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Review article

Designing nature's nanocarriers: advances and challenges in functionalizing bacterial extracellular vesicles☆

Varunaa Sri Hemanth Kumar [a], Hannah C. Zierden [a,b,c,d,*], Sara Molinari [a,b,c,*]

[a] Fischell Department of Bioengineering, University of Maryland, College Park, MD 20742, United States of America
[b] Department of Chemical & Biomolecular Engineering, University of Maryland, College Park, MD 20742, United States of America
[c] Robert E. Fischell Institute for Biomedical Devices, University of Maryland, College Park, MD 20742, United States of America
[d] Department of Obstetrics, Gynecology and Reproductive Sciences, University of Maryland School of Medicine, Baltimore, MD 21201, United States of America

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ABSTRACT

Bacterial extracellular vesicles (bEVs) are nanoscale, membrane-bound particles naturally secreted by bacteria and are increasingly being explored as therapeutic carriers. Their small size, ability to encapsulate and protect cargo, inherent bioactivity, and compatibility make them attractive candidates for therapeutic development. However, precise and reproducible functionalization remains a central challenge for clinical translation. Post-isolation physical and chemical modification methods enable cargo loading and surface conjugation but are often limited by membrane disruption, heterogeneous functionalization, and scale-up constraints. In contrast, genetic engineering approaches program vesicle composition during biogenesis, enabling controllable luminal loading, surface display, and hypervesiculation. In this review, we examine luminal targeting strategies (secretion pathway-mediated loading, lipoprotein anchoring, and membrane scaffold fusions), surface display platforms (ClyA, Lpp-OmpA hybrids, autotransporters, and ice nucleation protein), and genetic yield-enhancement mechanisms. Finally, we outline key translational considerations to advance bEVs toward clinically viable, programmable delivery systems.

1. Introduction

Cellular communication regulates all biological processes [1,2]. These communication pathways have inspired innovation in drug-delivery approaches for decades, with recent growing interest in extracellular vesicles as a key mode of cellular signaling. Extracellular vesicles (EVs) are membrane-bound, cell-derived, nano-sized particles that carry diverse cargoes to facilitate long-distance communication in the body [3]. All cell types produce EVs, and fundamental studies regarding EV function have revealed these particles to have potential as next-generation therapeutic carriers [4]. Their biocompatibility, low immunogenicity, and inherent ability to cross biological barriers make them attractive as both delivery vehicles and standalone therapeutic agents, while their natural targeting capacity and stability in biological fluids further distinguish them from synthetic nanocarrier platforms [5–7]. Bacterial EVs (bEVs), in particular, show promise as a therapeutic platform for treating a range of human diseases [8].

bEVs are naturally secreted by both Gram-positive and Gram-negative bacteria. Depending on the bacterial origin and biogenesis pathway, bEVs are classified into several subtypes based on biogenesis: outer membrane vesicles (OMVs), explosive outer membrane vesicles (EOMVs), outer-inner membrane vesicles (OIMVs), and cytoplasmic membrane vesicles (CMVs) (Fig. 1) [9]. bEVs, an umbrella term for all bacteria-derived vesicles, are naturally enriched with a diverse array of biomolecules, including proteins, nucleic acids, lipids, and metabolites. These cargo signatures play a key role in microbial communication, immune modulation, and pathogenesis [3,8,10–12]. bEV cargoes reflect the molecular landscape of parent cells [9], and the packaging provided by the bEV membrane protects these cargoes from enzymatic degradation, supporting the stable delivery of functional payloads [13]. In addition to potentially probiotic cargoes, bEVs have unique structural and functional attributes that make them well-suited for drug delivery. First, the small size of bEVs (100–300 nm) allows them to diffuse across mucosal, epithelial, and endothelial barriers and to distribute throughout the body, enabling long-distance communication [10,14]. Additionally, the lipid bilayer membrane enables fusion with host cell

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* Corresponding authors at: Fischell Department of Bioengineering, University of Maryland, College Park, MD 20742, United States of America.
E-mail addresses: hzierden@umd.edu (H.C. Zierden), saramol@umd.edu (S. Molinari).

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membranes and facilitates direct delivery of luminal cargo [11]. Beyond membrane fusion, bEVs can also enter host cells through multiple endocytic pathways, including receptor-mediated endocytosis, clathrin- or caveolin-dependent uptake, macropinocytosis, and other non-receptor-mediated internalization mechanisms [11,15–17]. Following internalization, luminal cargo must still escape the endosomal or phagosome compartment to reach the cytosol. Many of these characteristics, such as simultaneous surface ligand display and protected luminal cargo delivery, are difficult to achieve with synthetic nanoparticles due to their limited structural complexity [18,19]. Together, these attributes position bEVs as a versatile delivery platform capable of addressing therapeutic challenges across a wide range of applications [20].

Therapeutic and engineered bEVs are commonly derived from probiotic and commensal bacteria, such as *Escherichia coli* Nissle 1917, *Lactobacillus* spp., and *Bifidobacterium* spp. due to their favorable safety profiles and host compatibility [21]. In addition, vesicles from symbiotic and environmentally associated species, including *Vibrio fischeri*, have been widely studied for their roles in host-microbe communication and vesicle-mediated signaling [22,23]. Compared with mammalian and plant-derived EVs, bEVs offer several advantages for large-scale cGMP manufacturing, including rapid bacterial growth kinetics, lower production costs, and greater amenability to genetic manipulation, enabling more reproducible and scalable bioprocessing workflows [24]. Despite these advantages, several key challenges limit the clinical translation of bEV therapeutics, including heterogeneity in bEV composition, potential immunotoxicity, and limited cargo loading efficiency [17,18].

Nonetheless, bEVs bridge a critical gap between synthetic and cellular therapeutics, enabling the development of programmable, biologically integrated solutions for precision medicine. This review highlights advances and challenges associated with bEV-based therapeutics, with a particular emphasis on genetic engineering approaches to load, functionalize, and enhance the production of bEVs.

2. Overview of bacterial extracellular vesicles

2.1. Biogenesis

Gram-negative bacteria produce bEVs through two distinct biogenesis mechanisms that influence composition and function. Outer membrane blebbing occurs when the outer membrane of a Gram-negative microbe protrudes and pinches off to form outer membrane vesicles (OMVs). This occurs in response to various environmental and physiological stimuli, including temperature fluctuations, nutrient limitation, oxidative stress, quorum sensing, and exposure to envelope-targeting antibiotics [19,25–29]. Explosive cell lysis results from endolysin-mediated degradation of the peptidoglycan layer, thereby rupturing the bacterial envelope. This process releases explosive outer membrane vesicles (EOMVs), which are derived exclusively from the outer membrane, as well as outer-inner membrane vesicles (OIMVs), which incorporate components from both the inner and outer membranes due to the violent nature of lysis. These mechanistic differences carry important implications for engineering, as each pathway generates vesicles with distinct cargo profiles and membrane compositions [9].

Gram-positive bacteria, which lack an outer membrane and possess a thick peptidoglycan cell wall, produce bEVs through a less well-understood process. They are produced through a phenomenon termed “bubbling cell death,” in which the plasma membrane protrudes through weakened areas of the cell wall, giving rise to cytoplasmic membrane vesicles (CMVs) [9]. These vesicles originate from the inner membrane and can carry cytosolic and membrane-associated components. Future work may consider how bacterial morphology or aspect ratio impacts bEV formation or composition relevant to their use as therapeutic carriers.

In addition to vesicle budding and lysis-associated pathways, both Gram-negative and Gram-positive bacteria form membrane-derived nanotube structures that facilitate intercellular exchange of proteins, nucleic acids, metabolites, and other cytoplasmic components [30]. Although the relationship between bacterial nanotubes and bEV biogenesis remains incompletely understood, recent evidence suggests that nanotube extrusion may arise from membrane destabilization during cell stress or death, paralleling mechanisms of vesicle release [31]. Therefore, nanotubes represent a complementary route of extracellular cargo transfer relevant to bEV formation and function [31].

The structural differences between Gram-negative and Gram-positive envelopes lead to distinct vesicle compositions and biogenesis pathways, posing unique challenges and opportunities for engineering in each bacterial class [9]. For the purposes of this review, OMVs, OIMVs, EOMVs, and CMVs are collectively referred to as bEVs.

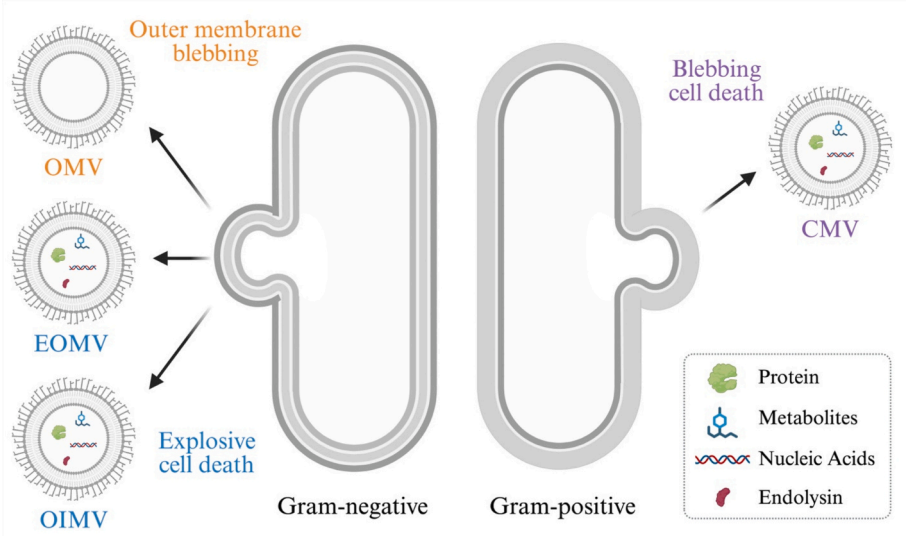

Fig. 1. Biogenesis pathways of bacterial extracellular vesicles in Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, outer membrane blebbing (orange) produces outer membrane vesicles (OMVs) containing only outer membrane and periplasmic content, while explosive cell death (blue) releases explosive outer membrane vesicles (EOMVs) and outer-inner membrane vesicles (OIMVs) that additionally carry cytoplasmic proteins, metabolites, nucleic acids, and endolysin. In Gram-positive bacteria, explosive blebbing cell death (purple) produces cytoplasmic membrane vesicles (CMVs) containing similar cytoplasmic cargo. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. bEV cargo and phenotypes

Naturally incorporated cargo and surface characteristics differ substantially between Gram-negative and Gram-positive bEVs as a direct consequence of differing membrane structures and biogenesis mechanisms [9,32]. Gram-negative bEVs have an outer membrane-derived lipid bilayer enriched in lipopolysaccharide (LPS), outer membrane proteins, lipoproteins, and periplasmic components [9,13,32]. Additionally, lysis-derived vesicles encapsulate cytoplasmic proteins and nucleic acids [9,13,32]. The presence and structural heterogeneity of LPS strongly influence vesicle surface charge, immunogenicity, and interactions with host cells, while native outer membrane proteins provide inherent surface functionality but limited control over orientation or density [9,13,32]. In contrast, Gram-positive bacteria produce cytoplasmic membrane vesicles that lack LPS and instead display phospholipids and cell wall-associated components, such as lipoteichoic acids and surface-anchored proteins [9,32,33]. The luminal contents of Gram-positive-derived bEVs reflect cytoplasmic and inner membrane-associated materials that traverse the thick peptidoglycan layer during vesicle formation [9,32,33]. These differences result in distinct cargo profiles, surface chemistries, and immune recognition properties, which shape the potential of bEVs to be used for drug delivery applications.

