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Outer membrane vesicles (OMVs) and their therapeutic potential as anti-infectious agents

Mariana Ottaiano Gonçalves [a,b], Paula Maria Pincela Lins [a,c], Giulia Kassab [a,d], Vanderlei Bagnato [a,e], Valtencir Zucolotto [a,*]

[a] São Carlos Institute of Physics, University of São Paulo, São Carlos, SP, Brazil
[b] Federal University of São Carlos, São Carlos, SP, Brazil
[c] Biomedical Research Institute (BIOMED), Hasselt University, Agoralaan 3590 Diepenbeek, Belgium
[d] Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada
[e] Biomedical Engineering Department, Texas A&M University, College Station, USA

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ABSTRACT

Outer membrane vesicles (OMVs) derived from Gram-negative bacteria have emerged as promising therapeutic agents for combating infectious diseases. These small, spherical structures carry diverse cargo molecules, including virulence factors, antigens, and immunomodulatory molecules. OMVs derived from pathogenic bacteria can be engineered to deliver antimicrobial peptides, antibiotics, or specific antigens, eliciting targeted immune responses against bacterial pathogens. Similarly, OMVs can deliver viral antigens, facilitating immune recognition and clearance of viral infections. Furthermore, OMVs can be engineered to encapsulate antifungal agents or fungal antigens, enabling targeted delivery and immune activation against fungal pathogens. The cargo-carrying capacity and immunostimulatory properties of OMVs make them valuable tools for developing effective treatments against infectious diseases. This review also discusses the challenges and future directions in applying OMVs as therapeutic agents, highlighting the need for further research and development to harness their full potential in clinical applications. Overall, OMVs represent a promising avenue for developing novel therapeutic strategies against infectious diseases, offering targeted and immunomodulatory strategies for combating bacterial, viral, and fungal infections.

1. Introduction

Extracellular vesicles are produced by both eukaryotic and prokaryotic cells.Among these, exosomes originating from eukaryotes play vital roles in intercellular communication, diagnostics, and drug delivery [1]. In prokaryotes, these vesicles are known as membrane vesicles, and for Gram-negative bacteria, precisely, they are termed outer membrane vesicles (OMVs) [2]. OMVs are known for their roles in cell-to-cell communication, biofilm formation, pathogenesis, and stress responses [3].

OMVs are small structures released by bacteria as part of their normal growth process, ranging from approximately 20 to 250 nm. They are crucial players in bacterial survival, evolution, and pathogenesis [4]. Research has unveiled their role in biofilm formation, virulence factor transfer, and DNA transmission among bacteria, highlighting their importance in bacterial communities and ecosystems [5]. OMVs have also been found to influence host immunity, with studies revealing their involvement in supporting immune system maturation and facilitating infection and inflammation in the host. Broadening our understanding of OMV interactions with other bacteria and host cells opens avenues for OMV-based therapeutics, including vaccine design and genetic engineering-based treatments, promising novel approaches in the fight against infectious diseases [6,7].

This review provides an overview of bacterial OMVs, focusing on their biogenesis, functions, applications, and isolation methods. We explore the current understanding of OMVs, cargo composition, formation, and their roles in bacterial physiology and pathogenesis. We discuss the potential applications of OMVs mainly in vaccine development and drug delivery. Additionally, we highlight various methods available for the isolation and purification of OMVs, providing insights into their advantages and limitations. Understanding the mechanisms of OMVs production, cargo selection, and their impact on bacterial and

* Corresponding author.
E-mail address: zuco@ifsc.usp.br (V. Zucolotto).

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host systems will pave the way for innovative approaches to fight bacterial infections and improve biotechnological advancements.

2. Outer membrane vesicles (OMVs)

a. Composition

The envelope of Gram-negative bacteria contains two membranes – the outer membrane and the cytoplasmic membrane - and a layer of peptidoglycan (PG) as a periplasmic space between them (Fig. 1). The cytoplasmic membrane is made of a phospholipid bilayer responsible for the osmotic barrier, electrons, enzyme delivery, and lipid biosynthesis [8]. Similarly, the outer membrane is composed of two leaflets: the exterior one made of lipopolysaccharides (LPS), composed of lipid A portion, oligosaccharides, and O-antigen; and the interior one made of phospholipids. The lipid A portion is stabilized by divalent cation binding and serves as lipid anchoring of fatty acids [9]. Furthermore, crosslink interactions help the envelope to be stable. Some important crosslinks are the non-covalent bond between PG and the outer membrane protein A (OmpA), the also non-covalent interaction between PG and the peptidoglycan-associated lipoprotein (Tol-Pal) complex that crosses the two membranes, and the periplasmic space; and the covalent bond of lipoproteins in the outer membrane and PG [10].

OMVs' lipid bilayers comprise sphingolipids, glycerophospholipids, and serine-dipeptide lipids. Sphingolipids constitute over half of the total lipid content, notably ethanolamine phosphoceramide (EPC) and inositol phosphoceramide (IPC) as the predominant types. Sphingolipids are crucial polar lipids with diverse structures, playing essential roles in maintaining the cell membrane balance and participating in various cell signaling processes [11]. Glycerophospholipids comprise a polar head group attached to a glycerol backbone and up to two fatty acyl chains. They play an important role in the survival of bacteria and their interaction, providing stability, fluidity, and permeability [12,13].

OMVs are enriched in outer membrane (OM) elements and periplasmic proteins, and their cytoplasmic content can vary depending on their biogenesis route [14]. Integral membrane proteins, lipoproteins, and less frequently, peripherally attached soluble proteins are standard components across OMVs variants. In addition, affected by environmental stimuli, different genes are expressed in order to produce important molecules that will deal with the threatening environment, and may be packed into OMVs [15]. For example, *Helicobacter pylori* OMVs enriched with the catalase KatA were more protective against oxidative damage through H_2O_2 hydrolysis than the bacteria's outer membrane alone [16]. In high temperatures, OMVs remove misfolded proteins and cell debris from inside the bacteria, increasing its chance of survival [17]. Because of inheriting features of the parental bacteria, OMVs can present the binding site for phage infection, so the probability of infecting the bacteria is lower. Also, OMVs can capture some phage particles before the replication happens [18].

