Biofilm 8 (2024) 100216

Contents lists available at ScienceDirect

Biofilm

journal homepage: www.sciencedirect.com/journal/biofilm

Bacterial extracellular vesicle: A non-negligible component in biofilm life cycle and challenges in biofilm treatments

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ARTICLE INFO

Keywords:
Adaptive response
Biofilm formation
Anti-Biofilm
Transportation
Horizontal gene transfer

ABSTRACT

Bacterial biofilms, especially those formed by pathogens, have been increasingly impacting human health. Bacterial extracellular vesicle (bEV), a kind of spherical membranous structure released by bacteria, has not only been reported to be a component of the biofilm matrix but also plays a non-negligible role in the biofilm life cycle. Nevertheless, a comprehensive overview of the bEVs functions in biofilms remains elusive. In this review, we summarize the biogenesis and distinctive features characterizing bEVs, and consolidate the current literature on their functions and proposed mechanisms in the biofilm life cycle. Furthermore, we emphasize the formidable challenges associated with vesicle interference in biofilm treatments. The primary objective of this review is to raise awareness regarding the functions of bEVs in the biofilm life cycle and lay the groundwork for the development of novel therapeutic strategies to control or even eliminate bacterial biofilms.

1. Introduction

Bacterial biofilms are aggregates of bacteria encapsulated by self-produced extracellular polymer matrix (EPS) that functions as a shield to help the bacteria adapt to the living environment [1,2]. However, biofilm formation by pathogenic bacteria poses significant concerns due to their impact on human health and economic losses. Statistical reports from the National Institutes of Health (NIH) have indicated that biofilms are responsible for 65 % of all microbial clinical infections and 80 % of chronic infections [3]. In 2019, the estimated global cost of biofilm-related medical expenses and public health implications amounted to $386.8 billion [4]. Among the numerous challenges associated with bacterial biofilms, the development of antibiotic resistance stands out as a significant obstacle that compromises the effectiveness of biofilm treatments [5]. The extensive research on biofilms has thus expanded from a focus solely on biofilm formation to encompass the entire life cycle, from initial attachment to biofilm formation and subsequent detachment [1,6]. However, the detrimental impact of biofilms on the successful treatment of human infections necessitates a thorough understanding of the underlying mechanisms. This ongoing and challenging endeavor is crucial in addressing the persistent issue of biofilms and improving overall treatment outcomes.

Since the life cycle and resistance of bacterial biofilms are extensively studied (see reviews [1,5,7,8], this review aims to expand the existing knowledge by focusing specifically on an essential component of the biofilm matrix: bacterial extracellular vesicles (bEVs) [9–12]. bEVs are evolutionarily conserved sub-cellular structures that naturally derived from bacterial cells [13,14], contributing to inter- and intra-species bacterial communication such as biofilm development, virulence, and colonization [15–17]. Therefore, illustrating the function of bEVs in biofilms is critical to elucidate interactions between bacteria and explore potential strategies to prevent, inhibit, and eradicate

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https://doi.org/10.1016/j.biofilm.2024.100216
Received 7 April 2024; Received in revised form 23 July 2024; Accepted 24 July 2024
Available online 25 July 2024

biofilms. Growing evidence has highlighted the direct role of bEVs in influencing biofilms by actively participating in the biofilm life cycle [18–26]. This review provides an overview of the features and biogenesis of bEVs, as well as the current understanding of the functions of bEVs in the biofilm life cycle. We also address the challenges associated with bEV interference in biofilm treatments. Collectively, the updated information will provide valuable insights into the field of bEVs and may assist in designing effective control measures against bacterial biofilms.

2. Overview of bEVs in gram-negative and gram-positive bacteria: biogenesis and features

The understanding of bEV biogenesis and features is essential for investigating their role in biofilms, as their components and production have been found to influence biofilm development [19,23]. The difference in the structure of Gram-negative and Gram-positive bacteria leads to variations in vesicle features and their biogenesis processes (Fig. 1). Gram-negative bacteria mainly release outer membrane vesicles (OMVs), while vesicles originated from the cytoplasmic membrane referred to as membrane vesicles (MVs) are mainly released by