Beyond biogenesis, bEV characteristics are shaped by the isolation methods used to purify bacterial cultures. Ultracentrifugation remains widely used for its simplicity, but often produces broader, more heterogeneous particle size distributions and greater co-isolation of protein aggregates than size-exclusion chromatography (SEC), which produces smaller, more structurally homogeneous bEV populations with significantly improved batch-to-batch reproducibility, although this improved homogeneity may vary by parent cell [34]. Tangential flow filtration (TFF) offers a gentler, more scalable alternative that reduces shear-induced aggregation relative to ultracentrifugation and has increasingly been paired with SEC or high-performance anion-exchange chromatography (HPAEC) to further improve purity. HPAEC has been shown to enhance both purity and anti-inflammatory efficacy of probiotic-derived bEV preparations [35,36]. Commercial isolation kits allow EV recovery within approximately 30 min but offer limited scalability [35].

Culture conditions add additional variability that shapes both bEV production and cargoes. Growth phase, media composition, temperature, pH, and oxygen availability alter bEV protein, lipid, and nucleic acid content, ultimately changing how bEVs interact with host cells, independent of isolation method [37]. Taken together, isolation method and culture condition represent two largely independent sources of variability.

Methodological variability, along with inconsistent reporting practices, make it difficult to compare bEVs across studies, limiting the translation of bEV-based therapeutics. To address this, the International Society for Extracellular Vesicles (ISEV) has published the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines, most recently updated as MISEV2023, which establish community standards for EV study design, execution, and reporting [38]. Because bacterial EVs lack the universal surface markers (e.g., CD63, CD81) used to validate mammalian EVs, purity assessment instead relies on a combination of physical characterization (transmission electron microscopy, nanoparticle tracking analysis), protein-level quality control, and species-specific markers. In recognition of these gaps, ISEV's Bacterial EVs Task Force is working specifically toward standardized nomenclature, purity markers, and preparation protocols tailored to bacterial systems, reflecting a broader field-wide push toward the kind of rigor these methodological comparisons demand.

3. Bacterial extracellular vesicles in drug delivery

Building on the diverse biogenesis pathways outlined in the previous section, selecting an appropriate functionalization strategy for engineered bEVs remains a central challenge. Existing approaches broadly fall into physical, chemical, and genetic categories, each associated with distinct advantages and limitations. Physical and chemical post-isolation strategies offer rapid, modular means to introduce cargo or surface functionalities; however, multiple studies demonstrate that these approaches can compromise membrane integrity, generate heterogeneous bEV populations, and exhibit limited reproducibility [37]. Such drawbacks are particularly problematic in translational settings, where dose consistency, scalable manufacturing, and regulatory compliance are essential considerations [37].

3.1. Non-genetic loading strategies

Physical post-isolation functionalization strategies are widely used for loading luminal cargo and for surface functionalization. These approaches rely on non-covalent, membrane-disruptive, or mechanical processes to enable cargo incorporation without genetic manipulation of the parent bacteria. Common physical methods include cargo acquisition during bacterial growth, passive incubation, sonication, electroporation, and extrusion (Fig. 2). Cargo size and physicochemical properties determine loading feasibility across all physical methods. Compared with mammalian EVs, bEVs tend to be smaller, reducing the luminal volume and constraining both the amount and size of cargo that can be loaded. Large biomolecules, such as high-molecular-weight proteins or long nucleic acid constructs, are disproportionately limited by this reduced luminal space. For nucleic acids, electrostatic repulsion between the negatively charged phosphate backbone and the anionic inner leaflet of the bEV membrane further impedes passive membrane crossing [39,40]. These constraints apply to passive incubation, sonication, electroporation, and extrusion methods, and should inform cargo selection and loading protocols (Fig. 2 and Table 1).

Passive incubation is the simplest and most used loading strategy, which can be achieved by culturing bacteria in the presence of therapeutic agents or by incubating bEVs with cargo after isolation (Fig. 2A). This approach is commonly used to load antibiotics into bEVs [41,42]. Hydrophilic molecules enter the periplasmic space through outer membrane porins. As the outer membrane buds outward during vesicle biogenesis, target cargoes are enclosed within bEVs via bulk entrapment [39]. In contrast, hydrophobic or amphipathic molecules partition directly into the lipid bilayer of the outer membrane and are co-packaged into bEVs as they are produced [43]. In the case of antibiotics, low concentrations may increase bEV production through envelope stress responses. Cell-wall-active antibiotics such as β-lactams (ceftriaxone, ampicillin) inhibit penicillin-binding proteins (PBPs), promoting outward membrane bulging and hypervesiculation [39]. Fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin) induce the bacterial SOS response, which has been shown to substantially increase bEV production independently of direct envelope disruption [44]. Passive incubation of antibiotics was used to engineer bEVs to treat gastrointestinal infections while reducing the total antibiotic dose [41].

Passive incubation of therapeutics after bEV isolation is used to load small-molecule hydrophobic cargoes (Fig. 2B). Two thermodynamically driven processes can incorporate hydrophobic molecules into the bEV membrane: membrane partitioning and passive diffusion [45]. Membrane partitioning is the dominant mechanism, in which hydrophobic and amphiphilic molecules preferentially migrate out of aqueous solution into the lipid bilayer, driven by the hydrophobic effect [46,47]. The partition coefficient (logP) is the key physicochemical predictor of this behavior; molecules with higher logP values exhibit greater affinity for the membrane phase relative to the aqueous phase and are stabilized within the bilayer interior through van der Waals interactions with the fatty acyl chains [45]. Therefore, hydrophobic cargo preferentially localizes within the membrane rather than the aqueous lumen. Passive diffusion plays a secondary role, particularly for moderately hydrophobic molecules that retain partial aqueous solubility. A concentration gradient between the incubation medium and the vesicle membrane drives net movement toward the bilayer until equilibrium is reached

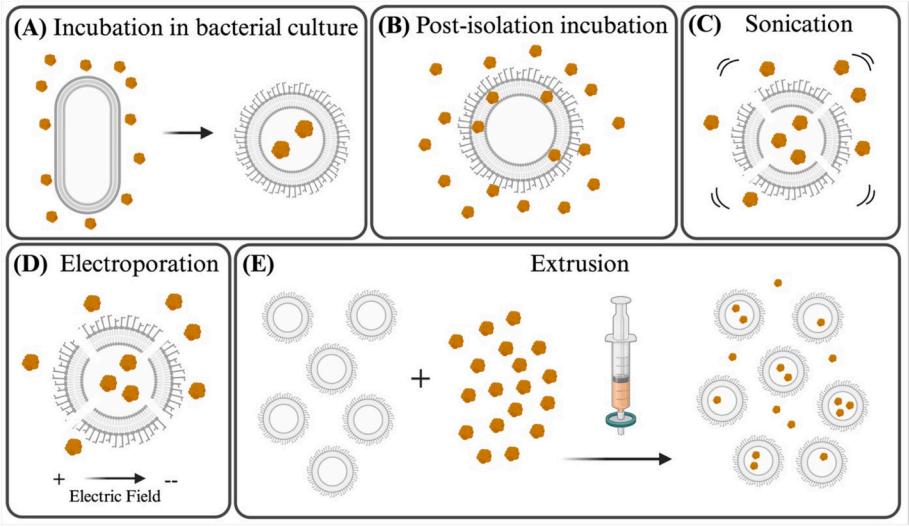

Fig. 2. Methods for loading cargo into isolated bEVs. (**A**) Incubation in bacterial culture, in which cargo (orange) is added directly to the bacterial culture and becomes encapsulated during natural vesicle biogenesis. (**B**) Post-isolation incubation, in which purified vesicles are passively incubated with cargo, allowing spontaneous membrane permeation and loading. (**C**) Sonication, in which ultrasonic energy transiently disrupts the vesicle membrane to allow cargo entry before resealing. (**D**) Electroporation, in which an applied electric field transiently permeabilizes the vesicle membrane to drive cargo influx. (**E**) Extrusion, in which pre-formed vesicles are mixed with cargo and mechanically forced through a membrane filter, promoting membrane fusion/reformation around the cargo.

[48]. This process is slow and temperature-dependent, contributing to the extended protocol times and variable loading efficiencies commonly observed. Notably, membrane saturation imposes an upper loading limit; once the bilayer reaches capacity, additional cargo cannot be incorporated, which partly explains the high drug concentrations required for passive loading workflows [4]. Passive incubation has been used to load small-molecule chemotherapeutics, such as doxorubicin, into bEVs, resulting in enhanced antitumor efficacy in non-small-cell lung cancer models [25]. Similarly, passive loading of hydrophobic compounds, such as curcumin, has been shown to suppress tumor development by modulating the polarization of tumor-associated macrophages. In one study, curcumin served as a model compound to demonstrate the broad applicability of passive loading to small-molecule therapeutics [26].

Passive incubation can also be used to functionalize the surface of bEVs. In one study, DSPE-PEG-paclitaxel and DSPE-PEG-mannose lipid-polymer conjugates were incubated with bEVs to enable tumor microenvironment-responsive prerelease and to target M2 macrophages. This approach enhanced siRNA delivery and was associated with tumor-associated macrophage repolarization, tumor suppression, immune activation, and remodeling of the tumor microenvironment in a triple-negative breast cancer model [55].

Passive loading is attractive due to its simple workflow and mild conditions, which help preserve vesicle integrity. However, passive loading preferentially favors small, hydrophobic cargoes, making it challenging to efficiently load large, hydrophilic, or unstable molecules that may not readily diffuse across the membrane or may degrade during prolonged incubation. In addition, passive loading often requires high initial drug concentrations, which can be problematic for costly therapeutics and frequently results in low and variable loading efficiencies, leading to inconsistent cargo dosage per vesicle [8]. Additionally, high concentrations of unencapsulated drug often remain in the surrounding medium following incubation, adding a necessary removal step prior to downstream applications [48,63]. Separating free drug from loaded bEVs without compromising vesicle yield or integrity is non-trivial, and no standardized guidelines currently exist for method selection [35,63]. SEC is widely regarded as the most vesicle-compatible option, resulting in bEVs with preserved membrane integrity and higher bEV recovery than ultracentrifugation [38,63,64]. Ultracentrifugation, though widely used, is associated with vesicle aggregation and mechanical stress, while density gradient centrifugation offers improved purity at the cost of substantially longer processing times [38,63,65]. Ultrafiltration is faster and accommodates larger volumes, but is susceptible to membrane fouling and yield losses from non-specific vesicle adhesion [63,66]. Dialysis is the simplest approach and preserves vesicle integrity, but is slow and may be ineffective for highly hydrophobic cargoes [48,63]. As with other methodological considerations, purification protocols must be tailored to cargo properties, required throughput, and downstream application [35].

To improve loading efficiency, active physical methods, such as electroporation and sonication, are often employed. Sonication uses acoustic energy to induce transient membrane deformation and pore formation, enabling efficient cargo loading (Fig. 2C). Key parameters include amplitude, pulse duration, on/off cycle intervals, and sample temperature. Excessive energy input may cause membrane protein denaturation, cargo degradation, and increased polydispersity, while insufficient energy fails to achieve adequate membrane disruption for loading [45,48,51,52]. Performing sonication in pulsed cycles on ice or in a temperature-controlled bath is widely recommended to dissipate heat and preserve both vesicle morphology and cargo activity [48,53]. This method was used to incorporate curcumin-loaded nanoparticles into bEVs, followed by passive co-incubation with bovine serum albumin to improve biocompatibility and tumor-targeting capability, resulting in enhanced therapeutic efficacy [54].

