The Role of Outer Membrane Vesicles against Bacteria

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Gram-negative bacteria are resistant to many commercialized antibiotics. The outer membrane (OM) of Gram-negative bacteria prevents the entry of such antibiotics. Outer membrane vesicles (OMV) are naturally released from the OM of Gram-negative bacteria for a range of purposes, including competition with other bacteria. OMV may carry, as part of the membrane or lumen, molecules with antibacterial activity. Such OMV can be exposed to and can fuse with the cell surface of different bacterial species.

outer membrane vesicles | antimicrobial activity | Gram-negative bacteria | Gram-positive bacteria

1. Introduction

Membrane vesicles (MV) are formed and released by a broad range of cells, from bacteria to human cells, with different nomenclature attributed according to the budding cell [1][2][3]. These MV are mostly spheres from the membrane of the cells with a large size range from 20 nm to 10 µm, depending on the donor cell [4][5]. The formation of vesicles occurs by vesiculation from living cells as a result of a disruption of the membrane caused by internal mechanisms or induced by an external signal. Depending on the donor, the bacterial MV have different functions, and the same cell can also produce MV with different functions. The main function of MV is the transport of different molecules, such as lipids, proteins, and nucleic acids. The molecules that constitute the cargo of the MV as well as their target will differ depending on the stimuli [6][7][8][9]. The MV show diverse functions from secretion of toxins and virulence factors to the modulation of the target cell, acquisition of nutrients and even resistance to stress [8][10][11][12].

Depending on their constitution and biogenesis, MV secreted by Gram-negative bacteria can be classified as outer membrane vesicles (OMV), outer-inner membrane vesicles, explosive outer membrane vesicles or tube-shaped membranous structures [13]. The most common MV from Gram-negative bacteria are the OMV, also named membrane blebs or outer membrane blebs [6][14]. MV released by Gram-positive bacteria include the cytoplasmic membrane vesicles and the tube-shaped membranous structures [13].

Antibiotic resistance is due to the bacteria's ability of adaptation to the action of antimicrobial molecules as well as to prevent them reaching the target site [15][16]. OMV have a natural role in antimicrobial resistance since they can act as a decoy or remove antibiotics that cross the cell wall, allowing bacteria to survive [6][16]. New approaches

have been developed to understand whether OMV may be used as a tool to deliver antibiotics against bacteria. Due to the composition similarity of OMV and the outer membrane (OM) of the cell wall of Gram-negative bacteria, delivery of OMV content into Gram-negative bacterial cells is more efficient; nonetheless, an antimicrobial effect can also be seen against Gram-positive bacteria. Additional advantages of OMV as antibiotic delivery tools include their stability, the cargo protection against enzymatic degradation, the ability to incorporate both hydrophilic and hydrophobic molecules and the capacity to selectively target other bacterial cells [17]. Several challenges have been reported including the need to optimize the loading of different antibiotics, as well as the binding of OMV with the desired target cell and resulting toxicity.

2. OMV Biogenesis in Gram-Negative Bacteria

Gram-negative bacteria have an envelope comprising an OM and an inner membrane (IM) with a periplasmic space in between, which contains a layer of peptidoglycan (PG). In the OM there are lipopolysaccharides (LPS) linked covalently by the lipidic moiety and proteins bound as β-barrels, while in the IM the proteins are bound as α-helical [18].

The destabilization of ligations in a bacterium's cell wall can lead to the detachment of the OM from the cell membrane. Consequently, the natural stabilization of the molecular charges allows the formation of the OMV [18] [19]. OMV are nanostructures with size range between 20 and 250 nm that are secreted from the bacteria's OM, being composed of phospholipids, LPS and outer membrane proteins (OMP) [19]. During formation, molecules such as nucleic acids and proteins from the periplasm and cytoplasm of the cells can be localized to the lumen of the vesicle, making it a vehicle for antibiotic resistance and virulence dissemination [18][20]. Some bacteria can also incorporate external antibiotics into vesicles, allowing isolation of antibiotics inside OMV and cell membrane stabilization [6].

The first step to the formation of OMV is the disruption of the connection OM-PG-IM without damage or loss of membrane integrity [18]. To explain the formation of OMV, three models have been created (**Figure 1**), which are not mutually exclusive: deficiency of lipoprotein (LPP) or its links in OM; increase of PG or other lipids residues; repulsion of negatively charged LPS [19].

