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Review on bacterial outer membrane vesicles: structure, vesicle formation, separation and biotechnological applications

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Abstract
Outer membrane vesicles (OMVs), shed by Gram-negative bacteria, are spherical nanostructures that play a pivotal role in bacterial communication and host-pathogen interactions. Comprising an outer membrane envelope and encapsulating a variety of bioactive molecules from their progenitor bacteria, OMVs facilitate material and informational exchange. This review delves into the recent advancements in OMV research, providing a comprehensive overview of their structure, biogenesis, and mechanisms of vesicle formation. It also explores their role in pathogenicity and the techniques for their enrichment and isolation. Furthermore, the review highlights the burgeoning applications of OMVs in the field of biomedicine, emphasizing their potential as diagnostic tools, vaccine candidates, and drug delivery vectors.

Keywords Outer membrane vesicles, Extracellular vesicles, Gram-negative bacteria, Biotechnological applications

Introduction

Secretion at the plasma membrane is an essential process that occurs in all life forms, enabling the organism to interact with its environment. It has been found that bacteria can achieve this goal by secreting nanoscale spherical vesicles [1, 2]. Both Gram-negative and certain types of Gram-positive bacteria are able to produce bacterial extracellular vesicles (EVs).

EVs also have different names in different organisms, depending on the cellular structure, Gram-positive bacteria produce EVs called membrane vesicles (MVs) [3]. The EVs secreted by Gram-negative bacteria in a budding manner are called outer membrane vesicles (OMVs) [2]. The bacterial cell membrane of Gram-negative bacteria is divided into cytoplasmic membrane and bacterial outer membrane (OM). The plasma membrane is composed of a phospholipid bilayer, while the OM is composed of an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide (LPS). LPS is composed of lipid a, core oligosaccharides, and O antigen. Between the two membranes is the periplasm, which contains the peptidoglycan (PG) layer and periplasmic proteins [4]. OMVs secreted by Gram-negative bacteria can spread over long distances and play biological roles in the environment and other cells, involving substance transport, gene transfer, and signal transduction [4].

OMVs carry a variety of pathogen-associated molecular patterns (PAMPs), such as endotoxin (LPS), PG, and bacterial DNA, which are capable of binding to pattern-recognition receptors (PRRs) of host cells, initiating an innate immune response, and initiating an inflammatory response [4]. For example, OMVs from *Salmonella* are

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able to activate macrophages and dendritic cells, increase MHC II expression and stimulate the secretion of tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12), thereby inducing $CD4^+$ T cell activation [5].

Vaccines and biotherapeutic technologies can greatly benefit from OMVs [6, 7]. Due to their unique structure and function, OMVs can be utilized as efficient drug carriers to allow combinations of immunotherapy and chemo-phototherapy to enhance the anticancer effect of drugs. As an example, OMVs are commonly used for tumor vaccination, and prior administration can enhance the vaccination's effectiveness. Furthermore, OMVs are highly biocompatible and possess a high capacity for drug delivery, are easy to modify and industrialize, and are expected to become bilayer nanocarriers resembling lipids [8]. In recent years, significant progress has been made in the study of OMVs, spherical microvesicles secreted by Gram-negative bacteria that play an important role in the biological functions of bacteria and their interactions with their hosts. This Review discusses the biological components, biological effects, pathogenic mechanisms, enrichment and isolation methods of OMVs and emphasizes their potential for biomedical applications.

The role of OMVs in the bacterial-host interaction was analyzed. The key mechanisms of OMVs-induced host inflammation and immune tolerance were discussed. Finally, the new progress of OMVs in biotherapeutic technologies such as vaccine design and drug-loaded nanoparticles were summarized.

Table 1 Structure and composition of OMV

Structure/composition	Description
Size	Typically at 20–400 nm
Membrane structure	It is composed of a lipid bilayer and is similar to the OM of bacteria
Lipid	Contains phospholipids, LPS, and other membrane-associated lipids
Protein	Contains a variety of transmembrane and OM proteins, which may include enzymes, channel proteins, and transporters
Nucleic acid	Sometimes it contains DNA or RNA, which may involve horizontal transfer of genes
Cytotoxins and signaling molecules	Can carry toxins, signaling molecules, and other bioactive molecules that affect host cells
Periplasmic protein	May contain proteins from the bacterial periplasmic space
Function	Material transport, gene transfer, signal transduction, immune regulation, etc.

Constituents of OMVs

Initially, OMVs were regarded only as growth artifacts or by-products associated with cell lysis [9]. When OMVs were detected in cerebrospinal fluid samples from patients with meningococcal infections [10], a turning point occurred, suggesting that OMVs are not only produced under laboratory conditions, but may also play a role in disease development. As a result of this discovery, researchers have been interested in studying the biogenesis and function of various bacterial OMVs. Pioneering research by Terry J Beveridge and his research team confirmed that OMVs conferred selective advantages on parental bacteria by promoting biofilm formation and playing an important role in genetic transformation and disease as transport carriers of DNA and virulence factors [11, 12].

OMVs are spherical nanoparticles with a lipid bilayer structure, with a diameter of about 20–400 nm, containing periplasmic cavity components, and are budding and separated from bacteria during active growth, rather than being a by-product of bacterial lysis [13]. OMVs are generated when a small portion of the OM expands from the bacterial cell wall, squeezes and releases. Proteomic and biochemical analyses have shown that OMVs contain many bacterial components, including DNA, RNA, LPS, proteins, enzymes, and PG [14, 15]. Thus, OMVs contain many biological substances found in the parent bacteria, but in a non-replicating form. Below, the components of OMVs are outlined (Table 1).

Nucleic acid

Renelli, M et al. [12] found that OMVs have not only luminal DNA, but also surface-related DNA. After DNAse digestion of exogenous DNA, the lumen contained only plasmid DNA, and both showed significant resistance. This finding suggests that the DNA in OMVs has some resistance to maintain its stability in the external environment. Various types of luminal DNA have been identified in a variety of bacteria, such as *Pseudomonas aeruginosa* [16], *influenzae* [17], and *Neisseria gonorrhoeae* [18]. Other studies have found that OMVs contain not only DNA, but also mRNA, microRNA and non-coding RNA. Cherie Blenkiron et al. [19] found that OMVs of uropathogenic strains derived from *Escherichia coli* (*UPEC*) contain a variety of RNAs, such as rRNA, tRNA and mRNA. Recent studies have shown that *Pseudomonas aeruginosa* can regulate the host immune response through sRNA contained in its OMVs.