Electroporation utilizes short electrical pulses to transiently permeabilize the vesicle membrane, facilitating the encapsulation of hydrophilic and charged biomolecules (Fig. 2D). The extent of membrane permeabilization is governed by field strength, which is a function of applied voltage and electrode geometry, as well as pulse capacitance, duration, and number. These parameters must be tuned to achieve sufficient membrane opening without inducing irreversible damage, vesicle aggregation, or cargo precipitation. Similarly, buffer composition affects loading, as the ionic strength and osmolarity of the electroporation medium directly influence vesicle recovery and effective loading efficiency [56]. Mild, low-voltage protocols that prioritize membrane resealing over maximal permeabilization have been shown to better preserve vesicle integrity and functional surface markers compared to high-field approaches [57]. This approach has been widely used for nucleic acid delivery, including siRNA-loaded bEVs for cancer vaccination and immunotherapy, in which electroporation of kinesin spindle

Table 1
Physical bEV functionalization strategies. Comparison of post-isolation physical loading methods used to incorporate cargo into bEVs, including incubation in bacterial culture, post-isolation passive incubation, sonication, electroporation, and extrusion.

Strategy	At a glance	Mechanism	Advantages	Disadvantages	Optimization parameters	Ref.
Incubation in bacterial culture	**Preferred Cargo**: Antibiotics **Cost**: Low **Expertise**: Low **Throughput**: Moderate **Usage**: Moderate	During active cell growth, antibiotics introduced into the culture medium accumulate in the periplasmic space or associate with outer membrane proteins. During bEV biogenesis these antibiotics are passively packaged into the vesicles.	• No extra processing steps required • Scalable with bacterial culture volume • Preserves native bEV membrane integrity	• Low control over loading efficiency • Limited to molecules compatible with bacterial growth conditions • Cargo can partition into the periplasm rather than the bEV lumen	• Growth phase • Drug concentration • Incubation duration • Culture conditions	[39,41–44]
Post-isolation incubation	**Preferred Cargo**: Small, hydrophobic molecules **Cost**: Low **Expertise**: Low **Throughput**: High **Usage**: High	Isolated bEVs are incubated directly with small hydrophobic molecules, which naturally partition into the lipid bilayer.	• Simple procedure; no specialized equipment required • Mild conditions preserve bEV membrane integrity • High encapsulation efficiencies achievable with ratio optimization	• Low encapsulation efficiency for large or highly hydrophilic molecules • Requires optimization of temperature, time, and drug: vesicle ratios • Difficult to remove unbound cargo completely	• Temperature • Duration • Drug: vesicle ratio • Agitation • Post-processing purification methods	[45–50]
Sonication	**Preferred Cargo**: Hydrophilic molecules **Cost**: Moderate **Expertise**: Moderate **Throughput**: Moderate **Usage**: Moderate	Ultrasonic waves transiently disrupt the bEV lipid bilayer, opening pores that allow hydrophilic molecules to diffuse into the vesicle lumen. The membrane then reseals during a recovery period, encapsulating the loaded cargo.	• Improves loading efficiency compared to passive incubation • Promotes transient membrane disruption enabling hydrophilic cargo entry • Can be used to coat synthetic nanoparticles with bEV membranes	• Membrane disruption can cause vesicle aggregation • Heterogeneous cargo loading across vesicle population • Excessive sonication duration degrades bEV structure • Requires specialized equipment	• Frequency • Duration • Cycle pattern • Bath temperature • Amplitude • Recovery conditions • Sample volume	[42,45,48,51–54]
Electroporation	**Preferred Cargo**: Charged molecules and nucleic acids **Cost**: Moderate - High **Expertise**: Moderate **Throughput**: Moderate **Usage**: Moderate	Short, high-voltage electrical pulses generate an electric field across the bEV membrane, opening pores that allow charged molecules to diffuse into the vesicle lumen. The membrane then reseals during a recovery period, encapsulating the loaded cargo.	• Well-characterized protocol for nucleic acids (DNA, RNA, siRNA) • Higher loading efficiency for charged molecules vs. other physical methods • Tunable parameters	• bEV membrane disruption and vesicle aggregation • Requires optimization for each cargo type • Specialized electroporation equipment required	• Voltage • Pulse duration • Number of pulses • Buffer composition • Recovery conditions • Cargo concentration	[55–58]
Extrusion	**Preferred Cargo**: Large, hydrophilic molecules **Cost**: Moderate **Expertise**: Moderate **Throughput**: Low **Usage**: Low	bEVs and cargo are *co*-extruded through polycarbonate membranes under mechanical pressure. The repeated deformation and reformation of the membrane as vesicles pass through drives passive cargo encapsulation.	• Can improve size uniformity of loaded vesicles • Enables encapsulation of larger cargo and nanoparticle complexes • Allows sequential surface modification during extrusion steps	• Repeated membrane disruption can cause vesicle aggregation and affect integrity • Reduced efficiency for some cargo types • Multi-step procedure increases process complexity • Requires extrusion apparatus and optimization of pore size	• Pore size • Number of passes • Temperature • Cargo: vesicle ratio • Sequential pore sizing	[59–62]

protein siRNA or Redd1 siRNA induced potent anti-tumor immune responses [55,58]. Comparative studies loading fluoroquinolones into bEVs demonstrated that active loading techniques, particularly low-voltage, single-pulse electroporation, achieve higher encapsulation efficiencies than passive incubation. However, this study reported that sonication yielded more consistent loading results than electroporation [42].

Extrusion-based methods rely on repeated mechanical forcing of vesicles and cargo through nanoporous membranes to promote membrane fusion and cargo incorporation (Fig. 2E). Critical parameters include membrane pore size, number of extrusion cycles, and applied pressure. Sequential extrusion through progressively smaller pore sizes has been shown to improve both loading efficiency and size homogeneity, while excessive cycles or pressures can induce membrane stress and reduce vesicle recovery [59]. Membrane clogging during repeated passes is a practical limitation that must be anticipated, particularly when extruding dense bEV preparations or viscous cargo formulations [60]. Extrusion has been used to generate modular nanoplatforms by physically integrating outer membrane vesicles with nanoparticles for cancer immunotherapy, enabling coordinated activation of innate and adaptive immune responses [61]. Similarly, extrusion of bEVs with DSPE-PEG-modified agents through 200 nm polycarbonate membranes has been reported to facilitate photothermal-immune synergistic therapy [62].

Physical post-isolation functionalization strategies have also been applied to Gram-positive bEVs, underscoring their broad versatility. Doxorubicin-loaded *Lactobacillus*-derived bEVs prepared using passive incubation, electroporation, or sonication exhibited approximately six-fold greater antibacterial activity against *Staphylococcus* spp. compared with free doxorubicin, demonstrating the capacity of bEVs to enhance drug delivery even in organisms lacking an outer membrane [67]. While Gram-negative bEVs possess an outer membrane which provides both a lipid barrier for hydrophobic partitioning and an aqueous luminal compartment for hydrophilic cargo, Gram-positive bEVs are bounded by a single phospholipid membrane, resulting in a simpler membrane and smaller luminal volume [9,33,68]. The absence of an outer membrane and periplasmic space may reduce the barrier function that protects luminal cargo during electroporation- or sonication-induced permeabilization, potentially increasing susceptibility to membrane damage or cargo leakage under equivalent energy inputs. At the same time, the Gram-positive membrane may simplify passive diffusion-based loading of hydrophobic small molecules, as cargo need only cross one lipid bilayer rather than navigate an outer membrane, periplasm, and inner membrane. Passive incubation, electroporation, and sonication were all successfully used to load doxorubicin into *Lactobacillus*-derived bEVs, suggesting these methods are broadly transferable across bacterial classes [67].

Overall, physical functionalization strategies offer versatile, potentially scalable approaches for engineering bEV-based therapeutic systems, but the choice of method requires careful consideration of cargo properties, functional requirements, and translational constraints. While passive incubation is best suited for small, low-cost, hydrophobic molecules when preservation of membrane integrity is a priority, it is limited by variable loading efficiencies. Electroporation enables higher encapsulation of hydrophilic or charged cargoes but is often associated with membrane disruption and increased heterogeneity. Sonication can achieve comparably high loading efficiencies with improved reproducibility, although at the cost of partial structural perturbation [42]. Extrusion offers an additional means of enhancing loading uniformity and vesicle homogeneity, but its mechanical stress and scalability limitations must also be considered. Collectively, these trade-offs highlight the importance of matching physical loading strategies to the intended therapeutic cargo and downstream application. Despite their widespread use, the limitations of physical functionalization strategies, including membrane disruption, heterogeneous loading, and scalability challenges, restrict their applicability in settings requiring precise and reproducible bEV engineering. As a result, chemical functionalization approaches have emerged as complementary strategies that offer greater control over cargo attachment and surface modification through defined chemical interactions [69].

3.2. Chemical functionalization

Chemical functionalization strategies are widely used for surface engineering of bEVs. These approaches rely on covalent or affinity-based reactions with functional groups naturally present on the vesicle surface, enabling cargo attachment without genetic modification of the parent bacteria. Chemical functionalization is particularly attractive for workflows that prioritize speed, modularity, and compatibility with diverse bacterial strains (Fig. 3 and Table 2) [69].

Covalent conjugation represents the most widely applied chemical strategy for bEV surface functionalization (Fig. 3A). The most common approach targets surface amines, which are abundant on lysine residues of outer membrane proteins. This is typically achieved using carbodiimide chemistry, most often EDC/NHS, which activates carboxyl groups on the bEV surface that can react with primary amines present on target ligands [69]. This method is popular because it works in water, uses inexpensive reagents, and requires minimal chemical expertise. It also allows a variety of cargoes, such as peptides, polymers, small-molecule drugs, and immune-modulating ligands, to be attached directly to bEV surfaces [70,71]. Amine-based conjugation has been widely applied in immunotherapy studies. One study developed a platform that leveraged NHS chemistry to install linker groups on bEVs, enabling them to capture tumor antigens released in situ while simultaneously delivering the immunoregulatory drug 1-methyl-tryptophan, thereby enhancing antitumor immune responses [72]. Another study generated antigen-capturing bEVs that trafficked efficiently to lymph nodes and promoted strong cytotoxic T-cell responses [73]. Despite its ease of use, amine-directed chemistry suffers from limited precision because lysine is widely distributed across the bEV surface, resulting in random ligand placement and batch-to-batch variability that can affect targeting performance and reproducibility.

Thiol-directed modification offers greater chemical selectivity by exploiting the reaction between surface-exposed cysteine thiols and maleimide-functionalized molecules. This reaction forms a stable thioether bond under mild, aqueous conditions and is more selective than amine labeling, since thiols are less abundant on bEV surfaces [74,75]. Although thiol density is typically low and susceptible to oxidative inactivation, which limits overall efficiency, thiol chemistry enables more sophisticated designs that are difficult to achieve with amine-based approaches [76]. For example, bEVs were coated with drug-loaded albumin through a matrix metalloproteinase-cleavable thiol linker. In circulation, the albumin shell masked bEV immunogenicity and prolonged blood residence time, whereas in the tumor microenvironment, the linker was selectively cleaved, releasing both bEVs and the drug locally [77]. Thiol chemistry has also been used to construct more

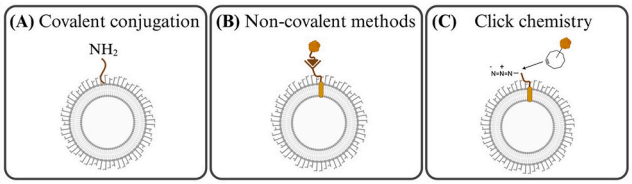

Fig. 3. Chemical conjugation strategies for post-isolation cargo attachment to vesicles. (**A**) Covalent conjugation, using functional groups on the vesicle surface for direct chemical crosslinking of cargo. (**B**) Non-covalent methods, in which cargo is attached via affinity-based or electrostatic interactions between a surface-anchored linker and the cargo molecule. (**C**) Click chemistry, in which bioorthogonal reactive groups added on the vesicle surface and cargo react selectively to form a stable covalent linkage.