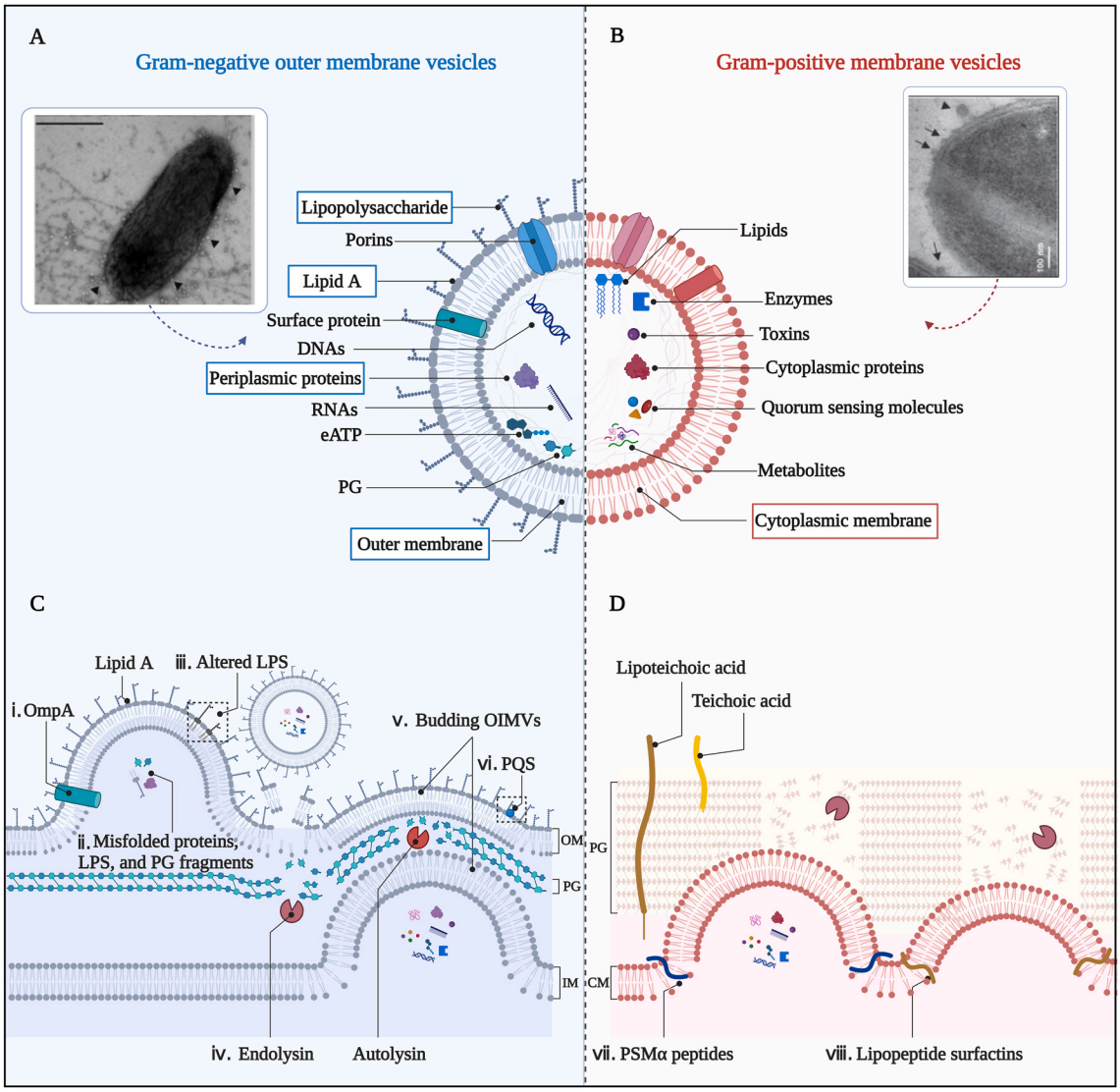

Fig. 1. Biogenesis and features of Gram-negative and Gram-positive bacteria vesicles. (A) The feature of Gram-negative vesicles (*Pseudomonas aeruginosa*) is reprinted with permission from Ref. [12]. (B) The feature of Gram-positive bacteria (*Staphylococcus aureus*) vesicles is reprinted with permission from Ref. [119]. Left: vesicles in *P. aeruginosa* biofilms, right: vesicles released by *S. aureus*. The contents in the blue box and red box are exclusively found in Gram-negative and Gram-positive bacteria vesicles, respectively. (C) Biogenesis model for vesicles in Gram-negative bacteria, including (i) the loss of linkages that bound the outer membrane and peptidoglycan, like outer membrane protein A (OmpA) [27]; (ii) the releasement of membrane stress by transporting misfolded proteins, LPS, and peptidoglycan (PG) fragments from periplasm [28]; (iii) membrane bending influenced by LPS composition [29]; (iv) endolysin Lys-mediated explosive cell lysis [31], (v) outer-inner membrane vesicles (OIMVs) originated from inner membrane are released by autolysin degradation of peptidoglycan [30]; (vi) PQS interacts with LPS [52]. Similarly, in Gram-positive bacteria (D), a decrease in turgor pressure, peptidoglycan cross linkers, the autolysin activity, and lipid geometry also facilitate vesicle blebbing in Gram-positive bacteria. (vii) Phenol-soluble modulins (PSMs), especially PSMα peptides targeting the cytoplasmic membrane combined with cell wall degradation by autolysin Sle1 [34]. (viii) Alternatively, lipopeptide surfactins disrupt membranes [35,36]. PQS: *Pseudomonas* quinolone signal. eATP: extracellular adenosine triphosphate. OM: outer membrane, PG: peptidoglycan; IM: inner membrane, CM: cytoplasmic membrane. The Blue ring presents vesicles derived from Gram-negative bacteria. The red ring presents vesicles derived from Gram-positive bacteria. The box indicates the site of action. The black LPS presents the altered LPS. All figures were created with BioRender.com. Adapted with permission from Ref. [27]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Gram-positive bacteria.

The proposed biogenesis models for bEVs involve different mechanisms (Fig. 1C and D). In Gram-negative bacteria, these mechanisms include: (i) releasement of membrane stress. bEVs are formed by transporting misfolded proteins, lipopolysaccharide (LPS), and peptidoglycan (PG) fragments from the periplasm, thus relieving membrane stress; (ii) loss of linkages. The loss of linkages that bind the outer membrane and peptidoglycan, such as the loss of outer membrane protein A (OmpA) can contribute to bEV release; (iii) membrane bending. The composition of LPS can influence membrane bending, which promotes the formation of bEVs; (iv) outer-inner membrane vesicles (OIMVs). OIMVs, originating from the inner membrane, can be released through the degradation of peptidoglycan by autolysin; (vi) endolysin Lys-mediated explosive cell lysis. Endolysin Lys can induce explosive cell lysis, leading to the release of bEVs; (vii) interaction with *Pseudomonas* quinolone signal (PQS). PQS can interact with lipid A of LPS, inducing membrane curvature and contributing to vesicle production not only in Gram-negative but also in Gram-positive bacteria [27–33]. Similarly, in Gram-positive bacteria, vesicle blebbing is facilitated by factors such as decreased turgor pressure, peptidoglycan cross-linkers, autolysin activity, and lipid geometry. Additional factors include phenol-soluble modulins (PSMs), particularly PSMα peptides that target the cytoplasmic membrane, autolysin Sle1-mediated degradation of the cell wall, and lipopeptide surfactins that disrupt membranes [34–36].

