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REVIEW

Outer Membrane Vesicles: Biogenesis, Functions, and Issues

Rokas Juodeikis,[a] **Simon R. Carding**[a,b]

[a]Quadram Institute Bioscience, Norwich, United Kingdom
[b]Norwich Medical School, University of East Anglia, Norwich, United Kingdom

SUMMARY This review focuses on nonlytic outer membrane vesicles (OMVs), a subtype of bacterial extracellular vesicles (BEVs) produced by Gram-negative organisms focusing on the mechanisms of their biogenesis, cargo, and function. Throughout, we highlight issues concerning the characterization of OMVs and distinguishing them from other types of BEVs. We also highlight the shortcomings of commonly used methodologies for the study of BEVs that impact the interpretation of their functionality and suggest solutions to standardize protocols for OMV studies.

KEYWORDS outer membrane vesicles

INTRODUCTION

Bacterial extracellular vesicles (BEVs) are small membranous vesicles released by bacteria which vary in origin, size, composition, and function. Recently, classification of

Address correspondence to Simon R. Carding, simon.carding@quadram.ac.uk.

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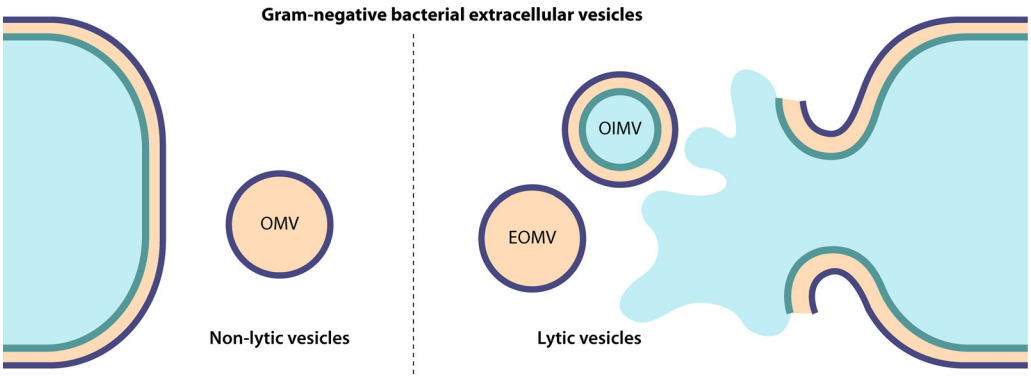

FIG 1 Three different types of bacterial extracellular vesicles produced by Gram-negative organisms via nonlytic and lytic mechanisms. OMV, outer membrane vesicle; EOMV, explosive outer membrane vesicle; OIMV, outer inner membrane vesicle; dotted line, inner membrane; dashed line, outer membrane; blue, cytoplasmic content; red, periplasmic content.

BEVs into four different types based on their membrane composition and origin has been proposed (1).

Three BEV types are produced by Gram-negative organisms: outer membrane vesicles (OMVs), generated by nonlytic release of the outer membrane; explosive outer membrane vesicles (EOMVs), generated by lytic release of the outer membrane; and outer inner membrane vesicles (OIMVs), generated by corelease of the inner and outer membranes (Fig. 1).

Due to their similar size, different types of BEVs copurify, leading to difficulties in assigning activities to individual subtypes and generalization of function that may only be attributed to a single type. An example of this is the perceived presence of DNA in OMVs, with its unequivocal presence only confirmed for other types of BEVs, such as OIMVs (2). This issue is discussed in more detail later.

OMVs generated by nonlytic methods can contain specific cargo and be considered an extension of the cell, or an extracellular bacterial organelle. Focusing on the generation and function of OMVs as well as general issues with commonly used methodologies, we highlight that OMV production is variable and dependent on the producing organism and its environment.

We also review the literature on functional studies investigating OMVs which normally do not distinguish between the types of vesicles present with the functions reported perhaps being attributable to other types of BEVs.

BIOGENESIS

Bacterial Physiology and Membrane Structure

Discussion of the formation of nonlytic OMVs requires an appreciation of the structure of the bacterial envelope. Two types of bacterial envelopes are recognized: Gram-negative and Gram-positive. Gram-positive envelopes are composed of the inner membrane (IM) and a peptidoglycan layer (PG) while Gram-negative cells are further encased in an outer membrane (OM) (Fig. 2A).

The IM is a phospholipid (PL) bilayer which confines the cytoplasm of the cell. Important processes, such as energy generation as well as lipid and PG biosynthesis are carried out on the IM. PG in combination with multiple IM proteins link the two layers together. The PG layer is made of repeating units of β-(1, 4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by peptide side chains and is often referred to as murein (3). This covalently linked structure provides rigidity to the cell and counteracts the osmotic pressure of the cytoplasm. The OM is an asymmetric bilayer with the inner leaflet containing PL while the outer layer principally contains lipopolysaccharides (LPS). Usually, LPS consists of two phosphorylated glucosamines bound to multiple fatty acids (lipid A) connected to a short chain of species-dependent

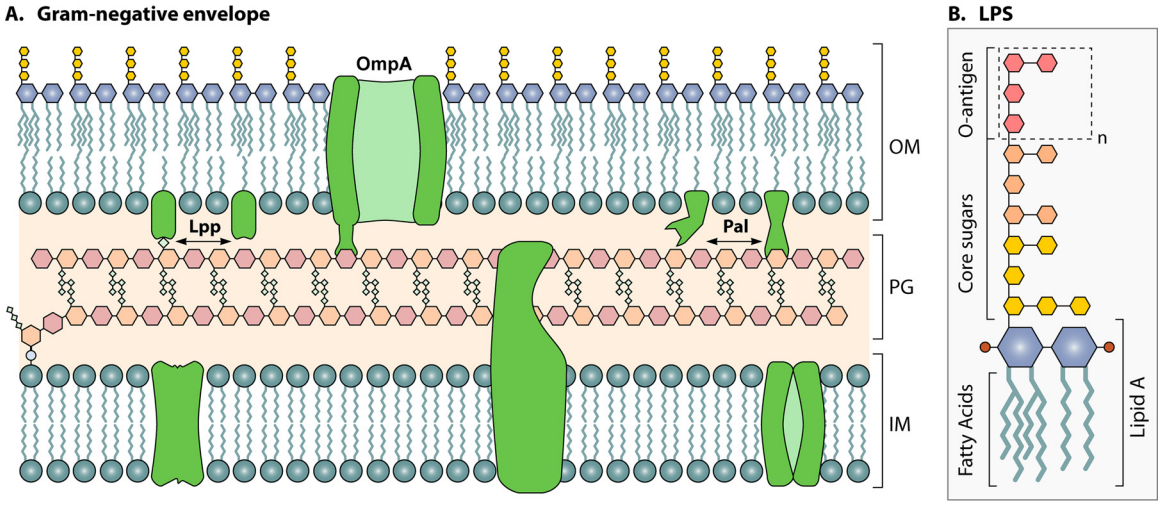

FIG 2 Gram-negative envelope and LPS structures. (A) In Gram-negative organisms, the envelope is made of the inner phospholipid membrane (IM) linked to the peptidoglycan layer (PG) of sugar polymers cross-linked via amino acids encased in the asymmetric outer membrane (OM) bilayer, where the inner leaflet contains phospholipids, and the outer leaflet is made of LPS. The different layers contain a variety of proteins (green), some of which cross-link the layers together providing structural rigidity. Three OM proteins are highlighted: the lipoprotein Lpp that covalently links PG and OM; the porin OmpA; and the lipoprotein Pal that binds PG noncovalently. (B) Lipopolysaccharide (LPS) contains several variable structural elements depending on the organism and environmental conditions. The Lipid A moiety contains a phosphorylated (blue circles) disaccharide attached to the hydrophobic fatty acids. Lipid A is decorated by a complex oligosaccharide referred to as core sugars, which displays the repetitive glycan polymer termed the O-antigen.

sugars (core sugars) followed by a long chain of repeating sugar units (O-antigen) (Fig. 2B). Most of the proteins that interact with the OM are either membrane-spanning beta-barrel proteins (OMPs) or lipidated proteins referred to as OM lipoproteins. To provide structural integrity, some OMPs and lipoproteins bind the PG layer no-covalently, while the lipoprotein Lpp can form a covalent linker between these layers (4–8).

OMV Generation Pathways

OMVs are generated when the OM is released from the cell. Of the two generally accepted methods for OMV formation, lytic (during cell lysis) and nonlytic (via outer membrane blebbing) we will focus our discussion on the latter and the four different mechanisms that have been described that require the release of peptidoglycan. These are, the reduction in local OM-PG connections, increase in local OM curvature, increase in periplasmic pressure, and flagellar release (Fig. 3).

The outer membrane-peptidoglycan connection. Since the OM is covalently linked to the PG layer and, via this connection, to the IM, this connection needs to be broken in order to release OM while excluding the IM. In the case of lytic OMVs, this is achieved by breakage of the PG layer as seen in *Pseudomonas aeruginosa* endolysin-triggered lysis. Such breakage leads to cell death and the release of a variety of BEVs, including lytic OMVs. For the generation of nonlytic OMVs, vesiculation depends on either breakage of this connection or increased spacing between the OM-PG linkage points (Fig. 3A).

Multiple studies have shown that weakening the OM-PG connection via gene deletion leads to hypervesiculation (9–12). However, these studies likely investigated mixtures of BEVs, as demonstrated by the presence of cytoplasmic and IM proteins in those that investigated the protein content of the vesicles produced (10). Recent evidence supports this view, as the increase in vesiculation in some of these mutants has been attributed to other types of BEVs, such as OIMVs, which likely result from cellular stress or the inability to manage the osmotic pressure (13). Below we discuss the proteins suggested to impact vesiculation, envelope stability, and OM vesiculation.

