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Review article

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Composition and functions of bacterial membrane vesicles

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Abstract

Extracellular vesicles are produced by species across all domains of life, suggesting that vesiculation represents a fundamental principle of living matter. In Gram-negative bacteria, membrane vesicles (MVs) can originate either from blebs of the outer membrane or from endolysin-triggered explosive cell lysis, which is often induced by genotoxic stress. Although less is known about the mechanisms of vesiculation in Gram-positive and Gram-neutral bacteria, recent research has shown that both lysis and blebbing mechanisms also exist in these organisms. Evidence has accumulated over the past years that different biogenesis routes lead to distinct types of MV with varied structure and composition. In this Review, we discuss the different types of MV and their potential cargo packaging mechanisms. We summarize current knowledge regarding how MV composition determines their various functions including support of bacterial growth via the disposal of waste material, nutrient scavenging, export of bioactive molecules, DNA transfer, neutralization of phages, antibiotics and bactericidal functions, delivery of virulence factors and toxins to host cells and inflammatory and immunomodulatory effects. We also discuss the advantages of MV-mediated secretion compared with classic bacterial secretion systems and we introduce the concept of quantal secretion.

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Introduction

Extracellular vesicles are produced by species across all domains of life, suggesting that vesiculation is a fundamental process of living matter[1]. Bacterial membrane vesicles (MVs), which are typically 40–400 nm in diameter, carry specific cargos and are therefore considered to represent a unique bacterial secretion pathway[2]. MVs were first shown to originate from blebs of the outer membrane (OM) of Gram-negative bacteria and have therefore often been referred to as outer membrane vesicles (OMVs). More recent work has provided evidence that Gram-negative bacteria can produce different types of MV and that many Gram-positive bacteria and bacteria that do not Gram stain also release MVs[3]. Moreover, it has been demonstrated that MVs are not only produced by living cells but can also originate from endolysin-triggered cell lysis[3–5], but whether this is a bona fide MV biogenesis pathway is widely debated in the field. Given that phages are the most abundant form of life on Earth[6], it is tempting to speculate that lytic phages triggering explosive cell lysis may be the main drivers of MV biogenesis in nature. Several recent studies have provided further evidence that the role of cell lysis in MV formation has previously been underappreciated[7–12]. In this Review, we summarize current knowledge regarding how the different biogenesis pathways affect MV composition and function.

Biogenesis and types of membrane vesicle

Gram-negative bacteria

MVs can be formed via two principal routes in Gram-negative bacteria[3,13], either via blebbing of the OM (B-type MVs) or via explosive cell lysis and concomitant curling and self-annealing of shattered membrane fragments[4] (E-type MVs) (Fig. 1). Explosive cell lysis is triggered by genotoxic stress that activates the expression of prophage-derived endolysins, which degrade the bacterial peptidoglycan layer[4,5,12]. More recently, it has been demonstrated that lytic phages cause not only explosive cell lysis but also MV formation as a result of blebbing owing to the binding of phages to the OM[9]. The different biogenesis routes give rise to particular MV types that have distinct structures and contents (Fig. 1), which eventually determine their functions. For example, OMVs of Gram-negative bacteria can be enriched for hydrophobic compounds, denatured proteins or peptidoglycan as a consequence of different mechanisms causing OM blebbing. On the contrary, OMVs are thought to be free of cytosolic contents such as DNA, RNA and ATP, which are considered characteristic contents of cytoplasmic membrane vesicles (CMVs) of Gram-positive bacteria and E-type MVs of Gram-negative bacteria. The only B-type MVs that can contain these cytosolic contents are outer–inner MVs (OIMVs). These double membrane MVs, which were first observed in culture supernatants of *Shewanella vesiculosa* M7, were thought to be formed by the combined protrusion of the inner membrane and OM through holes in the peptidoglycan layer, such that cytoplasmic contents get entrapped within the MVs[14]. A recent, more detailed analysis of *S. vesiculosa* M7 vesiculation showed that MV production in this strain is greatly increased in the late exponential to stationary phase owing to prophage-dependent explosive cell lysis[11]. This not only gives rise to explosive outer MVs but also to a new type of MV, referred to as explosive outer–inner MVs, which differ from OIMVs that originate from blebbing. These novel MVs have unusual structures with often more than one vesicle inside a larger vesicle or irregularly shaped inner vesicles (Fig. 1).

Gram-positive bacteria

Although numerous studies demonstrated that Gram-positive bacteria produce CMVs, the underlying biogenesis mechanisms were only recently unravelled (reviewed elsewhere[15,16]). In analogy to explosive cell lysis in *Pseudomonas aeruginosa*, it was shown that expression of an endolysin encoded by a defective prophage triggers formation of CMVs in *Bacillus subtilis*[5]. Although in both organisms the enzymatic activities of the endolysins weaken the peptidoglycan, the consequences are different: whereas *P. aeruginosa* cells round up and explode, *B. subtilis* cells protrude cytoplasmic membrane (CM) material through holes in the peptidoglycan, which are then released as explosive CMVs (ECMVs). Although Gram-negative cells completely disintegrate during this process, the thick Gram-positive cell wall of *B. subtilis* is not entirely hydrolysed; however, most cells die owing to the loss of CM integrity, as indicated by the formation of ghost cells containing intracellular MVs. For this reason, this mechanism, which was shown to stimulate CMV formation in other Gram-positive bacteria as well[17,18], was named 'bubbling cell death'. Akin to explosive cell lysis in Gram-negative bacteria, bubbling cell death can be induced by exposure to DNA-damaging antibiotics such as ciprofloxacin[5,19]. In the case of *Lacticaseibacillus casei*, no specific stimulation is required, as the spontaneous induction rate of its prophage is sufficient for the production of high amounts of ECMVs under normal culture conditions[20]. More recent work has shown that in addition to phage-derived endolysins, autolysins that are normally required for the separation of daughter cells can also induce bubbling cell death under various stress conditions in *B. subtilis*[21]. Given that both endolysins and autolysins have been identified in ECMVs, this suggests that bubbling cell death seems to be an important mechanism of CMV formation in many Gram-positive bacteria[22–27]. CMV formation can also be stimulated by weakening treatment of the cell envelope with peptidoglycan-hydrolysing enzymes[28–31] or β-lactam antibiotics[19,32]. Although subinhibitory concentrations of these agents allow the release of CMVs through the leaky cell wall, they can also trigger cell lysis at higher concentrations.

CMV biogenesis in *Staphylococcus aureus* is proposed to occur via a blebbing mechanism, which involves the disruption of the CM by amphipathic, α-helical, phenol-soluble modulins. Subsequently, autolysins, which weaken the crosslinking of the peptidoglycan, modulate CMV release through the cell wall[32,33]. Although this study reported only a minimal impact of CMV formation on viability, the surfactant-like phenol-soluble modulins have also been shown to induce the release of membrane lipoproteins and cytoplasmic proteins through cell lysis[34].

Archaea and bacteria with rare cell walls

MV formation has also been observed in Gram-neutral bacteria belonging to the genus *Mycobacterium*[35,36], in bacteria that have no cell wall including members of the genera *Acholeplasma*[37] and *Mycoplasma*[38], in bacteria with a very thick and compact Gram-negative cell wall as it is the case with some cyanobacteria[39,40] and in archaea[41], which generally lack peptidoglycan but are often surrounded by a crystalline protein S-layer. As knowledge on the underlying vesiculation mechanisms in these microorganisms is scarce, and owing to limited information on their cargo and functions being available, we limit our Review to focus on MVs produced by Gram-positive and Gram-negative bacteria. The interested reader is referred to a recent review[1] that provides an extensive overview on extracellular MVs in the three domains of life and beyond.

Membrane vesicle composition and cargo packaging mechanisms

There is a large body of evidence suggesting that the composition of MVs is different from their parent bacterial cells. This cargo selectivity is commonly used as an argument that MVs represent a bona fide secretion pathway that depends on a specific packaging mechanism.