These studies have proved that OMVs can not only serve as genetic information carriers but also as genetic information transmitters. However, little is known about the biological effects of DNA and RNA delivered to host cells through OMVs. In the case of the *Pseudomonas aeruginosa* OMVs, sRNAs are capable of targeting host

mRNA, inhibiting cytokine secretion such as IL-8 and neutrophil infiltration, as well as reducing host immunity [20]. Additionally, sRNA4518698, sRNA2316613, and sRNA809738, the 3 most abundant sRNAs in OMVs, are non-coding RNA fragments that are resistant to external RNase degradation [21].

Phospholipid

The lipids contained in OMVs are mainly phospholipids and LPS, among which phospholipids are similar to OM [22]. A study by Hoekstra et al. [23] showed that OMVs of *Escherichia coli* have a phospholipid profile similar to that of OM. Subsequently, Kato et al. [24] identified LPS, phosphatidylethanolamine and cardiolipin as the main lipid components of the vesicles, similar to the main lipid components of the OM, by using thin-layer chromatography. However, quantitative lipidomic analysis of different classes of phospholipids has not yet been performed and will be one of the future research directions.

In addition, the curvature of the OMVs membrane is much higher than that of its bacterial parent; therefore, it may be characterized by a different composition of various phospholipids [25]. Tashiro et al. [26] found that phosphatidylethanolamine was abundant in OM, while phosphatidylethanolamine was found in OMVs. In addition, the relative number of saturated fatty acyl chains is higher in OMVs compared to OM, making OMVs more rigid than the OM.

LPS

Lipopolysaccharide (LPS) is an important component of the OM of Gram-negative bacteria, and it plays a key role in the formation and function of OMVs [27]. It has been found that not all LPS of bacteria are present in OMVs, but only some LPS are present in OMVs. LPS may play an important role in the biogenesis of OMVs. According to Kadurugamuwa et al. [28] the OMVs of *Pseudomonas aeruginosa* consists predominantly of the negatively charged B-band LPS rather than the more neutral A band. The results of this study suggest that LPS may be important to the biogenesis of OMVs. LPS is not only present in OMVs, but also plays a key role in its formation. Certain chemical forms of LPS, such as pentanoylated LPS produced by PagL, are known to increase the number and size of OMVs released by bacteria [29]. This suggests that modification of LPS can stimulate OMVs synthesis. In addition, the enrichment of LPS in OMVs is critical for OMVs formation, and the LPS commonly detected in OMVs is predominantly the B band form of charged polysaccharides.

Regulation and generation mechanisms of OMVs

Bacterial OMVs have been extensively studied for decades, and the compositional and structural similarities between bacterial OMVs and OM have been recognized, providing some clues to the biogenesis of OMVs [30]. OMVs are basically formed by OM through multiple pathways to out-budding and acquire many biomolecules from OM and periplasm, such as proteins, genetic material, and virulence factors [31]. Therefore, it is crucial to understand how bacterial cell membranes are covalently cross-linked and how membrane stability and fluidity are maintained to maintain bacterial viability [30]. However, there is still no well-established model or universal mechanism to fully elucidate the mechanism of OMVs biogenesis. Currently, research has focused on the following several models to explain how OMVs are formed.

Membrane cross-linking and OMVs formation

OMVs formation is closely associated with reduced cross-linking between the OM and PG of Gram-negative bacteria. Outer membrane protein A (OmpA), Braun lipoprotein (Lpp), and the TOL-PAL complex are the major membrane proteins that stabilize the OM through protein-protein or protein-PG interactions [32].

OmpA is an OM porin protein that contains a periplasmic binding site with diaminopyrenic acid (DAP) composed of PG and plays an important role in anchoring the OM to the bacterial cell wall [33]. It has been reported that the production of OMVs is regulated by OmpA, and the loss of OmpA induces hypervesiculation in many bacterial species. For example, *Salmonella typhi* genes (commonly referred to as *zzz* genes) were found, and these genes correspond to ompA, nlpI, and others. Some *zzz* deletions affected the protein content of *Salmonella typhi* derived OMVs. In addition, *zzz* genes are involved in OMVs biogenesis and regulate different properties such as OMVs size [1]. In some bacteria, Lpp is an abundant OM protein, one third of which is covalently cross-linked to PG and acts as a molecular backbone to link OM to the PGlayer [34]. It has been suggested that the absence or loss of function of Lpp may result in weakened attachment of the bacterial outer membrane to the peptidoglycan layer, thus making the outer membrane more susceptible to bulge to form OMVs. For example, it has been found in several studies that bacterial mutants lacking Lpp exhibit increased OMVs release. This phenomenon may be due to the disruption of the connection between the outer membrane and the cell wall, leading to local instability of the outer membrane, which in turn promotes the formation and release of OMVs [35]. The TOL-PAL complex is a bacterial division component consisting of five proteins: ToLA, ToLB, ToLQ, ToLR, and PAL, which can link the OM to the PG layer and the cytoplasmic membrane (IM) layer through

protein-protein and protein-protein interactions [36]. For example, *S. Choleraesuis* ToLA, ToLB and ToLR genes are involved in OMVs biogenesis, and OMVs envelope integrity is impaired when ToLA, ToLB and ToLR are mutated [37]. Additional, ToLA, ToLQ, and ToLR form complexes in the inner membrane, while ToLB is a periplasmic protein that interacts with Lpp, OmpA, and PAL. It has been found [38] that the TOL-PAL complex contributes to the invagination of the OM and the stability of the cell membrane and interacts with PG. Carmen Schwechheimer et al. [32] found that minor modifications of PG remodeling and cross-linking regulate the production of OMVs, which is inversely proportional to the level of bound Lpp (Fig. 1A), that is, when local membrane cross-linking is destroyed or reduced, a small part of the OM can bud outward, leading to the formation of OMVs.

