Nanomaterials and molecular transporters to overcome the bacterial envelope barrier: towards advanced delivery of antibiotics

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Abstract

With the dramatic consequences of bacterial resistance to antibiotics, nanomaterials and molecular transporters have started to be investigated as alternative antibacterials or anti-

infective carrier systems to improve the internalization of bactericidal drugs. However, the capability of nanomaterials/molecular transporters to overcome the bacterial cell envelope is poorly understood. It is critical to consider the sophisticated architecture of bacterial envelopes and reflect how nanomaterials/molecular transporters can interact with these envelopes, being the major aim of this review. The first part of this manuscript overviews the permeability of bacterial envelopes and how it limits the internalization of common antibiotic and novel oligonucleotide drugs. Subsequently we critically discuss the mechanisms that allow nanomaterials/molecular transporters to overcome the bacterial envelopes, focusing on the most promising ones to this end – siderophores, cyclodextrins, metal nanoparticles, antimicrobial/cell-penetrating peptides and fusogenic liposomes. This review may stimulate drug delivery and microbiology scientists in designing effective nanomaterials/molecular transporters against bacterial infections.

Keywords: bacteria, antibiotics, oligonucleotides, siderophores, metal nanoparticles, antimicrobial peptides, cell-penetrating peptides, liposomes, nanomedicines

Abbreviations: OM: outer membrane; CM: cytoplasmic membrane; OMP: Outer membrane protein; LP: lipoprotein; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; WTA: wall teichoic acids; LTA: lipoteichoic acids; CD: cyclodextrin; TBDT: TonB-dependent transport; pmf: proton motive force; ABC: ATP-binding cassette; NAM: Nucleic acid mimic; PNA: peptide nucleic acids; PMO: phosphorodiamidate morpholino oligomers; LNA: locked nucleic acids; 2'OMe: 2'-OMethyl RNA; PO: phosphodiester linkages; PS: phosphorothioate linkages; MIC: minimum inhibitory concentration; NP: Nanoparticles; ROS: reactive oxygen species; LSPR: surface plasmon resonance; TEM: transmission electron microscope; AMP: Antimicrobial peptide; CPP: Cell-

penetrating peptide; SNAPPs: structurally nanoengineered antimicrobial peptide polymers; PAMAM: Polyamidoamine; a.a.: amino acid; X: 6-aminohexanoic acid; B: β-alanine; K: Lysine; F: Phenylalanine; R: Arginine; Q: Glutamine; I: Isoleucine; W: Tryptophan; N: Asparagine; CNS: Carbon nanostructures; CNT: carbon nanotubes; DOPE: dioleoylphosphatidylethanolamine; DMPG: dimiristoylphosphatidylglycerol; DPPC: dipalmitoylphosphatidylcholine; DSPC: distearoylglycerophosphocholine; DOTAP: dioleoyltrimethylammoniumpropane; DMPC: dimyristoylphosphocholine; DHP: dihexadecylhydrogenphosphate; DPPS: dipalmitoylphosphatidylserine; Chol: cholesterol

Contents:

1. Introduction

The ability to control infections with antibiotics has had a major impact on human and animal life over the last century [1]. However, the antibiotic era is threatened by resistance of bacteria to antibiotics and a very short pharmaceutical pipeline of new antibiotics [2]. Infections caused by resistant bacteria are already responsible for nearly 50 000 annual deaths in Europe and the United States [3, 4].

The bacterial cell envelope is a sophisticated permeability barrier that can hinder the internalization/accumulation of antibiotics in bacterial cells, being a major cause of bacterial resistance to antibiotics [5]. Poor antibiotic internalization could be solved if antibiotics were "carried" into bacterial cells. Bacterial resistance to antibiotics can also arise from the expression of specific genes of resistance [6-8]. This could be solved by controlling gene expression in bacteria through antisense oligonucleotides [9]. However, oligonucleotides generally fail to be internalized in bacteria [10]. Nanomaterials and molecular transporters able to successfully interact with the bacterial envelopes are interesting to carry oligonucleotides and poorly internalized antibiotics across the bacterial envelope. Also, these carriers may prevent drug efflux from the bacterial cells, as intracellular delivery of a high dosage of drug into bacteria may overwhelm the efflux pumps [11, 12]. The efflux of antibiotics will not be detailed here since it has already been extensive reviewed [13-16]. Instead of serving as carriers of bactericidal drugs into bacteria, certain nanomaterials and peptides interact with the bacterial envelope so extensively that they can disrupt it, thus serving as bactericidal *per se* to replace antibiotics. Clearly, the potential of nanomaterials and molecular transporters to overcome the bacterial envelope depends on the ability of the materials/transporters to efficiently interact with the different bacterial envelopes.