It's also postulated that these vesicles have heterogeneous populations that need further exploring. The variability in size and composition among bacterial extracellular vesicle populations is becoming increasingly evident [19]. This diversity is attributed to distinct biogenesis pathways and influenced by external factors such as growth stage, medium composition, and sample collection methods [20]. Moreover, the concentration and composition of virulence factors appear to differ in various fractions of the same bacteria-derived OMVs [21]. These findings highlight the impact of multiple factors on OMVs heterogeneity, emphasizing its importance due to potential differences in cargo composition and target host cells, leading to distinct biological effects [22].

b. Biogenesis

Different mechanisms describe OMVs biogenesis, and each gives rise to OMVs with distinct structures and contents, which ultimately determine their functions [2]. OMVs can be formed either by lytic pathways (during cell lysis and self-coiling of broken membrane fragments) or nonlytic pathways (blebbing of the outer membrane, without cell lysis). Explosive cell lysis is triggered by genotoxic stress when genes are damaged by physical and/or chemical conditions, activating the expression of endolysins that degrade the bacterial peptidoglycan layer. Then, the broken fragments of bacterial membrane reorganize into vesicles with random protein cargo [10,23]. On the other hand, for

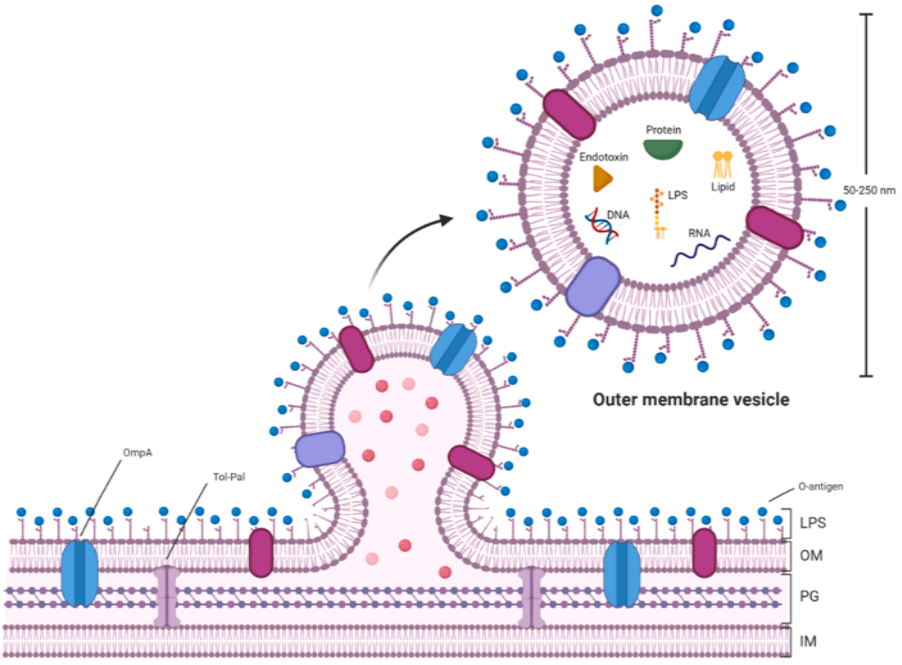

Fig. 1. Composition and origin of OMVs. Shown is the cell envelope of Gram-negative bacteria, consisting of the outer membrane (OM), with an exterior leaflet of lipopolysaccharide (LPS), and the inner membrane (IM), made of a phospholipid bilayer. In the periplasmic space, between the two membranes, we can find peptidoglycan (PG) layer and periplasmic proteins. Created with biorender.com.

OMVs that bud from the outer membrane, biogenesis may occur due to peptidoglycan fragments or misfolded proteins that accumulate in the periplasmic space, creating a turgor pressure and displacing crosslinks between lipoproteins of the outer membrane and the underlying PG layer; or changes in LPS composition which cause dissociation of the outer and inner membrane in specific zones. The consequence is an asymmetric expansion of the outer leaflet in relation to the inner leaflet, contributing to the detachment of the outer membrane from the cell wall and consequent pinching into a vesicle [10].

One example of a non-lytic pathway is based on a self-produced small molecule found in *Pseudomonas aeruginosa*, the *Pseudomonas* quinolone signal (2-heptyl-3-hydroxy-4-quinolone; PQS). Aside from its other functions, PQS is one of the multiple signaling molecules responsible for delivering virulence factors, facilitating cell-cell communication, phenomenon known as quorum-sensing, and modulating host immune responses [24]. The strongly hydrophobic interaction of PQS with the lipid A portion of the LPS layer of the outer membrane weakens its affinity with the phospholipid layer by sequestering divalent cations such as Mg^{2+} and Ca^{2+}, causing an anionic repulsion between neighboring LPS molecules. This repulsion generates an asymmetric expansion of the outer leaflet compared to the inner leaflet, contributing to membrane curvature and subsequent pinch-off [4]. The importance of PQS was investigated in studies where cell mutants lacking PQS-specific receptors were shown to produce a reduced number of vesicles. Besides, the addiction of PQS receptors in these mutant cells and PQS-null cells (like red blood cells) restored its production [10].

c. *Function*

OMVs play a multifaceted role in bacterial survival by contributing to communication, virulence, stress response, secretion, and biofilm formation. Their ability to transport diverse molecules makes them essential for bacterial adaptation, survival, and successful colonization in various ecological niches, including both environmental and host environments. They have an important role against antibiotics, acting like traps that bind and absorb them. Besides, OMVs are essential to transport pathogenic toxins, virulence factors, resistance genes and degradative enzymes [4,25].

