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REVIEW

Bacterial extracellular vesicles in osteoarthritis: a new bridge of the gut-joint axis

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ABSTRACT

Osteoarthritis (OA) is a degenerative joint disease primarily characterized by cartilage degeneration. Increasing evidence suggests that there is an interplay between the gut microbiota (GM) and joint health, known as the gut-joint axis. In recent years, with the advancement of bacterial extracellular vesicles (BEVs) research, its role in the gut-joint axis has attracted increasing attention. BEVs are phospholipid bilayer nanocarriers with sizes ranging from 20 to 400 nm, which can deliver various bioactive molecules to modulate the intestinal and joint microenvironments. Due to nanoscale structure, low toxicity, high drug loading capacity, good biocompatibility, easy modification, and industrialization, BEVs are promising for OA treatment. Here, we reviewed the research status of BEVs including biogenies, classification, structures, and composition, and summarized the cargo within BEVs that is associated with OA. The application of natural BEVs in the treatment of OA is the core, and the separation, purification, and modification strategies for engineered BEVs are summarized. Finally, we provide an overview and prospects of the role of BEVs in the clinical diagnosis and treatment of OA. We hope that a comprehensive understanding of BEVs will provide new solutions for OA treatment.

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Introduction

Osteoarthritis (OA), characterized by progressive cartilage degradation, subchondral bone sclerosis, synovial inflammation, and osteophyte formation, represents the most prevalent form of degenerative joint disease worldwide.[1,2] Cardinal signs include joint pain, stiffness and swelling lead to millions of people around the world suffer from OA and prevalence rate rise steeply with age in both sexes during their whole lifetime, which creates a huge socioeconomic burden globally.[3,4] Despite its high incidence, the elusive nature of OA pathogenesis has prompted extensive research efforts toward effective therapies. An ideal pharmacological intervention for OA must alleviate symptoms, reverse joint structural damage, and restore functionality.[5] Yet, the scarcity of knowledge regarding disease mechanisms hinders the development of disease-modifying OA drugs for clinical use.[6] Therefore, current first-line drugs for the treatment of OA include non-steroidal anti-inflammatory drugs (NSAIDs),[7] which are beset by issues of low bioavailability and potential long-term toxicity. This necessitates the exploration of innovative, efficacious, and safe therapeutic strategies for OA.

The National Institutes of Health's Human Microbiome Project, along with extensive cross-sectional research, has uncovered a spectrum of associations – both recognized and previously unknown – linking the gut microbiota (GM) to human health.[8,9] GM dysbiosis can cause immune dysregulation and inflammatory disorders such as inflammatory bowel disease, systemic inflammatory arthritis (rheumatoid arthritis and spondyloarthritis).[10] Our knowledge of the GM in joint diseases has increased particularly after the

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development of metagenomics analysis and high-throughput sequencing techniques.[11] Emerging studies highlight its significant role of GM in bone metabolism and the etiology of OA.[12] And there is an accumulation of evidence on the link between the GM and OA (Table 1). The concept of a "gut-joint axis" has been introduced to describe the interaction between the GM and joint health. Historically, it was believed that the metabolites produced by the GM could enter systemic circulation and contribute to OA development.[20–22] In previous research, the role of single-microbial-derived molecules in OA has been emphasized. However, the regulation of joint health by the gut microbiota is not mediated by a single substance, but rather through the combined action of multiple molecules. Recent studies indicate that extracellular vesicles (EVs) secreted by GM may exert a special influence on distant organs by transferring molecules into host cells and modulating host signaling pathways.[23]

Bacterial extracellular vesicles (BEVs) are nano-sized, lipid-membrane-enclosed particles secreted by both *Gram-negative* (G^-) and select *Gram-positive* (G^+) bacteria. They play a pivotal role in bacteria-host communication, facilitating the transfer and modulation of various bacterial components, including nucleic acids (DNA, miRNA, and siRNA), proteins, lipids, and metabolites.[24] BEVs' functional diversity, non-replicability, low toxicity, and ability to traverse biological barriers confer upon them significant potential for biomedical applications.[25,26] Technological advancements have enabled the functionalization of BEV surfaces with biomolecules, allowing for the targeted delivery of therapeutic agents, thanks to their stable phospholipid bilayers.[27,28] BEVs offer distinct advantages over mammalian cell-derived extracellular vesicles (MEVs), such as higher extraction rates and cost-effectiveness, due to bacteria's rapid growth and the sophistication of high-density culture methods like fed-batch fermentation, which enhances their

Table 1. Published studies relating to microbiota and osteoarthritis.

Year	Study population/ experimental animals	Design	Sample size	OA type	Findings	Conclusion	Reference
2024	Mice	Animal study	34	DMM model	Sexually dimorphic severity of OA in mice was observed, with opposite-sex microbiome transplantation showing potential to reverse this disparity	Differential composition of the GM can influence the progression of OA.	13
2023	The DIGItal COhort Design (DIGICOD)	Cross-sectional study	416	symptomatic hand OA	Tryptophan metabolites disturbance is associated with erosive HOA and pain	Microbiome is a potential therapeutic target for OA related knee joint pain	14
2023	Hospital based study and mice	Cross-sectional study and mice	78/36[a]	Knee OA and DMM model	The gut microbiota of osteoarthritis mice can produce metabolites related to the development of osteoarthritis	Microbial metabolites are an important component of the gut-joint axis	15
2023	Xiangya OA Study	Cross-sectional study	1501	Symptomatic hand OA (SHOA)	Participants with SHOA had significantly altered microbial functions related to tryptophan metabolism and tryptophan metabolites	The role of the microbiome and tryptophan metabolites function were observed in OA	16
2022	Johnston County Osteoarthritis Study	Cross-sectional study	92	Obesity-Related OA	Untargeted metabolomics analysis indicated that OA cases had significant perturbations in microbial metabolites including propionic acid, indoles, and other tryptophan metabolites.	Disorders in gut microbiota composition and gut metabolism are associated with obesity related osteoarthritis	17
2021	Xiangya OA Study	Cross-sectional study	1388	SHOA	Patients with SHOA exhibited higher relative abundances of Bilophila and Desulfovibrio genera, and a lower relative abundance of Roseburia genus	Alterations in the composition of the GM were observed in symptomatic hand OA.	18
2020	Hospital based study and mice	Case-control study and animal study	20/42[b]	MLI model	Transplantation of fecal microbiota from human donors with impaired metabolism accelerated OA in mice	Direct GM-OA connection exists	19

DMM, destabilized medial meniscus; MLI, meniscal ligamentous injury.
[a]The numbers of participants and mouse were 78 and 36, respectively;
[b]The numbers of participants and mouse were 20 and 42, respectively.

scalability for production.[23,29–31] Consequently, engineered BEVs are emerging as a promising platform for targeted therapies.[32] Both naturally occurring and engineered BEVs could significantly influence the gut-joint axis, potentially modulating the onset and progression of OA (Figure 1).

In this review, we delve into recent advancements concerning GM-derived BEVs, comparing the characteristics of BEVs from G^- and G^+ bacteria within the GM. Subsequently, based on the literature, we explored the cargo within BEVs that may impact osteoarthritis and categorized them into three major classes: Membrane-associated cargo, Cytoplasmic cargo and Genetic cargo. Following this, we explore the potential mechanisms of naturally occurring BEVs within the gut-joint axis, encompassing their roles in gut permeability and Immune-inflammatory response. The review culminates with an in-depth examination of the therapeutic prospects of engineered BEVs for OA management. We posit that BEVs represent a novel conduit within the gut-joint axis, holding promise as a source of groundbreaking technologies for OA treatment.

Overview of BEVs secreted by GM

The GM, composed of bacteria (with the four predominant phyla being *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*), viruses, fungi,

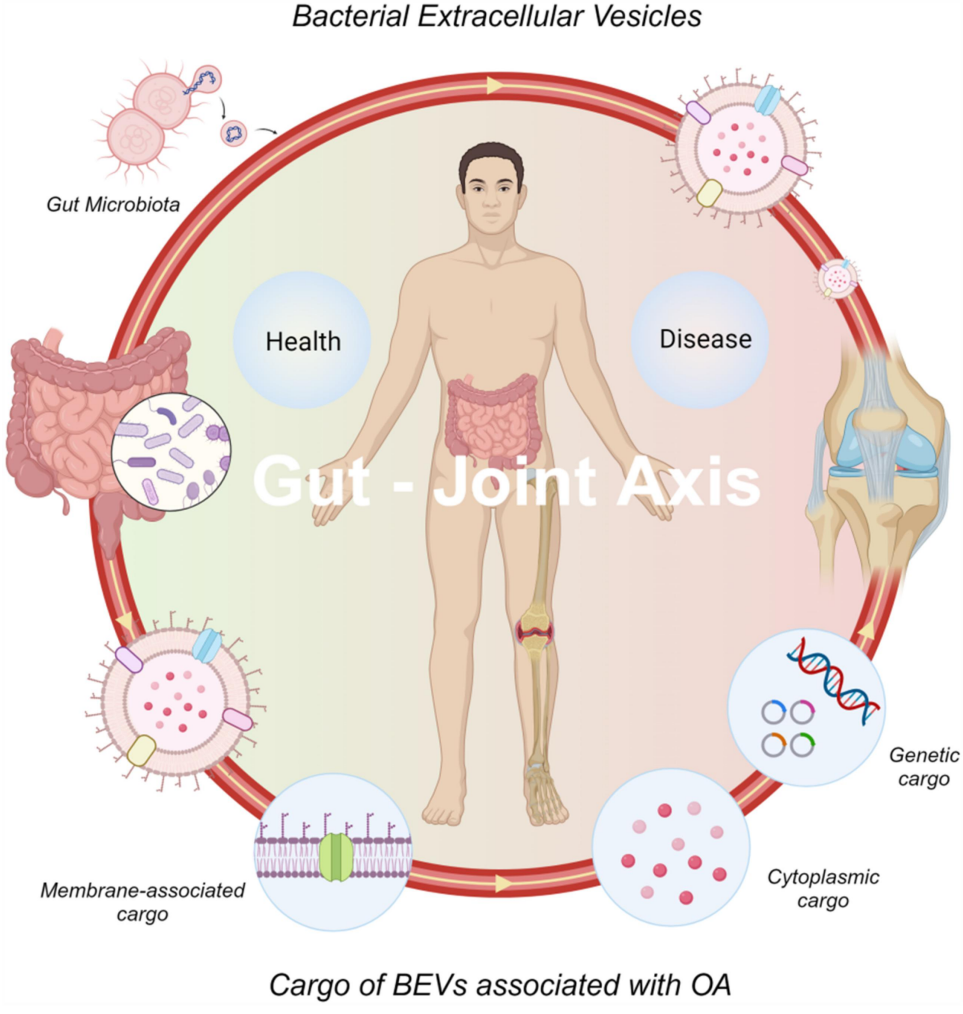

Figure 1. The GM and joint health are interconnected, known as the "gut-joint axis". Under the gut-joint axis model, extracellular vesicles secreted by the GM may play a significant role in this axis. BEVs are not only small membrane-enclosed structures rich in bacterial-derived molecules but also natural nanoparticles. BEVs carry various oa-related cargoes, including membrane-associated cargo, cytoplasmic cargo, and genetic cargo. Natural BEVs and tailored engineered BEVs hold significant importance and promise in the treatment of OA. In summary, BEVs represent a new bridge in the gut-joint axis. The figure was created with https://app.bioreunder.com/.

