Engineering strategies and biomedical applications of bacterial extracellular vesicles

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ABSTRACT

Bacterial extracellular vesicles (BEVs) are emerging as promising therapeutic agents and drug delivery vehicles due to their unique properties. These nanoscale vesicles possess stable membrane structure and naturally encapsulate a variety of bioactive molecules, making them versatile tools in biomedical applications. However, clinical translation of BEVs faces challenges such as insufficient display of disease-specific antigens, excessive toxicity, and rapid clearance. Addressing these issues is crucial for the clinical translation of BEVs. In this review, we discuss recent advances in BEV engineering strategies aimed at addressing these limitations and expanding their therapeutic applications. We highlight approaches for loading exogenous cargo into BEVs, detoxification strategies, and the latest progress in the application of engineered BEVs for treating infectious diseases, cancer, and other disorders. Despite promising preclinical results, clinical translation is hindered by safety concerns, standardisation difficulties, and scalability issues. Future research should focus on optimising detoxification processes, establishing global standardisation, and improving production methods to facilitate successful clinical translation of engineered BEVs. This review provides insights into the current status and future perspectives of BEV engineering for therapeutic applications.

Keywords:
Bacterial extracellular vesicles; Disease treatment; Engineering; Vaccine

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1. Introduction

Bacterial extracellular vesicles (BEVs) are bacterial-derived nanovesicles with diameters ranging from 20 to 400 nm that carry nucleic acids, virulence factors, membrane proteins and other bioactive molecules.[1] Due to their particulate composition and abundance of immunostimulatory proteins, BEVs enhance the expression of cytokines and costimulatory molecules, facilitate antigen presentation, and elicit durable immune responses,[1-3] with two BEV-derived vaccines in Phase I and II trials demonstrating their potential as vaccines.[4,5] Besides, BEVs can accumulate in tumour tissues and stimulate the production of the anti-tumour cytokine interferon-γ, which mobilises anti-tumour cells to generate a robust anti-tumour response.[6,7] Additionally, BEVs possess inherent characteristics such as high stability, excellent biocompatibility, and efficient traversal of biological barriers. Taken together, BEVs are regarded as ideal adjuvants and drug carriers in various pathological conditions, with a particular emphasis on infectious diseases and cancers. Despite the benefits, research on the biomedical application of BEVs faces several challenges at present. Firstly, the insufficient content of antigens carried by BEVs, especially disease-specific antigens, limits their application in various diseases.[8] Secondly, BEVs carry an excessive amount of virulence factors such as lipopolysaccharide and Porin B,[9,10] triggering an overly strong inflammatory response and, consequently, resulting in toxic side effects. Lastly, in most cases, BEVs provoke antibody-mediated clearance, leading to the premature elimination of BEVs before they can manifest their therapeutic effects.[11] These shortcomings may diminish expectations for the application of BEVs in drug delivery and disease treatment, thereby impeding the clinical translation of BEVs.

As research on BEVs advances and technological capabilities develop, the engineering of BEVs emerges as a promising solution to address existing challenges. Initially, engineering

strategies can effectively promote BEVs to activate immunity by introducing more disease-specific antigens and repositioning antigens effectively. Subsequently, engineering strategies can mitigate the virulence factors inherent in natural BEVs, thereby reducing their toxicity and improving safety. Lastly, engineering strategies offer avenues to enhance BEVs stability through surface modification or encapsulation, resulting in reduced clearance rates within circulation. Consequently, engineering strategies facilitate the optimisation of drug dosages, ensuring therapeutic efficacy while minimising potential adverse effects.

This review used searches in PubMed with the keywords "engineered", "bacteria", "vesicles", and "membrane", and summarised recent advances in strategies for BEV engineering and their biomedical applications from 2018 to the present.

2. Biogenesis, components, and characteristics of bacterial extracellular vesicles

BEVs can be synthesised by both Gram-positive and -negative bacteria through multiple pathways, leading to variations in their composition and cargo (**Figure 1**), thereby imparting distinct properties.

Gram-negative bacteria employ two main methodologies for BEV production: membrane blebbing and explosive cell lysis. Membrane blebbing involves the generation of outer membrane vesicles (OMVs) through outer membrane derivations, constituting the most classical BEV type.[1] Originating from the outer membrane, OMVs are enriched with outer membrane proteins, lipid components like lipopolysaccharide, nucleic acid, virulence factors, and other components, implicating their role in the bacterial secretion process.[12,13] Additionally, explosive cell lysis leads to the generation of outer-inner membrane vesicles (OIMVs) and explosive outer-membrane vesicles.[1] OIMVs are produced as a result of explosive cell lysis, which causes the inner membrane to protrude and wrap into part of the cytoplasm and then dissociate from the cell surface along with the surrounding outer membrane.[1,14] Consequently, OIMVs have both inner and outer membranes, and they carry intracellular components such as plasmid DNA.[15] Explosive outer-membrane vesicles are similar to OMVs in composition, but due to explosive cell lysis, explosive outer-membrane vesicles randomly carry cytoplasmic components including chromosomal DNA and endolysins.[1]

Gram-positive bacteria produce cytoplasmic membrane vesicles (CMVs) through a process known as bubbling cell death.[1] CMVs are derived from the inner membrane and encompass components from both the inner membrane and the cytosol.[16] In comparing OMVs and CMVs, a notable distinction lies in their content and formation mechanisms. CMVs may naturally include endolysins and entire phages due to their mode of formation, whereas OMVs do not internalise phages, though they can adsorb them on their surface[1]. Thus, CMVs can encapsulate phages and phage-related enzymes, while OMVs are generally devoid of these internal elements.

Due to their distinctive components, various types of BEVs manifest unique characteristics. OIMV and CMV encapsulate intracellular components, including DNA and hydrolases, facilitating DNA transfer[17] and bactericidal activity against other bacterial species.[18] This capacity promotes horizontal gene transfer and expedites bacterial evolution.[19] Additionally, OMVs contribute to the regulation of signalling within bacterial populations by carrying signalling molecules on their membrane surfaces.[20]

In the context of human interaction, the antigens conveyed by BEVs, such as pathogen-associated molecular pattern and immunostimulatory proteins, endow BEVs with the characteristic of immune stimulation. This stimulation, in turn, induces the release of various cytokines, such as IL-10 and TNF-α, promoting antigen presentation and activating dendritic cells.[21] Through active participation in intercellular communication, BEVs also contribute significantly to triggering subsequent immune responses.[22]

Considering these distinctive and interconnected characteristics, BEVs emerge as promising candidates as both drug carriers and biological adjuvants in the treatment of diverse diseases.

3. Strategies for bacterial extracellular vesicle engineering

Following the discussion of the biogenesis, components, and characteristics of BEVs, the subsequent section explores the strategies used in BEV engineering. Recent research highlights the clinical promise of natural BEVs for treating diseases, attributed to their stability, traversal capabilities, and bioactive components. Nevertheless, there are challenges in the practical implementation of natural BEVs, including inadequate immune responses against antibiotic resistance, suboptimal drug delivery, and systemic inflammatory risks. To address these challenges, engineering strategies offer promising avenues to reduce the toxicity of natural BEVs while harnessing their advantageous features, including targeting capabilities and immunostimulation. Two main strategies for engineered BEVs have emerged: one involves the combination of foreign materials with BEVs to achieve targeted delivery and optimise material utilisation, while the other focuses on detoxifying BEVs to reduce systemic inflammatory responses.