Periplasmic accumulation and OMVs formation

Some scholars have proposed that the formation of OMVs is related to the accumulation of periplasmic contents [32]. With the increasing PG fragments in the pericytoplasm, Gram-negative bacteria can exert swelling pressure on the OM, which leads to OM budding and the formation of OMVs [39] (Fig. 1B). Japanese scholars found in the study of *Porphyromonas gingivalis* [40] that by building the autolysin mutant of *Porphyromonas gingivalis* and comparing it with the wild-type strain 381, the results showed that the lack of autolysin activity prevented *Porphyromonas gingivalis* from degrading PG fragments in the periplasm, so that PG fragments accumulated in the periplasm and eventually produced more OMVs. Other scholars have found that the amount of protein accumulation in the cell envelope is directly related to the amount of OMVs released [41]. When the cell stress is damaged, it leads to increased protein accumulation in the envelope.

LPS remodeling and OMVs formation

Some scholars have found that changing the LPS content of the bacterial OM can affect the formation of OMVs [29] (Fig. 1C). Membrane vesicles were isolated from three isogenic LPS mutants of the Gram-negative bacterium *Pseudomonas aeruginosa* PAO1 and compared with the wild type. *Pseudomonas aeruginosa* is known to

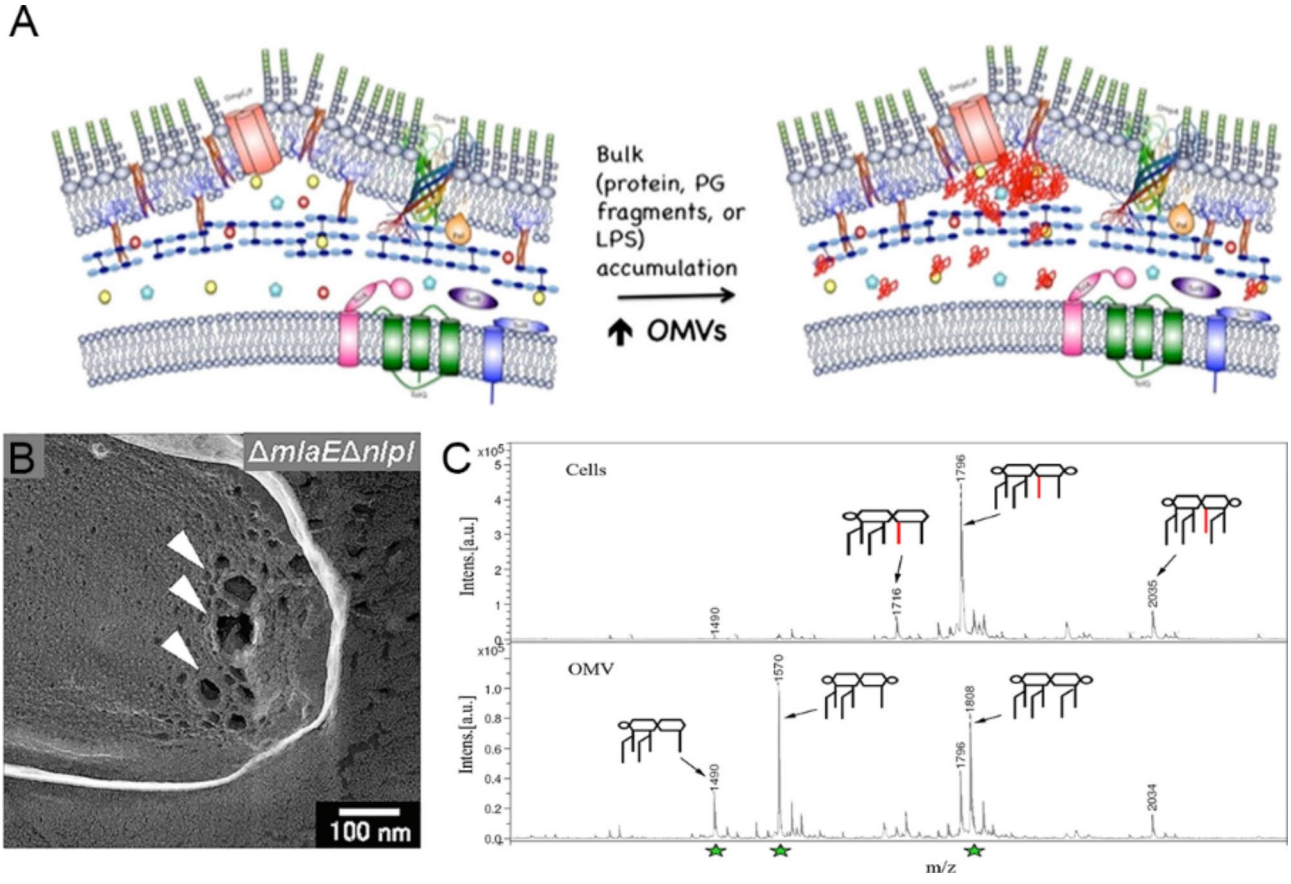

Fig. 1 (**A**) The inverse relationship between the production of OMVs and the level of covalently crosslinked Lpp. (**B**) Significant holes observed in the cell wall through quick-freeze deep-etch electron microscopy represent key structural features for periplasmic accumulation and the formation of OMVs. (**C**) When PagL is expressed in *Salmonella Typhimurium*, deacylated LPS is selectively enriched in OMVs, revealing a direct link between LPS remodeling and the formation of OMVs

produce two LPS types containing different O-polysaccharides, namely A with LPS (neutral charge) and B with LPS (anionic charge) [28]. However, it was found that the B-only LPS mutant produced more OMVs than the wild type, the mutant only produced A-band LPS, and the resulting vesicles were very different in size, mass and total protein [42]. At the same time, wild-type strains and these mutant strains secrete different OMVs, suggesting that the overproduction of OMVs by strains producing B-only LPS may be the result of an envelope stress coping mechanism [43]. Interestingly, only B-band LPS was detected in OMVs, a finding that led to the hypothesis that OMVs arise in the more endotoxin-rich region of the B-band and that the OM bends to mitigate charge repulsion between them [28]. In addition, LPS remodeling can also affect the production of OMVs. One example is that LPS remodeling is important for the formation of OMVs in *Salmonella* [29]. The addition of specific modifications of LPS (e.g., acetylation) to the outer membrane increases membrane instability (e.g., altered flexibility and fluidity). *Pseudomonas* quinolone signal (PQS) can enhance the anion repulsion on the bacterial surface and bind to LPS. Additionally, deacylation of anionic lipopolysaccharide (A-LPS) may further increase membrane curvature by up-regulating the production of some lobular lipids, leading to local bending of the OM, which in turn increases the formation of OMVs and their diameter. Modification by LPS may also affect the permeability of the outer membrane and thus the formation of OMVs [44, 45].