OMVs enhance bacterial resistance to antimicrobials by facilitating the transfer of antibiotic resistance enzymes, such as carbapenemases, β-lactamases and proteases, between bacteria and acting as decoys that bind to or absorb antimicrobial agents, reducing their effectiveness [26]. For example, OMVs from carbapenem-resistant *Klebsiella pneumoniae* can transfer the $_{bla}$KPC-2 gene to susceptible strains, rendering them resistant to imipenem and meropenem. Although this transmission did not occur in *Escherichia coli*, the findings indicate that OMVs are crucial in spreading antibiotic resistance and point to a potential therapeutic target to contain resistant infections [27]. On the other hand, another study showed that OMVs from carbapenem-resistant *K. pneumoniae* degrade the antibiotic imipenem, protecting *P. aeruginosa* against antibiotic treatment. Furthermore, at low concentrations, these OMVs favored the emergence of resistant subpopulations due to mutations in the OprD gene, without the need for acquisition of external resistance genes [28].

OMVs carry and deliver virulence factors such as toxins, enzymes, and adhesins. These factors can enhance the pathogenicity of bacteria by interacting with host cells and modulating the host's immune response [25]. Virulence factors like intimin, needed for bacterial adhesion to host epithelial cells, and toxins such as Shiga and cholera toxin have been demonstrated to be present respectively in *E. coli* and *Vibrio cholerae* OMVs, and available for horizontal gene transfer. These elements can also be delivered to the host by pathogenic species' OMVs, allowing pathogenesis establishment through antimicrobial resistance, increased virulence, modulation of the immune response, and cell invasion, adherence, and damage [26].

This ability to share a specific function with the microbial community can also be observed for nutrient acquisition. OMVs can contain enzymes that degrade extracellular compounds, facilitating the breakdown of complex nutrients into simpler forms that can be more easily absorbed by bacteria [29]. Furthermore, they can transport various nutrients, including lipids, proteins, and nucleic acids, essential for bacterial growth and survival. These vesicles facilitate the transfer of these nutrients across the bacterial outer membrane, ensuring that bacteria can access the necessary resources even in nutrient-poor environments [30]. For instance, iron is a vital nutrient for many bacteria but is often limited in the environment. OMVs can play a role in iron acquisition by transporting iron-binding proteins and siderophores, which are molecules that bind and transport iron [31]. A study demonstrated that OMVs are crucial in acquiring and transporting iron by *Cupriavidus necator*. The bacterium uses the type VI secretion system (T6SS) to release the TeoL protein, which binds to the LPS of OMVs, allowing its capture by specific receptors on the cell membrane (CubA and CstR). When recruited, these OMVs contain iron and provide this essential element to *C. necator*, promoting its growth in conditions of low iron availability. Furthermore, the study suggests that bacteria may widely use this OMV recruitment mechanism to overcome nutritional limitations and obtain competitive advantages in the environment [32].

OMVs can easily interact with other species to work together to survive and facilitate the biofilm formation and the quorum sensing process. In the same way that *P. aeruginosa* has the PQS molecule, other species have their signal pathways to enable communication inside the bacterial community, such as C16-HSL in *Pseudomonas denitrificans* and the CAI-1 in *V. cholerae.* OMVs collect signal molecules released from the cell and deliver them to a target bacterium, promoting changes in gene expression that can benefit all the species inside the microbiome environment, including those arranged in a biofilm matrix [33]. OMVs are essential for bacterial adhesion in biofilms for the same or other strains. For example, the adhesion of multiple oral microorganisms in dental plaque was enabled by *Porphyromonas gingivalis* OMVs. Moreover, OMVs from *H. pylori* contain a protein responsible for cell-cell and cell-surface junctions, promoting the aggregation of the cells in the matrix [26].

OMVs of different species can harm both Gram-positive and Gram-negative. Differences in peptidoglycan composition and the enzyme cargo of OMVs help bacteria distinguish between self and non-self populations and target non-self bacteria for destruction, making the process more specific and compelling. Besides virulence factors and toxins, OMVs can also encapsulate antimicrobial factors, such as degradative enzymes and bacteriocin, leading the bacteria to death [34]. OMVs from *P. aeruginosa* have been shown to inhibit the growth of *Acinetobacter baumannii*, a Gram-negative bacterium, by delivering virulence factors and other bioactive molecules [35]. OMVs from *Lysobacter* secrete endopeptidase L5, capable of degrading competing Gram-negative species. For example, *P. gingivalis* can eliminate even competing biofilms, composed of *Streptococcus gordonii* through OMV-derived gingipains [7].

3. Interaction with the immune system

OMVs are crucial in modulating the immune system by transporting virulence factors, such as lipopolysaccharides, proteins, and toxins, to host cells and immune cells. They can activate pattern recognition receptors (PRRs) on immune cells, triggering excessive inflammatory responses, leading to immune exhaustion or dysfunction [29]. In addition, OMVs can induce apoptosis in immune cells, modulate immune checkpoint pathways, interfere with antigen presentation by dendritic cells, and influence cytokine secretion, promoting immune response polarization and contributing to immunosuppression and immune dysregulation [36,37].

As OMVs have a structure that helps the development of pathogenesis and survival of the parental bacteria, they can interact with different cells of the host's immune system [33,38]. Macrophages, neutrophils, and dendritic cells (DCs) express Pattern Recognition Receptors (PRRs)

that directly interact with microbial structures like LPS, PG, Lipid A, DNA, RNA, and toxins, found in OMVs as well. There are two main types of PRRs able to detect microorganisms: toll-like receptors (TLR) are transmembrane proteins capable of recognizing pathogen-associated molecular patterns (PAMPs) from both Gram-positive and Gram-negative bacteria; and NOD-like receptors that are located in the cytoplasm of host cells and bind mainly to dipeptides present in the bacterial PG layer [39]. With this interaction, the activation and signaling of the innate immune system starts, by releasing inflammatory cytokines, chemokines and antimicrobial molecules [40]. Many studies investigate the interactions between OMVs and different cells from the innate immune system such as macrophages [41,42], neutrophils [43] and DC [44].