4. Strategies for loading exogenous cargo into bacterial extracellular vesicles

Strategies for loading exogenous cargo into BEVs include biological strategies, physical strategies, and chemical strategies[7,8,23-59] (**Table 1** and **Figure 2**). The majority of these technologies were developed between 2020 and 2022, while the earliest employed technology, common genetic engineering, dates back to 1996[60] (**Figure 3**). Many of these strategies, such as sonication and electroporation, are adaptations from liposome engineering. Given the structural similarities between

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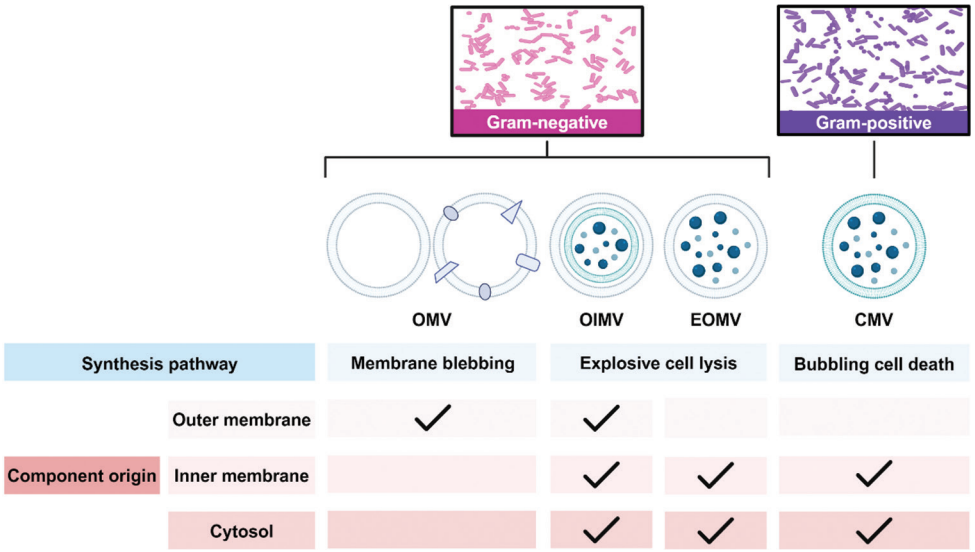

Figure 1. Biogenesis and component of BEVs. BEVs can be synthesised by both Gram-positive and -negative bacteria through different pathways, leading to variations in their composition. Created with BioRender.com.
Abbreviations: BEV: Bacterial extracellular vesicle; CMV: Cytoplasmic membrane vesicle; EOMV: Explosive outer-membrane vesicle; OIMV: Outer-inner membrane vesicle; OMV: Outer membrane vesicle.

Table 1. Strategies for loading exogenous cargo into BEVs

Strategy	Method	Advantage	Disadvantage	Reference
Biological strategy	Common genetic engineering	No damage to membrane structure	Low efficiency; Potential toxicity	23-32
	Recombinant cytolysin A	No damage to membrane structure; Locate to the membrane surface	Potential toxicity	7, 8, 33-35
	Lipoprotein-OmpA system	No damage to membrane structure; Locate to the membrane surface	Potential toxicity	36-40
Physical strategy	Co-extrusion	High efficiency; Easy operation	Damage to membrane structure	41-48
	Electroporation	High efficiency	Damage to membrane structure; Rely on complex physical equipment	24, 49, 50
	Sonication	High efficiency; Easy operation	Damage to membrane structure	47, 51-53
	Incubation	Simple and straightforward	Exclusively for small lipophilic compounds	54-56
	pH gradient manipulation	High efficiency	Antigen type limitation; Hard to operate	33
	Vortex	Quick and efficient	Limited to small volumes; Damage to membrane structure	57
Chemical strategy	Palmitic acid modification	Increased stability	Potential for increased non-specific binding	26, 31
	Tag/Catcher system	High binding specificity; Rapid preparation	Low reaction rate; Non-specific binding	58, 59
	Biotin-avidin system	Rapid preparation	Suboptimal cost-effectiveness	40
	Archaeal L7Ae and a box C/D RNA system	Display mRNA efficiently	Restriction of cargo type	34

Abbreviation: BEV: Bacterial extracellular vesicle.

liposomes and BEVs, particularly their lipid bilayer membranes, these techniques have been repurposed for use in BEVs. Among these strategies, biological strategies stand as the most prevalent approach, characterised by its mature technology and satisfactory loading efficiency. Nonetheless, it is susceptible to potential toxicity owing to the possibility of triggering unforeseen genomic alterations or side effects. In contrast, physical strategies emerge as a more efficient, straightforward, and feasible strategy. However, the direct passage of cargo through the membrane into BEVs may compromise membrane integrity. Moreover, chemical strategies enable independent modification of both cargo and BEVs, facilitating their convenient assembly and large-scale preparation in a short time frame. Nevertheless, the materials utilised for modification typically incur high costs and are prone to aggregation.

4.1. Biological strategies

In biological strategies, the most prevalent method is common genetic engineering. As a conventional approach, a plasmid vector carrying target genes is constructed and transformed into bacteria, which synthesis the cargo and express it on BEVs that are subsequently extracted.[23-25,32] Gujrati et al.[23] produced

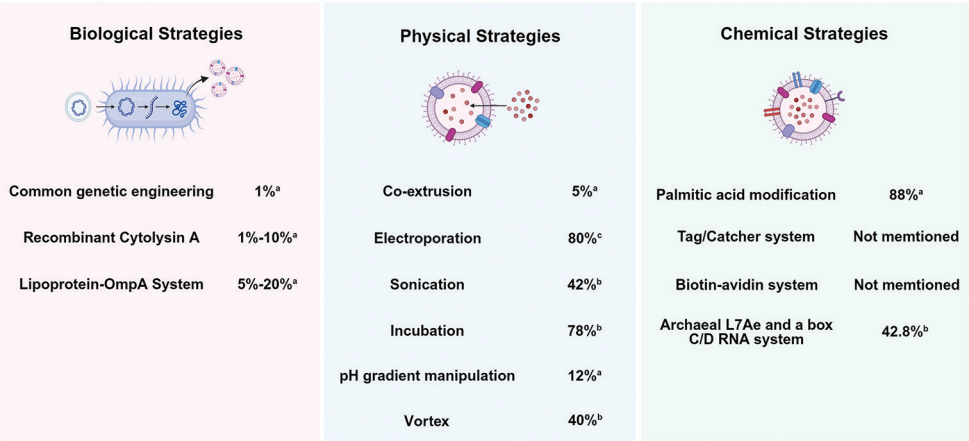

Figure 2. Strategies for loading exogenous cargo into BEVs and their loading efficiency. Due to the different engineering strategies, the methods for calculating loading efficiency are inconsistent, making direct comparisons challenging. Additionally, some articles do not report loading efficiency, resulting in incomplete data. [a]The ratio of the amount of cargo within BEVs to the total amount of BEVs. [b]The ratio of the amount of cargo loaded into BEVs to the initial amount of cargo added. [c]The electrotransformation efficiency. Created with BioRender.com.
Abbreviation: BEV: Bacterial extracellular vesicle.

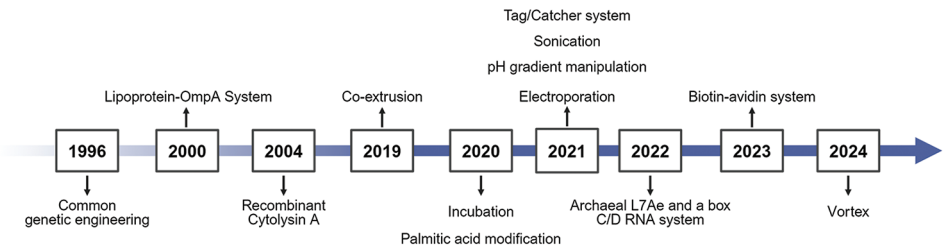

Figure 3. Timeline of various engineered BEV technologies. Originating in 1996, common genetic engineering was the earliest of these technologies, while most others were developed between 2020 and 2022. Created with BioRender.com.
Abbreviation: BEV: Bacterial extracellular vesicle.

BEVs encapsulating biopolymer-melanin using a bacterial strain expressing a tyrosinase transgene. However, BEVs engineered through this strategy often exhibit a relatively low level of cargo expression and display. To overcome this limitation, improved methods have been proposed.