As mentioned previously, covalent linkage of OM to PG is dependent on Lpp (6, 7). Lpp is evenly distributed across the cell and is attached to the PG every 10 to 12 repeating units (7, 14, 15). Considering the length of a hexose is around 1 nm, and PG

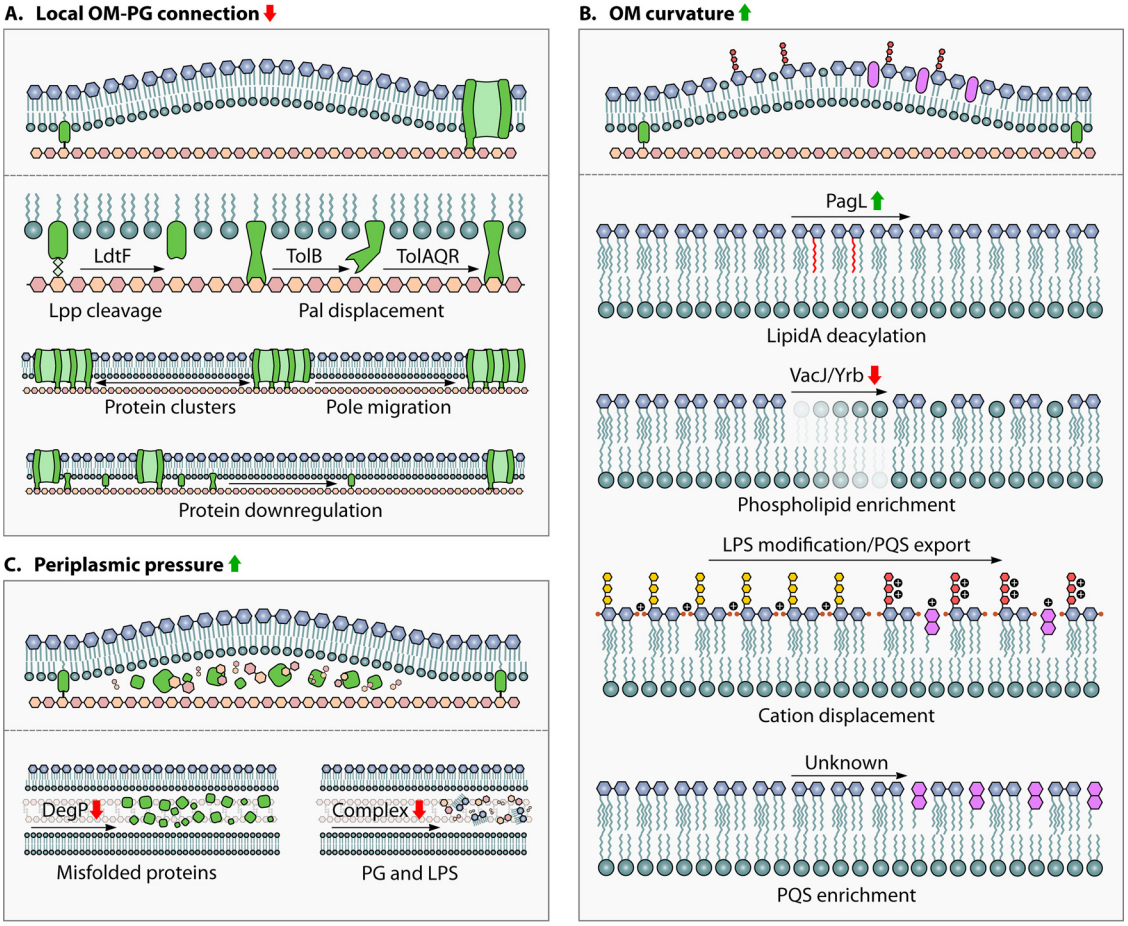

FIG 3 Mechanisms activating OMV production. Red arrows indicate downregulation of processes/proteins, while green arrows indicate increases, all of which lead to vesiculation. (A) Reduction of local OM-PG linkages. Lpp covalently links the OM-PG layers which can be cleaved by a specific periplasmic enzyme LdtF. Similarly, OM protein Pal binds PG; however, this binding can be displaced by TolB. TolB binding can be reversed by the TolAQR complex which is localized to the division septum. Additionally, OM proteins form protein clusters leading to naturally occurring zones of lower OM-PG connectivity, which are further enlarged by the migration of OM proteins to cell poles during growth. Finally, OM protein downregulation can reduce the number of OM-PG connections. (B) Increase in OM curvature. Lipid A deacylation by PagL can lead to increased OM curvature due to the change in the shape of the molecule. A similar effect is observed when the outer leaflet of the OM is enriched in phospholipids due to the downregulation of the VacJ/Yrb phospholipid transporter. Displacement of stabilizing cations by charged LPS or chelating molecules, such as PQS, can affect membrane curvature. Insertion of PQS in the outer leaflet of the OM also affects membrane curvature due to the shape of the molecule. (C) Increase in periplasmic pressure. Downregulation of the periplasmic protease/chaperon DegP leads to the build-up of misfolded proteins in the periplasm. Multiple gene deletions can lead to the build-up of periplasmic PG or LPS.

monomers comprise 2 hexoses, the spacing of OM-PG linkage via Lpp would be at least 20 nm. Furthermore, Lpp exists in equilibrium between the PG-bound and unbound forms, with evidence of a protein (LdtF) able to cleave Lpp-PG links, suggesting that this connection is more fluid than previously believed (16–19). Notably, deletion of *lpp* or disruption of its PG binding function leads to an increase in vesiculation; however, the vesicles observed are likely due to nonspecific release resulting from OM instability and are therefore more closely related to lytic OMVs (20–23). Subtle reductions of Lpp-PG linkage also induce vesiculation, while the inverse is true when such connections are increased (24). However, as these changes are generated by mutations in genes involved in PG synthesis, the effect may be due to overall stability of the PG rather than the OM-PG connection (24–26).

Although Lpp is the major OM-PG cross-linking protein, its deletion can be rescued by overproduction of Pal, whereas overproduction of Lpp does not rescue the *pal* mutant strain (12). Pal is an OM lipoprotein that noncovalently binds the PG layer and is part of the Tol-Pal system comprising five proteins: three inner membrane components

(TolQ, TolR, and TolA), the periplasmic TolB, and the OM lipoprotein Pal (27–29). The Tol-Pal complex is involved in lipid homeostasis and cell division where one of its roles is to capture and localize Pal to the division site (30–34). Pal binding to the PG can be interrupted by TolB, while TolB is only released from Pal by the TolAQR complex (34–36). During cell division, the TolAQR complex localizes to the cell poles, thereby effectively concentrating the Pal protein at the cell division site and reducing the Pal-PG connections at other sites. Mutations affecting the Tol-Pal system leads to a significant increase in vesicle generation. However, as with *lpp* mutants, such strains have a leaky and unstable OM, therefore the increase in vesiculation is likely due to an increase in the generation of vesicles resembling lytic BEVs (12, 21, 22, 37, 38). Furthermore, as the Pal-PG connection is dynamic, there is no need for its specific release during OMV generation.

OMPs cover most of the OM and, rather than being evenly distributed, form clusters governed by protein-protein interactions (39–42). These clusters tend to form around the β-barrel assembly machine (Bam) complex, which inserts new OMPs into the OM (43–45). During cell division, a variety of OMPs have been shown to migrate to cell poles (43). Multiple proteomic studies reveal that the majority of OMPs show lower relative concentrations in BEVs compared to the OM, suggesting their preferred exclusion or specific enrichment of other proteins (46–51). Given the ability of OMPs to migrate and their relative exclusion in BEVs, vesiculation may occur between these OMP islands which would stabilize the OM-PG connection. The inclusion of low concentrations of OMPs into the vesicles could be explained by the observed diffusion of OMPs from these islands (39–42).

A commonly studied OMP deletion, *ompA*, leads to hypervesiculation and alterations in vesicle cargo in different bacteria (10, 21, 52–54). OmpA binds the PG layer in a noncovalent manner and this binding is suggested to be Lpp-dependent (4, 55). As with *lpp* deletion, such deletion affects the OM stability and likely leads to the generation of other types of BEVs rather than the wild-type OMVs. This is supported by the observed change in the protein composition and size of such vesicles.

Mutant libraries have been used to identify proteins involved in OMV generation, however, to date no mutants resulting in the complete or undetectable absence of vesicle production have been generated. Most of these studies show that deletion of genes involved in maintaining the PG-OM connection increase vesiculation; however, they also show a significant change in the protein composition and size of such vesicles, suggesting new combinations of BEVs are generated. Notably, many studies claim that the mutants do not cause OM instability. However, the stability assays routinely used are direct cell lysis or growth inhibition, which are not necessarily good indicators of OM stress. A more complex study investigated the deletion of all the major candidate genes (*lpp*, *pal*, and *ompA*) and showed they effect OM stiffness in a similar manner to EDTA, supporting the view that the observed vesiculation arises from membrane instability and the resulting reduction in OM rigidity (56). This study also suggested that due to the mechanical characteristics of the OM during growth, the budding of OM occurs spontaneously (56).