LPS from *E. coli* OMVs and TLR4 from endothelial lung cells increased the stimulation of NF-kB leading to the expression of CXCL1, IL-8, TNF-α, and CD54 in mice [43]. Likewise, OMVs from commensal and probiotic *E. coli* induced the production of proinflammatory interleukins such as IL-6 and IL-8 by activating NOD-1 signaling pathways in intestinal epithelial cells [39]. Furthermore, genes responsible for the production of OMVs were effective in inhibiting the complement system and in the consequent survival of *Salmonella enterica* in host cells. The PagC protein can bind and inhibit the C3 component of the complement system, through the recruitment of the complement-inhibiting Factor H. Since the *pagC* gene plays an important role in OMVs' biogenesis, this mechanism allows the generation of vesicles enriched with the PagC protein, impairing in an upregulated way the bactericidal action of the complement system against *Salmonella* spp [45].

Sphingolipids (SL) present within *P. gingivalis* OMVs suppressed the inflammatory response in periodontal disease, in a contact-dependent manner. Specifically, SL-null mutants showed a hyperinflammatory response, with high production of TNF-α, IL-1β, IL-6, and IL-8. SL-containing OMVs changed their protein cargo and induced a mild inflammatory response in macrophages. SL-containing OMVs reduced the production of gingipains, important proteinases that mediate pathogenesis, and decreased the expression of the important innate receptor TLR2 in macrophages [46]. Macrophages are crucial both in innate and adaptative immune response, since they are great phagocytes, participate in antigen-presentation for T cells, and release cytokines that activate other cells [47]. OMVs also play an important role in DC recruitment, activation and maturation, essential for antigen presentation and thus the occurrence of and efficient adaptative immune response [48]. OMVs extracted from intestinal commensal *Bacteroides vulgatus* induced a DC tolerance, in a state of semi-maturation, due to the activation of TLR2 and TLR4, which leads to a reduced expression of pro-inflammatory cytokines and important molecules for T cell activation and maturation. By mediating immune-system silencing properties, colitis and other intestinal diseases can be prevented in a non-cellular-dependent manner [49]. Furthermore, neutrophil recruitment was impaired by the reduced expression of its chemoattractant β-defensin hBD-2 caused by *Moraxella catarrhalis* OMVs in A549 epithelial cells. In case of infection, the migration of neutrophils to the lung tissue is a significant step for the microbe-killing strategy, cell activation, and release of pro-inflammatory cytokines [50]. Similarly, OMVs can interact with components of the adaptative immune system due to the ability to recognize millions of different molecules and create a "memory" to generate a faster response to repeated exposures; specialization for clearing different types of pathogens [7].

OMVs have also been explored to develop new vaccines and anti-infectious treatments. For example, OMVs are fundamental in the activation of the immune system from vaccines such as pertussis, in which the immunization through the intranasal route increases the mucosal thickness, activates the response of antibodies type IgA and IgG, of memory B cells resident in the lung and of Th17-related cytokines, which avoids the colonization of the bacteria *Bordetella pertussis* in lungs, trachea and nose, the mayor transmission routes of the disease [51]. In another study, OMVs from *E. coli* were loaded with the essential fibroblast growth factor (BFGF) through the genetic recombination technique. It enabled the production of autoantibodies anti-BFGF, which were essential in tumor suppression, tumor cell apoptosis induction, tumor immunologic barriers reversion, and promotion of specific cytotoxic T lymphocytes. OMVs can also be used as a delivery system in vaccines to induce the production of antibodies against autoantigens, helping the improvement of antitumor therapy [52].

4. OMV applications against infectious diseases

OMVs have gained attention at first for being used as adjuvants against infectious diseases, including bacterial, viral, and fungal infections. This is primarily due to their ability to stimulate the immune system through PAMPs on their surfaces [53]. Further studies showed that they can also be used as therapeutic agents, since OMVs can be engineered to carry specific cargos, including drugs and small interfering RNA (siRNA), providing a novel platform for targeted drug delivery [54]. OMVs as delivery systems hold promise in fighting infectious diseases (Table 1) by harnessing their cargo-carrying capacity and immunostimulatory properties, opening new avenues for developing effective treatments [19]. Depending on the purpose of the application, one type of bacteria can be more beneficial than others. For example, *P. aeruginosa* OMVs facilitate gene transfer between bacteria which can be used as a carrier of antibiotics for bacterial infection [55]. *E. coli* can be of added value to tackle a viral infection as their OMVs can be easily engineered to express viral receptor to inhibit viral replication [56]. In the case of fighting a fungi infection, *Lysobacter enzymogenes* OMVs can be used as fungicides as their parental bacteria has a naturally fungicidal behavior [57]. Depending on the location of the infection, some types are more prefered than others. For example, probiotic-derived OMVs from bacteria like *Lactobacillus* offer immune tolerance and compatibility with mucosal surfaces, making them ideal for targeting fungal and bacterial infections in the gut [58,59]. The type of infection and its location will determine the bacterial species and OMV engineering strategy used, these are detailed below. In the case of bacterial infections, engineered OMVs can be loaded with antimicrobial peptides, antibiotics, or specific antigens to induce an immune response against the pathogen. Similarly, OMVs can deliver viral antigens, triggering an immune response to combat viral infections. Furthermore, OMVs can be engineered to encapsulate antifungal agents or fungal antigens, enabling targeted delivery and immune activation against fungal pathogens [60,61].

a. Bacterial targets

An OMVs-based vaccine is available for human immunization against serogroup B meningococcal disease. The 4-component meningococcal serogroup B (MenB) vaccine, known as 4CMenB or Bexsero, is the first broadly protective, protein-based MenB vaccine to be licensed [76]. It contains recombinant proteins and OMVs and has been approved for use in Europe, Australia, and other countries. Clinical trials have demonstrated its immunogenicity against a range of serogroup B strains, and global evidence confirms its effectiveness and impact, with vaccine effectiveness ranging from 71–95 % against invasive MenB disease [77]. OMV-based vaccines have shown efficacy, tolerability, and safety, significantly reducing disease rates observed in vaccinated populations [53].