One strategy involves the utilisation of recombinant cytolysin A (ClyA). ClyA, a pore-forming toxin produced by certain bacteria within the *Enterobacteriaceae* family, has been investigated for its potential as an ideal scaffold for loading exogenous proteins onto BEVs.[61] Research has demonstrated that recombinant ClyA can be expressed on the surface of BEVs through genetic engineering, offering an efficient means of loading exogenous cargo.[61] For example, Gao et al.[33] engineered a recombinant protein comprising ClyA fused to the Arg-Gly-Asp (RGD). In another study, the mouse programmed death-1 (PD-1) ectodomain was fused to ClyA and the expression of recombinant protein in BEV was confirmed by Western blot analysis.[7] The fusion of exogenous cargo with ClyA enhances its presentation on the BEV surface, rendering it more recognisable and accessible to immune cells.[7,8,33]

Another strategy is the lipoprotein-OmpA system (Lpp-OmpA system), specifically designed for implementation in the Gram-negative bacterium *Escherichia coli* (*E. coli*). The Lpp-OmpA system comprises a lipoprotein (Lpp) domain and an outer membrane protein A (OmpA) domain.[62] Given that OmpA is a transmembrane protein naturally expressed on the surface of BEVs, the OmpA domain plays a crucial role in facilitating the expression of exogenous cargo. Simultaneously, the Lpp domain functions to target and anchor the cargo to the outer membrane.[62] Irene et al.[37] demonstrated that lipidated recombinant proteins engineered by Lpp-OmpA system were expressed at higher level compared to their nonlipidated counterparts. Integration of the genes encoding exogenous cargo with the Lpp-OmpA system enhances cargo expression on the BEV surface.[36-38]

Collectively, both recombinant ClyA and the Lpp-OmpA system share the advantage of facilitating antigen presentation on the BEV surface. Recombinant ClyA simplifies the production process and is compatible with antigens of various structures.[61] However, potential side effects may arise due to the inherent toxicity of ClyA.[61] Furthermore, the lipoprotein-OmpA system efficiently delivers cargo to antigen-presenting cells.[63] Nevertheless, it may interfere with the expression and presentation of antigens[37] and add complexity to the modification process.

4.2. Physical strategies

Common physical strategies include co-extrusion, electroporation, sonication, incubation and pH gradient manipulation. Among these, the co-extrusion strategy stands out as the most frequently employed. The co-extrusion strategy involves the mixture of exogenous cargo with BEVs, followed

by extrusion of the mixture through a 200 nm polycarbonate porous membrane using an extruder. In Chen et al.'s study[44] the cargo and BEVs were mixed and then extruded to generate BEV-coated nanoparticles. This approach is notably efficient in loading cargo into BEVs, with its procedural simplicity contributing to its broad application.[41-43] However, like other strategies for directly loading exogenous cargo into BEVs, the co-extrusion method poses the risk of damaging the membrane structure of BEVs.

In addition to co-extrusion, there are other strategies used to load cargo into BEVs. First, electroporation is a method used to create pores in the BEV membrane by applying an electric field, facilitating the entry of antigens into the BEV and effectively enhancing antigen loading. For example, Pan et al.[24] loaded PD-1 into BEVs by electroporation, and the electrotransformation efficiency reached 80%. However, the damage to the membrane structure caused by electroporation may adversely affect the activity of BEVs, and the reliance on complex physical equipment makes its clinical application challenging.[26] Second, sonication increases the permeability of the BEV membrane, allowing antigens to enter BEVs, and offers the advantage of simple and rapid operation.[47,51-53] Zhai et al.[47] found that the tumour-targeted cargo was loaded into BEVs by sonication to achieve more effective treatment. Similarly, sonication was used to load ceftriaxone into BEVs for the treatment of bacterial meningitis.[52] Nonetheless, sonication may impact BEV structure and activity. Third, incubation offers the advantages of simple and easy operation with minimal damage to BEVs.[54-56] For example, Kuerban et al.[54] incubated doxorubicin (DOX) with BEVs to load it into BEVs. Despite this advantage, sonication is exclusively for small lipophilic compounds. Additionally, pH gradient manipulation has high loading efficiency but is primarily used for loading amphiphilic substances, with specific requirements for the nature of the cargo.[33] Gao et al.[33] developed a method to load DOX inside BEVs via the pH gradient-mediated drug loading. Moreover, the operation of pH gradient manipulation is intricate and demands high precision, which hinders its clinical application. Finally, vortex involves rapid circular motion and is generally a brief process, which is quick and efficient. Wang et al.[57] combined BEVs and the cargo by vortex for 30 minutes. However, vortex is not suitable for mixing larger volumes, and may cause damage to the structure of BEVs.

4.3. Chemical strategies

Chemical strategies involve the binding of modified BEVs' membrane and exogenous cargo by connection pairs. Upon mixing the modified BEVs and cargo, they form a binding interaction facilitated by the connection pairs. Common strategies include palmitic acid modification, the Tag/Catcher system, the biotin-avidin system and the archaeal L7Ae and a box C/D RNA system.

In the strategy of palmitic acid modification, palmitic acid serves as a linker, enabling the stable anchoring of drugs to the surface or interior of BEVs.[26,31] This strategy increased the stability of BEVs and it is easy to operate. For example, Peng et al.[26] anchored RGD to BEVs by inserting the palmitic acid on RGD to into the phospholipid layers of BEVs, as Gu et al.[31] did in another study. Despite these advantages, palmitic acid modification carries the potential risk of increased non-specific binding. Similar to palmitic acid modification, the Tag/Catcher system is also widely utilised. The Tag/Catcher system comprises a peptide-protein pair that can form an isopeptide bond with its partner. Notably, this system includes SpyTag/SpyCatcher and SnoopTag/SnoopCatcher pairs derived from *Streptococcus pyogenes* and *Streptococcus pneumoniae*, respectively.[64] When the Tag/Catcher pairs are combined, the reaction occurs spontaneously, displaying good specificity across a wide range of conditions.[65] The binding specificity of SpyTag/SpyCatcher and SnoopTag/SnoopCatcher allows their concurrent application, enabling the attachment of two distinct cargos to the same BEV.[58] This "plug and display" system streamlines the production procedure of cargo-loaded BEVs, facilitating rapid preparation and cost control. Compared to the Tag/Catcher system, the biotin-avidin system and the archaeal L7Ae and a box C/D RNA system are less frequently applied. The biotin-avidin system allows for rapid preparation, but its high cost of raw materials limits cost control in industrial production. For example, Weyant et al.[40] develop a platform based on biotin-avidin system that enables the rapid and simplified assembly of antigen-studded BEVs for use as vaccines against pathogenic threats. In addition, the archaeal L7Ae and a box C/D RNA system are primarily utilised for loading mRNA, exhibiting high loading efficiency, but are not suitable for other cargo. For instance, BEVs modified with L7Ae can rapidly adsorb box C/D sequence-labelled mRNA antigens and deliver them into dendritic cells.[34]

5. Detoxification strategies for bacterial extracellular vesicles

BEVs transport PAMPs that engage with pattern recognition receptors on host cells, initiating signal transduction pathways and inflammatory cascades, resulting in the synthesis of inflammatory mediators, recruitment of inflammatory cells and subsequent induction of inflammatory responses.[1] Nevertheless, natural BEVs often contain excessive virulence factors, leading to exaggerated inflammatory responses that can cause multi-organ damage and even fatalities.[6] Therefore, detoxification of BEVs is essential to modulate the inflammatory response within a controlled range. Strategies for on-demand detoxification are proposed, including knocking-out or silencing of virulent genes, lysozyme treatment, detergent treatment, extracting outer membrane, shell coating, ligand binding, and BEV-based hydrogel system[7,11,23,24,26,27,30-32,36,37,39,45,50,54,56,59,66,67] (**Table 2** and **Figure 4**).