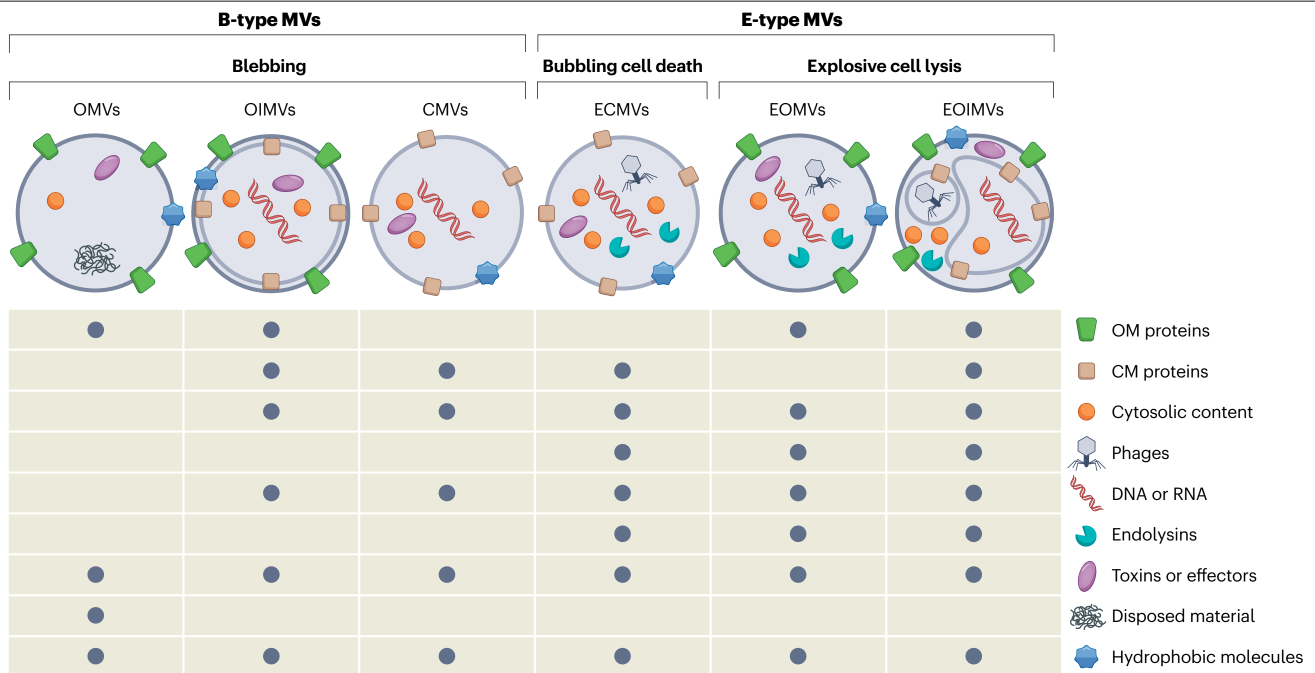


Fig. 1 | Membrane vesicle types with different structures and compositions have different biogenesis routes. Gram-negative bacteria produce membrane vesicles (MVs) either via blebbing of the outer membrane (OM) or via explosive cell lysis. OM blebbing resulting from cell envelope disturbances such as imbalanced peptidoglycan biosynthesis, the accumulation of denatured proteins or the intercalation of hydrophobic molecules into the OM leads to the production of outer membrane vesicles (OMVs). Thus, the OMV cargo is devoid of cytoplasmic components but enriched for periplasmic proteins and OM constituents. Outer–inner MVs (OIMVs) are formed by a blebbing mechanism that starts with the weakening of the bacterial peptidoglycan layer by an autolysin and the subsequent protrusion of the inner membrane into the periplasm. Cytoplasmic contents then enter the vesicle, which is eventually pinched off from the cell surface, together with the surrounding OM. Vesicle production mediated by explosive cell lysis is triggered by a phage-derived endolysin that degrades the peptidoglycan layer of the cell. The cell then rounds up, explodes and the shattered membrane fragments self-anneal to form explosive outer–inner membrane vesicles (EOIMVs) and explosive outer membrane vesicles (EOMVs). In contrast to OMVs, these E-type vesicles contain cytosolic components, including genomic DNA. Endolysin also contributes to vesicle production in Gram-positive bacteria by triggering bubbling cell death, giving rise to cytoplasmic membrane vesicles via lysis (ECMVs). ECMVs can also form as a result of stress-induced autolysis of Gram-positive bacteria[21] or as a consequence of peptidoglycan hydrolysis[5] by exogenous endolysins or antibiotics inhibiting peptidoglycan biosynthesis. These cytoplasmic membrane vesicles (CMVs) do not carry endolysins (not shown). In *Staphylococcus aureus*, CMVs can also be formed via a blebbing mechanism that involves the disruption of the cytoplasmic membrane by phenol-soluble modulins and the subsequent release of CMVs through the cell wall after weakening of the peptidoglycan crosslinking by autolysins[32]. CM, cytoplasmic membrane.

Although the search for such a cargo sorting mechanism is ongoing, some factors that affect MV composition have been identified. In Gram-negative bacteria, the mechanism of MV biogenesis seems to be the greatest determinant of cargo selectivity. For example, OMVs should by definition be devoid of cytoplasmic components but can be enriched in components of the OM, periplasmic proteins and possibly peptidoglycan or hydrophobic molecules (Fig. 1). On the contrary, OIMVs and E-type MVs can contain cytoplasmic components in addition to cell envelope material. However, simple geometric considerations of surface-to-volume ratios of bacterial cells and MVs show that, independent of the biogenesis route, the loading capacity of MVs for cytoplasmic and soluble periplasmic proteins is very limited (Box 1). As a consequence, all MV types are enriched for integral membrane proteins, lipoproteins and to a lesser degree peripherally attached soluble proteins. In a recent study, *Escherichia coli* MVs produced during conditions of oxidative stress showed a preferential retention of OM integral proteins compared with lipoproteins. The mechanistic basis for this cargo selectivity was suggested to result from differences in envelope tethering, that is, proteins that are anchored in the peptidoglycan layer or that form larger cell envelope-associated complexes are preferentially retained in the cell, and consequently these proteins do not become part of the MV cargo[42].

CMVs were shown to contain cytosolic proteins, nucleic acids (RNA and DNA) as well as secreted proteins[22,27,43,44] (Fig. 1). Although CMV cargo selectivity has been observed for various Gram-positive bacteria[27,45,46], the underlying mechanism is unclear. For *S. aureus*, it has been shown that CMVs are enriched for positively charged proteins. This suggests a passive sorting mechanism on the basis of electrostatic interactions of negatively charged microdomains at the cytoplasmic membrane surface with charged proteins at the site of extracellular vesicle formation[43]. Whether MVs originating from bubbling cell death (ECMVs) or via a blebbing mechanism (CMVs) differ in their cytosolic contents remains to be investigated.

An important factor affecting MV composition is the physiological state of the cell at the time of MV formation[7,47]. For example, MV formation by *P. aeruginosa* is strongly induced when cells grow as a biofilm,

Box 1

Increased surface area to volume ratio limits cargo capacity of membrane vesicles

Although bacteria exhibit a robust surface area (SA) to volume (V) homeostasis (SA:V), the SA:V changes when cells alter their size or morphology[189]. This drastically reduces the loading capacities of membrane vesicles (MVs) for soluble contents, whereas membrane-associated components are much less affected. For example, the volume of a rod-shaped *Escherichia coli* cell (approximated as a cylinder and two half spheres) growing in rich lysogeny broth medium is approximately 4.6 μm^3 (cell length (l) of 3.9 μm and cell width (w) of 1.3 μm; $V = \pi w^2(\frac{l}{4} - \frac{w}{12})$) (ref. [190]) and has a calculated surface area ($SA = \pi l w$) of 16 μm^2 and thus an SA:V ratio of about 3.5 μm^{-1}. Assuming that the entire outer membrane of the cell is used for the production of MVs with an average diameter of 100 nm, approximately 500 MVs with a surface area of $3.1\times10^{-2}\ \mu m^2$ and a volume of about $5.2\times10^{-4}\ \mu m^3$ would be formed. The SA:V of the MV would increase to about 60 μm^{-1}. The entire volume of these MVs would add up to $2.6\times10^{-1}\ \mu m^3$ and consequently, <6% of the soluble contents of the cell's cytoplasmic and periplasmic space can potentially be packaged into MVs, whereas the remainder would be released into the supernatant. By contrast, all membrane-associated compounds would still be present in the membranes of the MVs formed and thus the ratio of membrane associated to cytosolic and periplasmic proteins increases about 15-fold. For a given volume, the object with the smallest SA is a ball and we wondered whether the reduced loading capacities of MVs would also apply to spherical bacteria. If we assume that a spherical *Staphylococcus aureus* cell with a diameter of 1 μm forms MVs of 100 nm in diameter, using the same equations as discussed earlier, the ratio of membrane-associated to cytoplasmic proteins increases about 10-fold.

yet the protein content of planktonic and biofilm-derived MVs was shown to be surprisingly different and reflected the differing physiological states of planktonic and sessile cells rather than differences in MV packaging[48]. Likewise, analysis of the mRNA content of planktonic *P. aeruginosa* cells and MVs revealed that MVs are strongly enriched for mRNAs that are typically expressed as part of the SOS response, indicating that these MVs originate from explosive cell lysis of a small subpopulation[4,8]. In this case, the apparent cargo selectivity of MVs is an indicator of their mechanism of biogenesis rather than of specific cargo packaging. In conclusion, although it remains to be shown that a specific MV sorting machinery exists, there have been several alternative mechanisms identified that at least in part explain the observed cargo selectivity of cytosolic and cell envelope-associated contents of MVs.