Therefore, studies have been carried out to expand the applications of this kind of vaccine platform. For example, OspA is a lipoprotein found on the surface of *Borrelia burgdorferi*, the bacterium responsible for causing Lyme borreliosis, transmitted by *Ixodes* ticks. OMVs derived from genetically modified *Neisseria meningitidis* could express OspA as a heterologous antigen. Mice were challenged after vaccination with OspA-OMV constructs to determine whether the induced antibodies could protect against *B. burgdorferi* infection. This new strategy was compared to the classic vaccination of OspA with aluminum hydroxide ($Al(OH)_3$)

Table 1
Outer membrane vesicles used for prevention and therapy against different microorganisms.

Target organism	Target Specie	OMV Source	Pre-Modification	Post Modification	Application	Ref
Bacteria Virus	*Acinetobacter baumanii*	*Acinetobacter baumanii*	–	Vaccine assembly	Intranasal vaccine candidate and immunization	[62]
	Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	–	Formulation with aluminium phosphate adjuvant	Intramuscular vaccine candidate and immunization	[63]
	Acinetobacter baumanii	*Acinetobacter baumanii, Escherichia coli* and *Salmonella Enteriditis*	Treatment with LysP53	Vaccine assembly	Intransal and intramuscular vaccine candidate and immunization	[64]
	Borrelia burgdorferi	*Neisseria meningiditis*	Expression of OspA	Vaccine assembly	Vaccine candidate and immunization	[65]
	Bordetella pertussis	*Bordetella pertussis*	PRN deficiency	Vaccine assembly	Comparative proteomic and immunogenic study between samples obtained from planktonic and biofilm cultures	[66]
	Streptococcus mutans	*Burkholderia thailandensis*	–	–	Antibacterial activity against planktonic and *S. mutans* biofilm	[67]
	Escherichia coli, Enterobacter cloacae and *Klebisiella pneumoniae*	*Escherichia coli*	–	Complex with cholera toxin	Intranasal vaccine candidate and immunization	[68]
	Influenzavirus A (H1N1) + MERS-CoV	*Escherichia coli*	Transformation with H1-type haemagglutinin (HA) from H1N1pdm09 + receptor binding domain (Rrbd) from MERS-CoV	Vaccine assembly	Vaccine candidate and immunization	[69]
	Influenzavirus A (H1N1 pHINI, H1N1, PR8 and H5N2)	*Escherichia coli*	Lipid A structure moiety of lipopolysaccharide	Vaccine assembly	Intranasal vaccine candidate and immunization	[70]
	SARS-CoV-2	*Neisseria meningiditis*	–	Complex with recombinant receptor-binding domain (rRBD) from SARS-CoV-2 Spike protein	Intranasal vaccine candidate and immunization	[71]
	SARS-CoV-2	*Vibrio cholerae* and *Escherichia coli*	Deletion of one functional secondary lipid A acyltransferase, OmpA, subunits A and B in *V. cholerae* and heat-labile enterotoxin subunit in *E. coli*	Complex with recombinant receptor-binding domain (rRBD) from SARS-CoV-2 Spike protein	Vaccine candidate and immunization	[72]
	SARS-CoV-2	*Neisseria meningiditis*	Streptomycin resistant	Complex with recombinant Spike protein	Intranasal vaccine candidate and immunization	[73]
Fungi	*Fusarium subglutinans* and *Saccharomyces cerevisiae*	*Lysobacter enzymogenes*	–	–	Evaluation of antifungal activity	[74]
	Candida albicans	*Salmonella enterica* serovar Typhi	–	–	Evaluation of the effect of OMVs in polymyxin B and limonene resistance	[23]
	Candida albicans	*Moraxella catarrhalis*	–	–	Evaluation of the effect of OMVs in polymyxin B and fluconazole (Diflucan) resistance in yeasts	[75]

Abbreviations: sRNA: small RNA; Omp: outer membrane protein A; MERS-CoV: Middle East Respiratory Syndrome Coronavirus; SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2.

and to a combination of soluble OspA and empty OMVs. The authors determined that vaccination with the OspA-OMV construct is able to induce a robust humoral immune response, comparable with the current alternatives. They showed that OMVs offer significant adjuvant power comparable to $Al(OH)_3$ to provide excellent protection against Lyme borreliosis [65].

OMVs derived from *E. coli* and three Multi-Drug Resistant Gram-Negative Organisms (MDRGNO) strains, known for causing severe infections in humans, were administered intranasally to mice [68]. This administration was carried out both with and without the use of cholera toxin as an adjuvant. The intranasal vaccination with OMVs successfully triggered robust humoral immune responses specific to the corresponding MDRGNO strains in mice. Importantly, no indications of local or systemic toxicity were observed. Intranasal vaccination with OMVs could potentially serve as a quick, simple, and safe approach to enhance antibody levels against Gram-negative bacterial strains that adhere to the outer surface of viable bacteria. This strategy can potentially improve patient survival and aid in preventing infections caused by problematic Gram-negative bacteria [68].

Another improved vaccine candidate against pertussis has been studied, using OMVs derived from a biofilm-forming strain of *B. pertussis* (OMVbiof). This approach represents a third-generation vaccine, in which the strain rapidly releases OMVs and overexpresses specific proteins that serve as antigens. The vaccine demonstrated greater immunogenicity compared to OMVplank (OMVs derived from planktonic bacteria), elicited a strong T-cell response, and conferred long-lasting immunity [78].

