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Review Article

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Gram-Negative Bacteria's Outer Membrane Vesicles

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ABSTRACT

Outer membrane vesicles (OMVs) are spherical bilayered nanoparticles derived from the outer layer of Gram-negative bacteria. Bacteria communicate with nearby bacteria, their environment, and the cells of their host by secreting OMVs, which are essential for their survival. OMVs also play a critical role in bacterial pathogenesis since they are loaded with virulence factors, toxins, and enzymes. OMVs may modulate the immune response of the host by initiating inflammation through cytokine production and activating the innate immune response. OMVs also contribute to the resistance of bacteria to antibiotics by carrying antibiotic-degrading enzymes and acting as natural protection barriers. Concerns have also been raised regarding OMVs mediating the transfer of antibiotic resistance. Due to their advantageous properties, OMVs are attractive platforms for vaccine discovery and drug delivery research. In this review, we discuss the fundamental structure and biogenesis mechanisms of OMVs as well as their multifaceted roles in bacterial infection pathogenesis and host immune responses. We also discuss application examples of OMVs.

Keywords: Gram-negative bacteria; Outer membrane vesicles; Pathogenesis; Virulence

INTRODUCTION

Extracellular vesicles produced by both eukaryotes and prokaryotic cells continuously interact with their environment. Exosomes are microvesicles derived from eukaryotic cells. Their roles in intercellular communication and usage potential in clinical applications such as diagnostic methods and drug delivery vehicles have been widely investigated [1, 2]. Extracellular vesicles of prokaryotic cells are called membrane vesicles. While membrane vesicles are secreted by both Gram-positive as well as Gram-negative bacterial cells, the term "outer-membrane vesicles" (OMVs) is specifically used to refer to vesicles from Gram-negative bacteria that are enclosed naturally by an outer membrane.

OMVs from Gram-negative bacteria were first described in *Escherichia coli* in 1965. Since then, OMVs of all Gram-negative bacterial species identified to date have been determined, and are now considered a ubiquitous secretion process [3, 4]. Initially, OMVs were presumed to be merely lysed bacterial debris. However, their delicate production mechanism and function as a selective secretion process have since been proven [5]. OMVs of *Neisseria meningitidis* detected in the cerebrospinal fluid of an infant with meningococcus infection drew attention

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to the role of OMVs in the pathogenesis of Gram-negative organism infections [6, 7]. However, general models of OMV biogenesis are still lacking and regulation and selective cargo mechanisms remain unknown despite decades of research in this area. A major challenge in research on OMVs is the difficulty of purifying true OMV from artifacts, which leads to low yields of OMVs from large bacterial culture volumes. However, bona fide OMVs are produced via an orchestrated process and play multifaceted roles in bacterial survival, and the clinical utility of OMVs as vaccines and drug delivery vehicles is actively investigated.

In this review, we discuss recent advances in our understanding of OMV biogenesis mechanisms as well as the roles of OMVs in bacterial infection pathogenesis and host immune responses. We also discuss application examples of OMVs.

STRUCTURE AND BIOGENESIS OF OMVS

OMVs are single-membrane bilayered spherical particles ranging from 20 to 300 nm in size. OMVs are derived from the cell envelope of Gram-negative bacteria [8] (**Fig. 1**, **2**). A good understanding of the cell wall structure of Gram-negative bacteria is therefore essential to understand the architecture of the OMVs. The cell envelope of Gram-negative bacteria consists of an outer membrane, a cytoplasmic membrane, and a periplasmic space. The outer membrane (OM) of Gram-negative bacteria is distinct from that of Gram-positive bacteria, in that it buds out and forms the OMV membrane. OM is asymmetrical, with the outer leaflet riched in lipopolysaccharide (LPS), and the inner leaflet primarily made up of phospholipids. The cytoplasmic membrane is composed of a phospholipid bilayer [9]. The transmembrane proteins of OM are referred to as outermembrane proteins (OMPs) and

