The tremendous biomedical potential of bacterial extracellular vesicles

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Abstract

Bacterial extracellular vesicles (bEVs) are nano-sized particles delimited by a lipid membrane and filled with bacteria-derived components. bEVs play important roles in the physiology and pathogenesis of the bacteria, and in bacteria-bacteria and bacteria-host interactions. Interestingly, recent advances in biotechnology made it possible to engineer the surface of bEVs and decorate it with diverse biomolecules and nanoparticles. bEVs have gained tremendous interest in a wide range of biomedical fields and are currently evaluated as vaccines, cancer immunotherapy agents and drug delivery vehicles. However, significant hurdles in terms of safety, efficacy and mass production need to be addressed to enable their full clinical potential. Here, we review recent advances and remaining obstacles regarding the use of bEVs in different biomedical applications and discuss paths toward clinical translation.

A general Introduction to Bacterial Extracellular Vesicles

Bacterial extracellular vesicles (bEVs) are small biological entities surrounded by a proteolipid bilayer and carry various biological molecules from the parental bacteria. It has to be emphasized that bacterial isolates, either derived from a Gram-negative or a Gram-positive bacteria, produce several bEV (sub)types which may differ in composition and content. Currently, the necessary technology to analyze the cargo of every single (b)EV is not available [1]. bEVs from Gram-negative bacteria, called outer membrane vesicles (OMVs), originate from the outer membrane, carry both periplasmic and cytoplasmic components, and have a diameter of ~20-250 nm. bEVs from Gram-positive bacteria, known as cytoplasmic membrane vesicles (CMVs), are derived from the cytoplasmic membrane, contain substances from the cytosol and have a diameter of ~20-400 nm. **Figure 1** illustrates the differences between OMVs and CMVs. All bEVs play an important role in the interaction of bacteria with each other and with the host [2].

The biogenesis of CMVs remains a poorly understood process. The current supported mechanism involves the action of peptidoglycan-damaging enzymes that trigger **bubbling cell death** (see **Glossary**), i.e. explosive cell lysis mediated through the activity of an endolysin [3, 4]. For OMVs, different biogenesis pathways have been reported and those pathways are summarized in **BOX 1**. Once bEVs are released by the bacteria into the extracellular space, they can activate intracellular signaling via ligand-receptor interactions and/or be internalized by the target cell via either endocytosis, phagocytosis, micropinocytosis or membrane fusion [5, 6]. Thereafter, the bEVs release their cargo into the cytoplasmic space and can induce, depending on the cargo, effects ranging from either suppression to activation of the immune response (**Figure 2**) [5]. For example, OMVs released by *Pseudomonas aeruginosa* or *Salmonella enterica* have the ability to reduce the inflammatory response [7]. In contrast to these immunosuppressive OMVs, *Escherichia coli* (*E. coli*)-derived OMVs can induce pro-inflammatory response in the recipient cells [8]. Moreover, bEVs have also been reported to play a role in the development and progression of several diseases such as bacterial

infections [9, 10], pulmonary fibrosis [11], and Alzheimer's disease (AD) [12]). Of note, bEV populations originating from the same strain but generated by different biogenesis pathways can have distinct compositions and therefore different effects on the host cell [2].

As emphasized above, bEVs can extensively influence and modify the behavior of recipient cells depending on their biological cargo. This tremendous potential and the possibility to engineer the surface of bEVs make them attractive for different applications leading to an increasing amount of preclinical and clinical studies [13]. Moreover, in 1987, the first OMV-based vaccine was licensed for use in Cuba against *Neisseria meningitidis* (*N. meningitidis*) serogroup B (MenB) and was subsequently approved in Norway and New Zealand [14]. In 2013, a multi-component MenB OMV-based vaccine, 4CMenB (Bexsero) was approved for human use by the EMA and the FDA [14]. Despite this success, significant hurdles such as reducing reactogenicity and improving homogeneity, stability and scalability [5, 14], still need to be taken in order to make the full transition towards the clinic.

In the current review, we first highlight the importance of the bEV purity as this is the premise of exploiting its application potential. This includes describing the different separation technologies and the problems related to the use of EV-containing matrix. Next, we give a complete overview of the recent progress, future potential but also the remaining challenges of bEVs for different biomedical applications. To end, we focus on another potential avenue for bEVs, namely their use as diagnostic biomarkers [15, 16].

The First Step: Isolation and Purification of the bEVs

bEV Heterogeneity

It is becoming increasingly clear that bEV populations are very heterogeneous in size and composition, even if they are isolated from pure bacterial cultures [4]. This high degree of heterogeneity may partially be due to different biogenesis pathways, but also external factors like growth stage, medium composition, sample collection and so on affect the observed heterogeneity [17-19]. For example, biophysical (i.e. size and shape) and proteomic

analyses of *Helicobacter pylori* (*H. pylori*) OMVs isolated from different growth stages showed that both size and composition differ between different growth stages [17]. Additionally, the concentration and composition of virulence factors can appear to differ in the different OMV fractions produced by *H. pylori* [19].

The studies described above underscore that bEV heterogeneity is affected by many factors. This heterogeneity is extremely important as different bEV subsets may contain a different composition of cargo and may target different host cells leading to distinct biological effects [6, 20]. For the further development of the bEV applications, it is of utmost importance to address the bEV heterogeneity in more detail. To be able to do so, sensitive techniques to isolate and analyze the different subpopulations are essential. In this regard, novel micro- and nanotechnological tools have opened a new era of single-particle detection and analysis and have been successfully applied to EV research [21, 22]. Once the required tools, e.g. specific antibodies, are available, this could also be applied to bEVs. Yet to date, it is difficult to physically separate the bEV subtypes so the heterogeneity of bEVs reveals a layer of complexity that remains to be kept in mind when interpreting results of different studies.

bEV Isolation and Purity: a Major Hurdle to Overcome

High bEV quality and purity are prerequisite in order to translate research findings into actual clinical practice. To meet this requirement, many techniques have been developed to isolate and purify bEVs [23]. These techniques include commonly used approaches such as ultracentrifugation (UC), ultrafiltration (UF), precipitation and size-exclusion chromatography (SEC), as well as some less frequently including affinity isolation and density gradient centrifugation (DGC) (**Figure 3**). Different isolation methods used to isolate bEVs from bacterial cultures and biofluids have their own strengths and weaknesses and those futures are summarized in **Figure 3**.

However, high-efficiency isolation of bEVs and the separation of them from matrix contaminants is still needed to ensure an accurate interpretation of the biological functions of (a subpopulation of) bEVs. In most cases, the

mentioned techniques acquire sufficient yield and purity as most of the culture medium has a low number of contaminants with similar density and size of the bEVs (e.g., non-bacterial EVs and lipoproteins). However, in complex matrices, such as biofluids and serum-supplemented medium, combining different purification methods based on complementary principles will be imperative to remove contaminants like EVs and lipoproteins [24].

It is worth noting that fastidious bacteria such as *N. meningitidis* and *H. pylori* need to grow in serum-containing media [25, 26]. In these specific cases, EV-depleted serum can be used but this is often too expensive for large-scale experimental studies. Moreover, EVs are often not completely removed in these EV-depleted sera and this varies dependent on the method used and/or the commercial supplier. Consequently, it is essential to develop standardized methods to isolate and purify bEVs from EV-containing matrix and perform the necessary quality controls on the bEV purity. Currently, only one strategy is described to specifically separate bacterial from non-bacterial EVs in biofluids by combining UC, SEC, and DGC (**Figure 4**) [24]. Of note, this method is relatively time-consuming (~23 hours) and iodixanol may be present in the final bEV sample, making it a difficult method to use in research or clinical applications. In this context, an affinity strategy that uses specific (b)EV capture molecules such as antibodies or **aptamers** can provide a rapid method to isolate high-purity (b)EVs from complex matrices [27-29]. However, more efforts are needed to design bEV-specific capture molecules or to develop simpler and more effective purification methods.

bEVs as Novel Therapeutics and Diagnostics: Perspectives and Challenges

bEVs have tremendous potential in many biomedical applications. Here, we discuss the perspectives and challenges of this per application field (**Figure 5**, Key Figure). **Table 1** summarizes the preclinical and clinical studies of bEVs in biomedical applications in 2017-2021.

bEVs as Vaccination Agents

In recent years, the potential of bEVs as vaccines against bacterial pathogens

has been extensively investigated. bEVs inherit several **pathogen-associated molecular patterns (PAMPs)** including antigens from their parental bacteria and consequently can induce an immune response against pathogens [14].

bEV-based candidate vaccines are being developed at the preclinical level against many pathogens, such as *Vibrio cholerae* (*V. cholera*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Bordetella pertussis* (*B. pertussis*), *Salmonella* Typhimurium (*S.* Typhimurium) and *Mycobacterium tuberculosis* and have been proven to elicit humoral and cellular immune responses [14]. For example, *B. pertussis* OMVs isolated by UC are able to induce a robust antibody response in mice with serum IgG levels comparable to those obtained with the current approved whole cell *B. pertussis* vaccine [30]. At the clinical level, the first OMV-based vaccine was licensed in 1987 for use in Cuba against MenB and these OMVs have been successfully employed as vaccines in controlling a MenB outbreak in New Zealand [14].