To begin with, a widely employed strategy for detoxification involves knocking-out or silencing of virulent genes. This genetic intervention prevents the synthesis and secretion of toxic substances while retaining other component crucial for targeted delivery and immune stimulation.[23,24,32] For example, Flagella-deficient *E. coli Nissle 1917* was created by knocking out the *flhD* gene, which reduces its toxicity.[29] In another study, *E. coli* was made less endotoxic by inactivating the *msbB* gene.[23] Although this genetic approach enables precise detoxification,

Table 2. Detoxification strategies for BEVs

Strategy	Advantage	Disadvantage	Reference
Knocking-out or silencing virulent genes	High specificity	Hindrance caused by gene interactions	7, 23, 24, 27, 30, 32, 36, 37, 50, 59
Lysozyme treatment	Removing toxic components from the membrane; Simple operation	Damage to membrane structure	26, 31
Detergent treatment	Removing endotoxin	Targeting only one specific toxin	45
Extracting the outer membrane	Removing cytoplasm	Damage to membrane structure	39, 66
Shell coating	Protecting BEVs; The shell can be modified	Weaken the presentation effect of BEVs	11, 56
Ligand binding	High specificity; Removing endotoxin	Suboptimal cost-effectiveness	54
BEV-based hydrogel system	Localised release of BEVs; Decreased systemic toxicity	Exclusively for treating superficial solid tumours	67

Abbreviation: BEV: Bacterial extracellular vesicle.

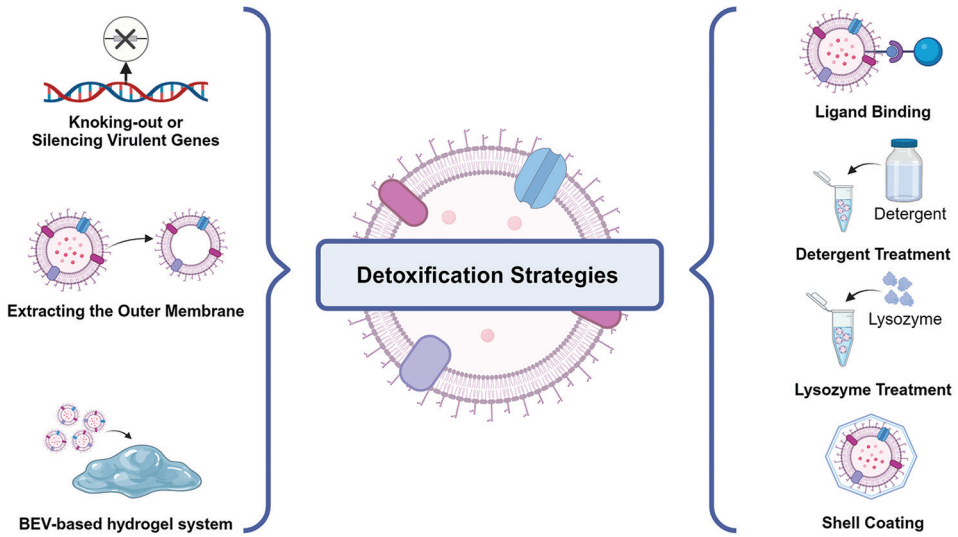

Figure 4. Detoxification strategies for BEVs. Strategies for on-demand detoxification include knockout or silencing of virulent genes, extracting the outer membrane, BEV-based hydrogel system, ligand binding, detergent treatment, lysozyme treatment, and shell coating. Created with BioRender.com. Abbreviation: BEV: Bacterial extracellular vesicle.

the interaction between genes may sometimes impede the complete removal of toxic substances. Additionally, lysozyme treatment is a widely used strategy for removing toxic components from the BEV membrane due to its simplicity of operation.[26,31] For instance, Peng et al.[26] incubated BEVs with lysozyme while shaking for detoxification. Similarly, BEVs were depleted of toxic outer membrane components by lysozyme treatment to generate a safe drug delivery system.[31] However, this treatment may damage the BEV membrane by removing outer membrane components. Similar to lysozyme treatment, detergent treatment offers a simple and highly specific method for removing endotoxins, but it is not effective against other toxins.[45] Moreover, the strategy of extracting the outer membrane effectively removes cytoplasm by disrupting the BEV structure and reassembling it via sonication.[39,66] For example, Park et al.[66] created BEVs by extracting the outer membrane, resulting in very few cytosolic components, no RNA or DNA, and no systemic pro-inflammatory cytokine responses. Nevertheless, this strategy may damage the membrane structure, thus impacting the integrity and biological activity of BEVs. Furthermore, shell coating involves affixing a protective shell to the BEV to shield it from damage prior to the target site.[11,56] Simultaneously, the shell can be modified with biological agents, such as folic acid and indocyanine green,[11] to provide BEVs with additional advantages. However, the integration of shell coating in the packaging process may diminish the efficiency of BEV presentation. Also, ligand binding exhibits high specificity in endotoxin removal.[54] However, ligands are costly and pose challenges in managing industrial production costs. Lastly, the BEV-based hydrogel system incorporates BEVs into a thermosensitive hydrogel. This system enables the localised release of BEVs, thereby reducing systemic toxicity.[67] Nevertheless, it is more suitable for treating superficial solid tumours rather than internal organ tumours.

6. Engineered bacterial extracellular vesicles for disease treatment

Having outlined the strategies for BEV engineering, the next section reviews the application of engineered BEVs in disease treatment. As potential drug carriers and biological adjuvants, natural BEVs have been extensively studied in various diseases. However, natural BEVs exhibit limitations, including the absence of disease-specific antigens and the potential for varying degrees of side effects. Consequently, engineering

strategies are employed to address these weaknesses and expand their applicability. Engineered BEVs exhibit favourable characteristics, including commendable biocompatibility, moderate immunostimulatory properties, and low toxicity, which enable their application in a broader range of diseases. Notably, there is substantial research interest in the use of engineered BEVs, particularly in the domains of infection and tumour treatment. Recent advances in the applications of engineered BEVs for disease treatment are comprehensively outlined in the following section.

6.1. Engineered bacterial extracellular vesicles for anti-infection therapy

Compared to natural BEVs, engineered BEVs have an elevated antigen display level and reduced toxic effects. In the context of anti-infection therapy, the incorporation of multiple antigens or biological agents into BEVs is pursued to enhance their immunostimulatory effects. Two distinct strategies are employed for engineered BEVs in anti-infection therapy. The first involves the direct use of BEVs derived from pathogenic bacteria, while the second entails loading disease-related antigens into BEVs sourced from heterologous bacteria[25,27,29,30,32,36-40,44,52,59] (**Figure 5** and **Table 3**).

6.1.1. Engineered bacterial extracellular vesicles from pathogenic bacteria

Natural BEVs extracted from pathogenic bacteria carry antigens specific to the parental bacteria, eliciting targeted immune responses. However, there are inherent limitations. On one hand, natural BEVs can provoke restricted immune responses, leading to insufficient protective efficacy. On the other hand, they may induce excessive inflammatory reactions, increasing the risk of systemic inflammatory responses. Consequently, engineering strategies have been developed to address these issues. To improve immune protection, genetic engineering techniques are employed to load multiple disease-related antigens into BEVs, thereby inducing a polyvalent immune response against the pathogenic bacteria. Wang et al.[32] demonstrated that BEVs purified from a *Staphylococcus aureus* mutant that is genetically engineered to express detoxified cytolysins can elicit cytolysin-neutralising antibodies and protect mice in a lethal sepsis model. In addition, the incorporation of biomaterials, such as photosensitisers, into BEVs allows for their combined application with engineered BEVs and other therapeutic modalities, such as photothermal therapy (PTT), to amplify immune responses. Chen et al.[44] encapsulated indocyanine green in BEVs, enabling them to induce lysosomal escape upon laser irradiation. In mitigating the toxic effects of natural BEVs, detoxification strategies are employed to diminish cytoplasmic components prone to triggering excessive inflammatory reactions. BEVs detoxified by extracting the outer membrane activated the immune system without causing severe immunotoxicity.[39]

6.1.2. Engineered bacterial extracellular vesicles from heterologous bacteria

By loading pathogenic antigens from different variants into heterologous BEVs, the development of multivalent vaccines can be realised, providing enhanced protection against infections.[37,38,59] *E. coli* is commonly chosen as a donor bacterium due to its rapid reproduction, capacity to accept foreign plasmids, and well-defined genetic background. For example, BEVs derived from *E. coli* were loaded with *Staphylococcus aureus* protective antigens to elicit high, saturating antigen-specific antibody titres.[37] In another study, Nakao et al.[29] bioengineered BEVs derived from *E. coli* to display pneumococcal capsular polysaccharide, resulting