Table 2
Chemical bEV functionalization strategies. Overview of post-isolation chemical conjugation approaches for bEV surface functionalization, encompassing covalent conjugation, non-covalent methods, and click chemistry.

Strategy	At a glance	Mechanism	Advantages	Disadvantages	Optimization parameters	Ref.
Covalent conjugation	**Preferred Molecules**: Targeting ligands, antibodies, peptides bearing amine or carboxyl groups **Cost**: Moderate **Expertise**: Moderate **Throughput**: Moderate **Usage**: Moderate	Amine, carboxyl, or thiol groups exposed on the surface of bEV membrane proteins are covalently crosslinked to molecules using bifunctional reagents.	• Stable cargo attachment via covalent bond • Enables modular, customizable ligand conjugation to bEV surface • Compatible with a wide range of functional groups and cargo types • Well-established chemistry with predictable reaction outcomes	• Limited precision in attachment sites • Risk of altering membrane proteins • May require sequential reactions and extra purification to remove excess crosslinker • Potential loss of bEV structural integrity at high reagent concentrations	• EDC activation pH • Excess of crosslinker • Temperature • Reaction time • Post-processing purification methods	[69–81]
Non-covalent methods	**Preferred Molecules**: PEGylated molecules, DSPE-lipid conjugates, targeting antibodies, charged polymers **Cost**: Low **Expertise**: Low **Throughput**: High **Usage**: High	Cargo associates with the bEV surface via reversible non-covalent interactions through a variety of mechanisms, including hydrophobic lipid anchor insertion, electrostatic adsorption, and streptavidin-biotin bridging.	• Allows reversible functionalization • Avoids harsh chemical reagents that may compromise bEV membrane integrity • Straightforward implementation without extensive chemical modification • Lipid anchor insertion preserves native surface protein accessibility	• Cargo dissociation under dilution or physiological conditions in vivo • Reduced stability in circulation compared to covalent approaches • Lower control over orientation and density of attached molecules • Potential competition between inserted lipid anchors and endogenous membrane components	• Temperature • Lipid: vesicle ratio • Incubation time • Post-processing purification methods	[82,83]
Click chemistry	**Preferred Molecules**: Targeting ligands, fluorescent probes, and antibodies **Cost**: Moderate - High **Expertise**: Moderate - High **Throughput**: Moderate **Usage**: Moderate	Bioorthogonal chemistry uses highly specific chemical groups that are pre-attached to both the bEV surface and the cargo, so that when they come into contact, they selectively bind to form a stable conjugate.	• High selectivity • Efficient reaction kinetics with high conjugation yield under mild conditions • Copper-free chemistry avoids metal cytotoxicity, making it compatible with live-cell applications.	• Complex experimental design and optimization • Higher reagent costs • Metabolic azide labeling requires bacteria to be grown for 48–72 h in a sugar solution containing azide groups. • Additional purification steps necessary post-functionalization	• Excess of crosslinker • Temperature • pH	[84–86]

complex systems, such as self-propelled bEV nanorobots with enhanced tumor penetration and immune modulation, illustrating how relatively simple chemical reactions can enable advanced biological functions [78].

Carboxyl-group modification provides a third route for chemical functionalization. Carboxyl groups are commonly found in lipopolysaccharide sugar residues and phospholipid head groups and can be activated via EDC/NHS chemistry to react with amine-containing ligands [69,79]. Compared with amine labeling, carboxyl-directed conjugation often proceeds under milder conditions and can offer improved tunability, although efficiency depends strongly on the accessibility of the carboxyl groups [69,80]. One group demonstrated this approach in the system, using carboxyl groups on hyaluronidase-expressing bEVs to attach boronic-acid ligands that enhanced tumor targeting and tissue penetration of siRNA cargoes. In this case, chemical modification complemented the bEVs' intrinsic immunostimulatory properties while adding targeting functionality that would be difficult to encode genetically [81].

Non-covalent methods enable surface functionalization through reversible interactions (e.g., hydrophobic insertion, electrostatic adsorption, or affinity binding), often avoiding harsh reagents that can compromise membrane integrity (Fig. 3B). One widely used non-covalent approach is lipid-mediated "post-insertion," in which amphiphiles (e.g., PEG-lipids or ligand-lipids) are inserted into preformed vesicle membranes via hydrophobic partitioning. One study demonstrated a post-insertion workflow on bacteria-derived EVs, highlighting its operational simplicity for surface engineering [82]. Non-covalent (affinity) display has also been used for rapid cargo presentation, where bEVs were engineered to display the RNA-binding protein L7Ae, enabling rapid adsorption of box C/D-tagged mRNA onto the bEV surface, thereby creating personalized mRNA vaccine formulations [83].

Click chemistry provides a higher-control alternative that combines the mildness of many non-covalent workflows with the durability of covalent attachment (Fig. 3C). These reactions (especially copper-free SPAAC) enable site-selective, orthogonal conjugation under physiological conditions once azides/alkynes are installed on the vesicle surface. In one example, azide groups were attached to bEVs (via metabolic labeling) and then SPAAC was used to attach a PEG layer, thereby reducing immunogenicity and enabling stimulus-triggered release [84]. Click chemistry has also been used to attach targeting ligands with high specificity. One study conjugated an azide-modified kidney-targeting peptide for precise renal delivery [85]. Click reactions have also been leveraged to build bEV-nanoparticle hybrids, where covalent coupling of functionalized bEVs to gold nanoparticles provides an example of modular composite designs [86].

Overall, chemical functionalization strategies offer versatile, modular approaches for engineering bEVs, but their successful application requires careful consideration of surface chemistry, cargo properties, and downstream translational constraints. Beyond conjugation chemistry, downstream purification represents a significant burden for chemically functionalized bEVs. Removal of unreacted crosslinkers, excess small-molecule reagents (e.g., EDC/NHS, maleimide- or azide-bearing linkers), and unconjugated cargo typically requires additional purification steps, which can reduce final bEV recovery [35,63,66]. Moreover, each of these purification methods introduces biases in the recovered bEVs, as described in Sections 2.2 and 3.1 [35,38]. Furthermore, chemical modification suffers from a lack of site-specificity. Reactive chemistries may engage native surface groups on endogenous membrane proteins, potentially masking or altering proteins that contribute to bEV targeting or immunomodulatory properties [75,76,87]. Chemical modification approaches implemented post-isolation also share key limitations with physical functionalization methods, including product heterogeneity, sensitivity to processing conditions, and challenges in scalability and reproducibility [69]. These constraints motivate a critical evaluation of physical and chemical functionalization strategies, particularly for applications that require precise, robust, and clinically translatable bEV engineering.

3.3. Challenges in post-isolation bEV functionalization

Despite the broad range of physical and chemical strategies available for post-isolation engineering of bEVs, several shared challenges limit their robustness, reproducibility, and translational potential. Physical functionalization approaches rely on membrane disruption to enable cargo loading. Electroporation, sonication, and extrusion disrupt membrane integrity to facilitate encapsulation, but this disruption can also result in irreversible membrane damage, vesicle aggregation, and reduced recovery of intact, functional bEVs. While such effects may be manageable at a small scale, they become increasingly problematic during scale-up, where reproducible vesicle integrity and yield are required. Even comparatively mild approaches, such as passive incubation, suffer from low and variable loading efficiencies that complicate dose control and reproducibility [8,42].

These challenges can be mitigated using rational selection of loading approaches. A recently proposed descriptor-guided decision framework for EV loading uses cargo physicochemical properties to predict the most effective loading method. For example, lipophilic, low-polarity cargoes are best suited to passive incubation via membrane partitioning, whereas polar, hydrogen-bond-rich or charged cargoes require permeabilization-based or mechanical disruption methods [45]. Vesicle source further constrains this choice in bEV systems, where the thick peptidoglycan layer that is characteristic of Gram-positive bEVs can limit penetration and consistency of mechanically disruptive methods relative to Gram-negative bEVs [9,33]. Taken together, cargo descriptors and parent cell structure offer a practical, generalizable starting point for method selection.

Heterogeneity is an additional challenge that spans both physical and chemical functionalization strategies. Physical methods often generate broad distributions of cargo content per vesicle [8]. Similarly, non-selective chemical conjugation produces heterogeneous ligand densities and orientations [69–71]. This variability may impact targeting efficiency and pharmacokinetics, posing significant barriers to standardization and translation. To improve homogeneity, upstream process control, including defined and reproducible culture media, standardized growth-phase harvesting, and controlled bioprocessing approaches such as batch-refeed culturing, may improve production consistency, as biogenesis is an inherent source of variability. For example, glycine supplementation during *E. coli* culture substantially increases bEV protein and lipid content while simultaneously reducing endotoxin activity, illustrating how a single upstream intervention can address multiple sources of heterogeneity at once [88]. Analogous batch-control strategies developed for mammalian EV production, such as batch-refeed culturing to sustain constant-quality output across production cycles, may offer a translatable framework for bEV manufacturing [89].

Cargo loading heterogeneity represents a distinct but equally consequential source of dose inconsistency. Standardizing protocol parameters such as the drug-to-vesicle ratio, incubation duration, and temperature reduces run-to-run encapsulation variability [48]. Post-loading removal of unencapsulated free drug by SEC or density gradient centrifugation further narrows the distribution of drug content across the vesicle population, improving dose homogeneity prior to downstream use [40].

From a manufacturing perspective, post-isolation functionalization workflows introduce added process complexity [8,90]. Moreover, the use of specialized reagents or reactive chemistries in some chemical approaches introduces additional safety and regulatory considerations that may limit scalability. Where feasible, consolidating functionalization into fewer processing steps can reduce bEV variability, though this may limit the complexity of the resulting therapeutic. Nonetheless, no single strategy fully resolves heterogeneity across cargo loading, surface functionalization, and batch consistency, underscoring the need for

combined process-analytical and engineering approaches to produce clinical-grade bEV-based therapeutics.

Collectively, these challenges highlight fundamental limitations of physical and chemical functionalization strategies for bEV-based drug delivery. While such approaches offer flexibility and rapid prototyping, their impacts on vesicle integrity, heterogeneity, cargo stability, and manufacturability limit their translatability. These considerations motivate increased interest in genetic functionalization strategies that enable cargo loading and surface functionalization during vesicle formation.

4. Genetic engineering for drug delivery applications

Genetic engineering strategies have emerged as viable opportunities to functionalize bEVs by encoding cargoes and surface modifications directly within the parent bacteria. By shifting functionalization from external modification to endogenous vesicle assembly, genetic approaches offer the potential for improved uniformity, reproducibility, and scalability [4]. Given their central importance to the rational design of bEV-based systems, the following section focuses on genetic engineering strategies for bEV functionalization, highlighting emerging approaches that enable precise control over vesicle properties and performance.