It is possible that during growth, certain areas become naturally depleted of the OM-PG connection thereby leading to vesiculation due to turgor pressure with no requirement for a specific protein to release this connection. This is supported by evidence of vesiculation occurring during the exponential growth phase (57). Furthermore, as discussed later, hypervesiculation can be achieved without reducing Lpp-PG connections. It is likely that the OM-PG connection plays an inhibitory role in nonlytic OMV formation, as observed by the decrease in vesiculation when Lpp-PG cross-linking is increased, although its specific cleavage does not appear to be required for vesicle release (24). When utilising mutant strains to study OMV biogenesis better characterization of vesicles needs to be undertaken to distinguish OMVs from other types of BEVs.

Outer membrane structure. The OM is an asymmetric bilayer with the inner leaflet made up of PL while the outer layer is principally LPS. Modification of this asymmetric

bilayer can induce membrane curvature (Fig. 3B). Indeed, multiple LPS modifications have been reported to induce OMV generation without the reliance on PG cleavage, including mutations in genes involved in LPS biosynthesis, such as those responsible for core polysaccharide synthesis (9, 49, 58).

Furthermore, deacylation of LPS fatty acids can activate vesiculation (59). Deacylation leads to penta-acylated LPS, which is predicted to lead to a more fluid OM (60). Additionally, a decrease in the number of hydrophobic sidechains may allow the molecule to adopt a cone shape to promote membrane curvature. Notably, strains lacking such activity still produce vesicles, albeit at a reduced rate. However, some bacterial species such as *Bacteroides* and *Porphyromonas* only contain penta-acylated LPS, putting into question the direct effect of this modification on vesiculation (61). On the other hand, in *Citrobacter rodentium* addition of phosphoethanolamine to LPS stabilises the OM, leading to a decrease in OMV formation (58).

Theoretically, creating an imbalance between the inner and outer layers of the OM would cause curvature, whereby enlargement of the outer layer or the depletion of the inner layer would lead to vesiculation. This appears to hold true, as the controlled increase of PL in the outer leaflet of the OM induces vesiculation in both *Haemophilus influenzae* and *Vibrio cholerae* (62). This was also observed for *Escherichia coli* OMV formation (9, 63). Studies using the fluorescent dye FM4-64 suggest that the increase in PL within the LPS layer leads to a decrease in the stiffness of the OM, mimicking the effects of *lpp*, *pal*, or *ompA* deletions or EDTA treatment (56, 64). Sulphur depletion induces OMV formation in *Neisseria meningitidis* which may be a result of a general increase in PL synthesis (65).

Furthermore, insertion of *Pseudomonas* quinolone signal (PQS) into the OM can activate vesiculation. Initially, PQS was suggested to be a key molecule for OMV generation with its interaction with LPS leading to curvature via enrichment of the LPS layer or repulsion introduced by its negative charge (66–69). Further evidence supporting this demonstrates the requirement of PQS export to the OM to induce vesiculation (70). However, other studies suggest that PQS is not necessary for OMV generation in *P. aeruginosa* and instead only affects their release under limited conditions (71–73). These contradictory findings can be explained by the existence of multiple routes for vesicle release, of which PQS-activated release is but one.

A key aspect to consider is the availability of divalent cations, such as magnesium and calcium, which bind LPS molecules leading to stabilisation of the OM (74, 75). EDTA chelates such divalent cations thereby leading to vesiculation and a decrease in OM rigidity as observed for *lpp*, *pal*, and *ompA* deletions (56, 76). It has been suggested that negatively charged molecules, such as PQS, may bind cations thereby leading to a decrease in OM rigidity and vesiculation in a similar manner (67). Furthermore, *P. aeruginosa* OMVs are enriched in the negatively charged B-band LPS which can interact with magnesium and calcium cations (77–79). However, strains lacking the capacity to produce this type of LPS were still able to produce OMVs, albeit with an altered size and protein composition (80). The modified LPS types differed in their polysaccharide component. Complete removal of the polysaccharide component leads to the generation of large OMVs although the protein compositions were comparable to that of the wild-type strain (80). This once again supports the view of multiple vesiculation mechanisms present in *P. aeruginosa*. Similar results have been observed in *Porphyromonas gingivalis*, which also produces a negatively charged LPS subtype enriched in OMVs (81–83). *Bacteroides thetaiotaomicron* preferentially packages lipoproteins into its OMVs based on a surface-exposed acidic targeting sequence (47). In a similar manner to negatively charged oligosaccharides, such charged amino acids may interfere with divalent cation stabilisation of LPS, leading to vesiculation. Alternatively, the negative charge may lead to repulsion between the phosphate groups on the LPS, thereby expanding the outer layer of the OM and inducing vesiculation.

Temperature could also play a role in OMV production since membrane fluidity changes with temperature (84). *Escherichia coli* shows increased vesiculation with increasing temperature, while no such effect was observed for *P. aeruginosa* (72, 85). Furthermore, the opposite

trend has been observed for *Serratia marcescens*, *Bartonella henselae*, and *Shewanella livingstonensis* (49, 86, 87). Bacteria can increase their membrane fluidity in response to decreases in temperature via desaturation of the fatty acids found on both PL and LPS (84). Such desaturated fatty acids have been detected in *Pseudomonas syringae* OMVs, which may be a route for the removal of such lipids in order to decrease membrane fluidity (88). On the other hand, *P. aeruginosa* and *Prochlorococcus* MED4 OMVs are enriched in saturated fatty acids, while *Acinetobacter baylyi* OMVs had no observable differences in saturation levels, suggesting that vesiculation in these strains is independent of fatty acid saturation levels (89–91). These contrasting findings suggest the existence of more complicated and unknown pathways.

Periplasmic enrichment. OMV generation can be activated by the enrichment of misfolded proteins and other molecules within the periplasm (Fig. 3C) (11, 23, 72, 85, 92–95).

In *E. coli*, deletion of the *degP* gene encoding the periplasmic protease/chaperone responsible for removing unfolded and misfolded proteins leads to increased vesiculation (85). Notably, increased vesiculation was not dependent on the level of OM-PG cross-linking by Lpp (93). Additionally, the release of vesicles was further enhanced by PL imbalance in the OM, suggesting these mechanisms act independently to induce vesiculation (63). Furthermore, mutants accumulating large PG fragments or LPS in the periplasm also show increased vesiculation without reduction in Lpp-PG connections (23). However, the physiological relevance of such accumulation is questioned by the fact that multiple genes need to be mutated to achieve these effects and also stress response pathways leading to increased vesiculation are not discounted (23, 96–98). The authors suggest that the observed hypervesiculation results from increases in periplasmic pressure and is independent of the OM-PG linkage.

In *P. aeruginosa* depletion of a protein involved in OMP synthesis results in the accumulation of unfolded OMPs in the periplasm leading to increased vesiculation (95). However, as discussed previously, depletion of OMPs can lead to vesiculation independent of periplasmic enrichment. Significantly, deletion of periplasmic proteases, resulting in the build-up of misfolded proteins in the periplasm, does lead to an increase OMV generation in this bacterium in a PQS-independent manner (92). As discussed earlier, PQS can induce vesiculation in *P. aeruginosa*.

In *V. cholerae*, a small noncoding RNA which downregulates *ompA* expression is produced during membrane stress resulting from the build-up of unfolded proteins in the periplasm, leading to an increase in OMV generation (53). This increase may be due to a synergistic effect of the increased periplasmic pressure, caused by misfolded proteins, and the reduction in OM-PG connection resulting from the decrease in OmpA. The generation of OMVs is beneficial for bacteria in relieving membrane stress by exporting misfolded proteins. Similar signaling mechanisms have been described in *E. coli* and *Salmonella enterica* serovar Typhimurium; however, to date, this specific signaling mechanism has not been linked to increased OMV production in these organisms (99, 100).

Flagellar release. Bacteria which produce a LPS sheathed flagellum can release OMVs via flagellar rotation (101–103). As the PG layer is predicted to be absent within the structure, the shearing force generated by the rotation of the flagellum may be enough to release the vesicles. A variety of microbes, including the pathogens *V. cholerae* and *Helicobacter pylori*, utilize a sheathed flagellum and package virulence factors into OMVs (48, 104–106). However, OMVs released via the sheathed flagellum may be distinct from those generated by other pathways as there is evidence of the proteome and lipid content of the flagellar sheath being distinct from that of the OM (104).

OMV biogenesis conclusions. Overall, OMV biogenesis appears to rely on multiple different mechanisms which may be species and growth condition specific. Multiple studies guided by mutant gene libraries have focused on the OM-PG connection; however, this does not appear to be necessary for OMV release in all cases. More recently the ability to induce vesiculation via OM modification or periplasmic enrichment of molecules without affecting the OM-PG connection as well as the lack of specific hydrolases for the release of OMVs suggests that this is not a prerequisite for their generation and leads to enrichment of other types of BEVs. In studies investigating vesicle biogenesis, identification of the

types of vesicles produced needs to be improved, as changes in production may be attributable to other types of BEVs. In addition to the pathways discussed in detail here, it is noteworthy that OMVs can form complex structures, such as chains reported in *Shewanella oneidensis* and *Myxococcus xanthus* as well as nanopods, and OMVs encased in S-layer pods produced by *Delftia acidovorans* (107–110).

OMV CARGO

It is likely that the vesicles generated by different routes contain different cargo. In this section we discuss the different methods of protein loading into OMVs as well as links between OMV production and content.

