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Unlocking the potential of bacterial membrane vesicles: Advances in isolation techniques and biomedical applications

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ABSTRACT

Bacterial membrane vesicles (MVs) are nanosized, lipid bilayer-enclosed structures released by both Gram-positive (commonly referred as MVS, bacterial membrane vesicles) and Gram-negative bacteria (commonly referred as OMVs, outer membrane vesicles and outer-inner membrane vesicles (OIMVs)). They carry proteins, lipids, nucleic acids, and metabolites, playing key roles in intercellular communication, host–pathogen interactions, and immune modulation. Owing to these properties, MVs are attracting growing interest as versatile tools for vaccine development, drug delivery, and diagnostic applications. However, their translation remains constrained by low production yields, potential toxicity, and the lack of standardized isolation and characterization methods. This review summarizes current knowledge on MV composition and biogenesis, with a particular focus on emerging strategies for their isolation and engineering, and highlights their biomedical potential while outlining the challenges that must be overcome to enable future clinical applications.

1. Introduction

The interaction of organisms with their environment and other living beings is a fundamental aspect of life, occurring across all life forms. This interaction often relies on the secretion and uptake of molecules from the surrounding milieu. In the bacterial world, this form of communication, particularly dependent on cell population density, is known as *quorum sensing*. The canonical model of *quorum sensing* assumes that signaling molecules freely diffuse across cellular membranes. However, many of these molecules are hydrophobic and do not easily diffuse in aqueous environments, leading to their impaired release and consequent accumulation within cells [1]. Recent evidence suggests that some of these signaling molecules may instead be transported between cells via membrane vesicles (MVs) [2,3].

MVs were first identified in 1965 in *Escherichia coli* [2] but they were initially considered cellular debris or byproducts of membrane turnover processes after binary fission [3]. Subsequent research revealed the presence of MVs in many other gram-negative bacteria, including *Vibrio cholerae* [4], *Salmonella entérica* [5], and *Haemophilus influenzae* [6]. More recently, MVs were identified in gram-positive bacteria [7], challenging earlier assumptions about their distribution across bacterial taxa.

In recent years, interest in MVs has grown significantly, with numerous studies addressing their biogenesis, composition, and cellular functions [3,8]. Several comprehensive reviews have already covered these fundamental aspects [8–24]. Building on this body of knowledge, the present review focuses specifically on the methodological and translational dimensions of bacterial MVs. In particular, we aim to provide an updated overview of the approaches used for their isolation and characterization, and to critically assess their potential as biomedical tools in comparison with conventional therapeutic and diagnostic approaches.

To this end, we will first summarize the current understanding of MVs in terms of composition, biogenesis, and intercellular communication in both prokaryotic and eukaryotic systems. We will then examine in detail the strategies available for MVs isolation, an essential step for advancing extracellular vesicle research across biological domains. Finally, we will discuss emerging biomedical applications,

Abbreviations: MVS, Bacterial membrane vesicles Gram-positive bacteria; OMVs, Outer membrane vesicles Gram-negative bacteria; OIMVs, Outer-inner membrane vesicles Gram-negative bacteria; LPS, Lipopolysaccharide; PQS, Pseudomonas quinolone signal; UC, Ultracentrifugation; SEC, Size exclusion chromatography; TFF, Tangential flow filtration; UF, Ultrafiltration; PQS, Pseudomonas Quinolone Signal.

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highlight the main challenges that hinder clinical translation, and outline future perspectives, thereby defining the scope and objectives of this review.

2. MVs composition

MVs are spherical nanoparticles with a lipid bilayer structure, ranging in size from 20 to 300 nm [25–27]. Their composition reflects the assembly of their parental bacterial membrane [26], encompassing membrane proteins, lipopolysaccharides in Gram-negative bacteria [28,29] phospholipids, and various intracellular components [30] such as nucleic acids (DNA, RNA) [31,32], cell wall fragments, metabolites, signaling molecules, toxins [33,34], and proteins from the periplasmic space in Gram-negative bacteria and from the cytoplasmic space [28,34,35] (Fig. 1). Since MVs originate from bacterial membranes, their nomenclature differs based on bacterial classification (Fig. 1): MVs from Gram-negative bacteria are referred to as outer membrane vesicles, whereas those from Gram-positive bacteria are simply called membrane vesicles [19]. For simplicity, we will use the general term "MV" throughout this review, regardless of their bacterial origin.

The cargo of MVs is highly variable, depending on the bacterial species analyzed, the mechanisms underlying their formation, stress conditions suffered, and external factors such as the growth medium and interactions with other microorganisms present in the environment [35]. Despite similarities, notable differences have been observed between the protein content of MVs and that from their parent bacteria (Fig. 1) [36]. Most MV-associated proteins are derived from the cytoplasm or plasma membrane, with fewer contributions from the cell wall or extracellular binding proteins [37]. Some membrane proteins, such as lipoproteins (e.g., Lpp and OmpA), are less abundant in MVs, suggesting that these vesicles may originate from specific membrane microdomains, which could provide insights into their biogenic pathways [38].

Research on Gram-positive MVs is less extensive than that on Gram-negative MVs, and their protein composition remains under debate. Related to interactions between the bacteria and their human hosts, pathogenic bacterial MVs contain virulence factors and toxins [39,40], while commensal bacterial MV proteins might have a variety of protective effects acting as probiotics [41–43]. One example of the proteins found in MVs are endolysins and autolysins, which have been found in Gram-positive bacteria; these peptidoglycan-hydrolyzing enzymes have an antimicrobial effect against other bacteria [44–46].

MVs also carry genetic material, including DNA and RNA, which suggests their role in horizontal gene transfer [47]. This was initially demonstrated in *Ruminococcus spp.*, where wild-type-derived MVs enabled knockout mutants to synthesize missing crystalline cellulose-degrading proteins [48]. Additionally, MVs from *Streptococcus mutans* were found to contain extracellular DNA codifying for biofilm formation [49,50]. Moreover, MVs can mediate the horizontal transfer of antibiotic resistance genes, enabling resistance transmission between bacterial species [50].

Lipids are critical components of MVs, with phosphoglycerolipids being the most abundant, followed by glycerolipids and lipopolysaccharides (LPS) in Gram-negative bacteria [38,51]. Specific lipid aggregation patterns have been identified, including enrichment in short-chain saturated and unsaturated fatty acids [37,52,53], as well as in cylindrical fatty acids such as phosphatidylglycerol and phosphatidylethanolamine [54]. These patterns may influence membrane curvature and fluidity, facilitating the formation and release of MVs, particularly in Gram-positive bacteria [36,54]. The metabolic profile of MVs also adapts to environmental conditions. Metabolites such as ornithine, citrate, inositol, phenylalanine, citric acid, pyruvate, betaine, trehalose, and L-carnitine have been identified within MVs [8,52]. However, the metabolomics of MVs remain poorly understood, warranting further investigation.

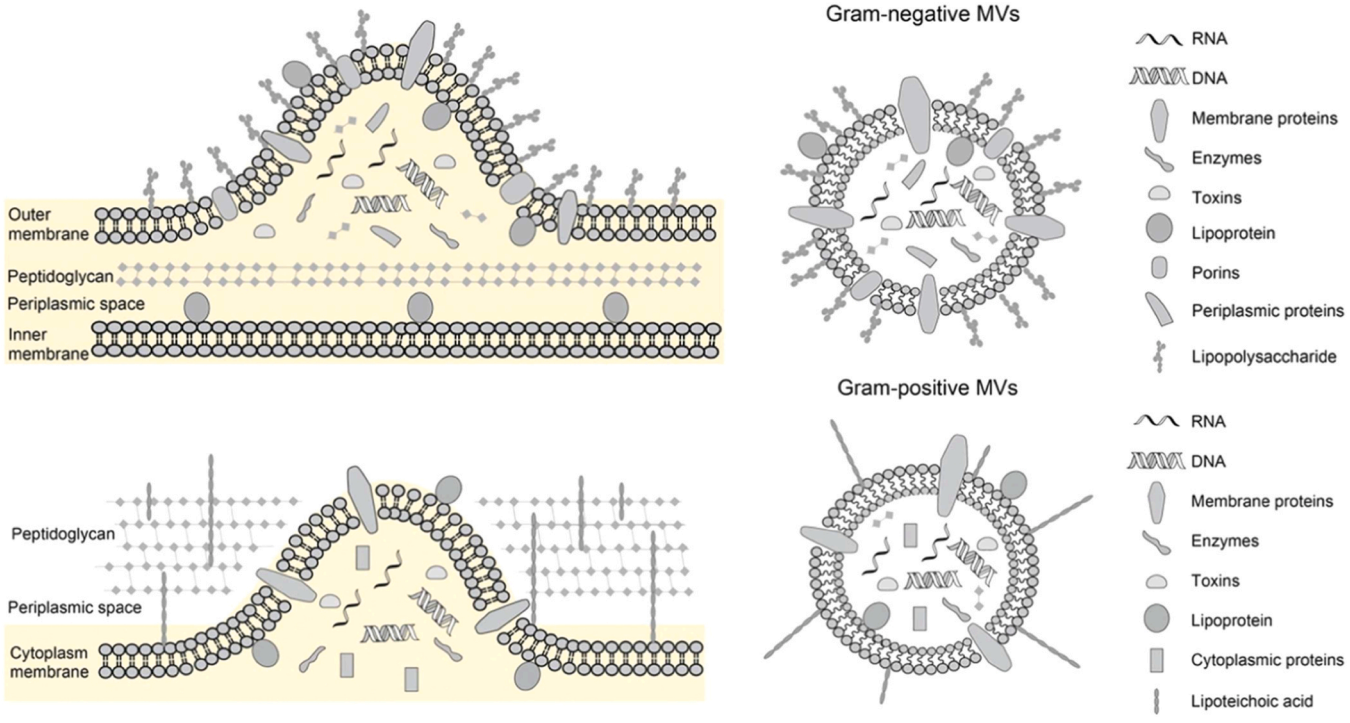

Fig. 1. Architecture and composition of Gram-negative and Gram-positive MVs differ in their membrane origin and contents. Gram-negative MVs derive from the outer membrane, containing LPS, outer-membrane proteins, periplasmic components, cytoplasmic proteins, and nucleic acids. In contrast, Gram-positive MVs originate from the cytoplasmic membrane and include lipoproteins, cytoplasmic proteins, enzymes, toxins, and nucleic acids. Reprinted with permission from Elsevier -Copyright 2024 [36].

