Membrane Vesicles Derived from Gut Microbiota

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Multidrug-resistant (MDR) superbugs can breach the blood–brain barrier (BBB), leading to a continuous barrage of pro-inflammatory modulators and induction of severe infection-related pathologies, including meningitis and brain abscess. Both broad-spectrum or species-specific antibiotics (β-lactamase inhibitors, polymyxins, vancomycin, meropenem, plazomicin, and sarecycline) and biocompatible poly (lactic-co-glycolic acid) (PLGA) nanoparticles have been used to treat these infections. However, new therapeutic platforms with a broad impact that do not exert off-target deleterious effects are needed. Membrane vesicles or extracellular vesicles (EVs) are lipid bilayer-enclosed particles with therapeutic potential owing to their ability to circumvent BBB constraints. Bacteria-derived EVs (bEVs) from gut microbiota are efficient transporters that can penetrate the central nervous system. In fact, bEVs can be remodeled via surface modification and CRISPR/Cas editing and, thus, represent a novel platform for conferring protection against infections breaching the BBB.

blood–brain barrier extracellular vesicles gut microbiota

1. Introduction

The blood–brain barrier (BBB) plays a central role in the unique and complex microenvironment of the central nervous system (CNS) [1]. In particular, it restricts the entry of drugs and other exogenous molecules, including host immune cells [2] and infectious pathogens [3]. Nevertheless, opportunistic pathogens can occasionally breach the BBB and cause serious illnesses, including meningitis and brain abscess [4]. Although the occurrence of CNS infection is relatively rare, chronic malignancies can result in serious neurological disorders [5]. Drug-resistant pathogens, including *Acinetobacter baumannii*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Streptococcus* spp. can enter via the respiratory tract and mucosa and breach the BBB [6]. In the BBB, pathogens tightly regulate intrinsic virulence mechanisms via drug-resistance pumps [7] and biofilm formation [8]. *Acinetobacter* spp., *Klebsiella*, and *S. aureus* further modulate the expression of proinflammatory cytokines [9] and movement of immune cells, thereby destabilizing the endothelial lining and tight junctions of the BBB [10]. However, due to the complexity of the brain microenvironment and its associated endothelial tight junctions, transport of effective antimicrobials and therapies is challenging [11]. In fact, the physiological nature of the CNS environment prevents 90–95% of antimicrobials from progressing toward drug development [12]. Various nanoparticles (NPs), especially liposomal NPs [13][14], and their derivatives (e.g., polysaccharide and polyester NPs) [15][16] are considered effective and innovative drugs against pathogens that invade the BBB. However, NP-associated toxicity

[17] and dose-dependent mortality [18] seriously limit their application. It is, therefore, necessary to consider alternatives, particularly those that can mimic non-immunogenic biological entities [19].

Membrane vesicles or extracellular vesicles (EVs) play crucial roles in polymicrobial interkingdom communication [20]. Microbial evolution involves the continuous transfer of metabolites via nanosized vesicles that carry important biomolecules, virulence factors, and membrane receptors of the cells from which they originate [21] to proximal and distant cells via blood and lymphatic systems. These vesicles range in size from 20 to 400 nm. The release of EVs is a general phenomenon performed by many cell types, including those of eukaryotes, Gram-negative/-positive bacteria, and archaea [22], as a means of communicating with other cells. In particular, bEVs have been characterized as the delivery vehicles of host–microbe interactions, responsible for the delivery of signaling molecules, such as autoinducers, virulence factors [23][24], and antibiotic genes [25][26]. In contrast to pathogen–host interactions, mucosal- or gut microbiota-derived bEVs contribute to homeostasis, immune system regulation, bowel movements, and the gut–brain axis [27]. Based on their immunomodulatory properties, gut microbiota-derived bEVs are currently employed in therapies aimed at promoting both humoral and cell-mediated responses [28]. Among them, tuning probiotic-derived bEVs, for interactions between interstitial cells and the gut–brain axis, represents a novel strategy for promoting immune responses during infectious disease [29]. Furthermore, this strategy can benefit from the ease of fermentation culture techniques, potential application of probiotics, and mucoadhesive encapsulation [30][31]. Moreover, combining functional biomaterials with active bEVs has the potential to target autoimmune inflammatory dispositions and treat severe chronic infections [32]. More specifically, beneficial gut microbiota-derived bEVs are a promising tool to regulate the gut–brain axis by reducing inflammation and restoring immunity [33], creating a benchmark for the targeted delivery of drugs to the CNS. However, currently, most EV-based drugs are derived from eukaryotic systems, including those for cancer [34], gastric disorders, and polymicrobial infections, due to the various challenges related to bEVs [35]. Nevertheless, genetically modifying bEVs via surface remodeling [36] to target neurotransmitters and quorum sensing (QS) inhibitors, and through CRISPR/Cas system-based modifications [37], has the potential to provide novel noninvasive therapies against BBB infections [38].