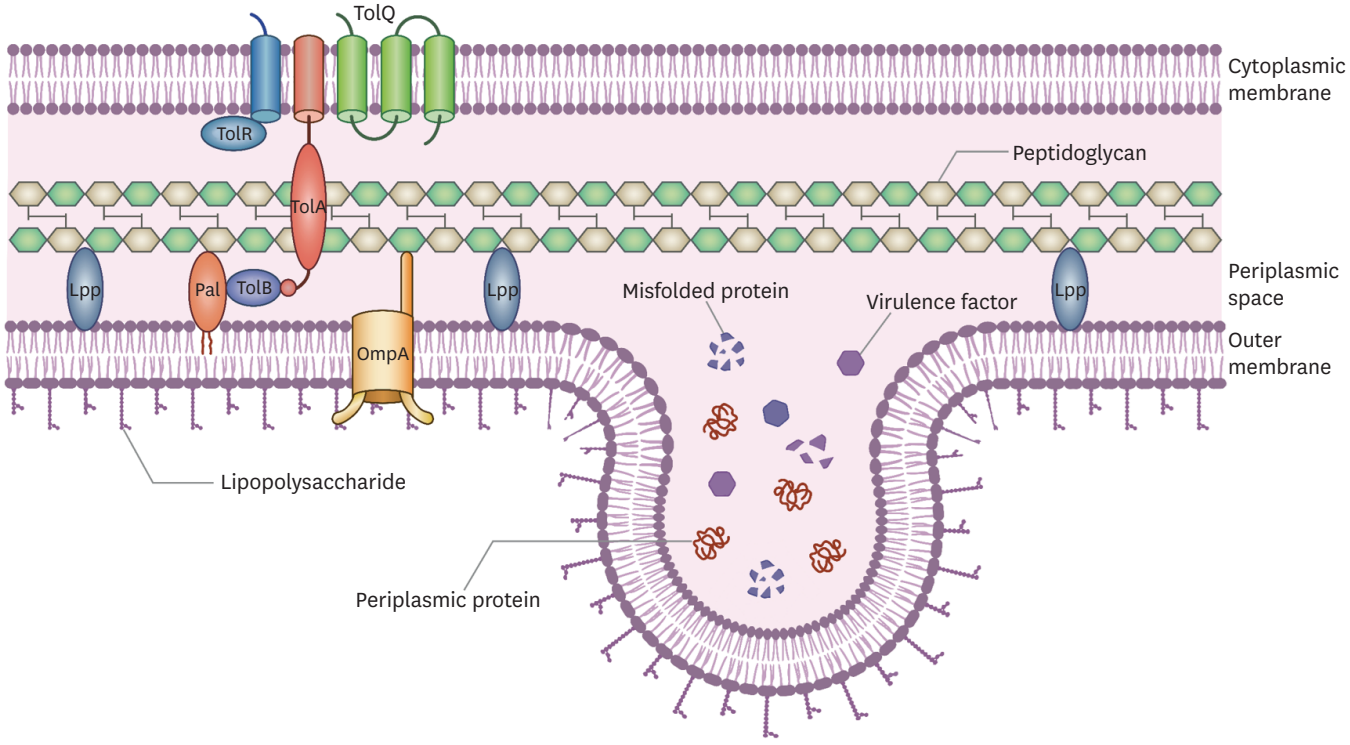

Figure 1. This figure depicts cell wall envelope of the Gram-negative bacteria and biogenesis of Outer membrane vesicles (OMV). Gram-negative bacteria consists of two membrane. Outer leaflet of the outer membrane (OM) is composed of lipopolysaccharide (LPS). To stabilize membrane integrity, Braun's lipoprotein in the OM crosslinks OM layer and peptidoglycan (PG). Also Outer membrane protein A non-covalently binds with PG layer. The Tol-Pal complex which is consisted with cytoplasmic proteins, periplasmic protein, and outer membrane lipoprotein (peptidoglycan-associated lipoprotein) binds cell wall envelope together. OMV is generated from the OM by several mechanisms including reducing cell wall envelope crosslinks, LPS remodeling, and bilayer couple model by increasing OM fluidity. Periplasmic proteins including various virulence factors and cellular waste such as misfolded proteins are packaged into OMVs as cargos and secreted.