Nanomaterials and molecular transporters have been widely explored in mammalian cells which mostly take them up by endocytosis [17]. Differently, it is believed that bacteria do not endocytose (except a restricted group belonging to the phyla Planctomycetes [18]) and the use of nanomaterials/molecular transporters in bacteria is mostly based on a trial and error approach [1, 11, 12, 19-21]. An in-depth discussion on how the different layers of bacterial envelopes may be potential barriers is pivotal to boost the rational development of more efficient nanomaterials/molecular transporters in the combat of bacterial infections. Although there are some interesting reports on the recent use of nanomaterials to manage infections [1, 11, 12, 19-

21], to the best of our knowledge the interaction of nanomaterials with bacteria has never been addressed.

In this review we first summarize the general structure of the bacterial envelope of Gram-negative bacteria and Gram-positive bacteria of the typical Firmicutes phylum (Actinobacteria with a different cell wall, such as that of the genus *Mycobacterium, Nocardia, Rhodococcus* and *Corynebacterium*, are beyond the scope of this review). We then consider the permeability of these envelopes to external compounds and how it limits the internalization of common antibiotics and novel oligonucleotide drugs into bacteria. Subsequently, the nanomaterials and molecular transporters with potential to overcome the bacterial envelope, either by direct penetration or intracellular delivery of antibiotic/oligonucleotide drugs, are discussed. A special focus is given to the cell wall, i.e. the outermost layer of the bacterial envelope that determines the bacterial envelope permeability and the initial interaction with foreign compounds and materials. Seemingly much more research has been reported on the transport in Gram-negative bacteria compared to Gram-positive bacteria; nevertheless, this review aims for a global overview, including the relevant Gram-positive teichoic acids which are seldom considered. Finally, our conclusions and future perspectives are presented.

2. Bacterial cell envelope barrier

Bacteria, like mammalian cells, have their cytosol surrounded by a symmetric bilayer composed of amphiphilic phospholipids – the **cytoplasmic membrane** [22]. However, because they are unicellular organisms that often inhabit hostile environments, bacteria developed an extra cell wall that surrounds and protects the cytoplasmic membrane [23]. The **cell wall** provides protection against osmotic pressure and mechanical damage, while allowing permeation of key substrates for bacterial metabolism and communication with the environment

[24, 25]. Together, the cell wall and the cytoplasmic membrane compose the **bacterial cell envelope**. The cell wall, in turn, is subdivided into different layers. There is often lack of precision in the field on the reference to the different bacterial envelope layers. In this section, these envelope layers and their permeability will be described for Gram-negative and Gram-positive bacteria of the typical Firmicutes phylum, Figure 1 and 2. The permeability of these envelopes is overviewed according to the current knowledge (which is still limited), with a focus on the transport through the cell wall – the critical intake barrier.

2.1 Gram-negative bacteria

The **cell wall** of Gram-negative bacteria comprises the outer membrane (OM) and the periplasmic space composed of a thin layer of peptidoglycan, Figure 1 [26]. Since the OM is determinat to define the permeability to foreign compounds, a particular focus is given below to the OM.

2.1.1 Outer membrane

The **OM** is not a common phospholipid bilayer, but rather an asymmetric lipid bilayer – it is composed of phospholipids only in the inner leaflet and mostly lipopolysaccharide (LPS) in the outer leaflet [24, 27, 28]. In Gram-negative bacteria the major membrane **phospholipid** is phosphatidylethanolamine (PE), followed by phosphatidylglycerol (PG) and smaller amounts of cardiolipin [29, 30] (Figure 1). The **LPS** is a nonfluidic amphiphilic structure composed of three covalently-linked regions (Figure 1). LPS has a net negative charge, higher than the usual negatively-charged phospholipids, and it is held in position at the OM surface by divalent cations (Figure 1) [23, 24, 27]. The presence of LPS in the OM decreases its permeability to hydrophobic compounds (Figure 1) up to 50-100 times (as calculated from the oxidation's rate of a steroid probe in the cytosol of Gram-negative bacteria with respectively intact and LPS-

deficient OM) [23, 24, 27, 31, 32]. Hydrophobic substrates that succeed in crossing the LPS are expected to become internalized into the cytosol by diffusion through the lipid bilayers of the outer and/or cytoplasmic membranes. In addition, the OM contains embedded proteins, mainly the outer membrane proteins (OMPs; which are integral proteins spanning the entire OM) and lipoproteins (LPs; which are mainly embeded in the cytoplasmic leaflet of the OM) (Figure 1) [23]. The function of LPs is not yet completely resolved, they may act as enzymes and transporters [33]. Differently, the OMPs form aqueous channels cucial for bacterial intake of nutrients [23].