Besides, bEVs are a good source of bacterial antigen. Their PAMPs enable the interaction with antigen presenting cells (APCs) and the bEVs itself can be phagocytosed by APCs. All of this makes bEVs of use as an adjuvant [23]. For example, recent studies reported that co-immunization of an influenza vaccine with *E. coli* OMVs obtained by combination of ammonium sulfate precipitation and DGC, enhanced the antigen-specific humoral and cellular immune responses in mice [31-33]. Compared to conventional adjuvants such as aluminum hydroxide (alum) and CpG DNA, *Burkholderia pseudomallei* OMVs isolated by ammonium sulfate precipitation and DGC promote the humoral and cellular immune responses against ovalbumin [34] and *S.* Typhimurium [35] in mice. Moreover, an increased specific antibody response is induced upon co-administration of pneumococcal protein antigens and MenB OMV adjuvant, however, its efficacy is not superior to alum adjuvant [36]. This latter finding indicates that the adjuvant capacity of bEVs may be bacterial specific.

One of the challenges to develop bEVs as vaccines is to find an efficient strategy to engineer the parental bacteria to enhance the production and immunogenicity of bEVs and lower the potential reactogenicity. Moreover,

there is an increasing interest in the influence of bEV heterogeneity on the immunization effect. More precisely, the size, shape, and rigidity of the particles affect cell uptake, antigen presentation, and immune activation [37]. Different strategies are currently under investigation to address these challenges and are discussed below:

To enhance immunogenicity and reduce endotoxicity, the **generalized modules for membrane antigens (GMMA)** technology has been developed. This technology incorporates heterologous antigens into the vesicular compartment and alter the acylation process of lipid A to produce penta-acylated LPS with reduced endotoxicity and maintain immunodominant O antigen component of the LPS [38]. This engineered vaccine is well tolerated and able to elicit antibodies against *Shigella sonnei* (*S. sonnei*) in healthy adults [39, 40]. The same technology is now under investigation to design a quadrivalent vaccine containing *S. sonnei* and three of the *Shigella flexneri* serotypes that can provide overall coverage for up to 88% of all *Shigella* strains [41]. Based on the GMMA-technology, a recent study decorated OMVs with heterologous antigens by channeling these antigens to the lipoprotein transport machinery. This approach allows accumulation of the lipidated antigens in the vesicular compartment [42]. Moreover, the same research group generated an *E. coli* mutant that directly eliminated 59 of the endogenous proteins in order to improve the loading capacity of the desired antigens via display on the OMV surface resulting in an elevated immune response [43]. Recently, a more flexible vaccine platform based on genetic engineering and **plug-and-display** technology has been established to display heterologous antigens [44]. Specifically, this platform provides different plasmid-encoded polysaccharide biosynthetic pathways that can be readily transformed into *E. coli*, enabling rapid development of personalized multivalent OMV-based vaccines. However, another study indicates that GMMA technology used to display LPS O-antigen in *Salmonella* strains exhibits a higher immunization effect in mice compared to the plug-and-display system [45]. The difference in immunogenicity might be explained by the fact that GMMA may constitute a more immunogenic format for presenting antigens to the immune system and resulting in an increased specific antibody

response [45].

To make uniformly sized and stable bEVs, Zhang and colleagues coated *E. coli* DH5α OMVs onto gold nanoparticles to make them specific and similar in size and shape. Compared to natural OMVs, this resulted in a more robust antigen-specific immune response in mice, reflected by a longer lasting response with a higher avidity [46]. Consistent with this finding, coating *K. pneumoniae* OMVs onto bovine serum albumin-based nanoparticles induces dendritic cell maturation and specific antibody responses [47]. In addition to coating bEVs onto surface of nanoparticles, loading *E. coli* OMVs into nanoparticles (e.g., zein and chitosan-based nanoparticle) also enhances the ability of the bEVs to induce immune responses in animals [48-50].

Taken together, the above studies show that coating or loading of bEVs on/in nanoparticles enhances their immune effect by improving their stability and antigen presentation efficiency. However, it needs to be kept in mind that different types of nanoparticles may have an additional influence on the characteristics of an induced immune response. Consequently, the selection of the optimal nanoparticles is of utmost importance. A way to circumvent this problem is to generate bacterium-membrane-formed nanovesicles by using **nitrogen cavitation**. Such artificially assembled double-layered membrane vesicles positively affect stability and immunogenicity without the presence of nanoparticles [51].

bEVs as Cancer Immunotherapy Agents

The use of bacteria-associated substances for cancer treatment dates back to the early 1890s, when Dr William Coley injected a mixture of weakened bacteria solution to treat cancer patients [52]. As the attenuated bacteria may still pose a potential risk for infection, non-cellular bEVs are naturally non-replicating and have emerged as a safer alternative. bEVs contain a large number of components, including various immunostimulatory molecules from the parental bacteria [5], making them a potential therapy to treat cancer.

Kim and colleagues first reported the use of different bEVs as cancer immunotherapeutic agent in 2017 [53]. They showed that bEVs specifically accumulated in tumor tissues, subsequently induce antitumor immune

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responses by mediating IFN-γ signaling pathways in mice. However, IFN-γ is also known to upregulate immunosuppressive factors such as immune checkpoint inhibitors in the **tumor microenvironment (TME)** [54]. To counteract the upregulation of immunosuppressive factors, a recent study developed engineered *E. coli* OMVs containing the ectodomain of the immune checkpoint PD1 on its surface. This increases the accumulation of OMVs at the tumor site and exerts the PDL1 blockade effect. These engineered OMVs induced an enhanced anti-tumor immune responses compared to treatment with natural OMVs reflected by a ~1.5-fold increase in pro-inflammatory cytokine levels in serum and tumor tissue and a 1.5-fold impairment of tumor growth in mice [55].

Another way to go is by decorating the membrane of bEVs with tumor antigens to induce an immune response against that specific tumor antigen. Unfortunately, tumor antigens are very diverse and vary considerably between patients, hampering the of use natural or single antigen-decorated bEVs as generic therapy for different patients [56]. In contrast, modified bEVs that simultaneously display multiple tumor-specific/relevant epitopes have the possibility to be effective in a broader range of patients [57, 58]. The plug-and-play technology described above is already used to create bEVs that display different tumor antigens [59].

Although bEVs elicit an effective antitumor immune response, combination therapy is recommended to further enhance the tumor immunotherapeutic potential in order to completely eradicate the tumor and prevent tumor recurrence and metastasis such as loading **chemotherapeutic** drugs (e.g., tegafur and doxorubicin) in bEVs [60, 61] or co-treatment with anti-PD-1 immunotherapy [62] or photothermal therapy [63] or adding a photosensitizer agent such as indocyanine green and polydopamine to the bEVs. The latter facilitates photothermal-induced **immunogenic cell death** which in turn amplifies the anti-tumor immune response in mice [64-66]. All these studies highlight that combining traditional treatment strategies with natural or modified bEVs may greatly improve the efficiency of cancer treatment.

bEVs as Drug Delivery Vehicles

(b)EVs are also explored for the delivery of therapeutic payloads to specific cells or tissues, harnessing their cell-targeting abilities. Compared to the most common and well-investigated synthetic nanocarriers, namely liposomes, (b)EVs are similar in terms of size, shape and structure but have more complex bilayers that contain various lipids and proteins as well as internal cargo and surface-associated molecules; some of them aid in cell targeting [67]. These intrinsic features give (b)EV several advantages over liposomes in the context of safe and effective drug delivery (summarized in **Table 2**). Compared to EVs, bEVs are in specific cases more favorable drug vehicles as they can be more easily customized and they can be produced in large quantities by using bacterial fermentation vessels [59, 68]. However, in order to make the step to the clinic, bEVs still need to be further improved in terms of drug loading capacity, targeting capability and blood circulation stability.

Currently, there are two general strategies used to load bEVs, i.e. post- and pre-loading. **Table 3** summarizes the advantages and disadvantages of methods for drug loading into (b)EVs. Despite the different loading approaches, the low loading efficiency is still a major challenge.

Although bEVs play a vital role in transporting biomolecules to specific distant sites for their parental bacteria, their targeting capacity still needs improvement before it can be used in the clinic. One way is to add targeting molecules to the bEVs. E.g. coating OMVs from *Salmonella* or *E. coli* with Arg-Gly-Asp peptide lead to a 2.5-fold and 11-fold increase in tumor-targeting ability in mice, respectively [60, 69]. However, in some cases, this strategy may not be enough to mediate robust specific targeting as non-target cells may also express similar receptors. To overcome this, a **eukaryotic-prokaryotic vesicle (EPV)** nanoplatform has been designed [65, 66, 70]. This platform is constructed by fusing bEVs with tumor cell membranes and vesicles. Such assembled EPV integrates various tumor-associated antigens and enables a higher (up to 10-fold) tumor-specific accumulation than natural bEV in mice [66]. A comparable but indirect strategy uses circulatory cells to take up bEVs and to subsequently deliver these bEVs to target cells. The potential of this strategy is shown by using anti-CD11b antibody-decorated

nanoparticles that target circulating neutrophils and subsequently accumulate into mouse tumors [71]. To improve the efficiency of bEV uptake by neutrophils, a more generalized method is established based on pathogen-mimicking **nano-pathogenoids (NPNs)** cloaked with bEVs. The neutrophil targeting efficacies of these bEV-cloaked NPNs in the peripheral blood of mice can reach ~41% [72], compared to ~30% when CD11b-decorated nanoparticles are used [71].

The stability of bEVs in blood circulation is also an important factor to be able to increase targeting and retention. Firstly, PEGylation can be used to improve bEVs stability in blood circulation. However, this strategy has also compromised their targeting ability toward the tumor tissue [60]. To avoid this issue, a recent study proposes to encapsulate OMVs with a nanoshell of calcium phosphate (CaP), which is a pH sensitive controlled-release material [64]. The CaP-shielded OMVs not only help to neutralize the acidic TME but also retain further accumulation of the bEVs at tumor sites through an improved circulation time. This effect can be further enhanced by doping tumor target ligands such as folic acid into the CaP shells, thereby facilitating active targeting to tumors in mice [64].