Others

In addition to the above categories of models, there are the following categories: (1) aggregation of membrane lipids. Because the biological properties of membrane lipids determine membrane curvature and fluidity, membrane lipids may play a key role in OMVs biogenesis [46]. The curvature of the OMVs membrane is about 14 times greater than that of the OM, suggesting that OMVs biogenesis requires energy expenditure to significantly bend the OMVs membrane. It has been found [45, 47] that membrane curvature is closely related to the aggregation of lipid molecules, and the aggregation of some lipid molecules can cause the local outward bending of the OM and eventually form OMVs. (2) double-layer coupling model. This model mechanism argues that membrane curvature triggered by the insertion of biomolecules into the outer leaflet of the OM, and that the outer leaflet changes more rapidly than the inner leaflet, leading to the budding of the OM and the formation of OMVs. Quinolone PQS of *Pseudomonas aeruginosa* induces the formation of OMVs through the mechanism of asymmetric expansion of the outer leaflet of the OM [48]. However, it is limited by the fact that PQS is produced only by *Pseudomonas aeruginosa* and thus is species specific. (3) VacJ/YRB ABC transport system. This transport system is involved in the transport of phospholipids in OM, and disruption of this system leads to excessive secretion of OMVs [49]. Recent studies have reported [50] that inhibition or deletion of VacJ/YRB can lead to the accumulation of phospholipids in OM and increase the production of OMVs in *Haemophilus influenzae* and *Vibrio cholerae*, and the OMVs secreted by the mutant contain twice as many phospholipids as the wild type. This mode of OMVs formation may be a general mechanism applicable to a variety of Gram-negative bacteria.

Enrichment and separation of OMVs

OMVs play a role in a variety of disease changes and may become one of the diagnostic methods of diseases [51]. It has been found [13] that even OMVs isolated from pure bacterial cultures are heterogeneous in size and molecular composition. The results of a recent study on *Hp*-OMVs [52] showed that the size, protein composition and immunogenicity of OMVs produced by different growth stages of *Hp* are not comparable. The enrichment and separation of OMVs is the prerequisite for the translation of biomedicine and clinical medicine. Therefore, accurate qualitative and quantitative analysis of OMVs is extremely important. Here, we summarize several common enrichment and separation methods and analyze their advantages and disadvantages (Table 2).

Ultracentrifugation

Ultracentrifugation (UC) is the most commonly used method for enrichment and isolation of OMVs. The principle is to separate OMVs by multistage centrifugation using differential centrifugal force depending on particle size and density. First, bacteria and bacterial debris were removed with a low centrifugal force, and the supernatant was filtered with a 0.45 μm filter, then concentrated with a 100 kDa ultrafiltration tube, then filtered with a 0.22 μm filter, and finally ultrafiltration with a high centrifugal force (150000 g) for 3 h to isolate OMVs [53]. Recently, other studies have exploited the property of small RNAs (sRNAs) packaged in bacterial OMVs to enter host cells and modulate innate immune responses, and further explored the mechanism of OMVs in modulating host responses by purifying OMVs from *Pseudomonas aeruginosa* by OptiPrep density gradient ultrahigh-speed centrifugation [54]. This method is well-established, but it has some limitations, including long experimental time, need for high-speed centrifugation, and limited efficiency and purity. Crude OMVs can be further purified by gradient ultracentrifugation, and the separation depends on the mass density and size; iodixanol and sucrose are commonly used density media [55–57].

Table 2 Advantages and disadvantages of OMVs extraction methods

Extraction method	Description	Advantages	Disadvantages
Ultrafiltration	Most of the non-OMVs protein fractions were selectively filtered using 50–100 kDa membrane ultrafiltration.	Easy to operate and can concentrate OMVS.	It may not be possible to completely remove macromolecular impurities.
Ultracentrifugation	Purification of OMVs is achieved using different centrifugal forces, typically including medium- and high-speed centrifugation to remove large impurities, followed by ultrafiltration, and finally ultracentrifugation.	high purity and is the most commonly used method for separating OMVs.	The operation is complex, time-consuming and costly.
Density Gradient Centrifugation	OMVs were separated by ultracentrifugation using a density gradient medium such as iodixanol or sucrose.	High purity for further purification of OMVs.	The operation is complex and requires specific density gradient media.
Gradient Filtration	The efficient separation of OMVs by gradient filtration demonstrated the superiority of the new method in OMVS separation compared with the conventional ultracentrifugation method.	high efficiency and maintained the biological activity of OMVs.	Specific filter material may be required.
Protein Precipitation	Proteins were precipitated using, for example, ammonium sulfate, and then OMVS was collected by centrifugation.	OMVs can be condensed for easy operation.	Precipitant impurities may be introduced.
Affinity Chromatography	The affinity of specific molecules on the surface of OMVs for the chromatographic medium was utilized for separation.	Highly selective for specific enrichment of OMVS.	Specific affinity chromatography media are required and can be costly.
Field Flow Fractionation	Separation using differences in hydrodynamic radii.	High resolution for effective separation of particles of different sizes.	The equipment is costly and complicated to operate.
Microfluidics	Separation of OMVs using microfluidics.	Fluid dynamics conditions can be precisely controlled for highly efficient separation.	The equipment is costly and technically demanding.

Ultrafiltration

The principle of Ultrafiltration (UF) is a technology that uses pressure as the driving force to selectively filter nanoscale substances according to morphology and molecular size. In brief, after removal of bacteria and debris, the supernatant was filtered through 0.45 μm and 0.22 μm filters, and the supernatant was collected and ultrafiltrated through a 100 kDa ultrafiltration tube. The ultrafiltrate was defined as OMVs. Ultrafiltration can be combined with ultrafiltration to jointly extract OMVs [56]. The advantage is that the experiment is fast and easy to operate, and the disadvantage is that the purity is general.

