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Engineered Bacterial Extracellular Vesicles: Developments, Challenges, and Opportunities

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ABSTRACT

The abundant microbe-associated molecular patterns (MAMPs) and nanoscale structures of bacterial extracellular vesicles (bEVs) collectively facilitate their versatile biological activities. Building on these inherent properties, engineering methods encompassing physical, chemical, and genetic modifications have been strategically employed to enhance the functional diversity of bEVs. Therefore, bEVs are being explored as innovative and promising platforms for developing immunotherapeutic strategies targeting diverse pathological states. To establish a foundational understanding of bEVs, we first summarized their biogenesis, classification, structures and biomolecular constituents of bEVs. This review discusses techniques for bEV production and modification and explores the immunological characteristics and effects of engineered bEVs, along with their biomedical applications. Special attention is devoted to advanced engineering approaches and outlining the challenges and emerging avenues in the development of engineered bEVs. This review aims to systematically construct an evidence-based and comprehensive framework that promotes translational optimization and clinical implementation of engineered bEVs, thereby maximizing their application potential in the biomedical field.

1. Introduction

In 2011, the generic term "extracellular vesicles" (EVs) was proposed to define all cell-derived lipid bilayer-enclosed membranous structures [1,2]. In general, spontaneously forming EV populations consist of distinct subpopulations that vary in size, morphology, composition, and biogenesis [2]. EVs are now recognized as an alternative mode of intercellular signaling and contribute to the maintenance of microenvironmental homeostasis, by systematically transporting multidimensional bioactive substances such as proteins, lipids and nucleic acids to target cells [3]. Notably, the functions of EVs are not limited to the horizontal transfer of biological cargoes, but also include signaling at the recipient cell surface, nutritional support, clearance of cellular material, and redistribution of interstitial fluid or extracellular matrix. This has highlighted the vital role of EVs in numerous physiological and pathological processes, disease biomarkers, therapeutic agents, and drug delivery vehicles showing enormous potential [4].

Bacterial extracellular vesicles (bEVs) can be secreted by both Gram-negative and Gram-positive bacteria via different biogenesis mechanisms [5–7]. Bacterial vesicles are further specifically termed according to their origin or preparation method [8,9]. For instance, cytoplasmic membrane vesicles (CMVs) refer to natural bEVs from Gram-positive bacteria, whereas outer membrane vesicles (OMVs) are naturally released by Gram-negative bacteria [5,7]. Beyond these native forms, two types of synthetic bEVs (SyBVs) have been developed: ① double-membrane vesicles (DMVs) formed through chemical or physical disruption-induced self-assembly of bacterial cells [10], and ② protoplast-derived inner membrane nanovesicles (PDNVs) [11–13]. With the exception of PDNV, all other bacterial vesicles encapsulate diverse bacteria components within spherical structures measuring 20 to 400 nm [11,14], Proteomics and biochemical analyses have profiled multifunctional cargoes in bEVs, including outer membrane-associated and periplasmic proteins, enzymes, polysaccharides, nucleic acids, peptidoglycans, and metabolites [15–17]. These nanosized structures, together with various cargo compositions enable bEVs to be actively recognized and absorbed by immune cells, thereby initiating immune responses.

Compared with conventional synthetic nanomaterials, bEVs exhibit superior biocompatibility, physiological functions, loading

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capacity, ease of modification and industrialization, collectively positioning them as a promising drug-delivery platform for biomedical applications. Specifically, the ease of modification allows bEVs to encapsulate small-molecule therapeutic drugs or synthesize targeting substances such as antibodies and enzymes, through the genetic editing of parental bacteria [18,19]. Simultaneously, the inherent membrane stability preserves cargo integrity (such as genetic tools and functional molecules) against enzymatic degradation or hydrolysis during delivery [18,20]. This ensures the structural integrity and functional activity of the cargo delivered to the targeted site [21]. The compatibility of BEVs extends to hybrid systems, in which functional materials are introduced to enable synergistic treatment and improve efficiency [22]. In terms of bioactivity, bEVs play an important role in regulating the gut microbiota and maintaining immune homeostasis. Emerging evidence highlights bEVs and their dual bioactivities in gut microbiota modulation and the maintenance of immune homeostasis [23,24]. For example, *Lactobacillus* CMVs and *Akkermansia muciniphila* OMVs can reverse gut microbiota dysbiosis and maintain immune homeostasis and thus can be used as immune regulators to ameliorate colitis and induce the apoptosis of colorectal cancer cells [25–27]. Furthermore, bEVs exhibit anti-microbial potential against pathogens such as *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecium* and human immunodeficiency virus 1 (HIV-1) [24,28,29], while natively facilitating intercellular communication through gene transfer and protein transport mechanisms [30,31].

In addition to the diverse physiological activities derived from packed cargo, bEVs are characterized by distinct functional roles as a secretion system. Notably, OMVs from Gram-negative are the only known example of a type 0 secretion system (T0SS) [32]. This system protects unstable biomolecules from extracellular enzymes and aqueous environments, distinguishing it from conventional secretion systems [33]. The concentration-dependent secretion dynamics of the T0SS demonstrate unique advantages for the transport of lipids, hydrophobic molecules, insoluble substances, and virulence factors [32,34]. For bioactive molecules that require high local concentrations, OMV-mediated concentration ensures high potency and maintains biologically relevant stoichiometric requirements without the need for bulk secretion [33]. In addition, this system enables the coordinated delivery of multiple functionally complementary components to the target cell/tissue, potentially synergistically amplifying their activity [34]. Despite these excellent system properties and promising therapeutic effects, natural bEVs face clinical barriers such as low extraction efficiency and production complexity. Recent advances in bioengineering techniques are devoted to overcoming these limitations through strategic modifications, enhancing functional versatility for biomedical applications. However, current research exploring improved production efficiency and therapeutic effectiveness of engineered bEVs is limited.

This review systematically examines the classes, biogenesis mechanisms, structures and molecular compositions of bEVs. We then present bacterial engineering strategies (parental bacteria modification) and post-isolation vesicle engineering approaches and highlight their applications. Finally, we critically discuss the major challenges and prospects in optimizing engineered bEV platforms. These developments collectively suggest that these engineered bEVs hold promise as valuable reservoirs of functional nanomaterials in the biomedical field.

2. Generation of different bEVs

Based on membrane compositions and origins, bEVs can be categorized into two primary categories: natural and SyBVs. Natural bEVs further subdivide into OMVs and CMVs, whereas SyBV includes DMVs and PDNVs (Fig. 1 and Table 1).

2.1. OMVs

Gram-negative bacteria possess a distinct membrane structure comprising multiple layers, including the outer membrane, inner membrane, peptidoglycan layers and periplasm [35]. The outer membrane is predominantly composed of membrane proteins and lipopolysaccharides (LPS), whereas the inner membrane consists primarily of phospholipids. The peptidoglycan layer, positioned between the inner and outer membranes, is supported by proteins within the periplasmic cavity that establish connections with the outer membrane. Under stable conditions, these interactions maintain structural integrity but can be weakened by bacterial autoregulation or external disturbances. Weakened connections result in the separation of the outer membrane from the peptidoglycan layer, leading to the detachment of the outer membrane and the formation of OMVs through budding [7].

OMVs are nanoscale spherical lipid bilayers formed from bacterial outer membrane (20–250 nm in diameter). Theoretically, their molecular composition mirrors that of outer bacterial membranes. Research evidence has confirmed the presence of crucial outer membrane components in OMVs, including outer membrane proteins, LPS, and phospholipids. In addition, OMVs also encapsulate periplasmic components and nucleic acids [36].

OMVs have been utilized for diverse applications including vaccine development, drug delivery, cancer immunotherapy and antimicrobial therapy. For example, OMVs derived from *Neisseria meningitidis* (*N. meningitidis*) have been successfully used in the licensed meningococcal B vaccine vaccine (4CMenB) (Bexsero, USA), demonstrating their potential as an immunogenic platform capable of triggering robust immune responses [37]. In addition, advances in genetic engineering have enabled the modification of OMVs to eliminate endotoxicity and enhance cargo specificity, thereby expanding their therapeutic scope [7]. This review provides a detailed discussion on how diverse engineering approaches can be leveraged to unlock the multifaceted applications of OMVs. In general, the regulation of OMV formation involves processes of outer membrane destabilization, such as the aggregation of relevant components on the outer membrane, alteration of bacterial membrane fluidity, and disruption of peptidoglycan cross-linking [7,38].

2.2. CMVs

CMVs, ranging from 20 to 400 nm in diameter, are single-membrane vesicles naturally released from the plasma membrane of Gram-positive bacteria. These vesicles encapsulate cytoplasmic membrane components along with soluble cytoplasmic contents (e.g., nucleic acids and proteins) [39]. The structural framework of Gram-positive bacteria features a robust cell wall comprising peptidoglycan and lipoteichoic acids (LTAs), firmly anchored to the cell membrane by diacylglycerols. CMV biogenesis is initiated under swelling pressure, extending from the inner membrane, eventually being liberated into the periplasmic space and traversing the peptidoglycan layer. The thick peptidoglycan layer of the cell wall serves as a barrier to the release of CMVs. During vesicle budding, staphylococcal alpha-type phenol-soluble modulin (αPSM) can facilitate CMV release from the plasma membrane by enhancing membrane fluidity [40]. Genetic regulators and transcription factors including general stress transcription factor (σ^B), *spo0A*, and *sfp* further modulate CMV formation in *Listeria monocytogenes*, *Clostridium perfringens*, and *Bacillus subtilis*, respectively [41–44]. In *S. aureus*, autolysin-mediated pore formation disrupts peptidoglycan cross-linking and promotes CMV generation [45].