OMPs can serve either as passive diffusion channels (general porins and specific channels) or active transporters (Figure 2).

General porins (or simply porins), the most abundant proteins of the OM, allow the internalization of small hydrophilic substrates (including sugars, amino acids and ions) that are available in high concentrations and can thus passively diffuse down the concentration gradient through porins (Figure 2) [16, 34-37]. Porins do not bind the transported compounds; rather, porins are relatively unspecific and mostly discriminate their substrates by size, although charge may also play a role [28]. According to the crystallography studies on the better known classical porins (OmpF, OmpC and PhoE) present in *Escherichia coli* (*E. coli*), OmpF is typically considered the largest porin – a pore size of 0.7 nm and an exclusion limit of about 600 Da [16, 28, 30]. OmpF allows the diffusion of both cationic and uncharged molecules, while OmpC is slightly cation selective, and PhoE is slightly anion selective [30, 38-41]. Another exceptionally large porin named OmpG (with a pore diameter of 1.5 nm that can be constricted to 0.8 nm) was later found to exist in *E. coli* (and other bacteria), but only in rather small amounts [42]. The Gram-negative species investigated so far possess general porins with a permeability mostly similar to that of the general porins of *E. coli*, except *P. aeruginosa* which has a

significantly lower permeability since its major OMP channel (OprF) exists as an open channel only at very low levels [22, 28, 35, 43].

Hydrophilic substrates needed for bacterial growth are not always available in sufficiently high concentrations to diffuse fast through general porins [30]. Thus, **specific passive OMP channels** exist that can bind substrates, with low affinities, in order to preferentially facilitate their passive diffusion (Figure 2) [34, 44]. They can also allow the unspecific flow of compounds, such as amino acids and carbohydrates, that are scarce in the environment [23, 28, 36]. Like in general porins, transport through specific passive OMP channels is still driven by the concentration gradient and is thus energy-independent [34]. The best well-known specific channels are LamB and Tsx from *E. coli* [16, 36]. The LamB channel (which pore is 0.5-0.6 nm in diameter) is dedicated to the transport of maltose and larger malto-oligosaccharides, which need to be first converted in a linear form to fit the LamB channel [23, 28, 36, 45]. In addition, ScrY, a channel homologous to LamB but larger (≈ 0.85 nm pore diameter) allows the specific transport of several sugars such as sucrose, in some *E. coli* and *Salmonella* strains [28, 30, 46]. The Tsx channel, on the other hand, is involved in the specific transport of nucleosides and deoxynucleosides; free bases or nucleoside monophosphates (nucleotides) are not internalized [30, 46]. Tsx has several distinct binding sites in the channel; the part that binds the base moiety of nucleosides is only 0.3-0.5 nm wide, but the part that binds the sugar moiety is 0.7-0.8 nm wide [46].

Other noteworthy specific passive OMP channels were recently discovered that only open upon the presence of their substrate, without the need of energy, named **ligand-gated channels** (Figure 2). This way the passive diffusion of a cyclic oligosaccharide, which is too big to pass through the classical OMP passive channels, was found possible without linearization (needed for malto-oligosaccharides to cross LamB) [45]. In particular, α-cyclodextrin (α-CD), with a cylindrical bulky structure of 973 Da and an outer diameter of 1.37 nm, was internalized via

the CymA channel in *Klebsiella oxytoca* (a species closely related to *Klebsiella pneumoniae*) which, in the open state, has a diameter of around 1.1-1.4 nm [47, 48]. Orthologues of CymA are present in the Enterobacteriaceae and Vibrionaceae, although the channel permeability has only been studied in *Klebsiella oxytoca* [47].

Another ligand-gated channel is dedicated to the transport of hydrophobic long fatty acids, therefore contradicting the general understanding that only molecules with a hydrophilic surface can diffuse through OMP channels [44, 49]. In particular, oleic acid, 283 Da, could be internalized by FadL channels, which are widespread among Gram-negative bacteria, but better studied for *E. coli* [44, 49]. Differently from hydrophilic compounds, the hydrophobic fatty acids are not transported across the membrane via the polar central part of the channels, but rather through a lateral opening (of 0.8 nm in diameter) from where the fatty acids diffuse laterally in the OM (Figure 2) [37, 44].