2. Blood–Brain Barrier (BBB): A Roadblock to Invading Pathogens

The endothelial layer of the BBB selectively transports immune cells and other metabolites involved in maintaining the functional stability of the nervous system [39]. However, during the neonatal period, in some cases, the BBB can shield pathogens, resulting in a breach of the protective layer and subsequent serious disorders and infection [40]. Endogenous markers, such as pathogen-associated molecular patterns and small molecular motifs conserved within a class of microbes [41], are recognized by endothelial receptors of the BBB. This recognition results in an immunological burst at the target site [42] that can breach the endothelial lining. Moreover, the complicated structure of the CNS limits the access of several antimicrobial agents to the nervous system [43], however, facilitating the transport of lipophilic drugs with a molecular weight <400 Da [44] that form fewer than eight hydrogen bonds via lipid-mediated free diffusion [45], into the bloodstream via the transcellular route [46]. As efficient drugs, antiepileptics

(e.g., diazepam and phenytoin) [47][48], PLGA-coated nanoparticles, and laser-assisted therapies (e.g., focused ultrasound and interstitial thermal therapy) are commercially available [49][50]. However, these therapies do not guarantee the long-term potency of drugs because the microbial flora is constantly evolving, either through horizontal gene transfer or cell-to-cell communication, resulting in reduced susceptibility to certain drugs [51].

3. Multidrug-Resistant (MDR) Superbugs: A Prominent Case Involving the BBB

Infections caused by MDR superbugs have emerged as a major threat to global health in the post-antibiotic era, especially in the 21st century [52]. The Centre for Disease Control and World Health Organization have predicted that there will be ~2 million cases of MDR infections and 27,000 related deaths per year by 2050 in Asia, Africa, and North America [53]. Carbapenem and colistin are the most widely used last-resort antibiotics against bacterial infections [54]; however, by the late 2000s, drug resistance exhibited an unexpected increase in mortality associated with hospital-acquired infections by 40–60% [55]. Pan-drug resistant *A. baumannii* is routinely reported in patients with meningitis [56][57][58] and has acquired resistance to most antibiotic therapies, including colistin and tetracycline [59][60]. Although combined treatment with gentamicin and meropenem is efficient, the reduction rate of infection is <17–19% [61] given that the BBB limits the permeability of drugs and the continuous administration of drugs further increases the probability of resistance [62][63]. Moreover, frequently screened drug-resistant pathogens (*A. baumannii* [64] and *E. coli* [65]), few routinely screened pathogens (*N. meningitidis* and *Streptococcus* spp.) [66] [67], other neuroinvasive pathogens (*Haemophilus influenzae*) [68], and *Chlamydophila pneumoniae* [69] not only disrupt the tight junctions of the BBB but also induce leakage between tight junctions and vascular endothelial cells [70]. For example, Gram-positive *L. monocytogenes*, *Staphylococcus* spp., and *Streptococcus pneumoniae* elevate the levels of proinflammatory cytokines and disrupt the endothelial lining in the CNS [71], thus creating a path of invasion for opportunistic pathogens.

4. Bacteria-Derived EVs (bEVs): Nanoscale Vesicles

bEVs have been studied since the early 1960s when lipid-like structures released from *E. coli* were discovered as a means to transport secondary metabolites and intrinsic biomolecules to the communicating host [72]. After the discovery of bEV production from Gram-positive bacteria, such as *Bacillus subtilis*, *Mycobacterium tuberculosis*, *S. aureus*, and *Streptococcus* spp., bEV release is regarded as a general phenomenon carried out by bacteria that has an important role in cell-to-cell communication and disease progression during gastric cancers and tuberculosis [73][74]. Cell-to-cell communication by bEVs involves internalization via the endothelial layer, micropinocytosis, and endocytosis by utilizing invasion proteins at the host–pathogen interface [75]. Certain pathways, such as the stress induced network, cause bEVs to function as anti-phagocytosis bodies, evading phagocytosis and weakening the clearing mechanism via the host immune response [76]. *M. tuberculosis* is a classic example of pathogen evasion of the innate immune responses; that is, it infects phagocytic and inhibits phagosome maturation. Moreover, Athman et al. [77] discovered that *Mycobacterium* bEVs produce lipoglycans and lipoproteins that play an important role in regulating the host immune response and facilitating persistent infection.