The versatility and wide range of sources make CMVs an emerging platform for biomedical and biotechnological applications. CMVs can be isolated from Gram-positive bacteria or synthetically
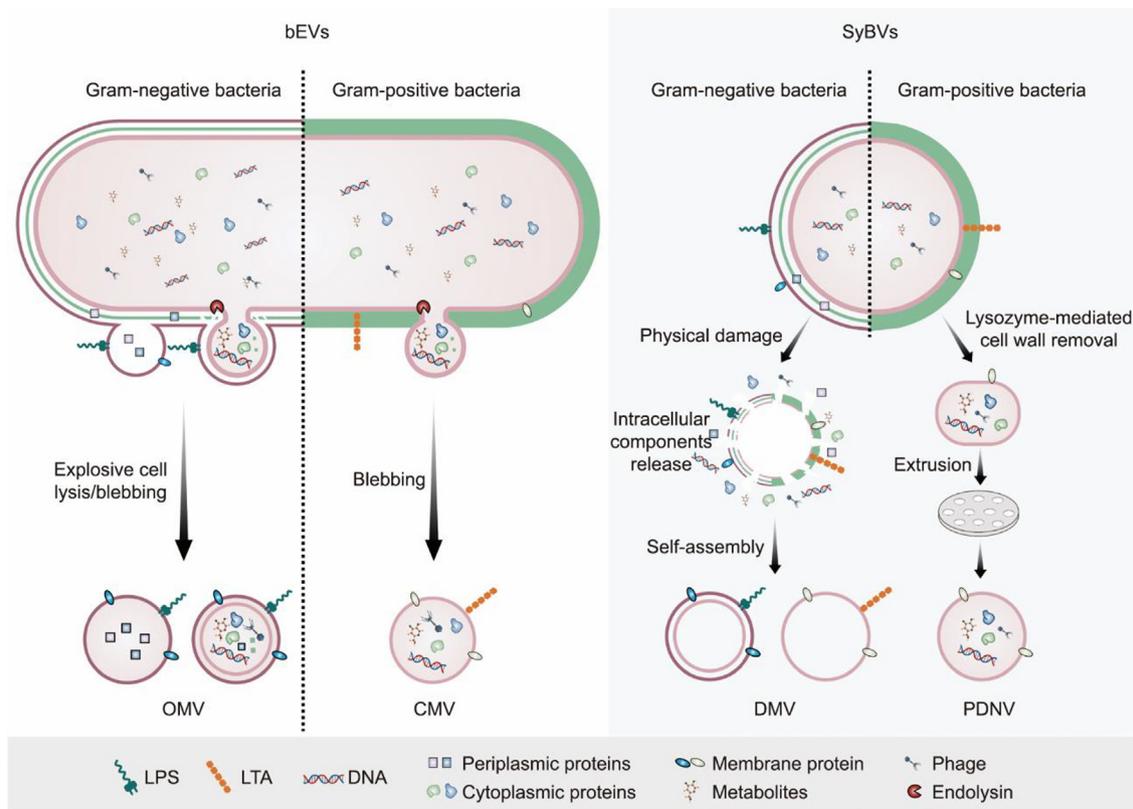
Fig. 1. Generation, structure and molecular composition of diverse bEVs. The multilayered membrane structure of Gram-negative bacteria refers to an inner membrane, an outer membrane and a peptidoglycan layer. In contrast, the membrane structure of Gram-positive bacteria is mainly characterized by a single lipid membrane and a thick cell wall layer consisting of peptidoglycan and lipophosphatidic acid (LTA). EVs derived from both Gram-positive and Gram-negative bacteria may contain a variety of components originating from their parent bacteria, such as proteins, lipids, lipoproteins, DNA, and RNA. Generally, EVs secreted by Gram-negative bacteria are referred to as OMVs due to their origin from the outer membrane. In contrast, those produced by Gram-positive bacteria are called CMVs. In addition to the naturally produced formation, SyBVs are an artificially introduced mode for bEV production. One feasible approach involves the physical disruption of bacterial cells to release substantial contents, followed by self-assembly of the bilayer membrane to form a DMV. Another method employs lysozyme to remove the outer membrane and peptidoglycan layer and result in protoplasts, which are then subjected to physical squeezing to generate the PDNVs. LPS: lipopolysaccharides.

Table 1
The overview of bEVs with different origins.

Type	Origins	Size	Membrane structure	Content	Potential mode of formation	Features
Natural bEV						
OMV	Gram-negative bacteria	20–250 nm	Multiple layers • Outer membrane • Peptidoglycan layer • Inner membrane • Periplasm	Phospholipids Proteins Nucleic acids Virulence factors (e.g., LPS) Metal ions Signaling molecules Metabolites	Outer membrane separation due to its weakened adhesion with peptidoglycan layer Accumulation of outer membrane components Changed the fluidity of bacterial membrane Reduced cross-linking of peptidoglycans	Containing periplasmic components Containing LPS
CMV	Gram-positive bacteria	20–400 nm	Bilayers • A layer of lipid membrane • A cell wall of peptidoglycan and lipoteichoic acid	Nucleic acids Proteins Lipids Enzymes Toxins	Outgrowth of exposed lipid membrane outgrowth led by disrupted peptidoglycan layers Introduction of signaling molecules, autolysin and antibiotics	Without LPS Without periplasmic components
Synthetic SyBV						
DMV	Damaged bacterial membrane	Average size 250 nm	Inner and outer lipid membranes Two membrane linkers of peptidoglycans	Components and periplasm of parental bacterial membrane Antigens	Released bacterial contents by physical fragmentation and self-assembly formation of bilayer membranes	Containing many antigens required for vaccine development
PDNV	Gram-negative or Gram-positive bacteria	20–400 nm	Inner membrane	Bacterial cytoplasmic protein Antigens	Protoplasts physically squeezed through grille	Manageable in size Free of toxic outer membrane components, cell debris, and protein aggregates

designed to encapsulate specific cargoes such as proteins, nucleic acids, or small molecules for targeted delivery [40]. Their applications range from drug delivery systems (as biocompatible nanocarriers) to synthetic biology (as minimalistic cellular mimics for studying biological processes) [46]. Advances in related engineering technologies can overcome the limitations of conventional vesicle technology and position CMVs as promising tools for personalized medicine and therapeutic innovation.

2.3. SyBVs

To tackle the limitations of naturally secreted OMVs (undesirable yields and immunogenicity), SyBVs consisting mainly of DMVs and PDNVs have been developed. The production of SyBVs, including DMVs, typically begins with the growth and harvesting of bacterial cultures. DMV production typically involves bacterial culture expansion followed by high-pressure homogenization to disrupt cell membranes and release cellular contents, triggering the spontaneous self-assembly of membrane fragments into biomimetic vesicles [12]. These (20–250 nm) DMVs which are close in size to the source OMVs retain the parental membrane components but have reduced protein/nucleic acid content [38]. Importantly, given the ability to unload intracellular proteins and nucleic acids, DMVs exhibit higher physiological safety than OMVs while preserving OMV-like reactogenicity and adjuvant properties [47]. In addition to high-pressure homogenization, using high pH to remove cytoplasmic components and detoxify membrane glycoproteins could be another potential promising strategy for enhancing the safety of natural OMVs. Specifically, protoplasmic spheres obtained by treatment with lysozyme and EDTA to remove the cell walls were treated with Tris-HCl (pH 8.0) to disrupt the intact structure, form membrane sheets and eliminate the cytoplasmic components. Subsequently, buoyant density-gradient ultracentrifugation is employed to obtain purified membrane sheets which were further exposed to ultrasonic energy to fabricate SyBV [13,48]. These vesicles are similar in morphology and diameter to natural OMV and are characterized by low contaminant concentration, low nucleic acid loads and relatively few cytoplasmic proteins [13].

PDNVs are fabricated by enzymatically degrading the cell wall of Gram-positive or the outer membrane of Gram-negative bacteria using lysozyme, followed by mechanical extrusion of the resulting protoplasts into 100–800 nm vesicles. PDNVs are characterized by their manageable size, absence of toxic outer membrane components, cell debris, protein aggregates and other contaminants. Notably, PDNVs exhibit lower toxicity compared to naturally secreted bEVs due to the removal of pyrogens (e.g., LPS), but still induce comparable or even stronger immune responses [11,13,49,50]. A recent study has engineered hybrid PDNVs from commensal strains *Akkermansia muciniphila*, *Bifidobacterium longum*, and *Bifidobacterium breve*. Compared to single-source PDNVs, these novel nano-functional units exhibit enhanced ability to target tumors, facilitate dendritic cell maturation, and coordinate anti-tumour immunity [50]. Hybrid PDVNs display higher production efficiency, shorter preparation process and better stability. More specifically, after more than 10 h of separation/purification, the yield of OMVs generally does not exceed 5 mg in 1 L bacteria (OD$_{600}$: 0.6–1.0), whereas the yield of vesicular protein amount of hybrid PDVNs can easily reach 40 mg within 5 h [50].