Valuable hydrophilic substrates that are available only in extremely low amounts (such as micronutrients) need to be bound with high affinity by **active (transport) OMPs** to be internalized (Figure 2). The transport by such OMPs occurs against the concentration gradient, thus requiring energy expense [34]. Active OMPs, named TonB-dependent transporters (**TBDTs**), form large channels generally used for the uptake of iron complexes (normally up to 1000 Da) and vitamin B12 (around 1355 Da) [34, 37]. TBDTs are present in much lower amounts than passive channels and only open when triggered by substrate binding, similarly to ligand-gated passive channels [23, 28, 47]. However, differently from ligand-gated diffusion channels, energy from the proton motive force (pmf) of the cytoplasmic membrane (as the OM is not energized) has to be transmitted to the TBDT to open the channel [47], which explains that the TBDT in the OM is part of a complex system that spans the envelope (Figure 1) [28, 50]. For the active intake of iron, iron is typically in the form of complexes formed by iron chelating siderophores [50]. The siderophores are transported as a whole via the OM

transporters into the periplasm where they bind to a periplasmic binding protein and only in the cytoplasm the iron is released from the complex [50, 51]. Also vitamin B12, containing a Co^{2+}-ion in a corrin ring, is transported by bacteria as a whole [50]. Recently, it was found that besides iron and vitamin B12, the TonB-dependent transport can also be used to take up other substrates such as nickel and carbohydrates, but with much lower affinity [50]. Also colicins (proteinaceous toxins) can be taken up by TBDTs, as studied for colicin M transported via the TBDT named FhuA in *E. coli* – the only example of a protein import by *E. coli* [52]. The FhuA pore diameter is around 2.5 nm in the fully open state, while colicin M is 3 to 4 nm of diameter in the folded state, so it first needs to unfold to become (fully or partially) imported [52, 53].

2.1.2 Periplasm and cytoplasmic membrane

Compounds that cross the OM will encounter the periplasmic space located in between the outer and cytoplasmic membrane (CM) (Figure 1) [27, 30]. The **periplasm** is crucial for cell's structure maintenance, nutrition and protection against potentially harmful compounds [23, 24, 27, 30]. As such, the periplasm is densely populated with transport proteins (involved in the transport of sugars, amino acids, vitamins and inorganic ions) and enzymes that degrade potentially harmful compounds and participate in envelope biogenesis (such as phosphatases, nucleases, proteases and β-lactamases) [23, 24, 27, 30]. Besides proteins, the periplasmic space possesses a high concentration of small molecules (such as amino acids, mono- and oligosaccharides and biosynthetic precursors and degradation products of peptidoglycan) which results in a space of significantly higher viscosity than the bacterial cytosol [24]. This gel-like periplasm can, therefore, considerably retard diffusion; protein diffusion was slowed down up to 3.5 times when compared to diffusion in the cytosol, as measured for the periplasmic and cytoplasmic green fluorescent protein in *E. coli* by fluorescence recovery after photobleaching [54].

The periplasmic space comprises a thin layer of **peptidoglycan** (Figure 1). Peptidoglycan is a rigid polymer that provides structure, mechanical protection and osmoregulation [23, 24, 27]. It is a disaccharide composed of alternating units of N-acetylglucosamine and N-acetyl-muramic acid cross-linked by short peptide chains with variable composition [23, 27]. These form a coarse mesh that will normally offer little resistance to diffusion [22, 30]. The peptidoglycan mesh pore size is similar in Gram-negative and Gram-positive bacteria and it is thought to be around 2-3 nm, as estimated for *E. coli*, *B. subtillus* and *B. megaterium* (Figure 1) [55-57]. Using this pore size, it was calculated that peptidoglycan should be permeable to globular uncharged hydrophilic proteins up to 22-24 kDa and 50 kDa, respectively for unstretched peptidoglycan (isolated from bacteria) and stretched peptidoglycan (resembling growing bacterial cells) [56]. Nevertheless, doubts remain about the peptidoglycan permeability; moreover it is known that it can depend on the bacteria, the bacterial growth rate and the degree of peptidoglycan's cross-linking [58, 59]. For example, *Helicobacter pylori* was hypothesized to have a larger peptidoglycan's mesh than *E. coli* [60].