Precipitation

The Precipitation method is divided into charge method (protamine precipitation method, sodium acetate precipitation method) and hydrophilic method (polymer precipitation method) according to different principles [58]. At present, commercial kits have been used for OMVs enrichment, with the advantages of simple operation and the disadvantages of high price and lack of specificity. Deregibus MC et al. were proposed EVs loading-based precipitation from biological fluids and cell supernatants. ζ potential analysis revealed that EVs have a negative charge that allows interaction with positively charged molecules such as protamine. Protamine has been shown to induce EVs precipitation from biological fluids and cell culture media by avoiding ultracentrifugation. When protamine-induced precipitation was performed in a polymer matrix such as PEG 35,000 Da, EVs recovery was improved and pellets were easily resuspended [59]. Using metal precipitation combined with size-exclusion chromatography, Won S et al. demonstrated that large-scale production of *Escherichia coli* OMVs increased $CD8^+$ T cell infiltration and activation by inducing $CD8^+$ T cell infiltration and activation, Immunotherapy with anti-PD-1 antibody showed synergistic antitumor activity [60].

Size exclusion chromatography

Size exclusion chromatography (SEC) achieves separation based on molecular size rather than molecular weight. By applying columns filled with porous polymer microspheres, soluble components and molecules of small radius are allowed to enter the microspheres temporarily, while macromolecules are eluted earlier from the column [61]. The advantages are high yield and wide elution range, while the disadvantages are low specificity and expensive kits that are difficult to be used on a large scale.

Field flow classification method

The experimental principle of the field-flow fractionation method is to apply a force field perpendicular to the sample flow to achieve a separation based on size and molecular weight. When the sample flows in the channel, the fluid moves slower at the boundary than at the

center of the flow due to laminar flow. When a vertical force field is applied, the analyte in the sample is driven in the direction of the boundary. Brownian motion creates a counteracting motion such that smaller particles tend to reach an equilibrium position at a distance from the boundary [62, 63]. This method has a wide separation spectrum and elution range. The disadvantage is that the experiment takes a long time and requires specialized equipment.

Functional role of OMVs

OMVs contain a variety of specific biomolecules and special structures, leading to their various physiological and pathobiological functions. Studies have found that OMVs play an important role in bacterial survival, bacterial communication, bacterial pathogenesis and immune regulation [22].

Role of OMVs in bacterial survival

OMVs play an important role in the physiology of Gram-negative bacteria [64]. OMVs are indispensable for the survival of Gram-negative bacteria. In the case of stress, bacteria make the cell membrane vesiculate and increase the production of OMVs to help bacteria survive under stress conditions [65]. Because the membrane composition of OMVs is essentially the same as OM, vesicles can serve as potential decoys to divert the attack away from the parental bacteria when they are under targeted attack. For example, when bacteria are treated with lytic phage, vesiculation of the bacterial OM increases and a large number of decoy vesicles are produced, which can help bacteria avoid infection by phage [66]. OMVs can also bind or absorb certain antibacterial molecules (such as complement and antibiotics), inactivate or transport these molecules from bacteria, and finally achieve the role of protecting bacteria [67] (Fig. 2A). For example, *Moraxella catarrhalis* OMVs promote *Haemophilus influenzae* survival in complement-mediated challenge [68]. Other studies have found that OMVs produced by one bacterium can increase the resistance of other bacterial antibiotics. Viveka Schaar et al. [69] found that OMVs of *Moraxella catarrhalis* carry β-lactamase and promote the survival of Streptococcus pneumoniae and *Haemophilus influenzae* by inactivating amoxicillin, which emphasized the ecological role of OMVs in microorganisms.

Role of OMVs in interbacterial communication

OMVs are known to contain proteins, lipids, and nucleic acids that play important roles in intercellular communication. OMVs are secretory and delivery systems that transmit bacterial products and interact with the environment. Lauren M Mashburn et al. [70] reported in a study published in Nature that the signaling molecule 2-heptanyl-3-hydroxy-4-quinolone (PQS) of *Pseudomonas aeruginosa* was introduced into OMVs, and that removal of these OMVs from the bacterial group would