OMVs can also act against bacterial biofilms, disrupting their integrity, inhibiting their growth and potentially eradicating [67]. *Burkholderia thailandensis* OMVs are effective against several species of

bacteria and fungi. Therefore, Wang et al. (2021) tested the antibiofilm activity of *B. thailandensis* OMVs against planktonic and pre-formed *Streptococcus mutans* biofilms. The treatment significantly reduced cell viability, overall biomass and biofilm integrity in a dose-dependent response. This activity was further improved when the antibiotic gentamicin was delivered, acting synergistically [67].

b. Viral targets

The Spike protein of SARS-CoV-2 is linked to OMVs extracted from the genetically-edited *N. meningitidis*, by anchoring it to an LPS-binding peptide sequence (mCramp). The study was conducted in rats and hamsters, comparing intranasal and intramuscular administration. The animals were protected from viral replication after two doses of the vaccine, showed no lung damage or adverse reactions, and a strong humoral immune response was induced. Another study compared OMVs of *V. cholerae* and Enterotoxigenic *E. coli,* genetically modified for the lack of enterotoxins, decorated with the receptor binding domain (RBD) of the Spike protein of SARS-CoV-2. The intranasal administration promoted the induction of a robust immune response against OMV components and a large number of functional antibodies against the Spike protein [72].

There are several studies that demonstrate the ability of OMVs to increase the protection of the immune response induced by existing vaccines [70]. A bivalent vaccine was developed against Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and Influenza A virus (H1N1). An *E. coli* strain was modified to express the H1-type haemagglutinin (HA) of the H1N1 and the receptor binding domain (RBD) of the MERS-CoV virus. These expressed antigens were incorporated into the released OMVs. The antigen-specific response was synergistically enhanced by the cellular activation triggered by LPS, resulting in a robust immune reaction in mice and high levels of antibodies against both viruses [69].

A vaccine candidate for the Zika Virus was studied using *N. meningiditis* OMVs added to contaminated *Aedes albopictus* cells, which allows the fusion of the virus and the vesicle to be used as in vaccines (Fig. 2). With this fusion, viral particles were added with the antigens present in OMVs, triggering a robust immune response of the host. Moreover, the authors measured the response in vaccinated mice through the production of specific antibodies, the presence of neutralizing antibodies, and the expression of inflammatory chemokines. The immunization trials showed a reduced number of viral particles, and an increased number of cytokines, chemokines and antibodies produced, indicating a stronger immunization. The success in producing antibodies and the ability of soro-neutralization indicated a very efficient OMVs vaccine formulation against the virus [79].

c. Fungal targets

Few studies have reported the use of OMVs against fungal diseases. OMVs extracted from bacteria that naturally express fungicidal molecules can inherit this characteristic and also show antifungal activity [74]. *Lysobacter enzymogenes* C3 can produce a complex called heat-stable antifungal factor (HSAF), with some antibiotic compounds also identified in OMVs extracted from this bacterium. The antifungal activity was tested against *Saccharomyces cerevisiae* and *Fusarium subglutinans* and showed a significant ability to inhibit fungal growth. After heat treatment, the activity remained intact, while HSAF-null mutants showed no fungicidal ability [74].

Bacterial OMVs can also protect yeasts from host cells and increase their virulence, explaining the dynamics of mixed infections and how these interactions can act synergistically. Marchant *et al.* tested whether OMVs derived from different *S. enterica* serovar Typhi mutants could protect the bacteria from the action of the antimicrobial polymyxin B. Similarly, they decided to verify if this action was confirmed for other agents that act on microbial membranes. Only the presence of the OMVs was enough to increase the MIC value of limonene, tested against *Candida albicans*, protecting the yeast from the oxidative stress induced by this monoterpene [23]. In another study, *M. catarrhalis* OMVs decreased the susceptibility of *C. albicans* to antimicrobials such as polymyxin B and fluconazole (Diflucan), inhibiting their fungicidal activity against pathogenic yeasts. These results show how the interactions of different microorganisms collaborate for the survival and/or worsening of the infection in the host. Future studies are required to develop new agents preventing and treating co-infections [75].

5. Strategies for application: isolation and characterization

Many techniques for isolation, purification, and characterization of OMVs have been developed to allow the exploitation of their properties in potential applications. Minimum Information for Studies of Extracellular Vesicles (MISEV) 2023 is the third iteration of the guidelines, providing recommendations and guidance for extracellular vesicles (EV) -related studies. Published by the International Society for Extracellular

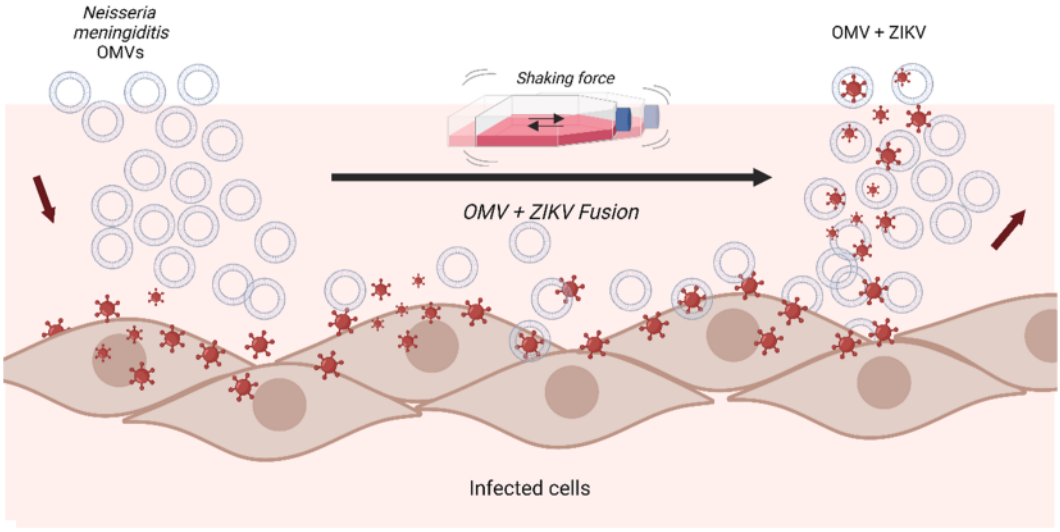

Fig. 2. Schematic representation of candidate vaccine production. *N. meningiditis* OMVs (gray circles) are added to infected cells of *Aedes albopictus.* The mechanical shaking force promotes the fusion of Zika Virus particles (red circles) and OMVs. These new particles were used in a vaccine candidate and immunization trials in mice demonstrated robust antibody production and activation of cellular immune responses [79]. Created by BioRender.com.