Cell envelope-associated content

Many reports have shown that MVs from bacterial pathogens carry toxins or virulence factors to manipulate the physiology of the host cell[49]. Although there is only anecdotal, and mostly microscopic evidence for MV release in vivo[50,51], MVs have been isolated from human body fluids[52], and their production was shown to be increased during host colonization, probably owing to stress-induced vesiculation[53]. For this reason, bacterial MVs have been considered 'long distance weapons' as they allow the delivery of high amounts of effectors into host tissues that are not colonized by the parent bacteria[54–56]. Moreover, the association of virulence factors with MVs not only protects them from degradation but can also increase their stability and prolong their activity, as in the case of the enterohaemorrhagic *E. coli* haemolysin[57,58]. In Gram-negative pathogens, virulence factors are often associated with bacterial surface components, for example, the heat-labile enterotoxins of *E. coli*[59], or exhibit periplasmic localization and accumulation as an intermediate step in the export process, as is the case for cholera toxin, the PrtV protease in *Vibrio cholerae* or Shiga toxin from pathogenic *E. coli* strains[56,58,60]. These examples suggest that the enrichment of virulence factors in MVs may not reflect an active export mechanism, but rather indicate that the production of cell envelope-associated virulence factors and MV release are linked. It is noteworthy that many of the MV-associated virulence factors are encoded by prophages (Box 2) or exhibit bacterial surface binding properties.

MVs are strongly enriched for components of the cell envelope, including peptidoglycan, membrane proteins, lipoproteins, lipopolysaccharides (LPS), lipooligosaccharides (LOS) and (lipo)-teichoic acids depending on the Gram status of the bacterium. Although several of these components have immunomodulatory potency, this effect is best investigated for LPS and LOS (discussed subsequently). The lipid composition of the membrane determines membrane curvature and fluidity and thus influences MV biogenesis, particularly the formation of B-type MVs. Consequently, the lipid composition of an MV can be different from that of the cell membrane, as has been reported for various membrane lipids in phylogenetically diverse bacteria[27,61–63]. MVs from *P. aeruginosa* and *Porphyromonas gingivalis* were shown to be enriched for LPS types with negatively charged O-antigen chains[64,65]. It has been suggested that the repulsive interactions between negatively charged O-antigen chains destabilize the OM and thereby facilitate OMV formation[64]. In the case of *P. gingivalis*, it was also shown that packaging of the virulence factor gingipains and the exclusion of abundant OM proteins are dependent on negatively charged LPS. The release of unfavourable LPS types via MVs was recognized as a novel strategy to efficiently remodel the bacterial cell surface according to extracellular or intracellular lifestyle or during environmental transitions[53,66]. OM remodelling through deacylation of lipid A in *Salmonella enterica* subsp. *enterica* serovar Typhimurium and phospholipid accumulation in the outer leaflet of the OM of *Haemophilus influenzae* and *V. cholerae* were shown to induce OM curvature that causes hypervesiculation in these organisms[61,67].

Proteins anchoring the OM to the peptidoglycan layer in Gram-negative bacteria, such as the highly conserved lipoprotein Lpp[68] or the peptidoglycan-anchored proteins RmpM, MtrE and PilQ in *Neisseria meningitidis*[69], are not detected in MVs. Conversely, OM porins are enriched in MV proteomes and have been implicated in diverse functions. Host cell internalization of *V. cholerae* MVs relies on the OM porins OmpU and OmpT[58]. MVs of *P. aeruginosa* are enriched for the porin OprF, a homologue of OmpA, which seems to affect MV formation through reduction of the *Pseudomonas* quinolone signal (PQS) levels rather than tethering the OM to peptidoglycan[70]. Moreover, OprF facilitates adhesion to the host mucosa, bacterial cell surfaces and the biofilm matrix via protein–exopolysaccharide interactions[71]. A recent model proposes two conformations of OprF, comprising a bacterial surface-associated closed state as well as an open form

lacking the peptidoglycan linkage, which is preferentially found in MVs, suggesting that the conformational state of the effector affects its packaging efficacy[71]. The MV-associated porins PorB and OmpA of *Neisseria gonorrhoeae* and *Acinetobacter baumannii*, respectively, are virulence factors targeting mitochondria[72,73]. MVs from β-lactam-resistant bacteria often carry high concentrations of β-lactamases[74,75], which are typical periplasmic enzymes in Gram-negative bacteria but are secreted by Gram-positive bacteria. Within the MV lumen, the enzyme is protected from degradation and shielded from neutralization by the antibodies of the host[76]. β-Lactamase-carrying MVs promote survival not only of the donor species but also of the residual bacterial community, notably in the context of co-infections[75,77]. A recent clinical study reports the failure of amoxicillin therapy in patients with group A *Streptococcus pyogenes* pharyngotonsillitis owing to the presence of resistant *H. influenzae* secreting β-lactamase-containing MVs[78]. Of particular concern are new variants of membrane-anchored metallo-β-lactamases capable of inactivating last resort carbapenems. The New Delhi metallo-β-lactamase NDM-1 maintains activity under conditions of metal depletion and its membrane anchor facilitates secretion via MVs, promoting survival of otherwise susceptible bacteria at nearby infection sites[79,80].

Cytoplasmic material

On the basis of their origin, the cytoplasmic content in Gram-positive MVs is not very surprising. Indeed, several proteome studies of Gram-positive MVs suggest that the majority of MV-associated proteins have a cytoplasmic localization followed by extracellular and membrane proteins[22,81]. In general, Gram-positive CMV cargos show a high abundance of proteins linked to metabolism. MVs derived from Gram-negative bacteria have also been reported to contain cytoplasmic material. The bacterial nucleoid represents an intrinsic cytoplasmic constituent that in *E. coli* takes up approximately 20% of the cell volume[82]. Many bacteria also contain plasmids and other mobile genetic elements. To accommodate the observation that MVs can contain DNA[83,84], a new type of MV was proposed, namely, double bilayered OIMVs[64]. The existence of this type of vesicle was subsequently confirmed by examination of *S. vesiculosa* M7 supernatants by cryo-electron microscopy[14], which showed that most of the DNA was within OIMVs rather that OMVs, which are also produced by this strain. An association of DNA with MVs that possess a double bilayer has also been reported for several other bacteria[85]. Explosive cell lysis liberates large amounts of DNA fragments, which can be entrapped by the concomitantly produced membrane fragments and thus represents another mechanism for the formation of DNA-containing MVs[4]. The fact that the chromosomal DNA cargo of MVs is highly fragmented suggests that DNA contained within these MVs originates from dead cells[86,87]. E-type MVs were shown to carry higher amounts of DNA and had a higher frequency of horizontal gene transfer compared with OMVs[11,19,88]. Although many studies have reported that DNA is present in OMVs, it seems possible that the investigated OMV samples may unintentionally have also contained OIMVs or E-type MVs. A frequently discussed study showed that OMVs can contain plasmid but not chromosomal DNA[86]. This study

Box 2

The various links among bacterial toxins, phages and membrane vesicles

Temperate phages can switch between dormant (lysogenic) and productive (lytic) states. In the lysogenic state, the phage is integrated into the bacterial host genome and is transmitted to daughter cells at each cell division without causing cell death or the production of phage particles. Many prophages enter the lytic state under environmental conditions that cause DNA damage and thus activate the SOS response, such as exposure to DNA-damaging agents such as certain antibiotics or ultraviolet radiation. The SOS response induces the expression of lytic genes that promote DNA replication, phage particle assembly, DNA packaging, host DNA degradation and eventually bacterial lysis. Temperate prophages are often associated with increased bacterial virulence, as they can carry pathogenicity determinants[191,192], many of which encode toxins that are typical cargos of membrane vesicles (MVs), including the cholera toxin in *Vibrio cholerae*, the Shiga toxin, the cytolethal distending toxin and the type II heat-labile enterotoxins in pathogenic *Escherichia coli* strains[54]. Expression of the Shiga and cholera toxins was shown to be dependent on prophage induction[193,194]. A link between prophage induction and toxin expression has also been reported for the lysogenic bacteriophage ΦSa3ms of the Gram-positive bacterium *Staphylococcus aureus*, which encodes three enterotoxins (SEA, SEG and SEK) as well as the fibrinolytic enzyme staphylokinase (Sak)[195]. Although SEA was shown to be contained in cytoplasmic MVs[196], the presence of other enterotoxins within cytoplasmic MVs seems to be variable[197]. These examples support the idea that genotoxic stress not only induces production of phage-encoded toxins but also triggers cell lysis and thereby ensures that under stress conditions these toxins are packaged, secreted and delivered to their host cells via E-type MVs. Another important virulence factor of *E. coli* is the pore-forming cytolysin A (ClyA), which has been demonstrated to be exported from the bacterial cell in outer membrane vesicles (OMVs), where it adopts a cytolytically active, oligomeric conformation[198]. Although ClyA is neither encoded by a prophage nor is its production induced by the SOS response, a haemolytic phenotype on blood agar was only observed when lysis of the bacteria was triggered by the inducing agent mitomycin C. It has been suggested that OMV-based release of ClyA does not permit export of a sufficient amount of the toxin to allow for detectable haemolysis on blood agar plates[199]. It therefore seems likely that under genotoxic stress ClyA is exported by E-type MVs, whereas unstressed cells release this cytolysin mainly via OMVs. Importantly, given that some antibiotics such as ciprofloxacin and trimethoprim induce the SOS response and consequently toxin production and the formation of E-type MVs in many pathogens carrying toxin-encoding prophages, their use for the treatment of infected patients has been discouraged[192,200].