bEVs are heterogeneous in size, of which OMVs are typically between 10 and 300 nm in diameter [37], OIMVs are 60–160 nm [30] and the diameter of MVs ranges from 200 to 400 nm [37]. Vesicles transport various cellular components, such as lipids, proteins, genetic material (DNA/RNA), extracellular adenosine triphosphate (eATP), and signaling molecules [14,16,23,38,39]. Vesicles of Gram-negative bacteria carry LPS and OmpA specifically, whereas in Gram-positive bacteria, lipoteichoic acid is contained [37]. The encapsulation of vesicle cargoes is not a random packaging but a well-regulated process, proved by preferentially packaging OM lipoproteins into OMVs [40] and enriching specific RNA categories (eg., small RNAs and messenger RNAs) into vesicles from both Gram-negative and Gram-positive pathogenic bacteria [41,42].

3. Functions and mechanisms of bEVs in the biofilm life cycle

Growing evidence has shown that bEVs when exogenously added directly influenced inter- and intra-species interactions during various stages of biofilms (Table 1), including initiation, growth and maturation, and dispersion stages [1,19,20,23,43]. Besides, substantial reports have indicated disparities in size, quantity, and composition between biofilm and planktonic-derived vesicles (bEVs). These differences offer valuable insights into exploring the connection between bEVs and biofilms [11,23,44]. Thus, in this section, we aim to outline the known mechanisms underlying the functions of bEVs in the biofilm life cycle.

3.1. bEVs affect cell aggregation and attachment

The ability of bacteria to attach to cell/substrate surfaces is crucial for biofilm formation. Considerable evidence supports the notion that bacteria preferentially adhere to nonpolar and low-energy hydrophobic surfaces, which facilitates the formation of robust biofilms [45–48]. Interestingly, it has been found that the release of vesicles can impact bacterial adhesion and further enhance biofilm formation by modulating cell hydrophobicity [43,49,50] (Fig. 2 A).

According to prior research, various molecules, including proteins and lipids, can influence the surface hydrophobicity of bacterial cells, thus impacting the properties of the cell surface [51]. This understanding sets the stage for exploring the intriguing role of vesicles, which are postulated to provide biological advantages for hydrophobic interactions via their luminal or membrane-anchored contents [12,38,52]. For example, the presence of OMPs with hydrophobic pockets, serving as binding sites for hydrophobic molecules and facilitating their transport across the outer membrane, could contribute to increased cell surface hydrophobicity in *Pseudomonas putida* [43,49,53]. Additionally, adding MVs can enhance the relative ratio of proteins to carbohydrates and influence the non-ionization of carboxyl groups in fatty acids in the *Staphylococcus aureus* biofilm matrix, thus promoting biofilm formation by enhancing hydrophobic interactions between MVs and bacterial cells [50,54]. Further exploration is needed to ascertain which specific components of the vesicles modulate hydrophobicity and elucidate the mechanism by which vesicles promote biofilm formation through their impact on hydrophobicity.

Besides hydrophobic interaction, extracellular DNA (eDNA) has been

Table 1
The functions of bEVs in biofilms and proposed mechanisms.

Species/Strain	Method	Description	Mechanism	Reference
Gram-negative bacteria				
Helicobacter pylori	Addition of the OMVfraction	Biofilm formation was enhanced in a dose-dependent manner	–	[102]
Pseudomonas aeruginosa	Exogenous addition of purified OMVs	Only $PaAP^{+}$ OMVs exhibit antibiofilm activity	Endogenous protease	[120]
Francisella tularensis	Supplementing purified vesicles in cell suspension	Biofilm formation was enhanced	Providing protection against fluoroquinolone	[121]
Aeromonas strain	Exogenous addition of purified OMVs	The biofilm-forming ability was enhanced in a dose-dependent manner	Proteins presented in the OMVs	[25]
Burkholderia thailandensis	Exogenous addition of purified OMVs	Inhibit biofilm biomass and integrity in *Streptococcus mutans*	–	[24]
Pseudomonas aeruginosa	Compared the phospholipid components between biofilm and planktonic culture	Membrane is significantly more rigid in biofilms	Reprogramming of membrane fluidity	[23]
Vibrio cholerae	Exogenous addition	Increase cell-aggregate formation	Carrying outer membrane proteins	[73]
Gram-positive bacteria				
Staphylococcus aureus	Exogenous addition of cell-free supernatant	Inhibit *Acinetobacter baumannii*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, and *E. coli* strains to form biofilms	Modifying the surface to be more hydrophilic	[122]
Staphylococcus aureus	EVs supernatant cultured in the presence of vancomycin	Biofilm formation increased	Increasing surface hydrophobicity	[50]
Streptococcus mutans	Inoculated with MVs at various concentrations at pH 6.0	Biofilm formation increased	Glucosyltransferases and extracellular DNA (eDNA)	[22]
Lacticaseibacillus casei	Exogenous addition of purified vesicles	Affect the early stages of *S. Enteritidis* biofilm development and prevented attachment of bacteria to polystyrene surfaces	Peptidoglycan hydrolases	[21]

