ISOLATION OF BACTERIAL EXTRACELLULAR VESICLES: WHAT'S DIFFERENT AND WHAT'S THE SAME?

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The isolation and analysis of bacterial extracellular vesicles (bEVs) requires an understanding of bacterial biology, and an appreciation for how bEVs compare and contrast with their well-studied eukaryotic EV counterparts. bEV research is still a very nascent field with many complexities, arising from the fact that EVs may be produced from hundreds of different species, both of which (species composition and EVs) can change rapidly. Despite this, the study of bEVs is a worthwhile endeavour, as bEVs represent an important biotechnological platform that shows promise in many medical applications.

ESSENTIALS OF BACTERIAL BIOLOGY FOR EXTRACELLULAR VESICLE ISOLATION

Bacteria and archaea are part of the prokaryote domain. The cell structure of bacterial cells differs substantially from that of eukaryotic cells (which are found in animals). Although prokaryotes are unicellular organisms which do not have a nucleus, organelles, or cytoskeleton, they contain the genetic material (genomic DNA and RNA) and machinery required for biomolecule synthesis, metabolism, and other cellular activities. Prokaryotic cells are smaller than their eukaryotic counterparts, with sizes ranging from approximately 0.5 to 2 µm. Bacterial growth is fast; whereas mammalian cells can have duplication times of 18-72 hours, prokaryotes can typically duplicate in 20 minutes, enabling full-grown cultures (i.e. saturated with cells) in 8-10 hours. This rapid growth is suited to changing environmental cues, allowing bacteria to respond quickly via complex gene expression regulatory networks.

Although bacteria have a common cellular backbone, there are significant structural differences in the cell wall which enable bacteria to be broadly categorised into groups, such as Gram positive, Gram negative or Acid-fast bacteria. Importantly, these cell wall differences can significantly impact the production of cell membrane-derived nanostructures, such as EVs.

BACTERIAL EXTRACELLULAR VESICLES: TERMINOLOGY, COMPOSITION, AND BIOGENESIS

As bEVs were first identified in Gram Negative bacteria, they were historically named Outer Membrane Vesicles (OMVs) due to their presumed cellular origin. Conversely, more recently discovered Gram Positive bEVs were called Membrane Vesicles, using the same concept of cellular origin. However, it is now known that bEVs are not only released by prokaryotes, but also all domains of life, including fungi, protozoa, and plant cells. Thus, in this document, we use the terminology 'bEVs' to refer indistinguishably to all EVs derived from bacteria, falling under the umbrella EV definition established by MISEV guidelines[1]. bEVs are lipidic nanostructures (~25-300 nm) derived from parental bacterial cells and may contain a diverse molecular composition, such as nucleic acids (DNA, RNA), proteins, lipids, organic molecules, etc [2]. More recently, it has been established that bEVs can be produced by different biogenesis mechanisms, where some EVs may be derived from blebbing cells, while others are derived from the process of cell lysis. Each of these groups may contain cargo from different cellular origins: membrane-enriched cargo (in the membrane-blebbing type) and membrane/cytosolic-enriched cargo (in the cell-lysis type)[3]. In this way, the predominant mechanism of bEV biogenesis is modulated by the cell's response to the environment, thereby impacting bEV composition.

THE IMPORTANCE OF PRE-bEV ISOLATION VARIABLES

As mentioned earlier, the fast pace of bacterial growth can create significant challenges for bEV investigations. The quick cellular response to environmental cues also triggers a rapid turnover of cargo adjusted-bEVs capable of coping with new conditions or stimuli, resulting in a heterogeneous bEV population heavily influenced by experimental-dependent factors. Culture conditions for bacterial growth can vary significantly by:

- culture media (e.g. rich, minimal, physiological)
- predominant bacterial growth mode (cells grown as biofilms or suspensions)
- growth stage (e.g. log, stationary)
- temperature (range of 4 to 37°C)
- oxygen levels (e.g. aerobic, microaerobic, anaerobic) or
- the presence or absence of a specific stimulus.