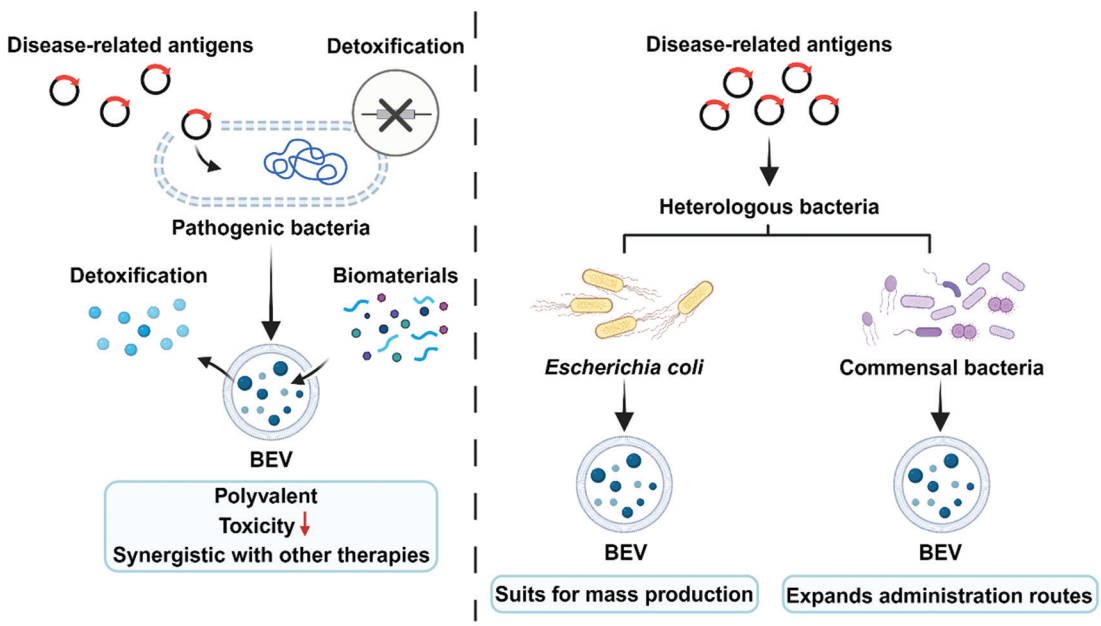

Figure 5. Engineered BEVs for anti-infection therapy. BEVs derived from pathogenic bacteria are engineered to carry multiple disease antigens, inducing polyvalent immune responses. Biomaterials are incorporated for combined therapy to enhance immune responses. Furthermore, loading pathogenic antigens into BEVs derived from heterologous bacteria offers additional advantages. BEVs derived from *Escherichia coli* are suitable for mass production, while those from commensal bacteria enable potential administration routes beyond tradition. Created with BioRender.com. Abbreviation: BEVs: Bacterial extracellular vesicles.

Table 3. Engineered BEVs for anti-infection therapy

Types of infection	BEV resource	Engineering method	Cargo	Administration route	Therapeutic outcome	Reference
Unlimited	*Bacteroides thetaiotaomicron*	Common genetic engineering	Antigens from pathogens	Mucosal administration	Deliver antigens directly to the target tissue; Reduce drug dosage	25
	Escherichia coli	Minimise endogenous proteins; Lipoprotein-OmpA system	Antigens from pathogens	Subcutaneous injection	Raise the expression of heterologous proteins and the stability of BEVs	36
	Escherichia coli	Common genetic engineering	Poly-N-acetyl-D-glucosamine	Intravenous injection	Induce protective immunity against the broad range of pathogens that produce surface poly-N-acetyl-D-glucosamine	27
	Escherichia coli	Lipoprotein-OmpA system; Biotin-avidin system	Antigens of various chemical nature	Subcutaneous injection	Suitable for most antigens; Simple and rapid preparation	40
Escherichia coli	*Escherichia coli*	Sonication; Incubation	Ceftriaxone; meso-tetra-(4-carboxyphenyl) porphine	Intravenous injection	Overcome drug resistance; Rapid administration	52
Staphylococcus aureus	*Staphylococcus aureus*	Common genetic engineering	HlaH35L; LukE	Subcutaneous injection	Induce effective; Specific immunity	32
	Staphylococcus aureus	Co-extrusion	Indocyanine green-loaded magnetic mesoporous silica nanoparticles	Subcutaneous injection	Enhance photothermal effects; Effective against drug-resistant *Staphylococcus aureus*	44
	Escherichia coli	Lipoprotein-OmpA system	FhuD2; HlaH35L; LukE; SpAKKAA; Csa1A	Intraperitoneal injection; Intravenous injection; Subcutaneous injection	Induce innate immunity; Improve the intensity of immune responses	37
	Escherichia coli	Lipoprotein-OmpA system	ClfAY338A; Luke; SpAKKAA; HlaH35L	Intraperitoneal injection	Induce effective specific immunity; Suitable for the preparation of polyvalent vaccines	38
	Escherichia coli	Tag/Catcher system	EsxA; Sbi; SpA	Subcutaneous injection	Simple and rapid preparation; High intensity of specific immunity	59
Streptococcus pneumoniae	*Escherichia coli*	Common genetic engineering	Serotype 14 pneumococcal capsular polysaccharides	Subcutaneous injection	Induce stronger immunological responses than pneumococcal vaccines; Applicable for mice of different ages	29
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	Extract the outer membrane	–	Intraperitoneal injection	Induce effective specific immunity; Reduce toxic effects	39
SARS-CoV-2	*Escherichia coli*	Extract the outer membrane; Lipoprotein-OmpA System	S1 protein	Intraperitoneal injection	Induce effective specific immunity; Reduce toxic effects	39
Burkholderia pseudomallei	*Staphylococcus aureus*	Common genetic engineering	pdhB-hcp1	Subcutaneous injection; Intramuscular injection; Intraperitoneal injection	Induce effective specific immunity; Defend against fatal infection	30

Abbreviations: BEV: Bacterial extracellular vesicle; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

in robust production of specific IgG. Nevertheless, certain commensal bacteria are preferred for their ease in delivering antigens or drugs from symbiotic sites, such as the mucosa, leveraging host-microbe interaction pathways. Carvalho et al.[25] extracted BEVs from genetically engineered *Bacteroides thetaiotaomicron* to deliver biologics to mucosal sites and protect against infection and injury. Moreover, the low toxicity profile of commensal bacteria opens up potential delivery routes beyond traditional methods (intraperitoneal injection, subcutaneous injection, intravenous injection, etc.), offering avenues conducive to the precision medicine applications of BEVs. Furthermore, it has been established that genetic minimisation of endogenous proteins in *E. coli* is advantageous for optimising the expression of heterologous proteins, intensifying immune stimulation, and ensuring the stability of BEVs.[36]

6.2. Engineered bacterial extracellular vesicles for cancer therapy

Leveraging the enhanced permeability and retention effect, BEVs can passively accumulate within tumours, undergoing recognition and internalisation by antigen-presenting cells, thereby activating a heightened immune response.[6] Engineered BEVs, loading with an array of antigens, drugs, and biomaterials, exhibit the potential to induce more efficacious anti-tumour immune responses[7,8,11,23,24,26,28,31,33-35,41-43,45-49,51,54-58,67] (**Figure 6** and **Table 4**).

