptake in *S. aureus* infected cells compared to the OMVs which were

obtained from *E. coli*. These results suggest high selectivity of bacterial OMVs for infected cells and could be harnessed for the development of nano-biotic sepsis management (98).

Free antibiotics have poor accumulation at infections sites (99) therefore requiring high doses to achieve an effective bactericidal concentration. Long term use of high doses lead to adverse events (100) and over time, prolonged use of antibiotics leads to drug resistance by bacteria (101). It is therefore important of develop antibacterial drug delivery platforms that can deliver effective doses to target sites. Huang and colleges secreted OMVs from *Acinetobacter baumannii* loaded with high concentrations of antibiotics via efflux pump method. Theses OMVs penetrated and killed enterotoxigenic *E. coli* (ETEC) infection in mice model of intestinal infection. They demonstrated that quinolone loaded OMVs effectively reduced bacterial infection versus the free antibiotic of the same concentration. For example, levofloxacin at a low dose of 1.4×10^{11} particles/kg effectively reduced the CFU in ETEC infected mice model of intestinal infection compared to the same dose of free levofloxacin (102). Their study showed that OMVs have good biocompatibility and high specificity. OMVs are therefore a novel way to effectively deliver antibiotics without the risk of adverse effects associated with antibiotics.

5.Future Perspective of Utilizing OMV for Vaccination and Drug Delivery

OMVs are spontaneously shed from Germ-negative bacteria during exponential phase both in vitro and in vivo (103). OMVs typically ranges from approximately 10 nm to 300 nm in diameter which possess the optimal size to be internalized by host cells (28). OMVs components are typically representative of the outer surface of parental cells. OMVs consist of lipid bilayer and contain biological active products, such as nucleic acids, toxins, proteins, and lipids. OMVs are

associated with a variety of biological functions including, pathogenesis, biofilm formation, antibiotic resistance transfer, modulation of host immune response, interspecies communication, DNA transfer, and virulence factors delivery into the cytosol of the host cell (10, 21, 45, 57, 104, 105). Interestingly, some specific proteins have been shown to be enriched or excluded from OMVs suggesting a specific sorting mechanism(s) for these proteins (68, 106, 107).

The composition of OMVs and their mechanism of cargo delivery hold great promise as cargo delivery vehicles. In 2013 a Meningococcal Group B vaccine (4CMenB), Bexsero[®], was approved in Europe and Australia, which is formulated with vesicles from the *Neisseria meningitidis* New Zealand strain contains a bacterial OMV component (108).

Nanovaccines have advantages compared to conventional vaccines against infectious diseases. They can induce a protective immune response via efficient display of specific antigens and lymph node accumulation. During the ongoing coronavirus disease 2019 pandemic (COVID-19), lipid nanoparticle vaccination has proven the effectiveness of nanovaccines in defense against the pathogens. OMVs are becoming increasingly popular and have become a highly effective vaccine platform due to several attractive features, including but not limited to, the intrinsic adjuvanticity (45, 69), the ease of their surface decoration with heterologous antigens (109-111), the simplicity of their production (112), and the ability of the vesicles to accumulate in lymph nodes.

Thus, nano-sized and lipid membrane vesicles of Germ-negative bacteria are an ideal platform for broad applications in vaccine designs and drug delivery. However, there is also a pressing need for further investigation and creating safer and more effective OMV-based platforms towards human health.

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Figure Legends

Figure 1. (a) Cryo-TEM micrograph of OMVs. OMVs are spherical in shape and range from 10-300 nm in size. (b) A schematic illustrating the biogenesis of OMVs production in flagellin-deficient *S. typhimurium*. Scale bars = 200 nm. Adapted with permission from (113).

Figure 2. Schematic representation of the structure composition of OMVs and their potential in biomedical applications. OMVs packed with a variety of PAMPs, including LPS, lipoprotein, DNA, RNA, peptidoglycans. Adapted with permission from (83). Copyright (2020) Elsevier.

Figure 3. Schematic representation of the biogenesis of OMVs. Adapted with permission from (11). Copyright (2022) Springer Nature.

Figure 4. Observation and characterization of OMV by Cryo-TEM from three strains of Gram-negative bacteria. (A) OMV from *Neisseria gonorrhoeae*. (B) OMV from *Pseudomonas aeruginosa*. (C) OMV from *Acinetobacter baumannii*. All images showing spherical bilayered membrane vesicles surrounded with outer membrane (OM) and inner membrane which corresponding to the plasma membrane (PM), separated by thin layer of peptidoglycan (PG). Bar = 100 nm. Adapted with permission from (43). Copyright (2015) Pérez-Cruz et al.

Figure 5. Strategies for cargo loading into OMVs Adapted with permission from (114). Copyright (2021) Pharmaceutics.

Figure 6. (a) Schematic representation of cargo loading into OMVs via electroporation technique (b) TEM images of empty OMVs and siRNA@M-/PTX-CA-OMVs displayed a Bowl-like bilayer structure (scale bar = 100 nm). (d) Nanoparticle tracking analysis (NTA) demonstrated that the average size of empty OMVs and siRNA@M-/PTX-CA-OMVs were (104 ± 12.3 nm) (130 ± 15.6). Adapted with permission from (65). Copyright (2022) American Chemical Society.

Figure 7. Schematic representation of the process of drug release from siRNA@M-/PTX-CA-OMVs and the interaction of their components with different cell types in TEM. First, PTX released at 6.8 pH. Secondly, the rest of the system will be taken up by macrophages which induce suppression of tumor. Adapted with permission from (65). Copyright (2022) American Chemical Society.

Figure 8. Schematic representation of the production and purification of OMV-PD1 from engineered *E. coli* and their use as effective anti-tumor agents. The injection of OMV-PD1 causes: (1) activation of the immune response and (2) immune checkpoint blockade with PDI. Adapted with permission from (66). Copyright (2022) American Chemical Society.

Table Legends

Table 1. Isolation methods of OMVs. Adapted with permission from (115). Copyright (2021) Zhu et al.

Table 2. Common antigens for application of OMVs vaccine