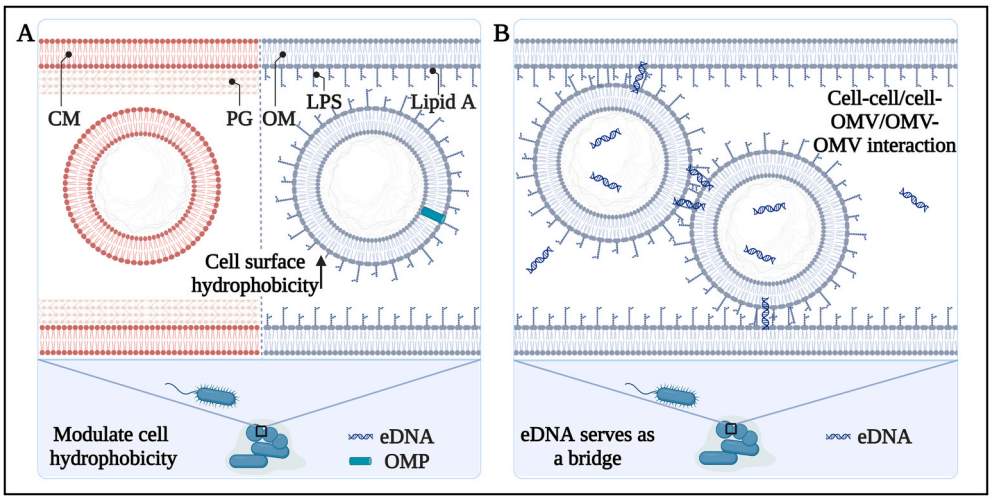

Fig. 2. bEVs affect cell aggregation and attachment. (A) Vesicle components can affect cell aggregation by modulating membrane hydrophobicity. Left: in *Staphylococcus aureus*, the adding of MVs can enhance the relative ratio of proteins to carbohydrates and influence the non-ionization of carboxyl groups in fatty acids in the biofilm matrix, thus promoting biofilm formation by enhancing hydrophobic interactions between MVs and bacterial cells. Right: in *Pseudomonas putida*, OMPs with hydrophobic pockets could serve as binding sites for hydrophobic molecules and contribute to increased cell surface hydrophobicity. (B) Membrane-encapsuled/anchored eDNA serve as bridges for OMV-OMV and OMV-cell interactions and increase cell adherence. eDNA, extracellular DNA. OMPs, outer membrane proteins. OMV, outer membrane vesicle. The Box indicates the site of action. The red ring presents vesicles derived from Gram-positive bacteria. CM: cytoplasmic membrane, PG: peptidoglycan, OM: outer membrane, LPS: lipopolysaccharide. The blue ring presents vesicles derived from Gram-negative bacteria. The up arrow indicates that the increased hydrophobicity caused by vesicles is associated with elevated cell aggregation and attachment. All figures were created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

widely accepted as an essential contributor to biofilm formation, adhesion, and structural maintenance [55–57]. In the early stage of biofilms, eDNA can penetrate the repulsive electric double layer to enhance adhesion, while adhesion is achieved through acid-base interactions [58]. While eDNA has been proposed to act as a bridge for interactions between OMVs and cells within the biofilm, as well as between OMVs themselves [59,60] (Fig. 2 B). The interactions between vesicles and DNA occur through salt-bridging and electrostatic interactions, resulting in an increased negative charge that can influence potential interactions with the extracellular polymer matrix (EPS) [22,59,60].

3.2. bEVs affect growth and accumulation of attached/aggregating cells

Following the initial attachment or aggregation, bacterial colonies expand by growing and recruiting the adjacent cells. In this process, bEVs can serve as an alternative strategy to meet nutrient requirements, provide adhesive factors, and facilitate bacterial communication, particularly through the carrying of QS molecules.

3.2.1. bEVs provide alternative source of nutrients and proteins as adhesive factors

bEVs can scavenge nutrients such as eATP and iron, which are essential in mediating the transition from a planktonic state to a biofilm state and result in biofilm-associated persistence and infection (Fig. 3 A) [2,17,61–64]. Much interest has thus been focused on the relationship between bEVs, eATP/iron, and biofilm formation.

Notably, eATP is encapsulated within vesicles as a cytoplasmic component and benefits from the protection provided against apyrase treatment [23,65]. eATPs are accumulated when bacteria switch to biofilm-forming state, which can induce cell lysis and releasement of eDNA, leading to an increment of bacterial attachment and biofilm formation [66]. This could be the reason why the level of vesicle-encapsulated eATPs in biofilms is approximately two-fold higher compared to that of planktonic bacteria in *Pseudomonas aeruginosa* [23]. In addition to being released by bacteria, eATP can also be released by damaged host tissues during bacterial infections [66]. Therefore, future studies should be conducted to investigate the mechanism of eATP encapsulation by vesicles and compare it with the role of free eATP in biofilm development. On the other hand, bacteria can recruit vesicles when iron is limited (predominantly found in OMVs), and capture ferric iron by vesicle-associated siderophores. These siderophores can bind and transport free iron, delivering it to bacteria through recognition of specific cell surface receptors and may induce biofilm-related regulatory systems (eg., QS) [67–69]. Take the example of PQS in *P. aeruginosa*, iron can be chelated by PQS and directly bind to TseF, a type VI secretion system effector involved in iron uptake [69]. The Fe^{3+}-PQS complex, along with TseF, can be incorporated into OMVs and subsequently recognized by the FptA (Fe(III)-pyochelin) receptor, and/or the OprF porin on the membrane [69]. In *P. aeruginosa*, the majority of matrix-associated proteins were found in OMVs, particularly in biofilm-derived OMVs [67]. These proteins include outer membrane receptor proteins involved in iron acquisition.