Taken together, the outer shell of bEVs is a flexible structure that can be used to maximize the efficiency of drug delivery by improving bEV stability and decorating it with targeting molecules. Exciting new avenues, including the fusion of drug-loaded liposomes with EVs and/or bEVs to further improve drug loading capabilities, are also being explored in the EV-field [73]. Notably, the production of designer EVs by implanted cells has recently been reported [74]. This technique offers a new route for *in vivo* production of engineered (b)EVs inside the body. These techniques make (b)EV favorable in the delivery of novel drugs, such as therapeutic nucleic acids.

bEVs as Antibacterial Agents and Targets

Besides vaccines, antibiotics are the most important type of antibacterial agent. In recent years, however, more and more bacterial strains are becoming resistant to antibiotics, highlighting the urgent need to rethink our way of combating bacterial infections.

The innate antibiotic properties of bEVs in interspecies competition have also attracted interest in the use of natural bEVs as antibacterial agents. For example, myxobacterial-derived OMVs show the ability to inhibit the growth of *E. coli* and *Staphylococcus aureus* in cultures [75, 76]. This property may be attributed to the presence of anti-bacterial **cystobactamids**. In addition to encapsulation of anti-bacterial molecules, *Lysobacter enzymogenes*-derived OMVs have been observed to naturally encapsulate anti-fungal molecules chitinase and also show the ability to inhibit fungal growth in cultures [77]. CMVs from *Lactobacillus plantarum* also show the ability to protect against vancomycin-resistant *Enterococcus faecium* infection by up-regulating host defense genes such as *clec-60* in Caenorhabditis elegans [78]. More recently, *H. pylori* OMV membrane-coated nanoparticles showed the ability to bind with gastric epithelial cells (AGS) and reduce *H. pylori* adhesion in AGS cultures [79]. This indicates that bacterium-mimicking nanoparticles, obtained by coating the nanoparticles with membrane molecules of bEVs confer an anti-adhesion property against the parental bacteria.

From another perspective, bEVs play a vital role in bacterial physiology and pathogenesis [2]. Therefore, targeting bEV production by the bacteria or protein export to bEV may provide a new antibacterial therapeutic strategy [80]. Although this idea has been proposed for several years, no study has so far shown the viability of this strategy. The complexity of the bEV composition and incomplete understanding of bEV biogenesis may be the major challenges. Thus, more techniques and knowledge are required before we can fully elaborate on this treatment avenue.

bEVs as Diagnostic Biomarkers

In the last 20 years, EVs have been extensively investigated as biomarkers to diagnose and monitor different diseases [67], in contrast to bEVs; partially due to the remaining methodological challenges discussed above. However, accumulating evidence suggests that disease-associated microbiome changes may be reflected in biofluids bEV levels and composition. Consequently, the presence of specific bEVs in biofluids such as serum can be associated with a specific state of infection making bEVs attractive as

biomarkers for clinical diagnosis [16, 81].

So far, metagenomics and metabolomics have indicated the association between bEVs and diseases such as AD [82], cancers [81], allergies [83], and respiratory diseases [84, 85], which makes the bEV population a promising tool for diagnosing these diseases. In addition, a combination of metagenomic and metabolomic analysis of vesicles isolated from faeces demonstrates the correlation between microbial changes and metabolic alternations within the vesicles population, indicating gut microbes-derived bEVs carry dynamic changes in the metabolic information that reflect the host's health state [86].

Despite these promising results, implementation in the clinical lab remains challenging. One of the reasons is the requirement for special instruments, such as the MiSeq system and Gas chromatography, as well as bioinformatic tools to analyze the obtained data [87]. To address these challenges, more efforts are needed to lower the requirements for the technological platform and make data interpretation easier. In this regard, Han and colleagues introduced a method for genomic DNA qPCR and methylation analysis of vesicle extract and indicated that bEVs is a good discriminators for periodontitis [15]. Of note, the actual presence of EVs in crude bEV extracts may interfere with the interpretation of the results. Taken together, a better isolation of pure bEVs or specific bacterial bEVs from biologic matrices will likely facilitate the development of detection techniques and improve the sensitivity and specificity of the bEV as a diagnostic tool.

More recently, a strategy was proposed to isolate pure bEVs from biofluids by the combination of UC, SEC, and DGC. This technique associated LPS-positive bEVs present in plasma with impaired barrier integrity in patients diagnosed with IBD, HIV, and cancer therapy induced-intestinal mucositis [16]. Unfortunately, the proposed assay is complex and time-consuming and thereby limiting its clinical utility. Another promising capture and detection technique for bEVs is the aptamer-based detection platform. Although this technology shows high sensitivity to detect as low as 25 ng/ml bEVs in bacterial cultures, further validation in clinical samples is warranted [88].

In addition, an enzyme-linked immunosorbent assay-based analysis of anti-bEV antibodies has been developed as a diagnostic tool for lung disease. For this, pure bEVs isolated from bacterial cultures are used to capture antibodies in human serum and to determine the titers of anti-bEV antibodies as a diagnostic indicator [89]. However, the most important disadvantage of antibody-based serological tests is that they do not distinguish between an active infection and a previously resolved infection. Antibody levels can persist in the blood of individuals cured of bacterial infection for long periods and this may lead to a false-positive result.

The Unmet Needs for bEVs to Become Successful in the Clinic

Although there is tremendous potential of bEVs in different biomedical applications, some unresolved issues are hampering the further development of applications in the clinic. Major concerns are regarding the safety, reproducibility, stability, and scalability of the approach.

Safety

Safety is the biggest hurdle to take in order to bring bEVs to the clinic. The main component of OMVs, namely LPS, not only induces immune responses but also induces reactogenicity [23]. In addition, other components such as outer membrane proteins and lipoproteins can also induce systemic inflammatory responses [90]. Currently, some strategies have been introduced to obtain bEVs with a low level of LPS or other components toxicity (summarized in **Box 2**).

Unfortunately, LPS-deficient OMVs exert a lower degree of immunogenicity compared to OMVs with normal LPS levels [91]. Consequently, a new challenge arises: find the ultimate balance between low toxicity and high immunogenicity. To this end, high-throughput screenings are needed to look into the synergistic effect of different pattern recognition receptor agonists. In this way, the best combination of adjuvants can be determined [92]. In addition, comprehensive safety assessments, including absorption, distribution, metabolism, and excretion tests, as well as

pharmacokinetic/toxicokinetic studies, are important for a complete understanding of the toxicity of bEV-based drugs [93].

Reproducibility

bEV research remains highly challenging due to the lack of standardized preparatory and analytical methods. Indeed, the huge heterogeneity and purity of isolated bEV populations caused by different production and isolation techniques hamper the repeatability and reproducibility of results between different studies [17-19, 94]. Additionally, the different methods used to quantify bEV protein and quantity can alter experimental outcomes [94]. Unfortunately, these are ongoing problems, as we currently still lack the necessary techniques and guidelines to perform uniform bEV studies. To promote in-depth research and clinical translation we urgently need standardized guidelines like those that are available for studies with EVs [95, 96].

Stability

The stability of the bEVs *in vivo* poses another big challenge. Indeed, the *in vivo* biodistribution of naïve bEVs shows a swift clearance and preferential accumulation in the mononuclear phagocytic system (containing the liver, lung, and spleen), followed by a rapid elimination and/or phagocytation [26, 97]. So, an important hurdle to take is to find out how we can modify bEVs to extend their circulation time and improve their accumulation at the site of interest. Surface modifications of bEVs with hydrophilic moieties, such as polyethylene glycol (PEG), or the decoration of bEVs with nanoparticles can be interesting avenues [46, 47, 60]. Moreover, the incorporation of PEG reduces the interaction of bEVs with non-target cells. Next to this, the direct fusion of bEVs with cell target peptides enhances bEV targeting to a specific cell population [60, 65]. Unfortunately, such modification may alter the surface composition and consequently the *in vivo* behavior of the bEVs.

Scalability

Scalability is crucial in order to ensure an economically favorable production process. Although we can easily grow bacteria in large quantities by using big

fermentation vessels, the amount of bEVs released from bacteria is still not sufficient for making their cost-effective mass production [23]. To optimize the bEV production, different culture systems are currently under investigation [18, 98]. However, it is important to keep in mind that the bEV composition may differ dependent on the used culture condition. Interestingly, engineered bacteria with a compromised envelope have been successfully used to increase bEV secretion [99, 100]. The further development and search for new appropriate approaches to cost-effectively scale-up bEV production are indispensable.

Concluding Remarks and Future Perspectives

bEVs are of high interest for novel therapeutic and diagnostic applications. They are more favorable than their parental bacteria as they have higher biocompatibility and lower risks of malignancy. Additionally, their complex composition of bioactive molecules and their capacity to cross cellular barriers and infiltrate tissues make them suited to support different biomedical uses. However, extensive efforts are still needed to resolve outstanding issues (see **Outstanding Questions**) in order to make the step to the clinic. The most important unmet challenges are regarding (I) cost-effective and rapid bEV production and separation (II) standardized analytical methods and production procedures, and (III) safe and effective modification strategies to improve bEV functionality and to lower toxicity.