also provided evidence that broken OMVs can encapsulate extracellular plasmid DNA and proposed this as an alternative mechanism of how DNA is packaged into OMVs. Interestingly, this mechanism is very similar to MV formation by explosive cell lysis, in which the released DNA is captured by recircularizing membrane fragments. The analysis of total MV-associated DNA has shown that the sequences cover the entire genome[4,11], whereas enrichment for some specific chromosomal regions was observed in studies investigating the luminal DNA fractions[89,90]. Whether the over-representation of certain DNA regions affects DNA transfer or immunogenic properties of MVs is an interesting topic for future research. Given that the cargo of CMVs contains large amounts of cytosolic contents, it is not surprising that many studies demonstrated the presence of DNA in CMVs[44,91–93]. Interestingly, a recent study showed that CMVs originating from phage lysis have a generally higher DNA content than CMVs originating via a blebbing mechanism[19].

RNA is another typical cytosolic component that is present in CMVs[94,95] as well as in E-type MVs[96,97] of Gram-negative bacteria but is expected to be absent from OMVs. In fact, the finding that the mRNA of *P. aeruginosa* MVs is enriched for genes encoded by the pyocin gene cluster of a prophage region initially sparked the idea of explosive cell lysis as a novel MV biogenesis mechanism[4]. Recent work confirmed that MV-associated mRNAs are often encoded by genes located in prophage regions[8,97–99], providing evidence that mRNA is mostly released by E-type MVs. Noteworthy, mRNA is only a very minor constituent of the MV luminal RNA cargo, which was shown to consist mainly of ribosomal RNA, transfer RNA (tRNA) and small RNA (sRNA)[8,94,100]. When compared with the RNA composition of bacterial cells, MVs were found to be specifically enriched for tRNAs. Interestingly, *P. aeruginosa* PA14 MVs contain tRNA-derived fragments that can attenuate the immune response of the host[96]. Although it is well established that sRNAs have an important role in the post-transcriptional regulation of diverse functions in bacteria[101], it remains to be elucidated whether they have a role in MV-mediated interbacterial or interkingdom interactions[8,94,102].

Cargo transport and delivery

Cargo transport

MVs seem to be particularly important for the secretion of hydrophobic molecules, including membrane-associated proteins, toxins and virulence factors, which have poor solubility in water. A recent metabolomics study showed that MVs of two *Prochlorococcus* strains were specifically enriched for nonpolar metabolites[103], supporting the idea that MVs also serve as vehicles for the secretion of small hydrophobic molecules. When the export of such molecules via classic secretion systems may result in the molecule not being able to dissociate from the cell envelope and disperse into the surrounding environment, their packaging into MVs would allow their secretion and also their dispersal in aqueous environments (Fig. 2). Interestingly, a recent study that used time-lapse fluorescence microscopy to visualize the movement of individual MVs labelled with a fluorescent dye did not support a role for extracellular vesicles as long-distance messengers, as MVs mostly dispersed along the bacterial surface with rare diffusion into the intercellular space[104]. However, as this study investigated MV trafficking in *E. coli* biofilms grown statically on an agar surface, the role of fluid flow, which prevails in most aqueous habitats, has not been considered. For example, MVs released by oral and intestinal bacteria were shown to contribute to inflammation in the central nervous system, implying a risk for developing neurodegenerative disorders, such as Alzheimer disease[105]. Mechanistic studies on how MVs cross mucosal epithelial barriers are limited, but given the fact that MVs are efficiently internalized by epithelial and endothelial cells, translocation might follow the same pathways[106]. MVs produced by pathogens of the oral cavity and respiratory tract may enter the bloodstream in the case of advanced disease progression. MVs from invasive *H. influenzae* type B increase blood–brain permeability and MVs *of Aggregatibacter actinomycetemcomitans* can cross the blood–brain barrier[107,108]. *P. gingivalis* MVs show high abundance of gingipains, which act as proteases that loosen connections between endothelial cells and may subsequently facilitate crossing of the blood–brain barrier where they induce inflammatory responses. Indeed, gingipain inhibitors reduced nervous system inflammation in a mouse model for oral *P. gingivalis* infection[109].

In addition to increased solubilization, the luminal contents and membrane-embedded molecules of MVs are also protected against degradation (Fig. 2). Many virulence factors, toxins and enzymes associated with MVs were shown to be protected against proteolysis[55,58,110,111]. Likewise, MV-associated DNA and RNA were shown to be protected from enzymatic degradation[86,89,96]. MVs were also shown to protect their cargo against environmental stress, such as heating and freezing, and to prolong the activity of enzymes[112]. Another often-overlooked feature of MV-mediated secretion is that the delivered components are concentrated in MVs, such that the fusion of a single MV with its target cell often delivers a sufficiently high amount of a molecule to ensure its bioactivity (Figs. 2 and 3). This phenomenon was first described for *Paracoccus* sp., which releases the hydrophobic quorum sensing (QS) signal *N*-hexadecanoyl-L-homoserine lactone (C16-HSL) via MVs[113]. The amount of signal molecules associated with one MV was found to be much higher than the critical concentration required for triggering a QS response. Hence, the cargo of a single MV is sufficient to induce a response in another cell, which may cause bistable gene expression, that is, two bacterial populations that are either induced or uninduced. In contrast to the classic diffusion-based QS model, in which the signal concentration decreases with increasing distance to the producing cell to a level that is too low to activate another bacterium, MV-associated signals can travel over long distances and still induce gene expression in another cell (Fig. 3). This may be particularly valuable for trafficking hydrophobic signals in aqueous environments. Importantly, although secreted signals would be infinitely diluted, their packaging into MVs ensures that a high concentration of the signal is delivered. We refer to this phenomenon as quantal secretion, and we propose that it is not limited to signal molecules but applies to any bioactive cargo molecule. For example, packaging antibiotics or toxins into MVs would ensure that a lethal dose of the compound is delivered to the target cell[114].

Specificity of cargo delivery

Evidence suggests that fusion of MVs with bacterial cells shows some degree of specificity[103,113,115] (Fig. 2). However, the underlying mechanisms of target specificity are not well understood and seem to be multifaceted. It has been hypothesized that the propensity of an MV to associate with a particular bacterium may be influenced by its specific cell envelope structure. Specifically, surface charge (zeta potential)[116] and surface hydrophobicity[117] can influence the affinity of an MV for a particular cell type. Specific ligand–receptor interactions between MVs and target cells can also affect specificity of MV delivery. In *P. aeruginosa*, TseF, an effector that is incorporated into MVs via a type VI secretion system (T6SS), directs the MVs to their parent cells