Additionally, vesicle components, particularly adhesion proteins can influence the matrix development and stability of the biofilm structure (Fig. 3B). Apart from being secreted into the matrix via the type II secretion system (T2SS), OMVs can provide another source of adhesive factors, such as rugosity and biofilm structure modulator A (RbmA), RbmC, and biofilm-associated protein 1 (Bap1) (Fig. 3B) [70,71]. RbmA plays a crucial role in the initial stages of biofilm development by facilitating the attachment of subsequent cells to the founder cell [72]. OMVs carry matrix proteins RbmA, RbmC, Bap1, and *Vibrio* polysaccharide (VPS), in which VPS can directly interact with these proteins and contribute to the biofilm architecture [71–73]. These findings provide direct evidence of the involvement of OMVs-associated proteins in cell-cell and cell-surface adhesion at growing biofilms in *Vibrio cholerae*.

3.2.2. bEVs facilitate quorum sensing communication

Quorum sensing (QS) is a bacterial communication system that allows cells to coordinate their behavior when reach threshold population density. Actually, signal-receiving bacteria can sense QS molecules that freely diffuse across complex environments and trigger QS-based biofilm development, including in biofilm formation and dispersion [75,76]. In this section, we want to emphasize the function of QS in biofilm formation, especially the involvement of bEVs.