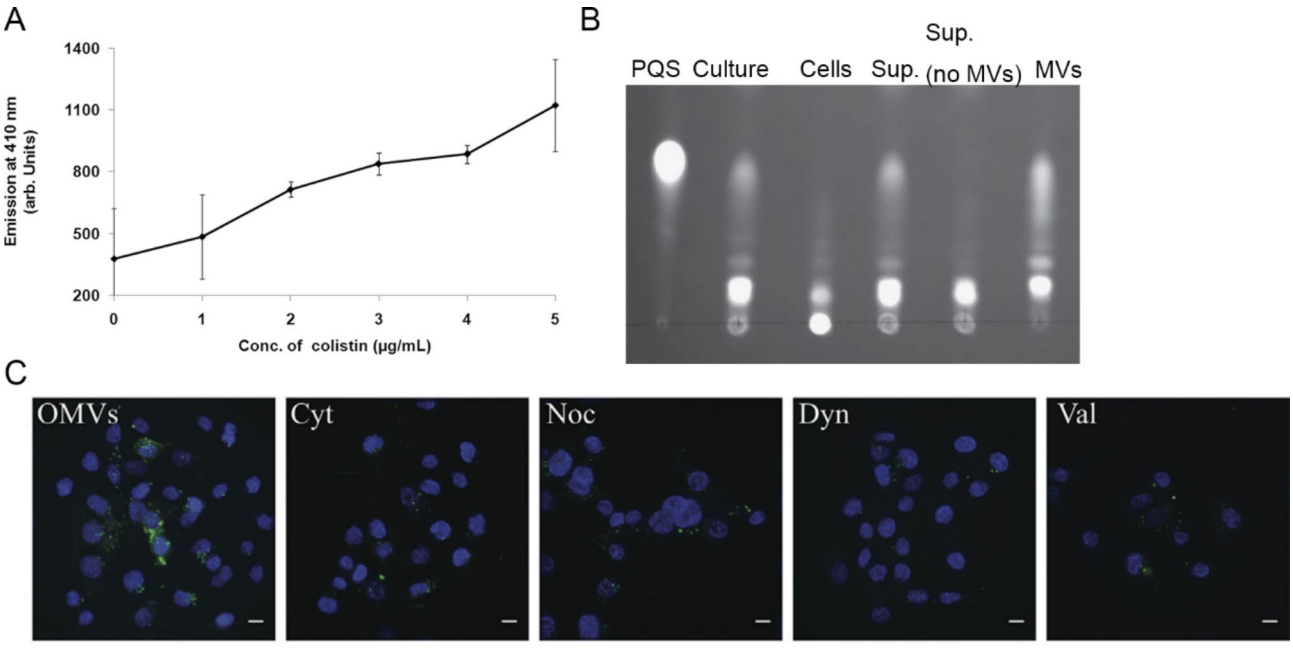

Fig. 2 (**A**) Effect of OMVs on bacterial survival: At different concentrations of colistin, OMVs protected P. syringae Lz4W bacteria from inhibition by adsorption of antibiotics, as indicated by the increase in NPN fluorescence intensity. (**B**) Thin-layer chromatography (TLC) analysis revealed how *Pseudomonas aeruginosa* packages the signaling molecule PQS into OM vesicles (OMVs) for effective cellular communication and coordination of population behavior among bacteria. (**C**) Co-culture of pretreated AGS cells with DiO-labeled *Hp* OMVs showed that OMVs enter the cells through multiple endocytosis pathways that may trigger inflammation and pathogenesis

Table 3 Mechanisms of OMV-induced host immune responses

Mechanism Type	Description	Host Cellular Response
Endocytosis	OMVs enter host cells through macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis.	Promotes the production of inflammatory factors such as IL-8.
Proteins and Lipids	Membrane proteins and lipids carried by OMVs can activate pattern recognition receptors (such as TLRs) on host cells.	Activates inflammatory and immune signaling pathways.
Immunostimulatory Molecules	Molecules such as lipopolysaccharides (LPS) and PG can strongly activate the host's immune response.	Induces the expression of inflammatory cytokines and the recruitment of immune cells.
DNA and RNA	Nucleic acids in OMVs can be recognized by innate immune receptors in host cells, such as NOD-like receptors.	Promotes the production of type I interferons and other inflammatory mediators.
Bacterial Toxins	Some OMVs may carry bacterial toxins that can directly damage host cells or modulate host immune responses.	Causes cell death, inflammation, and tissue damage.
Antigen Presentation	OMVs can be captured by antigen-presenting cells such as dendritic cells, thereby activating T cells.	dendritic cells, thereby activating T cells.Activates adaptive immune responses, including cytotoxic T cells and helper T cells.

stop intercellular communication and inhibit PQS-controlled group behavior (Fig. 2B). It was also found that PQS actively mediates not only its own entry into OMVs but also other antimicrobial quinolines produced by *P. aeruginosa*. In addition, OMVs can selectively interact with bacteria in order to transfer their contents to target bacteria [71, 72].

Pathogenic role of OMVs

Recent studies have shown [73] that OMVs are related to pathogenesis, which can transmit virulence factors to cause host damage, promote bacterial colonization in the host, and weaken the immune response, leading to immune escape. In contrast to soluble secretion methods, OMVs provide a unique mode of secretion for pathogens by protecting virulence determinants from host proteases and concentrating them for host cell delivery. In addition, OMVs can simultaneously deliver multiple virulence factors and confer antibiotic resistance. O'Donoghue EJ et al. [74], when studying the mechanism of OMVs entering host cells, proposed that the pathways of OMVs entering host cells include endocytosis (macroendocytosis, clathrin-mediated, caveolin-mediated and non-caveolin, non-clathrin-mediated endocytosis) and membrane fusion. A study of *Hp*-OMVs found [75] that smaller OMVs with sizes ranging from 20 to 100 nm preferentially enter host cells through caveolin-mediated endocytosis (Fig. 2C), whereas larger OMVs with sizes ranging from 90 to 450 nm enter host epithelial cells through macroendocytosis and endocytosis. Smaller OMVs contain fewer bacterial proteins than larger OMVs.

Virulence factors in OMVs include adhesins and toxins [76], which not only provide stability but also increase concentration compared with free toxins [77]. Thus, OMVs secreted by bacteria can enable more translocation of virulence factors into host cells and induce more severe responses. It has been found [78] that the ClyA protein is a pore-forming cytotoxin expressed by *Escherichia coli* and some other enterobacteria. ClyA forms oligomeric pore assemblies in OMVs, which exhibit higher cytotoxicity against mammalian cells compared to ClyA protein purified from bacterial periplasm. It has also been found [79] that hemolysin is mainly related to bacterial OMVs and the induction of vacuolar rupture containing OMVs, which increases the exposure of LPS to cytosolic sensors, and hemolysin is largely bound to OMVs, resulting in more severe disease.

Mechanisms by OMVs induce host inflammatory and immune responses

OMVs can trigger inflammatory responses in host tissues, but the specific mechanisms are still unclear. Recent research has advanced into how OMVs affect the immune response. The key mechanisms by which OMVs induce host inflammation are discussed below (Table 3).

OMVs interact with epithelial cells

The mucosal tissues of the body surface skin, respiratory tract, and digestive tract play a mechanical role in blocking the invading microorganisms. The mucosal epithelium is shown to be the first line of host defense. OMVs of pathogens can interact with host epithelial cells, leading to cytokine production [80], cell proliferation, apoptosis [81], and induction of immune responses [82].

It is known that OMVs carry a variety of immunogenic molecules such as LPS, flagella and PG, and the bacterial components of pathogen associated molecular patterns (PAMPs) contained in OMVs can be recognized by the host immune system and play a pro-inflammatory and immunomodulatory role [83]. OMVs, as carriers, can deliver LPS into the cytoplasm and play a key role in activating the host defense system [84]. The content and composition of OMVs are different among different bacteria, so the mechanisms by which OMVs from different bacteria initiate PRR signaling are different. In a study on *Hp*, it was reported that *Hp* rapidly activated MAPK and transcription factors NF-κB and AP-1 in gastric epithelial cells after host attachment, indicating that OMVs

can induce PRR signaling [85]. A large number of studies have reported that Toll-like receptor 4 (TLR4) plays an important role. For example, OMVs of *Pseudomonas aeruginosa* regulate host immune response by targeting TLR4 signaling pathway, resulting in increased expression of IL-1β and IL-6 [86].