3. Biogenesis

The selective inclusion of bacterial cargo into MVs remains poorly understood [27,55]. Several models have been proposed to explain MVs production through distinct pathways [44,56], which may account for the heterogeneity in composition, cargo and functions of MVs [38] (Fig. 2). Biogenetic routes also differ between Gram-negative and Gram-positive bacteria, reflecting their architectural differences [27]. In general, Gram-negative MVs have been studied more extensively due to the complexity of their outer membrane.

3.1. Proposed models in Gram-negative bacteria

Several models have been proposed to explain the formation of outer MVs in Gram-negative bacteria. These include: 1) reduced cross-linking between the outer membrane and peptidoglycan, 2) accumulation of periplasmic material, 3) alterations in lipopolysaccharide composition, 4) asymmetric expansion of the outer membrane leaflets, and 5) selective cargo sorting. Each of these models emphasizes different structural or functional aspects of the bacterial envelope and provides complementary perspectives on MV biogenesis.

One of the earliest models links vesiculation to a decrease in the cross-linking between the outer membrane and the underlying peptidoglycan layer [55,57,58] (Fig. 2). This effect is often observed when proteins such as Lpp, OmpA or the Tol-Pal complex, which normally stabilize the Outer Membrane-Peptidoglycan interaction, are inactivated or deleted [59–61]. Under these conditions, the outer membrane loses part of its anchoring, which favors its deformation and the release of vesicles. However, this model faces some limitations, as mutant strains frequently display compromised membrane integrity, making it

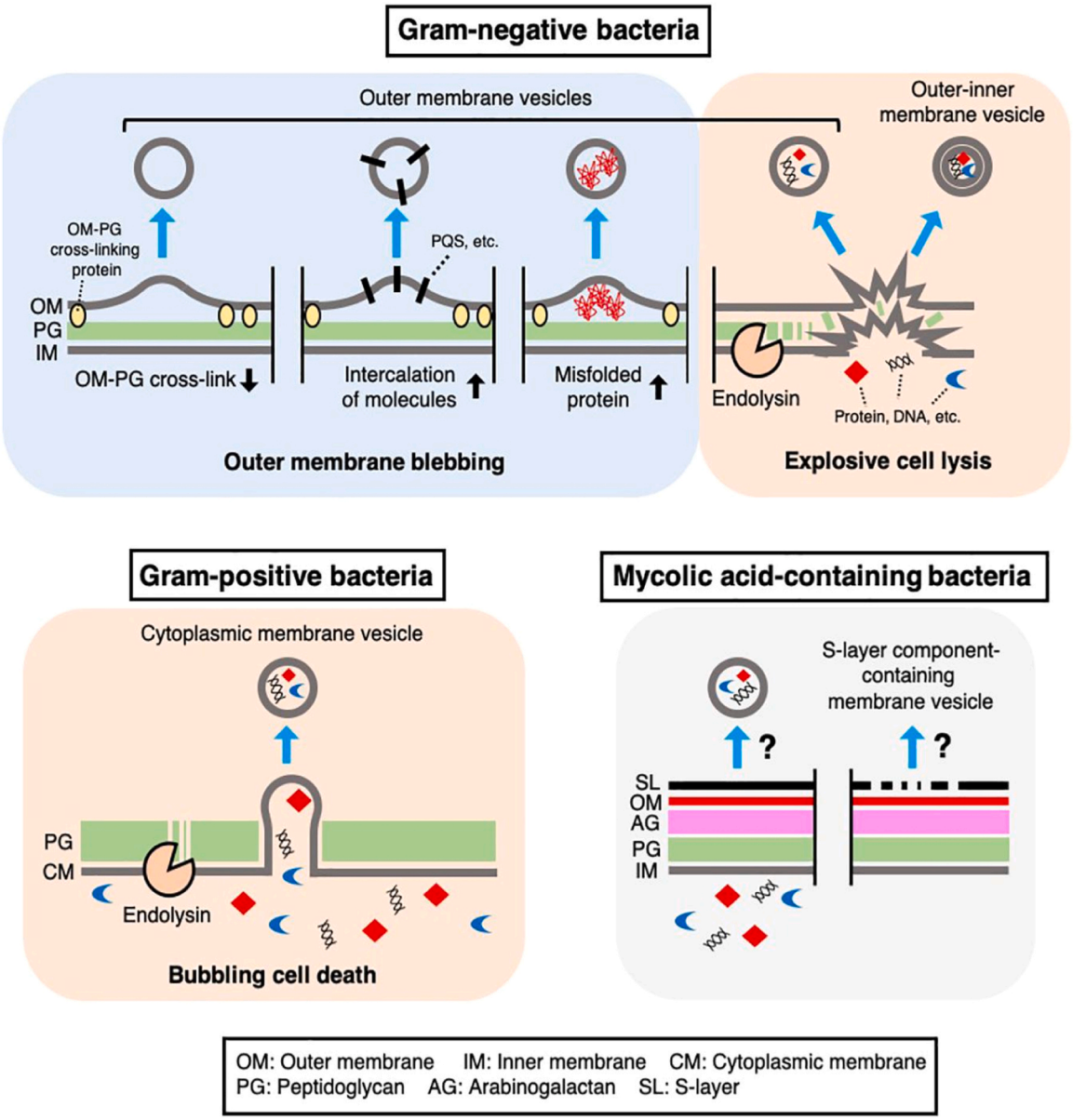

Fig. 2. Mechanisms of MV biogenesis. In Gram-negative bacteria, OMVs and OIMVs form through outer membrane blebbing or explosive cell lysis. Blebbing occurs due to structural changes in the cell envelope, such as reduced cross-linking between the outer membrane and peptidoglycan, intercalation of molecules like PQS, or accumulation of misfolded proteins. Explosive cell lysis is driven by phage-derived endolysin, which degrades the cell wall. In Gram-positive bacteria, cytoplasmic membrane vesicles are produced via bubbling cell death, where endolysin disrupts the peptidoglycan, allowing the cytoplasmic membrane to protrude. In mycolic acid-containing bacteria, MV formation mechanisms remain unclear, but MVs have been shown to carry inner membrane lipids and envelope-associated proteins. Reprinted with permission from Frontiers -Copyright 2020. *Open access article under a Creative Commons license* [38].

difficult to distinguish true MVs production from structural defects [55].

Another proposed model focuses on the accumulation of periplasmic material [55,62] (Fig. 2), such as peptidoglycan fragments [57,58,63] or misfolded proteins [38,64]. In this model, MVs act as a stress-relief mechanism, allowing the bacterium to expel toxic or excessive material from the periplasm. A classic example is *Porphyromonas gingivalis mutants* lacking autolysin, which are unable to degrade peptidoglycan fragments; as a consequence, these bacteria accumulate periplasmic debris and significantly increase MVs output [65]. This mechanism reinforces the idea of MVs as adaptive responses to periplasmic stress [66].

A third model highlights the contribution of LPS. MVs in Gram negative bacteria are often enriched in anionic LPS compared with the bacterial membrane, suggesting that vesiculation originates from domains with altered lipid composition [67,68]. By selectively budding from these sites, bacteria may alleviate electrostatic repulsion between negatively charged LPS units [68–70] and simultaneously remove unfavorable LPS species from the membrane [38].

Another proposed mechanism is based on asymmetric expansion of the outer leaflet of the outer membrane. When biomolecules such as phospholipids or the Pseudomonas Quinolone Signal (PQS), a quorum-sensing molecule produced by *P. aeruginosa* that regulates virulence gene expression and promotes MVs biogenesis, insert into the outer leaflet, they destabilize the bilayer, causing the outer leaflet to expand more rapidly than the inner one. This imbalance results in membrane curvature and vesicle budding [55,71](Fig. 2). In this context, phospholipids play a particularly important role, since their accumulation in the outer leaflet enhances membrane blebbing and vesicle release [72,73].

Finally, selective cargo loading has been attributed to protein-sorting mechanisms that resemble those described for eukaryotic EVs. This would resemble the role of galectin as protein sorter in exosomes derived from mammalian cells [74]. It has been suggested that an as-yet-unidentified factor, possibly a chaperone, directs proteins to specific outer membrane regions destined to form MVs [55,75]. Such a system would explain the enrichment of virulence factors in pathogenic bacteria [76], glycoside hydrolases in commensals [77], or hydrolytic enzymes in predatory bacteria [78]. In support of this idea, Bacteroidetes lipoproteins contain a negatively charged amino acid motif (Q-K-D-D-E) located just downstream of the lipidated cysteine residue, which serves as a signal for selective targeting to vesicles [79,80]. This motif provides compelling evidence that protein sorting into MVs can be genetically encoded and highly specific.

All these models describe MVs formation as a detachment of the outer membrane bleb [81] and this was initially thought to be the only mechanism responsible for MV biogenesis [35], but recent studies have uncovered another possible model, called explosive cell lysis [56,82], where the shattered membrane derived from lysed cells rounds up and forms MVs [82]. This process is triggered by endolysin, a peptidoglycan degrading enzyme, that is upregulated under DNA damaging stress conditions such as in biofilms or under anoxic conditions [1,35]. Explosive cell lysis would also explain the biogenesis of outer-inner membrane vesicles, which consist of two membrane layers (from both the outer and the inner membranes) along with periplasmic material and cytoplasmic material such as proteins, DNA or RNA [1,35,57](Fig. 2).