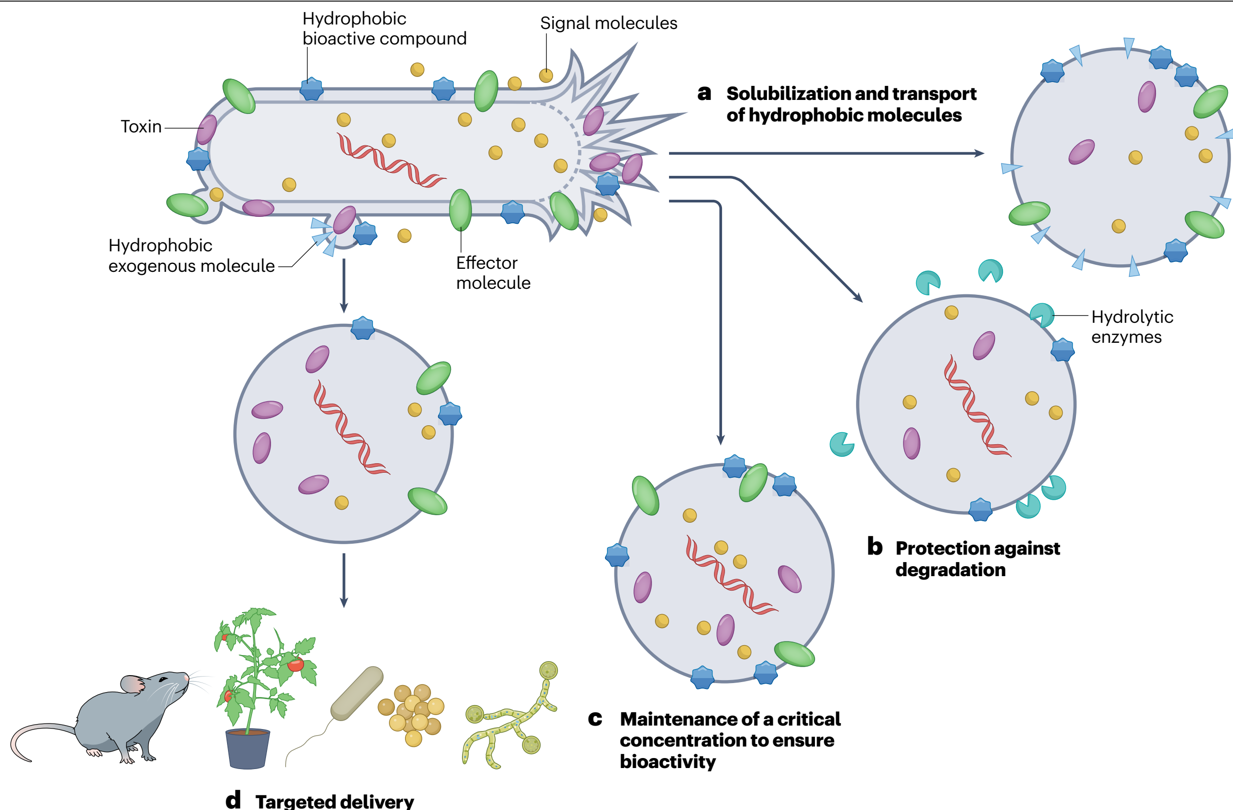


Fig. 2 | Membrane vesicle cargo transport and delivery. a, Hydrophobic molecules with poor solubility can be dispersed and might be transported over long distances in aqueous systems via membrane vesicles (MVs). **b**, The luminal contents and membrane-embedded molecules are protected from degradation by hydrolytic enzymes and adverse physiochemical conditions. **c**, MVs can concentrate bioactive components such that the fusion of a single MV with its target cell can directly deliver a sufficient amount of the molecule to ensure its bioactivity, a phenomenon referred to as quantal secretion (see also Fig. 3). **d**, MVs may have a preference to specifically fuse with certain types of cell allowing for targeted MV cargo delivery. These characteristics apply for all vesicle types independent of their biogenesis routes and thus no particular MV type is depicted, or solely attributed to these functions. However, the cargos of the different MV types differ depending on their biogenesis routes. Although the example shown is based on MVs produced by Gram-negative bacteria, most advantages of cargo delivery also apply for CMVs formed by Gram-positive bacteria.

by binding to the surface receptors FptA and OprF[118]. This system has been suggested to facilitate the acquisition of MV-associated iron by a yet unidentified mechanism. Likewise, in *Cupriavidus necator*, the T6SS-secreted LPS-binding effector TeoL is incorporated into MVs to allow their recruitment by the parent cell via the OM receptors CubA and CstR[119]. This LPS-mediated mechanism enables bacteria to recruit MVs derived from different species and is not only used for iron acquisition but also provides a fitness benefit for interbacterial competition, stress resistance and horizontal gene transfer. MVs can also deliver their cargos to eukaryotic cells via different routes, as described in the following section.

Functions of membrane vesicles

Disposal of waste material and surface remodelling

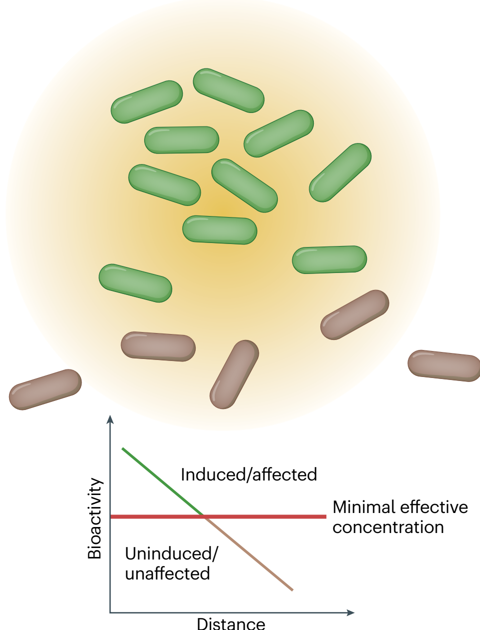
Several reports have demonstrated that OMV production can relieve membrane stress caused by the accumulation of misfolded proteins, peptidoglycan fragments or LPS in the periplasmic space[87,120,121] (Fig. 4). As this typically occurs when cells are stressed, increased vesiculation is considered to be a response to overcome adverse environmental conditions[122].

Nutrient acquisition

MVs can carry receptors that bind nutrients and deliver them to bacterial target cells. The best-investigated example is iron that can bind to MV-associated siderophores. Under iron-limiting conditions, such iron-loaded MVs have a crucial role in bacterial iron acquisition[115,118]. The involvement of MVs in iron acquisition seems to be particularly important for hydrophobic siderophores such as mycobactin of *Mycobacterium tuberculosis*, which is released and dispersed in the environment via MVs[123]. MVs can also carry hydrolytic enzymes that are essential for polysaccharide utilization by some bacteria[124,125], as well as cytosolic metabolites such as vitamins, amino acids and components of the carbon metabolism, that can support bacterial growth[39,103,126].

Neutralization of phages and antibiotics

Agents that bind to bacterial membranes will also adsorb to MVs (Fig. 4). Hence, MVs not only neutralize membrane-targeting antibiotics such as polymyxin, colistin and daptomycin[19,127] but also provide protection against host-defence factors such as antimicrobial peptides from mammalian tissue and complement system factors of the blood[46,128].



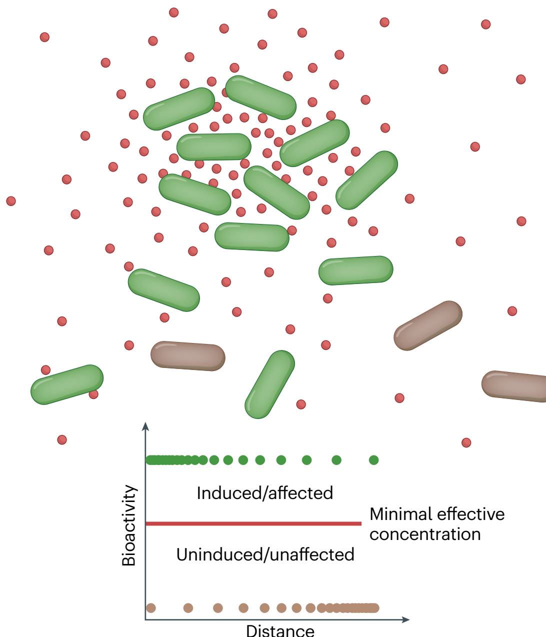


Fig. 3 | Membrane vesicles as quantal delivery systems. a, The concentration of bioactive molecules that are secreted by classic transport systems rapidly decreases with increasing distance to the producing cell as a consequence of diffusion into the surrounding environment. Below a certain minimal effective concentration, the compound will no longer show biological activity. This threshold concentration reflects the calling distance in the case of signal molecules used for cell-to-cell communication or the minimal inhibition concentration in the case of antimicrobial compounds. Importantly, as there is a gradient of the bioactive molecule, neighbouring cells will be exposed to different levels of the compound and thus will be differentially affected. **b**, Bioactive molecules that are released by the aid of membrane vesicles (MVs) are not only protected from degradation, and therefore might be capable of travelling over long distances, but are also concentrated such that the fusion of a single MV with its target cell is sufficient to deliver a dose that is above the minimal effective concentration. As a consequence, two distinct cell populations are formed, namely induced or affected and uninduced or unaffected cells. Although the concentration of MVs will decrease with distance, the high concentration will ensure that even a very distant cell can be fully affected. In the case of cell-to-cell communication, this can be compared with a message in a bottle[188] whereas in the case of antibiotics, this phenomenon has been referred to as death in a sphere[114]. All types of MV can serve as vehicles for quantal secretion.

In addition, MVs were shown to serve as decoys that can inactivate phages[127,129].