and archaea, plays a pivotal role in the regulation of human health and disease.[33] Human GM constitutes a sophisticated ecosystem, subject to the continual introduction of microbes from environmental sources. Variability in GM composition among individuals is influenced by a multitude of factors, including host genetics, diet, environmental exposures, and the prevailing state of the microbiome.[34–36] Bacteria within the GM are categorized as G^+ or G^- based on their cellular morphology, structural characteristics, and responses to staining procedures. It is a well-documented phenomenon that bacteria, akin to mammalian cells, naturally produce EVs. Initially, BEVs were perceived as mere byproducts of cell decay. However, the revelation that EVs are produced by living bacteria has underscored the ubiquity of vesiculation as an intrinsic biological process. Nonetheless, discrepancies exist in the formation mechanisms and the cargo of BEVs between G^+ and G^- bacteria, attributable to their distinct cellular architectures and physiological functions (Figure 2).[23]

Gram-negative bacterial extracellular vesicles

The G^- bacterial envelope is structured in three layers: the outer membrane (OM), the periplasmic space, and the cytoplasmic membrane.[37] G^- BEVs were first observed in an auxotrophic *Escherichia coli* strain in the 1960s,[38] and in 1995, it was reported that *Pseudomonas aeruginosa* releases

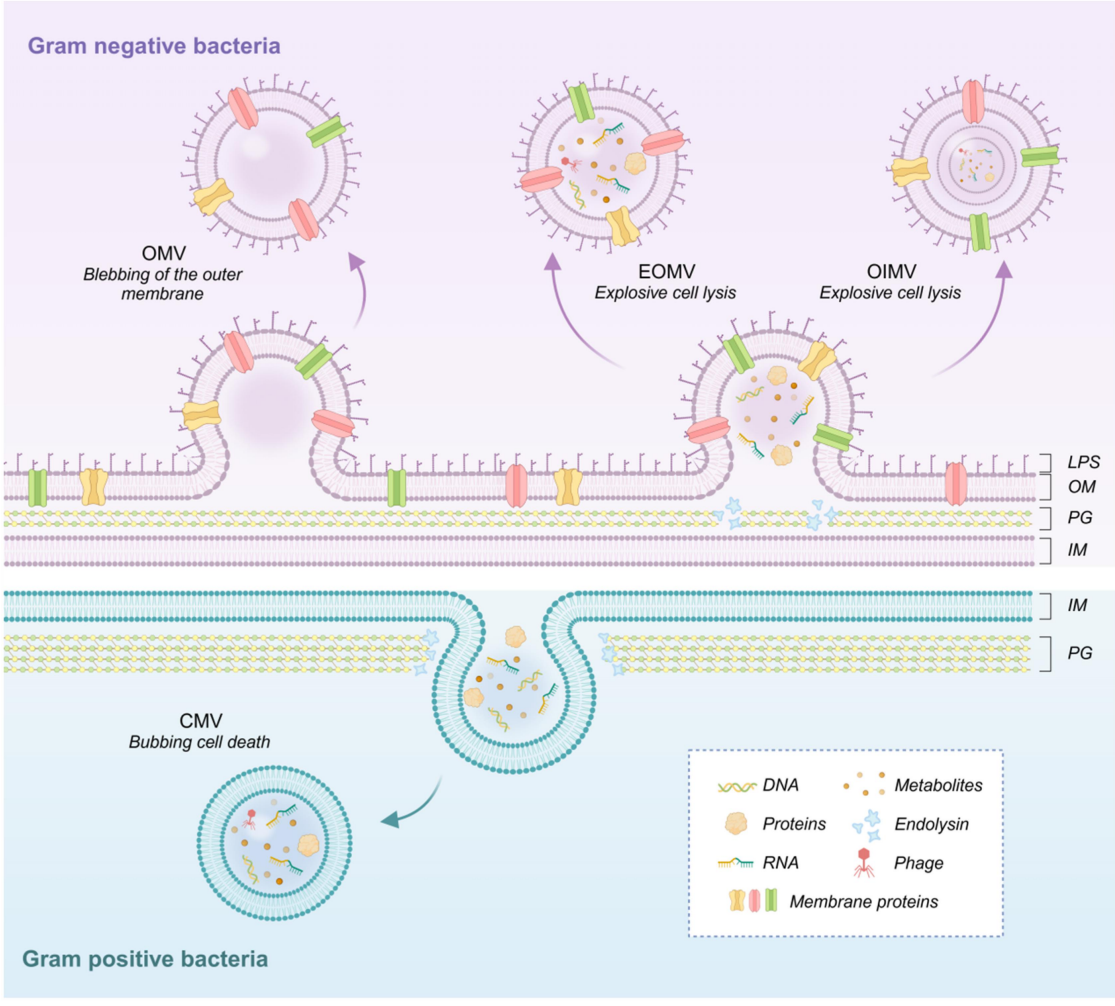

Figure 2. The biogenesis, classification and composition of bacterial extracellular vesicles. The G^- bacteria can produce OMVs through blebbing of the outer membrane and can also generate OIMVs and EOMVs through explosive cell lysis. G^+ bacteria produce CMVs through the process of bubbling cell death. BEVs contain various contents, including nucleic acids (DNA, RNA), proteins, and metabolites. IM: inner membrane, OM: outer membrane, PG: peptidoglycan, LPS: Lipopolysaccharide. The figure was created with https://app.Biore under.com/.

EVs during normal growth. These EVs, secreted by various G^- bacteria, are spherical bilayers with diameters ranging from 20 to 200 nm.[39] Research on G^- BEVs has advanced steadily, with recent studies contributing to the identification of their biogenesis and components.

G^- BEVs can be formed through two primary mechanisms: blebbing of the outer membrane (B-type) or explosive cell lysis followed by the curling and self-annealing of shattered membrane fragments (E-type).[40] Current hypotheses suggest that factors such as the reduction of OM-peptidoglycan cross-links, periplasmic accumulation, lipopolysaccharide (LPS) remodeling, and the insertion of hydrophobic molecules are crucial in enhancing outer membrane fragility, thereby facilitating the vesicular budding process.[41] OMVs, formed by the outward budding of the outer membrane without damaging the inner membrane, are believed to be devoid of cytosolic contents such as DNA, RNA, and ATP.[42] They are typically laden with outer membrane proteins and lipids, which are hallmarks of B-type membrane vesicles characteristic of G^- bacteria. The lipid constituents of OMVs are predominantly phospholipids and LPS.[43] The proteome of OMVs, as revealed through mass spectrometry-based proteomic analysis, is diverse, with over 3,500 distinct proteins identified to date.[44] These proteins include porins for solute transport, transmembrane channel proteins, and factors associated with bacterial virulence. The specific cargo of OMVs is influenced by a variety of factors, including the bacterial species and the environmental conditions during vesicle production.[45]

Interestingly, Outer-inner membrane vesicles (OIMVs) have been observed to contain cytosolic material. This phenomenon was first described in *Shewanella vesiculosa M7*, where both the inner and outer membranes protrude to encapsulate a portion of the cytoplasm, resulting in double-membrane vesicles.[46] Researchers hypothesize that this phenomenon is due to the disruption of the peptidoglycan（PG） layer, leading to co-budding of the outer and inner membranes. However, the budding model fails to explain how DNA fragments of the bacterial chromosome are packaged into these vesicles. Indeed, the presence of chromosomal DNA within vesicles is considered an indicator of cellular lysis. Consequently, the prevailing view is that OIMVs are generated through explosive cell lysis. Under genotoxic stress, the expression of prophage-derived endolysins is activated, leading to the degradation of the bacterial peptidoglycan layer. This degradation causes the inner membrane to bulge into the periplasm, resulting in the formation of OIMV and explosive outer-membrane vesicles (EOMVs).[47] The antibiotic ciprofloxacin can induce the SOS response, thereby triggering endolysin expression and promoting explosive cell lysis.[48] During the formation of E-type membrane vesicles (MVs), an interaction occurs between phages and the OM, which often results in E-type BEVs containing phage-derived components. EOMVs may also encapsulate a variety of cytoplasmic components, such as membrane proteins, DNA, and RNA.[42] Recent studies have identified a novel subtype of MVs produced by *S. vesiculosa M7*, known as explosive outer-inner membrane vesicles.[49] These vesicles exhibit distinctive structures, sometimes featuring multiple vesicles nested within a larger vesicle or having irregularly shaped internal vesicles.