3.2. Proposed models in Gram-positive bacteria

In Gram-positive bacteria, the mechanism of vesicle formation is fundamentally different, as these microorganisms lack an outer membrane and are surrounded by a thick, multilayered peptidoglycan wall [83]. For many years, this rigid barrier was thought to preclude vesiculation, but accumulated evidence now confirms that Gram-positive bacteria do release membrane-derived vesicles. The most widely accepted model attributes this process to the activity of enzymes that locally weaken the peptidoglycan layer, allowing the cytoplasmic membrane to protrude through small pores [57,84] (Fig. 2). Among these enzymes, endolysins, peptidoglycan-degrading proteins often associated with phage activity, play a central role [85]. Their action disrupts the mechanical integrity of the cell wall, generating pressure imbalances that promote localized bulging of the cytoplasmic membrane and the subsequent release of vesicles (Fig. 2). This process, known as bubbling cell death [44], appears to occur in cells that lose viability but retain their overall morphology. In this scenario, the cell envelope remains largely intact, forming so-called “ghost cells,” while intracellular contents and vesicles are expelled into the surrounding environment [1,85]. Although the process leads to cell death, it differs from explosive cell lysis observed in Gram-negative bacteria, as the bacterial shape is preserved and the release appears more controlled. The resulting vesicles, often enriched in cytoplasmic and membrane-associated components, reflect the physiological state of the cell at the time of vesiculation.

The accumulation of certain RNA supports the idea that the content of MVs reflects the physiology of the cells [1]. Environmental and enzymatic factors seem to modulate this process significantly. Stressors such as antibiotics, nutrient deprivation, or oxidative stress can stimulate vesicle release, suggesting that vesiculation serves as an adaptive response mechanism. The vesicles produced through this pathway can carry a diverse molecular cargo, including proteins, lipids, and nucleic acids, which may contribute to intercellular communication, stress tolerance, or virulence [35,57]. Taken all together, MVs can be generated from different routes either by blebbing of the membrane or by cell lysis and would result in the generation of different types of MVs [44] (Fig. 2). This would mean that bacteria have selective MV formation pathways in order to produce distinct MVs having specific roles. Consequently, vesicle formation in Gram-positive bacteria is now recognized as a dynamic, regulated process rather than a byproduct of cell damage, highlighting the evolutionary convergence between Gram-positive and Gram-negative species to produce vesicles as versatile tools for interaction and adaptation.

3.3. Environmental and Physiological Stressors Affecting MV Production

MVs production is influenced by multiple environmental and physiological stressors, which can profoundly affect their abundance, molecular composition, and biological roles. During host colonization, pathogens encounter stressors such as nutrient deficiency, antibiotics, and immune responses. In these conditions, MVs act as a bacterial defense mechanism, mitigating stress and promoting survival. These vesicles help mitigate the detrimental effects of antibiotics and facilitate the transfer of resistance and virulence factors (Table 1). Antibiotics are a well-established trigger of MV formation (Table 1). For instance, β-lactam antibiotics like flucloxacillin and ceftaroline increase MVs production in *Staphylococcus aureus* by weakening the peptidoglycan layer, independent of prophages [45]. Conversely, DNA-damaging agents and antibiotics inducing the SOS response stimulate vesicle formation in lysogenic strains through phage lysis mechanisms, resulting in MVs with high DNA content [45]. Beyond antibiotics, specific chemical compounds such as glycine can also modulate MV production. In *E. coli* Nissle 1917, glycine supplementation induces morphological changes and quasi-lysis, leading to increased MV yields with altered protein profiles and reduced endotoxin activity [56]. Osmotic stress, caused by high salt concentrations, leads to vesicle production in *Listeria monocytogenes* [86] and *Pseudomonas putida* [87], potentially as an adaptation to environmental changes. Additionally, chemical compounds like EDTA [87] and detergents (SDS [88], deoxycholate [89]) resulted also in increased vesiculation.

Other stressors, including environmental and chemical factors, also have a significant impact on MV production (Table 1). For instance, acidic pH enhances vesiculation in *S. mutans* [90], *S. enterica* [91], *Francisella tularensis* [92], and *Pseudomona aeruginosa* [92,93] with both vesicle size and protein composition varying depending on the species and specific conditions. A mildly acidic pH induces significant changes

Table 1
Stimuli affecting MV production in different strains.

Stimuli	Effect	Bacteria	Reference
Mitomycin C	Induces the SOS response and increases vesicle formation through endolysin-triggered cell death	*S. aureus*	[45]
Flucloxacillin	Weakens the cell wall and stimulates MV production through a phage-independent blebbing mechanism	*S. aureus*	[45]
Ceftaroline fosamil (Zinforo®)	Weakens the cell wall and stimulates MV production through a phage-independent blebbing mechanism	*S. aureus*	[45]
Glycine	Glycine significantly enhances MV production	*E. coli*	[56]
NaCl-osmotic	Increase in vesiculation rate	*L. monocytogenes*	[86]
NaCl-osmotic	Induce to production of small size MV	*P. putida*	[87]
EDTA	Induce to production of small size MV	*P. putida*	[87]
pH	At mildly acidic pH,	*S. mutans*	[90]
pH	vesiculation rates increase	*S. enterica*	[91]
pH	several-fold compared to those at neutral pH	*F. tularensis*	[92]
pH	MVs produced in mildly acidic medium exhibit a significantly higher lipid-to-protein ratio and a substantially greater surface negative charge compared to those produced at neutral pH	*P. aeruginosa*	[93,98]
Temperature	Increase in vesiculation rate and significant change in composition cargo	*P. putida*	[87]
Temperature	Increase in vesiculation rate and significant protein cargo changes	*F. tularensis*	[92]
Oxidative stress	Increase in vesiculation rate and significant protein cargo changes	*Campylobacter jejuni*	[94]
Oxidative stress	Increase in vesiculation rate	*P. aeruginosa PAO1*	[95,96]
Cysteine depletion	Increase in vesiculation rate and oxidative stress	*N. meningitidis*	[97]

in bacterial MVs characteristics, including an increased number of vesicles per colony-forming unit, a higher lipid-to-protein ratio, and a significantly more negative surface charge compared to that at neutral pH. Thermal stress, both at high and low temperatures, has been shown to induce hypervesiculation with modifications in membrane lipid and protein composition. High-temperature stress can trigger the overproduction of MVs in bacteria, increasing membrane hydrophobicity and enhancing biofilm formation [87]. In addition, heat stress can lead to the release of MVs enriched in enzymes involved in the biosynthesis and in the remodeling of envelope components, underscoring the central role of cell envelope dynamics in vesicle production [92]. Oxidative stress caused by agents such as hydrogen peroxide, free radicals, presence of Fe^{3+} ions, depletion of electron donor molecules (e.g. glutathione) further increase MV production. This response is often linked to the accumulation of misfolded proteins or damage to the membrane integrity [94–96]. Moreover, nutrient depletion, including cysteine [97] or sulfate deprivation, also triggers substantial vesiculation, as observed in *Neisseria meningitidis*. These stimuli highlight the dynamic role of bacterial MVs when responding to stress, underscoring their potential as immunomodulatory agents and biotechnological tools. Collectively, these stress stimuli not only contribute to bacterial adaptation and survival, for example, by providing protection against membrane-targeting antibiotics, but also reveal the multifaceted role of MVs as mediators of bacterial communication and potential platforms for immunomodulation and therapeutics delivery in biotechnological applications.

4. Roles and functions

MVs play diverse roles in bacterial physiology and interactions (Fig. 3), including communication among bacterial populations, genetic exchange, transport of virulence factors, survival under stress, colonization, and predation.

As previously mentioned, one of the earliest roles identified for MVs is their involvement in *quorum sensing*, a cell-to-cell communication process mediated by signaling molecules that regulates gene expression in response to population density. As mentioned before hydrophobic signaling molecules often face limitations in diffusing freely through aqueous environments, leading to their accumulation within cells [1]. MVs provide a protective and hydrophobic interior environment that enhances the solubility and bioavailability of these molecules [99], enabling an alternative transport mechanism for long-distance signaling. By encapsulating such compounds, MVs help maintain effective concentrations over time and space, preventing the rapid dilution that occurs in free-diffusion models [1]. For instance, PQS molecules, which are too large and hydrophobic to diffuse efficiently on their own, are transported within MVs in *Pseudomonas aeruginosa* [99]. Interestingly, PQS not only relies on MVs for delivery but also promotes further MV production by their intercalation into the outer membrane [99].

In the context of pathogenesis, MVs function as long-distance delivery vehicles, transporting a wide variety of bioactive molecules involved in bacterial adhesion, invasion, and antimicrobial resistance. Toxins such as cytolysin A and heat-labile toxin have been identified in MVs produced by enterotoxigenic *Escherichia coli* [100–102], while cytotoxic necrotizing factor 1 was found in MVs from uropathogenic *E. coli* [103]. MVs from *P. gingivalis* [67,104] are enriched in gingipains, whereas those from *Pseudomonas aeruginosa* contain degradative and pore-forming enzymes such as peptidoglycan hydrolase, phospholipase C, alkaline phosphatase, proteases, elastase, and hemolysin [68,105]. Similarly, MVs from *Salmonella Typhimurium* carry cytolethal distending toxin [106], and those from *Vibrio cholerae* contain cholera toxin, the pore-forming toxin Vibrio cytolysin proteases and metalloproteases [33,107,108]. MVs also carry hemagglutinins and heat shock proteins, which facilitate host cell attachment, invasion [78], and bacterial aggregation [109–111]. In addition, bacterial DNA, including virulence genes [112,113], can be packaged within MVs. The presence of immunogenic components such as LPS, flagellin, and peptidoglycan further enables MVs to stimulate host immune responses [114,115].