In this way, subtle changes in these variables, sometime not perceived by the user, can be sensed by the cells resulting in bEVs with different compositions. The importance of culture variables in bEV composition has started to be addressed, with studies showing how different culture conditions can impact the bEV composition, so far studied in detail at the protein [4] and RNA level [5,6]. Broad quantification of bEV cargo has also shown composition variability due to culture conditions (**Figure 1A**). Moreover, culture variables can affect the size of bEVs, with the consecutive impact found in studies downstream. This was shown by bEVs collected at different bacterial growth stages, which presented different bEV size heterogeneity [7] (**Figure 1B**) and were preferentially taken up by different host cell entry mechanisms [8]. Therefore, when isolating bEVs, it is therefore strongly recommended to keep a thorough and comprehensive record of culture variables as a way to decrease inter-batch variability and improve the reproducibility of resulting bEV preparations.

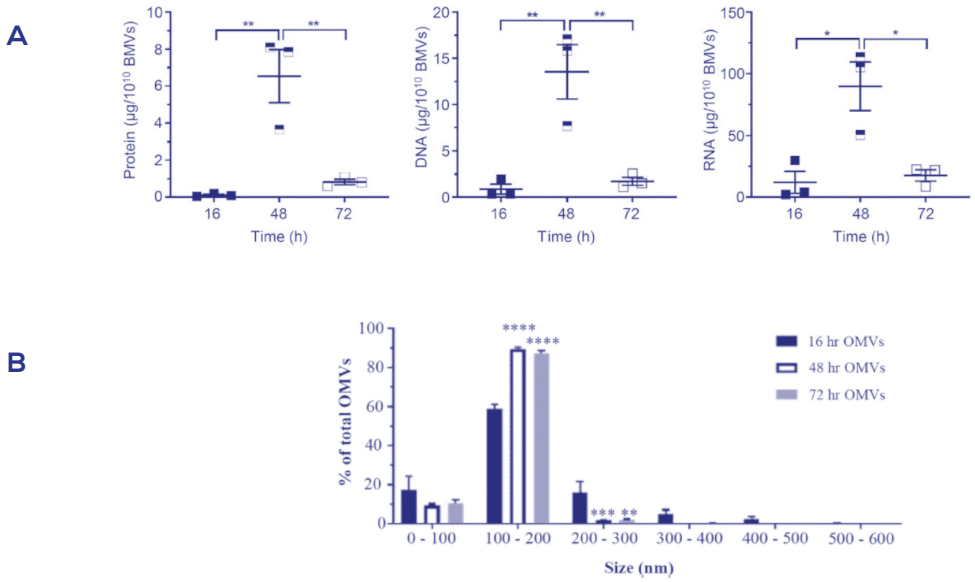

Figure 1. *Variability in bEV composition due to culture conditions. Differences in the amount of protein, DNA and RNA (A)[28] and particle size distribution (B)[7] in bEVs harvested from Helicobacter pylori grown at 16 h (early-exponential growth phase), 48 h (late-exponential growth phase) and 72 h (stationary growth phase).*

HOW TO OBTAIN CELL-FREE bEV SAMPLES

Bacterial culture conditioned media (CCM) derived from cells growing in suspension (as opposed to biofilm grown cells) will have an abundant number of cells. Common methods to remove cells from CCM includes centrifugation, filtration, or both. Although bacterial cells can have different shapes, cell sizes in a bacterial monoculture will be known and homogeneous. Most centrifugation protocols used to pellet down bacterial cells include centrifugation speeds ranging from 4,000 to 10,000 x g for 10 to 20 minutes. However, depending on the production of extracellular components by the cells, the consistency of the pellet can be tighter or looser, making the transfer of a complete cell-free supernatant more difficult or less effective. Filtration methods are based on passing CCM through a microfiltration device capable of processing different sample volumes, or by using different forces to push the sample (e.g. syringe, vacuum or pump filters). Filtration pore sizes normally range from 0.2 to 0.4 µm; these are effective at retaining most bacterial cell sizes, regardless of their shape or the angle at which they pass through the filter (e.g. longitudinal passing of rod-shaped bacteria) – and they allow the majority of bEVs to pass to the filtrate. The biggest limitation of filtration when removing bacterial cells is the clogging of the filter membrane, due to the abundance of bacterial cells and secreted components in the CCM. Therefore, a combination of centrifugation and filtration has shown to improve the overall efficiency of cell depletion: centrifugation removes the majority of cells and produces a clarified supernatant which can be filtered to remove the remaining cells with minimised clogging of the filter membrane.