OMVs interact with immune cells

Studies have found that OMVs penetrate the host mucosal epithelial cells and interact with a variety of submucosal immune cells (e.g. Dendritic cells (DCs), macrophages, etc.) [87]. As antigen-presenting cells (APCs), DC has the ability to absorb and process antigens, and then present them to T cells, thereby activating adaptive immunity [88]. DC can be regulated by OMVs, Lim Y et al. found that *Porphyromonas gingivalis* OMVs induced the expression of pro-inflammatory cytokines IL-1β, IL-6, IL-23 and IL-12p70 in BMDCs. However, the poor detection of proinflammatory cytokines in *T. denticola* OMVs-induced BMDCs may be attributed to post-translational degradation due to the highly proteolytic nature of the OMVs [89]. OMVs from a variety of pathogens can induce DC maturation and cytokine production. OMVs of *Salmonella typhimurium* stimulated antigen presenting cells in vitro at levels similar to those induced by bacteria, and OMVs-stimulated DC showed increased expression of MHC-II and CD86 and increased production of proinflammatory mediators NO, TNF-α, and IL-12. Moreover, DC maturation induced by OMVs was both TLR4 signaling pathway-dependent and TLR4 signaling pathway-independent, indicating that DC maturation induced by OMVs of *Salmonella typhimurium* containing LPS was not entirely dependent on TLR4 signaling pathway [90]. It has also been found that bactericidal/permeability increasing protein (BPI), as a host immune factor, can not only kill and eliminate Gram-negative bacteria, but also play a role in neutralizing endotoxin [91]. *Neisseria meningitidis* OMVs can promote the presentation of BPI to DC by binding to BPI, and OMVs can also be internalized by DC. These findings suggest that OMVs are able to induce DC maturation and promote antigen presentation.

OMVs can also mediate the occurrence and development of diseases through macrophages. It has been reported that OMVs can regulate macrophages and induce immune responses by binding to their cell surface PRRs [92]. In their study of the effect of *Legionella pneumophila* OMVs on macrophages, Jung AL et al. found that treatment of THP-1 human macrophages with *Legionella pneumophila* OMVs induced a TLR2-mediated proinflammatory response in vitro. Meanwhile, the pre-treatment of THP-1 cells with OMVs prior to infection significantly diminished the replication of Legionella pneumophila within THP-1 cells [93]. OMVs promote macrophages to produce proinflammatory cytokines. For example, the effect of OMVs released by *Hp* SS1 on macrophage RAW 264.7 cells was shown to induce Th2 cell immune response and secrete a large amount of IL-10 and IL-4 [94]. OMVs can activate macrophages to induce adaptive immune responses. It is known [95] that *Porphyromonas gingivalis* is one of the bacterial species most closely associated with periodontitis and that macrophages can mount an immune response against bacteria and their products at an early stage. Macrophages were tested with *Porphyromonas gingivalis* and its OMVs, respectively. It was found that macrophages stimulated with OMVs produced high levels of TNF-α, IL-12p70, IL-6, IL-10, IFN-β and NO. These results indicate that *Porphyromonas gingivalis* and its OMVs have different inflammatory phenotypes on macrophages, and thus have different effects on the development of chronic periodontitis.

OMVs activate adaptive immune responses

The activation of APCs mediated by OMVs serves as the cornerstone for the subsequent engagement of lymphoid cells, encompassing both B and T cells [96]. As an example, Alaniz RC et al. reported that *S. enterica serovar Typhimurium* OMVs activates DC that present *Salmonella* specific antigens, and this antigen presentation activates $CD4^+$ T, which in turn activates B cells to produce *Salmonella* specific antibodies. These antibodies induce protection from subsequent *Salmonella* infection in mice [90]. As another example, *Hp* OMVs regulated heme oxygenase-1 (HO-1) expression through two different pathways in DC, Akt-Nrf2 and mTOR-IKK-NF-κB signaling. HO-1 is important for adaptive immune responses. Moreover, after induction, increased HO-1 expression in DC may modulate the inflammatory response during *Hp* infection [97].

Role of OMVs in chronic infection

In chronic infections, bacteria often evade host immune attack through a variety of mechanisms, including regulating host immune tolerance. Among them, biofilm formation is a survival strategy that promotes bacterial persistence by protecting bacteria from the host immune system and antimicrobial agents. For example, *Hp* continuously regulates the host immune response through derived OMVs, so that the host immune system gradually favors an immune tolerance state and achieves its own long-term survival [98]. Pathogens can also avoid being recognized and cleared by the host immune system through various mechanisms, forming immune escape. A typical example is the effect of *Hp* OMVs on the autophagy process in host cells. Studies have found that *Hp* OMVs can inhibit autophagy in hepatic stellate cells, and this inhibitory effect may be achieved by changing the

expression levels of autophagy-related genes. Autophagy is an important cellular self-protection mechanism capable of removing damaged organelles and invading pathogens. By inhibiting the autophagy process of host cells, *Hp* OMVs effectively reduce the ability of host cells to clear bacteria, thereby increasing the survival of bacteria in the host [99].

Application of OMVs in biotechnology

In recent years, a burgeoning body of research on OMVs has revealed their diverse biological properties and significant potential in various fields, including vaccine development, adjuvant formulation, drug delivery systems, and cancer therapeutics. These studies underscore the versatility and promise of OMVs as cutting-edge tools in biomedical research and clinical applications.