The priority includes optimizing the culture system for large-scale production and developing protocols for reliable and reproducible isolation of bEVs based on morphological (i.e., size and shape), biophysical (e.g., surface charge and density), and biochemical composition (e.g., specific surface and internal markers) properties. Additionally, developing cost-effective and straightforward bEV isolation techniques is a major economic factor to enable clinical translation. In diagnostic applications, the miniaturization of bEV separation approaches that enable efficient and robust isolation from limited biological samples is also important to develop fast and sensitive detection platforms. Second, ample attention to fundamental studies should be given to unveil the physiological functions of bEV (sub)populations differing in membrane

markers and cargo. We need to have a better understanding of the impact of endogenous gene modification and exogenous conditions on the biochemical features of bEVs. This will ensure more consistent quality and efficacy between batches of bEVs. This is not only important to be able to compare different studies, but also necessary to meet regulatory requirements. Finally, endotoxin should be reduced to ensure safety in clinical use. Meanwhile, how to maintain the efficacy of bEV immunogenicity while removing the endotoxins needs further in-depth studies. It is important to find a good balance between low toxicity and high immunogenicity. Additionally, the modified (either physical, chemical, or genetic) bEVs with the detoxified and controllable composition based on the comprehensive understanding of their active ingredients would provide a robust approach to improve immunogenicity and reduce the toxicity of bEVs.

Overall, despite the profound challenges, an emerging field of bEVs for diagnosis and treatment will introduce a new frontier in medical treatment strategies.

Tables

Table 1. Preclinical and Clinical Studies of Bacteria Extracellular Vesicles (bEVs) in Biomedical Applications in 2017-2021.

Application	Bacteria	OMV type[a]	Composition	Target	Status	Refs
Vaccines/adjuvants	*S. sonnei* 1790	mdOMV	GMMA with OAg (1790GAHB)	*S. sonnei* infection	Phase I in Europe Phase II in Africa	[39] [40]
	MenB	dOMV	4CMenB OMV (Bexsero)	*N. gonorrhoea* infection	Preclinical	[101]
	MenB	dOMV	OMV with adjuvants	MenB infection	Preclinical	[25, 102]
	MenB	(md+d)OMV	ΔporAΔporB MenB OMV	MenB infection	Preclinical	[103]
	MenB	nOMV	Polyhistidine triad protein D; OMV and alum as adjuvants	*S. pneumoniae* infection	Preclinical	[36]
	MenX	dOMV	OMV	MenX infection	Preclinical	[104]
	N. gonorrhoea FA1090	mdOMV	OMV with IL-12	*N. gonorrhoea* infection	Preclinical	[105]
	S. Typhimurium 2189 and *S.* Enteritidis 618	mdOMV	GMMA with OAg	*Salmonella* infection	Preclinical	[45]
	S. Typhimurium P-102 and IDH3162, and *S.* Enteritidis 520833 and IDH1125	nOMV	OMV	*Salmonella* infection	Preclinical	[106]
	V. cholerae O1 El Tor and VC492	nOMV	OMV	*V. cholerae* infection	Preclinical	[107, 108]

B. pertussis B1917	nOMV	OMV	*B. pertussis* infection	Preclinical	[109]
E. coli JC8031	glyOMV	OMV with poly-N-acetyl-d-glucosamine	*S. aureus* and *F. tularensis* infection	Preclinical	[110]
E. coli HK100 and BL21	(md+gly)OMV	Δ*ompA*Δ*msbB*Δ*pagP* mutant OMV expressing *S. aureus* Ags	*S. aureus* infection	Preclinical	[42]
E. coli W3110	mdOMV	Δ*msbB*/Δ*pagP* mutant OMV	Influenza	Preclinical	[32, 33]
E. coli MC4100	mdOMV	Δ*nlpI* mutant OMV expressing ClyA-M2e4xHet	Influenza	Preclinical	[100]
E. coli DH10B	mdOMV	*E. coli* mutant OMV expressing HA and RBD	H1N1 and MERS-CoV infection	Preclinical	[111]
S. Typhi BRD948	mdOMV	GMMA with heterologous *S.* Typhi Vi Ag and homologous O:2 OAg	*Salmonella* infection	Preclinical	[112]
S. Typhimurium S100, *S.* Choleraesuis S340 and *S.* Enteritidis S246	mdOMV	Δ*fliC* and Δ*fljB* mutant OMV	*Salmonella* infection	Preclinical	[35]
S. Typhimurium χ3761	mdOMV	Mutant expressing *Orientia tsutsugamushi* Ags	APEC and *S.* Enteritidis infection	Preclinical	[113]
S. Enteritidis LQSE1714	mdOMV	OMV expressing OmpF and OmpP	*S.* Enteritidis infection	Preclinical	[114]
APEC O1, O2 and O78	nOMV	OMV	APEC infection	Preclinical	[115, 116]
E. coli F4 and F18	cOMV	OMV coated into/on NP	ETEC infection	Preclinical	[48-50]

	P. aeruginosa PA-103	sMV	Bacterial membrane assembled nanovesicles	*P. aeruginosa* infection	Preclinical	[51]
	S. aureus RN4220, Newman, N315, Mu50 and ATCC 25923	mdCMV	Δ*agr* CMV expressing dengue virus Ag	dengue virus infection	Preclinical	[117]
	S. aureus S29213, BW15 and BWMR26	cOMV	OMV coating ICG-loaded magnetic mesoporous silica NPs	*S. aureus* infection	Preclinical	[118]
	G. anatis 12656-12	nOMV	OMV	*G. anatis* infection	Preclinical	[119]
	B. melitensis 16M	nOMV	OMV with Poly(I:C)	*B. melitensis* infection	Preclinical	[120]
	A. baumannii ATCC 19606	mdOMV	Δ*lpxD* mutant OMV	*A. baumannii* infection	Preclinical	[121]
	A. baumannii ATCC17978 and LAC-4	nOMV	OMV with aluminum phosphate adjuvant	*A. baumannii* infection	Preclinical	[122]
	H. pylori 7.13	nOMV	OMV	*H. pyflori* infection	Preclinical	[123, 124]
	Francisellaceae NCIMB14265[T]	nOMV	OMV	*Francisellaceae* infection	Preclinical	[125]
	B. abortus S19	mdOMV	Δ*per* mutant OMV	*B. abortus* infection	Preclinical	[126]
Cancer immunotherapy agents	*E. coli* W3110	nOMV	OMV	CT26, B16BL6 and 4T1	Preclinical	[53]
	E. coli HK100	mdOMV	Mutant OMV expressing epidermal growth factor receptor variant III	B16F10	Preclinical	[58]

	E. coli DH5α	mdOMV	Mutant OMV expressing fibroblast growth factor	B16F10 and TC-1	Preclinical	[57]
	E. coli W3110	mdOMV	Mutant OMV expressing ectodomain of programmed death 1	B16 and CT26	Preclinical	[55]
	E. coli	mdOMV	Mutant OMV expressing RGP and ICG	B16F10 and A375	Preclinical	[69, 127]
	E. coli Rosetta (DE3)	mdOMV	Mutant OMV plug-and-display tumor antigens	B16-F10, MC38, Pan 02, and B16-OVA	Preclinical	[59]
	E. coli T1	cOMV	OMV coated on NPs	EMT6, EMT-EGFP and CT26	Preclinical	[72]
	E. coli DH5α	sMV	Fusing tumor cell membrane and *E. coli* OMV, and coated on NPs	B16F10	Preclinical	[66, 70]
	E. coli	sMV	Synthetic vesicles of *E. coli* outer membrane	B16F10	Preclinical	[62]
	S. Typhimurium	(md+c)OMV	Attenuated *S.* Typhimurium OMV coated on drug-loaded polymeric micelles	B16F10	Preclinical	[60]
	Salmonella	sMV	Fusing melanoma cytomembrane vesicles and attenuated *Salmonella* OMV	B16F10 and 4T1	Preclinical	[65]
	K. pneumonia ACCC 60095	cOMV	Doxorubicin-loaded OMV	A549	Preclinical	[61]

	V. cholera C6706 , *S. flexneri* 301, *E. coli* DH5α AND bl21	cOMV	OMV coated into calcium phosphate shell	4T1 and CT26	Preclinical	[64]
	S. Typhimurium, *S. aureus* and *VNP20009*	mdOMV	Δ*pG* mutant OMV	4T1 and CT26	Preclinical	[63]
Drug delivery vehicles	*B. thetaiotaomicron* GH290, GH484, GH486, GH474 and GH503, and *E. coli* Rosetta 2(DE3)	mdOMV	Mutant OMV expressing/delivering OmpA/SseB, IAV or KGF-2	Virus infection and colitis	Preclinical	[128]
	B. thetaiotaomicron VPI-5482 and *E. coli* J53/R751	mdOMV	Mutant OMV expressing/delivering F1 and V plague antigens	Plague	Preclinical	[129]
	E. coli	mdOMV	Mutant OMV expressing RGP and ICG	B16F10	Preclinical	[69]
	E. coli	cOMV	OMV coated on fluorouracil-loaded NPs	Caco-2	Preclinical	[130]
	S. aureus (ATCC 25923) and *E. coli* (ATCC 25922)	cOMV	OMV coated on antibiotic-loaded NPs	*S. aureus* infection	Preclinical	[131]
Antibacterial agents	*L. plantarum* WCFS1	nCMV	CMV	Vancomycin-resistant *E. faecium* infection	Preclinical	[78]
	V. cholerae O1 El Tor Ogawa HC1037	nOMV	OMV	*Bacteriophage* infection	Preclinical	[132]
	Myxobacteria SBSr073,	nOMV	OMV	*E. coli* infection	Preclinical	[75, 76]