Various antigens can be loaded into engineered BEVs, enabling targeted delivery to antigen-presenting cells. Specifically, engineered BEVs loaded with PD-1 can bind to the programmed death ligand-1 (PD-L1) in tumour cells to block the PD-1/PD-L1 signalling pathway. This inhibition can alleviate the suppressive influence of tumour cells on T cells and amplify the cytotoxic T lymphocyte killing effect. For instance, Pan et al.[24] developed BEVs loaded with a PD-1 plasmid to achieve self-blockade of PD-L1 in tumour cells. Similarly, BEVs modified by the insertion of the ectodomain of PD-1 can bind to PD-L1 on the tumour cell surface, thereby protecting T cells from the PD-1/PD-L1 immune inhibitory axis.[7] Furthermore, the engineered BEVs loaded with RGD peptide,[33,43] basic fibroblast growth factor,[28] and other molecules associated with tumour microenvironment formation contribute to improving the tumour microenvironment. This involves remodelling the tumour stroma and inhibiting angiogenesis, facilitating improved accessibility of drugs and immune cells to tumour cells, thereby augmenting their therapeutic impact.

In addition, engineered BEVs find application in drug loading. Engineered BEVs can be loaded with chemotherapy drugs, such as DOX, adriamycin, and tegafur, to enhance drug targeting and accumulation within tumour tissues, thereby improving drug efficacy and reducing systemic toxicity.[33,43,46] For instance, the loading of DOX into BEVs enhances its delivery to tumor microenvironments, resulting in a significant inhibition of tumour growth.[33] Likewise, BEVs loaded with tegafur can initiate an antitumoral response and directly kill cancer cells.[43] Additionally, engineered BEVs can be loaded with diverse biomaterials to enhance the accumulation of these materials in tumour tissues. When employed in conjunction with various cancer therapies like PTT[11,41,51] and radiotherapy,[35] this combination enhances both the biomaterial accumulation in tumour tissues and anti-tumour immunity. Among those therapies, PTT stands out as the most extensively researched when combined with BEVs.

PTT represents a non-invasive approach to tumour treatment, utilising photothermal agents (PTAs) to convert light energy into heat energy. This localised heating initiates biochemical reactions, effectively eradicating tumours. Despite PTT's non-invasive, precise, and efficient characteristics, certain limitations persist. Firstly, the limited transmission ability of lasers restricts the effectiveness of PTT in treating deep-seated tumour tissues.[68] Additionally, challenges arise in

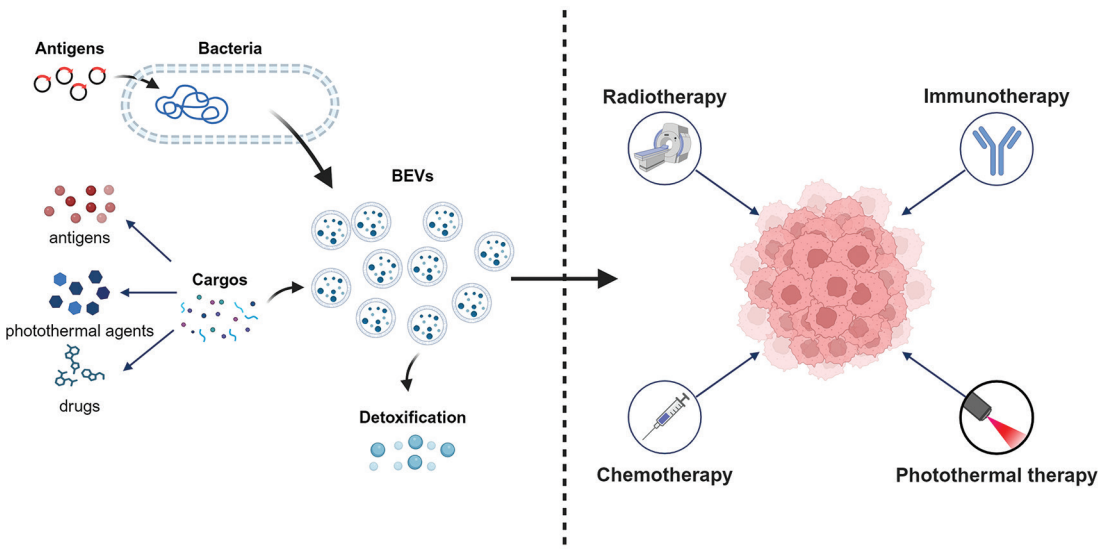

Figure 6. Engineered BEVs for cancer therapy. Engineered BEVs can carry a variety of antigens, drugs, and photothermal agents to elicit more potent anti-tumour immune responses. Additionally, they can synergise with other therapies, such as immunotherapy, chemotherapy, photothermal therapy, and radiotherapy, to exert a more robust anti-tumour effect. Created with BioRender.com.
Abbreviation: BEV: Bacterial extracellular vesicle.

Table 4. Engineered BEVs for cancer therapy

Therapy	BEV resource	Engineering method	Cargo	tumour model	Therapeutic outcome	Reference
Immunotherapy	*Escherichia coli*	Tag/Catcher system	ADP-dependent glucokinase	B16-OVA model; MC38 model	Elicit efficient anti-tumour immunity; Rapid preparation	58
		Archaeal L7Ae and a box C/D RNA system	Listeriolysin O; ADP-dependent glucokinase	B16-OVA model; MC38 model	Elicit efficient antigen-specific anti-tumour immunity	34
		Common genetic engineering	Basic fibroblast growth factor	B16-F10 model; TC-1 model	Inhibit angiogenesis in tumour tissues; Induce tumour cell apoptosis	28
		Recombinant cytolysin A	IL-10; Tumour-specific antigen E7	B16-F10 model; TC-1 model	Stimulate the generation of tumour-specific $CD8^+$ T cells	8
		Recombinant cytolysin A	PD1	B16-F10 model; CT26 model	Block the PD1/PD-L1 interaction	7
		Common genetic engineering; Electroporation	LyP1 polypeptide; PD-1	4T1 model; B16 model; CT26 model	Block the PD1/PD-L1 interaction; Activate CTLs	24
		Common genetic engineering; pH gradient manipulation	RGD	B16-F10 model	Bind integrin αvβ3; Inhibit tumour angiogenesis and etastasis	33
	Salmonella	Vortex	Human Papillomavirus-associated E7 antigen incorporating nine arginine residues	TC-1 model	Generate systemic E7-specific $CD8^+$ T cells; Exhibit promising anti-tumour effects	57
	Fusobacterium nucleatum	Co-extrusion	Metronidazole; Fe-doped ZIF-90	4T1 model	Eliminate *Fusobacterium nucleatum*; Induce ICD of cancer cells	48
	Escherichia coli	–	Thermal-sensitive hydrogel	4T1 model; B16-F10 model; CT26 model	Rapid tumour eradication; Decreased systemic toxicity	67
Chemotherapy	*Escherichia coli; Magnetospirillum gryphiswaldense*	Common genetic engineering; pH gradient manipulation; Co-extrusion	RGD; Doxorubicin	B16-F10 model; 4T1 model	Directly target tumour vasculature and tumour cells; Inhibit tumour growth	33, 46
	Salmonella	Co-extrusion	RGD; Tegafur	B16-F10 model	Inhibit tumour growth and lung metastasis	43
	Escherichia coli	Co-extrusion	Poly (lactic-co-glycolic acid); Adriamycin	231Br model	Endow cargo with prolonged circulation; Intracranial interstitial distribution; High biocompatibility	45
	Klebsiella pneumonia	Incubation	Doxorubicin	A549 model	Improve pharmacokinetic profile; Elicit appropriate immune responses	54
Photothermal therapy	*Escherichia coli*	Common genetic engineering	Tyrosinase	4T1 model	Target specific tissues; Enhance photothermal effects	23
	Escherichia coli	Co-extrusion	Polymer poly (ethylene glycol)-b-poly (lactic-co-glycolic acid)	EMT6 model	Promote targeting efficacy	41
	Escherichia coli	Sonication	Fe_3O_4-MnO_2	B16-F10 model	Enhance photothermal effects	51
	Escherichia coli; Salmonella	Co-extrusion; Common genetic engineering; Covalent binding	ICG	4T1 model; B16-F10 model; A375 model	Promote T cells infiltration; Enhance photothermal effects	11, 26, 42

(Cont'd...)