Gram-positive bacterial extracellular vesicles

G^+ bacteria are characterized by the absence of an outer membrane and feature a singular lipid bilayer encased by a substantial cell wall. This cell wall is primarily composed of peptidoglycan and lipoteichoic acids.[50] The dense cell wall of G^+ bacteria greatly impedes the secretion of EVs, leading to reduced production levels.[51] Historically, the prevailing view was that membrane-derived vesicles were unable to penetrate the surface barrier of G^+ bacteria.[51,52] Despite the initial observation of EVs in *Bacillus* species in 1990,[53] substantial research into these structures did not immediately follow. A seminal study by Lee et al. in 2009 marked the first isolation of G^+ BEVs from the supernatant of *Staphylococcus aureus* cultures.[54] These EVs produced by G^+ bacteria are categorized as cytoplasmic membrane vesicles (CMVs).[55]

Our comprehension of the precise mechanisms that facilitate the genesis and extrusion of EVs in G^+ bacteria is still not fully developed. Over time, investigators have proposed three complementary hypotheses to explain the passage of EVs through

the substantial cell walls of G^+ bacteria[52]: 1) EVs, originating from the cytoplasmic membrane, may generate sufficient turgor pressure to cause the cell wall to yield, thereby releasing the EVs.[56,57] 2) It is theorized that concurrently released proteolytic enzymes could temporarily modify the cell wall's composition, thereby affording an avenue for EVs to exit.[54] 3) There is a hypothesis suggesting the presence of dedicated proteinaceous conduits or pores on the cell wall, which may serve as a conduit for the EVs' transit.[56,57]

Recently, there has been significant progress in understanding the biogenesis mechanisms of G^+ BEVs. In the majority of G^+ bacteria, including *Bacillus subtilis*, CMVs are predominantly produced via a process termed "budding cell death".[47] During this process, endolysins, which are encoded by defective prophages, weaken the PG layer, enabling cells to extrude cytoplasmic membrane material through the cell wall apertures. These structures are then liberated as explosive CMVs, a process that often results in cell death.[42] In addition to phage-derived endolysins, studies have shown that antibiotics capable of weakening the PG layer, such as β-lactams, as well as DNA-damaging antibiotics like ciprofloxacin, and autolysins, can initiate budding cell death and promote CMV formation.[58–60] CMVs have been found to encapsulate a variety of cargoes, encompassing fatty acids, phospholipids, cytoplasmic proteins, RNA, chromosomal DNA, endolysins, and virulence factors.[61] Specially, in *Staphylococcus aureus*, CMVs are generated through a budding process. Here, amphipathic, α-helical phenol-soluble modulins interfere with the CM, and autolysins reduce the cross-linking within the peptidoglycan layers. This sequence of events allows for the extrusion of CMVs through the cell wall.[59,62]

We propose that different formation pathways lead to distinct types of BEVs. The biogenesis mechanisms of BEVs appear to be the primary determinant of cargo selectivity (Table 2). For instance, OMVs are formed by the budding of the OM in G^- bacteria and, therefore, should not contain cytoplasmic components. Their main cargo includes OM constituents, periplasmic proteins, and possibly peptidoglycan fragments. The intrinsic characteristics of the bacteria, along with specific biogenesis mechanisms, influence the composition of vesicle cargo. This also determines the key substances within different BEVs that may regulate OA.

Cargo of BEVs associated with OA

The connection between the GM and OA has become a hot topic of research. BEVs have been isolated from human body fluids, and their production is shown to increase during host colonization due to stress induction.[63,64] Based on this, BEVs are considered "long-range weapons" that allow for the delivery of a large number of effectors into host tissues without the colonization by the parent bacteria. Therefore, it is necessary to categorize the cargo components within BEVs based on existing studies to explore the relationship between BEVs and OA. The selective enrichment of BEV cargo implies a degree of specificity in EV biogenesis, suggesting a regulated rather than random process. Nonetheless, the detailed mechanisms that govern the selective inclusion of cargo during vesicle formation are not fully understood. During the secretion phase, BEVs carry bacterial products, thereby protecting both their cargo and membrane-embedded molecules. In the translocation phase, BEVs navigate to distal joints via multiple pathways, fusing with

Table 2. BEV types, their routes of formation and their compositions.

GM type	BEV type	Mechanism	OM proteins	CM proteins	Cytosolic content	DNA or RNA	Toxins and effectors	Phages
Gram-negative	OMV	Blebbing	+	-[a]	-	-[b]	+	-
	OIMV	Explosive cell lysis	+	+	+	+	+	+
	EOMV	Explosive cell lysis	+	-	+	+	+	+
Gram-positive	CMV	Bubbing cell death[c]	-	+	+	+	+	+

+,present; –,absent; OMV, outer-membrane vesicle; OIMV, outer-inner membrane vesicle; EOMV, explosive outer-membrane vesicle; CMV, cytoplasmic membrane vesicle; [a]Occasionally, cytoplasmic proteins have been observed in OMVs, but it is not known how these proteins are packaged into OMVs. [b]Several reports have demonstrated the presence of plasmid DNA in OMVs, but we therefore speculate that these nucleic acids are mainly associated with OIMVs and EOMVs. [c]Vesicles originating from the inner (or cytoplasmic) membrane have also been reported in Gram-positive bacteria, but the underlying formation route is unclear.

specific cells to deliver bacterial regulatory molecules. During the internalization phase, these vesicles concentrate sufficient bioactive components to initiate specific biological responses. The varied cargo of BEVs are crucial for defining their myriad biological functions. Acting as carriers of bacterial cytoplasmic contents and membrane-associated molecules, BEVs contribute to the regulation of OA by the GM. This section summarizes the key molecules within BEVs that are involved in the modulation of OA.

Membrane-associated cargo

According to the Gram status of bacteria, BEVs are enriched with cell envelope components, including peptidoglycan, membrane proteins, lipoproteins, LPS, lipooligosaccharides, and teichoic acids.[65,66] These components serve as critical mediators in the regulation of OA by BEVs, linking the GM to joint pathologies.[67] Among these components, LPS has been the most extensively studied and shown the most promising results in relation to OA. LPS, a key circulatory inflammatory biomarker and a significant component of the outer membrane of G^- bacteria, acts as a potent pro-inflammatory mediator. It facilitates increased intestinal permeability, colloquially referred to as "leaky gut," thereby gaining access to systemic circulation and triggering systemic inflammation.[68] Corroborating evidence supports a link between elevated serum LPS levels and both the radiographic severity and clinical symptoms of OA.[69] Notably, an animal study has shown a diminished severity of post-traumatic OA in germ-free mice, which exhibit a marked decrease in LPS binding protein.[70] Additionally, research by *Kefeng Li et al.* suggests that OA symptoms can be mitigated through moderate exercise, which reduces circulating LPS levels.[71] LPS is implicated in OA pathogenesis, notably through the activation of the complement pathway in chondrocytes. The current understanding posits a two-hit model: the initial event involves priming of joint tissue macrophages by LPS via toll-like receptor signaling, followed by the activation of macrophage inflammatory pathways in response to damage-associated molecular patterns emitted from compromised joint tissues.[68,69,72] LPS is known to activate the innate immune system and to regulate the expression of diverse cytokines through several pathways.

Research on other cargo components is relatively scarce. Research by *Rabia Moentadj* and colleagues has demonstrated that PG-polysaccharide complexes from rheumatoid arthritis patients are capable of inducing arthritis in a murine model.[73] Additionally, research indicates that the PG cargo contained within BEVs can activate synovial fibroblasts by interacting with Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). The engagement leads to an upregulation of pro-inflammatory cytokines and the activation of matrix metalloproteinases (MMPs).[74] Recent findings suggest that PG contributes to OA progression by enhancing systemic innate immunity.[75] The significant role of membrane-associated cargo in the pathogenesis and advancement of OA is widely acknowledged. However, the functions of many related substances remain unclear and require further investigation.

Cytoplasmic cargo

The biosynthesis process of BEVs indicates that they typically encapsulate bacterial cytoplasmic components, including a variety of bioactive molecules. Research analyzing fecal samples from individuals with obesity-related OA has revealed significant alterations in microbial metabolites, such as propionic acid, indoles, and various tryptophan metabolites.[17] Tryptophan metabolites and short-chain fatty acids are among the most extensively studied metabolites originating from the GM. Recent research has highlighted a divergence in metabolite profiles between healthy individuals and those with OA, particularly in amino acids and their derivatives.[76] Observational studies have reported that individuals with symptomatic hand OA exhibit elevated levels of certain tryptophan metabolites, such as 5-hydroxyindoleacetic acid, 5-OH-tryptophan, and 5-hydroxytryptophol, while showing decreased levels of others, including indole-3-lactic acid, indole-3-aldehyde, and 3-hydroxyanthranilic acid, when contrasted with unaffected individuals.[14,16] Disruptions in tryptophan metabolism and shifts in GM-mediated tryptophan biosynthesis due to gut dysbiosis have been linked to symptomatic hand OA and pain. Short-

chain fatty acids, including propionate and butyrate, are predominantly generated by anaerobic bacteria from the *Firmicutes phylum*, which are known for their anti-inflammatory and immunoregulatory properties. *Keun-Hyung Cho* and colleagues demonstrated changes in the GM in a rat model of OA induced by monosodium iodoacetate, where *Eubacterium*, a notable SCFA producer, was absent in the affected group.[77] The administration of live *Lactobacillus* and quercetin has been shown to elevate butyrate levels, thereby mitigating OA progression through the modulation of the gut environment and enhancement of autophagic flux.[77,78] Not only do BEVs carry metabolites, but they also transport specific virulence factors. Certain virulence factors exhibit periplasmic localization and accumulation during the biogenesis of BEVs, such as cholera toxin and the PrtV protease in *Vibrio cholerae*, or Shiga toxin in pathogenic *E. coli* strains.[79–81] Accumulating evidence indicates that intra-articular administration of botulinum toxin type A can effectively reduce pain and inflammation in both osteoarthritic rats and clinical patients.[82–85] Botulinum toxin type A exhibits notable chondroprotection. It can mitigate the progression of OA by inhibiting chondrocyte ferroptosis via the SLC7A11/GPX4 axis. These findings provide detailed evidence supporting the role of BEVs in the regulation of OA.[86] However, some virulence factors are associated with cell surface components, such as the heat-labile enterotoxin in *E.coli* .[87] These represent two types of virulence factor loading mechanisms in G^- bacteria. These bioactive molecules not only link intestinal health to OA but also hold considerable promise as targets for therapeutic intervention. These findings offer compelling evidence that BEVs serve as a potential target within the gut-joint axis for managing OA.

Genetic cargo

Cartilage was traditionally considered a sterile environment. Nonetheless, recent findings have detected GM DNA, including species such as *Porphyromonas* and *Bacteroides*, in the synovium and cartilage of individuals with OA.[88] The research revealed significant alterations in cartilage microbial DNA profiles, characterized by an increase in G^- bacteria and changes across multiple microbial taxa, coinciding with the onset and progression of OA.[89] A study utilizing animal models explored the gastrointestinal source of cartilage microbial DNA, showing that these DNA patterns can manifest quickly, within 48 hours post-inoculation of germ-free mice with a GM.[90] The mechanism by which this DNA accesses the cartilage is still not fully understood.

Bacterial chromosome-derived and plasmid-derived DNA, as well as RNA, can be carried by certain special types of BEVs. As mentioned above, outer-inner membrane vesicles (OIMVs) and explosive outer-membrane vesicles (EOMVs) secreted by Gram-negative bacteria, along with cytoplasmic membrane vesicles (CMVs) secreted by Gram-positive bacteria, are typically capable of carrying genetic cargo. This characteristic is referred to as DNA transfer. Stimulating Acinetobacter baumannii with a sublethal concentration of gentamicin results in the production of more BEVs and achieves higher efficiency of DNA transfer.[91] These vesicles protect the genetic material cargo from degradation during transport. In OA, the vasculature becomes more abundant and dilated, potentially enabling the migration of BEVs from the blood into the articular cartilage through the tidemark's fissures.[92] Local dysbiosis, potentially triggered by the infiltration of pathogenic bacteria DNA from the subchondral bone marrow, could influence chondrocyte gene expression and contribute to the pathogenesis of OA.