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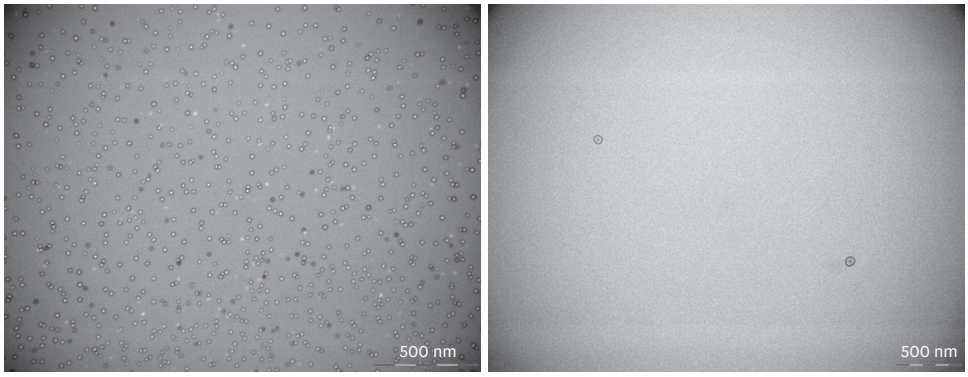

Figure 2. Transmission electron microscope-images of purified outer membrane vesicles derived from clinical isolates of *Pseudomonas aeruginosa* with magnification of × 80,000.

serve as porins or structural roles. Outer membrane protein A (OmpA) contains a periplasmic peptidoglycan (PG) binding site [10]. The space between the outer and cytoplasmic membranes is called the periplasmic space and contains a thin PG layer and periplasmic proteins. The PG layer serves as a skeleton for bacterial cells and protects against osmotic and shear stresses. Periplasmic proteins are densely packed in the periplasmic space, which is more viscous than the cytoplasm. Various crosslinks are present to maintain the stability of the cell envelope. Braun's lipoprotein (Lpp) in the OM is covalently crosslinked with PG, and staples the PG layers together [11]. OmpA non-covalently binds with diaminopimelic acid (DAP) in the PG layer [12]. The transmembrane of the Tol-Pal complex enables OM to encompass the periplasmic space and cytoplasmic membrane [12-14].

The OM should be free from crosslinks with PG, bulge outward to form budding vesicles, and finally detach to form OMVs. Several mechanisms have been proposed to explain OMV production and regulation; however, a definite mechanism still needs to be elucidated. Modulation of the envelope crosslink model is one of the earliest OMV biogenesis models proposed and has been widely investigated. Lpp and OmpA mutants in *E. coli*, *Salmonella enterica*, *Vibrio cholera,* and *Pseudomonas aeruginosa* were demonstrated to increase the fluidity of OM and lead to hypervesiculation [15, 16]. In addition, the weakening of interactions between PG layers called DAP-DAP crosslinks leads to an increase in Lpp-PG crosslinks and thereby results in a decrease in OMV production [17, 18]. Studies on the Lpp-independent OMVs production pathway have also been conducted. *Porphyromonas gingivalis* mutants lacking autolysin that cleaves PG amide bonds were found to lead to an increase in OMV production and thus motivated further studies on periplasmic accumulation [19]. Misfolded protein accumulation in the periplasmic space was also found to induce OMV production independent of Lpp crosslinks [18].

OMVs biogenesis based on alteration of the LPS content of the OM has also been reported in *P. aeruginosa*. This LPS remodeling process results in a selective type of anionic B-band LPS, which is detected in OMVs based on the mechanism of electronic charge repulsion [20, 21]. Further studies on the modulation of OM lipid content have been carried out using the LPS-binding molecule *Pseudomonas* quinolone signal (PQS). PQS constitutes a quorum sensing system of *P. aeruginosa* and serves as a bacterial intercellular communication system. PQS is secreted to the outside of the cell and subsequently engages with the outer leaflet of OM to cause an expansion and changes in curvature thus resulting in increased production of OMVs [22-24].