DNA transfer

DNA transfer is one of the best-documented phenotypic traits of MVs, as demonstrated for diverse bacteria[84,116,130,131] (Fig. 4). Although increasing evidence shows that the association of DNA with MVs is a consequence of explosive cell lysis, OIMV formation or encapsulation of extracellular DNA (eDNA) by broken OMVs, little is known regarding how MV-associated DNA is taken up by the recipient cell. In naturally competent bacteria, the uptake of MV-associated DNA was shown to be dependent on the competence machineries of the recipients. In *Acinetobacter baylyi*, DNA-containing MVs are lysed on contact with the OM of the bacterium followed by type IV pilus-mediated import of DNA[130]. A slightly different mechanism has been proposed for *Thermus thermophilus*. In this case, eDNA originating from cell lysis is thought to be tightly adsorbed to the surface of MVs and that the MV presents this surface-associated DNA to the competence apparatus of bacteria, which import it into the cytoplasm[132]. It is noteworthy that MV-associated DNA is mostly bound to the MV surface unless it is degraded by DNases and only a comparably small amount of DNA is found inside MVs[19,86,89]. The reason for this is that eDNA, which arises from bacterial lysis, is rapidly adsorbed by the OM. It remains unknown how, after fusion of the MV with a non-competent recipient, the associated DNA crosses the inner membrane to reach the cytoplasm. MVs isolated from various environments are enriched for viral sequences[39,133], and this would indicate that cell lysis is an important mechanism of MV formation in nature. As a result, E-type MVs are often decorated with phages and have also been observed inside MVs[5,88,134]. Given that standard protocols for the isolation of MVs do not easily separate phages from MVs, the observed DNA transfer in some of the reported studies could also be because of transduction.

MVs carrying DNA do not always promote transformation. A study showed that MVs isolated from the supernatant of a culture of *P. aeruginosa* PAO1 carrying plasmid pAK1900 contained plasmid but not chromosomal DNA in the lumen of the MVs[86]. However, MV-mediated transfer of the plasmid to *P. aeruginosa* PAO1 and *E. coli* was unsuccessful, and the authors speculated that this was due to the plasmid being unable to bypass the plasma membrane for efficient transformation.

Bacterial killing

A seminal study demonstrated that MVs of *P. aeruginosa* PAO1 can kill bacteria and proposed MVs as a conceptual new group of antibiotics[135]. A subsequent study reported that naturally produced MVs isolated from

15 Gram-negative bacteria exhibited bactericidal effects against various Gram-positive bacteria as well as *E. coli* K12 and *P. aeruginosa*[136]. The killing activity of such 'predatory' MVs was proposed to be due to cell lysis by peptidoglycan hydrolases associated with MVs, as visualized in zymograms[136]. Specifically, *P. aeruginosa* MVs were shown to carry a 26 kDa murein hydrolase, which was suggested to be required for MV-mediated killing of other bacteria. Recently, this 26 kDa major autolysin was identified as the AmphD3 amidase[137] and shown to actually have a role in cell wall recycling but not in bacterial killing, suggesting that the lytic activities observed on zymograms do not correlate with the bactericidal potential of MVs. A proteomics approach revealed that *P. aeruginosa* MVs are enriched for several autolysins that are not detectable on zymograms, suggesting that the killing activity of MVs may depend on the synergistic action of different enzymes[137]. In addition, antimicrobial metabolites associated with MVs could also contribute to their lethal activities. Such a situation is found with facultative predators, for example, members of the genera *Lysobacter* and *Myxococcus* that lyse and feed on microorganisms. These bacteria export a toxic cocktail of bioactive compounds and lytic enzymes via MVs to kill their preferred prey, which include bacteria, fungi and oomycetes[138,139] (Fig. 4). MVs of these bacteria were shown to be enriched for various hydrolytic enzymes, such as the chitin-degrading polysaccharide monooxygenase LeLPMO10A in *Lysobacter enzymogenes* OH11 (ref. [138]) or the lytic protease L5 in *Lysobacter* sp. XL1 (ref. [140]). For strain OH11, it has been suggested that LeLPMO10A may function as a 'wall opener' that enhances the action of antifungal compounds[138]. Whether the co-delivery of lytic enzymes and bioactive compounds is a general feature of predatory MVs remains to be elucidated.

Delivery of bioactive compounds

Small hydrophobic molecules can integrate into the OM of Gram-negative bacteria where they may induce membrane curvature into the cell envelope leading to the formation of OMVs through blebbing. This 'bilayer-couple' model was originally proposed for *P. aeruginosa*, in which the PQS mediates its own packaging and transport by stimulating OMV formation through intercalation into the OM[141,142]. Subsequently, several other bacterial signalling molecules were shown to be released via MVs, including C16-HSL in *Paracoccus denitrificans*[113] and the long-chain ketone CAI-1 in *Vibrio harveyi*[143]. The list of MV-secreted bioactive molecules is not limited to signal molecules and also includes many bioactive compounds with antibiotic or antifungal activities. In this context, it is interesting to note that the anti-staphylococcal activity of *P. aeruginosa* MVs was shown to be caused by PQS, which has antibiotic activity, and not peptidoglycan

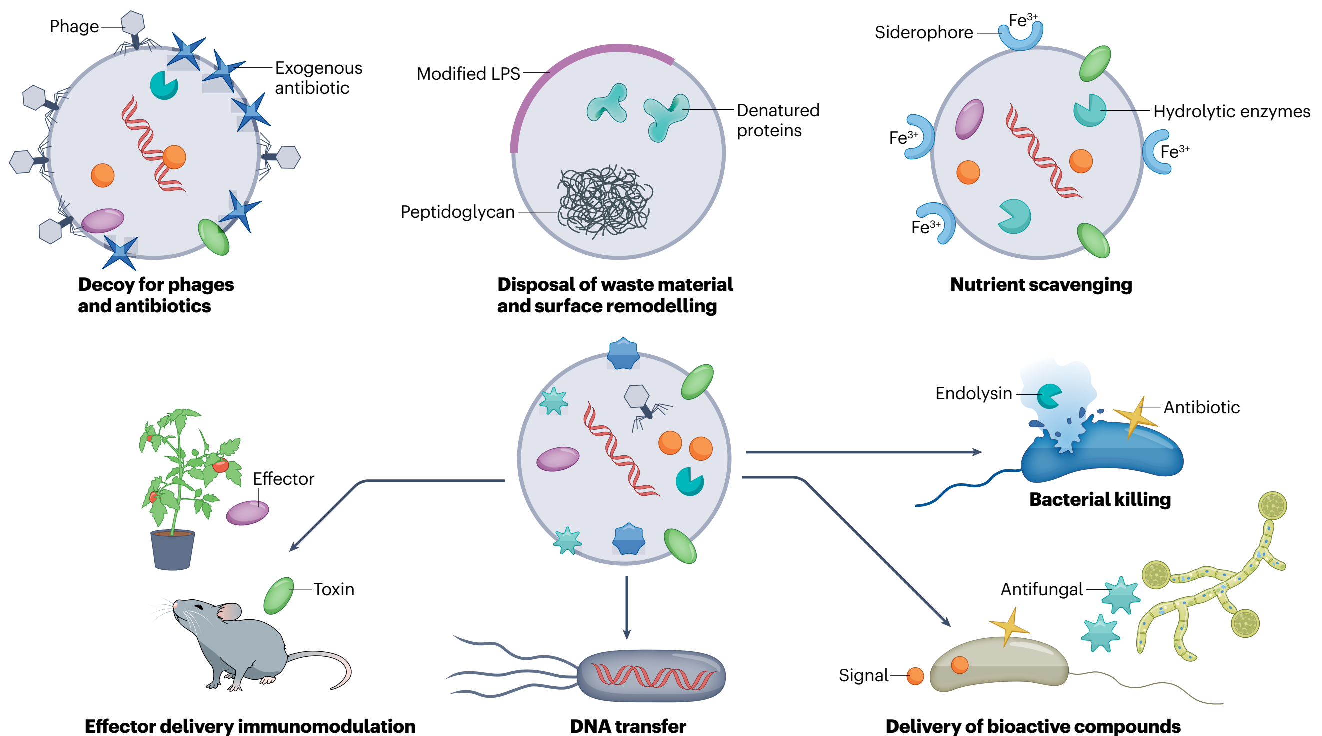


Fig. 4 | Membrane vesicles have diverse biological functions. Several functions are likely associated with all membrane vesicles (MVs) such as inactivation of phages, neutralization of externally added antibiotics, nutrient acquisition and bacterial killing (via antibiotic compounds enriched in B-type MVs and endolysins enriched in E-type MVs). Some functions may be more specific to a particular MV type. For instance, outer–inner MVs and E-type MVs will preferentially transfer DNA. Likewise, as in many bacteria, the expression of toxins and the formation of MVs are coordinated by the SOS response; E-type MVs may be particularly important for interactions with eukaryotic organisms and host immunomodulation. On the contrary, the disposal of denatured proteins, peptidoglycan and modified lipopolysaccharides (LPS) seems to be specific for outer membrane (OM) vesicles, which are formed on the accumulation of these molecules. Although all vesicle types can in principle bind and transport hydrophobic compounds, these may be particularly often associated with OM vesicles, which can be formed through the intercalation of hydrophobic compounds into the OM. Exogenous antibiotic (blue star) refers to any antibiotic that is added to a culture and is neutralized by MVs (such as membrane-targeting antibiotics). Antibiotics that are produced by the bacterium and are released via MVs are indicated by a yellow star.