Within the microbiota, MVs play diverse roles in modulating host immunity, promoting colonization, and acting as shared resources that benefit the entire bacterial community (Fig. 3). For instance, *Bacteroides* species secrete glycosylases and proteases via MVs to degrade complex polysaccharides and mucins, thereby providing accessible nutrients not only for themselves but also for other members of the gut microbiota [116,117]. *Bacteroides fragilis* also packages polysaccharide A in its MVs, which dampens host immune responses and enhances commensal colonization [118,119]. As it was mentioned before, MVs are also vectors for horizontal gene transfer, facilitating the exchange of metabolic enzymes, antibiotic resistance genes, and other functional traits [48,120]. For example, MVs from *Mycobacterium tuberculosis* contain the siderophore mycobactin, which chelates environmental iron and returns it to bacterial cells, enhancing survival under iron-limited conditions [121].

In stress response and envelope maintenance, MVs serve also as a mechanism to expel toxic compounds such as misfolded proteins, reactive oxygen species, or antimicrobial peptides [122,123]. They also act as decoys, binding to membrane-targeting antibiotics or phages, thereby protecting the bacterial cell [124]. In some species, such as *P. aeruginosa* and *Acinetobacter baumannii*, MVs carry enzymes like β-lactamases or

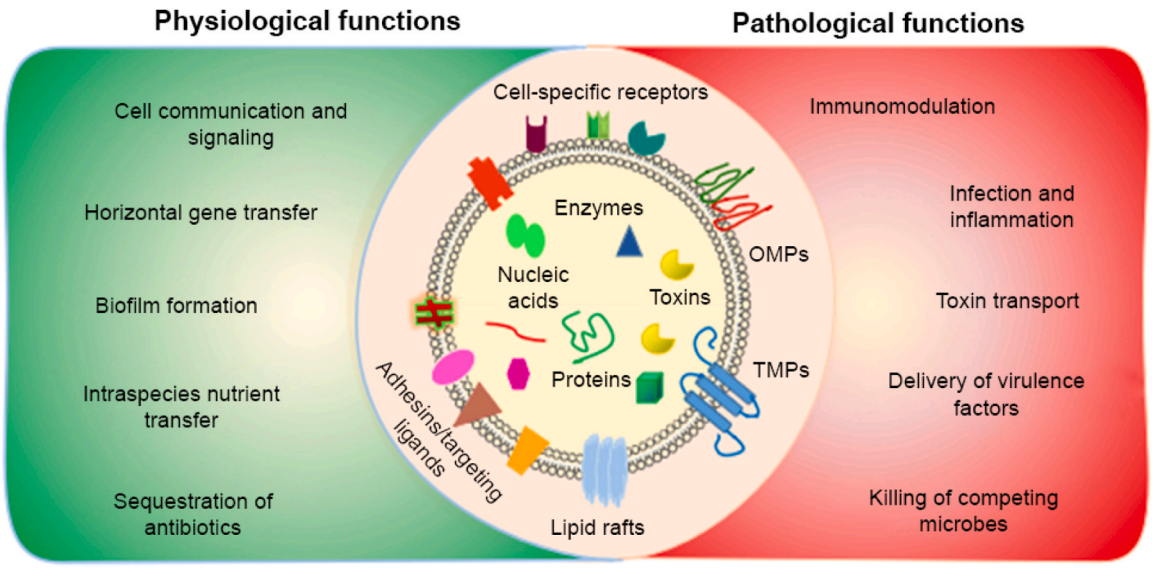

Fig. 3. Roles of membrane vesicles: include intracellular and extracellular communication, quorum sensing, horizontal gene transfer, interbacterial killing, toxin delivery, nutrient hydrolysis, and stress responses. OMP, outer membrane protein; TMP, transmembrane proteins. Reprinted with permission from Dovepress (2017). Open access article under a Creative Commons license [135].

carbapenem-hydrolases that inactivate antibiotics extracellularly [125–128]. Additionally, MVs have been shown to deliver bacterial RNAs capable of modulating host transcription through epigenetic mechanisms [129]. In biofilm formation and environmental colonization, MVs contribute to structure and mature bacterial communities by transporting extracellular matrix components such as exopolysaccharides and growth factors. These components enhance bacterial aggregation [18,35] and support multispecies interactions within biofilms, which facilitate cooperation, nutrient acquisition, and resilience [130]. Extracellular DNA, a key structural component of biofilms, can also be delivered via DNA-containing MVs [131].

Finally, MVs act as vectors for genetic exchange, mediating plasmid transfer, including those carrying antibiotic resistance genes, between bacterial cells [125,132]. Proteins involved in DNA uptake, such as integrative and conjugative elements or the Vir secretion system, may facilitate the incorporation of genetic material in the MVs [133,134]. In summary, bacterial MVs represent multifunctional entities involved in virulence, stress adaptation, interspecies communication, horizontal gene transfer, and biofilm dynamics modulation (Fig. 3). Their versatility underscores their critical role in microbial survival, host interactions, and ecological adaptability.

5. Internalization by prokaryotic and eukaryotic cells

The mechanisms underlying MV uptake by bacterial cells remain poorly understood. It has been proposed that membrane fusion is the primary mode of MVs internalization in bacterial cells [136–138]. When MVs come into contact with bacterial cells, the intermembrane hydration repulsion is reduced, leading to a decreased hydrophilicity in the intermembrane region as well as reduced surface density and polarity of the lipid bilayer [139–143]. Subsequently, divalent cations such as Mg^{2+} and Ca^{2+} can form salt bridges between membranes, modulating van der Waals forces and reducing electrostatic repulsion. These interactions ultimately facilitate membrane fusion between the bacterial cell membrane and the MVs [136,138,144,145].This model also suggests that microdomains within bacterial membranes play a role in facilitating fusion with MVs [146–150]. Lipids such as phosphatidylethanolamine and cardiolipin, which are present in both MVs and bacterial membranes, are thought to mediate lipid merging during the fusion process [143,151–153]. Additionally, proteins like the dynamin-like protein DynA, known for its role in lipid mixing, are hypothesized to also contribute to vesicle fusion [154].

In contrast, MV internalization by eukaryotic cells is better characterized and occurs via multiple pathways, including endocytosis, membrane fusion, and receptor-mediated signaling, with endocytosis being the predominant mechanism (Fig. 4). These vesicles often carry virulence factors like toxins, enzymes, and immunomodulatory molecules, allowing bacteria to manipulate host cell functions, suppress immune responses, and promote infection. This process encompasses several routes such as clathrin-mediated endocytosis, phagocytosis, macropinocytosis, and lipid raft–mediated uptake, including caveolae (Fig. 4) [28,155–159]. Similar to bacterial cells, lipid rafts in eukaryotic membranes play a critical role in MV uptake [76,156,160,161]. In some cases, specific cellular receptors are involved, for example, the monosialoganglioside receptor, which binds to the heat-labile enterotoxin present in MVs derived from enterotoxigenic *E. coli* [76,156,160,161]. Overall, the mechanism of MV uptake varies depending on both the bacterial species producing the vesicles and the targeted eukaryotic cell type [162]. Understanding these distinct internalization pathways in prokaryotic and eukaryotic systems is essential not only for elucidating bacterial pathogenesis and intercellular communication but also for advancing on the development of MV-based biotechnological applications, as it will be later discussed.

6. Engineering bacterial membrane vesicles: detoxification and isolation strategies, characterization and conservation methods

Pathogenic bacteria secrete a variety of toxic molecules that play essential roles in infection and virulence promotion [37]. Many of these compounds become incorporated into MVs during their biogenesis. Consequently, detoxification of these components is a fundamental prerequisite for the safe biomedical use of MVs [163]. A major limitation for the therapeutic use of MVs, particularly from Gram-negative bacteria, is their inherent toxicity. These vesicles naturally contain high levels of LPS and other Toll-like receptor agonists that strongly activate innate immune cells, potentially leading to uncontrolled inflammatory responses in vivo. Such hyperactivation can result in severe systemic effects, including sepsis, cardiac dysfunction, or acute lung injury, and, in extreme cases, even death [164,165]. Therefore, detoxification and purification of MVs are critical steps to enable their safe biomedical application. A schematic overview of the main stages involved in MV engineering, from isolation and purification to detoxification and