Synthetic biology enables bEV functionalization through the design of genetic cassettes, engineered DNA constructs that direct the expression of proteins, RNAs, and other elements of interest [91]. Each cassette includes four core parts: a promoter to initiate transcription, a ribosome binding site to initiate translation, a coding sequence specifying the protein product, and a terminator to end transcription [91]. These cassettes can be delivered to bacteria via two main strategies: plasmid-based expression and genomic integration [92,93]. Plasmid-based systems dominate early-stage bEV engineering work because they are easy to construct, transform with high efficiency, and pair with well-characterized, ready-to-use parts [92]. Inducible promoters on plasmids allow rapid prototyping and tunable, often high-level, expression [92]. Plasmid copy number further modulates expression strength, giving an additional lever for tuning cargo or surface-protein output [92]. While common plasmid assembly methods (Golden Gate, Gibson assembly, restriction-ligation cloning) differ in cloning efficiency, they ultimately converge on the same end goal: stitching defined DNA fragments into a functional plasmid backbone [94–97].

Plasmid DNA itself is introduced into the parent bacterium through one of several standard transformation methods. Heat-shock transformation, in which chemically competent cells take up plasmid DNA after a brief thermal pulse, is simple and widely used for laboratory strains such as *E. coli* but tends to be lower-efficiency and less suited to non-model or engineered production strains [98]. Electroporation, which uses a short high-voltage pulse to transiently permeabilize the cell envelope, achieves substantially higher transformation efficiency and is the more common choice for bEV-producing strains, particularly those engineered for hypervesiculation or heterologous surface/luminal cargo expression [99,100]. Conjugation, direct plasmid transfer from a donor bacterium via cell-to-cell contact, is used less often for standard laboratory production but becomes relevant for non-model or genetically intractable strains, including some probiotic and Gram-positive species, that transform poorly by heat shock or electroporation [101,102].

Plasmid systems come with trade-offs. The metabolic burden imposed by plasmid maintenance and strong protein overexpression can impair host fitness, alter vesicle biogenesis, and affect bEV cargo composition [103]. Plasmid retention also typically requires an antibiotic resistance cassette [92]. Loss of this plasmid can lead to population heterogeneity and reduced reproducibility. The reliance on antibiotics further raises biosafety and scalability concerns, particularly for therapeutic or industrial applications [104]. As with wildtype cultures, the timing of bEV harvest relative to culture growth is strain- and protocol-dependent. For genetically engineered strains expressing an inducible cargo or surface construct, induction is generally timed to mid-to-late exponential phase with harvest following at stationary phase, though exact windows vary by construct, promoter strength, and strain background [99,105].

To mitigate these limitations, genomic integration has emerged as a robust alternative. Here, synthetic cassettes are stably inserted into the host chromosome, ensuring uniform expression across the cell population without ongoing antibiotic selection. Genome integration can be achieved using suicide plasmids, the lambda red system, the ClosTron method, and the CRISPR-Cas system [106–110]. Genomic integration reduces metabolic burden, enhances batch-to-batch consistency, and is more compatible with regulatory frameworks relevant to clinical development and biomanufacturing [111,112]. Its primary limitation is that only a single genomic copy of the cassette is typically present, often necessitating additional promoter and codon optimization to match the expression levels achievable with multicopy plasmids [113].

Given the limited number of studies investigating the genetic functionalization of Gram-positive bEVs, this review primarily focuses on genetic engineering strategies developed for Gram-negative bEVs. This may be attributed to the fact that Gram-positive bacteria generally transform at lower efficiency than Gram-negative species [114]. In addition to their membrane structure, which makes genetic engineering difficult, Gram-positive species have fewer well-characterized promoters, plasmid origins, and other genetic parts [115]. CRISPR-Cas systems developed in *E. coli* often transfer poorly, showing strain-variable toxicity of the Cas effector protein and reduced recombination efficiency [114,115].

4.1. Luminal loading

Genetic luminal loading strategies exploit the natural biogenesis of bEVs, which inherently encapsulate periplasmic and membrane-associated components during vesicle formation [116]. Current genetic engineering cargo-loading approaches primarily rely on this mechanism and focus on directing recombinant proteins into the periplasm or periplasm-adjacent membrane compartments, thereby enabling their sequestration into nascent vesicles. These strategies can be broadly categorized into three mechanistic classes: (i) secretion pathway-mediated periplasmic targeting, (ii) lipoprotein-mediated membrane anchoring, and (iii) outer membrane protein-based scaffold fusion (Fig. 4 and Table 3) [12,99,100,117–119].

The most well-established strategy relies on N-terminal signal peptides that direct cargo proteins into the periplasm through native secretion pathways (Fig. 4A). In *E. coli*, these include the Sec (general secretory) pathway, which translocates proteins in an unfolded state via the SecYEG translocon, the twin-arginine translocation (Tat) pathway, which transports fully folded proteins and protein complexes using signal peptides containing a conserved twin-arginine motif [120], and the signal recognition particle (SRP) pathway, which mediates *co-*

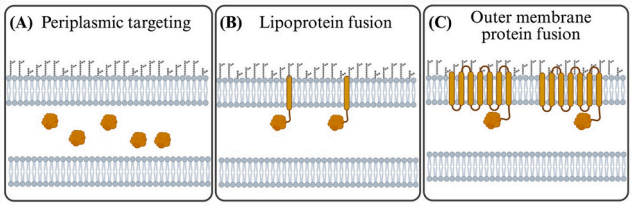

Fig. 4. Strategies for loading cargo into the vesicle lumen or periplasmic space. (**A**) Periplasmic targeting, in which cargo proteins are directed to the periplasm via a signal peptide, where they become entrapped in the vesicle lumen during vesicle formation. (**B**) Lipoprotein fusion, in which cargo is fused to a lipoprotein anchor that tethers it to the inner leaflet of the outer membrane, orienting it toward the vesicle lumen. (**C**) Outer membrane protein fusion, in which cargo is displayed on a cytoplasmic-facing loop of a multi-pass outer membrane protein, positioning it within the vesicle lumen.

Table 3
Genetic engineering strategies for bEV cargo loading. Summary of genetic approaches used to direct luminal cargo loading during bEV biogenesis, including periplasmic targeting via native secretion signal peptides, lipoprotein-mediated membrane anchoring, and outer membrane protein-based scaffold fusion.

Strategy	At a glance	Mechanism	Advantages	Disadvantages	Optimization parameters	Ref.
Periplasmic targeting	**Cargo Type**: Enzymes, antigens, toxins, fluorescent reporter proteins **Cost**: Low - Moderate **Expertise**: Moderate **Throughput**: Moderate **Usage**: High	A signal peptide is genetically fused to the cargo protein, directing it into the periplasmic space. During bEV biogenesis, periplasmic cargo is passively encapsulated within the vesicle lumen.	• Uniform luminal distribution of cargo • Maintains bEV membrane integrity • Cargo is protected from external proteolysis within the vesicle lumen	• Cargo yield impacted by secretion pathway capacity • Folding stress in the periplasm for disulfide-rich or aggregation-prone proteins • Variable loading efficiency across individual vesicles	• Signal peptide • Induction temperature • Expression level • Optical density at induction • Hyper-vesiculating strain	[99,100,105,120–128]
Lipoprotein fusion	**Cargo Type**: Enzymes requiring thermal/chemical stability **Cost**: Moderate **Expertise**: Moderate - High **Throughput**: Moderate **Usage**: Low	Cargo is fused to a lipoprotein signal sequence, which is cleaved after translation. The exposed cysteine is then modified to produce a lipid anchor that embeds stably into the inner face of the outer membrane. During bEV biogenesis, this anchored cargo is incorporated into the vesicle and presented on its inner surface.	• Stable membrane-anchored packaging • Thermal stability • Compatible with modular genetic fusion design • Co-production with native lipoproteins leverages established bacterial biogenesis machinery	• Membrane crowding can disrupt native outer membrane architecture • Limited display density • Relatively few documented applications compared to periplasmic targeting	• Signal sequence • Expression level • Linker design • Growth temperature	[12,105]
Outer membrane protein fusion	**Cargo Type**: Enzymes, therapeutic proteins, antigens **Cost**: Moderate **Expertise**: High **Throughput**: Moderate **Usage**: Moderate	Cargo is fused to inner-facing domains of outer membrane proteins. During bEV biogenesis, the cargo becomes positioned inside the vesicle lumen.	• Stable luminal anchoring • Enables the use of the SpyTag /SpyCatcher system for modular cargo loading.	• Potential disturbance of outer membrane architecture • Linker length between cargo and outer membrane protein scaffold requires optimization	• Expression levels • Linker length • Use β-barrel prediction tools (PRED-TMBB or BOMP) to select periplasm-facing insertion positions	[105,117,125,129,130]

translational targeting of nascent polypeptides to the inner membrane and can facilitate periplasmic exposure of membrane-associated cargoes [121,122]. All three pathways require an N-terminal signal peptide for the protein to be recognized and translocated. During translocation, the signal peptide is cleaved, releasing the cargo into the periplasm, yielding bEVs with therapeutic potential [123]. In one study, the authors used a Tat pathway to load bEVs with GFP [99]. This allowed visualization of bEV internalization [99]. This study revealed that overexpression of the Tat translocase complex (TatABCE) significantly enhanced periplasmic accumulation and vesicle loading efficiency, identifying secretion capacity as a key bottleneck in biosynthetic luminal loading [99]. Although most mechanistic characterization of these pathways comes from *E. coli*, Sec-dependent signal peptides have also been used to route heterologous cargo into the bEVs of other Gram-negative species. For example, using the native OmpA signal peptide from *Salmonella Typhi* efficiently directed cargo into bEVs from both wild-type and hyper-vesiculating strains [124].

Beyond reporter systems, periplasmic targeting has enabled diverse biomedical applications, including vaccine development and detoxification strategies. Signal peptide-directed expression of recombinant antigens using Sec signal peptides yields highly immunogenic bEVs that carry internalized antigens and elicit robust protective immune responses in vaccination models [125,126]. Similarly, signal peptide-mediated luminal loading of enzymatic cascades has been applied to oral detoxification platforms. Additionally, loaded bEVs administered through the gastrointestinal tract achieved systemic blood detoxification [123]. Beyond therapy, cytosolic and periplasmic accumulation strategies have also enabled imaging applications, such as optoacoustic (photoacoustic) imaging using melanin-producing bEVs generated by cytosolic expression of the *melA* gene, providing intrinsic contrast without the need for exogenous dyes [127]. Secretion pathway-mediated targeting is generally favored when homogeneous luminal distribution, high cargo accessibility, or release into the vesicle interior is required, making it particularly suitable for soluble enzymes, antigens, and imaging reporters. However, this approach is constrained by the finite capacity of the secretory machinery, susceptibility to stress induced by overexpression, and potential cargo misfolding or degradation in the oxidizing periplasmic environment [29,99,131].

A second major luminal loading strategy involves fusion of cargo proteins to bacterial lipoproteins, which localize to the inner leaflet of the outer membrane and are passively incorporated into vesicles during budding (Fig. 4B). In contrast to secretion-based approaches, lipoprotein-mediated anchoring can retain cargoes at the membrane-lumen interface, thereby enhancing stability for proteins sensitive to periplasmic redox stress or proteolytic degradation. The native lipoprotein SlyB has been used to anchor nanoluciferase (Nluc) to the

membrane-facing side of bEVs, achieving efficient luminal encapsulation without compromising protein activity or vesicle integrity [12,105]. Similarly, the lipoprotein SLP has been employed to load functional detoxifying enzymes, including phosphotriesterase (PTE) and diisopropylfluorophosphatase (DFPase), enabling the construction of bEV-based nanobioreactors for organophosphate degradation [105]. While lipoprotein anchoring is particularly advantageous for catalytically sensitive or multimeric enzymes that benefit from membrane proximity, cargo orientation and steric accessibility may be more constrained, and membrane crowding can limit achievable loading densities [105].