METHODS FOR bEV ISOLATION

Cell culture-based model systems are the backbone of research, they work to easily simulate cellular and biological processes across many different organisms. Naturally, bEVs derived from cell culture are the most studied sample, with bEVs derived from biofluids being a very nascent field.

As is the case for other similar nanostructures in suspension, CCM cell-free bEV samples can be processed with separation methods based on a single principle, or a combination of several. Bacterial cell culture is usually performed in large volumes of medium (> 200 mL); therefore, it is often necessary to concentrate the bEV-containing supernatant before an appropriate bEV separation method can be applied. The concentration of bEV sample is usually achieved by ultrafiltration techniques, with ultrafiltration devices of different processing capacities, fluid pressure forces or filter pore sizes. It is recommended to use an ultrafiltration filter pore size that allows the bulk part of contaminating proteins to be eliminated, while retaining large nanostructures/bEVs. Filters with a molecular weight cut-off (MWCO) of 100 kDa are commonly used [9–11], however higher MWCO of 300 kDa also work efficiently towards this objective.

As is the case for mammalian EVs, the most common bEV isolation technique is one based on ultracentrifugation (UC). Nevertheless, there is no consensus in the literature on the ultracentrifugation speed used to pellet bEVs – or to what extent bEV subpopulations are pelleted; published methods include ranges as wide as from 40,000 g to 400,000 x g for bEV isolation [6,12,13]. More studies are required to obtain a better understanding of the effects of UC on bEVs, in the same way that some of UC's detrimental effects on mammalian EVs' functionality have been shown [14].

Density Gradient Centrifugation (DGC) is a laborious and lengthy but highly regarded EV purification method that separates EVs from non-EV components by size and buoyant density. DGC has shown efficient separation of mammalian EVs from known and well-studied non-EV structures, like very low-density and low-density lipoproteins, abundant in plasma samples [1,15,16]. However, such method-dependent separation efficiency cannot be translated to the bEV field, as bEV purity (the extent of bEV separation from contamination) cannot be estimated due to the lack of defined universal non-EV contaminants for all bEV preparations. For now, bEV studies of non-EV contaminants from specific bacterial strains and growth conditions may provide insights on the efficiency of bEV isolation methods. For example, proteomic analysis of bEV preparations made with two isolation methods revealed candidate proteins that can be used as contamination markers as they are not truly bEV-associated and can be removed with a more stringent bEV isolation method. In this way, studies have shown that some proteins present in UC-pelleted bEVs (low purity method) are significantly decreased in abundance after DGC (high purity method) or, bEV purity markers which are enriched in bEVs after DGC and not with UC [4].

On the other hand, supporting the large variability seen in bEV composition, reports show that bEVs can be found in one, more, or any of the density layers of a gradient [17,18]. Moreover, a single change in culture conditions can result in a different DGC bEV distribution pattern, suggesting significant changes in bEV composition [17]. DGC layer(s) can be visualised to confirm EV enrichment using a preferred high-resolution microscopic technique, like transmission electron microscopy (TEM), cryoEM or by immunoblotting known markers [19]. However, some studies have shown that bEV-enriched DGC layers can be quickly identified due to their high levels of total protein and particle counts [17].