The bilayer couple model is based on a mechanism of phospholipid accumulation in the outer leaflet of the outer membrane [25]. The VacJ/Yrb ATP-binding cassette (ABC) is a phospholipid transporter that prevents phospholipid accumulation in the outer leaflet of the OM [9]. Mutants that lack the VacJ/Yrb ABC transport system were found to show increased phospholipid accumulation in the cell and thus increased OMV production in *Haemophilus influenzae* and *V. cholerae* [26]. Furthermore, the activity of the VacJ/Yrb system was also found to be regulated by the presence of certain conditions, such as an iron-limited or bile-salt-enriched environment. This suggests that OMV production is regulated by environmental nutrient conditions [26, 27].

FUNCTIONS OF OMVS IN BACTERIAL INFECTION PATHOGENESIS

1. Delivery of virulence factors and toxins

Bacteria secrete OMVs that are loaded with various types of molecules, such as enzymes, proteins, and even genomic materials, to be delivered to distant sites. Investigation of OMV cargo revealed an abundance of

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OMPs (OmpA, OmpC, and OmpF), periplasmic proteins, virulence factors, and nucleic acids. OMVs also carry proteins from various subcellular locations as well [28]. Accordingly, periplasmic proteins are more likely to be included in OMVs compared to proteins bound to the inner membrane.

The inclusion of toxins in OMVs plays a particularly significant role in bacterial infection pathogenesis. Bacteria can deliver virulence factors to distant sites via OMVs that protect them from degradation due to biochemical stress while avoiding cell-to-cell interactions [28, 29]. Enterotoxigenic *E. coli* (ETEC) produces several toxins, including heat-labile enterotoxin (LT) and cytolysin A (ClyA) [28, 30]. OMVs of ETEC deliver these toxins to mammalian cells and enhance their virulence by inducing oligomerization of toxins [31]. Leukotoxin and LPS that are tightly bound to the surface of OMVs also lead to more pronounced immunogenic effects on host cells than when presented alone [4, 32]. Cytotoxin necrotizing factor type 1 (CNF1) and Shiga toxin are found in OMVs of uropathogenic *E. coli* and *E. coli* O157:H7, respectively [33-35]. *V. cholerae* is a well-known pathogen that releases cholera toxin (CT) and secretes virulence factors via OMVs [36]. *P. aeruginosa* OMVs carry multiple virulence factors that cause degradation and pore formation [20]. OMVs of *P. aeruginosa* were also shown to have bacteriolytic effects on both Gram-negative and Gram-positive bacteria [37]. *Salmonella* typhimurium translocates the virulence PhoP/PhoQ regulon into host cells via OMVs, and thereby attenuates virulence in mice [38].

OMVs also release adhesion molecules to increase the adherence of bacteria to host tissues [39]. For example, *P. gingivalis* OMVs include hemagglutinins and heat shock proteins that are deeply involved in host cell attachment and bacterial aggregation by causing dental plaque [40, 41]. *P. aeruginosa* and *Bacteroides fragilis* OMVs contain aminopeptidase and hemagglutinin, respectively, and increase the adherence of bacteria to mammalian cells [42, 43]. However, OMVs containing adhesins may also act to compete with bacterial cells as well, since bacteria and OMVs use identical host-bacteria interaction mechanisms. For example, *Helicobacter pylori* OMVs were found to include less abundant adhesion molecules than the outer membrane [44].

2. Immunomodulatory activities

OMVs are internalized by host epithelial cells via direct fusion or diverse endocytosis mechanisms. OMVs of *P. aeruginosa* and *Legionella pneumophila* are internalized by the host cell through the actin remodeling process, which causes direct fusion and delivery of OMV cargo directly into the cytoplasm of the host cell [45, 46]. Clathrin- and caveola-mediated endocytosis pathways also participate in internalization, allowing large vesicles up to 80 - 120 nm to invade the host [47, 48]. Lipid rafts, which are microdomains in cell membranes rich in sphingolipids, mediate endocytosis and are responsible for the uptake of *H. inflenzae, P. aeruginosa,* and *P. gingivalis* OMVs [29, 42, 48, 49].