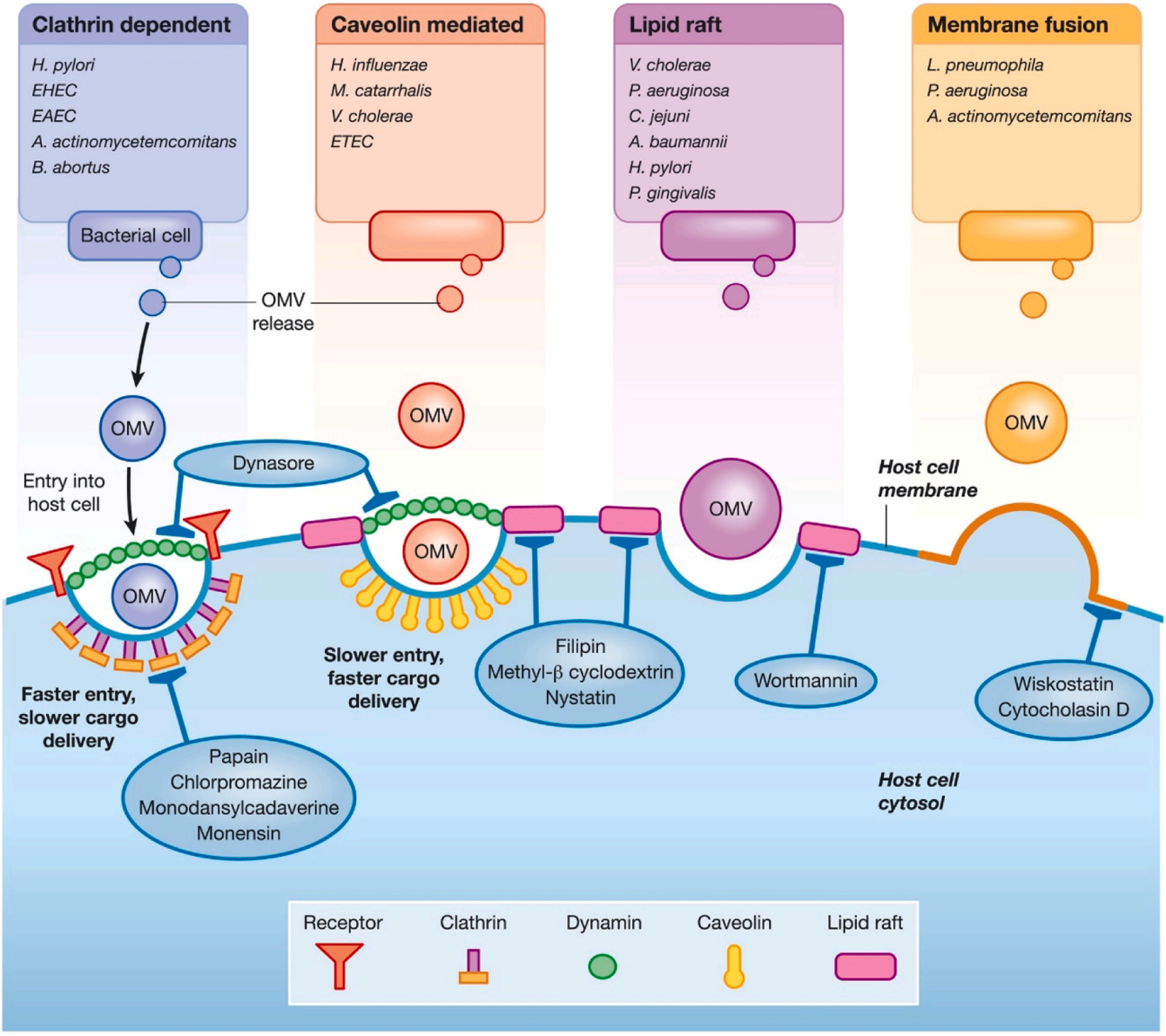

Fig. 4. Routes of MV entry into host eukaryotic cells. MVs from Gram-negative bacteria can enter host cells through various pathways, including clathrin-coated pits, caveolae formation, lipid rafts, or direct membrane fusion. Entry can be inhibited by compounds targeting these mechanisms, such as chlorpromazine (clathrin pit formation), papain (receptor degradation), monensin (proton gradient dissipation), monodansylcadaverine (receptor internalization), dynasore (dynamin GTPase inhibition), methyl-β-cyclodextrin (cholesterol extraction), filipin and nystatin (disruption of cholesterol-rich domains), wortmannin (phosphatidylinositol kinase inhibition), wiskostatin (actin polymerization regulation), and cytochalasin D (actin depolymerization). Reprinted with permission from Wiley –2016. Open access article under a Creative Commons license [155].

comprehensive characterization, is presented in Fig. 5 to provide an integrated view of the workflow and methodological options.

MV isolation is a labor-intensive process that typically requires large volumes of bacterial culture to compensate for the inherently low yield of vesicle production [166]. The resulting MVs display considerable variability in size, density, and composition, complicating downstream functional and omics analyses. This heterogeneity underscores the importance of optimized and standardized protocols for MV isolation, purification, and characterization to ensure reproducibility and functional relevance across studies. Commonly employed isolation techniques include ultracentrifugation (UC), tangential flow filtration (TFF), ultrafiltration (UF), and precipitation-based methods, while purification is typically achieved through density-gradient centrifugation or size-exclusion chromatography (SEC). These combined approaches balance yield, purity, and scalability depending on the intended application (Fig. 5).

6.1. *Detoxification approaches for MVs*

Beyond purification, several strategies have been developed to reduce the toxicity of MVs. Chemical and physical detoxification approaches focus on the removal or inactivation of toxic membrane components. A well-established method involves enzymatic digestion of *E. coli* [167] and *P. aeruginosa* [168] cells with lysozyme, followed by sonication to disrupt the membranes and a subsequent treatment with the ionic detergent Sarkosyl to selectively solubilize the inner membrane. The remaining outer membrane sheets can then be purified by buoyant density-gradient ultracentrifugation and fragmented by mild sonication to form synthetic MVs. These MVs retain the structural and compositional integrity of the outer membrane but exhibit markedly

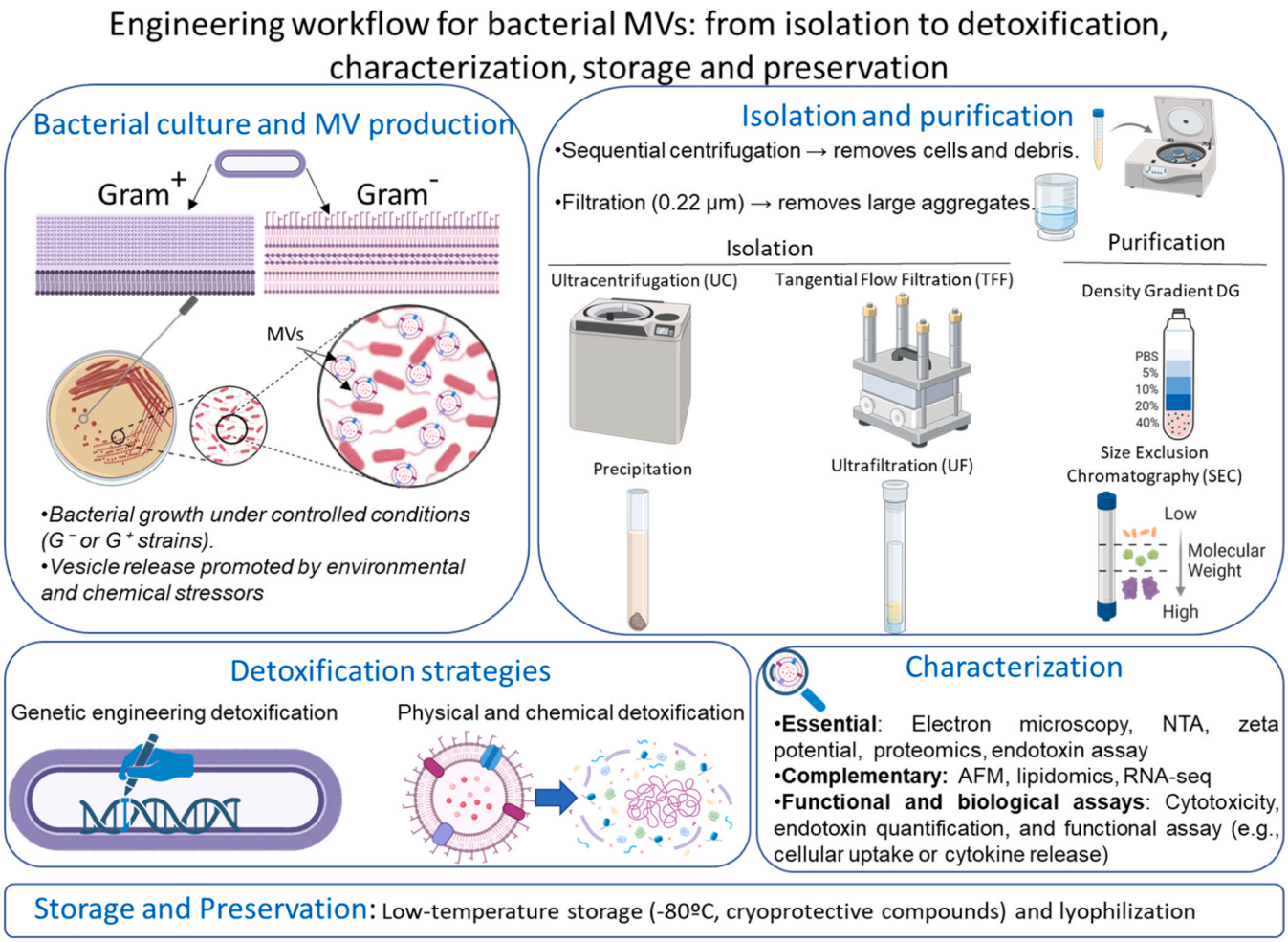

Fig. 5. Overview of the bacterial membrane vesicle (MV) engineering workflow. The process includes sequential stages of isolation, purification, and detoxification, followed by comprehensive characterization using complementary techniques. Storage and preservation strategies are also essential considerations to maintain MV stability and functionality over time. (Created with BioRender.com).

reduced endotoxicity. Similarly, detergent-based extraction using deoxycholate or Brij-96 can remove LPS and other pro-inflammatory molecules, though this treatment may also strip lipoproteins essential for immunogenic balance [55,169].

In parallel, genetic engineering has emerged as a precise and sustainable route to generate detoxified MVs directly from bacterial cultures. Since LPS, and specifically its lipid A component, is the primary driver of toxicity, modifying lipid A biosynthesis genes (such as lpxL1, lpxM, msbB, or pagP) can yield hypoacylated lipid A variants with diminished TLR4/MD2 activation [163]. This can be achieved by inactivating lipid A acyltransferases, reducing the expression of acylation enzymes, or introducing lipid A phosphatases that further decrease acylation [169–172]. For instance, Neisseria meningitidis lpxL1 mutants produce penta-acylated lipid A, resulting in MVs with substantially reduced endotoxic activity that no longer require detergent-based purification [173]. However, excessive genetic attenuation can sometimes impair bacterial growth or vesicle yield, as seen in certain mutants, emphasizing the need for balanced engineering strategies [163]. Recent approaches therefore target global regulators of virulence gene expression rather than single biosynthetic enzymes, achieving broader detoxification without compromising viability or productivity [55,163]. MVs can package numerous other virulence factors, such as bacterial adhesins, proteases, and cytotoxins. A common approach to reduce MV virulence involves the sequential deletion of genes encoding these factors. For instance, in *S. aureus*, key regulatory systems controlling virulence factor expression, such as Agr and the SaeR/S two-component system, can be disrupted to attenuate the pathogenic potential of the resulting MVs [46,174,175].