Proteomics and Targeting

To define the specific OMV protein content, multiple proteomic studies have been carried out. Notably, the majority of these likely investigate complex mixtures of BEVs (111). This is apparent by the presence of DNA in the majority of the analyzed samples, suggesting the presence of bacterial vesicles originating from cell lysis. The question of DNA and RNA incorporation into nonlytic OMVs remains unresolved. However, it has been demonstrated in some cases that the presence of DNA in isolated BEVs can originate from OIMV (2). Furthermore, the presence of endonuclease I in the periplasm questions the viability of DNA packaged into OMVs (112). Therefore, the presence of DNA in BEV purifications can be explained by the presence of OIMVs and as such will not be considered further here.

Although most proteomic experiments most likely investigate a mixture of vesicles, the data are consistent in showing the enrichment of specific proteins for which the existence of specific targeting mechanisms have been suggested (47, 50, 83). Selective packaging of lipoproteins into OMVs has been shown in *E. coli* (50). Furthermore, the enriched proteins were predicted not to interact with the PG layer. This was confirmed utilizing an OmpA mutant that could not interact with the PG, which led to its enrichment in OMVs. In *B. thetaiotaomicron*, the display of lipoproteins on the surface of the cell is guided by lipoprotein export signal (LES) amino acid sequences (113). Further research showed that lipoproteins containing this sequence are preferentially enriched in OMVs, linking surface display to OMV packaging (47). It is also possible that the negative charge of the LES sequence induces vesiculation (see previous discussion on the effect of charge on OMV generation). In *P. gingivalis*, an additional class of proteins, containing a conserved C-terminal domain (CTD-family), can be covalently attached to the OM via linkage to anionic LPS (114, 115). As discussed previously, anionic LPS is enriched in OMVs generated by this organism. Therefore, it is not surprising that CTD-family proteins, together with some lipoproteins, are specifically enriched in OMVs, while TonB-dependent transporters and proteins containing a peptidoglycan binding motif are specifically excluded (83, 116). Notably, multiple virulence factors are packaged into OMVs via the CTD-family targeting mechanism (83, 117).

Overall, it appears that lipoproteins as well as proteins that are not tethered to the PG layer are preferentially incorporated into OMVs. There does not appear to be a conserved mechanism of protein enrichment in vesicles as two related *Bacteroidetes* species, *B. thetaiotaomicron* and *P. gingivalis*, utilize different enrichment mechanisms. On the other hand, exclusion of proteins from OMVs may be conserved, guided by a direct linkage to the PG layer or by association with such proteins thereby limiting their incorporation into vesicles.

No protein has been identified as conserved cargo in OMVs from different species, suggesting different roles for vesicles produced by different organisms, or when exposed to different environmental conditions (51). For example, *P. gingivalis* packages proteases into OMVs allowing for the breakdown of host proteins, while gut commensal *Bacteroides* species package various hydrolases for the breakdown of large complex dietary polysaccharides (83, 118). The lack of conserved proteins also supports the presence of vesiculation mechanisms independent of OMV protein content.

Notably, as OMVs used in proteomic analysis are generated under controlled

laboratory conditions using specific growth media, the true capacity of proteins targeted to OMVs is best understood by defining the mechanisms that target individual proteins to the OMV and analyzing the bacterial genome for proteins fitting these targeting requirements. Such analysis will provide a better understanding of the true functional capacity of OMVs.

Links between OMV Biogenesis and Cargo

Multiple examples demonstrate that the biogenesis and composition of OMVs can be a linked process. A simple example is the removal of misfolded proteins from the periplasm via increased vesiculation (see "Periplasmic enrichment" section). We will now discuss further examples of this process.

Both *S.* Typhimurium and *V. cholerae* activate vesiculation to shed unfavourable OM LPS and proteins allowing for rapid adaptation to new environments (119, 120). This leads to the enrichment of the original LPS and/or OM proteins within the vesicles. Low pH conditions, mimicking that of an intracellular infection, induce vesiculation in *S.* Typhimurium (121). A further study reported a significant shift in LPS subtype composition of the OM and OMVs in response to low pH (119). Another study demonstrated a change in media pH leading to shifts in OMV protein composition with the incorporation of novel proteins (122). These results suggest that upon intracellular infection, *S.* Typhimurium uses OMVs to dispose of unfavourable OM LPS and incorporate new proteins allowing adaptation to a new environment. The cargo of such OMVs would therefore be significantly different from that of OMVs produced under neutral pH conditions. Notably, a similar increase in OMV generation followed by surface charge shift has been noted for *Pseudomonas putida* DOT-T1E after solvent induced stress (123).

A similar adaptation is seen in *V. cholerae* upon infection, which is linked to vesiculation induced by the enrichment of PL in the outer layer of the OM and is accompanied with the removal of the unfavourable OmpT on the cell surface (120). Curiously, OmpT participates in the uptake of *V. cholerae* OMVs by host cells (124). This vesiculation mechanism is also induced by iron limitation and may therefore be synergistic during infection, as iron limitation is a common resistance mechanism used by the host (62).

In *P. aeruginosa*, PQS activates OMV production and is incorporated into its membrane. However, as discussed previously, this bacterium can release OMVs via two additional PQS-independent routes (72, 92). PQS-loaded vesicles can contribute to different functions, including quorum sensing, immune regulation, and iron acquisition (125, 126). Under environmental stress, *P. aeruginosa* can release vesicles in a PQS independent manner, which is dependent on the presence of the negatively charged LPS subtype (72). Finally, the bacterium can also induce OM vesiculation to export misfolded proteins, which is independent of the other two mechanisms mentioned (92). It is also important to note that *P. aeruginosa* can produce lytic BEVs (127). Vesicles produced by these routes would be expected to have different cargoes. However, it is likely that all these processes are overlapping.

OMV FUNCTIONS

In BEV preparations it is difficult to distinguish the effect of OMVs from those of other types. Most of the research carried out to date does not investigate the type of vesicles studied and rely on limited purification. Furthermore, the culture media used has a big impact on BEV cargo. Currently, functional OMV studies rarely consider these effects.

We will focus our discussion here on functions which, with reasonable confidence, can be specifically assigned to nonlytic OMVs that relate to signaling, bacterial resistance, and metabolism. We will not discuss reported OMV function in relation to DNA transmission, as we associate internal DNA cargo with other types of BEVs (see "Proteomics and targeting" section).

Cellular Interactions

OMVs are effective delivery vehicles for signaling molecules due to their ability to

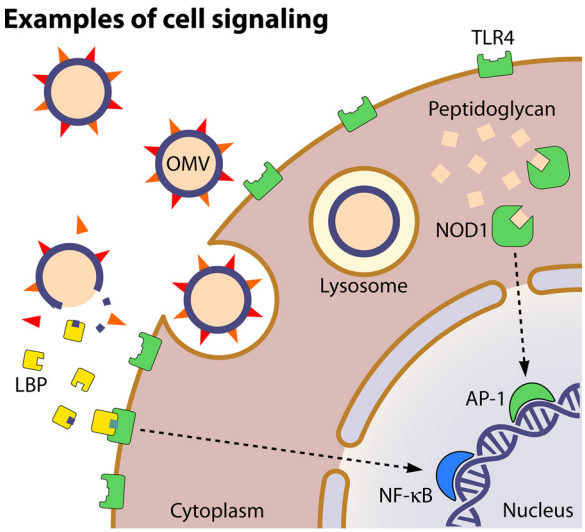

FIG 4 Examples of cellular immune system signaling pathways activated by OMVs. Lipopolysaccharide binding protein (LBP) binds LPS in OMVs and activates TLR4 receptors leading to activation of NF-κB. OMVs can also be acquired by cells leading to their degradation in lysosomes where released PG can be transported to the cytoplasm. Cytoplasmic PG is recognized by the NOD1 receptor, leading to the activation of AP-1 signaling. Notably, the activation of NF-κB and/or AP-1 leads to the production of inflammatory mediators and cytokines.

diffuse freely through complex environments and be acquired by boundary and barrier cells of the host. The majority of research focuses on host-pathogen interactions with some examples of commensal bacteria OMVs providing positive outcomes for the host.

Effects of OMV acquisition by the host primarily comes from studying OMV-immune cell interactions. Immune cells react to conserved microbe-associated molecular pattern (MAMP) molecules via a range of microbial pattern recognition receptor (PRR) molecules, including extracellular toll-like receptors (TLR) and intracellular nucleotide-binding oligomerization domain (NOD) proteins. PRR activation leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which facilitates the induction of inflammatory responses and proteins (e.g., cytokines) (Fig. 4) (128–130).

Gut bacteria. Pathogenic *E. coli* and *Shigella dysenteriae* package Shiga toxins in OMVs, which inhibit protein synthesis in host cells with toxin-containing *E. coli* OMVs, being sufficient to induce disease in a mouse model (131–134). Pathogenic *E. coli* can also package heat-labile toxin (LT) into OMVs, while *V. cholerae* packages the related cholera toxin (CT) (135, 136). These toxins alter cellular cAMP levels, leading to increased levels of water release into the gut lumen resulting in watery diarrhea (137). Notably, the *E. coli* toxin packaged in OMVs activates different host response pathways to that of the soluble toxin, although both ultimately lead to the release of interleukin (IL) 6 and tumor necrosis factor alpha (TNF-α) (138). Furthermore, the pore-forming *E. coli* toxins α-haemolysin and ClyA, known to induce cell death, are also present in OMVs (139, 140). Finally, pathogenic *E. coli* OMVs can contain cytotoxic necrotizing factor type 1 (CNF1) toxin which impairs neutrophil activity, while *V. cholerae* OMVs can contain the RTX toxin that affects the cellular actin cytoskeleton (141, 142).