OMVs trigger an inflammatory response in epithelial cells following the invasion. Increased levels of pro-inflammatory cytokines after processing of OMVs into host cell cultures have been observed in various pathogens as well. *H. pylori* OMVs showed dose-dependent production of interleukin-8 (IL-8) in gastric epithelial cells [50]. Likewise, OMVs of *P. aeruginosa* and *Klebsiella pneumoniae* that induce IL-1β and IL-8 in human alveolar epithelial cells have been described [51, 52]. OMVs that stimulate immune reactions have also been reported in mouse models. *Acinetobacter baumannii* OMVs were administered intratracheally to induce IL-1β and IL-6 cytokines in the lungs of mice [53].

The pro-inflammatory mechanism of OMV described above has been established based on the engagement of pathogen-associated molecular patterns (PAMPs) with host pattern recognition receptors (PRRs). PAMPs are highly conserved microbial determinants detected by host PRR, resulting in the induction of immune signaling. OMVs contain LPS, flagellin, peptidoglycan, lipoproteins, DNA, and RNA, which serve as PAMPs in the host, and activate PRR [54]. The signaling pathway of PRR differs between bacterial species depending on the components of the OMVs. *E. coli* OMVs induce Toll-like receptors 4 dependent IL-8 production [55]. *Neisseria gonorrhoeae* and *H. pylori* OMVs transduce signals in other PRRs such as nucleotide-binding oligomerization domain-containing protein 1 (NOD1) [56].

OMVs also exhibit immunosuppressive properties since inflammation induced by OMVs is not beneficial to bacteria. OMVs from *P. gingivalis* contain the cysteine proteinase gingipain, which degrades IL-8 [57]. In addition, OMVs of *N. meningitidis* induce the production of anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13. However, *N. meningitidis* OMVs also induce the production of pro-inflammatory cytokines IL-8, IL-1β, IL-6, and TNF, indicating that OMVs play a multifaceted role in both inflammation and immunosuppression [58]. Furthermore, OMVs of commensal bacteria in the gut are considered to promote the maturation of the immune system. *B. fragilis* releases capsular polysaccharides via their OMVs and results in enhanced activation of regulatory T cells and anti-inflammatory cytokine production, and prevention of experimental colitis [59].

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ROLES IN ANTIBIOTIC RESISTANCE

OMVs aid bacterial survival by hindering the activity of antibiotics. OMVs act as decoys that bind and absorb antimicrobial peptides or phages, and thereby function as physical barriers. Hence, antibiotic resistance is developed in bacteria. The addition of OMVs or hypervesiculating mutants of *E. coli* were found to result in the immediate development of resistance to the antimicrobial peptides polymyxin B, colistin, and phages [60]. In addition, the growth inhibitory effects of colistin and melittin on *Pseudomoas syringae* were found to be reversed by the addition of OMVs produced by the same organism. However, the protective effect of the vesicles on the organism was not observed against hydrophilic antibiotic streptomycin [61]. OMVs bind peptide antibiotics with high affinity, yet do not bind well to hydrophilic antibiotics [62, 63]. β-lactamase producing organisms are considered serious threats especially in hematologic malignancy units and intensive care unit [64, 65]. OMVs of these β-lactamase producing organisms carry hydrolases and protect bacteria from antibiotics [66]. Also β-lactamase associated with OMVs protects not only producer bacteria but also standing non-resistant pathogenic bacteria [67]. For example, OMVs from β-lactam-resistant *E. coli* degrade β-lactam antibiotics in a dose-dependent manner and rescue β-lactam-susceptible *E. coli* and other bacterial species from β-lactam antibiotic-induced growth inhibition [68, 69]. Similar findings have been reported in amoxicillin-resistant *Moraxella catarrhalis* OMVs as well. Active β-lactamase from *M. catarrhalis* OMVs was found to promote the survival of *H. influenzae* and *Streptococcus pneumoniae* [70].