	Cbv34 and Cbfe23					
	H. pylori SS1	cOMV	OMV coated on NPs	*H. pylori* colonization	Preclinical	[79]
	A. baumannii, ETEC, *P. aeruginosa* and *K. pneumoniae*	cOMV	Antibiotic-loaded OMV	ETEC infection	Preclinical	[133]
	B. thailandensis E264	nOMV	OMV	Drug-sensitive and drug-resistant bacteria and fungi	Preclinical	[134]
Diagnostic biomarkers	Microbiome	-	Metabolic alternations of OMVs in feces	AD	Preclinical	[82]
	Microbiome	-	Metagenomic alternations of OMVs in feces	Colorectal cancer	Preclinical	[86]
	Microbiome	-	Metagenomic alternations of OMVs in serum/urine	Ovarian cancer, benign ovarian tumor, AD, Asthma	Preclinical	[81, 83-85]
	Microbiome	-	LPS-positive OMVs in plasma and feces	Patients with intestinal barrier dysfunction	Preclinical	[16]
	Microbiome	-	Serum anti-bEV IgG ELISA	Lung disease	Preclinical	[89]
	Microbiome	-	DNA methylation of OMVs in salivary	Periodontitis	Preclinical	[15]

1

2   *Acinetobacter baumannii* (*A. baumannii*), *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*), *Bordetella pertussis* (*B. pertussis*), *Brucella abortus* (*B. abortus*),

1 *Brucella melitensis* (*B. melitensis*), *Burkholderia thailandensis* (*B. thailandensis*), *Escherichia coli* (*E. coli*), Enterotoxigenic *E. coli* (ETEC), *Gallibacterium*

2 *anatis* (*G. anatis*), H1-type haemagglutinin of the pandemic influenza A virus (H1N1) (HA), *Helicobacter pylori* (*H. pylori*), *Klebsiella pneumoniae* (*K.*

3 *pneumonia*), *Lactobacillus plantarum* (*L. plantarum*), *Neisseria gonorrhoeae* (*N. gonorrhoea*), Avian Pathogenic *E. coli* (APEC), *Pseudomonas aeruginosa* (*P.*

4 *aeruginosa*), *Salmonella* Enterica (*S.* Enteritidis), *Salmonella* Paratyphi (*S.* Paratyphi), *Salmonella* Typhimurium (*S.* Typhimurium), Serogroup B

5 Meningococcal (MenB), Serogroup X Meningococcal (MenX), *Shigella flexneri* (*S. flexneri*), *Shigella sonnei* (*S. sonnei*), *Staphylococcus aureus* (*S. aureus*),

6 *Vibrio cholerae* (*V. cholerae*).

7 Outer membrane vesicle (OMV), Natural outer membrane Vesicle (nOMV), Detergent-extracted outer membrane vesicle (dOMV), Mutant-derived outer

8 membrane vesicle (mdOMV), Glycoengineered outer membrane vesicle (glyOMV), Coated outer membrane vesicle (cOMV), Synthetic membrane vesicle

9 (sMV), Cytoplasmic membrane vesicle (CMV), Generalized modules for membrane antigens (GMMA), O-antigen (OAg), Nanoparticles (NPs),

10 Alzheimer's disease (AD); Receptor binding domain of the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (RBD), indocyanine green (ICG),

11 $\alpha_v\beta_3$ integrin peptide targeting ligand (RGP), Lipopolysaccharide (LPS), Enzyme linked immunosorbent assay (ELISA).

1

Table 2. Advantages and Disadvantages of Liposomes, Extracellular Vesicles (EVs)

and Bacterial EVs (bEVs) as a Drug Delivery System.

Carrier	Advantages	Disadvantages
Liposomes	• Scalable and cost-effective • Controlled and targeted drug release possible • Easy to modify the synthetic nanoparticle to custom specific requirements; e.g. loading of exogenous drug or increasing stability of the nanoparticle	• Less favorable for integration of multiple functional components • Low barrier crossing properties • Low cellular uptake • Rapid macrophage-mediated hepatic clearance • Toxicity and immunogenicity issues
EVs	• Good bioavailability and biocompatibility • Possibility of using cellular processes for drug loading and surface modifications • Easy to genetically modify cells to express multiple functional components • Sometimes inherent barrier crossing properties • Sometimes inherent targeting capacity • Non-immunogenic issues	• Heterogeneity issues • Lack of controlled release mechanism • Low stability in the circulatory system • Low efficiency of exogenous drug loading • Non-specific effects of natural EV cargo
bEVs	• Good bioavailability and biocompatibility • Possibility of using cellular processes for drug loading and surface modifications • Easier than EVs to genetically modify cells and also possible to express and package multiple functional components • Sometimes inherent barrier crossing barrier • Sometimes inherent and specific targeting • High scalability possible as bacteria	• Heterogeneity issues • Immunogenicity • Lack of controlled release mechanism • Low stability in the circulatory system • Low efficiency to load exogenous drugs • Non-specific effects of natural bEV cargo

	can be cultivated in fermentors	

1 **Table 3.** Advantages and Disadvantages of Drug-Loading Methods with (Bacterial) Extracellular Vesicles (EVs).

	Method	Drug	Principle	Advantage	Disadvantage	Scalability[a]	Refs
Cell manipulation	Drug treatment	Chemical compounds	Incubation of cells with a drug to obtain drug containing (b)EVs	• Relatively simple • No need for extra instruments • More efficient for hydrophobic drugs	• Low loading efficiency • Potential cytotoxicity from drugs	+++	e.g. [74, 133, 135]
	Passive loading	RNAs	Transfection of cells with sRNAs resulting in passive loading of EVs	• Relatively simple • Medium loading efficiency • No need for extra instruments	• Potential contamination by transfection reagent residues	+	e.g. [136]
	Active loading	Proteins, and RNAs	Genetic modified cells lead to the active loading of EVs	• High loading efficiency • Relatively homogeneous loading	• Specialized skills required	++	e.g. [137, 138]
Cell and (b)EV manipulation	Sonication	Chemical compounds, proteins, and RNAs	Ultrasound energy applied to cells or (b)EVs	• Medium loading efficiency • Applicable for e.g. small RNAs	• Membrane deformation • Not efficient for hydrophobic drugs	++	e.g. [70, 139]

	Electroporation	Chemical compounds, and RNAs	Short high-voltage pulses applied to cells or (b)EVs	• Possibility of loading with large molecules possible	• Low loading efficiency • Disrupts membrane integrity • Aggregation of vesicles	++	e.g. [140, 141]
(b)EV manipulation	Incubation	Chemical compounds and proteins	Incubation of (b)EVs with drug leads to passive loading of the EVs	• Relatively simple • No need for specific equipment • More efficient for hydrophobic drugs	• Low loading efficiency	++++	e.g. [61, 69, 142]
	Extrusion	Chemical compounds, proteins, and RNAs	Extrusion of (b)EVs via membrane filters with specific pore sizes	• High loading efficiency • Rapid	• Membrane deformation • Heterogeneous contents • Special equipment needed	++++	e.g. [130, 143]
	Dialysis	Chemical compounds, proteins, and RNAs	Diffusion of EVs via semipermeable membranes	• Relatively simple • No need for specific equipment	• Low loading efficiency • EV aggregation possible • Time-consuming	++	e.g. [144]

| | Freeze-thaw cycles | Chemical compounds and proteins | Energy alternation changes membrane stability of the EVs | • Relatively simple
• Membrane fusion of EVs possible
• Applicable for small molecules
• No need for specific instrument | • Low-medium loading efficiency compared to extrusion and sonication
• EV aggregation possible | +++ | e.g. [145] |
| | Saponification | Chemical compounds and proteins | Permeabilization of the EV membrane | • Relatively simple
• High loading efficiency
• No need for specific instrument | • Disrupts membrane integrity
• Chemical contamination | + | e.g. [139] |

1   [a]Potential for scalability rankings shown in arbitrary units using a range of 0-4 units.

Figure legends

Figure 1. Architecture and Composition of Bacterial Extracellular Vesicles (bEVs) Produced by Gram-negative (outer membrane vesicles; OMVs) and Gram-positive (cytoplasmic membrane vesicles; CMVs) Bacteria.

Figure 2. Cellular Activation and Uptake of Bacterial Extracellular Vesicles (bEVs). bEVs can bind to certain receptors such as toll-like receptor 2 and activate receptor-induced intracellular signaling in recipient cells. BEVs can also be taken up by recipient cells through direct membrane fusion or by using various endocytic routes including macropinocytosis, phagocytosis, endocytosis. Following entry into host cells, bEVs may enter or fuse with early endosomes and subsequently disintegrate and release their content into the cytoplasm. Alternatively, the bEV-containing early endosome can form late-endosome maturation and fuse with lysosomes resulting in degradation of the bEV content. The released bEV content into the cytosolic space can induce, depending on their cargo, pro- or anti-inflammatory response of the cell.