Role of natural BEVs in the gut-joint axis

Bacteria utilize vesicle secretion for a spectrum of purposes, such as outcompeting rivals, securing nutrients, neutralizing environmental toxins, augmenting pathogenicity, and enabling cell-to-cell communication.[55,93] There is a growing appreciation of the role of BEVs in microbial-host communication. BEVs act as cargo vessels, transporting a plethora of bioactive molecules to distant tissue cells in a safeguarded manner.[94–96] They are pivotal in the gut-joint axis, acting as a conduit that connects the gut and joint microenvironments.This provides an insight into exploring the relationship between the microbiota and joints: BEVs act as messengers of the gut-joint axis. As nanoscale entities, BEVs hold the potential to comprehensively

regulate OA through interactions with various bodily substances. To understand the specific roles of BEVs within the gut-joint axis, it is essential to address two fundamental questions: (1) How do BEVs manage to migrate from the gut to the joints? (2) Upon arrival in the joints, how do BEVs modulate joint health? In the gut, BEVs can control gut permeability, which is a prerequisite for their role as important messengers in the gut-joint axis. In the joints, BEVs modulate immune pro-inflammatory responses and joint homeostasis. These effects are the direct outcome of BEVs functioning as crucial messengers within the gut-joint axis. In a summary, BEVs control the progression of OA by regulating pathological mechanisms such as gut permeability, immune-inflammatory responses, and joint homeostasis.

Gut permeability

Within the gut, a layer of epithelial cells separates microbes from the underlying immune cells of the subepithelium, maintaining a critical equilibrium between intestinal barrier function and permeability. This epithelial barrier not only shields against the invasion of potential pathogens but also permits the essential transit of nutrients and water.[97] An increase in intestinal permeability coupled with a reduction in barrier integrity can precipitate a spectrum of intestinal and systemic disorders. A cross-sectional study among individuals with metabolic syndrome-related OA revealed a significant correlation between biomarkers of intestinal permeability and OA pathogenesis.[98] Intestinal permeability stands as a cornerstone in the gut-joint axis; its perturbation is known to intensify OA symptoms. Factors such as unhealthy lifestyles, chronic illnesses, and prolonged antibiotic use can induce GM dysbiosis, which in turn can disrupt tight junctions and alter gut permeability. This disruption may allow bacterial toxins, inflammatory mediators, and even gut bacteria to penetrate the submucosa. Considering that joint cartilage is avascular, its nourishment is chiefly sustained by synovial fluid and the blood supply from subchondral bone marrow. Consequently, toxins, inflammatory mediators, and microbes that gain entry to the systemic circulation through the compromised gut can impact the joint via two principal mechanisms: through cartilage fissures or via the vascular channels that arise from neoangiogenesis at the osteochondral junction.[99]

EVs secreted by the intestinal microbiota, which includes commensal bacteria, probiotics, and pathogens, play a pivotal role in modulating the intestinal microenvironment and overall host health. Recent research indicates that the impact of BEVs on intestinal barrier function is contingent upon their bacterial origin.[100] The majority of BEVs secreted by pathogens are known to compromise barrier function. After being internalized by intestinal epithelial cells, the LPS released from E. coli BL21 EVs leads to a reduction in the expression of E-cadherin. Feces-derived EVs decrease tight junction proteins (TJs), zonula occludens-1 (ZO-1), and occludin expression and enhance epithelial intestinal cell permeability via a non-muscular myosin light chain kinase (nmMLCK)-dependent pathway.[101] Elmi et al. discovered three proteases, HtrA, Cj0511, and Cj1365c, in the BEVs of *Campylobacter jejuni* which enhance the proteolytic capability of BEVs. These enzymes directly cleave intercellular tight junction proteins, thereby compromising the integrity of the barrier.[102] Additionally, BEVs can induce apoptosis in intestinal epithelial cells, as demonstrated by *F. nucleatum* EVs, which promote M1 macrophage polarization and trigger apoptosis via the RIPK1-caspase-3 pathway, further compromising the intestinal barrier.[103] In contrast, BEVs from commensal bacteria and probiotics are known to bolster the intestinal barrier. For example, *Propionibacterium freudenreichii CIRM-BIA 129* EVs are rich in surface layer protein B, which can interact with NF-κB to elicit anti-inflammatory responses.[104] The probiotic strain *Escherichia coli Nissle 1917* releases OMVs that protect against enteropathogenic *E. coli*-induced barrier disruption. These OMVs can be absorbed by intestinal epithelial cells, preventing the relocation of occludin and ZO-1 from tight junctions to intracellular sites, thereby preserving barrier integrity.[105] The beneficial effects of probiotic-derived BEVs also involve modulation of gene expression and the redistribution of tight junction proteins, enhancing intestinal permeability.

It is worth noting that the effects of BEVs are based on the characteristics of their corresponding

parent bacteria, the cargo they carry, and the physiological functions of the host. However, we cannot equate the effect of BEVs on intestinal permeability with the effect of their parent bacteria directly. BEVs offer a novel perspective for investigating bacterial influences on host health. Studies have shown that due to an age-related increase in intestinal permeability, the levels of circulating BEVs in the body also significantly rise. BEVs hold the potential to serve as novel biomarkers reflecting the health of the host's gut, bridging bacteria and host cells.BEVs could act as a double-edged sword, the consequences of which need to be further explored.[106]

Immune-inflammatory response

GM dysbiosis can alter the cargo within BEVs, disrupting the intricate equilibrium of pro-inflammatory and anti-inflammatory mediators and potentially contributing to joint structural degradation.[99] Under normal conditions, the GM is largely confined to the intestinal lumen and does not typically translocate to distant organs via intercellular pathways. Nonetheless, EVs from the GM can be readily internalized by intestinal epithelial cells, gain access to the systemic circulation, and effectively convey bacterial constituents to target cells. Research has revealed that OMVs derived from the microbiota have specific targeting abilities, enabling the selective delivery of diverse molecules, including peptidoglycans, lipids, proteins, and nucleic acids, to immune cells, with a particular affinity for macrophages and neutrophils.[107,108] BEVs are recognized and taken up by host cells through interactions involving Toll-like receptors (TLRs) and LPS-binding protein. Upon adhesion or binding to the cell surface, BEVs are primarily internalized into host cells via endocytosis. BEVs can also activate innate immune pattern recognition receptors within the host cell cytoplasm. NOD1 located in the cytoplasm, is capable of recognizing conserved structural motifs present within PG from almost all G^- bacteria. BEVs, therefore, play a pivotal role in the cross-species communication between the host and its microbiota. The pathogen-associated molecular patterns of BEVs enable them to bind to pattern recognition receptors located on the membrane surface and within the cytoplasm of both immune and nonimmune cells. This interaction activates downstream inflammatory signaling pathways and induces disruption of cartilage homeostasis. Analysis of the microbiome in OA patients has shown an elevated *Firmicutes* to *Bacteroidetes* phyla ratio and an increased prevalence of *Streptococcus* species.[109] A reduction in *Bacteroidetes* may result in diminished short-chain fatty acid levels, which could impede the differentiation of regulatory T cells. In contrast, heightened *Streptococcus* levels may trigger local or systemic inflammation by activating macrophages through LPS or other metabolites, utilizing pathways such as NF-κB or MAPK, or by engaging with LPS binding protein and CD14 to form active complexes.[99,110]

Under normal conditions, the GM is largely confined to the intestinal lumen and does not typically translocate to distant organs via intercellular pathways. Nonetheless, EVs from the GM can be readily internalized by intestinal epithelial cells, gain access to the systemic circulation, and effectively convey bacterial constituents to target cells. Research has revealed that OMVs derived from the microbiota have specific targeting abilities, enabling the selective delivery of diverse molecules, including peptidoglycans, lipids, proteins, and nucleic acids, to immune cells, with a particular affinity for macrophages and neutrophils.[107,108] BEVs, therefore, play a pivotal role in the cross-species communication between the host and its microbiota. In a murine model of collagen-induced arthritis, *F. nucleatum* exacerbates arthritis, with its OMVs being instrumental in this aggravation. The translocation of the FadA virulence factor from *F. nucleatum* OMVs to the joints results in FadA engaging with synovial macrophages. This engagement activates Rab5a GTPase, implicated in vesicular transport, and YB-1, a significant regulator in inflammatory pathways, thus initiating a localized inflammatory reaction.[111] Conversely, probiotic vesicles can play a role in ameliorating OA. Membrane vesicles derived from *Lactobacillus johnsonii* have the capacity to modulate the glutamine synthetase/mTORC1 axis in macrophages. They impede macrophage migration, M1-like polarization, and inflammatory mediators secretion, while simultaneously promoting the M2/M1

ratio in synovial macrophages. Therefore, the membrane vesicles from *L. johnsonii* exhibit a novel ability to alleviate inflammation, cartilage damage, and pain associated with OA.[112]

BEVs may also modulate chondrocytes. BEVs can directly influence chondrocyte metabolism, thereby contributing to cartilage protection and homeostasis. The aryl hydrocarbon receptor (AHR), a transcription factor present in the intestinal epithelium, influences the extent of cartilage degradation.[113] Kynurenine, a tryptophan metabolite, can activate AHR, and the kynurenine-AHR pathway has been shown to enhance the chondroprotective effects of mesenchymal stromal cells derived from human umbilical cord.[114] Furthermore, *Lactobacillus* species, known for their role in indole derivative production, have been implicated in mitigating chondrocyte inflammation and modulating extracellular matrix and NF-κB signaling pathways via AHR, as demonstrated by research conducted by Huangming Zhuang et al. .[115,116] Butyrate is recognized for its ability to stimulate autophagy through the AMPK and mTOR signaling pathways, thereby restoring compromised autophagy and reducing inflammatory cell death.[114] BEV, which bridge the GM and joint health, may present a promising therapeutic target for OA treatment. GM demonstrates the capacity to modulate Immune-inflammatory response and cartilage homeostasis within the host. This is achieved by their ability to secret BEVs which facilitate communication between bacteria and host cells.

These studies shed light on the regulatory mechanisms of natural BEVs within the gut-joint axis (Figure 3). On one hand, BEVs govern intestinal permeability, a critical factor that determines the potential for bacterial products to infiltrate the body and exert deleterious effects on joints. On the other hand, BEVs also play a role in modulating the host's Immune-inflammatory response, directly impacting cellular processes that influence joint structure. BEVs play a pivotal role by transporting enriched bioactive components from the gut to the joints, influencing each step along the gut-joint axis. Despite their importance, the role of BEVs has frequently been neglected in past research. This is precisely why we propose that BEVs serve as a novel bridge in the gut-joint axis. These findings establish a foundational basis for the prospective application of engineered BEVs, particularly those derived from probiotics, for their therapeutic BEVs in OA treatment. In conclusion, the exploration of engineered BEVs as a therapeutic strategy for OA has become a burgeoning area of research interest.