6.2. *Isolation and purification approaches for MVs*

Prior to any isolation method, the culture supernatant is subjected to sequential centrifugation to remove cells and debris, followed by filtration through a 0.22 µm pore membrane [176] (Fig. 5). While this step ensures sterility, it may decrease MV yield, as MVs exceeding 200 nm in diameter can be retained under certain conditions [177]. Currently, UC remains the most commonly used approach for bacterial MV isolation, frequently complemented or substituted by UF, SEC, or TFF depending on the intended application, required purity, and scalability [102,176,178]. Each isolation and purification technique offers distinct advantages and limitations (Table 2).

UC is widely used adopted for bacterial MV isolation due to its simplicity and capacity to handle large culture volumes. Nevertheless, centrifugation parameters differ considerably among bacterial species, reflecting variations in vesicle size, density, and biochemical composition; consequently, no universal protocol has been established [35,56,166,180,181]. Reported conditions for UC-based isolation of MVs range from 40,000 × g to 400,000 × g and 1–12 h, depending on the strain and medium composition, as summarized in Table 3, which compiles representative examples of speed, duration, and resulting yield for different bacterial models. High centrifugal forces can induce vesicle aggregation and inter-vesicle fusion, producing artificially enlarged

Table 2
Comparative analysis of different isolation procedures of bacterial membrane vesicles.

Method	Principle	Advantages	Limitations	Typical Yield/ Purity	References
Ultracentrifugation (UC)	Separation based on density and size using high g-forces	Widely used; allows bulk isolation; compatible with various sample types	Time-consuming; requires expensive equipment; potential vesicle deformation or aggregation; low reproducibility; Long processing times	Moderate yield; purity highly variable	See Table3
Density Gradient DG	Vesicles separated in density gradients (e.g., sucrose, iodixanol)	Improved purity; reduces contamination from protein aggregates and cell debris	Labor-intensive; gradient preparation variability; low scalability	High purity; low yield	[30,57, 179–184]
Size Exclusion Chromatography (SEC)	Separation by molecular size using porous beads	Preserves vesicle integrity; removes protein contaminants effectively; high reproducibility; time-efficient	Lower concentration output; limited scalability for large volumes	Moderate yield; high purity	[185,199]
Tangential Flow Filtration (TFF)	Separation using membranes under tangential flow	Scalable; gentle processing; suitable for large volumes	Membrane fouling; optimization needed for bacterial vesicles	High yield; moderate purity	[186]
Ultrafiltration (UF)	Filtration through specific pore-size membranes	Fast and simple; inexpensive	Possible vesicle deformation or loss; protein contamination	Moderate yield; low-to-moderate purity	[30,166, 187,188]
Precipitation (e.g., PEG)	Precipitation of vesicles and macromolecules	High recovery; easy to implement	Co-precipitation of proteins and polymers; limited purity	High yield; low purity	[189,190]

Table 3
Comparative analysis of different ultracentrifugation protocols reported.

Bacteria	Speed (g)	Time (h)	References
E.coli	40,000–210,000 g	1–4	[28,56,58,64,101–103,112, 113,115,124,195–202]
S. aureus	150,000 g	1–3	[45,46,174,187,203,204]
Salmonella spp.	100,000–150,000 g	2–4	[59,61,103,175,199]
V. cholerae	100,000–144,000 g	4–12	[33,75,205,206]
Neisseria spp.	40,000 g	1	[32,207,208]
P. aeruginosa	40,000–400,000 g	1–3	[28,32,60,68,70,99,126,136, 209]
Bordetella pertussis	100,000 g	2	[210,211]
Bacteroidetes spp.	27,000–100,000 g	0.67–2	[77,79,109,110]
P. gingivalis	27,000–213,000 g	0.67–3	[63,65,67,104,111,159]
Acinetobacter spp.	40,000–200,000 g	1–3	[32,128,132,212]
Streptococcus spp.	150,000–175,000 g	2–4	[49,213]

particle sizes and contamination with protein aggregates or cell debris [191–194]. The repeated pelleting of vesicles against tube walls may further compromise membrane integrity, particularly in samples from stressed or weakened cells [18,193]. To enhance sample purity, density gradient UC (using sucrose or iodixanol gradients) is frequently applied to separate vesicles according to their buoyant density [30,57, 179–183]. However, this refinement often results in reduced yield and prolonged processing times, up to 48 h, making it less suitable for large-scale preparations (Table 2). Despite these limitations, UC remains as the benchmark for analytical-scale MV isolation due to its robustness, affordability, and broad historical validation across different bacterial species (Table 3).

As current methods often fail short to sufficiently purify MVs from protein aggregates or membrane fragments (e.g., flagella or fimbriae) [105], additional purification approaches have been developed. UF and TFF represent gentler, membrane-based alternatives that use transmembrane pressure to concentrate and fractionate MVs by size (Table 2). Unlike UC, they avoid pelleting vesicles, minimizing aggregation and preserving their native structure. While both methods are strain-dependent and lack standardized conditions [30,166,187], TFF is particularly advantageous for its scalability, continuous operation, and compatibility with clinical-grade processing. In combination with SEC, TFF can increase vesicle recovery by 10–20 fold compared to UC while maintaining similar size distributions [186] (Table 2). Membrane fouling, however, remains as a practical limitation, especially when processing complex media, as it reduces recovery and enriches samples in smaller vesicles due to progressive pore blockage [166].

SEC enables gentle, size-based separation of MVs from soluble proteins and macromolecular contaminants by elution through a porous matrix. SEC has emerged as a powerful, high-purity method for separating MVs from soluble proteins and macromolecular contaminants based on their hydrodynamic radius [189]. Larger particles elute earlier from the porous matrix, while smaller molecules are retained longer. This gentle process preserves vesicle morphology and functional integrity, minimizes aggregation, and effectively removes free proteins and nucleic acids [185]. SEC is particularly advantageous when downstream biological or omics assays require minimal contamination from protein aggregates or free endotoxins. However, SEC is constrained by its low throughput, sample volume typically must not exceed **5 %** of column volume, and dilution effects that necessitate post-collection concentration [189,214]. Thus, SEC is useful when purity and reproducibility are prioritized over yield and is often integrated with TFF for scalable workflows (Table 2). SEC can also be combined with UF [188] and density gradient centrifugation to separate heterogeneously sized MVs (~300 nm and ~150 nm) [184].

Alternative or complementary strategies in MV isolation include polymer- or salt-based precipitation, which reduce solubility and induce vesicle pelleting after low-speed centrifugation [189,190]. Although these methods (e.g., polyethylene glycol precipitation) are simple and suitable for large-volume processing, co-precipitation of proteins and other macromolecules leads to low purity [190] (Table 2). Comparative studies indicate that UC yields higher recovery and more homogeneous particle size distributions than precipitation-based methods for both Gram-positive (Lactobacillus acidophilus) and Gram-negative (E. coli Nissle 1917) MVs, with the added advantage of reduced processing times [215]. Commercial kits designed for bacterial MVs (e.g., ExoQuick® TC, ExoBacteria®, qEV Izon Science)) [216] provide standardized, user-friendly alternatives, but their performance in bacterial systems remains less validated than in mammalian EVs, and they often combine polymer precipitation with filtration, thereby reproducing the same yield–purity trade-off. Other emerging strategies for MV purification include mixed-mode (multimodal) chromatography [214].

6.3. *Characterization techniques for MVs*

There are several characterization techniques that can be used to analyze MVs (Fig. 5). Total protein content, assessed via bicinchoninic acid (BCA) or Bradford assays, and lipid phosphate content, measured with the Malachite Green assay, correlate with MV concentration [56, 57,183]. Techniques such as Tricine sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE) and western blot analysis can also be performed to identify and determine MVs protein content [62,180,181,217]. For instance, OmpA, an abundant outer membrane porin, is a reliable marker for MV detection and can be analyzed via western blotting [218]. Also, the physicochemical characterization techniques routinely applied for nanoparticles are also used for MVs characterization, such as Dynamic Light Scattering, Zeta potential measurements, Nanoparticle Tracking Analysis and Scanning Electron Microscopy or Transmission Electron Microscopy imaging [30, 35] (Figs. 5–6). Nanoparticle Tracking Analysis is a technique that rapidly detects nanoparticles in solution by combining laser light scattering microscopy with a charge-coupled device camera that visualizes the colloidal particles present. Then, a software relates the rate of particle movement by Brownian motion of these detected nanoparticles to their size according to the Stokes–Einstein equation [219−221]. This allows for direct measurement of polydisperse samples, while measuring a large number of particles in a small time frame, resulting in extremely accurate measurements with little variance [219,221]. This technique can measure vesicles in a colloidal suspension as small as ~50 nm [222] and provides not only with particle size distributions but also with concentration measurements. AFM, SEM, cryo-TEM and TEM analysis are used to visually determine MV morphology and size [166,182,187, 216,223] (Fig. 6).

There are also methods like PCR or RT-qPCR that can generally be used to quantify the DNA and RNA content of MVs [31,45,103,120]. Omics technologies play a pivotal role in the comprehensive characterization of MVs, enabling detailed insights into their molecular composition and functional potential. Proteomics [39] allows the identification of vesicle-associated proteins, including virulence factors, transporters, and signaling molecules, while lipidomics [224] reveals the specific lipid profiles that define membrane structure and vesicle stability. Genomics and transcriptomics provide information on the nucleic acid content carried within MVs, which may influence host-pathogen interactions or horizontal gene transfer [50]. Together, these high-throughput approaches offer a complete understanding of MVs, supporting their potential use as biomarkers, therapeutic agents, or vaccine components

6.4. Storage and preservation of bacterial membrane vesicles

Long-term storage of MVs has not yet been standardized, and preservation conditions can markedly affect their structural integrity, biological activity, and reproducibility in downstream applications (Fig. 5). Most studies employ low-temperature storage conditions, typically at −80 °C, where MVs are frozen in phosphate-buffered saline or water, with or without the addition of cryoprotective compounds such as glycerol or trehalose. These additives help prevent vesicle aggregation and membrane disruption during freezing and thawing cycles.