SEC: A PROMISING AND EFFICIENT bEV ISOLATION TOOL

Size exclusion chromatography (SEC) has become positioned as a popular EV isolation method within the mammalian EV research community [20,21]. Advantages of SEC include simple and quick protocols, clean isolation and protection of EV physicochemical properties, the potential for standardisation, high reproducibility, and high specificity for EV separation. Despite popularity in eukaryotic EVs, SEC-based isolation of bEVs has only expanded in very recent years [22,23]. In particular, SEC-based qEV columns have become established as a reliable and convenient bEV isolation tool [4,10,17,24,25], with the qEV range accommodating different volume processing requirements and particle isolation ranges. In **Figure 2**, qEV columns show the separation of bEVs from soluble protein or aggregates, in early and late elution volumes, respectively [25]. qEV columns have also been compared to DGC, resulting in greater reproducibility in triplicate bEV preparations made with qEV columns (**Figure 3**) [17]. In addition, both qEV columns and DGC have demonstrated comparable levels of bEV protein depletion or enrichment when used for bEV purification [4]. Meanwhile, concentration by ultrafiltration combined with qEV isolation has shown efficient large-scale separation and reproducible bEV isolation in different bacterial species and strains [25].

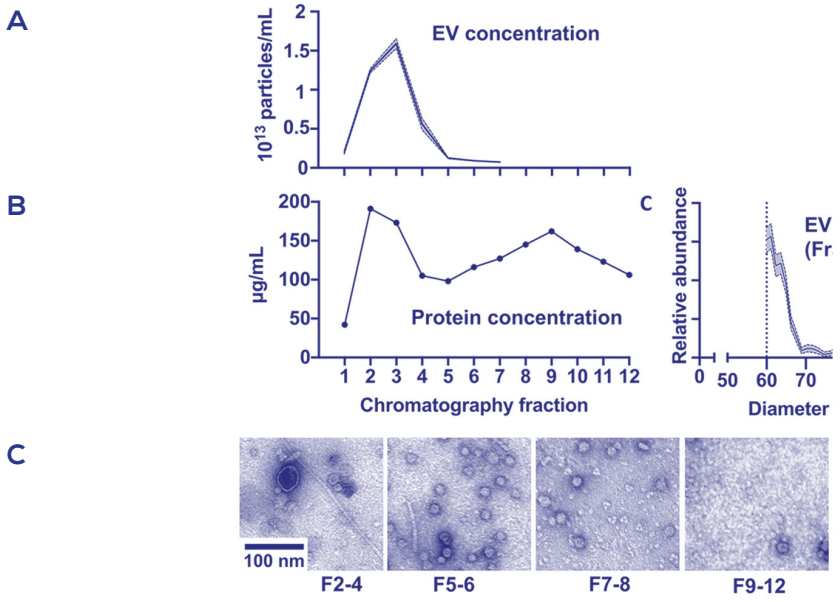

Figure 2. *Purification of bEVs by SEC qEV columns. Particle (**A**) and protein (**B**) concentration in collection volumes obtained from purifying Escherichia coli bEVs with qEV2 / 35 nm series. Transmission electron micrographs of collection volumes (**C**)* [25].

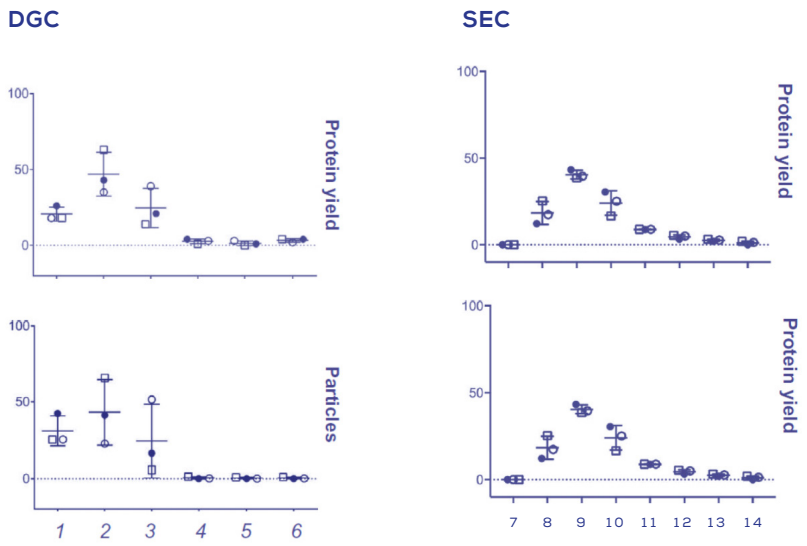

Figure 3. *Comparison of bEV isolation methods Density Gradient Centrifugation (DGC) and Size Exclusion Chromatography (SEC). Particle and protein distribution (as percentage of total recovered) of purified Uropathogenic Escherichia coli bEVs in density layers after DGC or collection volumes after SEC-qEV [17].*