In addition to the canonical model of QS [77], bEVs can package and

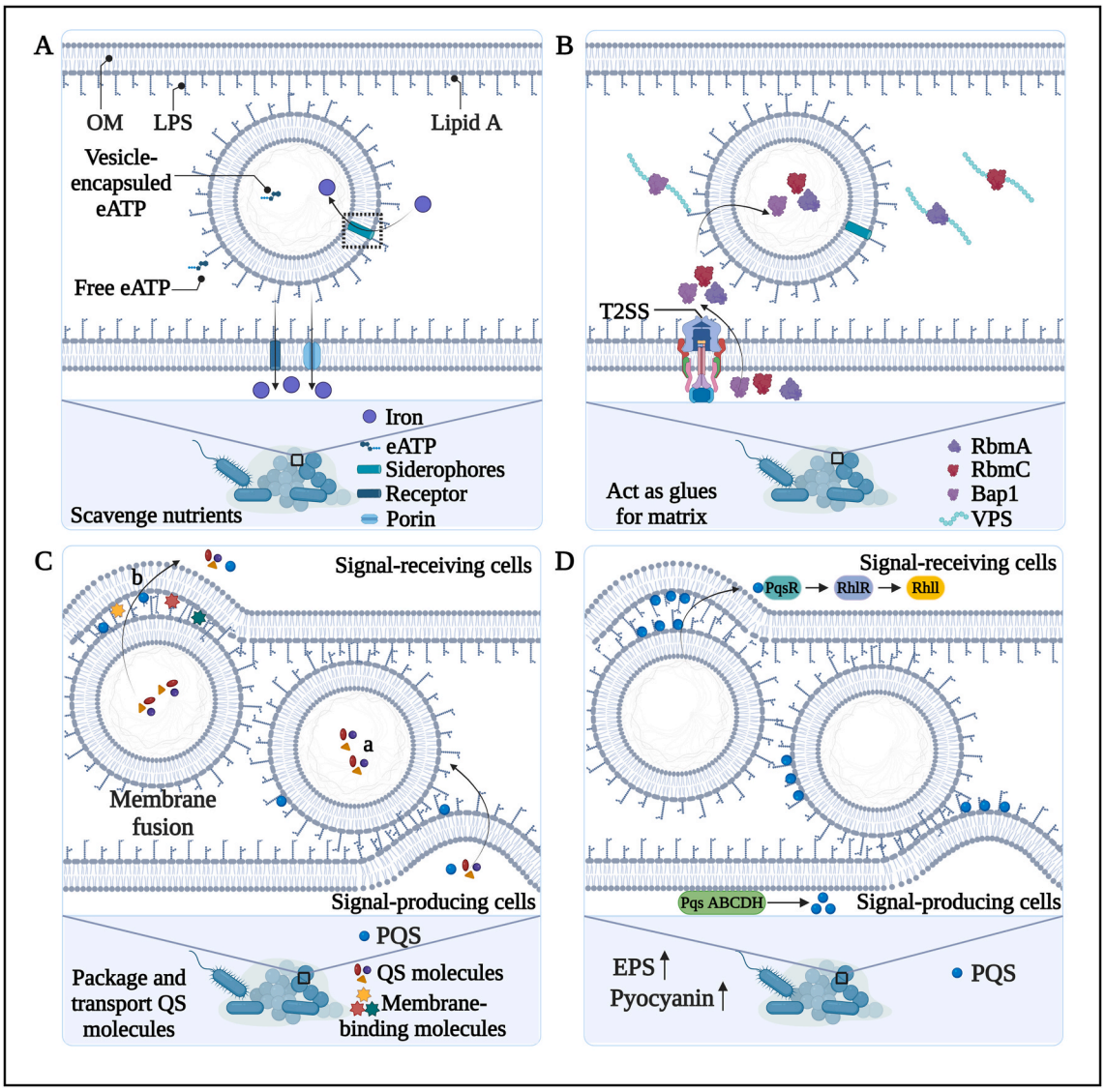

Fig. 3. bEVs affect growth and accumulation of attached/aggregating cells. (A) bEVs can scavenge nutrients such as eATP and iron. Bacteria can capture ferric iron by vesicle-associated siderophores and deliver into bacteria through recognition of cell surface receptor or porin involved in iron acquisition. (B) In *Vibrio cholerae*, adhesive factors RbmA, RbmC, and Bap1 that are secreted into the matrix via T2SS could be captured by OMVs. OMVs carry matrix proteins RbmA, RbmC, Bap1, and *Vibrio* polysaccharide (VPS), in which VPS can directly interact with these proteins and contribute to the biofilm architecture [71–73]. (C) bEVs facilitate quorum sensing communication by concentrating signal molecules (a) and providing unhindered passages (membrane fusion) (b). Membrane-binding compounds such as PQS, nisin, colistin, and polymyxin B can induce membrane fusion and facilitate fusion with recipient cells [87,88]. (D) In *P. aeruginosa* (*Pseudomonas aeruginosa*), PQS is produced by PqsABCDH and bound by PqsR receptor. PqsR-PQS activates *rhl R*and *rhlI* expression. PQS carried by vesicles can upregulate the *rhlRI* QS system and stimulate the secretion of EPS [79,84–86]. And PQS-containing OMVs also increase pyocyanin production, a secondary metabolite involved in biofilm formation [84,89]. eATP, extracellular adenosine triphosphate. T2SS, type II secretion system. VPS, *Vibrio* polysaccharide. RbmA, Rugosity and biofilm structure modulator A. RbmC, Rugosity and biofilm structure modulator C. Bap1, Biofilm-associated protein 1. QS, quorum sensing. EPS, extracellular polymer matrix. PQS: *Pseudomonas* quinolone signal. The box indicates the site of action. OM: outer membrane, LPS: lipopolysaccharide. The blue ring presents vesicles derived from Gram-negative bacteria. The dotted box indicates iron is captured by siderophores. The up arrow indicates that the production of EPS and pyocyanin is increased. All figures were created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transport QS molecules to assist QS response, providing two main advantages, namely unhindered transportation and enrichment of signal molecules [15,78,79] (Fig. 3C). Recently, a stochastic reaction-diffusion model of QS [15] provided further evidence supporting the notion that bEVs can enhance QS response by reducing the time and cell count required for activating the expression of QS-responsive genes. To facilitate the passage of hydrophobic QS molecules across the barrier of the cell envelope, bEVs can fuse with the target membrane in both Gram-positive and Gram-negative bacteria, thus providing unhindered transport [80–83]. Membrane-binding compounds such as PQS, nisin, colistin, and polymyxin B induce membrane curvature and facilitate fusion with recipient cells [87,88]. In *P. aeruginosa*, hydrophobic QS molecules like PQS (synthesized by pqsABCDH gene cluster) are packaged by OMVs due to their limited diffusion in an aqueous environment [84–86] (Fig. 3D). It is worth noting that *P. aeruginosa* OMVs are able to capture approximately 86 % of the PQS produced by the bacteria [84]. The PQS-containing OMVs play a significant role in the upregulation of the *rhlRI* QS system and the stimulation of EPS secretion. This process, in turn, promotes the formation of biofilm [79]. Besides, PQS-containing OMVs increases pyocyanin production, a secondary metabolite that is retained in the biofilm matrix by interacting with eDNA [84,89]. Moreover, the presence of CAI-1, a hydrophobic QS molecule, within

OMVs in *Vibrio harveyi* is capable of triggering an intracellular QS cascade even in cells that do not naturally produce CAI-1 [90]. Although further investigations are required to fully explore the impact of OMV-mediated communication on biofilm evaluation, this discovery underscores the involvement of vesicles in both inter-species and intra-species communication.

3.3. bEVs mediate disaggregation and detachment

Biofilms are in a constant state of dynamic balance, with approximately 30 % of the biofilm cells transitioning into planktonic cells through a process known as biofilm dispersion, resulting in the releasement of bacteria and the recurring formation of biofilms [6,91]. This dispersion process involves the degradation of the biofilm matrix, including extracellular polysaccharides, proteinaceous components (such as adhesins and amyloid fibers), and eDNA. Additionally, QS molecules, such as c-di-GMP, play a role in modulating the biological activities and physical interactions during dispersion [6].

Matrix-degrading enzymes are contained within bEVs and vesicles confer a protective effect for enzymatic activity (Fig. 4) [20,92–95]. For instance, proteomic analysis of *Salmonella pullorum* OMVs revealed the presence of enzymes like glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, and lactate dehydrogenase, suggesting their potential role in degrading carbohydrates and disrupting biofilm polysaccharides [96]. OMVs of *P. aeruginosa* package EPS-degrading enzymes that contribute to dispersal of biofilm [20]. PaAP (*Pseudomonas aeruginosa* aminopeptidase) has been found to facilitate the dispersal of established biofilm architecture not only in *P. aeruginosa* ΔPaAP, but also in *Klebsiella pneumoniae* [19]. PaAP brings about significant changes in the biofilm matrix in a vesicle-dependent manner and biofilm detachment is attributed to its indirect effect on OMV cargo [19,97]. It has been observed that endogenous protease activity is increased and localized within secreted OMVs [19]. Thus further research is required to determine whether the process involves one or multiple endogenous proteases and to understand the specific role of proteases in this mechanism. Moreover, *P. aeruginosa* exhibits elevated production of OMVs during biofilm dispersion, accompanied by increased synthesis of PQS compared to other stages [20]. Interestingly, in contrast to its role in biofilm formation (as shown in Fig. 3D), PQS regulates biofilm dispersion in a signal-independent manner (eg., induce OMV production) rather than its receptor PqsR, to orchestrate the dispersal process [20]. Further investigations are required to determine whether PQS-laden OMVs are involved in activities that contribute to biofilm dispersion, such as matrix degradation.

4. Challenges posed by bEVs in biofilm treatments: adaptive response against external stresses

Anti-biofilm strategies, including traditional disinfection/antibiotic treatments (eg., hydrogen peroxide/sodium hypochlorite and imipenem/ceftazidime), physical (eg., ultrasonic eradication and magnetic fields treatment), and biochemical (eg., phage lysins, degradative enzymes, metabolites, and nitric oxide) methods have been discussed [98–100]. However, previous reports suggest that vesicle biogenesis is a stress response process, which could not only alleviate the effect of anti-biofilm strategies but also strongly correlate with biofilm formation [54,101,102]. The following section will discuss the main challenges posed by bEVs during biofilm treatments, namely mediating horizontal gene transfer (HGT) and defense as decoys (Fig. 5).