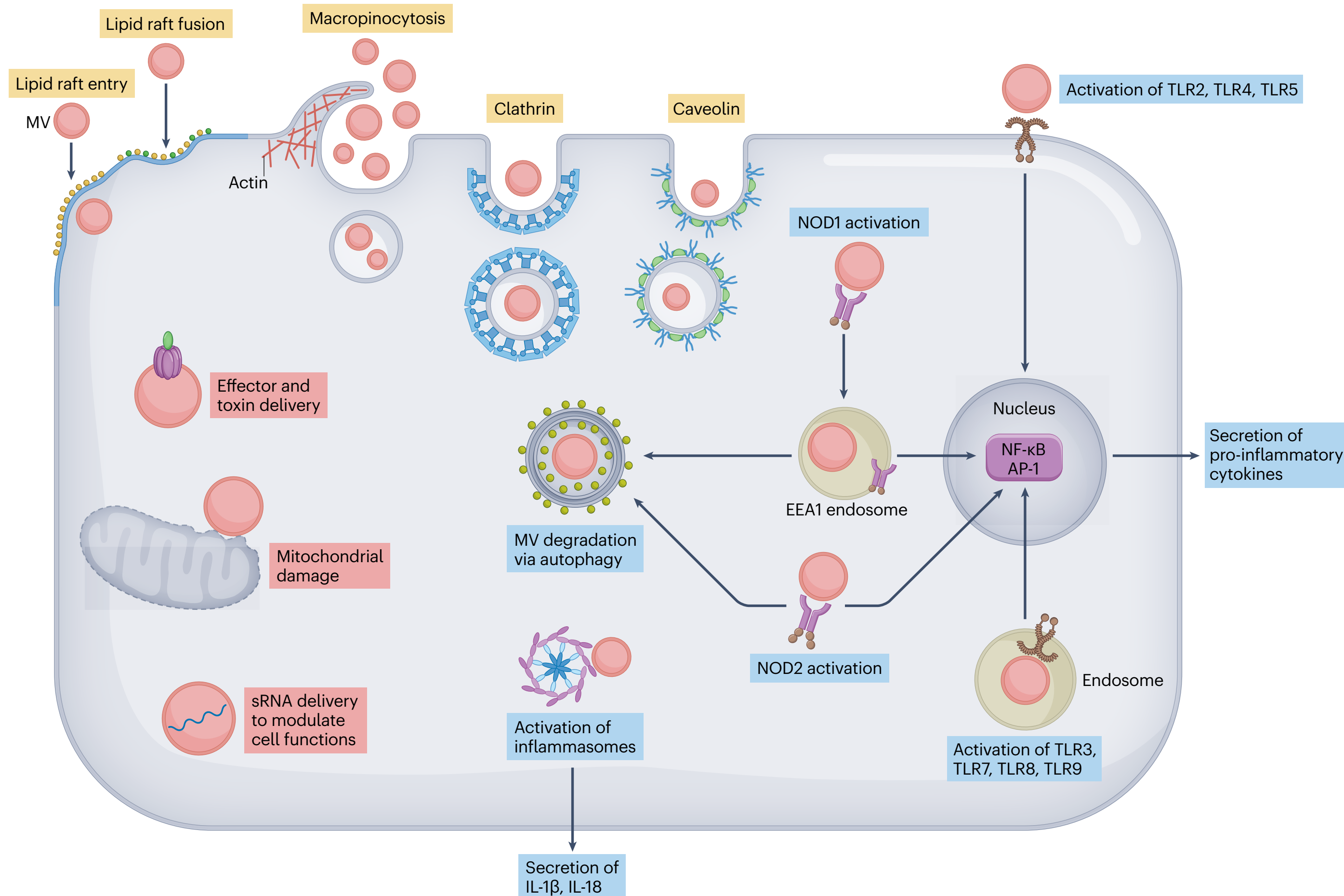


Fig. 5 | Bacterial membrane vesicles enter host cells to modulate immunity and mediate pathogenesis. Membrane vesicles (MVs) can enter non-phagocytic host cells via a range of mechanisms that include entry via lipid rafts, fusion with lipid rafts or entry via micropinocytosis, clathrin-mediated or caveolin-mediated endocytosis (indicated by yellow text boxes). Once within host cells, MVs can modulate host immunity by delivering bacterial effectors and immunomodulatory small RNA (sRNA) from their parent bacteria or by mediating mitochondrial damage (indicated by red text boxes). MVs and their cargo can be detected by host innate immune receptors resulting in the induction of a pro-inflammatory response (indicated by blue text boxes). This includes the activation of surface and endosomal-bound toll-like receptors (TLRs), the activation of cytoplasmic nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and 2 (NOD2) receptors and the activation of inflammasomes, which collectively results in the production of pro-inflammatory cytokines and chemokines. Once within host cells, MVs are cleared by the host cellular degradation autophagy pathway. AP-1, activator protein 1; EEA1, early endosome antigen 1; NF-κB, nuclear factor-κB.

lytic enzymes[144]. Likewise, *Burkholderia thailandensis* was shown to release MVs that contain the hydroxyalkylquinoline HMNQ and a long-chain rhamnolipid[145], both of which exhibit antimicrobial and antibiofilm properties against methicillin-resistant *S. aureus*. The purple pigment produced by *Chromobacterium violaceum*, violacein, which has broad antimicrobial activity, was also shown to be packaged into MVs[146]. The linear polyketides linearmycins A and B, which exhibit antifungal and antibacterial activity, are not only trafficked by MVs but also induce vesiculation in *Streptomyces* sp. Mg1 (ref. [147]). Another example is the bacteriocin micrococcin P1 synthesized by several Gram-positive bacteria. *Staphylococcus hominis* S34 secretes this hydrophobic compound into the supernatant where it is incorporated into MVs produced by the organism[148]. The MV-associated bacteriocin was found to be more active than the pure compound, possibly as a consequence of increased solubility, high compound concentration and optimized delivery. Although emerging evidence shows that the ability of MVs to kill other bacteria is often dependent on the presence of antimicrobial compounds, it remains to be explored whether MV-associated small molecules also affect plant and animal cells.

Host cell entry

MVs can enter host cells via different mechanisms involving fusion with the host cell membrane or by direct entry (Fig. 5). Fusion of MVs with host cells has been evidenced for a range of pathogens[55,149], and host cell lipid rafts can facilitate this process[150]. Direct entry of intact MVs into host epithelial cells may involve lipid rafts and cholesterol-rich membrane microdomains[51,55,151], or host uptake pathways involving micropinocytosis, clathrin-mediated or caveolin-mediated endocytosis[152], whereas MV entry into immune cells can occur via endocytic and phagocytic mechanisms[153]. More recently, MVs produced by

Gram-positive bacteria such as *S. aureus* have been reported to enter host epithelial cells via lipid rafts[154] and macrophages via endocytosis[45]. MV size and composition are key factors determining their entry into host cells. *Helicobacter pylori* MVs with a diameter of less than 100 nm predominantly enter host cells via caveolin-mediated endocytosis, whereas larger MVs use micropinocytosis and endocytosis mechanisms for cell entry[155]. The composition of MVs can also affect the efficiency of their uptake into non-phagocytic epithelial cells. For example, the presence of LPS O-antigen in *E. coli* MVs, or OmpU and OmpT porins in *V. cholerae* MVs, enhanced their uptake into host cells[58,156].