POST-bEV ISOLATION CONSIDERATIONS

The most common buffers used to resuspend purified bEVs are Phosphate-Buffered Saline (PBS), HEPES, and low strength Tris-HCl (20 mM) [19]. The storage stability of mammalian EVs has been studied extensively, and results can be translated to bEV preparations; for instance, bEVs are also delimited by a phospholipid bilayer. While cell wall-derived components may be contained in some bEVs, these components do not appear to confer resistance to temperature changes. As is the case for eukaryotic EVs, bEV-specific studies have shown that both storage at -80°C and freeze-drying methods provide better bEV recovery than storage at 20 or 4°C (**Figure 4**) [26].

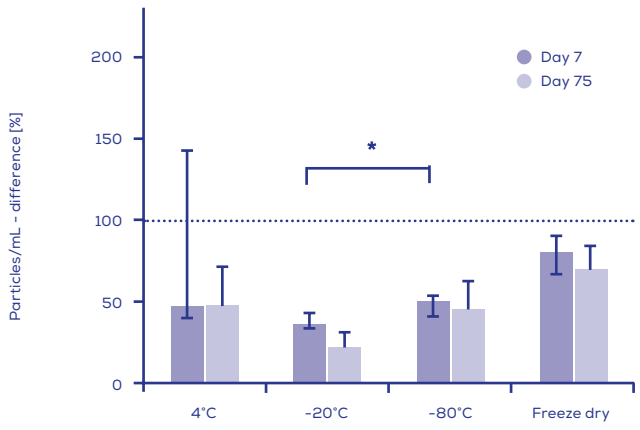

Figure 4. *Stability of bEV preparation with storage temperature and time. Particle concentration recovered after storing myxobacteria bEVs in PBS at 4°C, -20 ° C, -80 ° C or freeze dry during 7 or 75 days [26].*

It's worth mentioning that bEVs isolated from Gram Negative bacteria may naturally be attached to parental cell-derived endotoxin or lipopolysaccharide (LPS) molecules. However, LPS molecules can also be released in a soluble form to the extracellular space. Awareness of the presence of this highly immunogenic molecule in bEV preparations is critical to carrying out experiments with meaningful experimental controls, especially for functional studies. **Figure 5** highlights the concept, showing LPS found in bEV preparations from different culture conditions and strains of same species [10].