Vesicles (ISEV), these guidelines broadly cover the nomenclature, pre-processing variables, separation, and characterization of EVs (that include OMVs), as well as *in vitro* and *in vivo* analysis of EV release, uptake, and functions [80].

a. Bacterial culture and OMV isolation

According to MISEV 2023 (MISEV23), some general recommendations can be made despite the high diversity among bacteria and bacterial extracellular vesicles. To extract OMVs naturally produced by bacteria, the cell supernatant of bacterial cultures should be processed without using detergent cell disruption techniques. Also, culture conditions significantly influence OMVs yield and composition. Considerations such as media composition, oxygenation, culture format (*e.g.*, standing, shaking, bioreactor), and growth phase are crucial and should be reported [80]. The isolation process starts with bacterial culture centrifugation, followed by sterile filtering to eliminate the remaining microorganisms from the supernatant. Due to low OMVs concentrations in the medium, pre-concentration methods like precipitation and ultrafiltration can be employed, with final OMVs sedimentation achieved through high-speed centrifugation [81].

There are several methods for isolating OMVs, such as differential ultracentrifugation, density gradients, filtration, chromatography and immunoisolation. However, methods like ultracentrifugation and filtration may co-isolate unwanted extracellular components like flagella, fimbriae, pili, and large protein complexes or aggregates. Therefore, the MISEV23 suggests combining different isolation methods is more effective in obtaining OMVs with contaminants-free preparations than using any single method alone [82].

b. Composition and characterization

Several factors can alter OMVs' production, characteristics, and biological properties, including nutrient availability, stress conditions and growth state [81]. Therefore, the characterization of OMVs is essential to ensure rigor and reproducibility in the field. The recommendations from MISEV23 cover both qualitative and quantitative methods for characterizing OMVs. Quantitative techniques such as total protein or lipid quantification, protein markers, and nanoparticle tracking analysis (NTA) are also recommended by MISEV23 to determine OMVs concentration and size [83]. However, NTA's limitation in measuring total particles, including non-OMVs contaminants, often leads to overestimating OMVs numbers due to reporting all particles' hydrodynamic radius. Recent advancements like tunable resistive pulse sensing (TRPS) offer label-free, real-time detection and sizing, utilizing nanoscale pores for precise measurements. Changes in electric current are observed as particles pass through the nanopore, and the magnitude and frequency of these changes are used to calculate the size and concentration of particles in a sample. Flow cytometry has also been employed to quantify OMVs [84].

c. Structural characterization

Images of OMVs preparations should be provided using electron microscopy (TEM), atomic-force microscopy (AFM), or super-resolution microscopy. These images are used to complement size measurements obtained from NTA, as these methods offer insight into the densest portion of the OMVs membrane, aiding in distinguishing between OMVs and contaminants [84]. Various methods such as BCA assay, SDS/PAGE, Western blotting, ELISA, mass spectrometry, and colorimetric assays have been used to analyze OMVs composition, each providing specific insights such as protein concentration, qualitative analysis, protein conformation, proteomic analysis, and LPS content, with a comprehensive approach often combining these techniques [83]. Researchers are advised to characterize vesicles using multiple methods to ensure accurate size measurement and count in their OMVs preparations, which enhances the rigor, reproducibility, and validity of research outcomes [80].

In OMVs research, besides isolation and characterization methods, attention to statistical analysis, use of appropriate controls like uninoculated and OMV-depleted media, replication of findings with diverse bacterial strains and isolation methods, preference for primary host cell cultures from multiple donors, and validation through *in vivo* models are crucial to ensure reliability, reproducibility, and validity, thus enhancing the impact of findings in this rapidly evolving field [84]. Research including characterization studies is summarized in Table 2.

Most studies used ultracentrifugation, density gradient centrifugation, SEM, TEM, and NTA to prepare and characterize Avian Pathogenic *E. coli* (APEC) [85] and *Salmonella* [86] OMVs for nanovaccine development and immunotherapy, respectively, demonstrating their efficacy in protection against infections. Zwarycz *et al.* employed serial centrifugation coupled with mass spectrometry to understand proteomic variations among *Myxococcus xanthus* OMVs [87], while Bhar *et al.* used SEM, TEM, and protein quantification during growth phases to optimize OMVs production from *Enterobacter cloacae* [88]. Soltani et al. compared OMVs extraction methods from *B. pertussis*, highlighting a modified technique without ultracentrifugation that yielded similar results in size and immunogenicity, demonstrating its potential as an alternative method [89]. The treatment of *P. aeruginosa* OMVs with Tris–HCl, EDTA and deoxycholate, followed by serial centrifugation, guaranteed a higher amount of OMVs' protein content (up to 129 ug/mL) [90], compared to the standard protocol that includes centrifugation, filtration and ultracentrifugation of OMVs isolated from *B. pertussis* (2 ug/mL of protein content) [91]. A similar protocol using Tris–HCl, EDTA, sonication and centrifugation was used to a different strain of *B. pertussis* [66]. These studies collectively demonstrate the importance of utilizing combined techniques to enhance OMVs isolation, purification and characterization for application in fields such as vaccine development and immunotherapy.

6. Conclusion

This review summarizes current developments and technical advances about OMVs' protein cargo and biogenesis, and how these features can be explored to develop therapeutic tools for infectious diseases. OMVs play crucial roles in intercellular communication, microorganism survival, and pathogenesis, and can also act as vehicles for the delivery of biomolecules, facilitating horizontal gene transfer and cooperative behaviors. Further studies of their biogenesis, cargo composition, and interactions with host systems will reveal new insights into how their heterogeneity can be tuned for an enhanced effect against infectious diseases. To ensure rigor and reproducibility in OMVS isolation and characterization, MISEV23 proposed guidelines should be followed to improve the knowledge in the field. We conclude that OMVs are versatile therapeutics for infectious diseases.