The periplasm covers the **cytoplasmic membrane** which, differently from the OM, is a symmetric phospholipid bilayer (Figure 1) [30]. The phospholipid composition in the cytoplasmic membrane is similar to that of the inner OM's leaflet [23, 29, 30, 55]. Besides phospholipids, integral and peripheral proteins are also present in the cytoplasmic membrane [2]. These are either structural proteins or transport proteins involved in the passive or active transport of hydrophilic substrates into the cytosol [2]. For instance, most sugars such as maltose are carried by periplasmic proteins to the ATP-binding cassette (ABC) transporters in the cytoplasmic membrane [46]. For nucleosides transport, no periplasmic binding proteins are known and they are transported across the cytoplasmic membrane via the transporters NupC and NupG in *E. coli* mainly energized by the pmf [46, 61]. Differently, hydrophobic substrates

will pass through the hydrophobic lipid bilayer of the cytoplasmic membrane into the cytosol by simple diffusion.

2.2 Gram-positive bacteria

The cell wall that covers the cytoplasmic membrane in Gram-positive bacteria is markedly different from that of Gram-negative bacteria (Figure 1). The Gram-positive cell wall is composed of teichoic acids and peptidoglycan, containing also proteins.

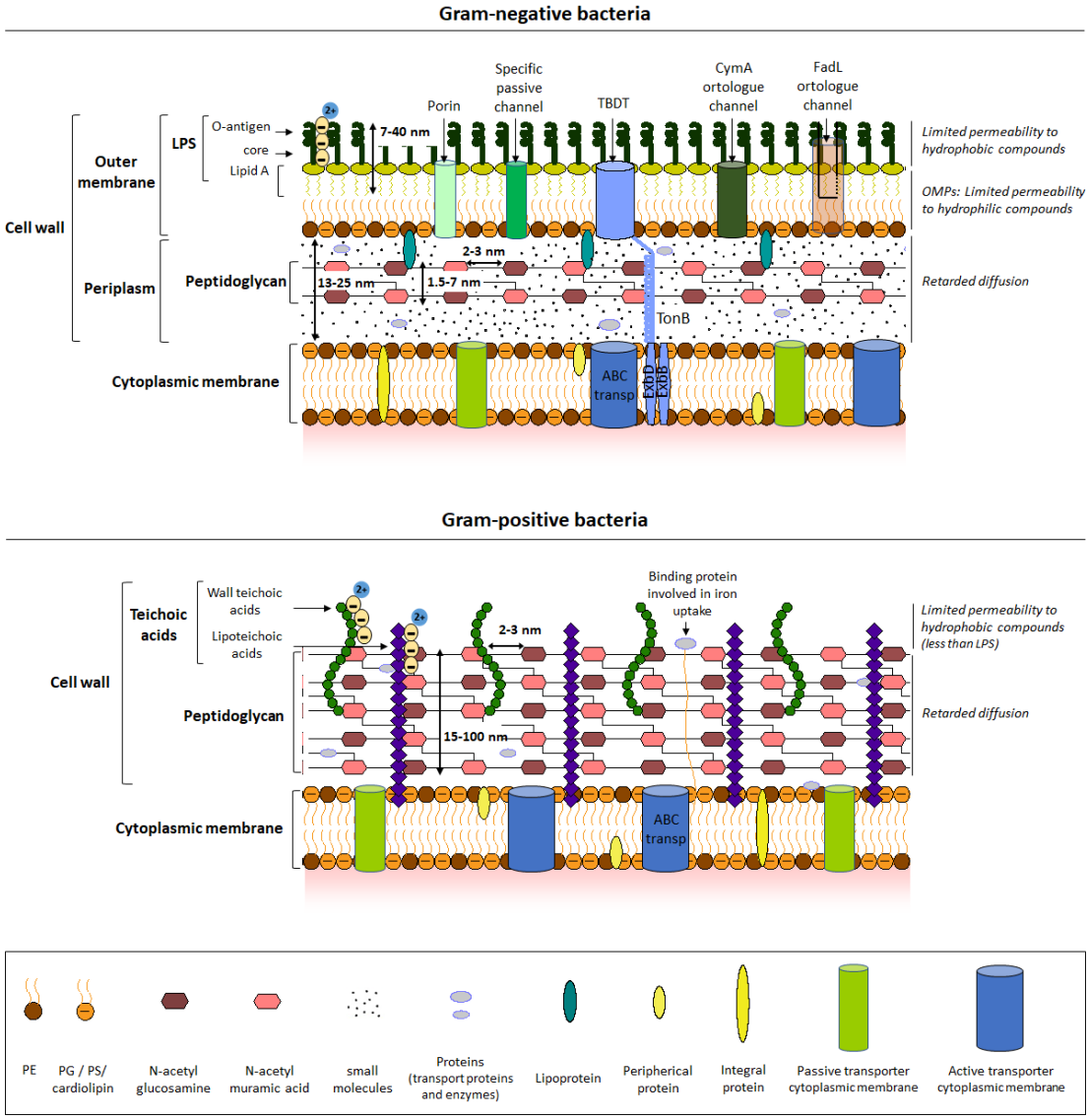
The cell wall is decorated with considerable amounts of anionic polysaccharides – the **teichoic acids** – that are in contact with the outer environment [23, 30]. Teichoic acids in a certain way relate to LPS in Gram-negative bacteria. Due to their anionic charge they bind metal cations (mainly Mg^{2+}, but also Ca^{2+} and K^{+}), regulating the envelope's rigidity and permeability [23, 30]. Teichoic acids are divided in wall teichoic acids (WTA), covalently attached to the peptidoglycan, and lipoteichoic acids (LTA), anchored to the head groups of the cytoplasmic membrane (Figure 1) [23, 62, 63]. Teichoic acids, in particular WTA, limit the permeation of hydrophobic compounds, in a similar way as LPS do but at a considerably lower extent [2, 23, 27, 28, 30, 34, 62].