Regrettably, existing studies primarily explore the unidirectional influence from the gut to the joints. While research has identified significant alterations in both the composition of gut microbiota and bacterial metabolites in the feces of OA patients,[117] the precise mechanisms driving these changes remain elusive. It is plausible that these phenomena are linked to EVs. Furthermore, the pathogenesis of OA remains unclear. Due to the relatively well-understood regulatory mechanisms of BEVs on the immune system, research has centered on immune-inflammatory responses. In recent years, studies have revealed that subchondral bone structural abnormalities and changes in the hypoxic joint environment can also contribute to OA.[118] However, there is currently a lack of direct evidence on how BEVs influence these factors, which could be a future hotspot for research on the regulation of the gut-joint axis by BEVs.

Current application of BEVs: a new strategy for OA treatment

OA is the most common joint disorder, yet its complex pathogenesis defies the existence of a treatment capable of slowing its progression.[1] The disease poses a significant burden to both patients and society,[3] underscoring an urgent need to uncover novel pathogenic mechanisms that may present new therapeutic targets. The preparation of nanoparticles for the treatment of OA has become a hot topic of research. EVs represent a natural form of nanobiomaterial with distinctive attributes. Their cell-free nature, combined with low toxicity, the capacity to carry drugs, and exceptional biocompatibility, positions EVs as a potential therapeutic platform for OA.[23] Compared to other nanoparticles, EVs possess unique advantages: (1) uptake by cells and (2) transport through tissues.[119] On one hand, the size, shape, and elasticity of nanoscale particles influence cellular uptake. EVs, which are composed of a phospholipid bilayer, have been shown to be more efficiently internalized by cells than stiff

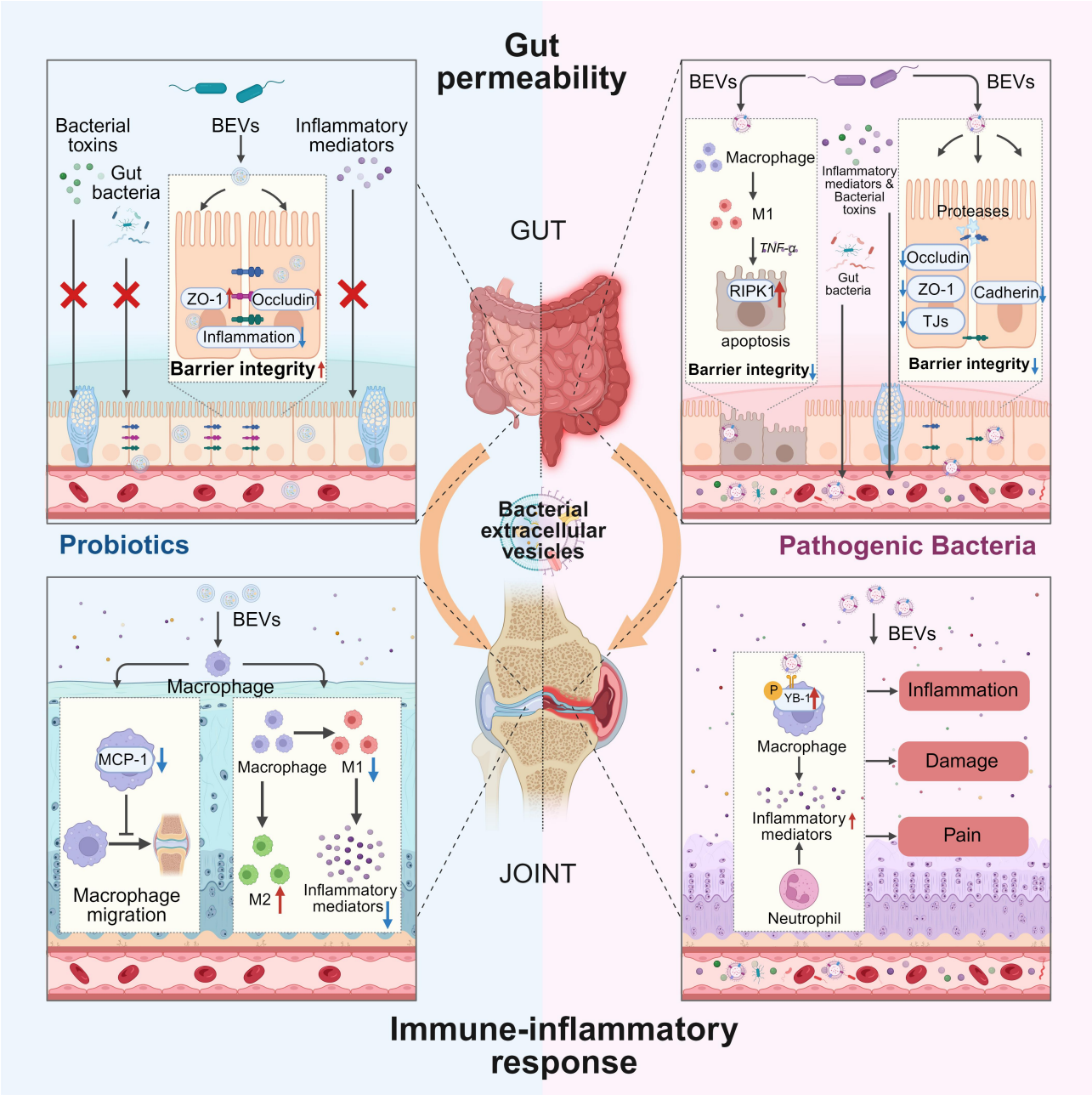

Figure 3. Summary of the role of bacterial extracellular vesicles in the gut-joint axis. The type and state of bacteria determine the functionality of BEVs. BEVs secreted by a dysregulated microbiota, or pathogenic bacteria may be a cause of OA, while BEVs secreted by probiotics could also be a solution for OA. In OA, BEVs can interact directly or indirectly with intestinal epithelial cells and immune cells, participating in the maintenance of the intestinal barrier and the regulation of anti-inflammatory responses. ZO-1: zonula occludens-1, RIPK: receptor-interacting protein kinase, tnf-α: tumor necrosis factor alpha, TJs: tight junction proteins, MCP-1: monocyte chemoattractant protein-1, YB-1: Y-box binding protein 1. The figure was created with https://app.bioreunder.com/.

nanoparticles.[118] On the other hand, the surfaces of EVs feature cellular membrane channel proteins that allow them to deform freely in response to environmental pressures.[120] This capability enables EVs to deliver their cargo more easily into the depths of tissues. EV deformation by fluid expulsion is a significant transport mechanism. However, there are challenges associated with EVs, including difficulties in extraction, limited targeting capabilities, and suboptimal therapeutic efficacy,[121] which impede their clinical translation. Consequently, research has increasingly concentrated on the engineering of MEVs to enhance their therapeutic potential for OA.[122,123] BEVs, in comparison to MEVs, offer advantages such as higher extraction efficiency and more straightforward engineering

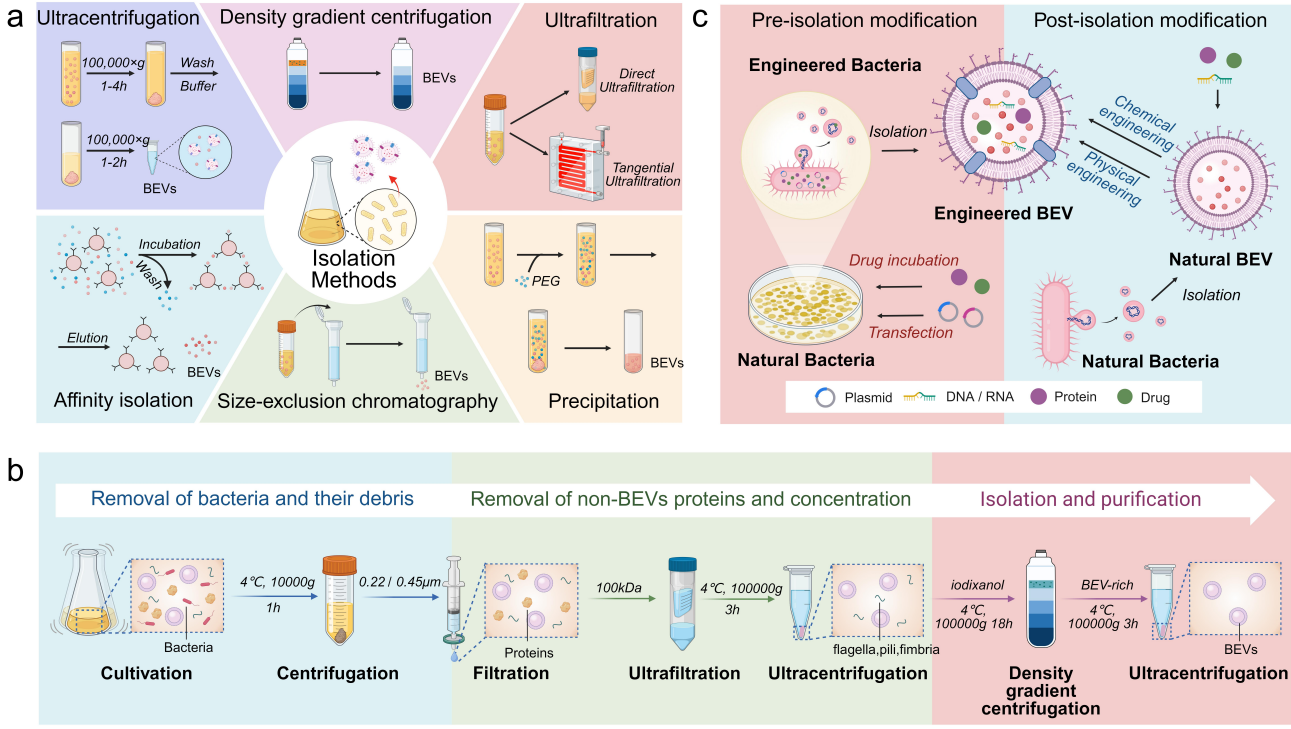

Figure 4. The engineering approaches for isolating and modifying BEVs. (a) Schematic diagram of the separation and purification procedures for several common EVs. Ultracentrifugation utilizes extremely high rotational speeds to generate gravitational forces that precipitate vesicles out of solution. Density gradient centrifugation involves creating a density gradient within the centrifuge tube, allowing vesicles of different densities to settle at distinct layers during centrifugation. Ultrafiltration uses membrane filters with specific molecular weight cut-offs. Pressure is applied to force the liquid through the membrane, retaining and concentrating vesicles larger than the pore size. Affinity isolation employs specific antibodies or other ligands that bind to surface markers on vesicles, enabling selective capture of target vesicles. Size-exclusion chromatography separates vesicles based on their size in a gel column, where larger molecules are eluted first, followed by smaller particles, suitable for separating vesicles irrespective of their density. Precipitation involves using chemical reagents such as polyethylene glycol (PEG) to alter solution conditions, causing vesicles to aggregate and precipitate. (b) A separation method applicable to the vast majority of bacteria, divided into three steps: (1) removal of bacteria and debris (2) removal of non-bev proteins and concentration (3) isolation and purification. (c) Engineering modification methods for BEVs, mainly divided into pre-isolation modification and post-isolation modification. That is, one involves the modification of donor bacteria responsible for BEV production, and the other involves direct surface modification of BEVs. The figure was created with https://app.bioreunder.com/.

modifications, attributes that have ignited significant interest in BEVs research.[23,26] In this section, we provide an overview of the current engineering strategies for the isolation and modification of BEVs.