The third class of luminal loading strategies employs outer membrane proteins or their truncated variants as scaffolds for cargo fusion (Fig. 4C). Truncated outer membrane protein A (OmpA) has been widely used as a luminal loading platform for incorporating peptides, enzymes, and antigens into bEVs [105,117,125,129]. This approach has been used to construct bEV-based nanobioreactors, such as OmpA-SpyTag scaffolds paired with SpyCatcher-fused PTE, enabling modular enzyme packaging with retained catalytic functionality [117]. Autotransporter-based systems further expand this toolkit: a modified Ag43β740 construct lacking the α-helical linker domain successfully delivered eGFP into bEVs, highlighting the versatility of autotransporter scaffolds for luminal cargo packaging [130].

Systematic comparative studies have revealed pronounced cargo-, anchor-, and architecture-dependent effects on loading efficiency and functional performance. In a comprehensive evaluation of enzyme packaging strategies, luminal loading of DFPase and PTE was assessed using periplasmic directors (MBP, BtuF), lipoprotein anchors (Lpp, SLP, SlyB), and membrane scaffolds (Lpp-OmpA). Lpp-OmpA-SpyTag constructs paired with SpyCatcher-fused enzymes enabled modular loading, while linker length modulation revealed additive effects on bEV hypersecretion and vesicle yield, independent of enzyme identity. Functional analyses demonstrated strong enzyme-specific dependencies. SpyTag-SpyCatcher systems supported high PTE activity but poor DFPase activity, whereas MBP-mediated targeting enabled moderate DFPase loading with effective PTE delivery. BtuF performed optimally for PTE, while SlyB preferentially supported DFPase activity. Linker length further emerged as a critical determinant of enzymatic performance, with pronounced cargo-specific sensitivity [105].

Beyond protein delivery, genetic luminal loading strategies have also been extended to nucleic acid therapeutics. RNA expression systems enabling intracellular accumulation and vesicular incorporation of plasmid-derived RNA have been used to generate bEVs carrying immune-modulatory cargoes, such as PD-L1 siRNA, thereby inducing antitumor immune responses by enhancing T-cell recognition of tumor antigens [132]. More recently, CRISPR-Cas9 systems have been refactored for bEV-based delivery. In these platforms, both Cas9 protein and single-guide RNA (sgRNA) are loaded into vesicles, enabling functional ribonucleoprotein delivery [133]. These findings highlight the potential of bEVs to serve as non-viral vehicles for programmable genome editing. Beyond direct gene silencing, bEV-mediated gene delivery has also been used to reprogram tumor cells. For example, engineered bEVs carrying genes encoding CXCL9 and IL-12 (OMV-C9IL12) induced tumor cells to secrete these immunostimulatory factors, thereby significantly enhancing T-cell chemotaxis and activation within the tumor microenvironment [133]. Together, these studies demonstrate that luminal nucleic acid loading can support diverse therapeutic strategies, including immune checkpoint silencing, genome editing, and cytokine-based tumor reprogramming.

Collectively, these biosynthetic luminal loading strategies highlight that effective genetic functionalization of bEVs is fundamentally a design-driven process rather than a one-size-fits-all solution. Secretion pathway-mediated targeting is best suited for soluble cargoes requiring uniform luminal distribution and high accessibility but is constrained by secretion capacity and periplasmic stress. Lipoprotein-mediated anchoring, though, provides enhanced stability and bypasses inner-membrane translocation, making it preferable for catalytically sensitive or multimeric enzymes, albeit it may limit cargo orientation and loading density. Outer membrane protein-based scaffold fusion provides the greatest architectural control and highest loading efficiencies but introduces additional design complexity and sensitivity to linker length and fusion topology. Importantly, comparative studies consistently demonstrate strong cargo-anchor-architecture dependencies, underscoring that rational selection of luminal loading strategies must balance cargo biophysics, functional performance, host burden, and scalability considerations. As bEV platforms move toward clinical translation, such decision frameworks will be critical for aligning molecular design with manufacturability, reproducibility, and regulatory requirements.

4.2. Surface functionalization

Genetic surface functionalization strategies exploit the enrichment of outer membrane proteins in bEVs to display peptides, proteins, or binding domains on the vesicle exterior. By encoding surface ligands directly into the vesicle biogenesis process, these approaches enable controlled stability that is difficult to achieve with post-isolation modification. Surface display strategies can be broadly grouped into four mechanistic classes based on their mode of membrane integration: (i) ClyA, (ii) OmpA and LppOmpA, (iii) autotransporter systems, and (iv) ice nucleation protein (Fig. 5 and Table 4). Each class offers distinct advantages depending on whether immune activation, ligand density, modularity, or structural robustness is prioritized.

Cytolysin A (ClyA) is one of the most widely used surface display platforms because of its strong intrinsic enrichment in bEVs (Fig. 5A). ClyA is a pore-forming protein that naturally inserts into the outer membrane, and the C-terminal fusion of cargoes reliably positions them on the vesicle surface [100]. These properties have made ClyA particularly effective for vaccine and immunotherapy applications, where

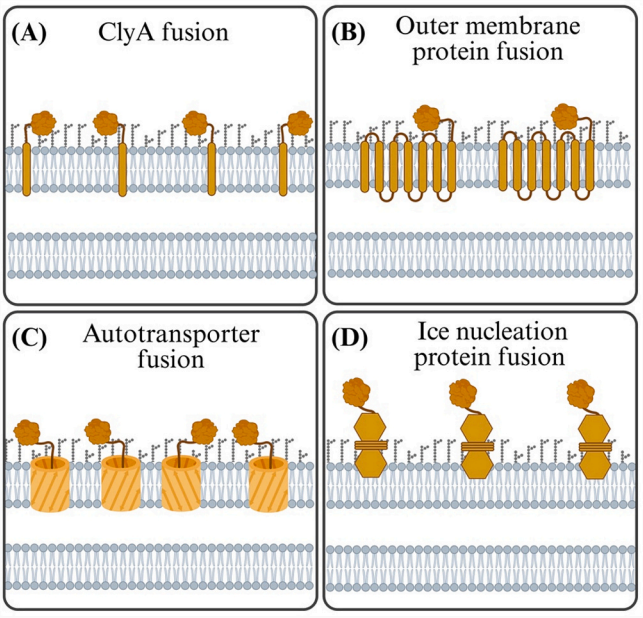

Fig. 5. Strategies for anchoring cargo to the bacterial vesicle surface via genetic fusion. (**A**) ClyA fusion, in which cargo proteins are displayed on the vesicle surface via fusion to the pore-forming toxin ClyA, a single-pass membrane anchor. (**B**) Outer membrane protein (OMP) fusion, in which cargo is inserted into a surface-exposed loop of a multi-pass β-barrel outer membrane protein for stable surface display. (**C**) Autotransporter fusion, in which cargo is fused to the passenger domain of an autotransporter and translocated through its own β-barrel translocation unit to the cell/vesicle surface. (**D**) Ice nucleation protein (INP) fusion, in which cargo is anchored via the membrane-spanning domain of ice nucleation protein for high-density surface display.

Table 4
Genetic surface functionalization strategies for bEVs. Comparison of genetically encoded surface display platforms for bEV engineering, including ClyA fusion, outer membrane protein loop fusion, autotransporter fusion, and ice nucleation protein fusion.

Strategy	At a glance	Mechanism	Advantages	Disadvantages	Optimization parameters	Ref.
ClyA fusion	**Displayed Peptides**: Antigens, enzymes **Cost**: Low - Moderate **Expertise**: Moderate **Throughput**: High **Usage**: High	Proteins are genetically fused to either end of the outer membrane protein ClyA. During bEV biogenesis, the fusion protein is incorporated into the vesicle and displayed on its outer surface.	• High surface display efficiency • Powerful immune activation properties immunotherapy • well-established in the literature	• May disrupt outer membrane integrity at high expression levels • Intrinsically immunogenic • Terminal fusion placement impacts cargo accessibility and must be empirically screened	• Fusion terminus • Expression level • Induction Optical density	[58,100,118,134–137]
Outer membrane protein loop fusion	**Displayed Peptides**: Short peptides, antigens, small targeting ligands **Cost**: Low - Moderate **Expertise**: Moderate **Throughput**: Moderate **Usage**: Moderate	Proteins are inserted into or fused to the outward-facing loops of outer membrane proteins. As these proteins are assembled into the outer membrane, the loops project outward and display the peptide. During bEV biogenesis, the fusion protein is incorporated into the vesicle and displayed on its outer surface.	• Stable surface anchoring via native OMP beta-barrel scaffold • Compatible with modular genetic fusion design using established molecular biology • Leverages native OMP biogenesis	• Overexpressed beta-barrel OMPs compete with native OMPs for Bam complex assembly, disrupting OM architecture • Linker length optimization required • Limited cargo size • Cargo must tolerate the oxidizing periplasmic environment during assembly	• Loop selection: structural analysis (AlphaFold, PDB) to identify surface-exposed loops tolerant of insertions • Linker design • Expression temperature • Cargo size	[100,129,138–140]
Autotransporter fusion	**Displayed Peptides**: Large or complex proteins **Cost**: Low - Moderate **Expertise**: Moderate - High **Throughput**: Moderate **Usage**: Moderate	Protein replaces or is fused to the passenger domain of the autotransporter. During bEV biogenesis, the fusion protein is incorporated into the vesicle and displayed on its outer surface.	• Supports display of large or complex cargo proteins that cannot fit into OMP surface loops • Passenger domain is fully exposed and accessible to substrate • Highly modular	• Potential disturbance of outer membrane architecture at high expression levels • Slower secretion kinetics for large cargo • Passenger domain size limit governed by periplasmic chaperone capacity	• Host strain • Growth medium • Growth temperature • Expression level • Expression temperature	
Ice nucleation protein fusion	**Displayed Peptides**: Enzymes **Cost**: Low - Moderate **Expertise**: High **Throughput**: Moderate **Usage**: Moderate	The ice nucleation hydrophobic N-terminus anchors into the outer membrane, while a central repeat domain acts as a rigid spacer that projects the fused protein away from the membrane surface for external accessibility. During bEV biogenesis, the fusion protein is incorporated into the vesicle and displayed on its outer surface.	• High-density surface display with strong, stable membrane anchoring • Central repeat domain projects cargo away from the membrane for better substrate accessibility • Supports multivalent presentation	• Large ice nucleation protein size imposes significant metabolic burden on the host and the outer membrane • Possible protein aggregation expression levels • Multi-enzyme scaffolding design is complex	• Expression level • Expression temperature	[12,141,142]

high antigen exposure and immune stimulation are desirable [118]. For example, ClyA has been used to display viral antigens such as the pre-fusion F protein of respiratory syncytial virus and the receptor-binding domain of SARS-CoV-2, resulting in bEV-based vaccines that elicit strong humoral and cellular immune responses [134,135]. In cancer immunotherapy, ClyA-fused affibodies and nanobodies targeting tumor-associated receptors have been employed to enhance antigen presentation and T-cell activation, including a highly cited cancer vaccine platform using ClyA-affibody constructs [58]. Beyond vaccination, ClyA-mediated display of PD-L1 antibodies and luciferase has been used to modulate immune checkpoints and to track vesicle biodistribution in solid tumor models, demonstrating the versatility of this anchor for

immune-focused applications [136]. However, ClyA's pore-forming activity and redox-sensitive vesicle sorting can perturb membrane integrity, making it less suitable for applications requiring minimal immunogenicity or prolonged systemic circulation [137]. Consequently, ClyA is most often selected when strong immune engagement outweighs concerns related to membrane disruption.