The stability of MVs under different temperature conditions (4 °C, −20 °C, −80 °C) and after lyophilization has been systematically evaluated, revealing that lyophilized preparations retain vesicle concentration and size distribution more effectively over time than frozen samples, even after storage periods ranging from 7 to 75 days [195,226]. Despite these insights, standardized protocols for MV preservation are still lacking. Factors such as buffer composition, ionic strength, and the presence of residual bacterial components may influence vesicle stability differently depending on the origin of the bacterial species. The development of harmonized storage guidelines, similar to those established for mammalian extracellular vesicles by the ISEV, will be essential to ensure data comparability and reproducibility across studies.

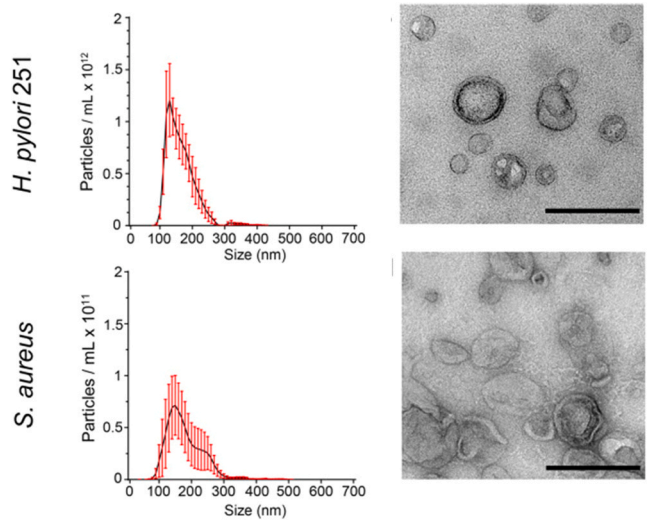

Fig. 6. NanoSight NTA and transmission electron microscopy images of MVS produced by Gram-negative H. pylori 251 and Gram-positive S. aureus. Reproduced with permission from ASM Journal-2021. Open access article under a Creative Commons license. [225].

7. Biomedical and biotechnological applications of MVs

As previously discussed, MVs hold significant promise for a wide range of medical applications [56,227,228], particularly in vaccine development and as drug delivery systems [163] (Fig. 7). Although, as it was mentioned before, the mechanisms underlying MV biogenesis are not yet fully elucidated [229], their intrinsic properties make them attractive candidates for biopharmaceutical use [27,163]. These include their inability to replicate, reducing the risk of bacterial contamination or infection [162], their relative ease of production compared to more complex delivery vectors such as liposomes or engineered nanoparticles, and their superior stability at physiological temperatures [27, 230−232]. MVs derived from Gram-negative and Gram-positive bacteria, as well as mycobacteria, can be isolated from large-scale cultures or bioreactors [18,121]. However, several challenges remain to be addressed. These include potential biotoxicity [233], limited immunogenicity in the context of vaccine development [174], and the mentioned inherently low production yield of naturally produced MVs [8]. Furthermore, current isolation and purification methods are often labor-intensive and require substantial processing volumes, which may hinder scalability and clinical translation [214].

MVs have shown potential as drug carriers due to their ability to actively target host immune cells (e.g., macrophages) or passively accumulate at infection sites [174,234] (Fig. 7). These targeting abilities make MVs a potential platform for drug delivery in bacterial-infection control, especially in infections caused by drug-resistant strains [163, 235]. This targeting ability together with the use of nanoparticulated antimicrobial carriers has been used for example to direct antibiotics to implant associated infections in vivo [236]. In that work, silica nanoparticles containing levofloxacin and cloaked with sonicated MVs and membranes derived from red blood cells showed superior antimicrobial efficacy, a reduction in the inflammatory response, and a modulation of the immunosuppressive microenvironment around the infected implant [236]. Even extracellular and intracellular pathogens have been targeted using siderophore-modified MVs loaded with lysostaphin and mupirocin in vivo using a muscle abscess infection model taking advantage of the targeting ability of the conjugated siderophore [237].

Packaging MVs with cargo serves several advantages including protection from degradation [113,195] and delivery at high concentrations over long distances [195] and they have also shown to retain enzymatic activity and antigenicity over long-term storage at low temperatures [195,207]. *S. aureus* infected mice, intravenously injected with rifampicin-loaded *S. aureus* derived MVs showed high antimicrobial effects, reducing the infection and showing tropism against macrophages [235]. MVs can also be used as vehicles for pathogens like viruses or other bacterial components with antimicrobial activity [25].

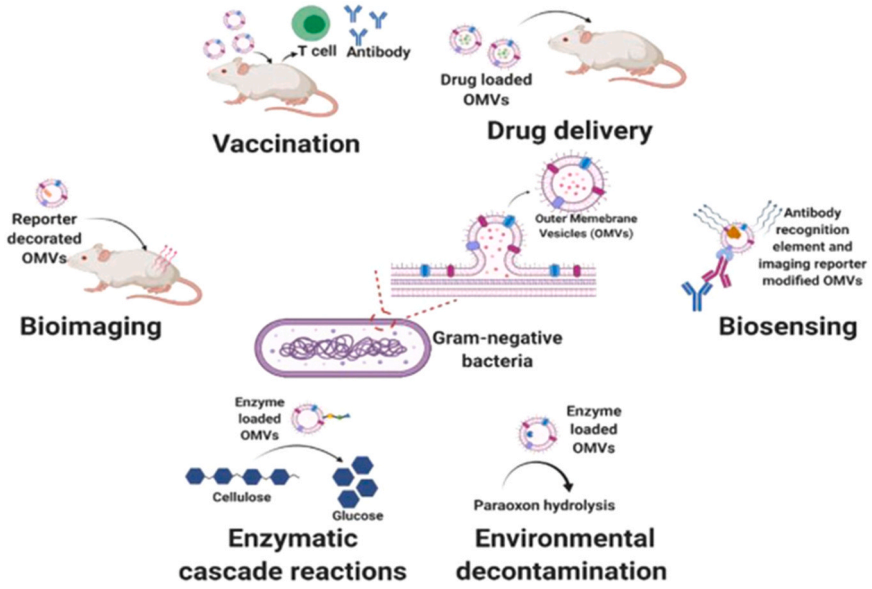

Fig. 7. Overview of outer membrane vesicles enabled bio-applications in vaccination, drug delivery, bioimaging, biosensing, enzymatic cascade reactions, and environmental decontamination. Note: Paraoxon (i.e., the active metabolite of the insecticide parathion) acts as an acetylcholinesterase-inhibiting insecticide. Reprinted with permission from John Wiley and Sons 2021 from [25].

This can help to fight drug resistance mechanisms of several clinically-important pathogenic species. For example, MVs from *P. aeruginosa* were shown to possess antimicrobial activity against *E. coli* and *S. aureus* [68,136,199]. *B. thailandensis* MVs contain peptidoglycan hydrolases, heat-stable small molecules and the biosurfactant rhamnolipid, and exhibited antimicrobial and antibiofilm activity against methicillin-resistant *S. aureus*, multidrug-resistant *A. baumannii*, *Candida albicans* and *Cryptococcus neoformans* [217]. However, the complexity, component inhomogeneity, and size heterogeneity of MVs entail the risks of using them rather than other synthetic delivery systems where those variables can be easily tuned [238].

Another reported application for MVs is their use as vaccines (Fig. 7), due to their ability to stimulate innate (cellular) and adaptive (humoral) immune responses and their ability to display antigens without the accompanying risks posed by metabolically active bacterial cells, such as those causing the related diseases [209,239–241]. Precisely, MVs contain bacterium-derived antigens and multiple pathogen-associated molecular patterns that can modulate the immune system [55]. Another advantage of MVs is their reduced size, which enables them entering into lymph vessels and being uptaken by antigen-presenting cells, which present them to T cells, leading to the generation of antigen-specific B cell responses [29,169,242]. Since MVs reflect the structure of the bacterial membrane, they display antigens in a native conformation. In this way, the neutralizing antibodies produced are more effective than purified antigens alone [208]. In addition, MVs have natural adjuvant properties. The MVs derived from Gram negative bacteria contain LPS, a potent activator of immune cells and inflammatory response [29,55]. This can also result in high vaccine formulated reactogenicity, but their adverse side effects are reduced compared to conventional adjuvants, such as aluminum, the cholera toxin, and diphtheria toxin [28,243,244]. For example, a purified malaria antigen vaccine with *E. coli* MV adjuvant raised antibody titers and cellular responses against the antigen equivalent to the same vaccine but with cholera toxin adjuvant, and with no significant side effects or weight loss in mice [201]. Similarly, vaccination with a novel purified influenza antigen in conjunction with an *E. coli* MV adjuvant generated higher titer mucosal antibodies and cell-mediated responses compared to the vaccination with the purified influenza antigen alone, in addition, no adverse side-effects were associated with the MV adjuvant regime [245].

MVs from numerous bacteria have been investigated as vaccine candidates. The vaccines against *N. meningitidis* group B (MenB) to prevent invasive meningococcal disease are the most successful MV-based vaccines developed to date [246–251] and vaccines against *V. cholerae, S. Typhimurium, Shigella flexneri, H. influenzae, Gallibacterium anatis, Pasteurella multocida, Mannheimia haemolytican, and Actinobacillus pleuropneumoniae* have been studied in animal models [175,205,206,251,252,253]. Also, vaccination with *Streptococcus pneumoniae* MVs elicited antibody production that was protective against pneumococcal infection [53]. Besides, MV vaccines have also proven efficient for antibiotic resistant strains of S. aureus [46,203], *B. pertussis* [210,211], *A. baumannii* [81,212], and so on.