The **peptidoglycan** in Gram-positive bacteria is similar in structure and porosity to that in Gram-negative bacteria [23, 24, 30]. However, to compensate the absence of an OM, in Gram-positive bacteria the peptidoglycan layer is significantly thicker and thus much more resistant to mechanical stress [23, 24, 30]. The thicker peptidoglycan layer can also retard the access of foreign compounds to the cytoplasmic membrane.

The Gram-positive's cell wall also comprises **proteins**, bound to the teichoic acids, the peptidoglycan, or the cytoplasmic membrane [23, 30, 64]. These proteins can be similar to the ones found in the periplasm of Gram-negative bacteria, being involved in defense, transport,

synthesis and turnover of peptidoglycan, adhesion to other bacteria and to their host for infection [23, 30, 64].

The **cytoplasmic membrane** of Gram-positive bacteria is similar to that of Gram-negative bacteria, differing only in the relative phospholipid composition – Gram-positive bacteria generally posess a lower amount of PE and a higher amount of PG than Gram-negative bacteria (Figure 1) [55, 65]. Besides PE and PG, smaller amounts of cardiolipin and eventually phosphatidylserine are also present [55, 65]. Like in Gram-negative bacteria, the cytoplasmic membrane contains peripheral and integral proteins, as well as passive and active protein channels for the internalization of hydrophilic compounds into the cytosol [2]. This includes the active transport of iron complexes, where a protein anchored on the cytoplasmic membrane (resembling the Gram-negative periplasmic binding protein) first binds the extracellular iron-siderophores and an ABC transporter brings the complex into the cytosol using ATP (Figure 1) [51]. Hydrophobic substrates, on the other hand, diffuse passively through the lipids of cytoplasmic membrane bilayer into the bacterial cytosol.



334

335 Figure 1. Structure and permeability of the bacterial envelope of Gram-negative and Gram-
336 positive bacteria of the typical Firmicutes phylum. Note that the TBDT transporter in the OM
337 of Gram-negative bacteria is part of a complex system that spans the envelope, composed of (i)
338 a specific TBDT at the OM that binds the substrate, (ii) the TonB complex (comprising the
339 TonB protein at the periplasmic space and ExbB and ExbD proteins at the cytoplasmic
340 membrane) that transduces the pmf of the cytoplasmic membrane for the conformational change
341 of TBDT, (iii) a protein in the periplasm (light purple circle on top of the 'ABC transp') that
342 captures the substrate that flows from the open TBDT channel and (iv) an ATP-binding cassette
343 (ABC) transporter that transports the substrate across the cytoplasmic membrane using ATP

[28, 50]. Also, LPS in Gram-negative bacteria is represented with its three regions: the lipid A (a lipid with a large number of saturated fatty acids), the core polyssacharide (a complex anionic oligosaccharide) and the O-antigen (a specific O-polysaccharide that differs between bacteria and can be highly antigenic) [22, 23, 27, 30]. LPS and teichoic acids are represented with their net negative charge (yellow circles) and the divalent cations attached (blue circles); the number of circles depicted are not representative and the divalent cations occur regularly along the LPS. Note that the peptidoglycan layer should be about 1.5-7 nm thick in Gram-negative bacteria [66, 67] and about 15-100 nm thick in Gram-positive bacteria [23, 67], while the peptidoglycan mesh is equally wide (2-3 nm) in both Gram types [55-57]. The thickness of LPS and periplasmic space in Gram-negative bacteria are believed to be 7-40 nm and 13-25 nm, respectively [27, 30, 68-70].