3. Engineering modification of bEVs

bEVs hold great promise as versatile platforms for a wide range of applications owing to the distinctive non-cell system, nanoscale structures, favourable drug-carrying capacity and biocompatibility [5]. However, clinical translation of natural bEVs comes with cer-

tain limitations, mainly including: ① batch-to-batch variations in the composition, yield, size and bioactivity primarily caused by fluctuations with the culture conditions; ② inherent biological variabilities in production processes involving living microorganisms to generate structurally complex bEVs [10,18]; ③ limited scalability due to technical constraints of industrial-scale production (e.g., low-temperature protein overexpression requirements) [10,51]; ④ poor targeting ability in the biomedical field, especially in the targeting of cancer cells and some hard-to-reach tissues [52]. Safety concerns also arise from the presence of toxic and harmful or immunogenic components in bEVs, such as endotoxins in the outer vesicles [38].

To address these limitations, engineering strategies aimed at improving bEVs yield, targeting, immunogenicity and batch homogeneity hold tremendous translational value. This review provides a summary of the engineering strategies for bEV modification tailored to diverse applications, encompassing both the engineering of parental bacteria and post-isolation modifications of bEVs (Table 2 [10,53–141]). Systematically organized research advancements will be structured around three pivotal aspects: bEV release induction, surface modification, and lumen modification (Fig. 2).

3.1. bEV release induction

Strategic approaches to increase bEV production have focused on bacterial culture optimization and engineering of parental strains (Fig. 3). Optimization of culture parameters involves two key elements: nutrient regulation and the induction of environmental stresses.

3.1.1. Culture optimization

Culture optimization encompasses three main aspects: adjustment of nutrient composition, physical and chemical stimulation. In cultures lacking certain nutrients, bacteria modulate bEV via distinct mechanisms. For instance, the exhaustion of iron and sulfate leads to the downregulation of the VacJ/Yrb transporter responsible for phospholipid transport, consequently inhibiting phospholipids accumulation in the outer membrane, disrupting membrane symmetry and increasing bEV release [53,54,142]. Similarly, cysteine exhaustion activates the oxidative stress pathway, which induces a wide range of bacterial stress factors that enhance bEV production [55]. Additionally, Mg^{2+} deficiency increases the levels of deacetylase PagL protein, which catalyzes the deacetylation of lipid A [38]. This enzymatic modification reduces the cross-sectional area of lipid A, inducing its inverted conical conformation, triggering membrane bulking, and ultimately facilitating bEV production [66,143].

Culture parameter modifications also dramatically affect bEV yield. Exposure to environmental stressors (e.g., extreme temperatures, acidic pH, oxidizing conditions, and ultraviolet (UV) irradiation) provokes the prompt release of bEV, which is an immediate protective response [63–65,143]. Specifically, high-temperature conditions not only increase membrane fluidity, but also facilitate the generation of quinolone-like *Pseudomonas* signals, that potently interact with LPS under heat stress to boost bEV production [56]. Conversely, low-temperature conditions have been shown to induce stress responses in some bacteria and promote bEV production [57]. Under unfavorable pH conditions for growth, the reorganization of the outer membrane during bEV formation helps maintain cell membrane integrity [58]. Notably, the high external pressure of dissolved oxygen governs the production of *N. meningitidis* bEV via the oxidative stress pathway [59]. OMV production by *N. meningitidis* can also be further regulated by a continuous culture system, achieving daily yields of up to 4.0 L and over 600 h, while maintaining product properties equivalent to conventional methods [144]. Since small variations in the culture process

Table 2
Overview of different engineering methods for bEVs.

Engineering approach	Object	Method	Purposes	References
Physical modification	*Escherichia coli* (*E. coli*); N. meningitidis; *Vibrio cholerae* (*V. cholerae*)	Depletion of sulfate, iron, or cysteine	Increase bEV production	[53–55]
	Pseudomonas aeruginosa (*P. aeruginosa*); *Serratia marcescens* (*S. marcescens*)	High/low temperature conditions		[56,57]
	Salmonella typhimurium (*S. typhimurium*)	Low pH conditions		[58]
	N. meningitidis	High dissolved oxygen tension		[59]
	Bordetella pertussis (*B. pertussis*); *Bordetella bronchiseptica* (*B. bronchiseptica*)	Sonication		[60–62]
	P. aeruginosa	Nitrogen cavitation		[10]
	Freshwater bacteria	UV		[63]
	Lactobacillus plantarum (*L. plantarum*)	Sonication and loaded with fucoxanthin	Increase bEV production Improve the effect of treating colitis	[64,65]
Chemical modification	*S. typhimurium*; *N. meningitidis*; *E. coli*	Treatment with detergents or chelating agents	Increase bEV production	[66–69]
	E. coli; *Acinetobacter baumannii* (*A. baumannii*); *P. aeruginosa*; *B. bronchiseptica*	Exposure to antibiotics or antimicrobial peptides		[70–75]
	S. typhimurium; *Brucella abortus* (*B. abortus*)	Covalently conjugate antigens to bEV via carbodiimide adipic acid and sulfhydryl-maleimide	Improve bEV immunogenicity	[76,77]
	S. typhimurium; *Shigella sonnei* (*S. sonnei*)	Covalently conjugate antigens to bEV via GMMA		[76,78,79]
	E. coli	Non-covalently conjugate antigens to bEV via SpyCatcher		[80,81]
		Receptor-ligand binding with RGD and RGP, and then covalently conjugate ICG		[82]
Membrane fusion and coating	*P. aeruginosa*; *E. coli*; *Salmonella*	Fusion bEV with eukaryotic membrane	Increase bEV targeting efficacy	[10,83–85]
	S. aureus; *Salmonella*; *E. coli*	Coating bEV with MSN/ICG and RGD peptide	Elicit specific immune responses	[86–88]
	E. coli	Coating bEV into CaP NPs, anti-CD11b antibody-decorated NPs and NPNs	Increase bEV targeting efficacy Increase bEV stability	[89–91]
	Klebsiella pneumoniae (*K. pneumoniae*); *E. coli*	Coating bEV onto BSA, MSN and 5-FU-based NPs	Increase bEV stability	[92,93]
	L. casei; *L. plantarum*	Coating bEV onto aldehyde/sulfate latex microparticles	Induce anti-inflammatory effects Ameliorate inflammation-induced loss of intestinal barrier function	[94]
	E. coli; *S. aureus*; *Helicobacter pylori* (*H. pylori*)	Coating bEV into PLGA, zein and chitosan-based NPs	Improve bEV stability and antigen presentation efficiency Increase bEV targeting efficacy	[95–99]
Genetic engineering	*E. coli*	Deletion of *tolRA*, *Lpp*, *degS*, *degP* gene Overexpression of OmpT and PagL protein	Increase bEV production	[100–102] [66,103]
	A. baumannii	Deletion of *OmpA* gene		[104]
	N. meningitidis	Expression of OspA Deletion of *PorA* and *PorB*	Improve bEV immunogenicity against meningitis	[105] [106]
	S. typhimurium	Expression of pneumococcal surface protein A (PspA)	Confer bEV immunogenicity against *Salmonella* infection	[107]
		Expression of Vi Ag and O:2 OAg via GMMA Deletion of flagellin		[108] [109]
		Deletion of *OmpA*, *OmpC*, and *OmpD*	Confer bEV immunogenicity against *S. enteritidis* and avian pathogenic *E. coli* O78 infection	[110]
	E. coli; *S. typhimurium*; *V. cholerae*; *N. meningitidis*	Expression of SARS-CoV-2 RBD and spike protein	Confer bEV immunogenicity against SARS-CoV-2 infection	[111–113]

Table 2 (*continued*)