These characteristics position MV-based vaccines as promising alternatives to traditional vaccine platforms. Nevertheless, several limitations must be considered. The immunogenicity and protective efficacy of MV vaccines can vary significantly depending on the bacterial species, and even the specific strain, used for vesicle production, potentially influencing the type and strength of the immune response elicited [27]. Additionally, MVs derived from Gram-negative bacteria inherently contain lipid A, the endotoxic component of LPS, which can trigger severe and potentially fatal inflammatory responses in the host [170]. Furthermore, many bacterial strains naturally produce MVs in low quantities, necessitating the cultivation of large volumes of pathogenic bacteria to obtain sufficient material [55]. To address these challenges, genetic engineering strategies have been employed to reduce endotoxicity and enhance MV yield, thus improving their safety and feasibility in clinical applications. As it was described in section 6, several strategies have been developed to reduce MVs toxicity. However, the potential of bioengineering extends beyond MV detoxification. One powerful strategy to expand the functionality of MVs involves the fusion of exogenous proteins with native MV-associated proteins, enabling the surface display or encapsulation of heterologous molecules. This approach allows for the design of tailored vesicles with enhanced therapeutic, immunogenic, or targeting properties (Fig. 7). For example, the *E. coli* protein ClyA has been successfully fused with a variety of antigens, such as green fluorescent protein, the ectodomain of influenza A matrix protein 2, and domain 4 of Bacillus anthracis protective antigen, to facilitate their surface display on MVs [197,202,254]. Similarly, the signal peptide of *E. coli* outer membrane protein A (OmpA) has been used to direct heterologous antigens to the periplasm, enabling their encapsulation into the lumen of MVs [255,256]. This strategy has been

applied for proteins from S *Streptococcus* [198] and *Chlamydia muridarum* protein HtrA [196], enhancing their loading efficiency and potential immunogenicity. Another strategy consists in transferring Capsule and O-antigen biosynthesis gene clusters from pathogenic bacteria to *E. coli* in order to display the glycans of choice as recombinant LPS in nonpathogenic bacterial MVs. These glycoengineered MVs can be purified and employed directly as conjugate vaccines. An example of this technology is the expression of the *S. pneumoniae* CPS gene cluster in *E. coli* [200] or the O-antigen polysaccharide gene cluster of *F. tularensis*, also in *E. coli* [172].

Cancer therapy is another niche where MVs can overcome several limitations due to their immunotherapeutic ability, which synergistically enhances the performance of traditional chemotherapeutic drugs [257]. Compared with most traditional drug delivery vehicles for cancer treatment, MVs have several unique advantages, like their large inner drug loading space, their structure that prevents leakage during host circulation, their natural cell targeting capabilities (surface proteins that recognize certain cell types [25,174,198,229,234,258,259]) and the immune-stimulating molecules which MVs carry, such as LPS, that can initiate an anti-tumor immune response [260]. Besides, MVs can also induce a durable antitumor immune response that inhibits tumor growth [229,261]. For example, MVs loaded with tegafur (i.e., a chemotherapeutic drug) showed a synergic effect between vesicles, that activated the immune system, and the anti-tumor drug; triggering direct tumor cell apoptosis [229]. In preclinical models Fe_3O_4-MnO_2 nanoparticles surface cloaked with MVs have recently demonstrated targeting ability to inflammatory tumor sites through neutrophil-mediated targeted delivery and the promotion of photothermally enhanced melanoma immunotherapy [262]. Cloaking polydopamine with *Salmonella strain VNP20009*-derived MVs has demonstrated superior antitumoral effects by combining the photothermal effects of polydopamine with the enhanced immune response triggered by the MVs passively targeting tumor-bearing mouse models and upregulating T cell infiltration and secretion levels of pro-inflammatory factors as well as antitumor related cytokines [263]. Recently bacterial protoplast-derived NVs carrying a CRISPR-Cas9 system has demonstrated their ability to target tumor associated macrophages (TAMs) and to remodel the tumor microenvironment by reprograming the TAMs towards an M1-like phenotype and inhibiting tumor growth *in vivo* [264]. Enzyme-powered OMV loaded with si-RNA and surface modified with cell-penetrating peptides able to target and penetrate solid bladder tumors have demonstrated their ability to inhibit surviving expression, a protein that promotes apoptosis inhibition in tumor cells [265].

Current chemotherapy indiscriminately affects all cells that rapidly multiply in the body, causing severe long-lasting side effects, and requiring a high dose to achieve an effect on the tumor due to their poor circulation half-life and rapid clearance [266]. Therefore, novel delivery platforms are being increasingly explored as vehicles for drugs and genes, or imaging agents to target diseased cells [27]. Considering the immunomodulatory role of MVs, the bacteria producing these MVs can be engineered to express cancer-specific epitopes or small noncoding RNAs [55,261,267], which can be packed inside those vesicles. One of the main strategies for MVs engineering in cancer therapy is the addition of cancer-targeting ligands on their surfaces, which bind overexpressed receptors in cancer cells, like HER2 [268] or the epidermal growth factor receptor [269]. Other strategies include the loading with anti-tumor siRNA directed against mRNA from key regulatory genes implicated in cancer progression [268,270] or anti-tumor drugs like doxorubicin or idarubicin [271].

As previously discussed, bacterial MVs hold promise for a variety of biomedical applications, including their use as imaging reporters (Fig. 7). When loaded with contrast agents such as luciferase (for bioluminescence imaging) or melanin (for optoacoustic imaging), MVs enable noninvasive *in vivo* visualization [187,197,202,254]. A representative example is the use of GFP-containing MVs for fluorescence microscopy [197]. These vesicles offer notable advantages for imaging applications, such as protection of the molecular cargo and the potential for cell-type-specific targeting through genetic engineering. Moreover, MVs can serve as multifunctional platforms for both imaging and therapeutic purposes, including thermal therapy [172,268].

8. Conclusion and prospects

Bacterial membrane vesicles represent a versatile and emerging platform with significant potential in biomedical and biotechnological applications. However, several fundamental challenges must still be addressed to fully harness their capabilities. Among the most pressing issues, a deeper understanding of the mechanisms underlying intraluminal cargo loading during MV biogenesis is needed. Elucidating how proteins, nucleic acids, and metabolites are selectively packaged in their interiors will be essential for designing MVs with defined and reproducible therapeutic or diagnostic functions. The complex composition of MVs also demands further exploration to identify the active substances responsible for their biological effects, which will aid in mitigating their pro-inflammatory and pathogenic properties for a safer clinical use. A clearer identification of the bioactive components and the molecular pathways responsible for their immunomodulatory, inflammatory, or pathogenic effects is needed to mitigate risks and engineer safe vesicles for human use.

A critical limitation in the field remains the lack of standardized, universally accepted protocols for MV isolation, purification, and quantification. The reliance on differential ultracentrifugation, although widespread due to its practicality, often leads to inconsistent yields and reduced purity levels. Density gradient ultracentrifugation is currently the method of choice for further purification, but its scalability is limited. Advances in alternative techniques such as size exclusion chromatography, tangential flow filtration, and affinity-based purification strategies may help to overcome these barriers. Interlaboratory validation and method harmonization are urgently required to improve reproducibility and accelerate regulatory acceptance. However, the transition from laboratory-scale to industrial-scale production remains as a major challenge. The inherent low yield of vesicle release, coupled with the need for high-volume bacterial cultures, complicates large-scale manufacturing. Moreover, detoxification processes, whether chemical, physical, or genetic, are often difficult to implement under conditions compatible with large-scale bioprocessing. Ensuring that detoxification protocols preserve vesicle integrity, biological activity, and batch-to-batch reproducibility remains as a crucial but unresolved bottleneck for their translation to clinical use.

To promote methodological rigor and harmonization, major efforts are being coordinated by international societies. Within the International Society for Extracellular Vesicles (ISEV), a Bacterial EVs Task Force was launched in 2021 [272] with the aim of standardizing nomenclature, preparation, characterization, and functional testing of bacterial EVs. A position paper [273] published by members of this society recommended to report the growth phase of bacteria when collecting EVs, to reduce the storage time before EVs isolation, to take into account that from in vivo or environmental sources bacterial EVs can be collected together with EVs from the host, and to exclude co-isolated non-vesicular components present in the medium including pili, flagellae, phage, protein, lipoprotein and nucleoprotein complexes [273]. In parallel, the Microbial Vesicles Task Force of the Chinese Society for Extracellular Vesicles has issued complementary guidelines, suggesting a unified nomenclature and providing specific considerations for vesicle collection from different fluids, pre-processing, and storage conditions, as well as discussing the strengths and limitations of current isolation and characterization approaches [274]. These consensus efforts represent a crucial step toward the development of global standards for bacterial MVs research.

The future of MV research will benefit greatly from the integration of cutting-edge analytical tools such as single-vesicle omics (proteomics, transcriptomics), high-resolution imaging, and nanoparticle tracking.

These technologies will provide unprecedented insights into MV heterogeneity, cargo sorting mechanisms, and intercellular communication pathways. The combination of vesicle profiling with CRISPR-based gene editing and synthetic biology offers exciting opportunities to customize the vesicle content and surface display for targeted delivery or immune modulation.

From a translational perspective, bioengineering strategies are already expanding the therapeutic applications of MVs. Modifying MVs to reduce endotoxicity, such as through lipid A deacylation or virulence factor deletion, combined with strategies to enhance targeting (e.g., surface display of ligands or antibodies), positions MVs as promising candidates for cancer immunotherapy, vaccination, and antimicrobial therapies. Engineered MVs have also shown potential as imaging agents and carriers for gene therapy, opening new horizons in personalized medicine.

Consequently, MVs offer a promising yet complex frontier for biomedical innovation. Continued efforts to understand their biology, improve their production, and tailor their functionalities through synthetic biology and nanotechnology will be crucial. With sustained interdisciplinary collaboration, bacterial MVs could evolve from experimental tools into clinically validated platforms in diagnostics, drug delivery, and next-generation vaccines.

CRediT authorship contribution statement

Manuel Arruebo: Writing – review & editing, Writing – original draft, Conceptualization. **Gonzalo Canalejo-Marco:** Writing – review & editing, Writing – original draft, Formal analysis. **Victor Sebastian:** Writing – review & editing, Writing – original draft, 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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Data availability

No data was used for the research described in the article.

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