Table 4. *(Continued)*

Therapy	BEV resource	Engineering method	Cargo	tumour model	Therapeutic outcome	Reference
	Escherichia coli	Electroporation	Maleimide groups; 1-Methyl-tryptophan	CT26 model	Enhance photothermal effects; Overcome Treg-mediate immunosuppressive microenvironment	49
	Salmonella	Sonication	Photothermal sensitive liposomes; Anti-CD7-9R	H22 model; 4T1 model	Blockade CD38; Improve T cell cytotoxicity	47
	Escherichia coli	Common genetic engineering; Covalent binding	Tumour necrosis factor related apoptosis-inducing ligand; Integrin αvβ3 specific ligands PEP; ICG	B16-F10 model	Enhance transdermal efficacy; Induce tumour cell apoptosis	31
	Rhodopseudomonas palustris	Incubation	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)]	B16-OVA model	Enhance photothermal effects and the presentation of antigens to APCs	55
	Escherichia coli	Common genetic engineering	Melanin	4T1 model	Enhance photothermal effects; Decreased systemic toxicity	56
Radiotherapy	*Escherichia coli*	Recombinant cytolysin A	Neutralising CD47 nanobody	B16-OVA model; MC38 model	Remodel TME; Induce T cell-mediated anti-tumour immunity	35

Abbreviations: ADP: Adenosine diphosphate; APC: Antigen-presenting cell; BEV: Bacterial extracellular vesicle; ICD: Immunogenic cell death; ICG: Indocyanine green; IL-10: Interleukin-10; OVA: Ovalbumin; PD1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; RGD: Arg-Gly-Asp; TME: Tumour microenvironment; Treg: Regulatory T cell; ZIF-90: Zeolitic imidazolate framework-90; αvβ3: Integrin alpha$_v$beta$_3$.

targeting and accumulating PTAs within tumour tissues, diminishing therapeutic efficacy. To tackle these obstacles, a novel method is necessary to improve the targeting and accumulation of PTAs within tumour tissues, a requirement fulfilled by engineered BEVs. Loading PTAs into engineered BEVs facilitates targeted delivery to tumour tissues through the enhanced permeability and retention effect of BEVs, thereby amplifying the photothermal effect in deeper tissues.[11,26,51] For example, Qing et al.[11] created indocyanine green-loaded BEVs to enhance the PTT effect against the 4T1 tumour, resulting in completely suppressed tumour growth. Similarly, BEVs loaded with MnO2 and Fe3O4 can enhance the therapeutic effect, preventing tumour growth and recurrence.[51] Moreover, the immunogenicity of the engineered BEVs promotes internalisation by tumour cells, enabling greater PTA penetration into tumour cells and consequently enhancing therapeutic efficacy.[42] While still in the early stages of experimental research, the combination of engineered BEVs and PTT holds substantial potential for future applications.

It is noteworthy that engineered BEVs can be employed not only independently but also in conjunction with tumour-derived extracellular vesicles to achieve enhanced anti-tumour effects. Park et al.[66] suggests that utilising detoxified engineered BEVs alongside tumour-derived extracellular vesicles enhances the immunogenicity of tumour-derived extracellular vesicles, serving as an adjuvant. This combination leads to a significant inhibition of tumour growth and metastasis, especially when applied in conjunction with anti-PD-1 immunotherapy.[66]

6.2. Engineered bacterial extracellular vesicles for other diseases

In addition to their applications in anti-infection and cancer therapies, engineered BEVs exhibit promising potential in addressing inflammatory bowel disease (IBD) and ischaemic stroke (**Figure 7**).

IBD is a group of chronic inflammatory disease of the gastrointestinal tract, including Crohn's disease and ulcerative colitis. Recent studies underscore the key role of the intestinal mucosal immune system and its interaction with the microbiota in IBD pathogenesis.[69] Given their capability to traverse the intestinal barrier and participate in microbiota-host communication, BEVs emerge as potential therapeutic agents for IBD. Notably, BEVs derived from *Lactobacillus Plantarum* that encapsulate fucoxanthin have demonstrated efficacy in alleviating colitis by modifying the colonic inflammatory response and reshaping gut microbiota communities.[53]

Ischaemic stroke, also known as cerebral infarction, poses a challenge for effective pharmacological intervention due to the inherent barriers in drug penetration through the blood-brain barrier (BBB). With the ability to traverse the BBB and modulate immune responses within the brain, BEVs are considered as potential therapeutic interventions for ischaemic stroke.[70] Loading pioglitazone into BEVs facilitates its transportation across the BBB, allowing accumulation in the ischaemic region and subsequent pioglitazone release, resulting in neuroprotection.[50] The ability to penetrate the BBB and their outstanding biocompatibility make BEVs more advantageous over standard neuroprotective agents.

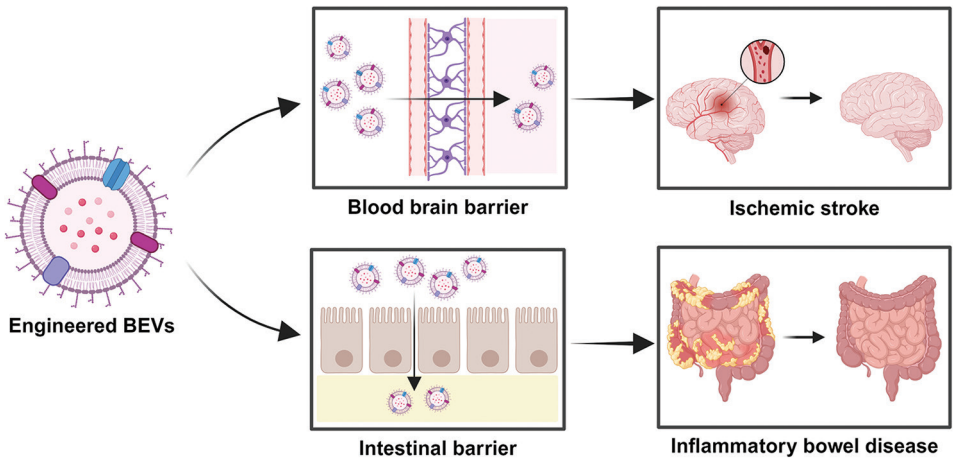

Figure 7. Engineered BEVs for other diseases. BEVs can cross the blood-brain barrier and offer neuroprotection in ischaemic stroke. Furthermore, BEVs have shown potential as therapeutic agents for inflammatory bowel disease due to their ability to cross the intestinal barrier and influence host-microbiota interactions. Created with BioRender.com.
Abbreviation: BEV: Bacterial extracellular vesicle.

7. Discussion

Natural BEVs, characterised by the presence of biofilm, offer stability and reduced cargo leakage, making them efficient drug carriers. Additionally, their bacterial origin allows for genetic modification and mass production, enhancing their potential as safe adjuvants and vectors. Despite these advantages, the inherent limitations of natural BEVs, such as low antigen display and high toxicity, necessitates the development of engineering strategies. The engineering strategies aims to increase antigen display levels, reduce toxicity, and improve targeting efficiency. The most common method for loading exogenous cargo into BEVs involves biological strategies, using technologies like recombinant ClyA and the Lpp-OmpA system. Additionally, physical strategies can directly load exogenous cargo into BEVs through methods such as co-extrusion, electroporation, and other methodologies. To detoxify natural BEVs, various approaches such as knockout or silencing of virulence genes, lysozyme treatment, and detergent treatment, among others, can be selectively employed to target specific toxic components. BEV engineering strategies expand the application potential of natural BEVs in disease treatment. In anti-infection treatment, engineered BEVs can present a diverse array of pathogenic antigens, offering improved protection against infections and providing solutions to the challenges of drug-resistant bacteria. In cancer therapy, engineered BEVs are versatile, accommodating a range of tumour-related antigens and biomaterials, thereby synergising with various therapeutic modalities to enhance anti-tumour responses. Moreover, engineered BEVs show promise in treating other pathological conditions, such as IBD and ischaemic stroke. The ongoing advancements in BEV engineering strategies suggest a promising future for their broader application in treating a variety of diseases.