Outer membrane protein A (OmpA) and its derivatives provide a more structurally stable alternative to pore-forming anchors (Fig. 5B). While native OmpA exhibits weaker bEV enrichment than ClyA, hybrid constructs combining the Lpp lipoprotein signal peptide with the OmpA transmembrane domain (Lpp-OmpA) improve surface localization and orientation [100,138]. These hybrids have become widely used in applications that require modularity and reduced membrane perturbation. Lpp-OmpA has been used to display monoavidin and enhanced monoavidin (eMA) on bEV surfaces, enabling modular vaccine platforms in which biotinylated antigens or antibodies can be captured post-secretion. This strategy decouples antigen production from vesicle biogenesis and allows rapid exchange of displayed ligands, which is particularly advantageous for multivalent or personalized vaccine design [139,140]. Similarly, Lpp-OmpA-His6 constructs have been used to enable affinity purification of bEVs, directly addressing downstream manufacturing and scalability challenges [129]. In another application, Lpp-OmpA-SpyCatcher003 [143] enabled orthogonal surface functionalization via binding to SpyTag003-fused nanobodies or immune ligands, supporting tumor-targeted vaccine and immunotherapy platforms [140].

Autotransporters (ATs) are a highly versatile class of surface display platforms that support large and complex cargoes (Fig. 5C) [144,145]. These proteins consist of an N-terminal Sec signal peptide, a customizable passenger domain, and a C-terminal β-barrel that anchors the construct in the outer membrane [144,145]. By inserting cargoes into defined positions within the passenger domain, ATs enable multivalent and spatially controlled surface presentation [146]. AT-based systems have been extensively applied in immunotherapy [119,139,147–152]. For instance, Hbp-based display of *Mycobacterium tuberculosis* antigens (ESAT6, Ag85B, Rv2660c) on bEVs derived from *E. coli and Salmonella* generated multivalent vaccine candidates capable of eliciting robust antigen-specific immune responses [147]. In cancer immunotherapy, AIDA-I-mediated autodisplay of anti-PSMA and anti-CD3 single-chain variable fragments enabled the construction of bispecific vesicles designed to physically bridge tumor cells and T cells, thereby enhancing cytotoxic immune engagement [148]. Autotransporters have also been used for modular antigen capture. In a comparative study evaluating eMA display using multiple anchors, including ClyA, Lpp-OmpA, Intimin, IgAP, and AIDA, several AT-based constructs achieved high surface expression [139]. However, functional binding assays revealed that IgAPβ-eMA and Lpp-OmpA outperformed higher-density displays in antigen capture, highlighting the importance of cargo accessibility and the local microenvironment over surface density alone [139]. IgAPβ-eMA and Lpp-OmpA were then further optimized, and Lpp-OmpA resulted in increased eMA surface display [139]. Lpp-OmpA was subsequently used to generate SNAP-OMVs decorated with biotinylated Sx-Cm-MOMP, and immunization studies demonstrated significantly elevated rCm-MOMP-specific IgG titers compared to PBS and blank SNAP-OMVs, confirming the platform's ability to elicit strong antigen-specific humoral responses [139]. These findings emphasize that AT systems offer flexibility and multivalency but require careful optimization to balance expression burden, folding efficiency, and functional performance.

The ice nucleation protein (INP) from *Pseudomonas syringae* provides a distinct surface display strategy optimized for high-density presentation (Fig. 5D). INP forms large, repetitive β-helical structures that strongly associate with the outer membrane and support multimeric cargo display [141]. INP-based displays have been particularly effective for biocatalytic and biosensing applications that benefit from high local enzyme concentrations. INP fusions have been used to generate bEV-based nanobiocatalysts that support multi-enzyme cascade reactions with enhanced efficiency, as well as Scaf3-based platforms for cancer cell imaging and biosensing [12,142]. These applications highlight INP's suitability for catalytic and diagnostic contexts, while its large size and expression burden may limit its use in therapeutic delivery.

Across surface functionalization strategies, comparative studies consistently demonstrate that optimal performance depends on the interplay between anchor choice, surface modification, and intended application. High surface density does not necessarily translate to superior function, as ligand orientation, accessibility, and membrane microenvironment strongly influence biological outcomes. ClyA-based systems excel in vaccine and immune-activating contexts; Lpp-OmpA hybrids offer modularity and manufacturability; autotransporters enable multivalent and complex displays; and INP supports high-density catalytic architectures. As with luminal loading, rational surface engineering of bEVs requires a design-driven approach that aligns molecular architecture with functional goals, host burden, and translational considerations. Notably, the repertoire of outer membrane anchoring systems extends well beyond the platforms currently adapted for bEV engineering [153]. Expanding systematic evaluation to these additional anchors may uncover display strategies that improve cargo compatibility, reduce host burden, or enhance manufacturability.

This contrasts with chemical surface conjugation, where display density and site specificity are constrained by whatever reactive groups happen to be exposed on the isolated vesicle, and where downstream purification to remove unreacted crosslinkers and excess reagent is itself a source of yield loss and compositional bias [35,38,63,66]. Genetic display avoids that purification burden, since the anchor and cargo are expressed as a single fusion construct during biogenesis, and the resulting vesicles carry no unconjugated small-molecule byproducts. In contrast, genetic constructs risk folding or translocation failure at the anchor itself, and instead of batch-to-batch variability from reaction efficiency, variability is introduced by expression level and host fitness. Both chemical and genetic paradigms face challenges with reproducibility, but genetic strategies move that control upstream, into strain design, potentially allowing for higher-level control.

4.3. Hypervesiculation

Efficient and reproducible vesicle production remains a major barrier to the clinical translation of bEV-based therapeutics. Native vesicle release is typically low and sensitive to culture conditions, limiting scalability and batch consistency [35,37,89,154]. As a result, substantial effort has focused on engineering hypervesiculating strains that increase output through defined genetic modifications. Hypervesiculation is not random membrane shedding but arises from predictable alterations in conserved features of bacterial envelope structure. These mechanisms can be grouped into four major categories: disruption of membrane anchoring, impairment of envelope protein quality control, protein crowding-induced membrane strain, and alteration of lipid architecture (Fig. 6). Although these mechanisms interact, they represent distinct routes to vesicle formation. Disruption of membrane anchoring reduces mechanical restraint on the outer membrane, whereas proteostasis imbalance increases periplasmic stress by accumulating misfolded proteins and envelope intermediates. In contrast, protein crowding reflects increased membrane occupancy by properly folded proteins, whereas lipid remodeling alters membrane curvature by changing outer membrane composition rather than by increasing protein load.

Among these, disruption of membrane anchoring represents one of the most direct and extensively characterized approaches (Fig. 6A) [55]. In Gram-negative bacteria, the outer membrane is physically tethered to the underlying peptidoglycan cell wall by structural complexes such as Tol-Pal and OmpA [155–160]. The Tol-Pal complex forms a trans envelope bridge in which TolA is anchored in the inner membrane and extends through the periplasm. TolB is a periplasmic beta-propeller protein that interacts with Pal, and Pal is an outer membrane

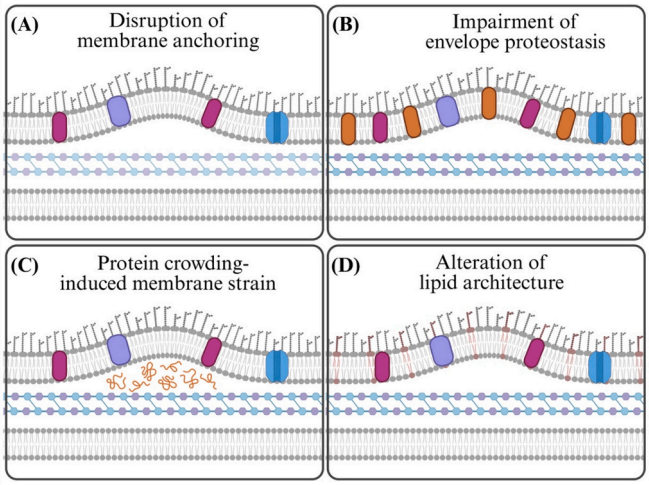

Fig. 6. Mechanisms of hypervesiculation. Schematic of processes proposed to increase bacterial vesicle production. (**A**) Disruption of membrane anchoring, in which loss of covalent or non-covalent links between the outer membrane and the underlying peptidoglycan/inner membrane allows the envelope to bulge outward. (**B**) Impairment of envelope proteostasis, whereby accumulation of misfolded or unfolded periplasmic proteins (orange) generates local turgor and drives membrane blebbing. (**C**) Protein crowding-induced membrane strain, in which dense clustering of membrane proteins combined with soluble periplasmic protein accumulation creates lateral pressure that curves the membrane outward. (**D**) Alteration of lipid architecture, in which changes to lipid composition or headgroup chemistry (dark red) locally alter membrane curvature and promote vesicle budding. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lipoprotein that directly binds peptidoglycan, completing the linkage [156,157]. OmpA contributes a complementary, independent tether through its C-terminal periplasmic domain, which binds directly to diaminopimelic acid residues in the peptidoglycan layer [159]. Lpp provides the most abundant covalent crosslink, anchoring the outer membrane to the peptidoglycan through a direct amide bond. Lpp crosslink density is a primary determinant of outer membrane constraint, and its reduction correlates quantitatively with increased vesicle production [158,161]. These linkages constrain membrane curvature and limit vesicle budding. Genetic disruption of these tethers consistently produces strong hypervesiculation phenotypes. Deletion of tolA, tolB, pal, or tolR in *E. coli* results in substantial increases in bEV production, and similar effects have been reported in other Gram-negative species. The critical parameters governing this approach are the density of Lpp-peptidoglycan crosslinks, the expression levels of individual Tol-Pal components, and the completeness of complex disruption. The deletion of any single component of the Tol-Pal complex can decouple the outer membrane from the cell wall and induce hypervesiculation, but the magnitude of the effect and the degree of outer membrane perturbation vary by gene. Importantly, tether disruption carries fitness tradeoffs. Deletion of pal or tolB can compromise outer membrane integrity, increase sensitivity to detergents and antimicrobials, and alter lipid asymmetry. These effects must be weighed against yield gains when selecting which tether to disrupt for a given production context [156,157]. Because this mechanism relies on conserved envelope architecture, it represents a broadly transferable yield-enhancement strategy. Tol-Pal-deficient strains have therefore been widely adopted in bEV-based therapeutic platforms due to their robust and genetically stable vesicle output [162]. Mechanistically, reducing membrane anchoring lowers the physical constraint on the outer membrane, facilitating spontaneous curvature and vesicle release. From a manufacturing perspective, this approach often yields large and reproducible increases in output.