OMV-mediated genomic transfer between microbial communities is also a concern due to the high prevalence of antibiotic resistance even in healthy carriers [71]. OMVs of *Acinetobacter* spp. and *E. coli* O157:H7 that transfer double-stranded DNA to intra- and inter-species have been reported [72]. OMVs from *A. baumannii* are also capable of transferring the OXA-24 carbapenemase gene, leading to further dissemination of antibiotic resistance in bacteria [73]. Recent studies on *K. pneumoniae* OMVs in hypervirulent and multidrug-resistant strains have also demonstrated plasmid horizontal gene transfer [74]. Plasmid exchange via OMVs generates hybrid clones of hypervirulent strains showing a hypermucoid and multidrug-resistant phenotype [75].

Interestingly, antibiotic use increases the production of OMVs as well. Antibiotics, including ciprofloxacin, meropenem, fosfomycin, and polymyxin B, were found to induce the production of OMVs in *E. coli* [76]. In addition, a study on multidrug-resistant *K. pneumoniae* demonstrated that the use of carbapenems exacerbates the secretion of OMVs, which promotes the production of pro-inflammatory cytokines [77].

CURRENT APPLICATION OF OMVS

OMVs are not capable of self-replication; however, they mimic the immunogenic properties of the producing bacteria, making them an attractive vaccine platform. OMVs also have a size advantage, which enables their entry into lymph vessels and uptake by antigen-presenting cells (APCs), in addition to strong adjuvant properties that stimulate both innate and adaptive immune responses, and high stability against biochemical stress [56, 78].

A successful OMV-based vaccine developed to date is the meningitis serogroup B vaccine BEXSERO® (GlaxoSmithKline, London, UK), which was approved by the Food and Drug Administration in 2016. Unlike other *N. meningitidis* serogroups that already have successful conjugate vaccines on the market, serogroup B capsules were not feasible since this polysaccharide was homologous to human brain tissue molecules [79]. To overcome this obstacle, researchers have focused on the outer membrane protein porin A (PorA), which is the main immunogenic molecule secreted by OMVs in *N. meningitidis*. Although PorA is highly variable between strains, OMVs derived from bioengineered strains expressing multiple PorA variants have been successfully developed [80]. OMV-based vaccines were also investigated for use against other pathogens, including *V. cholerae*, *S. typhimurium*, *Burkholderia* species, *H. pylori*, and *Bordetella pertussis*, yet none have progressed to clinical trials. *B. pertussis* acellular vaccine development was recently hampered by a reactogenic problem that prevents the effective reduction of pertussis infections. OMV-based vaccine from *B. pertussis* may provide a viable alternative to this end, due to the presence of many virulence factors in OMV of *B. pertussis*.

Besides bacterial vaccines, studies on the bioengineering of OMVs as cancer agents have also been carried out. OMVs can be engineered to express cancer-specific epitopes or to carry small non-coding RNAs [81]. The ability of OMVs to rapidly display antigens may lead to the development of personalized cancer vaccines [82]. Furthermore, OMVs induce a durable antitumor immune response and inhibit tumor growth in multiple tumor models by producing the antitumor cytokines and interferon-γ [83]. Their potential as drug delivery systems is also being investigated. Finally, doxorubicin-loaded *K. pneumoniae* OMVs showed tumor growth inhibition with favorable tolerability and pharmacokinetic profiles, thus showing great potential for tumor chemoimmunotherapy [84].

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CONCLUSION

In summary, we discussed OMVs' basic structure, suggested biogenesis mechanisms, and their functions and role in pathogenesis and antimicrobial resistance. To our most recent knowledge, the OMVs seem to serve a significant role in bacterial cells and bacterium-host interaction. However, current research on the therapeutic applications of OMV, such as vaccines or drug-delivery platforms, is still under progress. With deeper understanding of OMVs' biogenesis and selective cargo mechanisms, the value of OMVs as a future therapeutic tool may become revealed.

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Conflict of Interest
No conflict of interest.

Author Contributions
Conceptualization: JYK. Data curation: JYK, JWS, JSK. Resources: JWS, JSK. Supervision: JWS, SBK, YKY. Writing - original draft: JYK. Writing - review & editing: JYK, JWS, JSK, SBK, YKY, JWS.

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