From a functional perspective, pathogenic *E. coli* OMVs have been shown to induce invasive internalization of a defective mutant strain into intestinal epithelial cells, thereby enabling infection by parental bacteria (143). Additionally, when human microvascular endothelial cells were exposed to *E. coli* OMVs, an increase in endothelial intercellular adhesion molecule-1 (ICAM-1), E-selectin, and vascular cell adhesion molecule-1 was observed (144). Enhanced leukocyte binding in a NF-κB and TLR4-dependent manner was also reported in this study. Additionally, exposure of these OMVs to human umbilical vein endothelial cells (HUVECs) induces the production of tissue factor and the adhesion

molecules P-selectin and E-selectin while reducing the production of thrombomodulin leading to the increased formation of prothrombotic monocyte-platelet aggregates (145). Notably, only the increase in E-selectin was LPS dependent. Taken together, these experiments suggest that these OMVs can activate coagulation pathways in a protein-dependent manner which may play a role in the hypercoagulable response observed during sepsis.

In a similar manner, *S.* Typhimurium can deliver a variety of virulence factors to host cells via its OMVs (146). Exposure of dendritic cells and macrophages to these vesicles upregulates production of the class II major histocompatibility complex (MHC-II), TNF-α, IL-12, cluster of differentiation (CD) 86, and activation of $CD4^+$ T cells (147).

H. pylori OMVs can cause the formation of micronuclei, alteration of iron metabolism, and oxidative stress in human gastric epithelial cells (148). This effect is partially dependent on the OMV-bound VacA cytotoxin which increases OMV uptake (149). These vesicles also induce T cell apoptosis independently of VacA although its presence has a synergistic effect (150). Notably, the relative contribution of OMV-bound VacA compared to the soluble variant has been questioned and is not fully understood (151). When exposed to *H. pylori* OMVs, human peripheral blood mononuclear cells release IL-6 and IL-10 (150). These OMVs also induce the expression of cyclo-oxygenase-2 (COX-2) by monocytes and increase overall levels of prostaglandin E_2 and IL-10 and inhibit dendritic cell maturation via increased expression of heme oxygenase-1 (152, 153). Furthermore, these OMVs activate the peptidoglycan dependent NOD1 response in cell culture and in mice, leading to the activation of mitogen-activated protein kinase (MAPK) and activator protein 1 (AP-1) signaling (154, 155). In the case of *H. pylori*, such NOD1 activation also leads to the generation of antibacterial peptides (β-defensins) in HEK293 cells (156, 157). Furthermore, *H. pylori* OMVs containing the oncogenic CagA protein may contribute to cancer biogenesis via the activation of the cellular tyrosine phosphatase (SHP-2) and induction of IL-8 secretion (48). Activation of SHP-2 has also been shown to lead to a decrease in the production of β-defensin 3 thereby protecting the bacterial cells (157). Finally, the LPS found in *H. pylori* OMVs displays Lewis antigens, which can contribute to chronic immune stimulation in the host (158). Multiple OMV factors may therefore be involved in the increased cancer risk post-*H. pylori* infection (159).

Campylobacter jejuni releases cytolethal distending toxin via OMVs (160), which directly damages cellular DNA, leading to apoptosis (161). Additionally, the bacterium packages immunogenic proteins into the periplasm of the vesicles, which induce IL-8 and β-defensin 3 antimicrobial peptide production independent of the toxin presence (162).

Fusobacterium nucleatum is an opportunistic pathogen found in gastrointestinal, oral, and other infections (163). Proteomic analysis of OMVs from this organism shows the presence of a variety of potential virulence factors (164). In cocultures of macrophages and intestinal epithelial Caco-2 cells, these OMVs induce proinflammatory macrophage differentiation and epithelial barrier loss in a receptor-interacting serine/threonine-protein kinase 1 (RIPK1) dependent manner (165). *In vivo*, such damage can allow for bacterial translocation and penetration of peripheral tissues. These results were also confirmed in a colitis mouse model.

OMVs produced by the commensal gut bacterium *B. thetaiotaomicron* can cross the gut epithelial barrier of colitis prone mice in a sulfatase dependent manner resulting in intestinal inflammation (166). In the healthy colon OMVs stimulate mucosal dendritic cells to produce anti-inflammatory IL-10, while in the blood OMVs stimulate peripheral blood-derived dendritic cells to produce IL-10 and IL-6 (167). Notably, the release of IL-10 was significantly reduced when dendritic cells from Crohn's disease or ulcerative colitis patients were used. It has been shown that a closely related bacterium, *Bacteroides fragilis*, packages immunomodulatory capsular polysaccharide into OMVs which prevents the development of colitis (168). This is achieved by TLR2-mediated activation of dendritic cells leading to the enhancement of IL-10-dependent regulatory CD4 T cells and anti-inflammatory cytokine production. Similar immunomodulatory effects were

observed for *Bacteroides vulgatus* OMVs (169). On the other hand, OMVs from pathogenic *B. fragilis* have been shown to cause hemagglutination (170).

OMVs from commensal *E. coli* strains promote the secretion of anti-inflammatory IL-10 and activate NOD1 signaling pathways in Caco-2 cells leading to the secretion of IL-6 and IL-8 (171). In an experimental colitis mice model, these OMVs have been shown to produce anti-inflammatory and barrier enhancement effects which protect the host from colitis (172). OMVs from a different commensal and probiotic *E. coli* strain have been shown to induce TLR expression and the release of IL-8 in HT29-19A and Caco-2 cells (173). Additionally, these probiotic OMVs strengthen barrier function via a regulatory effect on tight junction proteins (174). Similar effects have been observed for OMVs produced by the gut commensal bacteria *Akkermansia muciniphila* (175, 176). Furthermore, vesicles released by this bacterium in the gut may also produce bone protective effects (177), and orally administered *A. muciniphila* OMVs can induce serotonin signaling in mice (178).

Oral bacteria. *P. gingivalis*, one of the causative agents of periodontitis, packages gingipains, trypsin-like cysteine proteinases, into OMVs (83). Gingipains are major virulence factors and contribute to multiple disease states, including a recently proposed role in the development of Alzheimer's Disease (179, 180). Specific examples of these proteases affecting the host immune system include the breakdown of IgG and IgM antibodies; degradation of complement factor 3 involved in the innate immunity and IL-8 chemokine; disruption of the interferon gamma (IFN-γ) signal transduction pathway; and the cleavage of the LPS receptor CD14 leading to reduced activation of macrophages in response to bacterial infection (181–184). These OMVs have been shown to induce strong TLR2 and TLR4 activation and, to a lesser extent, TLR7, TLR8, TLR9, NOD1, and NOD2 responses (185). Notably, TLR7, TLR8, and TLR9 recognize bacterial DNA and RNA. The presence of these molecules suggest different subpopulations of BEVs may have been present in these experiments. However, it is possible that nonlytic OMVs can bind and display DNA on their surface thereby resulting in the activation of these TLRs. Indeed, a large proportion of this DNA-mediated effect was abrogated by the addition of DNA degrading enzymes (185). The presence of this DNA may also be relevant to the role these OMVs play in biofilm production as discussed below. NOD1 and NOD2 activation suggests the intracellular delivery of peptidoglycan fragments via OMVs. Notably, NOD1 activation by OMVs has been reported for a variety of organisms (154, 185).

In a more specific manner, *P. gingivalis* OMVs can induce monocyte unresponsiveness to live bacteria in a TLR4- and mammalian target of rapamycin (mTOR)-dependent manner (186). Additionally, these OMVs have been shown to upregulate the expression of inducible nitric oxide synthase (iNOS) involved in inflammation, while suppressing expression of the antiatherogenic endothelial nitric oxide synthase (eNOS) (187–189). Additionally, these OMVs induced the formation of foam cells and platelet aggregation, both of which can further contribute to cardiovascular disease (190, 191). *P. gingivalis* OMVs also upregulate the attachment and invasion of *Tannerella forsythia* into epithelial cells (192). Notably, *P. gingivalis* and *T. forsythia* are commonly found together with *Treponema denticola* in periodontitis (193).

OMVs from *T. forsythia* may contribute to disease pathogenesis by promoting the release of inflammatory molecules, such as TNF-α, monocyte chemoattractant protein 1 (MCP-1), IL-6, and IL-8 (194). Like *P. gingivalis* OMVs, these vesicles also induce TLR2, TLR4, TLR7, TLR8, TLR9, NOD1, and NOD2 expression, although to a lower extent (185). *T. denticola* OMVs contain dentilisin, a chymotrypsin-like protease which enables the vesicles to degrade cellular tight junction proteins, promoting penetration of bacteria into deeper underlying tissue (195). These vesicles also activate TLR2 and TLR4, although to a lower extent than *P. gingivalis* or *T. forsythia* OMVs (185). *T. forsythia* LPS, referred to as lipooligosaccharide (LOS) due to the lack of the long O-antigen chain, present in these OMVs can induce a strong inflammatory response in fibroblasts associated with the release of IL-6, IL-8, MCP-1, prostaglandin E_2, and nitric oxide (196).

Additionally, the release of matrix metalloproteinase 3, an enzyme responsible for the degradation of the extracellular matrix, is also noted. Finally, *Aggregatibacter* (previously *Actinobacillus*) *actinomycetemcomitans*, which can also contribute to aggressive periodontitis, packages the cytotoxic GroEL-like protein and a lytic leukotoxin into OMVs (197, 198).