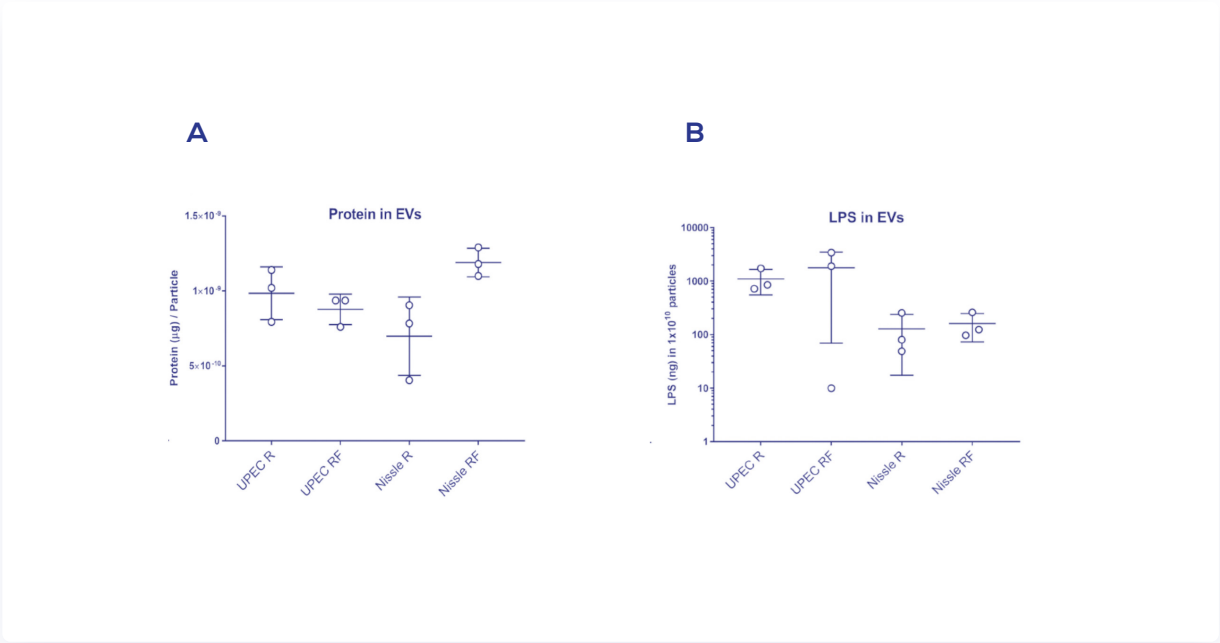

Figure 5. *Differences of molecular content in SEC qEV-purified bEVs isolated from E.coli Uropathogenic (UPEC) or probiotic (Nissle) strains grown with low iron (R) or high iron (RF). Protein content (A) and LPS content (B) in bEV preparations [10].*

The latent risk of incomplete cell removal or external environmental contamination during bEV isolation usually leads to sterility checks for bEV preparations. This is done by transferring an aliquot of bEV preparation to a culture plate and monitoring bacterial growth. It is recommended to discard the bEV preparation if bacterial contamination is confirmed. Contaminating bacterial cells release their own bEVs, which are nearly impossible to discriminate from bEVs of interest. Furthermore, contaminant-secreted LPS may linger in bEV preparations or consecutive downstream assays [10].

EV analysis is in general a controversial topic, as there are numerous EV analytical tools, targeting different EV signals/parameters and with significant variability in their sensitivity and specificity performance [27]. Total particle count and total protein amounts are usually considered exchangeable parameters applied to represent EV abundance and used to normalise EV treatments [1]. Nevertheless, recent bEV studies have pointed out the impact and differences of chosen protein-based over particle-based analytical assays in the resulting biological data and its interpretation. For example, 50 µg/mL of bEV protein quantified by different protein assays resulted in different biological outcomes (i.e. secretion of cytokine) when used to treat gastric adenocarcinoma cells (**Figure 6**) [28]. Thus, awareness of analytical options, variability and impact in downstream assays can enable better decision-making in experimental planning when working with bEVs.

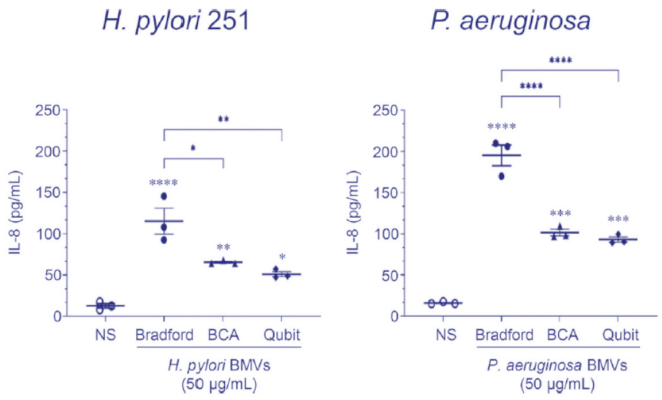

Figure 6. *Differences in downstream bEV functional assays depending on the chosen bEV protein quantification assay. Interleukin 8 secretion in AGS cells treated with 50 µg/mL of bEVs from Helicobacter pylori or Pseudomonas aeruginosa quantified with protein quantification assay Bradford, Lowry, BCA, or Qubit* [28].

bEV APPLICATIONS IN MEDICINE

bEVs offer many advantages to applications requiring mass production as they can be subject to easier genetic manipulation and their production is highly scalable. Furthermore, bEV production benefits from the existence of established biotechnological processes relevant to microbial cultures. The most promising applications of bEVs in human medicine can be found where their use is directed towards therapeutics, vaccines, or drug delivery vehicles.