Isolation and purification of BEVs

For biotechnological applications, BEVs must meet high purity standards, necessitating critical attention to their isolation and purification. A variety of separation techniques have been established to extract high-quality BEVs from bacterial culture broth. Standard isolation methods encompass ultracentrifugation, density gradient centrifugation, size-exclusion chromatography, ultrafiltration, precipitation, and affinity isolation (Figure 4a).[124,125] We detail the pros and cons of these methods in Table 3. Presently, no single technology suffices for BEV isolation; instead, a combination of methods, such as filtration and centrifugation, is often employed to effectively separate these vesicles. *Liu et al.* have crafted a versatile laboratory isolation method suitable for the majority of bacterial species (Figure 4**b**). The BEV recovery process begins with low-speed centrifugation (10,000 × g for 30 minutes) and filtration (0.22 or 0.45 μm, based on bacterial and vesicle dimensions) to eliminate intact bacteria and debris. Unwanted proteins associated with non-BEVs are removed by ultrafiltration through a 100 kDa membrane, followed by centrifugation (3,000 × g for 15 minutes).

Table 3. Overview of different BEVs isolation methods.

Method	Separation principle	Advantages	Disadvantages	Reference
Ultracentrifugation	Size and density	1. Simple extraction process 2. Great homogeneity	1. Limited efficiency and purity 2. Potential damage to BEVs	55
Density gradient centrifugation	Floatation speed	1. High purity 2. Possibility to identify subpopulations	1. High cost and time-consuming 2. Potential damage to BEVs	126
Size-exclusion chromatography	Hydrodynamic radius	1. High purity 2. Preserves vesicle integrity	1. Not suitablefor large sample 2. High time-consuming	127
Ultrafiltration	Hydrodynamic radius	1. Simple extraction process 2. Great homogeneity 3. Preserves vesicle integrity	Limited efficiency and purity	128
Precipitation	Solubility	1. Simple and economical extraction process 2. No requires specialized equipment	1. Limited efficiency and purity 2. High time-consuming 3. Impaired functional integrity	43
Affinity isolation	specific binding by receptor-ligand	High purity	1.High cost 2. low availability for BEVs	43

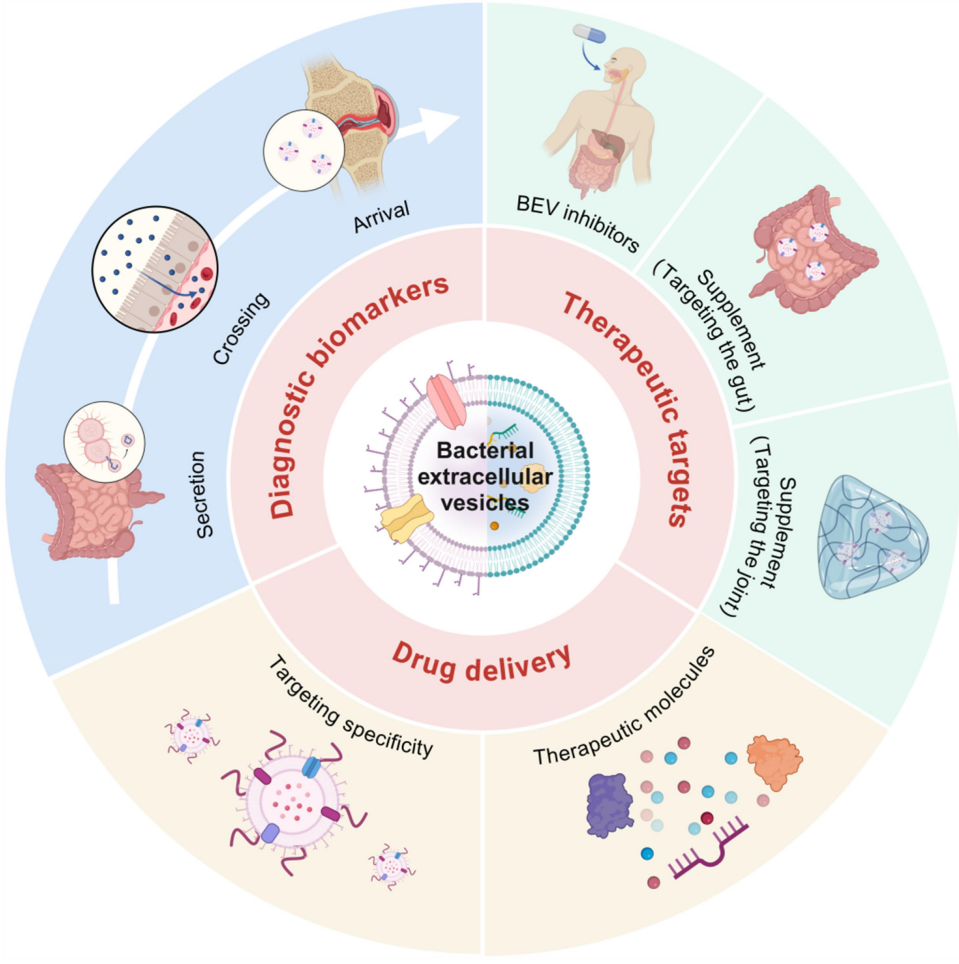

Figure 5. An overview of the application of BEV-based OA diagnosis and treatment. BEVs can serve as laboratory indicators for the early diagnosis of OA patients. Analysis of changes in the secretion and transport of BEVs can reflect the state of the GM and its contribution to disease. BEVs can offer new therapeutic strategies as treatment targets. This includes inhibiting BEVs produced as a result of dysbiosis, supplementing with BEVs derived from probiotics, and constructing novel biocompatible materials combined with EVs to directly target joints, thereby promoting cartilage repair and reducing inflammation. BEVs can enhance existing treatment methods by achieving targeted drug delivery through techniques such as genetic engineering and electroporation. https://app.bioreunder.com/

BEVs are then isolated via ultracentrifugation (100,000 × g for 18 hours) and density gradient centrifugation using iodixanol gradients. The BEV-enriched fraction is subsequently diluted with phosphate-buffered saline and subjected to ultracentrifugation at 100,000 × g for 3 hours. The physicochemical properties of BEVs are further characterized using transmission electron microscopy, nanoparticle tracking analysis, and western blotting.[129] Despite advancements in BEV isolation and purification protocols, the inherently low secretion yield and the complexity of the procedures pose challenges to the commercial viability of BEVs. We have outlined the ongoing efforts and strategies to mitigate these significant hurdles.

During culture, bacteria can spontaneously secrete BEVs into the media.[130] However, the yield from spontaneous secretion is typically inadequate to satisfy commercial requirements. To address this, various strategies are employed to augment BEV production for biotechnological applications.[131] One approach involves genetic manipulation through transgenic technology, which induces hypervesiculation by targeting genes that regulate membrane stability and stress-responsive chaperones.[132] Another method encompasses external stimuli, such as the use of detergents, sonication, and oxidative or temperature stress. Detergents, including deoxycholate and sodium dodecyl sulfate, are particularly prevalent in industrial applications due to their amphipathic nature, which facilitates vesiculation by interacting with bacterial membranes.[133] Techniques like sonication, vortexing, and the application of metal chelators, such as ethylenediaminetetraacetic acid (EDTA), can also destabilize cell membranes, thereby promoting vesicle release.[134] BEVs can be released as a protective mechanism by bacteria under stress conditions that threaten their survival. Leveraging this concept, the manipulation of culture medium composition – such as nutrient or growth factor deprivation and the addition of specific antibiotics – and growth conditions, including UV irradiation, pH adjustments, and temperature variations, can simulate hostile environments that enhance BEVs production.[135]

The current bioprocess for procuring BEVs encompasses two main stages: (1) the culture of bacteria, and (2) the subsequent isolation and purification of BEVs. The transition of BEVs-based technologies to industrial-scale production has faced challenges due to the high costs and the absence of standardized, reproducible methods. Consequently, bioreactor systems have been suggested as a solution to increase the culture volume from less than 1 L to over 80 L.[136] Research indicates that the use of bioreactors for continuous generation can yield BEVs concentrations as high as 4×10^{14} BEVs/L of culture per day.[137] This approach can be complemented by adjusting the culture medium composition and employing stressors to further boost BEV productivity. Moreover, bioreactor culture systems provide enhanced control over the microenvironment, which is vital for improving process reproducibility and ensuring the consistency of BEVs composition. On the other hand, optimizing the downstream processes for BEVs isolation and purification is equally critical. Laboratory-scale methods often have limitations when it comes to handling large volumes. For instance, ultracentrifugation, despite being a common technique, has several drawbacks, including extended processing times due to its batch nature and significant electrical power consumption.[138] To address these issues, the integration of tangential flow filtration with size-exclusion chromatography has emerged as a scalable, efficient method that aligns with Good Manufacturing Practices.[131] Tangential flow filtration enables the processing and concentration of large culture medium batches, while size-exclusion chromatography effectively removes impurities. This combined method can produce substantial quantities of functionally active BEVs, making it indispensable for industrial production and the exploration of new technologies and instrumentation.

Modification of BEVs

Nanotechnology has played a pivotal role in advancing targeted drug delivery systems. Beyond the realm of synthetic nanocarriers, EVs have garnered significant attention as therapeutic agents.[121] The strategic modification of BEVs is a crucial strategy for improving the precision of targeting and the efficacy of treatments.[139] Although research on the engineering of BEVs is in its nascent stages and lags

behind that of MEVs, the similarities in their biosynthetic pathways and membrane compositions allow for a high degree of versatility in engineering approaches. The primary methods for BEVs engineering are broadly divided into two categories: pre-isolation modifications and post-isolation modifications (Figure 4**c**).