Engineering approach	Object	Method	Purposes	References
	E. coli; *S. typhimurium*	Expression of *Mycobacterium tuberculosis* (*M. tuberculosis*) antigens ESAT6, Ag85B, and Rv2660c fused with autotransporter factor H-binding protein (fHbp)	Develop multivalent recombinant bacterial vector vaccines	[114,115]
	E. coli	Expression of *A. baumannii* Omp22 fused with ClyA	Confer bEV immunogenicity against *A. baumannii* infection	[116]
		Expression of SARS-CoV-2 RBD fused with ClyA	Confer bEV immunogenicity against SARS-CoV-2 infection	[117]
		Expression of M2e4xHet fused with ClyA	Elicit heterologous influenza protection	[118]
		Expression of OmpA fused with FLAG	Improve bEV immunogenicity	[119]
		Expression of *Streptococcus pneumoniae* (*S. pneumoniae*) CPS14 fused with glycoengineering	Confer bEV immunogenicity against pneumococci	[120,121]
		Expression of PD1 fused with ClyA	Induce antitumor immunity	[122]
		Expression of B16-M30 and epidermal growth factor receptor vIII (EGFRvIII) fused with fHbp		[123]
		Expression of bFGF		[88,124]
		Displaying engineered glycotopes and conserved surface polysaccharide	Elicit protective effect against tularemia	[125]
		Displaying conserved surface polysaccharide	Elicit broadly antimicrobial response	[100]
		Display *S. aureus* FhuD2 and Hla$_{H35L}$ proteins	Improve bEV immunogenicity	[126]
		Display streptococcal antigen with the OmpA leader sequence	Confer bEV immunogenicity against GAS lethal challenge	[127]
		Display *C. muridarum* HtrA	Neutralize Chlamydia infection	[128]
		Fusion of Trx with the human papillomavirus E7 during SyBVs formation	Enhance antitumor immunity	[129]
		Expression of HA with peptide linker RBD	Confer bEV immunogenicity against H1N1 and MERS-CoV infection	[130]
		Expression of lipid A 4′-phosphatase	Confer bEV immunogenicity against H1N1 influenza	[131,132]
	EcN	Overexpression of C–X–C motif chemokine receptor 4 (hCXCR4) fused with the ClyA and loaded with SOST siRNA	Increase bEV targeting efficacy Promote their osteogenic differentiation	[133]
		Incorporating RBD into bEV and display NG06 fused with ClyA	Confer bEV immunogenicity against SARS-CoV-2 infection	[134]
		Expression of quorum sensing-regulated PGase	Increase bEV production Enhance innate immune responses	[135]
	V. cholerae	Incorporating *E. coli* PhoA into bEV	Induce specific antibody response	[136]
	Salmonella	Delivering OAg with GMMA or glycoengineering	Improve bEV immunogenicity	[135]
	S. aureus	Expression of *S. aureus* antigens Hla$_{H35L}$, leukocidin ED (LukE), FhuD2, conserved staph antigen (Csa1A), and SpA$_{KKAA}$	Confer bEV immunogenicity against *S. aureus* infection	[138]
		Expression of dengue virus antigens Mntc, PdhB, PdhA, and Eno fused with FLAG tag	Confer bEV immunogenicity against dengue virus infection	[139]
	Bacteroides thetaiotaomicron (*B. thetaiotaomicron*); *E. coli*	Expression of OmpA/SseB or KGF-2	Elicit immunogenicity against virus infection and colitis	[140]
		Expression of F1 and V plague antigens	Elicit immunogenicity against plague	[141]

5-FU: 5-fluorouracil; bFGF: basic fibroblast growth factor; BSA: bovine serum albumin; CaP: calcium phosphate; ClyA: cytolysin A; CPS14: serotype 14 capsule; EcN: *E. coli* Nissle 1917; GAS: group A *Streptococcus*; GMMA: generalized modules for membrane antigens; HA: haemagglutinin; ICG: indocyanine green; KGF-2: keratinocyte growth factor-2; MERS: Middle East Respiratory Syndrome; MSN/ICG: magnetic mesoporous silica nanoparticles-indocyanine green; NPNs: pathogen-mimicking nanopathogenoids; NPs: nanoparticles; OAg: O antigen; OmpA: outer membrane protein A; OmpT: outer membrane protease T; OspA: outer surface protein A; PhoA: periplasmic alkaline phosphatase; PLGA: poly(lactic-*co*-glycolic acid); RBD: receptor-binding domain; Trx: thioredoxin; RGD: arginyl-glycyl-aspartic acid; RGP: α$_v$β$_3$ integrin targeting ligand; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; siRNA: small interfering RNA; UV: ultraviolet.
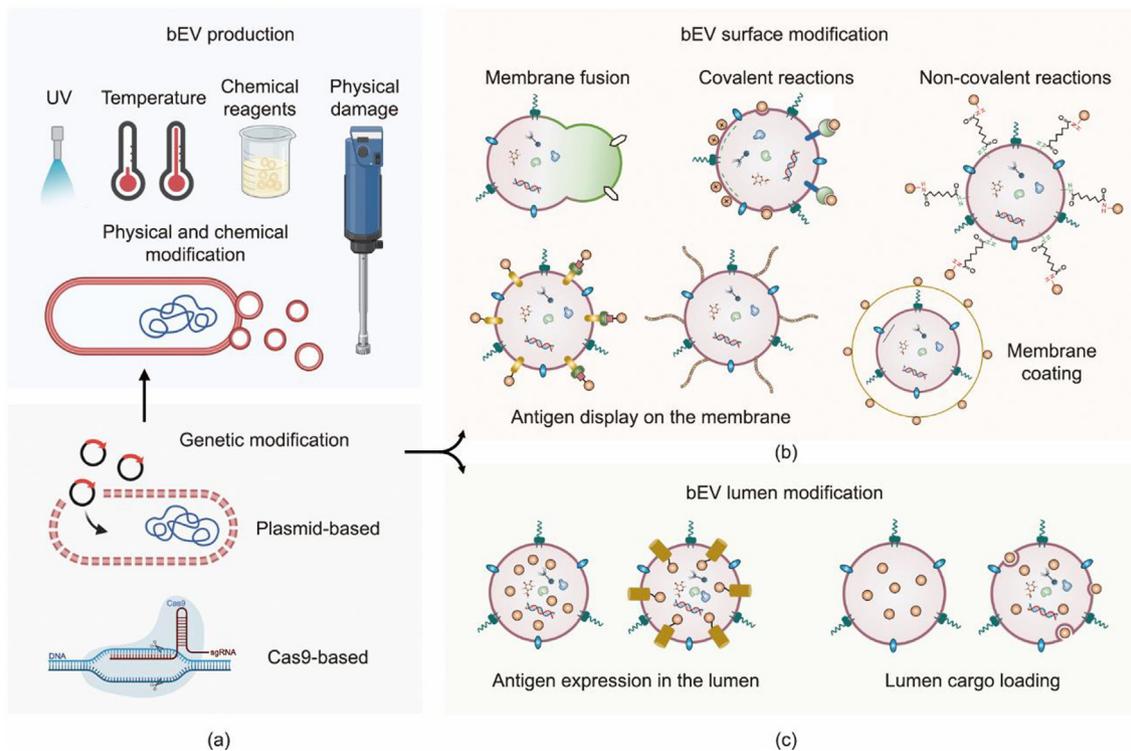
Fig. 2. Engineering approaches for modifying bEVs. The engineering approaches can be classified into three types based on the purposes, namely (a) bEV release production, (b) surface modification, and (c) lumen modification. To improve the bEV production, optimizing culture systems or inducing hypervesiculation via physical/chemical treatment and modifying gene expression related to the Tol-Pal system have been introduced. Genetic engineering can be used to display antigens on the bEV surface either directly or indirectly through fusion with the leading protein. In addition to genetic modification, membrane fusion/coating and chemical modifications are widely used approaches for membrane surface modification of bEVs. Similarly, genetic engineering can also be used to express antigens on the bEV lumen either directly or indirectly through fusion with the leading protein. By physical damage and chemical alteration, it is also feasible to load antigens and molecules within the interior space of bEVs. Cas9: clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9; sgRNA: small guide RNA; Temp.: temperature.
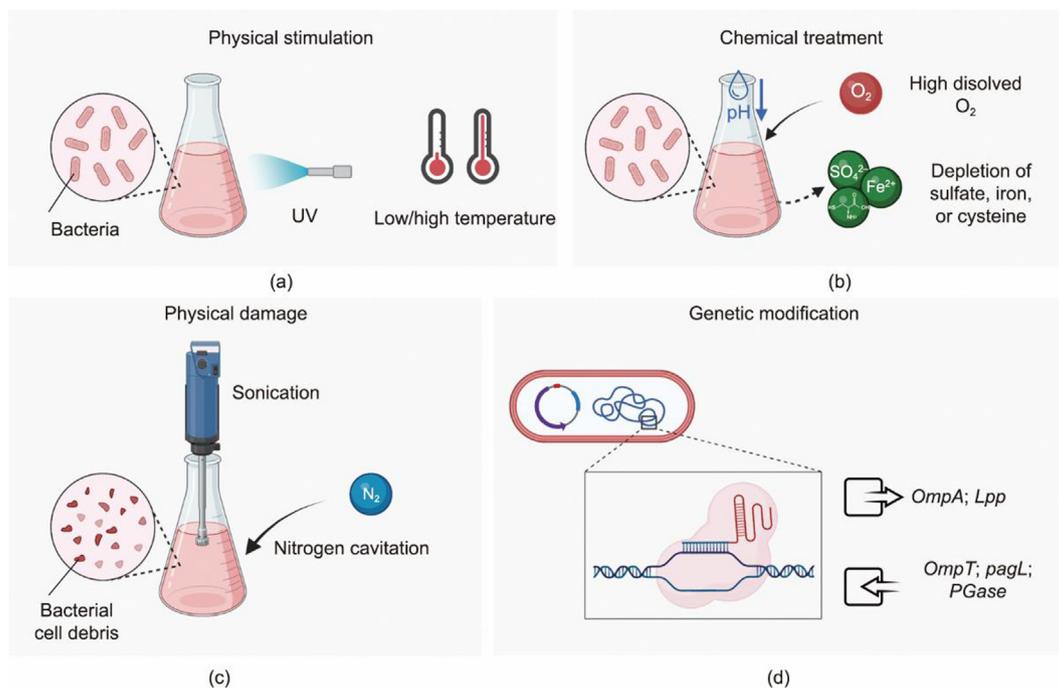

Fig. 3. Methods for promoting the production of bEVs. (a) Physical stimulation with abnormal or extreme environmental factors (e.g. low/high temperature and ultraviolet (UV)) induces substantial bEV releases as an instant self-protective response. (b) Chemical stimulation with abnormal or extreme environmental factors (e.g., low pH and oxidation) or depletion of nutrient content can induce substantial bEV releases as an instant self-protective response. (c) Physical sonication or nitrogen cavitation damage can induce bacterial cell debris reassembly of DMVs. (d) Genetically depletion of *OmpA* and *Lpp* genes diminishes the cross-linking between the outer membrane and the peptidoglycan layer, resulting in increased bEV production. The overexpression of *OmpT*, *pagL*, and *PGase* causes an inverted cone-shaped LPS and cleavage of peptidoglycan, enlarging the curvature of the bacterial outer membrane and leading to hypervesiculation.

considerably affect the quality and activity of bEVs, the optimization of parameters based on culture conditions must generally be systematically paired with a rigorous quality assessment.