Figure 3. Overview of Different Techniques to Isolate Bacterial Extracellular Vesicles (bEVs). **(A)** Differential ultracentrifugation (UF) is based on the difference in size of the bEVs compared to other components. The large-size debris is first removed at lower g forces. The soluble components are not affected by centrifugation, but other particles such as lipoproteins and protein aggregates may be co-pelleted with bEVs. **(B)** In ultrafiltration (UF), soluble proteins and particles smaller than the size cutoff (e.g., 10 kDa) are pushed through a filter. The bEVs are larger than the cutoff and are collected on top of the filter. **(C)** In the precipitation-based methods, the addition of precipitant induces clumping of bEVs, other particles and soluble proteins. The clumps will sediment and sedimentation can be accelerated by centrifugation. **(D)** In affinity isolation, bEVs are captured based on their immunophenotype or the presence of specific ligands on their surface (such as antibodies, aptamers and resin). The resin-based ExoBacteria™ kit are now commercially available and enables isolation of bEVs with a fast and simple workflow. However, the lack of specific bEV markers limits the development and popularization of this method. **(E)** Size exclusion chromatography (SEC) uses a porous matrix (dotted circles) that makes separation possible based on size. Soluble components and particles smaller than the size cutoff enter the porous matrix temporarily, whereas bEVs and particles

larger than the size cutoff do not , resulting in differences in elution time. **(F)** In density gradient centrifugation (DGC), separation is based on density and the different bEVs subpopulations will travel to their corresponding equilibrium density. [a]Performance shown in arbitrary units using a range from - to ++++.

Figure 4. The Principle of Combining Techniques for Bacterial Extracellular Vesicle (bEV) Isolation. The size and density range of components are obtained by combining size- and density-based separation of biological samples [24]. LPS, lipopolysaccharide; VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Figure 5. Overview of All Biomedical Applications of Bacterial Extracellular Vesicles (bEVs). **(1)** bEVs are usually excellent vaccines against their parent bacteria. They induce both humoral (i.e. antibody production) and cellular (i.e. T cell activation and cytokine release) immune responses in humans and animals. **(2)** bEVs are also evaluated as cancer immunotherapy agent to eradicate established tumor tissues. **(3)** bEVs can function as delivery vehicles for siRNA, chemotherapy drugs, and antibiotics to increase the efficiency of the anti-tumor **(a)** or anti-bacterial **(b)** treatment, respectively. **(4)** bEVs can also be used to inhibit the adhesion and infection of their parental pathogen to host cells by competitively binding with the target cells **(c)** and by inducing immune responses **(d)**. **(5)** The potential of bEVs as a diagnostic tool is also under investigation to detect or monitor bacterial infections.

Figure I. Currently Proposed Models for the Biogenesis of Outer Membrane Vesicles (OMVs). FA, fatty acid; Lpp, lipoprotein; LPS, lipopolysaccharide; PG, peptidoglycan; PQS, *Pseudomonas quinolone* signal.

1   **Text boxes**

Box 1. Currently Proposed Models for the Biogenesis of Outer Membrane Vesicles (OMVs)

(A) Disruption of peptidoglycan (PG)-lipoprotein (Lpp) crosslinks

PG endopeptidases and other enzymes that are involved in regulating PG breakdown and synthesis govern the ability of the envelope to form PG-Lpp crosslinks. When a defect occurs, the faster growth rate of the outer membrane (OM) than the underlying cell wall allows the OM to protrude and finally generate OMVs [3] (**Figure IA**).

(B) Accumulation of envelope components

A turgor pressure induced by the accumulation of misfolded proteins or envelope components (such as lipopolysaccharide (LPS) or PG fragments) promotes bulging of OMVs [146] (**Figure IB**).

(C) Enrichment of specific LPS in some areas

Some areas of the OM can become enriched in particular types of LPS, phospholipids, and/or specific LPS-associated molecules. These molecules have a propensity to bulge outwards owing to their atypical structures or charges [147] (**Figure IC**).

(D) Insertion of *Pseudomonas* quinolone signal (PQS)

Insertion of PQS into the outer leaflet of the OM can also induce membrane curvature and lead to OMV formation [148] (**Figure ID**).

(E) Downregulation of VacJ/Yrb ABC transporter

The VacJ/Yrb ABC transporter shuttles phospholipids from the OM back to the inner membrane. Downregulation of this transporter causes the accumulation of phospholipids in the outer leaflet of the OM promoting vesiculation [149] (**Figure IE**).

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3

4

Box 2. Safety Challenges of Bacterial Extracellular Vesicle (bEVs) in Biomedical Applications

Lipopolysaccharide (LPS)

• Genetic modify LPS synthesis related genes such as *msbA*, *msbB*, *lpxL1*, *lpxM* to reduce LPS production [23].

• Physical or chemical extraction of bEVs to selectively reduce the LPS content [90].

• Encapsulate OMVs with a pH-sensitive shell of CaP to avoid the induction of systemic inflammation prior to the delivery of the OMV to its site of interest [64].

Outer membrane proteins and lipoproteins

• Application of bacterial protoplast-derived nanovesicles as an alternative for drug delivery [143].

• Development of bacterium/bEV-mimicking vectors as adjuvants for cancer immunotherapy [62, 150].

1

2

Glossary

Adjuvant: An immunostimulatory vaccine additive that is associated with the antigen to enhance the immune response against the antigen.

Aptamer: Short oligonucleotides (with a length of 30-80 nucleobases) that bind to a specific target molecule.

Bubbling cell death: The formation of bubbles from the nucleus and release of this swelling bubbles to the cell surface that ultimately causes cell death.

Chemotherapy: Anti-cancer therapy using cytotoxic chemical substances. It has been applied to decrease the tumor burden, achieve prolonged disease control, and inhibit tumor recurrence.**Cystobactamids:** A novel natural class of antibiotics—myxobacteria-derived topoisomerase inhibitors—have broad-spectrum antibacterial activity.

Extracellular vesicles (EVs): A heterogeneous population of lipid bilayer-delimited versicles that are naturally released from cells; this includes e.g. exosomes and microvesicles.

Eukaryotic-prokaryotic vesicle (EPV): A hybrid eukaryotic-prokaryotic nanoplatform that is designed and constructed by fusing tumor cell-derived vesicles and bacterial extracellular vesicles.

Generalized modules for membrane antigens (GMMA): Gram-negative bacteria that are genetically engineered to enhance the production of OMVs through the disruption of the bacterial envelope integrity and to minimize their capacity to promote reactogenic responses once injected, e.g. through modification of the lipid A moiety of the LPS.

Immunogenic cell death: Any type of cell death eliciting an immune response. Both accidental cell death and regulated cell death can result in an immune response.

Nano-pathogenoids (NPNs): A miniaturized and simplified version of a pathogen produced *in vitro* that shows realistic micro-anatomy.

Nitrogen cavitation: This technique uses nitrogen to dissolve in the

cytoplasm of cells under high pressure. By then quickly releasing the pressure, the cell gets disrupted.

Pathogen-associated molecular patterns (PAMPs): Small molecular motifs conserved within a class of microbes, including single-stranded DNA (ssDNA), lipoproteins, polysaccharides, proteins, and small molecules, that can elicit cytokines and promote antigen presentation. These defined molecular entities are immune agonists that control the initial innate immune response and influence the downstream adaptive immune response against a target antigen.

Plug-and-play: A technology to isolate antigen and scaffold production through the modular assembly.

Tumor microenvironment (TME): The combined set of cellular and extracellular elements that comprise the native tumor niche. Tumor progression, metastasis, therapeutic responses, or drug resistance is profoundly influenced by the multidirectional interactions of different elements of the tumor microenvironment such as stromal cells (e.g., fibroblasts, endothelial cells, and mesenchymal stem cells), immune cells (e.g., infiltrating and resident macrophages, natural killer cells, dendritic cells, and T cells), and cancer cells.

Virulence factor: Bacteria-associated molecules that the bacteria need to manipulate and/or damage host cells, thereby increasing the probability of infection and disease.