Data availability

All data used in this review paper is available in the reference papers listed below.

Figures

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CRediT authorship contribution statement

Mariana Ottaiano Gonçalves: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Paula Maria Pincela Lins:**

Table 2
Isolation methods used to obtain higher OMV yields for different applications.

Bacteria	Culture Media	Isolation Method	Yield	Application	Characterization methods	Ref
Avian Pathogenic *Escherichia coli* (APEC)	Luria-Bertani (LB) Medium	Centrifugation (12,000 x *g*, 15 min, 4 °C), filtration (0.45 μm) and ultracentrifugation (150.000 x g, 2 h, 4 °C). Purification by discontinuous density centrifugation.	$1{,}4 \times 10^{10}$ particles/mL in NTA	Evaluate immunogenicity of OMVs in broiler chick model with *in vivo* and *in vitro* vaccination	SEM, TEM and NTA	[92]
Salmonella typhimurium	Luria-Bertani (LB) Medium	Centrifugation (4000 x *g*, 10 min), filtration (0.45 μm), ultracentrifugation (150 000 x *g*, 2 h, 4 °C)	23 μg of OMVs harvested from 1×10^{10} CFU	OMV-coated polymeric nanomedicine for immunotherapy in cancer and metastasis	Cryo-TEM, TEM, CLSM, SDS-Page and DLS	[86]
Myxococcus xanthus	Charcoal Yeast Extract (CYE) medium	Serial centrifugation (10 400 x *g*, 30 min, 4 times) and ultracentrifugation (100 000 x *g*, 80 min)	Not reported	Comparative proteomic analysis of OMVs from different strains	SDS-Page, LC-MS/MS, Bioinformatic classification and identification of proteins	[87]
Pectobacterium brasiliense	Luria-Bertani (LB) Medium	Serial centrifugation (16 000 x *g*, 20 min, 4 °C and 38 000, 1 h, 4 °C), filtration (0.22 μm), ultracentrifugation (145 000, 6 h, 4 °C, twice)	$1{,}4 \times 10^{10}$ particles/mL	Proteomic and virulence study of *P. brasiliense* OMVs	TEM, NTA, Bradford Assay and MS/MS	[93]
Enterobacter cloacae ATCC 13,047	Luria-Bertani (LB) Medium	Centrifugation (2300 x *g*, 20 min, 4 °C) and ultracentrifugation (150 000 x g, 2 h, 4 °C). The supernatant was filtrated (0.22 μm) and ultracentrifuged twice (150 000 x *g*, 2 h, 4 °C).	3 OMVs/CFU	Comparative proteomic analysis of OMVs	SEM, TEM, NTA, SDS-Page, MS/MS, Bioinformatic classification and identification of proteins	[88]
Bordetella pertussis vaccinal strain	Bordet-Gengou Agar (BGA)	Ultracentrifugation (150 000 x *g*, 2 h, 4 °C) Centrifugation (8000 x g, 30 min, 4 °C). Pellet was incubated at 25 °C, 30 min and sonicated for 10 min. Serial centrifugation (10 000 x *g*, 20 min, 4 °C and 60 000 x g, 2 h, 4 °C and 60 000 x g, 1 h, 4 °C). Filtration (0,22 μm)	600 to 800 OMVs proteins/mL	Comparison of different extraction methods	Protein assays (Bradford and BCA), TEM, SDS-Page and Werstern Blotting	[89]
Pseudomonas aeruginosa	Luria-Bertani (LB) Medium added with meropenem and amikacin	Treatment with Tris–HCl, EDTA and deoxycholate; Serial centrifugation (20 000 x *g*, 30 min and 125 000 x *g*, 2 h)	38, 5 to 129,5 μg/mL of protein content	Evaluation of OMV immunogenicity, protein concentration, size and cytokine profiles in PAO1	TEM, Bradford Assay and SDS-Page	[90]
Bordetella pertussis Tohama phase 1 strain	Bordet-Gengou Agar (BGA) and Stainer-Scholte Medium	Treatment with Tris–HCl, EDTA; Sonication (20 min, cool water); Serial centrifugations (10 000 x *g*, 20 min, 4 °C and 100 000 x g, 2 h, 4 °C)	Not reported	Comparison of OMV-based vaccine and commercial vaccines in controlling infections and inducing immunity	SEM, TEM, SDS-Page	[78]
Bordetella pertussis strain B213 and *Bordetella bronchiseptica* strain BB-D09	Bordet-Gengou Agar (BGA), SS, Verwey Mediums	Centrifugation (5000 x *g*, 10 min), filtration (0,45 μm), ultracentrifugation (40 000 x *g*, 2 h, 4 °C)	2 μg/L of protein content	Influence of heat shock treatment on OMV release	BCA Assay, Lowry DC protein assay, SDS-Page and TEM	[91]
Francisella tularensis holartica strain	Brain-Heart Infusion (BHI) Medium	Low-speed centrifugation, filtration (0,22 μm) and serial ultracentrifugation (100 000 x *g*, 90 min, 4 °C, and 100 000, 16–20 h, 4 °C)	Not reported	Effects of OMVs on host cells and pathogenesis	BCA assay	[94]

Abbreviations: SEM: Scanning Electron Microscopy; TEM: Transmission Electron Microscopy; NTA: Nanoparticle Tracking Analysis; CFU: Colony Formation Units; Cryo-TEM: Cryogenic Transmission Electron Microscopy; CLSM: Confocal Laser Scanning Microscopy; SDS-Page: Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis; DLS: Dynamic Light Scattering; LC/MS-MS: Liquid Chromatography-Mass Spectrometry; BCA Assay: Bicinchoninic acid assay; EDTA: Ethylenediaminetetraacetic Acid; SS: Salmonella-Shigella medium;.

Writing – original draft, Methodology, Conceptualization. **Giulia Kassab:** Writing – original draft, Conceptualization. **Vanderlei Bagnato:** Writing – review & editing, Conceptualization. **Valtencir Zucolotto:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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