3.1.2. Physical and chemical treatments

Physical and chemical interventions can alter the physiological state of bacteria to stimulate bEV production. Decontaminant treatments and chelating agent applications can destabilize bacterial membranes, thereby promoting bEV secretion [66,67]. On the other hand, detergents (e.g., deoxycholate or sodium dodecyl sulfate) can also eliminate outer membrane LPS of bEVs, thereby effectively minimizing LPS-triggered innate immune response [145]. Similarly, chelating agents, such as EDTA can neutralize the negative charge of LPS, inducing membrane destabilization and subsequent bEV secretion [68,69]. This effect is similar to that of detergent but is relatively mild, thereby retaining LPS and native cargo in bEVs [146].

Exposure to sub-lethal concentrations of antibiotics can activate protective stress responses and promote bEV production [70,71]. Ciprofloxacin induces DNA fragmentation and activates the SOS response, a system triggered by DNA damage. The activated genes consequentially suppress or inhibit cell division, temporarily affecting membrane status and thereby prompting bEV release [72]. Notably, eravacycline-induced *Acinetobacter baumannii* (*A. baumannii*) OMVs enrich outer membrane proteins and contain resistance-associated proteins such as adenosine triphosphate (ATP)-binding cassettes, suggesting that sublethal concentrations of antibiotics may contribute to antibiotic dissemination [73]. Antimicrobial peptides similarly stimulate bEV release, although their induced vesicles contain elevated phosphatidylglycerol levels, compromising their thermal stability [74,75].

Mechanical methods such as sonication enhance DMV yields by increasing membrane instability [60–62]. DMVs generated from bacterial membrane fragments may contain cargo that does not exist in naturally produced bEVs [60]. Although sonication results in higher DMV production, these vesicles are not fully representative of the *in vivo* protein composition of bEVs and thus require thorough assessment. Nitrogen cavitation enables the rapid preparation of *Pseudomonas aeruginosa* (*P. aeruginosa*)-derived nanovesicles [10]. The DMVs obtained were formed from the entire bacterial membrane and retained the integrity of the bacterial membrane. This method enables the scale-up of 15–20 mL cell suspensions to 10 L, while maintaining good reproducibility. In addition, because of the mild nature and protective effect of nitrogen, membrane proteins are not damaged and oxidation is avoided during the preparation process [10]. These advantages make the DMV an emerging technology in vaccine development [10]. Critically, all methods can shape the size, components, protein hydrolysis and stability of resulting bEVs, thereby directly determining the outcome of the immune responses triggered by bEVs [15,147,148].

3.1.3. Genetic engineering

Genetic engineering has also been used to modify bacteria to promote bEV secretion, with the main modification targeting molecules associated with outer membrane connections [149]. Due to the crucial role in connecting the outer membrane and peptidoglycan layer, the Tol-Pal system is commonly the target of engineered mutants to increase vesicle yield [100,150]. In *A. baumannii*, deletion of the outer membrane protein A (*OmpA*) gene disrupts its interaction with peptidoglycan-derived diaminoheptanedioic acid, weakening the outer membrane stability and promoting OMV secretion [104]. Similarly, the knockdown of the structural gene *Lpp* in *Escherichia coli* (*E. coli*) diminishes the binding between the outer constituents and the peptidoglycan layer, resulting in augmented bEV release [101]. In addition to disrupting the cell wall structure, the accumulation of the envelope compo-

nents (e.g., LPS and peptidoglycan debris) further exacerbates the stress on the outer membrane to foster bEV secretion [102]. For example, silencing genes related to the *VacJ/Yrb* transport system leads to the buildup of phospholipids in the outer membrane and induces bEV production [54,56]. Overexpression strategies have also been shown to be effective: up-regulation of outer membrane protease T (*OmpT*) enhances the bEV secretion by cleaving peptidoglycan-anchored proteins [103], whereas the overexpression of deacylase PagL and hydrolase (PGase) causes an inverted cone-shaped LPS and cleavage of peptidoglycan, collectively promoting membrane curvature and hypervesiculation [66,135].

Although genetic modifications enable spontaneous vesicle generation, concerns remain about differences between modified bEVs and their natural counterparts, potentially affecting their activity. As an illustration, bEVs generated by *degP* mutants differ notably from natural *E. coli* bEVs in terms of cargo composition, particularly in enriched periplasmic proteins [151]. In addition, multilamellar vesicles appeared in bEVs produced by the *Buttiauxella agrestis ΔtolB* mutant, while the entry of *E. coli ΔtolR* bEVs into epithelial Caco-2 cells was reduced [152,153]. Another drawback of genetic modifications is variability in the effectiveness of mutations across bacteria, necessitating dedicated research to identify specific mutations in different bEVs.

3.2. bEV surface modification

The three most widely used approaches for bEV membrane surface engineering are membrane fusion/coating, chemical modification, and genetic engineering. Compared to encapsulated antigens, antigen exposure on the vesicle surface is more advantageous for inducing antibodies by activating antigen-specific B cells (Fig. 4) [38].

3.2.1. Membrane fusion

The membrane fusion technique involves the mechanical extrusion-mediated integration with foreign membrane structures. This fusion imparts new functionalities to the bEVs, leading to potential therapeutic advantages [154]. Notably, bEVs exhibit the capability to merge prokaryotic membranes, thereby amplifying their therapeutic efficacies [10,83,84]. For example, bEVs–cancer EVs, hybrid membranes formed through membrane fusion technology, exhibit tumor-targeting properties and immunogenicity, enabling target delivery of coated poly lactic-*co*-glycolic acid (PLGA)–indocyanine green (ICG) nanoparticles (NPs) to tumor cells [84].

3.2.2. Membrane coating

Membrane coating stands out as nanotechnology with versatile applications in enhancing the capabilities, tissue targeting and scalability of EVs [155]. In this regard, Chen et al. [86] employed *S. aureus* CMVs-coated magnetic mesoporous silica nanoparticles (MSNs), which are loaded with ICG to enable targeted delivery of MSN–ICG to dendritic cells (DCs). Moreover, upon the incorporation of Arg–Gly–Asp peptides, the tumor-targeting capability of OMV in mice increased by 2.5 times and 11 times in *Salmonella* or *E. coli*, respectively [87,88]. Calcium phosphate (CaP) further enhanced this capability through pH-responsive controlled-release substance, while neutralizing the acidic tumor microenvironment (TME) and extending circulation time for reinforced tumor accumulation [89]. To amplify this property, the incorporation of folic acid, a tumor target ligand, into CaP shells promotes the active targeting of mouse tumor [89].