Respiratory tract bacteria. OMVs of the respiratory pathogen *P. aeruginosa* contains the Cif protein, which downregulates the expression of cystic fibrosis transmembrane conductance regulator, reducing chloride ion secretion, leading to the thickening of the mucus layer in the lungs, which facilitates bacterial colonization (199, 200). They also contain the hemolytic phospholipase C, which induces eukaryotic cell lysis (200, 201). Furthermore, the virulence-associated *P. aeruginosa* aminopeptidase (PaAP) is highly enriched in OMVs (202). PaAP found on these OMVs contributes to their attachment to cultured epithelial cells (203). These vesicles induce the production of IL-8 in lung epithelial cells and activate the NOD1 response in nonphagocytic cells (154, 202).

Legionella pneumophila, the causative agent of Legionnaire's disease, packages a variety of toxins into OMVs, including exclusive packaging of extracellular macrophage infectivity potentiator (Mip) into the vesicles (204). When these OMVs were incubated with alveolar epithelial cells, production of IL-6, IL-7, IL-8, IL-13, granulocyte colony-stimulating factor (GCSF), IFN-γ, and MCP-1 were induced. No shift in cytokine profile was observed for heat inactivated OMVs, suggesting that the observed effect was not due to an active protein effect.

OMVs from various other respiratory pathogens have been shown to induce cytokine release. OMVs from *Klebsiella pneumoniae* induce the production of proinflammatory IL-8 and IL-1β in epithelial cells and can induce a disease-like state in a neutropenic mouse model after intratracheal delivery (205). When exposed to epithelial cells, *H. influenzae* OMVs induce the production of IL-8 and the cathelicidin antimicrobial peptide (CAMP or LL-37 when in its active form), while *Acinetobacter baumannii* OMVs activate the expression of IL-6, IL-8, IL-1β, macrophage inflammatory protein-1α, and MCP-1 (206, 207). A similar response to *A. baumannii* OMVs was observed in the mouse lung.

Other respiratory pathogens have been shown to package toxins into OMVs. *Bordetella pertussis*, the causative agent of whooping cough, packages a variety of toxins, including its major virulence factor, adenylate cyclase-hemolysin, into OMVs (208). The respiratory pig pathogen *Actinobacillus pleuropneumoniae* packages proteases and the Apx toxin, which is associated with eukaryotic cell lysis (209). Finally, the opportunistic lung pathogen *Burkholderia cepacia* has also been reported to package a variety of virulence factors into its OMVs (210).

Other bacteria. *N. meningitidis* OMVs contain NarE, a homolog of the previously discussed LT and CT toxins (See "Gut bacteria" section) (211). These OMVs activate dendritic cells via TLR-2 and TLR-4, leading to cytokine (type I interferons, IL-6 and IL-10) production and the induction of B and T cell responses (212). A different study reported that monocyte-derived dendritic cells are activated by these OMVs, leading to CD80, CD83, CD86, and MHC class II expression and secretion of IL-8, chemokine C-C motif ligand 5 (CCL5), and interferon gamma-induced protein 10 (IP-10) (213). Additionally, the uptake of these vesicles by dendritic cells has been shown to be enhanced by the presence of bactericidal/permeability-increasing protein (213). When neutrophils were exposed to *N. meningitidis* OMVs, release of TNF-α, IL-8, IL-1β, macrophage inflammatory protein (MIP) 1α, and MIP-1β was observed, which was enhanced in the presence of IFN-γ (214). *N. meningitidis* adhesin A (NadA) is presented on these OMVs and shows increased immunogenicity of macrophages activating singaling of IL-6, IL-8, IL-10, IL-1β, IL-12p40, IL-12p70, TNF-α, MIP-1α, MCP-1, and CCL5, and production of CD80, CD86, MHC-II, and CD54 (215). In whole human blood, *N. meningitidis* OMVs induced inflammatory TNF-α, IL-6, IL-8, and IL-1β and anti-inflammatory IL-10 production (216). These OMVs also induced the production of prothrombotic tissue factor and plasminogen activator inhibitor 2 in isolated human monocytes, which are suggested to play a role in intravascular coagulation, microthrombosis, and organ dysfunction observed during bacterial infection (217).

Together, these extensive studies show that there is a wide range of molecules being activated in response to OMVs.

The OMVs of the childhood pathogen *Kingella kingae* contain a variety of virulence factors and possess hemolytic activity (218). When exposed to these vesicles, human osteoblasts and synovial cells activate the production of inflammatory human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6. Notably, GM-CSF has been suggested to be involved in joint damage observed during rheumatoid and septic arthritis, which are associated with infection by this bacterium. OMVs from *Brucella abortus*, the causative agent of brucellosis, have been shown to downregulate innate immune responses and promote bacterial internalization by human monocytes thereby contributing to the progression of infection (219). Specifically, these OMVs inhibit TNF-α and IL-8 responses in monocytes. *Borrelia burgdorferi*, the causative agent of Lyme disease, can deliver cholesterol and cholesterol-glycolipids to host cells via OMVs, which has been suggested to play a role in pathogenesis (220). Finally, the opportunistic pathogen *Serratia marcescens* also packages a variety of virulence factors into OMVs (49).

The common fish pathogen *Vibrio anguillarum* OMVs induce the production of inflammatory TNF-α, IL-1β, and IL-6 cytokines when injected into the host (221). Notably, these vesicles carry metalloprotease, hemolysin, and phospholipase activities, which may contribute to the pathogenicity of this bacterium.

Outside of animal-related microbes, *Xenorhabdus nematophilus* and *Photorhabdus luminescens* OMVs carry insecticidal activity and are cytotoxic to cultured Sf-21 insect cells, and *Xanthomonas campestris*, the causative agent for a variety of plant diseases, packages multiple virulence associated proteins into OMVs (222, 223). *P. syringae* and *Pseudomonas fluorescens* OMVs have been shown to activate plant immune responses and inhibit seedling growth (224).

Notably, OMVs may also be involved in bacterial quorum sensing. *P. aeruginosa* as well as *Paracoccus denitrificans* and *Vibrio harveyi* package hydrophobic bacterial quorum sensing molecules within OMVs, allowing for their diffusion and enabling bacterial cooperation (66, 225, 226). OMVs are also involved in other microbe-microbe interactions, such as metabolism, resistance, and biofilm formation which are discussed in a further section.

Cellular interactions discussion. As detailed in the previous section, OMVs play a significant role in a range of host processes that can have beneficial or detrimental outcomes. Of note, a variety of toxins can be packaged into vesicles. As vesicles can cross epithelial cell barriers and thereby diffuse from the active infection site, their role in systemic tissue damage during infection should be considered. Possible effects on coagulation, vascular health, and oncogenesis have already been identified; however, it is unclear if these, or any other mechanisms, play a significant role during infection, as the type and amount of OMVs released during infection is unknown and should be investigated.

Furthermore, a range of immunomodulatory effects are activated by OMVs. The most conserved is the response to LPS and PG found in OMVs; however, there is significant evidence for species-specific protein-mediated pathways. These immunomodulatory effects can be detrimental or beneficial. Beneficial effects include activation and arming of the immune response in response to an infection which has led to studies investigating their use as vaccine delivery vehicles (227).

Finally, there is emerging evidence of beneficial effects provided by OMVs generated by commensal bacteria, such as improved gut barrier function, bone protective effects, and promotion of immunoregulatory, homeostatic responses. Given the diversity of bacteria found in the gut and the fact that OMVs can cross the epithelial layer and access underlying cells, these interactions are likely to be just the tip of the iceberg.

Bacterial Resistance

OMVs enable bacterial survival mainly by breakdown and sequestration of antimicrobial molecules, particularly those affecting the OM and contributing to biofilm formation. Examples of these mechanisms are given in Fig. 5.

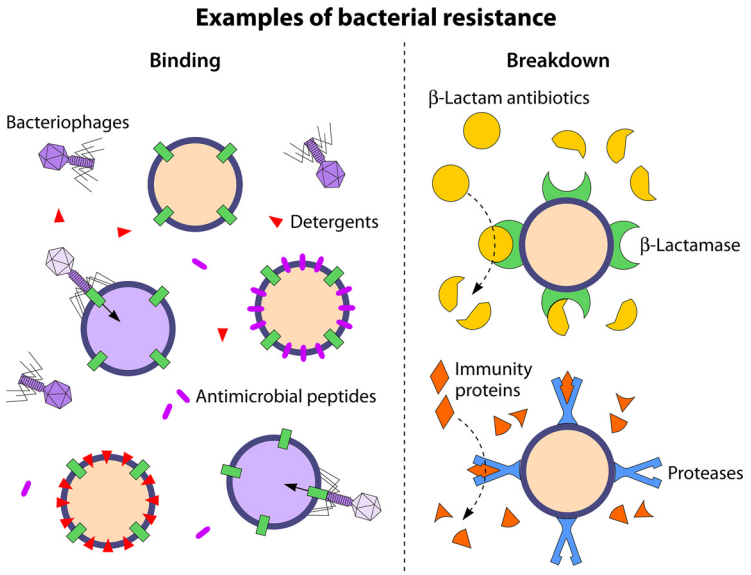

FIG 5 Examples of OMV-mediated bacterial resistance pathways. OMVs can act as off-targets for membrane targeting antimicrobials, such as bacteriophages, detergents, and antimicrobial peptides. Active OMV-associated enzymes such as β-lactamases and proteases degrade active antimicrobial compounds such as β-lactam antibiotics and a variety of host immunity factors, including immunoglobulins.