Immune stimulation and immunomodulation

The cargo of MVs can directly activate a broad range of host innate immune pattern recognition receptors (PRRs) to promote cytokine production, inflammation and programmed cell death (Fig. 5). Toll-like receptors (TLRs) are host innate PRRs that can be activated by various microorganism-associated molecular patterns contained within MVs (reviewed elsewhere[157]). The innate immune receptor TLR4 detects LPS and LOS, resulting in the activation of nuclear factor-κB and a pro-inflammatory response. TLR4 activation can be mediated by *E. coli* or *P. aeruginosa* MVs[158,159], and enterohaemorrhagic *E. coli* O157 MVs can additionally activate TLR5, to induce IL-8 production by epithelial cells[160]. TLR2 that detects lipoproteins can be activated by *Mycobacterium*-derived MVs, which mediate inflammation in the lungs of mice in a TLR2-dependent manner[161]. In addition, CMVs derived from a range of Gram-positive bacteria have the ability to activate TLRs and mediate pro-inflammatory cytokine responses[16], including *S. aureus* CMVs that can activate TLR2 signalling in epithelial cells[162] and macrophages[45]. Once inside the host cell, MVs can activate a broad range of intracellular TLRs. For example, *P. gingivalis* MVs activate TLR7, TLR8 and TLR9 via their RNA and DNA cargo, in addition to TLR2 and TLR4 (ref. [163]).

Glossary

Bistable gene expression
A regulatory system that results in the same gene being expressed in some cells and silenced in others to trigger stochastic switch-like transitions between cellular differentiation states.

Endolysins
Hydrolytic enzymes that are produced by bacteriophages to degrade the cell wall of the bacterial host during the final stage of the lytic cycle.

Intercalation
Reversible insertion of molecules into materials with layered structures such as the cellular membrane.

Pattern recognition receptors
PRRs. Germline-encoded innate immune receptors expressed by macrophages, dendritic cells and epithelial cells that recognize different types of pathogen-associated molecular pattern.

Quantal secretion
Secretion of molecule packages to ensure biological activity on delivery to target cells.

Quorum sensing
QS. A cell-to-cell communication mechanism in bacteria by which gene regulation is controlled in a population-dependent manner through the production and perception of signal molecules.

SOS response
A global regulatory system that allows bacteria to respond to DNA damage by arresting growth and inducing DNA repair and mutagenesis as well as controlling prophage induction.

S. aureus CMV-associated DNA and RNA activate TLR7, TLR8 and TLR9 in epithelial cells[162] in addition to TLR3, TLR7 and TLR9 in macrophages[93]. Given that the composition of MVs depends on culture conditions, careful considerations should be performed when comparing their immunostimulatory functions between bacterial strains and studies[164].

MVs can also modulate host immunity independent of PRRs to promote pathogenesis. For example, OmpA in *A. baumannii* MVs causes mitochondrial fragmentation and cytotoxicity[73], sphingolipids present in *P. gingivalis* MVs suppress cytokine responses in immune cells[165] and *Fusobacterium nucleatum* MVs transform macrophages to the M1 phenotype to enhance the development of periodontitis in a mouse model of disease[166]. Furthermore, a range of virulence factors can be delivered directly into host cells via MVs, and more recently, their contribution to delivering immunomodulatory sRNA into host cells to regulate host gene expression and immunity has been recognized[58,167–169]. This includes the ability of an sRNA identified in *P. aeruginosa* MVs to attenuate MV-induced IL-8 responses in human airway epithelial cells and neutrophil recruitment in a murine model[96]. Similarly, sRNAs contained in MVs produced by a range of periodontal pathogens[170], and a tRNA fragment contained within *H. pylori* OMVs[171], have been reported to impair cytokine responses in host cells. Collectively, these studies reveal the ability of MVs to deliver immunomodulatory cargo into host cells to modulate immunity and disease outcomes.

In addition to activating membrane-bound PRRs, intracellular MVs can also activate host cytosolic PRRs. The cytosolic innate immune receptor nucleotide-binding oligomerization domain-containing protein 1 (NOD1) detects a conserved structural motif present within peptidoglycan from almost all Gram-negative bacteria. MV-associated peptidoglycan was shown to enter epithelial cells and activate intracellular NOD1, resulting in the production of nuclear factor-κB, and the upregulation of human β-defensins 2 and 3 (ref. [151]), in addition to the activation of NOD2, which detects a conserved peptidoglycan motif common to both Gram-negative and Gram-positive bacteria[172,173]. Once within host epithelial cells, MVs are cleared from the host via the host cellular degradation pathway of autophagy in a NOD1-dependent manner[174]. A recent study reports the ability of *S. aureus*-produced CMVs to activate NOD2, resulting in cytokine production and clearance from the host via autophagy[162].

Cytosolic inflammasomes function to protect the host from pathogens by inducing cell death and initiating immunity. MVs produced by various pathogens can induce the activation of inflammasomes, which are multiprotein complexes that assemble in the host cell cytosol and involve caspases[175]. Recent data suggest that LPS associated with internalized MVs is recognized by cytosolic proteases caspase-4/11 and host guanylate-binding proteins involved in the NLRP3 inflammasome[176], which can trigger the secretion of IL-1β and IL-18, as well as pyroptosis resulting in endotoxin-related cell death. Similarly, PorB associated with *N. gonorrhoeae* MVs can cause loss of mitochondrial membrane potential and cell death in macrophages[72], and *S. aureus* CMVs can trigger inflammasome activation in macrophages[45].

MVs produced by commensals can also provide a selective advantage to their parent bacteria by killing competing bacteria, conferring a protective niche or facilitating immunoregulation in the host[177]. Similar to pathogen-derived MVs, commensal-derived MVs can enter host cells via different pathways, including clathrin-mediated[178] and dynamin-dependent endocytosis[179]. Commensal-derived MVs can also activate host PRRs[180,181] to elicit immunostimulatory or immunomodulatory effects[28] and to confer protection against experimental colitis[182]. In addition, MVs produced by the commensal *Bacteroides fragilis*

preferentially activate host PRRs compared with their parent bacterium, further implicating the contribution of commensal-derived MVs to modulating immunity in the gastrointestinal tract[183]. Moreover, microbiota-derived MVs can promote systemic antiviral immunity as a result of priming type I interferon responses owing to the detection of DNA containing MVs by cyclic GMP–AMP synthase[184]. These examples provide evidence that commensal MVs might be key contributors to maintaining host immunity and intestinal homeostasis.

Future perspectives

MV-based release of molecules in Gram-negative bacteria has been suggested to represent the type 0 secretion system (T0SS)[2]. In comparison to classic secretion systems that allow for the export of molecules into the extracellular space or the injection of effectors or DNA into target cells, MVs seem to be particularly valuable for the export of lipids, hydrophobic molecules, insoluble material and virulence factors, for which they are often enriched. Another advantage of the T0SS over other secretion systems is that it allows the export and specific delivery of a cocktail of molecules to their target cells, which may synergistically enhance their activities. Moreover, cargo compounds are concentrated in MVs and thus the T0SS may ensure that a biologically relevant dose is delivered, a phenomenon that we refer to as quantal secretion (Fig. 3). Although this concept can also possibly be applied to injection-based secretion systems, it is fundamentally different from all other secretion systems where the concentration of the secreted molecules is diluted with increasing distance to the cell. More recently, a novel holin and peptidoglycan hydrolase-dependent (type 10) secretion system (T10SS) has been proposed to be required for the release of a chitinase in *Serratia marcescens*[185]. Although the T10SS shows remarkable mechanistic similarity to endolysin-triggered MV formation and is often located within prophage genomic islands, evidence has been presented that cell lysis is not required for secretion[186]. In the current model, an L-Ala D-Glu endopeptidase, which enters the periplasmic space via pores formed by a holin-like protein, loosens the peptidoglycan crosslinking and thereby allows the release of the chitinase either via an unidentified OM channel or via packaging into OMVs. Further research is required to distinguish between these possibilities, to show that T10SS represents a novel OMV biogenesis route and to unambiguously rule out the possibility of cell lysis of a small subpopulation.

Given that most bacteria carry phage endolysins, cell lysis of a subpopulation of bacterial cells seems to be inevitable. Although particular growth conditions can favour certain MV biogenesis pathways[19,47,61,67], MV preparations will normally be heterogeneous and contain MVs originating from both blebbing and lysis[11,164,187]. As certain MV functions could be associated with an under-represented MV type, previous reports that attribute certain phenotypes solely to OMVs have to be interpreted with care. A recent study used high-resolution flow cytometry, transmission electron microscopy and cryo-electron microscopy to determine the amount and types of MVs secreted by *S. vesiculosa* M7T during different growth phases[11], leading to the discovery of a new E-type MV, explosive outer–inner MVs. Although this study showed that flow cytometry can be a valuable tool for the separation of relatively large MVs (>100 nm), currently it does not seem possible to separate smaller MV types. A main challenge in the field will therefore be the improvement of flow cytometric techniques as well as the development of novel methods to separate and isolate different MV types to determine their compositions and to analyse their functions. Once unambiguous profiles of all MV types are available, it may be possible to ascertain whether certain MV types are dominant in a particular environmental niche or clinical sample. This may not only shed light on the conditions bacteria experience in a particular habitat but may also allow us to draw conclusions about the functions of different MV types in nature.

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