Another comparable indirect approach involves the internalization by circulatory cells, followed by subsequent delivery to target cells [90]. The efficacy of this approach has been illustrated through the targeting of circulating neutrophils using NPs modified
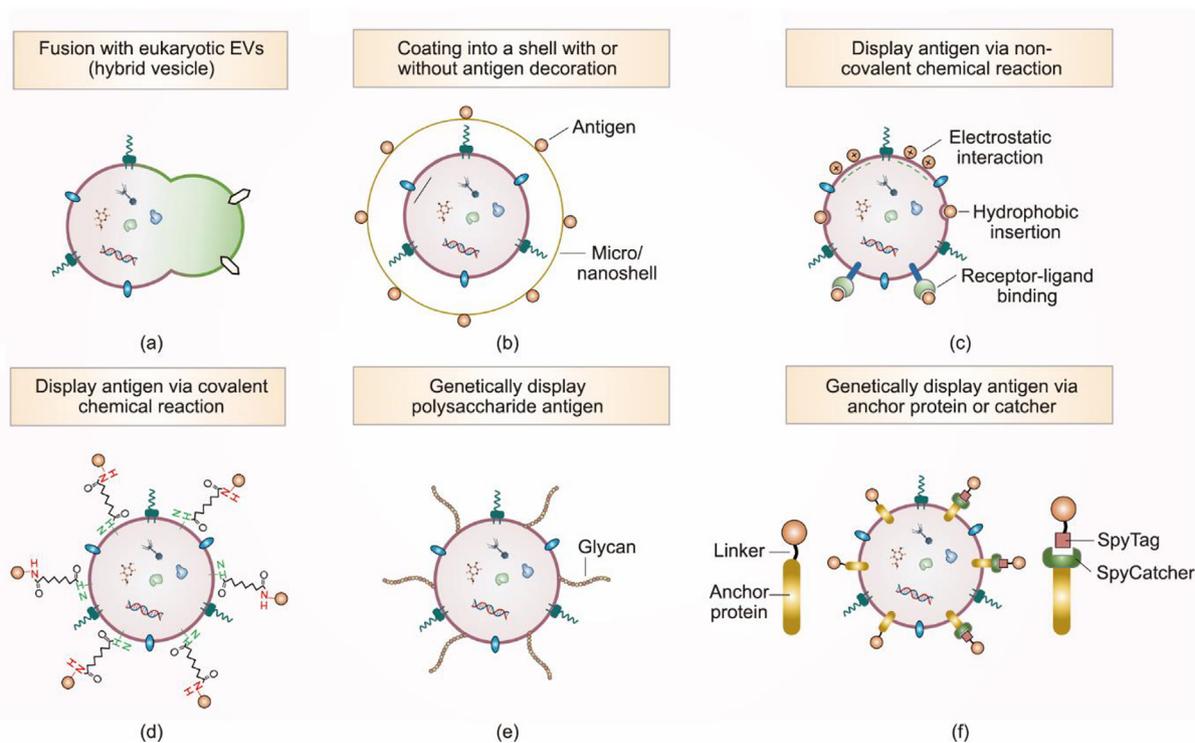
Fig. 4. Methods for bEV surface modification. (a) Fusion bEVs with eukaryotic EVs form hybrid vesicles. (b) Coating bEVs into a micro/nanoshell with or without antigen decoration. (c) Non-covalently modified bEVs via multivalent electrostatic interactions, receptor–ligand binding and hydrophobic insertion. (d) Covalently modified bEVs by click chemistry. (e) Displaying polysaccharides antigens on the surface of genetically engineered bEVs. (f) Covalently linking anchored protein–SpyCatcher and homologous 13-amino-acid peptide (SpyTag)–protein fusions to the bEV surface for antigen display.

with anti-CD11b antibodies, resulting in subsequent accumulation in mouse tumors [90]. To enhance the effectiveness of bEVs uptake by neutrophils, pathogen-mimicking nanopathogenoids (NPNs) is introduced to encapsulate bEVs. The neutrophil targeting efficiency of NPs using CD11b-modified NPNs in mouse peripheral blood was only 30%, whereas the targeting efficiency of bEV-enveloped NPNs was elevated up to 41% [90,91]. Compared to natural bEVs, the coating technology not only enhances the stability in blood circulation but also strengthens the immune response specific to antigens [87,156]. This feature extends to OMVs from *Klebsiella pneumoniae* (*K. pneumoniae*) and *E. coli* NPs such as bovine serum albumin (BSA), zein and chitosan-based NPs, which synergistically augment the bEVs-induced immune responses [92,95–97]. Similarly, coating CMVs from *Lactobacillus casei* (*L. casei*) and *Lactobacillus plantarum* (*L. plantarum*) onto aldehyde/sulfate latex microparticles induces anti-inflammatory effects and ameliorates inflammation-induced intestinal barrier dysfunction [94]. These enhanced immune responses may be attributed to the uniform size of the coated bEVs and increased *in vivo* diffuse capability [97]. The aforementioned evidence suggests that the immunological effects are augmented by increased bEVs antigen presentation capacity when bEVs are encapsulated or loaded onto NPs. However, the effects on the characteristics of the triggered immune response could vary on the type of NPs employed. To circumvent NP-associated variability, nitrogen cavitation-derived bacterial membrane nanovesicles are introduced to produce stable immunogenic DMV without the need for external NPs [10].

3.2.3. Chemical modification

Chemical modification is a common technique to anchor exogenous antigens or molecules onto the surface of bEVs. These approaches can be categorized into two primary types: the covalent and non-covalent reactions. Widely used covalent technologies for EV membranes include bioconjugation, click chemistry, and aldehyde amine condensation [38]. For antigen-specific targeting, chemical agents such as adipic acid, carbodiimide, and sulfhydryl-maleimide have been effectively utilized [76,77]. Nonetheless, these techniques require the pretreatment of bEVs and exogenous antigens before conjugation, entailing multiple procedures and potentially resulting in particle aggregation. To address these challenges, a refined chemical coupling approach, featuring a straightforward and safe detergent-free protocol for the high production of generalized modules of membrane antigens (GMMAs) carrying heterologous antigens, has been introduced [78,157]. This system employs two distinct chemical approaches—bis(sulfosuccinimidyl)suberate (BS3) crosslinking or reductive amination that enable direct coupling (of protein antigens and polysaccharides) via their lysine residues without requiring external antigen derivatization [76,79]. This flexibility makes the GMMA system convenient "plug-and-play" technology for developing conjugate vaccines against a wide range of pathogens. Notably, when targeting molecules lacking natural lysine residues (e.g., certain polysaccharides), an additional introduction of the amino/hydrazide linker is necessary [79].

In contrast to covalent approaches, non-covalent modifications can be achieved through strategically implemented multivalent electrostatic interactions, hydrophobic insertion or receptor-ligand binding [38]. A representative application includes arginyl-glycyl-aspartic acid (RGD) and $\alpha_v\beta_3$ integrin targeting ligand (RGP) to functionalize the surface of bEV, which subsequently conjugates with ICG through the fusion effect and electrostatic interaction [82]. Another novel strategy utilizes bEVs designed from surface-exposed SpyCatcher proteins that spontaneously form covalent bonds with target proteins fused to the complementary SpyTag, a homologous 13-amino-acid sequence [80]. This approach leverages the SpyCatcher structural domain

from the *Streptococcus pyogenes* surface protein to identify SpyTag. Upon identification, a covalent isopeptide connection is established between the side chain of aspartic acid in SpyTag and lysine in SpyCatcher [158]. This linkage system significantly enhances the bEV platform for showcasing antigens and achieves a more effective presentation than the cytolysin A (ClyA) fusion system [81]. The modular nature of this approach allows for the scalable production of bEVs with the flexibility to incorporate various target antigens onto the surface. Furthermore, micelles composed of phophatidylethanolamine–poly(ethylene glycol)–biotin (DSPE–PEG–biotin) or DSPE–PEG–folate (DSPE–PEG–FA) can be integrated into the outer membrane. The resulting biotinylated bEVs exhibit a specific binding affinity for various streptavidin-conjugated components, including streptavidin–dye conjugates and antigens–antibodies complexes. This interaction presents prospects for specific labelling and modulation of biological activities [159].

3.2.4. Genetic engineering

Genetic engineering of the bEV-forming bacteria could also be used for surface modification of bEVs to enhance immunogenicity [149]. A prevalent strategy involves the expression of recombinant proteins fused to carrier proteins that direct them to the external membrane and are present on the bEV surface [160]. Commonly employed leading proteins encompass ClyA, a type of pore-forming hemolytic protein, adhesin involved in diffuse adherence (AIDA-I) autotransporter domain, hemoglobin protease (Hbp) and fHbp of *N. meningitidis* [38]. Historically, the concept of surface antigen presentation originated from the autotransporter protein system. These proteins traverse via the Sec pathway in an unfolded state, after which periplasmic chaperones SurA and β-barrel assembly machinery A (BamA) mediate their folding, assembly, and translocation across the outer membrane [161]. Appropriately folded autotransporters firmly attach to the outer membrane via the C-terminal β structural domain. Various recombinant protein antigens can be produced in weakened strains of *S. Typhimurium* by merging with autotransporter Hbp of *E. coli*, and these antigens can be presented on the surface of the outer membrane [114]. The fusion antigens on the surface of *E. coli* OMVs utilized the Hbp autotransport system by substituting the side structural domains D1, D2, D4, and D5 with antigens from *Mycobacterium tuberculosis* (Mtb), specifically Ag85BC, Ag85BN, ESAT6, and Rv2660c [115]. However, the effectiveness of this antigen display approach may be limited when dealing with larger-sized protein fragments [162]. In addition, successful exposure of borrelial outer surface protein A (OspA) on the surface of *N. meningitidis* OMVs occurred through fusion with fHbp. When comparing the immunogenicity of *N. meningitidis* OMVs with internally expressed OspA, only the surface-exposed form can provoke OspA-specific [105]. Similarly, outer membrane proteins and LPS on the surface of *Salmonella* OMV can trigger a more severe immune response than vesicle-enveloped heterologous antigens [107].