Vaccine applications of OMVs

Vaccines are the clinical application of immunological theory, which activate the immune system of the body, induce specific immune response to pathogens, and reduce the infection rate and prevalence by simulating pathogens without causing related diseases [56]. OMVs have obvious advantages over regular vaccines: (1) OMVs are derived from parental bacteria, which are non-replicable, integrity and stability at different temperatures, and contain a variety of pathogen-related antigens. Regarding the non-replicability of OMVs, we would like to emphasize that this characteristic makes OMVs an ideal vaccine carrier as they do not cause unexpected disease transmission within the host, which is a key factor in their safety over live bacterial vaccines. Although OMVs are non-replicative, they can still trigger a robust immune response because they carry antigens from their bacterial parent strains, which can be recognized by the host's immune system, thereby eliciting an immune response [100]. (2) OMVs are vesicles with a size of 20–400 nm, which can effectively enter the body's lymphatic system and be taken up by antigen-presenting cells, causing the activation of host T cells [101]. To date, the most representative and successful OMVs vaccine is against *meningococcal type B* (*MenB*). This vaccine has successfully fought outbreaks of *MenB*-caused meningitis in Norway, Cuba and New Zealand [102]. In addition, OMVs vaccines against other pathogens, such as *Shigella flexneri* [103] and *Vibrio cholerae* [104], have entered the stage of animal model research. However, safety concerns need to be addressed before this naturally released OMVs can be widely used. It has been found [105] that lipid A, a glycolipid, is an endotoxic component of LPS, which can cause severe and even fatal inflammatory responses in the host. Researchers are addressing this concern through genetic engineering, such as modifying the structure of lipid A in OMVs to reduce endotoxicity. For instance, by deleting or modifying genes associated with lipid A biosynthesis, the endotoxicity of OMVs can be reduced while maintaining their immunogenicity. An example is the deletion of the arnT gene in *S. Gallinarum*, which reduces the cationic 4-aminoarabinose (Ara4N) on lipid A, altering its charge and stoichiometric properties, and directly reducing endotoxicity [106]. In recent years, with the development of biotechnology engineering, great progress has been made in solving such problems. The engineering of OMVs is not limited to lipid A modification; it also includes the targeted expression of heterologous proteins and peptides to the outer membrane or periplasm of an OMV-producing host strain through recombinant DNA technology and synthetic biology techniques [107]. This provides more flexibility and possibilities for vaccine development.

Adjuvant application of OMVs

Adjuvants can not only reduce the vaccine dose and promote the immune response induced by immunogen, but also regulate the body's immune response and play a key role in the formation of immune memory. Conventional vaccines are composed of attenuated or inactivated pathogens, which pose potential risks to the body's health. Adjuvants can overcome these deficiencies and produce strong immune protection [108], and can also improve immune efficacy, especially in newborns and the elderly [109]. The adjuvant properties of OMVs have been confirmed, including the inclusion of multiple PAMPs, non-replication mimicry of parental bacteria and induction of danger signals [110]. Timothy Prior et al. [111] found that OMVs were more effective than heat-inactivated and attenuated live bacteria in driving DC activation in vitro and in vivo. The antibody and B cell responses to codelivery of ovalbumin induced by OMVs as adjuvants were much greater than those induced by the adjuvants alum and CpG DNA. These results demonstrate that vaccines using OMVs as adjuvants can elicit stronger cellular and humoral immune responses. These results provide an experimental basis for the development of next-generation OMVs adjuvants. However, there are still many challenges in the large-scale production of OMVs adjuvants, such as the consistent production of OMVs and the high cost of OMVs adjuvant development. Therefore, the improvement and improvement of biotechnology is particularly important.

OMVs serve as transport vehicles

OMVs have great potential and advantages in clinical application as drug delivery vehicles. OMVs can target drugs to specific target cells and targets, and maintain drug activity during transport. There are two ways to load drugs to OMVs: in vivo and in vitro. In vivo loading is the loading of drugs into OMVs during the budding

process. It has been found [112] that when *Pseudomonas aeruginosa* PAO1 is treated with gentamicin, it releases membrane vesicles containing gentamicin and PG hydrolase, making the membrane vesicles bactericidal. However, in vitro loading is the loading of drugs into OMVs that have been isolated. A pioneering bioengineering technique [113] that utilizes bioderived vesicles as nanomedicines to achieve cell-specific drug delivery. In this study, a mutant *Escherichia coli* strain that exhibits lower endotoxicity to human cells and produces OMVs displaying a human epidermal growth factor receptor 2 (HER2)-specific affinity in the membrane as a targeting ligand was engineered. In animal models, systemic injection of siRNA-packaged OMVs caused targeted gene silencing and induced very significant tumor growth regression.

Over the years, with the application of OMVs production, characterization, targeting strategies and cargo loading technology, the delivery of therapeutic drugs based on OMVs can ensure the delivery of therapeutic agents across the body's biological barriers, such as impermeable biological barriers such as the blood-brain barrier, improve biocompatibility, increase solubility, metabolic stability and target specificity. Thus improving the efficacy of loading therapeutic agents [114].

Application of OMVs in cancer therapy

The application of OMVs in cancer treatment has been a research hotspot. The development of anti-tumor vaccines against OMVs is based on the fact that OMVs contain a variety of immune stimulatory molecules. Host immune cells can recognize and uptake OMVs to induce the activation of the immune system. Moreover, OMVs as nanoscale particles can continuously accumulate in tumors and induce local immune responses. Researchers use genetic engineering techniques to express foreign proteins in the vesicle cavity or on the surface of the vesicle membrane to trigger the desired immune response. Alternatively, microRNA and other substances can be injected into the vesicle cavity to achieve the expected immune effect or silence related genes and eventually kill cancer cells, while reducing the damage of side reactions to the body [115]. Recent studies have reported that OMVs can specifically target and accumulate in tumor tissues, promote the production of anti-tumor cytokines CXCL10 and IFN-γ, and effectively induce long-term anti-tumor immune response. However, most anti-tumor vaccines for OMVs are still in the stage of clinical trials [116], and there are still many difficulties to be solved urgently. A large number of basic and clinical studies are still needed before they are approved for marketing and large-scale clinical application.

Conclusions and prospects

In summary, it has been established that OMVs play an important role in the growth and survival of Gram-negative bacteria and in bacterial-bacterial and bacterial-host interactions. The formation of OMVs is a common, regulated process in Gram-negative bacteria, in which envelope proteins, including membrane binding proteins, lipids, immune stimulatory molecules and DNA and RNA, are secreted in concentrated form. These vesicles have great potential in vaccine development, as they can serve as vectors to stimulate an immune response in the host. An in-depth study of the biosynthetic mechanisms of OMVs and the design of vaccines enriched with specific antigens are essential for the development of more effective vaccines, which not only improves our understanding of these bacterial components, but also provides new strategies for the prevention and treatment of bacterial diseases.

Author contributions

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Declarations

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