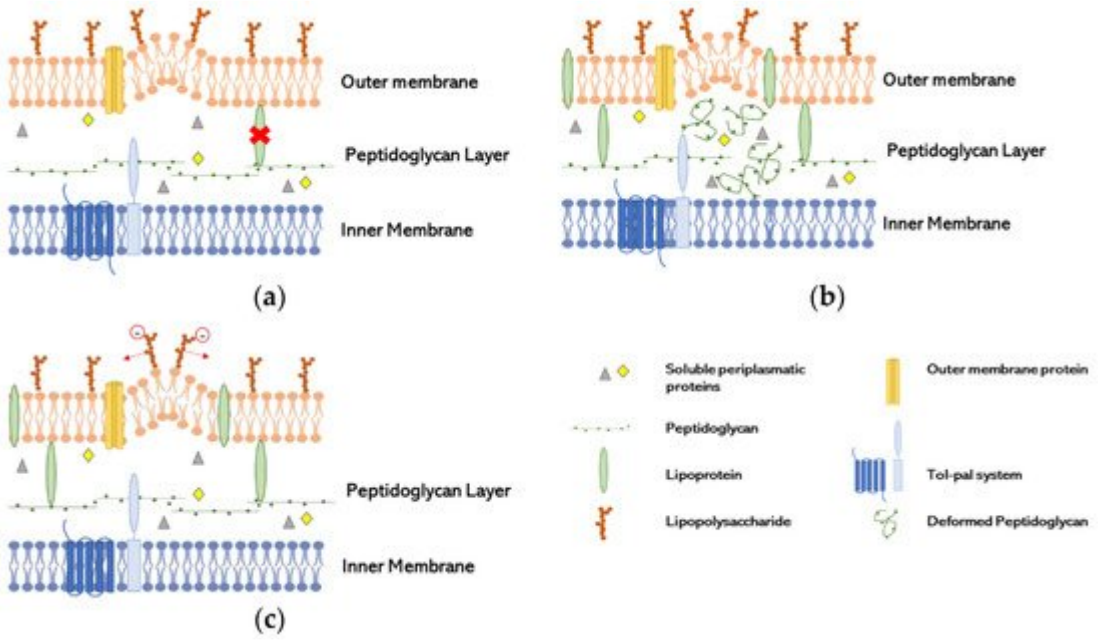

Figure 1. Models of OMV biogenesis (**a**) Vesiculation caused by the absence of lipoprotein links. (**b**) Vesiculation caused by accumulation of misfolded peptidoglycan. (**c**) Vesiculation caused by negatively charged lipopolysaccharide.

- LPP links deficiency (**Figure 1**a): the presence of LPP in the unbound form has been found in OMV, indicating that the covalent links were broken, or their distribution was not homogenous, since the conversion of free-form LPP into bound form is reversible [21]. These characteristics seems to be induced by the non-proportional growth of the OM compared with the PG layer [22]. The relation of the lack of link between OmpA and PG has been proven to be essential to the production of OMV in *Salmonella* spp. [23].

- Increase of misfolded PG (**Figure 1**b): Autolysins have a role in cleaving the covalent links of PG, resulting in cell wall remodeling. The lack of these enzymes increases the amount of peptides in periplasmatic space and other components leading to turgor pressure and therefore to OMV formation. Several studies explore the lack of autolysins to increase the concentration of proteins in the periplasmatic space and therefore converge to this model [24][25].

- Repulsion of negatively charged LPS (**Figure 1**c): A study suggested that the repulsion of negatively charge B-band LPS in cells exposed to gentamicin, with great affinity to LPS, induce the release of OMV as a way of antibiotic resistance in which gentamicin was incorporated into OMV. That repulsion increased the production of vesicles in *P. aeruginosa* [6].

3. Antimicrobial Activity of OMV

The OMV antimicrobial activity is appealing for treatment purposes, especially against Gram-negative bacteria since it allows bridging with its cell wall. During OMV biogenesis, some molecules with antibacterial activity will be

naturally included in the vesicle lumen. At the same time, several environmental inductors can enhance or contribute to the inclusion of those molecules into the OMV. In both cases, OMV can act as an antimicrobial agent.

3.1. OMV with Natural Antimicrobial Activity Cargo

The functions and roles of autolysin, also known as murein hydrolase [26][27], have being explored in *Bacillus* spp. [28]. Autolysins are usually PG-hydrolyzing endogenous enzymes that naturally lyse the peptide bridges in the PG layer [29], although there are some exceptions where it can have glycosidic activity [30]. There are several types of autolysins related with different mechanisms, such as protein and toxin secretion [31], flagellar formation [32][33], cell separation [34] and antibacterial activity associated to bacterial competition [12][35]. Its relation with vesicles and antimicrobial activity was observed for the first time in *P. aeruginosa* [6]. The presence of autolysins in OMV seem to be related with its normal location at the PG layer, being included in the cargo of the OMV during the bleb of the OM [6][26]. However, a recent study in *Lysobacter* spp. found that the distribution of the L5 enzyme, a bacteriolytic peptidase, may not be random, because it is only present in bacteria when this is exposed to a 30% sucrose medium. The study demonstrated that this autolysin is unevenly placed through the periplasmatic space, specifically where the vesiculation occurred and therefore L5 seems to be a factor in OMV biogenesis [36].

Hemolysin is another type of enzyme that is present in OMV from Gram-negative bacteria, such as *P. aeruginosa* and *E. coli* [6][37]. One hemolysin from *P. aeruginosa* has been shown to be responsible for co-regulation of protein secretion but also injection of toxin proteins into other Gram-negative bacteria, including *E. coli* [38], by type VI secretion system (T6SS) [39]. In addition, T6SS has been shown to be incorporated in OMV from P. aeruginosa [8]. The T6SS system allows the bacteria to compete through the delivery of toxins, that are capable of killing other bacteria [39].