Genetic engineering strategies are able to produce sophisticated surface modification of bEVs to enhance immunogenicity. A study engineered *E. coli* OMV surfaces by incorporating the ClyA-fused extracellular domain of programmed death 1 (PD1). This modification enhances OMV accumulation at the tumor location and a programmed death-ligand 1 (PD-L1) blockade, triggering more potent anti-tumor immune responses than the administration of natural OMVs. This was evidenced by 1.5-fold proinflammatory cytokine levels in both serum and tumor tissue, as well as corresponding to a 1.5-fold tumor growth impairment in mice [122]. Furthermore, the fusion expression of antigen with ClyA enables the presentation of larger proteins. In *E. coli* DH5α strain, Omp22 of *A. baumannii* was successfully displayed on the OMV surface [116]. Subsequently, after fusion with ClyA, the receptor-binding domain (RBD) of severe acute respiratory syndrome coronavirus 2 (SARS-

CoV-2) could still be presented and trigger antibody neutralization [117]. Due to the respiratory affinity of the SARS-CoV-2 virus, mucosal immunization is regarded as a highly promising approach.

Recent research has employed diverse approaches to showcase the RBD or spike protein on the surfaces of bEVs for the activation of intranasal immunity. These endeavors have proven successful in high titers of immunoglobulin G (IgG) and mucosal immunoglobulin A (IgA) with synergistic protective effects [111–113]. Furthermore, upon fusion with PrsA which functions as an anchor of lipoproteins on the inner membrane [163], epidermal growth factor (EGF) is presented on the surfaces of PDNVs in *E. coli* [164]. To optimize antigen presentation, researchers developed an *E. coli* mutant deficient in 59 endogenous proteins, which increased surface exposure on bEVs. This, in turn, elevates the loading capacity for targeted antigens and consequently augments the immune response [126].

In addition to protein components, glycoengineering methodologies facilitate the presentation of polysaccharide antigens on bEVs [165]. For instance, Price et al. [120] have constructed a modular plasmid encoding all essential glycosyltransferases, flippase and polymerase for synthesizing *S. pneumoniae* serotype 14 capsule (CPS14). Following the transformation of this plasmid into *E. coli* mutant deficient in O antigen-initiating glycosyltransferase, the generated capsular polysaccharide from *S. pneumoniae* was linked to the lipid A core of LPS, which enabled engineered OMVs released by *E. coli* to transport the CPS14 antigen. In another research, the Tularia O antigen polysaccharide (O-PS) was artificially produced within an O-PS-deficient *E. coli* strain. The exogenous O-PS prompted the assembly of the essential O-PS structure via lipid A core glycosylation. Glycosylated OMV produced by this process can protect mice against tularemia infection [125]. An alternative approach introduced, poly-*N*-acetyl-*D*-glucosamine (PNAG), a conserved carbohydrate polysaccharide immunogen expressed by multiple pathogens, has shown great research value. PNAG-displaying bEV elicited PNAG-specific antibody responses that proficiently facilitated the eradication of diverse PNAG-producing strains pathogens in both cellular and animal models, ultimately protecting against lethal infections [100]. These findings indicate that engineered polysaccharide-containing bEVs targeting universal polysaccharide antigens could induce a comprehensive immune defense against a range of pathogen-containing PNAG.

The ease of polysaccharide incorporation underscores the potential of bEVs as candidates in therapeutic applications. Both chemical modification and genetic methodologies enable the presentation of antigenic epitopes on bEVs to effectively boost immunogenicity, thereby offering substantial flexibility in vaccine development. Since the uptake of granules-containing antigens plays a crucial role in enabling antigen-presenting cells (APCs) and subsequent immune response, cautious selection of anchoring proteins or engineer techniques is imperative to ensure efficient connection and presentation of recombinant epitopes while maintaining normal bEV biogenesis or the viability of the parent bacteria.

3.3. bEV lumen modification

In addition to surface modification, heterologous antigens can be enveloped within bEVs by physical manipulation, chemical alteration and genetic engineering techniques. It is feasible to locate antigens and molecules with immunomodulatory properties in the interior space of bEVs (Fig. 5).

3.3.1. Physical engineering

Physical engineering techniques facilitate cargo loading into the bEV lumen. For example, SOST small interfering RNA (siRNA) was
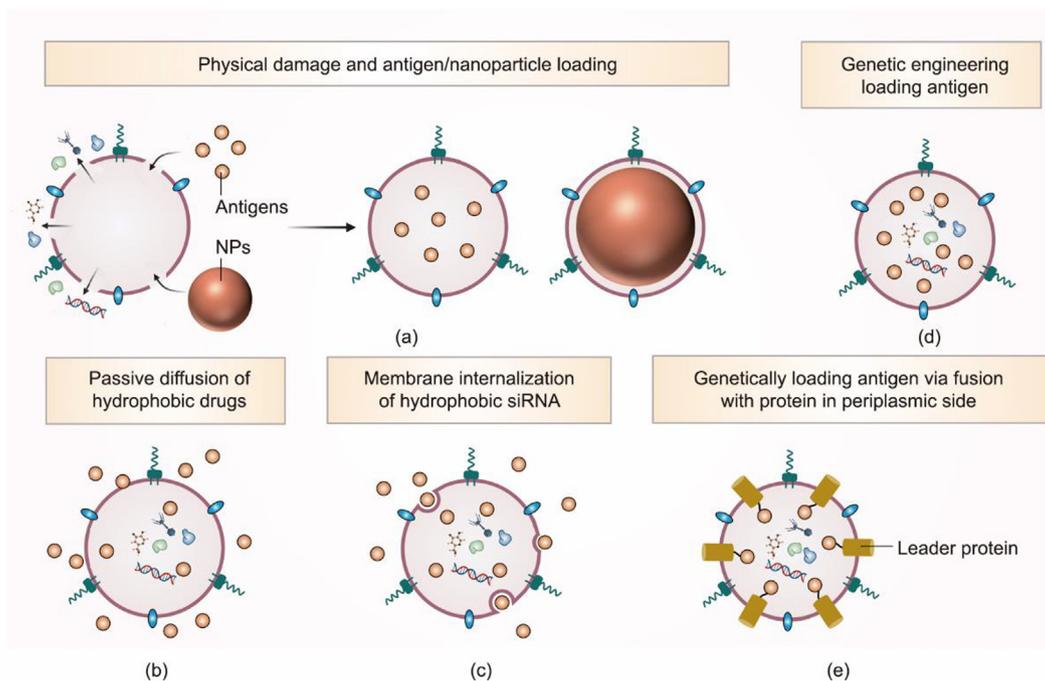
Fig. 5. Methods for bEV lumen modification. (a) Physical damage to bEVs via sonication and loading antigen or NPs into bEVs. (b, c) Loading hydrophobic drugs via (b) passive diffusion and (c) membrane internalization. (d) Introduction of genetic manipulation for loading antigens through periplasmic release. (e) Some leader proteins, such as pneumococcal surface protein A (PspA) and outer membrane protein A (OmpA), are utilized to achieve the internal loading of antigens. siRNA: small interfering RNA.

loaded into OMVs from probiotic *E. coli* Nissle 1917 (EcN) by electroporation, which regulates the WNT signaling pathway and stimulates osteogenic differentiation of bone marrow stromal cells (BMSCs) and ultimately ameliorates osteoporosis [133]. In addition, fucoxanthin loaded into *L. plantarum*-derived PDNVs using sonication and these vesicles improved the colonic inflammatory response [64]. Importantly, this engineering preparation strategy allows for the large-scale synthesis of membrane vesicles carrying therapeutic cargo.

3.3.2. Chemical engineering

Chemical treatment is another promising nanotechnology for loading therapeutics and enhancing the functionality of bEVs [166]. In this method, the therapeutic molecules need to be internalized into bEVs through their membrane. One non-invasive method for loading small drugs into bEVs is via co-incubation, which allows the loading of hydrophobic drugs through passive diffusion and has been successfully applied in loading fluoroquinolones [166]. In addition, modified siRNAs have been successfully loaded into EVs through conjugated with cholesterol for hydrophobic interactions and with single-stranded phosphorothioated for internalizing siRNA through EV membranes [167,168]. This study proposes that bEVs may utilize a mild, non-invasive encapsulation strategy for efficient siRNA loading, which could preserve the structural integrity and bioactivity of both vesicular carriers and therapeutic payloads.

3.3.3. Genetic engineering

Genetic modification of bEV-producing bacteria provides a way to enhance immunogenicity by directing the expression and periplasmic location of bioactive molecules [149]. The presence of antigens in the vesicle may activate cytotoxic T lymphocyte (CTL) responses and induce pathogen-specific antibodies [129]. A variety of antigens, such as the pneumococcal surface protein A (PspA), *Chlamydia muridarum* HtrA, streptococcal antigens and *E. coli* periplasmic alkaline phosphatase A (PhoA), have been

targeted to cavities of bEVs, triggering a protective immune response against pathogenic infections [107,127,128–136]. For example, PspA fused to N-terminal β-lactamase signal sequence is exported to the periplasm via the type II secretion system (T2SS) and incorporated into the interior of *S. Typhimurium* OMVs. OMVs elicit a modest antibody reaction and provide effective protection against *S. pneumoniae* [107]. In contrast, OMVs without PspA fail to induce specific antibody responses and confer immune defense [107]. Similarly, heterologously expression of *E. coli* PhoA in *V. cholerae* OMVs induced cholera-specific antibody production [136]. The incorporation of a streptococcal antigen into the OmpA leader sequence gives rise to a natural conformation on the periplasmic facet of *E. coli* OMVs, culminating in the generation of antibody titers with heightened functionality [127]. These findings suggest that internalized antigens may induce bEV structural changes post-inoculation, leading to the exposure or release of internal antigens that are subsequently recognized by APC to elicit a targeted antibody response. Moreover, during the synthetic SyBVs formation, the interaction between thioredoxin (Trx) and disulfide bond (Dsb) proteins in the periplasm leads to the enrichment of Trx fused with the E7 protein of the human papillomavirus (HPV) within the periplasm [129]. Taken together, the simplicity of genetic modification opens up diverse possibilities for loading antigens into bEVs.