Acknowledgments

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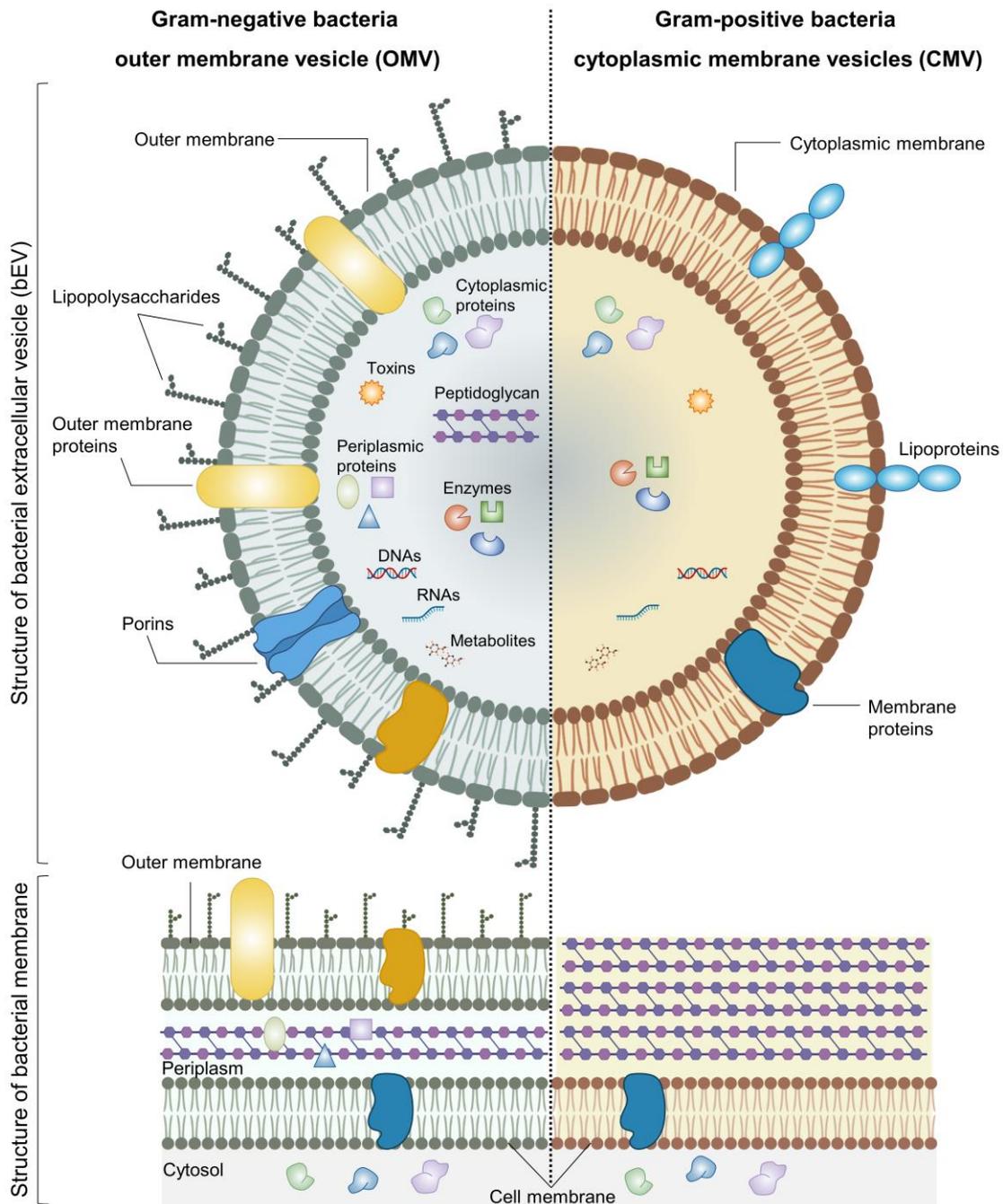
Figure 1. Architecture and composition of bacterial extracellular vesicles (bEVs) produced by Gram-negative (outer membrane vesicles; OMVs) and Gram-positive (cytoplasmic membrane vesicles; CMVs) bacteria.
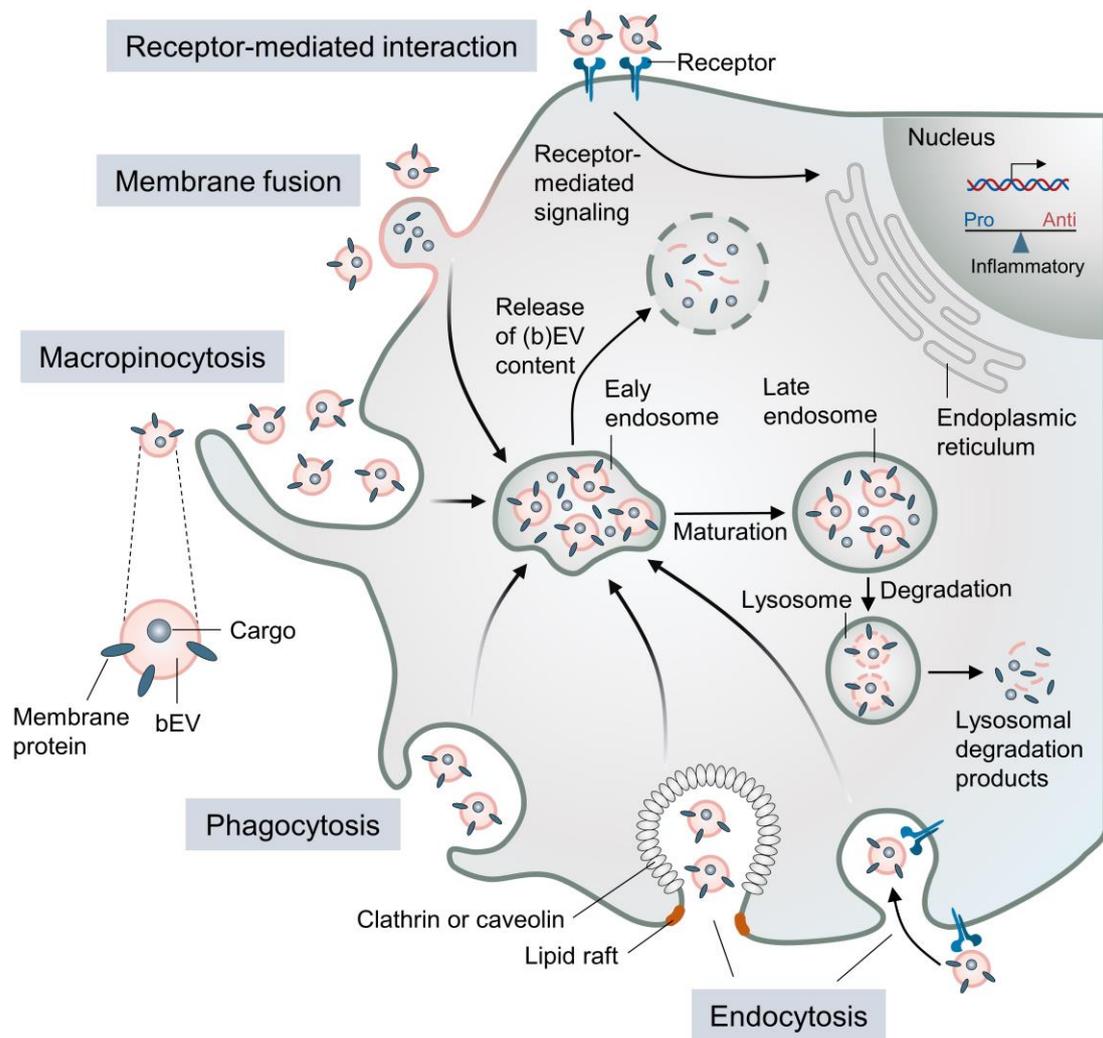
Figure 2. Cellular activation and uptake of bacterial extracellular vesicles (bEVs). bEVs can bind to certain receptors, such as Toll-like receptor 2, and activate receptor-induced intracellular signaling in recipient cells. bEVs can also be taken up by recipient cells through direct membrane fusion or by using various endocytic routes, including macropinocytosis, phagocytosis, and endocytosis. Following entry into host cells, bEVs may enter or fuse with early endosomes and subsequently disintegrate and release their content into the cytoplasm. Alternatively, the bEV-containing early endosome can form late-endosome maturations and fuse with lysosomes, resulting in degradation of the bEV content. The bEV content released into the cytosolic space can induce, depending on the cargo, pro- or anti-inflammatory responses of the cell.
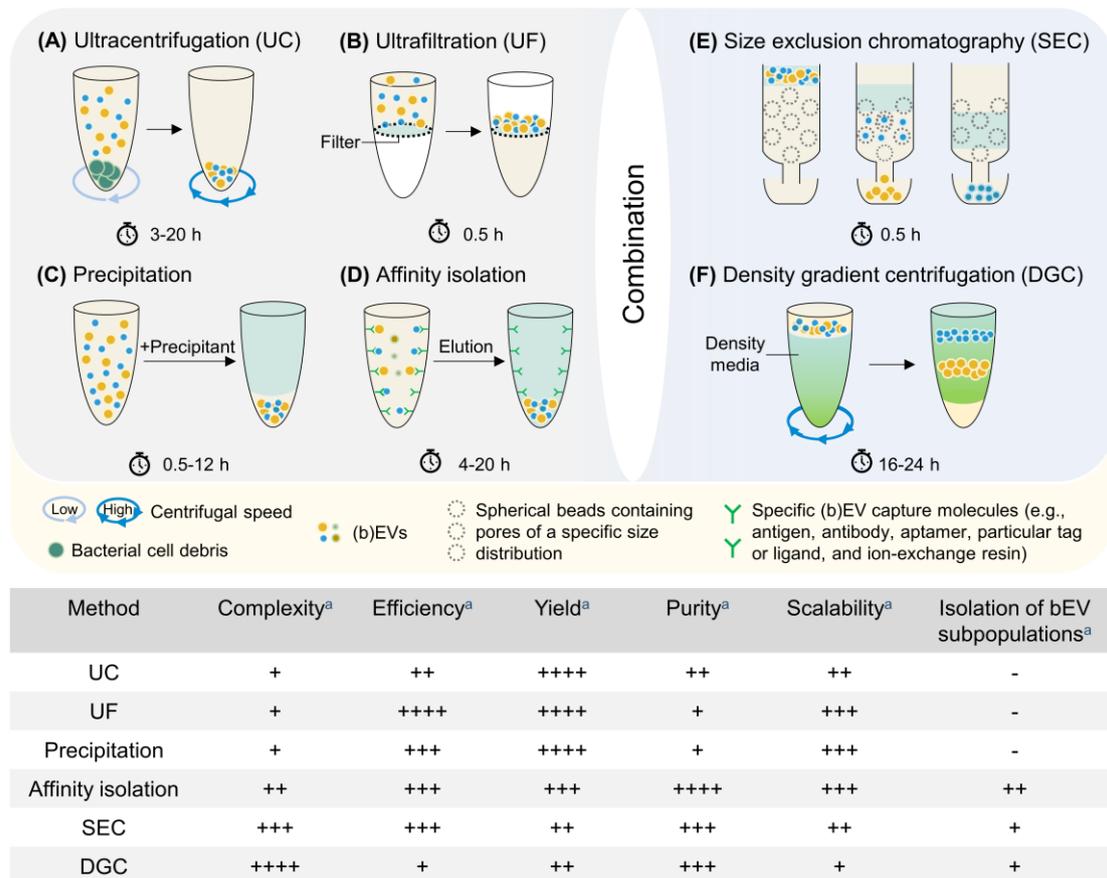
Method	Complexity[a]	Efficiency[a]	Yield[a]	Purity[a]	Scalability[a]	Isolation of bEV subpopulations[a]
UC	+	++	++++	++	++	-
UF	+	++++	++++	+	+++	-
Precipitation	+	+++	++++	+	+++	-
Affinity isolation	++	+++	+++	++++	+++	++
SEC	+++	+++	++	+++	++	+
DGC	++++	+	++	+++	+	+