3.2. OMV with Loaded Antimicrobial Cargo

There are two main ways to incorporate antibiotics into OMV: the passive loading, where the addition of the antibiotics during bacterial growth is enough to produce antibiotic-carrying OMV (aOMV), and the active loading approach, where the antibiotics are forced to enter or coat the OMV or OM of bacteria, so it can be part of the produced OMV.

3.2.1. Passive Loading

Passive loading methods use diffusion by osmotic gradient but only for hydrophobic positively charged small molecules. These molecules may pass through the lipophilic membrane because of their opposite charges and their similar affinity to water [40]. In these methods, only the components of the medium or environmental characteristics are changed to destabilise the membrane and allow the entrance of antibiotics and other molecules [40][41].

Up to now, only antibiotics that have been demonstrated to pass through the cell wall of Gram-negative bacteria have been inserted into the OMV. Addition of gentamicin to *P. aeruginosa* cells showed the production of OMV-

carrying gentamicin [6], which had antibacterial effects against both Gram-negative and Gram-positive bacteria species [12]. These gentamicin-carrying OMV showed antibacterial activity against *P. aeruginosa* 8803, which has a permeability-type resistance to gentamicin [12], highlighting the potential of OMV antimicrobial delivery to overcome resistance. More recently, different antibiotics, such as ceftriaxone, amikacin, azithromycin, ampicillin and levofloxacin, were loaded into *A. baumannii* OMV, which showed antibacterial effects against enterotoxigenic *E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa* without toxic activity in mice [42].

3.2.2. Active Loading

The active loading methods consist of forcing the entrance of the molecules into the vesicles, normally by physically damaging the cells or the vesicles. There are three main methods for active loading: electroporation, sonication, and extrusion, and they are already commonly used to produce vesicles from animal cells such as exosomes.

Electroporation is an electro-physical method that uses electron impulses to rearrange the OM of the cell with the consequent creation of pores [43][44]. This method is typically used to make cell transfections of DNA, RNA and proteins, however its ability to translocate large molecules leads to an instable cell wall and therefore more cell death by lysis and less efficacy [43]. This method has been successfully used to insert small interference RNA into OMV from *E. coli* [45].

Sonication uses ultrasound to compress and decompress cells in order to compromise membrane stability, followed by a second sonication to assemble the membrane fragments and allow the incorporation of external molecules [46]. Mild sonication has been used successfully to induce paclitaxel loading into exosomes from macrophages to treat cancer cells and also to load small RNA into extracellular vesicles from different cell lines [47] [48]. This method has already been used in *Haemophilus parasuis* to induce OMV production, however the protein content changed when compared to the natural OMV [49]. The use of sonication in bacterial studies is mostly used to induce cell lysis, and for detection of biofilms; studies related to OMV loading with sonication have not yet been performed [49][50][51][52].

Extrusion involves the mixture of cells and antibiotics added to a syringe extruder and then forced to pass through a porous membrane, under controlled temperature. The hydrostatic fluid pressure will disrupt the membrane, by increasing the axial tension, and allow the drug entrance at the same time that vesicles are formed; however, variation of size and zeta potential can occur [9][53]. Despite the yield in loading and forming vesicles with this method is high, the vesicles may not be homogenous and its impact in protein membrane structures is not clear, though higher pressures may lead to protein damage and cell death [53][54].

4. Conclusions

OMV from Gram-negative bacteria have innate antibacterial activity due to the incorporation of several enzymes such as lysins. The incorporation of non-natural molecules into OMV with additional antibacterial effect has been

shown. This suggests that a delivery system could be developed to overcome the OM barrier of the Gram-negative bacteria and that bacterial OMV can be used to deliver antibiotics to targeted populations of Gram-negative and -positive pathogenic bacteria. The OMV antimicrobial effect will depend on their cargo and the bacterial species targeted, as well as the resistance mechanism present in the target bacterial cells. The repurposing of antibiotics that are ineffective due to the permeability barrier of the cell wall of bacteria may also be possible with the use of OMV.

The industrial production of OMV can be enhanced by changing the growth conditions or through different techniques that force cell damage; an alternative to OMV production with active lumen content is coating of the OMV with nanoparticles. However, several technical challenges that hamper the use of OMV remain, such as the types of MV isolated, the purification yield, and the optimal technique to produce vesicles with desired lumen content, especially hydrophilic molecules which cannot enter by passive loading. The reduction of the LPS toxicity is another point that needs to be optimized in order to reduce the immunogenic potential of OMV; for instance, the engineering of strains with altered LPS [55] might be a solution to produce OMV with reduced cytotoxicity. There is also still the need to better understand the target populations, including the specifics of the interaction between OMV and the target as well as subsequent host cell reactions.

Overall, despite the challenges that must still be overcomed, OMV represent a cost-effective and safe drug delivery tool, representing a promising alternative for the treatment of bacterial infections caused by antibiotic-resistant bacteria and for the repurposing of antibiotics that are not usually effective against Gram-negative bacteria.

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