Further, it was found that *S. aureus*-derived bEVs contain super-antigens (protein A and lipase) that aid cells in phagocytosis evasion. Meanwhile, a proteomics study [78] found that immunoglobulin (IgG)-bound lipase and super-antigen (hydrogenated form of squalene; SQA) are presented in bEVs, thus highlighting the potential role of *S. aureus* in evading anti-phagocytic activity via super-antigens and lipase production. bEVs also have a basic role in exchange of genetic materials (DNA and RNA) through horizontal gene transfer, during which bEVs serve as a means of cell-to-cell communication within the same bacterial species [79]. Additionally, a study conducted on bEV cargo of *A. baumannii* reported the presence of a carbapenamse gene (bla_{OXA-24}) that increases the antibiotic susceptibility pattern against β-lactam antibiotics [80]. Similar studies on bEVs derived from *N. gonorrhoeae* [81] and *S. aureus* [82] have identified the presence of the outer membrane (OM) protein PorB and alpha toxins that transfer genetic materials, inducing apoptosis and host cell death.

bEVs released from the cell envelope of Gram-negative bacteria are so-called outer membrane vesicles (OMVs). The envelope is made up of three layers: the OM, cytoplasmic membrane, and the periplasmic space in between, which contains a layer of peptidoglycan (PG) [83]. An inside leaflet of phospholipids and an outer leaflet of lipopolysaccharide (LPS; also known as endotoxin) constitute the OM. LPS causes inflammatory responses in host cells [84], whereas the OM has a porous structure that aids in waste removal and nutrition uptake, and the peptidoglycan (PG) layer maintains the osmotic pressure of the cell and regulates the hostile environment (antibiotic stress) [85]. Gram-positive, unlike Gram-negative, bEVs are produced from cytoplasmic constituents via a blebbing mechanism; their genetic composition is comparable to that of Gram-negative bEVs, with the exception of the lipoprotein structure [86]. Apart from the normal mechanism of blebbing, prophage-encoded endolysins have also facilitated bEV release from Gram-negative and -positive bacteria. Studies on *Bacillus* spp. and *Staphylococcus* spp. have revealed that the prophage-encoded endolysin generates holes in the peptidoglycan cell wall, thus highlighting the potential role of these enzymes in bacterial cell wall lysis during mass production of bEVs [87][88].

5. Nanoscale bEVs as Potential Therapeutic Platforms

Recently, bioinspired NPs such as host (eukaryotic) EVs (hEV) and bEVs have shown promising effects against chronic infections [89][90]. Compared with their nanomaterial counterparts (liposomal NPs), bEVs provide increased drug delivery and efficient antigen-presenting properties [91][92][93]. Various microbes including *Helicobacter* spp., *Klebsiella pneumoniae*, *Lactobacillus* spp., *P. aeruginosa*, *S. aureus*, and *Streptococcus* spp. are involved in the transfer of metabolites between species for intracellular communication and are used in novel adjuvant-associated therapeutics as well as nano-sized vaccine delivery platforms for various infections [94][95].

hEVs have shown complexity of the yield coefficient, a high production cost, and limited downstream process, all of which limit their biomedical applications [96][97]. The continuous evaluation of EVs as potential tools against chronic infection has led to the development of bEVs derived from *Clostridium butyricum* [98] and *L. paracasei* [99]. Given that most chronic illnesses involve ‘dysbiosis’ of the gut microbiota, tuning the absorption capacity and nutrition digestion factors of the microbiome might influence the host–microbe physiological imbalance.

6. Unresolved Issues with Gut Microbiota-Derived bEVs in Modulating the Gut–Brain Axis: *Old Is Gold*

The continuous usage of antibiotics during BBB infections leads to prognosis of early psychosis and neurotoxicity [100]. Gut microbiota dysbiosis, a state where the physiological combinations of flora are transformed into pathological combinations [101] via continuous antibiotic administration, has been linked to neural abnormalities. This link is via the vagal nerve, which is associated with a lower response of neurotransmitters inducing systemic inflammation in the CNS [102]. These features highlight the importance of the gut–brain axis in modulating CNS homeostasis.

6.1. Gut–Brain Axis

The term '*Gut–Brain axis*' refers to a bidirectional network that includes multiple connections such as the vagus nerve (nervous control), immune coordination (epithelial and mucosal barrier), and secondary metabolite generation from microbes [103]. The complex architecture of the gut–brain axis entails the constant transit of neurotransmitters within the gastrointestinal (GI) tract, which, in turn, modulates the immune system, including macrophages and mast cells [104]. These immune cells boost neuron excitability and regulate the host's behavioral response. A recent study found that gut dysbiosis caused by a broad-spectrum antibiotic during traumatic brain injury (TBI) resulted in increased neuronal loss, suppressed neurogenesis, altered microglia and peripheral immune response, and modulated fear memory response, suggesting a role of gut microbiota in the recovery from TBI [105].