Impact on antimicrobial agents. *E. coli* OMVs protect cells from host antimicrobial peptides and phage infection by acting as decoys for their binding (85, 228). Similar effects have been described for *P. syringae*, *V. cholerae*, and *Moraxella catarrhalis* OMVs, which can sequester antimicrobial peptides, while phages binding to OMVs has been observed in a complex environment (88, 90, 229, 230). *M. catarrhalis* OMVs also interact with the complement system to reduce its active concentration and protect other microbes as demonstrated using *H. influenzae* (231). *N. gonorrhoeae* OMVs can bind and remove a range of bactericidal factors from human serum, while *N. meningitidis* OMVs bind bacteriostatic neutrophil extracellular traps and bactericidal/permeability-increasing protein produced during infection, thereby protecting the bacteria from their effects (213, 232, 233).

Membrane-dissolving agents can be used as efficient antimicrobials. *P. gingivalis* OMVs have been demonstrated to promote resistance of multiple bacteria to chlorhexidine, while *E. coli* OMVs protected against ethanol (85, 234). A toluene-tolerant strain of *Pseudomonas putida* IH-2000 utilizes OMVs to export toluene in order to generate resistance; no such export was observed in the wild-type strain (235). A similar effect has been observed for *P. putida* DOT-T1E and *P. putida* KT2440 strains after their exposure to long-chain alkanols (123, 236). Furthermore, pathogenic *Bartonella henselae* can utilize OMVs to protect against toxic levels of hemin present in its environment by loading the vesicles with a hemin-binding protein (86). Overall, these effects are achieved by binding of the antimicrobial compound to OMVs thereby effectively diluting the compound. As most of these effects are based on OM composition, they are likely transferable to OMVs generated by other organisms.

Beyond sequestration of bactericidal compounds, OMVs can contain protective enzymes. An active β-lactamase has been shown to be packaged into OMVs by *A. baumannii*, *C. rodentium*, *P. aeruginosa*, *M. catarrhalis*, *B. thetaiotaomicron*, *Stenotrophomonas maltophilia*, and *H. influenzae*, effectively inactivating β-lactam-based antibiotics in their environment (58, 237–242). Additionally, the encapsulation of β-lactamase in OMVs can protect the enzyme against inactivating antibodies (239). OMVs from *H. pylori* are enriched in catalase KatA, an antioxidant enzyme that protects bacteria from oxidative damage, a common strategy used by immune cells to counter infection (243). Finally, OMVs contain a range of proteases which, as discussed previously, can degrade a range of proteins involved in active immunity as well as signaling (181–184).

Biofilm formation. *P. gingivalis* OMVs cause bacterial aggregation and are part of their biofilm (244). This aggregation is suggested to be dependent on OMV-enriched

gingipain proteases, which have been shown to play an independent role in cell adhesion (245). Additionally, the *P. gingivalis* OMV-targeted iron uptake protein HmuY has also been shown to contribute to biofilm formation (246). *P. gingivalis* OMVs can also interact with extracellular DNA, which may influence biofilm structure (185). Functionally, these OMVs have been shown to play a role in complex biofilms formed between *P. gingivalis*, *T. denticola*, and *T. forsythia* (247). Furthermore, *P. gingivalis* OMVs alone are enough to induce aggregation of various other microbes in complex biofilms, including pathogenic *Staphylococcus aureus* (248, 249). Additionally, other organisms present in this complex biofilm, such as *T. forsythia*, can produce OMVs that contribute to its formation (194).

OMVs have also been demonstrated to be a constituent of biofilms generated by *P. aeruginosa*, *M. xanthus*, and several different *Aeromonas* species (250–255). Notably, *P. aeruginosa* vesicles contain a variety of factors that influence biofilm formation and architecture (200, 253, 254, 256). However, recent evidence suggests that these vesicles are a complex mixture of lytic BEVs arising from cell lysis (127). Similarly, it is not possible to say what, if any, contribution nonlytic OMVs provide *H. pylori* in biofilm generation, as the vesicles studied are also likely to be a mix of lytic BEVs (257). Finally, *S. maltophilia* and *Xanthomonas oryzae* excrete the protein Ax21 and its homologues via OMVs (241, 258). This group of proteins can contribute to biofilm formation; however, the direct role of OMVs in this process is unclear (259, 260).

Resistance discussion. Overall, OMVs protect against antimicrobial compounds, which target the bacterial cells by effectively diluting these compounds in the local environment thereby providing resistance to not only the producing organism, but also other organisms present in the environment. Furthermore, OMVs contribute to microbial resistance via their packaging of antimicrobial degrading enzymes, providing protection from a variety of antimicrobial compounds. Finally, biofilms are complex bacterial community structures which protect bacteria from environmental factors, and OMVs appear to play a structural role within these communities.

Metabolism

OMVs are involved in a variety of metabolic processes. Arguably these processes also contribute to cell signaling. However, there is limited research investigating such effects. Examples of OMV-mediated metabolic processes are given in Fig. 6.

Nutrient breakdown. *B. fragilis* and *B. thetaiotaomicron* OMVs contain a variety of metabolic enzymes, including numerous hydrolases, proteases, and phosphatases (170, 261, 262). These enzymes can release nutrients from complex and otherwise indigestible dietary glycans and host mucins for utilization by gut bacteria as well as the host (118). *Bacteroides* species utilize the starch utilization system (Sus) for the breakdown and uptake of complex sugars (263). In this system, SusC is a TonB-dependent transporter (TBDT) that transports nutrients through the OM and SusD, a surface-exposed lipoprotein that binds sugars and delivers them to SusC. Notably, SusD has been shown to be enriched in OMVs, while SusC is retained on the OM (47). This suggests a mechanism whereby hydrolases found on the surface of OMVs break down complex sugars, which is followed by the binding of the products by SusD and their delivery to bacterial cells via SusC. The cellulose-degrading bacterium, *Bacteroides succinogenes*, packages cellulase and a variety of hydrolases into OMVs (264). This allows for the enzymes to diffuse freely in the medium allowing for the degradation of the insoluble material. *X. campestris* also packages cellulase into OMVs, although its primary role may be in infection rather than metabolism, while *Pseudomonas putida* OMVs have been shown to contain lignin-degrading enzymes (223, 265).

As discussed previously, *P. gingivalis* and various other pathogenic organisms package proteases into OMVs (83). These protases can break down extracellular proteins into peptides and amino acids for utilization by the bacteria (179).

Iron acquisition. Iron is required for all living organisms, including bacteria. To import iron, bacteria primarily utilize TBDTs (266). *P. gingivalis* selectively enrich HmuY and IhtB heme-binding lipoproteins on the surface of OMVs, while retaining their partner TBDT on the

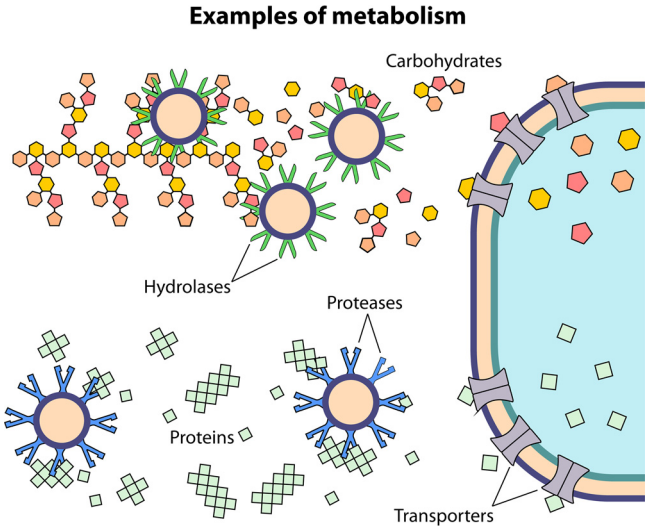

FIG 6 Examples of OMV mediated metabolism. Large complex diet- or host-derived extracellular carbohydrates and proteins can be broken down by hydrolases and proteases present on OMVs. The smaller breakdown products such as disaccharides and amino acids can then be transported and utilised by bacterial cells and the host.

OM (116). There appears to be a synergistic effect between HmuY and gingipain proteases, whereby HmuY binds heme released by the proteolytic degradation of hemoglobin (267). This suggests that OMVs can acquire micronutrients and deliver these to the bacterium.

Iron acquisition proteins are also found in the OMV proteomes of *M. catarrhalis* and *N. meningitidis* (268, 269). Additionally, *N. meningitidis* OMVs also contain zinc uptake proteins (269). However, both of these OMV proteomes included the associated TBDTs. Perhaps this is not surprising, as TBDTs require energy-dependent activation by other proteins to function. It is also possible that the presence of these proteins may be due to the contamination of vesicle preparations with OIMVs, which needs to be addressed by further experimentation.

PQS present in *P. aeruginosa* OMVs chelates iron and delivers it to the bacterium using a specialized uptake mechanism (126). The PQS-bound iron is transferred via a secreted protein to a specific OM receptor and transported into the periplasm. A similar mechanism is present in *Mycobacterium tuberculosis*. Under iron-limiting conditions the bacterium increases vesicle production and loads them with the iron siderophore mycobactin (270). Such vesicles can bind and deliver the iron to the organism. However, it is of note that *Mycobacteria* has a unique OM structure and therefore vesicles generated by this organism may fall into a different class of BEVs (271).

Other metabolic processes. *S. oneidensis* OMVs contribute to the formation of bacterial nanowires that comprise complex structures containing multiheme cytochromes which enable extracellular electron transfer required for cellular respiration (107, 108).