TBDT: TonB-dependent transporter; pmf: proton motive force; ABC transp: ATP-binding cassette transporter involved in the active iron uptake; LPS: lipopolysaccharide; PE: Phosphatidylethanolamine; PG: Phosphatidylglycerol.

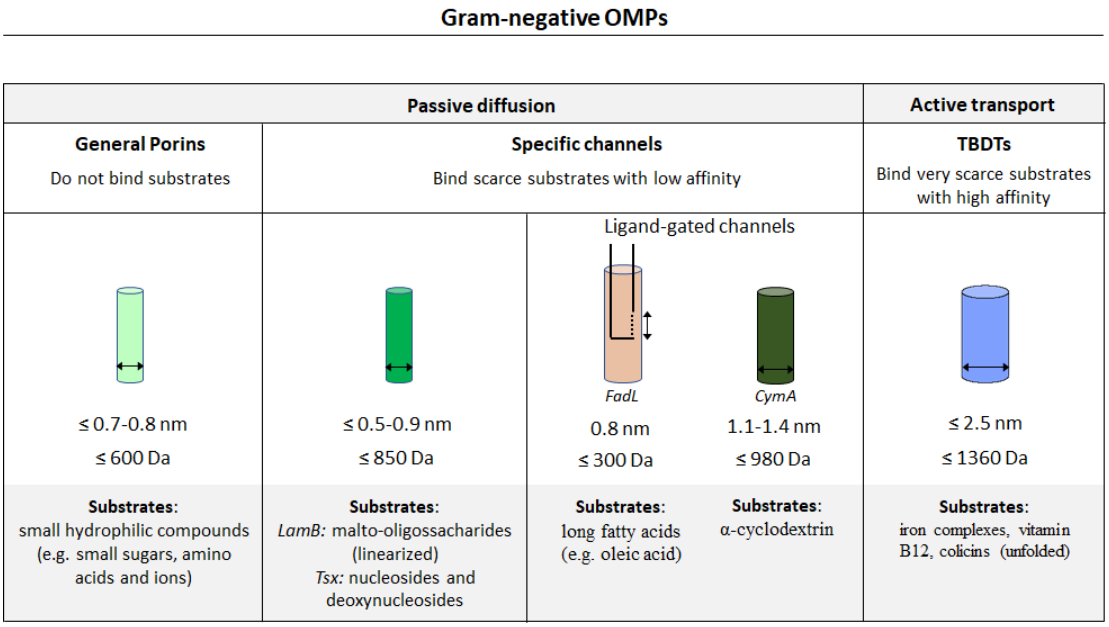


Figure 2. Channels formed by outer membrane proteins (OMPs) in Gram-negative bacteria. These can be passive diffusion channels, formed by general porins and specific channel, and active transporters, formed by TonB-dependent transporters (TBDTs). The diameter of the pores in the channels and the respective molecular weight exclusion (based on the substrates known to penetrate those channels) are presented. Specific channels are indicated in italic.

3. The bacterial cell envelope limits internalization of antibiotics and antibacterial oligonucleotides

The bacterial cell envelope and in particular the cell wall are stringent barriers that can significantly restrict and even completely hinder the penetration of anti-infective drugs into bacterial cells. In the subsequent sections it is discussed how the bacterial envelopes of Gram-negative and -positive bacteria may pose a barrier to traditional antibiotics (3.1) and to novel (antibacterial) oligonucleotides (3.2).

3.1 Internalization of antibiotics

Antibiotics may act at the bacterial envelope or in the bacterial cytosol [71, 72]. At the bacterial envelope, the peptidoglycan is a common target; the peptidoglycan's biosynthesis can be inhibited by β-lactam antibiotics (including penicillins, monobactams, cephalosporins and carbapenems) and glycopeptide antibiotics (vancomycin being the most common one) [73-75]. Intracellularly, antibiotics can target (i) DNA/RNA synthesis, as is the case of quinolones (e.g. ciprofloxacin), ansamycins (e.g. rifamycin), actinomycins, novobiocin and albicidin, (ii) protein synthesis, as is the case of tetracyclines, nitrofurans, macrolides (e.g. clarithromycin and erythromycin), aminoglycosides (e.g. tobramycin, streptomycin, kanamycin, gentamicin and amikacin), chloramphenicol, or fusidic acid and (iii) folate synthesis, being the case for sulphonamides and trimethoprim [71, 76].