Research has shown beneficial immunomodulatory properties of bEVs derived from bacterial probiotics. Work on an *Escherichia coli* Nissle strain, a known probiotic, has demonstrated that its bEVs can protect epithelial barrier disruption caused by Enteropathogenic *E. coli* [29]. In addition, Nissle bEVs can activate dendritic cells (DC) in a strain-specific manner, inducing DC differentiation into specific effector subsets of CD4+ T cells with a predominant role to fight against pathogens and resolve infection [30,31]. Thus, probiotic-derived bEVs can have significant potential as therapeutics delivered, for example, as functional food ingredients in diet supplements aimed to improve gut microbiome balance, or to help alleviate intestinal inflammatory conditions.

On the other hand, vaccines based on bEVs can be powerful candidates to generate innate and adaptive immune responses against a pathogen without the risk of the associated disease [32]. The vaccine-targeted pathogen can be native to the bEVs (parental cell) or a completely different one, like the process of incorporating heterologous antigens into bioengineered bEVs. In some cases, native bEVs present important immunogenic antigen(s) or have an overall antigen fingerprint similar to the parental cell, therefore their use in producing a protective antibody-based response is successful against infection with same bacterial species [32]. Among native bEV vaccines, the case of *Neisseria meningitidis* bEV vaccine stands out as one that has been applied in many countries [33]. Alternatively, there are several strategies to incorporate heterologous or foreign antigens in bEV vaccines with different efficiencies of antigen presentation, or preferential or type of immune responses induced (e.g., humoral or cellular). Options for antigen loading include luminal or surface location of the antigen in bEVs, endogenous (recombinant antigen expression in bacteria) or exogenous (addition after bEV purification) loading of antigen into bEV, passive or active procedures of loading of antigen into bEVs [34]. Also, multivalent vaccines can be generated using one or a combination of antigen loading strategies into a bEV backbone. As vaccines in general have been powerful public health tools to prevent and control infectious diseases, bEV vaccines present themselves as attractive candidates for a versatile and cost-effective platform.

Moreover, bEVs can also be great drug delivery vehicles. One main advantage is that some protein-recombinant expression systems have already been developed in bacteria, making it possible to introduce minor modifications to established protocols for the incorporation of the drug or molecule of interest. bEV drug-loading processes can occur during the bEV packaging or after bEV purification, just like the strategies mentioned for antigen incorporation for bEV-based vaccines. Approaches that modify the surface of bEVs are particularly of interest since they allow the attachment of tags for functionalised bEV isolation, or targeting moieties to improve in vivo cell delivery [35]. Researchers have demonstrated successful tumour growth regression in mice when treated with nanocarriers *E. coli* bEVs. These bEVs displayed on their surface the recombinant tumour-targeting antibody HER2 (human epidermal growth factor receptor 2) and in their lumen the active anti-cancer therapeutic component incorporated by electroporation: small interfering RNA (siRNA) targeting kinesin spindle protein (KSP) [36]. Alternatively, bEVs can be used to deliver encapsulated antibiotics to infecting bacterial cells. This is based on the affinity of bEVs for the bacterial cell membrane, and the ability of bEVs to protect the antibiotic from harsh environmental conditions [32]. In these ways, manipulated bEVs show superb potential in drug delivery, serving as vehicles for targets aimed at specific cells, such as cancer cells or bacterial cells.

In summary, a significant amount of specialised bEV research is needed to advance these applications. At the same time, they will all benefit from progress in overcoming common challenges, such as large-scale bEV production and isolation, removal of potential toxic contaminants like LPS, and overcoming inter-batch bEV variation in molecular composition and bEV shelf-life stability.

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