Pre-isolation modification entails the use of synthetic biology strategies to genetically alter donor bacteria, thereby producing bioderived nanomaterials in the form of BEVs with tailored biological functions.[124] This approach involves modifying donor cells prior to the secretion of EVs into the external environment, which can indirectly modify the cargo and membrane proteins of the vesicles. For instance, EVs derived from mesenchymal stem cells (MSCs) after incubation with paclitaxel have demonstrated anti-tumor effects. *Kim et al.* successfully transfected miR-584-5p into hMSCs using a lentivirus, with miR-584 being detectable in the subsequent EVs.[140] The majority of pre-isolation modification strategies concentrate on integrating targeting peptides and specific functional proteins into the bacterial membrane surface. This is achieved through plasmid-based and CRISPR-Cas9-based overexpression or knockout systems, which confer targeting or therapeutic capabilities upon the engineered BEVs. An innovative method for genetic modification involves cellular nano-perforation, where a monolayer of EV donor cells is cultured above a chip with a nano-channel array. Transient electrical pulses are then applied to transfect plasmid DNA from the buffer solution into the donor cells. Our research has identified that C-X-C motif chemokine receptor 4 (CXCR-4) has bone-targeting potential.[141] Building on this, we utilized plasmid transfection to enable probiotics to express the CXCR-4 protein on their surface. This technique resulted in the development of bone-targeting probiotic vesicles, which hold promise for the treatment of bone-related diseases.[124] Synthetic biology allows for the selective overexpression of proteins, cytokines, and growth factors, thereby modulating the disease microenvironment. For example, *Hay et al.* engineered Lactococcus lactis to overexpress bone morphogenetic protein-2 (BMP-2). These modified probiotics were capable of secreting substantial amounts of BMP-2, significantly enhancing the osteogenic differentiation of BMSCs.[142] The ongoing advancements in synthetic biology open up new avenues for bacterial modification, offering promising prospects for the effective development of therapeutic strategies for OA.

Post-isolation modification, which entails the direct alteration of EVs, is primarily categorized into physical and chemical engineering strategies. This approach offers greater flexibility compared to the modification of bacteria. Physical engineering encompasses techniques such as membrane fusion, membrane coating, and electroporation,[125] with electroporation being a widely utilized method. By adjusting the specific electroporation buffer and electrical parameters, it is possible to encapsulate a variety of substances within BEVs, including drugs, nucleotide sequences, plasmids, and others. *Alvarez-Erviti* optimized electroporation conditions (400 V and 125 μF in a buffer containing 1.15 mm potassium phosphate (pH 7.2), 25 mm potassium chloride, and 21% optiprep) to facilitate the transfer of GAPDH siRNA into MEVs.[143] Studies indicate that electroporation generally does not significantly alter the physical properties of MEVs, only minimally increasing the diameter of EVs. However, it is crucial to acknowledge that electroporation may result in low efficiency and potential damage to the vesicle structure. Chemical engineering involves both covalent and non-covalent reactions. Covalent reactions often rely on reactive groups present on the EV membrane, such as amino, carboxy, or thiol groups. Common covalent modification strategies include click chemistry, aldehyde-amine condensation, and bioconjugation. *Jia et al.* employed click chemistry to couple EV membranes with a neuraminidase-1 targeting peptide, achieving targeted delivery to glioblastoma and enhancing blood-brain barrier penetration.[144] Non-covalent reactions facilitate the attachment of peptide chains to the BEV membrane through mechanisms like hydrophobic insertion, multivalent electrostatic interactions, and receptor-ligand binding. Amphiphilic substances can be readily incorporated into the lipid bilayer of EVs through direct co-incubation. Zou et al. developed an aptamer-equipped exosome platform, functionalizing EVs loaded with chemotherapeutic drugs using a diacyllipid-aptamer conjugate, thus achieving targeted cancer delivery.[145] Nevertheless, the long-term stability,

safety, and biocompatibility of chemically modified BEVs necessitate further research.

Comprehensive prospects of BEVs in OA treatment: precision diagnosis and efficient therapy

The complex and profound relationship between the GM and human health is extensively documented. Emerging research underscores the pivotal role of BEVs in modulating the pathophysiology of various diseases. Although research on BEVs lags behind that of eukaryotic EVs, such as exosomes, studies on the natural functions of BEVs are advancing.[146] The similarities between BEVs and eukaryotic vesicles have accelerated the development of engineered BEVs for specific applications. The immunomodulatory characteristics of BEVs render them promising candidates for disease diagnostics, vaccine formulations, and therapeutic delivery systems.[23] In this section, we offer a concise overview of the potential applications of BEVs in the context of OA. We delve into their implications for OA management, focusing on their utility in diagnostics, therapeutic interventions, and as adjunctive treatments (Figure 5).

Diagnostic biomarkers

A multitude of diseases have been associated with the microbiota, sparking considerable interest in the use of BEVs as potential diagnostic biomarkers.[55] Researchers utilize a variety of sophisticated techniques to analyze BEVs isolated from human biological samples such as serum, urine, or feces. These methodologies encompass BEV metabolomics, (meta) genomics profiling, analysis of BEV properties, and quantification of BEV numbers. The goal of these analyses is to diagnose a spectrum of conditions, including cancer and allergic diseases. Results from a comprehensive case-control study indicate that BEV screening analysis may surpass fecal microbiota analysis in discerning between healthy individuals and those afflicted with inflammatory bowel disease (IBD).[147] BEVs are recognized as a vital link between the GM and the host's skeletal system. Recent investigations have discovered specific BEVs from the GM that are capable of traversing the intestinal mucosa to reach the bone marrow space. For example, BEVs secreted by *Akkermansia muciniphila* have been shown to access the bone marrow space within an hour following oral, rectal, or intravenous administration.[148] This groundbreaking finding establishes a theoretical basis for the use of BEVs in the detection of bone-related diseases. Consequently, the utilization of BEVs for diagnosing bone-related diseases is gaining momentum. The EV-bacteria aggregation assay is a valuable tool for diagnosing infectious osteomyelitis and rapidly identifying the causative microorganisms.[149]

As previously discussed, the GM and their BEVs play a central role in the pathogenesis of OA. This has led to the hypothesis that BEVs could be utilized as diagnostic biomarkers for the early screening of OA. There is evidence of a cross-talk between GM dysbiosis and the progression of OA.[150] Notably, fecal samples from OA patients show distinct variations in microbial composition, function, and phenotype compared to those from healthy individuals. In patients with OA, there is an observed increase in the abundance of *p_Bacteroidota*, *c_Bacteroidia*, and *o_Bacteroidales*, while the abundance of *s_Prevotella_copri* decreases compared to healthy controls. Among these, *Prevotella copri* has been identified as an effective discriminator for distinguishing OA in healthy individuals.[151] The imbalanced GM can produce abnormal metabolites that further disrupt multiple metabolic pathways associated with OA. The contents of BEVs fluctuate with changes in the bacterial status, and as significant secretions of bacteria, BEVs play a pivotal role in understanding bacterial functions at a systemic level.[152] Consequently, BEVs can, to some extent, reflect the patient's GM environment. When combined with the microbial changes commonly observed in OA patients, BEV-related information can offer insights into the presence of OA. Clinical diagnosis of OA primarily depends on medical history, physical examination, and radiological assessments, which lack laboratory indicators. BEVs have the potential to complement the diagnosis of OA alongside other metabolic markers. Moreover, radiological evidence of OA often emerges late, potentially missing the optimal treatment window. BEVs, being influenced by the GM, can exhibit changes in the early stages of OA, presenting a novel approach for early disease screening. The GM can secrete

BEVs into biological fluids. We can collect blood, saliva, urine, and synovial fluid from patients, isolate the BEVs, and then perform 16S rRNA sequencing or metagenomic sequencing. By comparing these results with those from healthy controls, the microbial characteristics of BEVs can reflect changes in the patient's microbiota, providing insights for the diagnosis of OA. This could effectively identify patients in the early stages of the disease, even before significant radiological changes occur, allowing for earlier intervention and improved treatment outcomes. Despite the potential of BEVs in OA diagnosis, it is crucial to emphasize that several challenges must be overcome. Since BEVs are isolated from biological fluids, there is heterogeneity within BEV populations, variability in isolation techniques, a lack of in-depth characterization of BEV cargo, and limited understanding of their interactions with host cells.[153] To enhance the clinical use of BEVs as diagnostic biomarkers, further research is essential to develop cost-effective and straightforward techniques for isolating and detecting BEVs.[55]

Therapeutic targets

BEVs present a promising therapeutic target for developing innovative treatment strategies for OA. Potential approaches include inhibiting BEVs generated by a dysbiotic GM or supplementing with probiotic BEVs. The BEV stands out as a promising target for therapeutic intervention in OA. Evidence suggests that depleting the GM can mitigate the progression of OA.[154] For instance, antibiotic treatment that precedes injury has been shown to enhance outcomes in post-traumatic OA in mice by reducing the GM.[155] BEVs that emerge from a dysbiotic GM can penetrate the intestinal barrier, trigger immune responses, and incite inflammation, all of which can worsen OA. Targeting the dysbiotic GM to curb BEV production might replicate these therapeutic effects. Several BEV inhibitors have been engineered to modulate the biological pathways of BEV biogenesis, thereby suppressing BEV secretion and reducing the dissemination of noxious components. Indole and its derivatives, for example, can hinder the production of OMVs by influencing the synthesis of Pseudomonas quinolone signal in *P. aeruginosa*.[156] Peptidyl arginine deiminases (PAD), a group of calcium-activated enzymes that facilitate BEV secretion, can be targeted by PAD-specific inhibitors to decrease the production of OMVs in *Escherichia coli*.[157] Compared to the indiscriminate depletion of the GM through antibiotics, the targeted regulation of dysbiotic bacteria using BEV inhibitors is less invasive and has fewer side effects, while also tackling the issue of antimicrobial resistance. Beyond disrupting BEV production, another potential therapeutic approach is to block the inflammatory response and immune activation stimulated by BEVs. Ethyl pyruvate, for instance, can inhibit various immune signals triggered by LPS and *E. coli* OMVs, including the activation of the NLRP3 inflammasome and caspase-11-mediated pyroptosis.[158] A range of compounds, such as N-acetyl-L-cysteine, budesonide, and fluticasone, have demonstrated broad inhibitory effects, quelling the pro-inflammatory response elicited by EVs from various bacterial sources.[159] Despite the development of several drugs that target BEVs, the mechanisms underlying the actions of most of these compounds are not fully understood and necessitate further investigation.