Bacteria prey on other bacteria for nutrients. The predatory *M. xanthus* utilizes OMVs to damage the envelope of their prey, which is likely achieved by hydrolases as well as antibiotics present within the vesicles (46, 272). Once the bacteria are killed, their nutrients can be utilized. OMVs produced by a variety of bacteria have been shown to carry similar bactericidal activity (46, 272–274). In most cases, this activity is suggested to arise from peptidoglycan hydrolase activity present in the membrane vesicles (275).

OMVs can also be used to export molecules, such as misfolded or redundant proteins, as well as unfavourable LPS, which contributes to overall cellular metabolism (119, 120). This can be considered both as a mechanism and a reason for OMV production (See "Links between OMV biogenesis and cargo" section).

Metabolism discussion. It is commonly assumed that hydrolases found on OMVs release sugars for other bacteria to use. While this may be true, specific sugar-binding proteins are also present on the vesicles and can bind any of the released sugars. To

release the sugars, specific receptors are required which are only present on the host cells, suggesting that these sugar scavenging mechanisms may be much more complex than currently assumed. In this sense, OMVs have similarities to siderophores. This theory is supported by the heme uptake mechanism in *P. gingivalis*, where active proteases and iron-binding proteins act on OMVs to release and bind heme, which is then delivered to the bacterium (267). Notably, only bacteria carrying the specific receptor can receive the bound nutrient, suggesting that only closely related bacteria would share these nutrients while sequestering them from unrelated organisms.

OMV Functions Discussion

OMVs have a wide range of associated functionality. However, this area of research is still at an early stage. Questions of subpopulations of different BEVs present and their individual roles and functions demonstrates the need to reinterpret the published studies and to perform future experiments with more highly purified populations of BEV subtypes. There is a considerable body of work on immune cell interactions with OMV from different organisms, although a better understanding of OMV components and identification of the subtypes involved in these interactions is still required. In particular, the effects of active proteins, such as metalloproteases found in vesicles, should be investigated further as these enzymes can have far reaching effects in the host and the bacterial environment.

Additionally, OMV cargo and function depends on the environment the bacterium is exposed to, and the question of how the OMV content shifts in response to a change in environmental conditions needs to be addressed in order to assign specific functions to OMVs. Functionally, OMVs from different organisms share some similarities, including acting as decoys for antimicrobials, their association with metabolic processes involving the breakdown of insoluble extracellular components, and specialized functions such as electron transport.

It is important to emphasise that functional studies often do not investigate OMVs in their natural environment and the amounts used may not relate to the physiological levels of OMVs normally produced in these environments which are technically challenging and difficult to measure.

DISCUSSION OF CURRENT BEV METHODOLOGY

Standard microbiology methods are commonly used to study BEVs. However, these are often not ideal for studying membrane vesicles, and as we have noted elsewhere in this article, the failure to acknowledge or fully account for the complexity of BEV preparations and presence of different subtypes is a major constraint in interpreting and defining pathways of biogenesis and function of OMVs. Here, we highlight in more detail some of the methodological issues that need to be considered.

Media

We observed that media components commonly used to derive BEVs (e.g., brain heart infusion, yeast extract, or peptone) contain lipid vesicles and other particles that copurify with BEVs (unpublished work). These complex ingredients are prepared from enzymatic digests of cell extracts which generate large numbers of membrane vesicles. Standard clarification steps carried out after the lysis do not separate the vesicles produced. Experiments on liposomes have shown that drying the extracts using the common spray-drying method and sterilisation using standard autoclaving procedures do not degrade such vesicles (276, 277). These extracts are then dried, reconstituted in water, and sterilised. Membrane vesicles and other nanoparticles containing complex ingredients are therefore carried over to bacterial cultures where it is likely that a large proportion of such structures survive within the culture and copurify with BEVs due to their similar size and the purification methods used. Such particles might not interfere with proteomics, even though media composition will dictate the proteome (48). However, when investigating biogenesis or biochemical composition or carrying out quantitative studies of OMVs, chemically defined media with minimal particle count should be utilized. We routinely utilize a basic phosphate-buffered media with cysteine as the sulfur source,

ammonium sulfate as the nitrogen source, and a simple sugar like glucose as the carbon source (278). Metals and other additives should be chosen based on their solubility in water and compatibility with the phosphate-buffered solution. Ideally, the developed media should then be analyzed for particles prior to experimentation.

Purification

Centrifugation is integral to isolating both cells and BEVs from culture media. However, centrifugation leads to significant cell compaction and cell surface damage, which can increase shedding of OM and lead to cell lysis (279). The extent of these may be influenced by the type and design features (rotors, bottles, etc.) of different centrifuges and the settings used. To limit such effects, cells should be harvested at the lowest possible speeds. Commonly, the supernatant is then ultracentrifuged to pellet the BEVs; however, this method can lead to contamination and altered vesicle states (see reference 13 discussion).

Another preferred method for BEV extraction is ultrafiltration, which provides a quicker method of purification and avoids issues arising from ultracentrifugation. A common problem when utilizing this method is clogging of the membrane, which can be avoided by using defined media and adding a filtration step postcentrifugation to remove any carry over of bacteria. Alternatively, crossflow filtration can be utilized to avoid clogging issues and to isolate vesicles from larger volumes. Furthermore, when utilising filtration, multiple wash steps should be used to dilute out contaminating material.

To achieve vesicles of higher purity, fractionation is required. Sucrose gradients are commonly utilized; however, in our experience, these can be technically challenging, time consuming, and difficult to replicate. Gravity flow size exclusion columns can be used to further purify the vesicles in a relatively simple, quick, and replicable manner. Prepacked columns are commercially available and those tailored specifically for membrane vesicle purification have recently become available.

Electron Microscopy

Due to their size, electron microscopy (EM) is required to visualize and study BEV generation. However, most EM experiments rely on chemical fixation, dehydration, and resin embedding of samples which introduce experimental artifacts and should therefore be interpreted with caution (280, 281). Additionally, protocols usually contain multiple centrifugation steps, which, as discussed above, may lead to surface damage and likely vesiculation, which may be misinterpreted as native vesicle generation (279). Alternative methods that do not rely on centrifugation should be utilized to avoid such artifacts. Ideally, studies concerning the characterization of BEVs should avoid chemical fixation and instead rely on cryofixation methods. Such methods conserve membranes and other ultrastructures more effectively (281, 282).

Furthermore, EM analysis often lacks quantitative information, only showing a single field of view selected by the author. Due to the variability and common occurrence of artifacts, care needs to be given when presenting data from such studies. When possible, data should be quantified and high-resolution overview images, showing populations of cells from biological replicates, need to be included.

Nanoparticle Tracking Analysis

Count and size measurements of BEVs are often carried out by nanoparticle tracking analysis (NTA) using instruments such as the NanoSight or ZetaView, both of which are based on the Brownian motion of particles in solution (recent comparison [283]).

When carrying out analysis using either of the instruments it is important to consider camera settings, as these impact observations and are a limitation of the method (283). However, if constant settings are used, such analysis is comparative, and the settings should be included in published articles. Furthermore, as the measurements are based on Brownian motion, the buffer and temperature used to measure the particles needs to remain constant. Ideally, a blank measurement of the buffer should also be carried out prior to measurement to exclude the presence of contaminating nanoparticles within the buffer. Additionally, vortexing of samples should be avoided, as this

can introduce bubbles which can register as particles. Finally, to improve the quantification, additional analysis of total protein and lipid should be carried out in parallel.

It is important to note that the hydrodynamic diameter observed using NTA analysis is not equal to vesicle diameter observed using EM, as these are two different measurements. A common explanation for this is shrinkage of the particles from dehydration during EM analysis. However, in our experience, the size difference is still apparent when cryoelectron microscopy is used (unpublished data). A possible explanation for this difference is the contribution of the O-antigen of LPS to the hydrodynamic diameter of the vesicle (284). Considering a glucose molecule is around 1 nm in length and, as an example, the *S.* Typhimurium O-antigen comprises 16 to >100 repeating tetrasaccharide units, the length of this polymer can be more than 400 nm (285). Indeed, it has been observed that the length of the O-antigen contributes to the hydrodynamic diameter of BEVs (284). As these polymers are not observed using regular EM methods, this is a possible explanation for the size differences observed using the different approaches, and as such requires further investigation. We advise that all future publications should refer to hydrodynamic diameter and vesicle diameter (or core diameter) as two separate vesicle characteristics.

More recently, instruments based on tunable resistive pulse sensing (TRPS) have become available, which analyze particles as they pass through tuneable nanopores. Comparison of this method to other commonly used quantification methods has been carried out using mammalian EVs, suggesting that there is significant variation between the methods used (286). To our knowledge, no comparative analysis of TRPS to other methods has yet been carried out using BEVs.

CONCLUSIONS

OMVs are a major constituent of BEV populations. However, due to their copurification with other different types of vesicles, methods for their generation and function can be misinterpreted. Furthermore, OMVs can be generated via multiple different mechanisms, which may be correlated with different functionality. Thus, the methods for generation and functional analyses of OMVs cannot be generalized as these likely differ based on the studied organism.

Due to their size, the analysis of BEVs is not straightforward. The effect of contaminating particles, purification, processing, and the method used to analyze these vesicles needs to be taken into consideration. We have outlined some common factors often ignored or not fully considered during experimentation and provided suggestions on how to alleviate some of these issues. We recognize that due to technical limitations, solutions to some of the mentioned issues are either not practical or not possible. However, consideration and appropriate controls should be utilized wherever possible to aid interpretation of generated data.

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