4.1. bEVs defend as decoys in anti-biofilm treatments

One of the main reasons for the hindering effect of bEVs on anti-biofilm strategies is their ability to act as decoys when exposed to external stresses, such as antibiotics, phages, and antimicrobial peptides. Vesicles provide a protective effect on cells against stresses, potentially by carrying porins and receptors [72,103,104]. Porins, which play a role in permeating antibiotics to intracellular targets, can trap antimicrobials and have been found to be anchored on bEVs [74,105,106]. For instance, the porins OprB, OprD, and OprE, which are dominant in OMVs of *P. aeruginosa* biofilms, have been reported to be the targets for antimicrobial peptidomimetics (synthesized based on the antimicrobial peptide protegrin I) that provides high efficacy against *P. aeruginosa* [12,107]. Among porins, the amino acid-specific one OprD has been found to be the main reason for carbapenem resistance and the sequestration OprD increases bacterial resistance [12]. In *V. cholerae*, Bap1 is found to interact with the integrin binding domain (LDV peptide) of an abundant OMP, OmpT porin, which traps antimicrobial peptide (AMP) LL-37 and

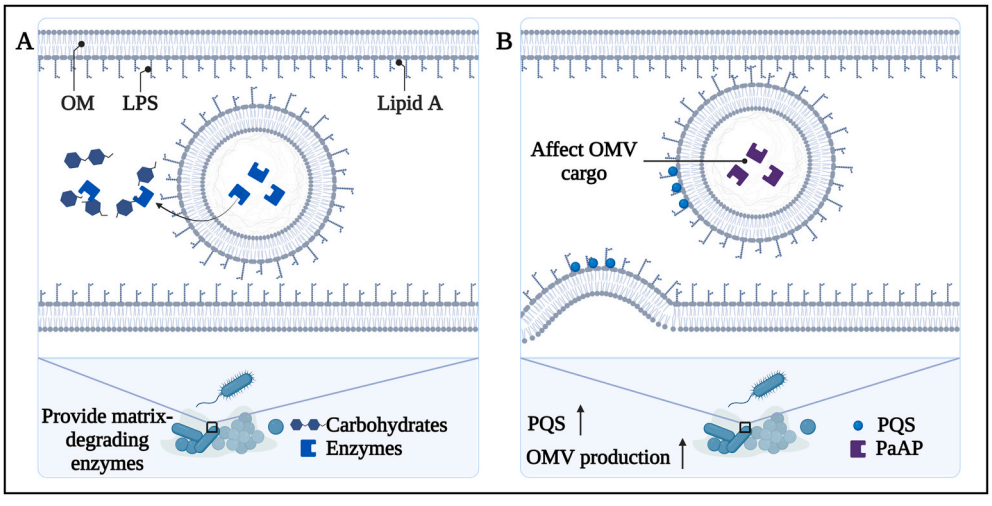

Fig. 4. bEVs mediate disaggregation and detachment. (A) Enzymes encapsulated by OMVs can degrade biofilm matrix. Enzymes like glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, and lactate dehydrogenase in *Salmonella pullorum* OMVs have potential role in degrading carbohydrates and disrupting biofilm polysaccharides [96]. (B) In *Pseudomonas aeruginosa*, PaAP mediates biofilm detachment by its indirect effect on OMV cargo [19,97]. PQS regulates biofilm dispersion in a signal-independent way and total PQS and OMVs production is significantly elevated during the dispersion stage [20]. OMV, outer membrane vesicle. PQS, *Pseudomonas* quinolone signal. PaAP, *Pseudomonas aeruginosa* aminopeptidase. The box indicates the site of action. OM: outer membrane, LPS: lipopolysaccharide. The blue ring presents vesicles derived from Gram-negative bacteria. The up arrow indicates that the production of PQS and OMVs is elevated. All figures were created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