Administration of probiotics to OA patients, such as *Lactobacillus* or *Bifidobacterium* strains, has demonstrated symptomatic relief.[160–163] Exogenous supplementation of BEVs for the treatment of OA can be approached from two angles: targeting the GM and directly targeting the joints. Research indicates that the transplantation of microbiota from metabolically impaired human donors into mice can accelerate OA progression.[19] There is evidence to suggest that modulating the GM through external interventions, such as probiotics, prebiotics, and fecal microbiota transplantation (FMT), may exert an influence on OA.[164] Prebiotics, probiotics, and postbiotics can selectively promote the growth of beneficial bacteria, thereby enhancing the production of GM that have beneficial effects on skeletal metabolism.[165] Nutrients from prebiotics can regulate the GM's composition and metabolism, potentially leading to improvements in OA symptoms and pain reduction. Among probiotics, the genus *Lactobacillus* has garnered significant interest. The presence of *Lactobacillus* and other bacterial species may influence the development of

OA.[166] For instance, a study by *Sim et al.* demonstrated that the oral administration of *Clostridium butyricum* to rats with OA effectively protected articular cartilage and diminished the quantity of fibrous tissue.[167] The supplementation with specific probiotics (such as *Lactobacillus paracasei*, *L. helveticus*, *L. casei*, *L. acidophilus*, and *Enterococcus faecium*) may offer benefits for skeletal health.[154] Probiotic BEVs might exert therapeutic effects similar to those of the probiotics themselves. Although research on the use of probiotic BEVs for treating OA is limited, studies on their role in treating other bone diseases like osteoporosis provide valuable insights. *Lactobacillus rhamnosus* BEVs, which contain various natural miRNAs, have been shown to promote osteogenesis and inhibit osteoclastogenesis in vitro.[168] FMT is a therapeutic procedure that involves the transfer of fecal matter from healthy donors to the distal gastrointestinal tracts of patients, aiming to treat diseases associated with microbiota imbalances. In a study involving diabetic mice, those treated with FMT exhibited decreased levels of GM-derived OMVs and experienced a reduction in the symptoms of diabetic nephropathy when compared to the untreated control group. Interestingly, the oral administration of fecal BEVs from diabetic animals has been shown to reverse these symptoms, indicating that the therapeutic benefits of FMT may be attributed to the vesicles secreted by the transplanted microbiota.[169] FMT, often referred to as a novel "organ transplant" technique, has the potential to regulate bone metabolism by correcting GM dysbiosis, normalizing intestinal metabolite levels, enhancing intestinal permeability, and modulating immune responses.[170] The oral administration of fBEVs from healthy individuals could elicit similar therapeutic effects as FMT. This approach is more convenient than the intricate screening and preparation process associated with FMT, and because it does not involve the introduction of foreign bacteria, it may result in fewer adverse reactions.[171]

External joint therapy can be enhanced by integrating EVs with biocompatible materials, such as hydrogels, which are then implanted into the affected joint areas. This approach aims to reinforce joint structure and mitigate the progression of OA. *Manyu Chen* and colleagues have developed an innovative application by encapsulating modified hybrid exosomes within methacrylic anhydride-modified hyaluronic hydrogel microspheres. These exosomes were designed to target chondrocytes and activate the FGF18 gene in OA chondrocytes at the genomic level in vivo, while the hydrogel component improved cartilage adhesion and lubrication. This cutting-edge biomaterial holds promise for promoting cartilage regeneration, reducing inflammation, and preventing the degradation of the extracellular matrix.[172] Although research on the use of BEVs in joint therapy is still limited, their potential in cartilage repair is significant. For instance, OMVs derived from *Lactobacillus johnsonii* have demonstrated the ability to alleviate synovitis, cartilage loss, and pain associated with OA.[112] The combination of probiotic BEVs with specialized materials may amplify the therapeutic effects of BEVs, as has been observed with MEVs.

Drug delivery

The unique avascular, dense, and sealed tissue structure of joints presents significant challenges for drug therapy in OA treatment.[7] To overcome these challenges, researchers have been developing nanoscale platforms for precise drug delivery over the past few decades. Traditional synthetic nanomaterials, such as metal-based nanoparticles, have been widely used as drug carriers. However, their exogenous nature can trigger immune recognition, which limits their ability to mimic complex intercellular interactions.[173] Consequently, cell membrane-coated nanoparticles (CNPs) have emerged as a promising alternative. CNPs are synthetic nanoparticles coated with natural cell membranes, endowing them with specific cellular properties like immune evasion and enhanced biocompatibility.[174,175] These nanoparticles can function effectively in complex biological environments and show great potential in biomedical applications. In this context, EVs have attracted attention as a targeted drug delivery system. EVs are nanoscale, spherical membrane structures with a phospholipid bilayer and an internal cargo space. These features confer low immunogenicity on EVs and enable them to carry a variety of bioactive molecules. Among EVs, BEVs are particularly attractive for their high production yields and ease of modification,

making them a promising platform for innovative drug delivery strategies.[23]

BEVs, as drug delivery vehicles, necessitate both targeting specificity and the capacity to encapsulate therapeutic molecules. For the pharmacotherapy of OA, intra-articular injection is commonly employed. However, due to the rapid metabolism of synovial fluid, the injected drugs often fail to remain effectively within the joint to achieve therapeutic concentrations. Therefore, when utilizing BEVs for the treatment of OA, it is often necessary to pair these with chondrocyte-targeting modifications. One approach involves targeting the extracellular matrix of cartilage. *Liu et al.* utilized genetic engineering to integrate collagen type II-targeting peptides onto the surface of MSC-derived EVs, thereby enhancing the affinity of these engineered vesicles for cartilage.[122] These cartilage-targeting EVs can adhere to cartilage structures over extended periods and effectively release their drug payloads. Another commonly used method is direct chondrocyte-targeting modification. Incorporating chondrocyte affinity peptides into the surface of engineered vesicles enables targeted delivery to chondrocytes.[172,176] These targeting peptides exhibit strong binding affinity to specific molecules in chondrocytes or the extracellular matrix. The modified vesicles can adhere to cartilage structures, thereby preventing drug loss caused by the metabolism of synovial fluid. Furthermore, the transient activation of subchondral osteoclasts has been recognized as a critical factor in the development of OA. Through plasmid transfection, the CXCR4 protein was introduced into *E. coli* BEVs, enabling these engineered BEVs to target bone tissue for drug delivery.[177] However, further investigation is required to determine if targeting bone tissue is equivalent to targeting subchondral bone specifically. Techniques such as genetic engineering and electroporation provide opportunities for BEVs to carry diverse payloads, including nucleic acids, proteins, metabolites, and more. The delivery of specific miRNA or siRNA to the joints has shown potential in alleviating OA symptoms. For example, when chondrocytes are treated with nanoparticles loaded with miR140 or MMP13 siRNA, the secretion of type II collagen is increased, effectively delaying joint cartilage damage.[123,178] Additionally, proteins like insulin-like growth factor 1 (IGF-1) and BMP-2, which are crucial for bone metabolism, have been shown to enhance chondrocyte activity and promote matrix formation.[7] These protein molecules offer valuable insights for the application of engineered BEVs in OA treatment.

The management of OA using BEVs holds great promise but also faces several challenges that need to be addressed. BEVs have unresolved biological mechanisms and unclarified endogenous contents. Moreover, the loading or expression of exogenous cargo in engineered BEVs is probability-dependent and hardly manipulable. To this end research efforts have been continuously made for natural and engineered BEVs to achieve scalable bioproduction, quality control, efficient packaging, and controlled release.[119]

Concluding remarks and outlook

In this review, we explore the role of BEVs in the gut-joint axis, specifically in the context of OA. OA is a prevalent and disabling joint condition that significantly affects the quality of life. Both experimental and clinical evidence indicate a correlation between the dysbiosis of the GM and the onset of OA. The GM plays a crucial role in maintaining joint health, and understanding the intricate interactions between the host and the microbiota is essential for a deeper comprehension of OA.[179] The GM does not directly interact with host cells; instead, it influences health through various mechanisms. Traditionally, substances implicated in regulating OA through the gut-joint axis are categorized into three main groups: membrane-associated cargo, cytoplasmic cargo and genetic cargo. However, these studies tend to emphasize the regulatory effect of a single substance, while in reality, the gut microbiota's modulation of OA involves the combined influence of multiple substances. No single molecule alone can fully control this process. Therefore, the relationship between the GM and OA should be analyzed from a more comprehensive perspective. An increasing body of evidence suggests that BEVs also play a significant role in this crosstalk between kingdoms.[107,180] In this review, we provide a comprehensive introduction to the concept of BEVs and compare the biosynthesis processes and cargo differences

between G^+ and G^- BEVs. This provides us with a new research avenue.

Bacteria-secreted EVs, known for their rich cargo, have the capacity to signal to the joints, thereby modulating host signaling pathways and cellular processes. The cargo they carry, including pathogen-associated molecular patterns and bioactive molecules, may be crucial for regulating osteoarthritis. With this understanding, we propose that BEVs are emerging as promising signaling carriers for long-distance interkingdom communication along the gut-joint axis. BEVs may exert substantial effects on both joint health and the pathology of OA. This review particularly emphasizes the roles of BEVs in regulating intestinal barrier integrity and immune-inflammatory responses. On one hand, dysregulated BEVs released from the GM have been shown to compromise the intestinal barrier and elicit abnormal immune-inflammatory responses. These actions can create a conducive environment for pathological events, suggesting a potentially causative role of BEVs in the development of OA. On the other hand, BEVs possess intrinsic therapeutic properties that are harnessed in probiotic therapy. Moreover, they can be engineered to improve outcomes in OA, offering a dual-edged potential: depending on the type and status of the bacteria, BEVs may either contribute to the disease process or serve as a therapeutic intervention.[181]

Given the regulatory mechanisms through which BEVs influence OA and the unique properties of these vesicles, we believe that BEVs offer considerable potential for the treatment of OA. We focus on utilizing BEVs for novel clinical treatment strategies, providing a summary of the most recent technologies and methodologies for the isolation, purification, and modification of BEVs. We innovatively posit that both naturally occurring and engineered BEVs offer extensive potential for application in the field of OA. Building on the current understanding of the physiological functions of BEVs, we delineate the prospective applications of BEVs as therapeutic agents in the screening, management, and adjunctive therapy of OA, all within the framework of the gut-joint axis. Although research on the relationship between BEVs and OA is still in its infancy, the ongoing exploration of BEV-based therapeutic strategies undoubtedly presents novel approaches for addressing OA and its associated complications. These efforts are instrumental in facilitating the clinical translation of BEVs for OA treatment.

Disclosure statement

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