In contrast to mechanical tether disruption, a second category of hypervesiculation arises from an imbalance in envelope proteostasis (Fig. 6B). Envelope proteostasis refers to the systems that maintain proper protein folding and quality control in the periplasm and outer membrane. Disruption of this balance can also drive hypervesiculation. Deletion of degP, a periplasmic protease involved in protein quality control, increases vesicle production in *E. coli*, particularly under heat stress conditions [163]. Similarly, deletion of *nlpI*, which encodes a lipoprotein adaptor that regulates periplasmic proteases and coordinates peptidoglycan hydrolase activity, also induces hypervesiculation. Loss of *nlpI* disrupts envelope protein turnover and cell wall remodeling homeostasis, leading to accumulation of envelope intermediates and localized envelope stress. Mechanistically, accumulation of misfolded proteins, peptidoglycan fragments, and lipopolysaccharide intermediates can increase periplasmic crowding and local envelope pressure. This buildup promotes outward bulging of the outer membrane and vesicle budding, particularly in regions where outer membrane-peptidoglycan crosslinks, such as Lpp, OmpA, or Tol-Pal, are sparse or transiently weakened. In these settings, vesicles are often enriched in misfolded protein cargo, supporting the idea that vesicle release functions as a protective pathway for removing toxic envelope components [159]. Importantly, not all hypervesiculation mechanisms operate through the same structural route. Some pathways correlate inversely with Lpp-peptidoglycan crosslink density, consistent with reduced mechanical restraint [158,161]. Others are largely Lpp-independent and appear to be driven primarily by the accumulation of periplasmic proteins, cell wall fragments, or LPS species [158,161]. These observations indicate that envelope proteostasis imbalance and crosslink density represent distinct but interacting modes of vesicle biogenesis. Unlike membrane tether disruption, proteostasis-driven hypervesiculation is inherently stress-associated. While it can increase yield, it may also alter vesicle composition and heterogeneity, thereby complicating therapeutic consistency [158,159,161].

Beyond stress-driven mechanisms, hypervesiculation can also result from increases in membrane occupancy. In this case, vesicle production rises not because proteins are misfolded, but because high-level expression of outer membrane components increases local membrane density and mechanical strain (Fig. 6C). Overexpression of outer membrane proteins, lipoproteins, or engineered fusion constructs can passively stimulate vesicle release by altering the physical balance of the outer membrane. Experimental evidence supports a direct relationship between protein crowding and bEV output [164]. For example, overexpression of β-barrel outer membrane proteins such as BamA or PhoE in a protein-depleted outer membrane produces up to approximately 30-fold more bEVs, with vesicle yield scaling proportionally to the level of β-barrel insertion [160]. This behavior is consistent with a membrane-crowding mechanism rather than a classical stress-response pathway. In these cells, the outer membrane architecture is perturbed, with altered ratios of outer membrane proteins, lipopolysaccharide, and phospholipids. The excess β-barrel proteins and associated phospholipids appear to promote membrane curvature and vesicle release while simultaneously relieving envelope strain [159]. More broadly, high local densities of outer membrane proteins or misassembled complexes can generate lateral pressure within the membrane, contributing to curvature and budding [32,159]. This crowding-based mechanism operates alongside other established models of vesicle biogenesis, including reduced outer-membrane peptidoglycan cross-linking, altered lipid asymmetry, and periplasmic pressure. Importantly, protein crowding-induced vesiculation can occur even when proteins are properly folded, distinguishing it from proteostasis-driven mechanisms. For translational applications, this mechanism is particularly relevant. Optimizing surface display constructs, cargo size, linker length, or membrane anchor design can inadvertently increase membrane occupancy and alter vesicle yield during strain development and scale-up. As a result, yield changes in engineered strains may reflect physical crowding effects rather than intentional hypervesiculation design.

In addition to protein-based mechanisms, alterations in outer membrane lipid architecture provide another powerful and translationally attractive strategy. Outer membrane lipid composition strongly influences membrane curvature and vesicle formation (Fig. 6D). Genetic modifications that alter lipid A acylation or disrupt lipid asymmetry can enhance vesicle budding while simultaneously improving safety profiles. Deletion of the *msbB* gene increases bEV production from *Brucella abortus* cultures and reduces endotoxic activity [162]. These strategies are particularly attractive in translational settings because they couple yield enhancement with reduced reactogenicity. Disruption of the VacJ/Yrb (Mla) lipid transport pathway represents another conserved approach [162,165]. Inactivation of *mlaA* or related components increases phospholipid accumulation in the outer membrane and promotes vesicle release [166]. Because lipid asymmetry pathways are conserved across Gram-negative bacteria, this strategy offers a rational and potentially transferable route to yield engineering. Despite their effects on yield and endotoxic activity, neither *msbB* deletion nor Mla pathway disruption substantially alters vesicle size distribution or gross morphology [167]. Hypervesiculating mutants produce more vesicles rather than structurally different ones. Interestingly, these changes to lipid structure led to differences in bEV cargoes. Mla-deficient bEVs are enriched in phospholipids relative to wild-type preparations, reflecting accumulation in the outer leaflet that drives vesicle budding, while protein cargo profiles remain broadly similar. Deletion of *msbB* shifts lipid A from hexa- to tetra-acylated species, reducing TLR4 agonist activity without disrupting outer membrane protein packaging [167]. Despite these observations, the downstream effects on bEV-host interactions are less well characterized, representing an important area for future research [168].

While the majority of well-defined hypervesiculation strategies have been developed in Gram-negative systems, related principles apply to Gram-positive bacteria. In these organisms, vesicle production depends on localized remodeling of the thick peptidoglycan cell wall, and reduced cross-linking has been associated with elevated vesicle release. Genetic modulation of peptidoglycan cross-linking in *Lactiplantibacillus plantarum* increased vesicle yield while preserving therapeutic efficacy [169]. However, compared to Gram-negative systems, robust and broadly applicable genetic hypervesiculation strategies remain less developed.

Taken together, these mechanisms illustrate that hypervesiculation can be achieved through multiple, predictable alterations in envelope structure and homeostasis. Nevertheless, increased yield is not universally advantageous. Mechanical tether disruption may affect membrane stability. Proteostasis-based approaches can enrich stress-associated cargo. Lipid remodeling may alter immunostimulatory profiles. Protein crowding strategies may introduce variability during scale-up. Accordingly, hypervesiculation should be treated as a tunable engineering parameter rather than a standalone objective. Optimal strain design will require balancing vesicle yield with growth kinetics, compositional consistency, immunogenicity, and manufacturability. Future progress in bEV translation will depend on modular integration of complementary hypervesiculation strategies while maintaining process control and regulatory safety.

5. Future directions and opportunities

Despite rapid advancements in engineering bEVs, several key challenges continue to limit their full potential, namely, heterogeneity. Without a standardized framework for evaluating and comparing functionalization strategies, inconsistencies are difficult to resolve. Compounding this issue are variations in host strains, cargo identities, and construct architectures, which obscure generalizable design principles and hinder cross-study comparisons. As discussed in previous sections, the rational selection of engineering approaches must consider cargo or surface group characteristics, application requirements, and vesicle stability. Cargo or surface group properties (i.e., molecular weight, charge, hydrophobicity, folding requirements, sensitivity to oxidative or proteolytic environments) must be the primary driver of strategy selection. Where small hydrophobic molecules favor passive physical loading, charged or hydrophilic nucleic acids require active disruption methods or genetic luminal loading, and conformationally sensitive enzymes may be better served by lipoprotein anchoring than by secretion pathway targeting. Application context further narrows the decision space. Vaccine and immunotherapy applications that depend on high surface antigen density and immune stimulation favor ClyA or autotransporter display platforms. These applications also leverage the innate immune response triggered by bacterial motifs, such as lipopolysaccharides (LPS), flagellin, and other pathogen-associated molecular patterns (PAMPs). Applications requiring prolonged systemic circulation and minimal immune activation call for reduced LPS content and surface-shielding strategies. Vesicle stability requirements, including storage conditions, administration route, and in vivo half-life expectations, must also be considered as engineering approaches may disrupt membrane stability. Establishing a decision framework, even informally, will help rationalize platform selection and reduce reliance on empirical trial-and-error approaches that currently dominate the field [49,154].

In addition to standardization challenges, bEVs require more complex purification and validation protocols than many synthetic nanocarriers. While this has historically limited the large-scale manufacturing of engineered bEVs, genetic engineering approaches may be able to address. However, loading limitations, immunogenicity, and the effects of genetic modifications on parent cell present challenges that future synthetic biology efforts should work to address.

Despite these challenges, genetic toolkits for bEV functionalization offer diverse capabilities in terms of expression strength, display density, and modularity. While each system comes with specific trade-offs, these systems have been widely applied to vaccine delivery, diagnostics, biosensing, and targeted therapies, systematic, side-by-side benchmarking under controlled conditions remains critically lacking. Establishing quantitative metrics for evaluating these tools would facilitate more rational platform selection and accelerate translational development. Future research efforts may reveal display platforms with improved cargo compatibility, reduced envelope stress, or enhanced manufacturability. Expanding the accessible design space will therefore be as important as optimizing existing systems.

Engineering bEVs from Gram-positive bacteria is another valuable area for future efforts. Recent studies suggest that hypervesiculation in Gram-positive bacteria is often driven by localized peptidoglycan weakening and autolysin dysregulation, processes that are frequently coupled to elevated cell death rather than controlled vesicle release [68]. While proof-of-concept work has demonstrated that genetically induced hypervesiculation can yield therapeutically active vesicles, robust, tunable strategies for decoupling bEV production from cytotoxicity remain largely underdeveloped. Addressing these limitations will be essential to harness the therapeutic potential of Gram-positive bEVs, particularly for probiotic and live biotherapeutic applications.

To overcome current challenges and unlock the full clinical potential of bEVs, a deeper understanding of how engineered bEVs interact with host biological systems is essential for guiding more effective and predictable design strategies. Equally important is elucidating how genetic functionalization and hypervesiculation mechanisms intersect at the level of envelope stress, cargo partitioning, and vesicle heterogeneity. Synthetic biology offers powerful tools for functionalization, enabling the creation of multifunctional vesicles that can carry diverse therapeutic cargoes. At the same time, optimizing production by refining culture conditions and introducing targeted genetic modifications is critical to address limitations in yield and cargo capacity, making large-scale manufacturing more feasible. Expanding the genetic toolkit for Gram-positive bacteria is another priority, as these strains remain underexplored due to their limited engineering accessibility compared to Gram-negative models. Advancing bEVs toward clinical application

will also require rigorous evaluation of pharmacokinetics, biodistribution, immunogenicity, and long-term safety. In parallel, combining bEVs with existing therapies, such as chemotherapy or photothermal therapy, could enhance treatment efficacy by leveraging synergistic effects. A particularly exciting direction involves engineering live therapeutic systems, where bacteria function as in situ drug factories that release bEVs directly at the target site, enabling localized and programmable drug delivery. Finally, interdisciplinary collaboration, drawing on automation, artificial intelligence, and systems biology, will be essential to streamline development pipelines and accelerate the translation of bEV technologies into real-world therapies.

As the field evolves, a unified, quantitative approach to evaluating bEV functionalization strategies, coupled with deeper biological insight and expanded genetic tools, will be essential for realizing the full promise of bEVs in synthetic biology and medicine.

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Declaration of AI-use

During the preparation of this work, the author(s) used ChatGPT (GPT-5 with a medium reasoning level) to edit text for flow and cohesiveness. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

CRediT authorship contribution statement

Varunaa Sri Hemanth Kumar: Writing – review & editing, Writing – original draft, Investigation. **Hannah C. Zierden:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization. **Sara Molinari:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Conceptualization.

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The authors declare no conflict of interest.

Data availability

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

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