Figure 3. Overview of different techniques to isolate bacterial extracellular vesicles (bEVs). (A) Differential ultracentrifugation (UF) is based on the difference in size of the bEVs compared with other components. Large-sized debris is removed first at lower g forces. The soluble components are not affected by centrifugation, but other particles, such as lipoproteins and protein aggregates, may be co-pelleted with bEVs. (B) In ultrafiltration (UF), soluble proteins and particles smaller than the size cutoff (e.g., 10 kDa) are pushed through a filter. The bEVs are larger than the cutoff and collect on top of the filter. (C) In precipitation-based methods, the addition of a precipitant induces clumping of bEVs, other particles, and soluble proteins. The clumps will sediment and sedimentation can be accelerated by centrifugation. (D) In affinity isolation, bEVs are captured based on their immunophenotype or the presence of specific ligands on their surface (such as antibodies, aptamers, and resin). The resin-based ExoBacteria™ kit is now commercially available and enables isolation of bEVs with a fast and simple workflow. However, the lack of specific bEV markers limits the development and popularization of this method. (E) Size exclusion chromatography (SEC) uses a porous matrix (dotted circles) that makes separation possible based on size. Soluble components and particles smaller than the size cutoff enter the porous matrix temporarily, whereas bEVs and particles larger than the size cutoff do not, resulting in differences in elution time. (F) In density gradient centrifugation (DGC), separation is based on density and the different bEV subpopulations travel to their corresponding equilibrium density. aPerformance is shown in arbitrary units using a range from – to ++++.
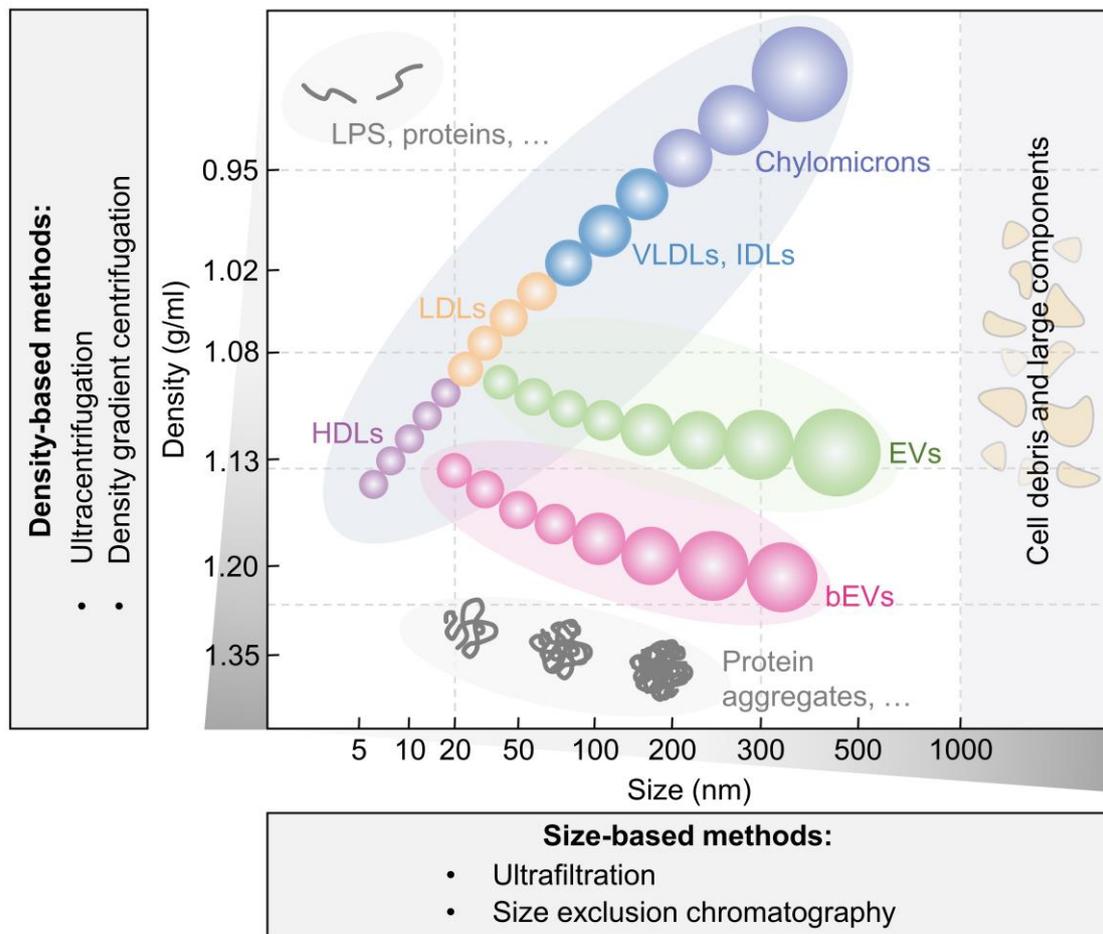
Figure 4. The principle of combining techniques for bacterial extracellular vesicle (bEV) isolation. The size and density ranges of components are obtained by combining size- and density-based separation of biological samples [24]. Abbreviations: HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LPS, lipopolysaccharide; VLDL, very low-density lipoprotein.
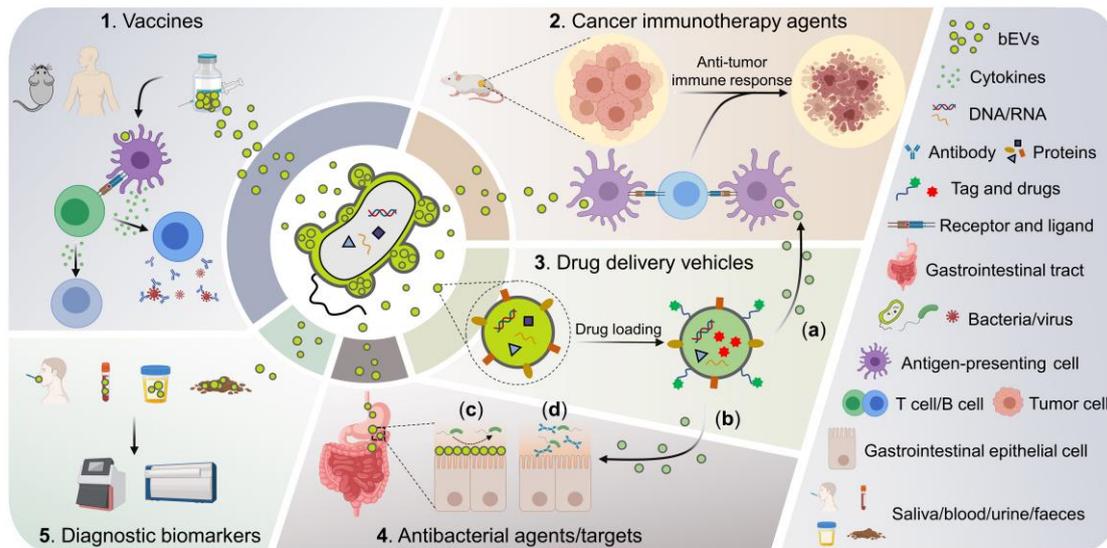
Figure 5. Key figure. Overview of all biomedical applications of bacterial extracellular vesicles (bEVs). (1) bEVs are usually excellent vaccines against their parent bacteria. They induce both humoral (i.e., antibody production) and cellular (i.e., T cell activation and cytokine release) immune responses in humans and animals. (2) bEVs are also being evaluated as cancer immunotherapy agents to eradicate established tumor tissues. (3) bEVs can function as delivery vehicles for small interfering (si)RNA, chemotherapy drugs, and antibiotics to increase the efficiency of the antitumor (a) or antibacterial (b) treatment, respectively. (4) bEVs can also be used to inhibit the adhesion and infection of their parental pathogen to host cells by competitively binding with the target cells (c) and by inducing immune responses (d). (5) The potential of bEVs as a diagnostic tool is also under investigation to detect or monitor bacterial infections.
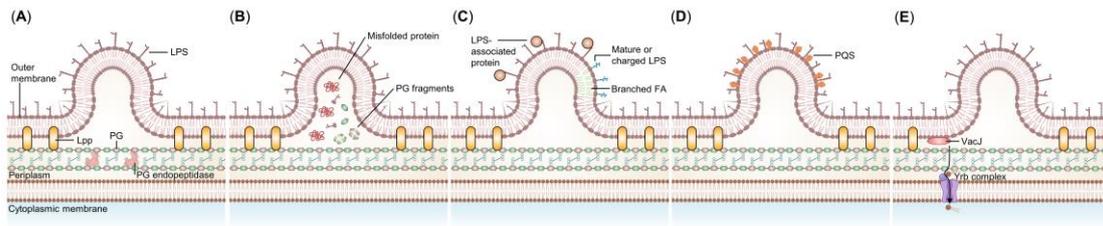
Figure I. Currently proposed models for the biogenesis of outer membrane vesicles (OMVs). Abbreviations: FA, fatty acid; Lpp, lipoprotein; LPS, lipopolysaccharide; PG, peptidoglycan; PQS, *Pseudomonas quinolone* signal.