Notably, similar to BEVs, bacterial ghosts (BGs) were considered as candidates for multivalent vaccines. BGs are non-living bacterial cell envelopes created through PhiX174 protein E-mediated lysis of Gram-negative bacteria.[71] This strategy ensures the preservation of the cell's outer membrane while expelling its cytoplasmic contents, thereby generating a structure that maintains the antigenic properties of the original bacterium. Ji et al.[72] demonstrated that hydrochloric acid-induced BGs from *Listeria monocytogenes* are promising vaccine candidates, as they are completely devoid of bacterial DNA and show reduced toxicity both *in vitro* and *in vivo*, mitigating adverse immune reactions from residual endotoxins. However, unlike BEVs, which are naturally secreted by bacteria, the production of BGs requires more intricate genetic manipulation and process control, potentially complicating large-scale manufacturing and quality assurance. Despite the promising antigenic profile of BGs, the relatively safer profile and simpler production process of BEVs often make them a more viable alternative for vaccine development.

While the advantages of engineered BEVs have been extensively validated in animal experiments, their clinical translation faces several challenges. Since the discovery of BEVs, only vaccines targeting Group B Meningococcus have advanced to clinical trials. These include Norwegian B:15:P1.7,16 MenBvac® OMV Vaccine, Cuban VA-MENGOC-BC®, Bexsero® 4 component Meningococcal B vaccine, Trumenba® 2 component Meningococcal B vaccine and MeNZB® OMV.[73] Of these, only Bexsero® and Trumenba® have received market approval. Both vaccines underwent extensive clinical testing for efficacy and safety before approval and continue to be monitored for safety post-approval.[74-77]

The primary obstacles hindering the clinical translation of engineered BEVs are as follows (**Figure 8**).

First, and most significantly, the safety of engineered BEVs is subject to considerable skepticism due to their bacterial origin and excessive immunogenicity. Despite substantial removal of toxic components through engineering, current technologies struggle to ensure complete detoxification, raising concerns of triggering severe systemic inflammatory responses leading

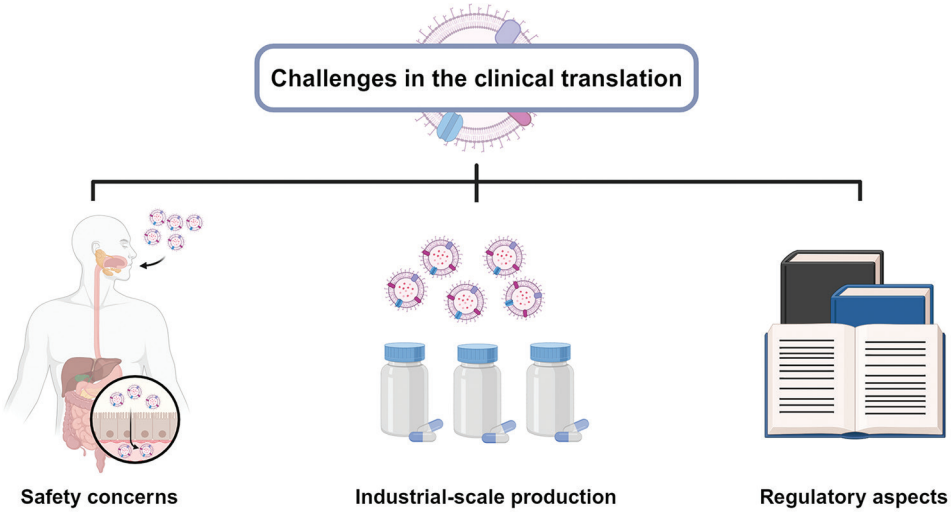

Figure 8. Challenges in the clinical translation of engineered BEVs. Despite the promising preclinical applications of engineering BEVs, challenges remain regarding the safety concerns, industrial-scale production, and regulatory aspects of engineered BEVs, which hinder their further clinical translation. Future research should prioritise conducting more comprehensive safety studies of engineered BEVs, refining production processes, and enhancing regulations governing BEV research, production, and use. Created with BioRender.com.
Abbreviation: BEV: Bacterial extracellular vesicle.

to patient deterioration or even death. In addition to the detoxification strategies previously discussed, several attempts have also been made to address the safety concerns associated with BEVs. One approach involves using attenuated strains for the production of BEVs. For example, Chen et al.[43] employed attenuated strains of *Salmonella* to extract BEVs, thus reducing their pathogenicity. Zhai et al.[47] also adopted this method. In another study, Kuerban et al.[54] focused on using attenuated *Klebsiella pneumoniae* for BEV extraction. Compared to their wild-type counterparts, these attenuated strains exhibit lower pathogenicity, making them relatively safer. Additionally, extracting BEVs from probiotics presents another promising strategy due to their inherent biocompatibility. Nakao et al.[29] utilised *E. coli Nissle 1917* for BEV extraction, as did Chen et al.[56] demonstrating the feasibility of this approach. Similarly, Liang et al.[53] utilised *Lactobacillus plantarum*, highlighting the variety of probiotic sources available for BEV extraction. Despite these efforts, future research should focus on optimising of BEV detoxification processes and prolonged, in-depth safety observations are imperative to ensure their harmlessness to humans. Additionally, more efficient novel detection techniques need to be introduced to better assess the biodistribution of BEVs.[78]

Second, compared to extracellular vesicles,[79] BEVs sourced from different bacterial strains exhibit heterogeneity, which presents challenges in establishing uniform standards for their production processes, quality control, and dosage regimens. To address these issues, several expert consensuses have been published in recent years. For example, the International Society for Extracellular Vesicles has published and updated the Minimal Information for Studies of Extracellular Vesicles, providing researchers with an updated snapshot of available approaches and their advantages and limitations for the production, separation, and characterisation of extracellular vesicles from multiple sources, including bacterial ones.[80] Likewise, the Chinese Society for Extracellular Vesicles has also published a position paper on BEVs to improve experimental reproducibility and interoperability in BEV research.[81] However, there is still a lack of internationally recognised guiding documents and effective supervision. To address these gaps, further research is required to delve into the production processes, quality control, and dosage regimens for BEVs. Additionally, there is a need for more guidelines and expert consensus to guide basic research and clinical translation. Moreover, it is essential to refine the relevant laws and regulations to standardise the research, production, and application of BEVs.

Finally, the current production processes for BEVs are complex and costly compared to traditional industrial methods, hindering large-scale production. To improve the yield of BEV production, several measures are taken, including genetic engineering and culture media treatment with stressors.[82] For example, Sawabe et al.[83] constructed an *E. coli Nissle 1917* strain with knockouts in the *mlaE* and *nlpI* genes, leading to increased BEV production. Similarly, Andreoni et al.[84] demonstrated that antibiotics can induce the formation of BEVs through different routes in *Staphylococcus aureus*. Going forward, it will be crucial to further optimise BEV production processes to reduce complexity and costs, which will be essential for advancing towards large-scale manufacturing.

8. Conclusions

In recent study, the application of engineered BEVs has addressed the issues of significant toxicity and low antigen presentation efficiency associated with natural BEVs, thereby expanding their preclinical applications. Engineered BEVs can load exogenous cargos through different strategies, enhancing antigen presentation efficiency and endowing the vesicles with novel properties. Furthermore, various detoxification strategies are utilised for engineered BEVs to remove toxic molecules and

reduce their loss in circulation, thereby improving their safety and targeted delivery capabilities. The scope of natural BEV applications has been broadened by engineered BEVs, which have shown promising preclinical results in treating infections, cancer, ischaemic stroke, and IBD. However, challenges remain regarding the safety, industrial-scale production, and regulatory aspects of engineered BEVs, which hinder their further clinical translation. Future research should focus on enhancing the safety of engineered BEVs, refining production processes, and promoting the development of international guidelines and regulatory frameworks.

However, the review has some limitations. For one, some data, such as loading efficiency, are difficult to obtain, resulting in gaps. Additionally, articles published before 2018 were not included in the analysis. In conclusion, this review provides a comprehensive overview of recent advances in BEV engineering strategies and their applications in disease treatment. The insights derived from these recent findings hold significant implications for the refinement and application of engineered BEVs.

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