3.4. Selection of bEVs engineering techniques

Different engineering strategies exhibit distinct characteristics and are suitable for specific applications, providing multiple options for the optimization of bEVs. The operational procedures for physical and chemical modification are relatively simple and have high flexibility, enabling rapid processing and functionalization of bEVs [5]. These approaches are particularly applicable to scenarios in which functionalized bEVs must be prepared in a short period. However, modified bEVs are less stable and may shed functional molecules in physiological environments. Additionally,

chemical reactions may introduce unintended toxicity or immune responses, thus limiting their application in certain biomedical applications [169,170]. Although physical methods eliminate the need for chemical reagents and minimize potential toxicity concerns, they may compromise the native structure and integrity of bEVs [171]. Moreover, physical and chemical modifications generally result in low loading efficiency and limited precision in cargo control [172].

In contrast, genetic engineering approaches based on gene expression regulation allow the targeted knockout of toxin-related genes to enhance biosafety [173]. This strategy demonstrates high precision and stability by fundamentally modifying bEVs at the genetic level, making it a promising option for clinical applications requiring long-term effects such as vaccine development [172,173]. However, owing to the requirement for in-depth knowledge of genetics and complex experimental designs, genetic engineering poses high technical barriers and extends the overall preparation time.

When classified according to functionalization sites, engineering strategies for bEVs can be divided into lumen modification and surface modifications. Lumen modification can effectively protect the cargo from enzymatic degradation and achieve intracellular release by encapsulating therapeutic molecules inside bEVs [174]. However, this is accompanied by limited loading efficiency, and the structural integrity of the membrane cannot be guaranteed [169]. Its relatively straightforward procedures make it broadly applicable to developing applications that require payload protection, but suffer from the limitation of insufficient target specificity [175].

In contrast, surface modifications allow precise modulation of bEVs biodistribution through membrane surface ligand engineering, which is essential for targeted therapy [175]. This strategy prolongs the circulation time and reduces immune clearance; however, its complex technological processes may alter the natural physicochemical properties of bEVs [175,176]. Both strategies demonstrate distinct advantages: luminal modification expertise on cargo protection, whereas surface modification excels in targeted delivery. The integration of the two strategies to build a multifunctional system is an important future development direction, that can optimize the therapeutic effect and reduce potential side effects in clinical applications, fully expanding the potential of bEVs applications in biomedicine [169].

Appropriate engineering strategies should be selected through a comprehensive evaluation of application-specific requirements (e.g., drug delivery versus immunotherapy), considering critical factors, such as modification precision, preparation time, cost-effectiveness, and product stability. For example, genetic engineering is generally preferred for vaccine development [173], whereas chemical and physical methods may be prioritized for urgent therapeutic delivery needs [177]. Collectively, whether classified by an engineering approach (e.g., genetic or chemical modification) or functional targeting site (lumen or surface modification), these strategies enhance the biomedical utility of bEVs. Thus, the optimal path forward is the integration of complementary engineering methods to construct multifunctional systems [178]. In clinical settings, such synergistic integration could enhance therapeutic functionality and outcomes while minimizing adverse effects.

4. Challenges and future directions for engineered bEV applications

Based on the plasticity of natural bEVs, modifications such as chemical and genetic engineering can further improve their performance in terms of product homogeneity, stability, productivity, drug loading, targeted delivery, multi-pathway synergism, and attenuation of potential side effects [38]. Advances in synthetic biology have enabled tailoring the design of engineered bacterial strains for a wide range of applications. One of the main applications of bEVs—the development of vaccines—is the best example of the application of engineered bEVs in the biomedical field. PorA, the main immunogenic protein of *N. meningitidis*, varies extensively among strains. This strain specificity makes it difficult to achieve desired vaccine efficacy [179]. Due to the presence of PorA in modifiable bEVS, Dutch researchers developed modifiable bEVs containing multiple *PorA* variants using genetically modified bacterial strains, creating a MenB vaccine [145,179]. This PorA-enriched bEV vaccine has been successful in combating meningitis outbreaks caused by MenB in areas such as Cuba, Norway, and New Zealand, with an effectiveness of 70% [179].

Engineered bEVs have significant advantages as carriers of heterologous recombinant proteins. The fusion of bEVs obtained from laboratory strains with natural bEVs proteins can effectively introduce heterologous antigenic proteins onto the surface of bEVs [180]. As an illustration, *E. coli*-derived ClyA, a commonly utilized implementation partner, has been successfully fused with the domain 4 moiety of *Bacillus anthracis* protective antigen and green fluorescent protein [181,182]. These genetic engineering strategies combined with plug-and-play technologies, have provided more flexible vaccine platforms for exposing heterologous antigens, which may dramatically increase vaccine efficacy. It has been proved that bEVs of *E. coli* DH5 with the addition of gold NPs elicit a stronger and longer-lasting specific immune response in mice [156]. Nevertheless, antigenic proteins that are not exposed to the surface may also trigger specific antigen–antibody reactions. This phenomenon occurs through the overexpression of target antigens in the periplasmic space, thereby increasing the chances of being included in bEVs [107]. The OmpA signal peptide of *E. coli* was successfully implemented with several overexpressed *Streptococcus*-derived proteins [127].

However, there are still several challenges in scaling-up bEVs from laboratory studies to clinical applications. First, although clinical studies have not yet detected toxicity triggered by the ingestion of bEVs, ensuring the non-carryover of immunogenic components such as biotoxins and LPSs remains a key priority for advancing the application of bEVs [47]. Current evidence has uncovered several virulence factors involved in invasion and diffusion into cells and tissues and their potential modes of action. Serine proteins and exfoliative toxins ease the disruption of physical barriers, while hyaluronate lyases and collagenases attack the host extracellular matrix [183–185]. Measures to mitigate such risks include knocking out toxin synthesis genes through genetic techniques, producing toxin-free bEVs through physical/chemical treatment methods, and using Gram-positive bacteria and toxin synthesis-deficient strains [51]. To streamline clinical translation, preliminary safety and efficacy evaluations should first be conducted in animal models to reduce the economic burden of subsequent clinical safety evaluations.

Another key obstacle to the clinical application of bEVs is batch-to-batch heterogeneity during the generation of bEVs. While numerous methods exist for the isolation and purification bEVs, standardized criteria are lacking [186,187]. Considering that subtle variations in the preparation of bEVs could affect the reproducibility of results, researchers in this field need to expedite the development of guidelines for key parameters and quality indicators (e.g., the continuously updated Minimal Information for Studies of Extracellular Vesicles (MISEV) launched by the International Society for Extracellular Vesicles (ISEV)) [188–190]. The required parameters should encompass include but not be limited to, the characteristics of the parental bacteria and bEVs (size, composition, purity, bioactivity, functional molecules, as well as biotoxin load, etc.), storage and transportation conditions, application scope

modes of ingestion, and safe dosage [191]. The technical prerequisites for standardized production and separation are accurate product detection methods, necessitating the development of absolute quantification techniques with multi-omics approaches (proteomic, transcriptomic, metagenomic, and lipidomic) to ensure good batch homogeneity for clinical use.

In addition, the limited scalability and technical complexity of bEV production remain major obstacles to the industrialization and clinical application of bEVs [192]. Furthermore, the ambiguous mechanisms of bEVs occurrence have not yet been considered the accumulation of nucleic acids. Genetic molecules are generally required to cross the periplasm and be encapsulated in a peptidoglycan layer to form bEVs. After cell lysis, RNA and DNA are released into the matrix, resulting in bEVs agent contamination. Moreover, current production technologies face challenges in achieving sufficient drug loading capacity and targeting specificity, which collectively hinders the industrialization and clinical application of bEVs [47]. Based on the main demand for efficient access to homogeneous and safe products, future research should focus on elucidating the mechanisms underlying bEV formation and optimizing large-scale bacterial cultivation parameters [14,189].

5. Conclusions

bEVs have emerged as important mediators of bacteria–host communications [30,193,194]. While challenges persist for full-scale application, bEV-based therapies have emerged as preferable techniques compared to their parental bacteria considering their distinctive cell-free system, nanoscale structure, excellent biocompatibility, and environmental friendliness. It is well-documented that some engineered bEVs hold substantial potential for diverse biological applications such as drug delivery for gene therapy and vaccine production. The substantial possibilities of engineered bEVs allow for their adoption as modular platforms for therapeutic development, greatly expanding their biomedical utility.

CRediT authorship contribution statement

Qiqiong Li: Writing – original draft, Visualization, Conceptualization. **Xinyang Chen:** Methodology. **Junhua Xie:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Shaoping Nie:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

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

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