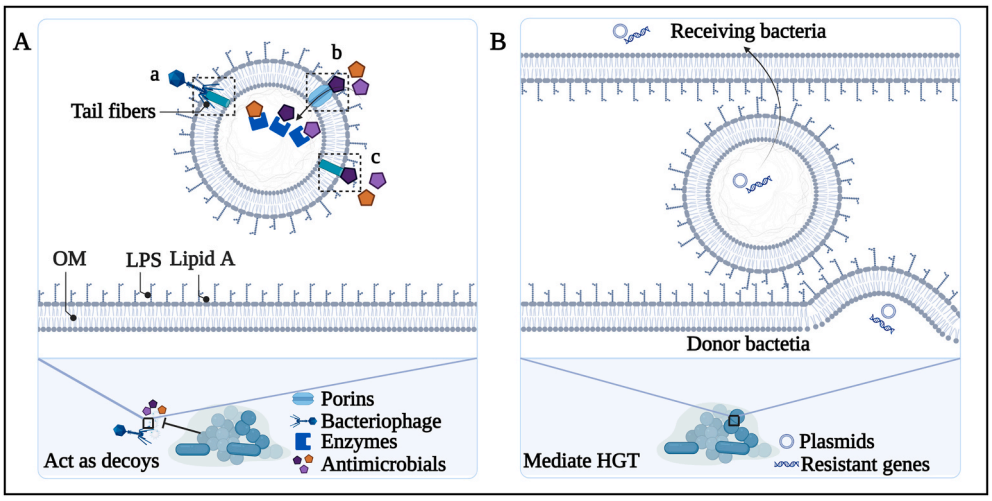

Fig. 5. Challenges posed by bEVs in biofilm treatments: adaptive response against external stresses. (A) bEVs defend as decoys when exposed to external stresses, such as phages and antimicrobial peptides. (a) Take an example in *V. cholerae*, the outer membrane porin protein OmpU carried by OMVs can neutralize phages by interacting with their tail fibers, impeding phage adsorption to bacterial surfaces [71,109,110].Vesicles provide a protective effect on cells against stresses, potentially by carrying porins (b) and receptors (c) [72,103,104]. Bap1 is found to interact with the integrin binding domain (LDV peptide) of an abundant OMP, OmpT porin, which traps antimicrobial peptide (AMP) LL-37 and plays a role in OMV-mediated AMP resistance [74]. (B) bEVs mediate HGT in biofilms. In *P. aeruginosa*, biofilm-released vesicles are more efficient at packaging and transferring plasmids carrying antibiotic resistant genes to recipient cells compared to the planktonic ones [44]. HGT, horizontal gene transfer. The box indicates the site of action. OM: outer membrane, LPS: lipopolysaccharide. The blue ring presents vesicles derived from Gram-negative bacteria. All figures were created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

plays a role in OMV-mediated AMP resistance [74]. On the other hand, phage treatments against biofilms, particularly in infectious biofilms, can be compromised due to the binding of phages with bEVs [104,108]. The outer membrane porin protein OmpU carried by OMVs can neutralize phages by interacting with their tail fibers, impeding phage adsorption to bacterial surfaces in *V. cholerae* [71,109,110]. The biofilm-derived OMVs in *P. aeruginosa* contained significantly more drug targets compared to the planktonic OMVs [111]. Further research should be conducted on biofilms to gain a better understanding of the neutralizing effects and resistance mediated by bEVs.

4.2. bEVs mediate HGT in biofilms

Numerous studies have demonstrated that bacterial cells encapsulated within the biofilm matrix are more resistant to external stresses compared to planktonic cells (see review [5]. One of the most important reasons is horizontal gene transfer (HGT) in biofilms, in which bEVs serve as the fourth mode of HGT apart from transformation, transduction, and conjugation [112]. The production of vesicles is found to be promoted by low doses of antibiotics [113]. Furthermore, under antibiotic concentrations that facilitate the emergence of resistant variants, the enhanced transport of bEVs effectively enables the transfer of resistant genes [114–116]. A recent study revealed that biofilm-released vesicles are more efficient at packaging and transferring plasmids carrying antibiotic-resistant genes to recipient *P. aeruginosa* compared to the planktonic ones [44]. One hypothesis suggests that the inclusion of competence proteins in bOMVs (biofilm-derived OMVs) has the potential to enhance the HGT efficiency [117,118]. Further studies analyzing the functionality and tracking of the genes carried by vesicles in biofilms are needed to elucidate the exact mechanisms involved.

5. Conclusions and perspectives

In summary, bEVs play a crucial role in facilitating bacterial communication in biofilms. They modify hydrophobicity/hydrophilicity, mediate cell-surface interactions, serve as nutrient sources, facilitate signal transportation, and reprogram membrane properties. Furthermore, bEVs contribute to the survival of biofilms by mediating resistance through HGT and serving as bait targets with anchored porins or receptors, which compromise the effects of treatments on biofilms.

In light of the above, it is crucial to recognize the significance of bEVs when devising effective alternative strategies for treating biofilms. Understanding the mechanism behind vesicle-mediated biofilm development can pave the way for the creation of anti-biofilm strategies that specifically target the cargo carried by these vesicles. These cargoes include eDNA, eATP, adhesive factors, and QS, etc. Utilizing corresponding degrading enzymes makes it possible to effectively target and disrupt these vesicle cargoes. This targeted approach holds great potential in combating biofilm formation. Further research and exploration in this field are of paramount importance for the advancement of effective anti-biofilm approaches. Understanding the relationship between bEV production and biofilm formation is crucial in addressing strategies employed by pathogens to counteract the detrimental effects of antibiotics and other antibacterial treatments. It is important to determine if biofilm formation induces changes in bEV composition and production or if bEVs play a role in remodeling biofilm structures under stress. To gain a comprehensive understanding of this dynamic, meticulous tracking and analysis of bEV cargoes in both planktonic and biofilm cells are crucial. These investigations will unravel the mechanisms involved, contributing to advancements in this field and the development of more effective strategies against biofilms and associated pathogens. Given the growing evidence of bEVs' significance in biofilms, it is critical to uncover the underlying mechanisms and explore new avenues for prevention and control of harmful biofilms.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Natural Science Foundation of China [32272424], the Guangdong Technological Innovation Strategy of Special Funds (Key Areas of Research and Development Program) [2018B020205003], and the GDAS' Special Project of Science and Technology Development [2019GDASYL-0201001, 2022GDASZH-

2022010101].

CRediT authorship contribution statement

Nuo Chen: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Yangfu Li:** Investigation, Formal analysis. **Xinmin Liang:** Investigation, Formal analysis. **Keyuan Qin:** Investigation, Formal analysis. **Ying Zhang:** Investigation, Formal analysis. **Juan Wang:** Writing – review & editing, Project administration. **Qingping Wu:** Writing – review & editing, Supervision, Software. **Tanushree B. Gupta:** Writing – review & editing, Validation, Formal analysis. **Yu Ding:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yu Ding reports financial support was provided by National Natural Science Foundation of China. Qingping Wu reports financial support was provided by Department of Science and Technology of Guangdong Province. Qingping Wu reports financial support was provided by Guangdong Academy of Sciences. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

All figures are created with the help from BioRender.com.

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