6.2. Gut Microbiota-Derived bEVs vs. Eukaryotic-Derived hEVs (Physiological Counterpart)

Generally, the use of hEVs is significantly limited by the yield coefficient and high-throughput screening. In addition, the current scenario for combating antibiotic resistance with chronic illness is not favored by the use of pathogen-derived bEVs, because the sudden release of pro-inflammatory factors by bacteria cannot be controlled [106]. In contrast, beneficial gut microbiota have shown the effective immune responses and efficient pathogen inhibition activity [107]. Moreover, bEVs from beneficial gut microbiota take a role in triggering inflammatory responses through LPS and lipoteichoic acid [108] and can cross the intestinal barrier, and have effective anti-inflammatory properties against chronic infections and gut dysbiosis [109]. The physiological features of hEVs differ significantly from gut microbiota-derived bEVs.

6.3. Problems Related to Gut Microbiota-Derived bEVs on BBB-Associated Diseases

The 'dysbiosis' condition in the gut microbiota environment by antibiotic usage has also shown certain detrimental impacts such as Alzheimer's disease, autism, and arthritis, all of which clearly demonstrate the mechanistic behavior and coordinated axis of mental health and intestinal mucosa [110]. A study by Lee et al. [111] showed that the release of bEVs from a gut pathogen *Paenalcaligenes hominis*, revealed movement of bEVs via the vagus

nerve, producing cognitive impairment in the nervous system. Another study using *Porphyromonas gingivalis*, an oral pathogen, demonstrated the importance of LPS-coated bEVs in the onset of Alzheimer's disease, emphasizing the role of protease and LPS in triggering the damage of collagen fibers, fibrinogen connective tissues, and induction of proinflammatory mediators in the transfer of bEVs that alter brain cognitive function [112]. The main drawback of bEVs derived from the gut microbiota is that they have a negative impact on memory, cognition, and neuroinflammation. Therefore, direct application of such bEVs may have both adverse and beneficial neurologic effects on CNS homeostasis.

6.4. Beneficial Roles of Probiotic-Derived bEVs on Gut–Brain-Axis Control

Considering the diverse array of gut microbiota from intestinal niches, probiotics including *Bifidobacterium* spp. and *Lactobacillus* spp. have been identified to create neurotransmitters (acetylcholine, gamma-aminobutyric acid (GABA), and serotonin), which continually control CNS homeostasis [113][114]. Overall, probiotics not only govern the bidirectional transit of biochemical signals, but also improve the host's behavioral response such as anxiety [115], depression, and stroke [116]. Apart from periodontal and gut pathogens, probiotics such as *Lactobacillus* spp. have influence on the motor neuron complex (M-N complex). This M-N complex includes the enteric nervous system (endocrine functions and secretion from intestinal mucosa) and the vagus nerve. *Lactobacillus* spp. normally modulates the neurotransmitter signals via the vagus nerve (intestinal nerve), involving sensory transmission of neuronal signals via the enteric nervous system to the CNS [114].

7. Conclusions

The effectiveness of EVs against various infectious pathogens has been highlighted in recent literatures. However, most of them have largely focused on developing therapeutics or drug delivery vehicles by utilizing either NPs or hEVs (exosomes). Although these agents are clinically significant, their utilization is limited by long-term toxicity and the related mortality, low immunogenic response, stability issues, cost of scaling up, fermentation culture conditions, and downstream processing. In contrast to hEVs, only a few FDA-approved therapeutic bEVs are available. This is due to either failed trials or a low therapeutic efficiency. The concept of 'postbiotics' has recently been evaluated as a source of nonviable bacterial supplements capable of regulating the gut–brain axis. That means, the use of probiotics alone may be limited in scope; however, it can be enhanced by tuning the active components of postbiotics to initiate the release of probiotics-derived bEVs or -enriched bEVs. Meanwhile, limitations of combining NPs with antimicrobial compounds have hampered their application for the treatment of infections; moreover, this strategy does not address safety issues related to BBB breach. Collectively, the work summarized in this review provides insights into the efficacy of probiotic-derived bEVs and the novel concept of 'postbiotics' as a potential tool for the development of therapeutic platforms to overcome drug resistance in pathogens causing neurological disorders.

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