Teichoic acids in Gram-positive bacteria and LPS in Gram-negative bacteria restrict the permeation of hydrophobic antibiotics (Figure 3a) [27, 28, 30, 62]. In particular, LPS can hinder the penetration of macrolides, rifamycins, novobiocin, or fusidic acid, contributing to the resistance of Gram-negative bacteria to these antibiotics (Figure 3b) [35, 72, 77, 78]. The peptidoglycan is generally believed not to be a significant barrier for the permeation of antibiotics [22]. Some antibiotics, such as aminoglycosides, may also "force" their penetration into bacteria by disturbing the bacterial cell wall, a mechanism known as "self-promoted uptake" [28]; studies on Gram-negative bacteria indeed suggest that these antibiotics may compete with divalent cations for binding to LPS, thus destabilizing LPS and forcing their own penetration [28].

After crossing the teichoic acids/LPS, the majority of antibiotics that are able to reach the cytosol do so by passive diffusion [36]. Only a minority of antibiotics, such as tobramycin, may be actively transported across the OM (Figure 3a and 3b) [36, 79]. Also 0.8 kDa rifamycin and 1 kDa albomycin (albomycin is comprised of an antibiotic moiety naturally covalently linked to Fe^{3+}-siderophores) are known to use the active TBDT channel named FhuA [36, 80].

Passive diffusion of antibiotics can either occur (i) across the lipid bilayers of bacterial membranes – being the case for most antibiotics (e.g. novobiocin, macrolides, tetracyclines and quinolones), since they have some degree of hydrophobicity, or (ii) across the Gram-negative OM's general porins and specific passive channels – being the case for the small hydrophilic antibiotics (such as ampicillin, amoxicillin, chloramphenicol, carbapenems, tetracyclines and quinolones), Figure 3b [16, 28, 35, 36]. Quinolones and tetracyclines, depending on the pH, may exist in an uncharged or charged form, respectively crossing the OM via lipid mediated diffusion or via the porins (Figure 3b) [28, 35, 81]. Penicillins, such as the zwitterionic ampicillin and amoxicillin, can be translocated via the general porin OmpF in *E. coli*; however, OmpF prevents anionic penicillins as carbenicillin to translocate, due to electrostatic repulsion between the anionic residues in the OmpF channel and the antibiotic [35, 82]. The carbapenem imipenem can also use the general OmpF to penetrate *E. coli* and the specific OprD channel (which uptakes basic amino acids and peptides structurally similar to this carbapenem molecule) in *P. aeruginosa* [38]. Albicidin, a relatively high molecular weight (~850 Da) antibiotic, can passively diffuse through the Gram-negative Tsx specific channel [36, 46]. Nevertheless, the size filtering effect of the OMP channels hinders or severally retards the diffusion of bigger hydrophilic antibiotics, as it is the case for glycopeptides like e.g. vancomycin (molecular weight of 1450-1500 Da) (Figure 3b) [22, 72, 74]. These antibiotics, together with lipophilic antibiotics which influx is restricted by LPS (as described above), are thus only active against Gram-positive bacteria (Figure 3b) [72]. Therefore, Gram-negative bacteria are intrinsically resistant to several antibiotics and among Gram-negative bacteria *P. aeruginosa* has a particularly low permeable OM [22, 28]. In addition, functional change or loss/significant reduction in the number of expressed porins in Gram-negative bacteria can further decrease the OM's permeability to antibiotics [35, 83]. Moreover, even antibiotics able to permeate the bacterial envelope in Gram-negative and -positive bacteria can become

inactivated by bacteria expressing specific genes of resistance that code for enzymes that modify/degrade the antibiotic, or for a competitive inhibitor of the antibiotic or for altered forms of the antibiotic's substrate [6, 72]. This antibiotic resistance crisis demands for novel antibacterial therapies.

3.2 Internalization of antibacterial oligonucleotides

To respond to the antibiotic crisis, antisense oligonucleotides could become a promising alternative as new antimicrobials. They act by specifically hybridizing *in situ* with complementary bacterial RNA and can, consequently, inhibit the expression of selected genes [10, 84, 85]. These can be essential bacterial genes, thus preventing bacterial growth, or genes involved in the resistance to antibiotics, hence restoring susceptibility of bacteria to antibiotics. This strategy could thus provide a potentially endless source of active antibacterials. Even if the bacterial target undergoes a point mutation that renders the oligonucleotide inactive, the oligonucleotide can be easily redesigned to become an effective drug again.

Among oligonucleotides, nucleic acid mimics (**NAMs**) are especially interesting, as, contrary to traditional DNA oligonucleotides, NAMs are composed of modified DNA or RNA sugars that make them resistant to endonuclease's degradation and improve their affinity towards RNA targets [86-89]. In particular, the charge neutral NAMs peptide nucleic acids (PNA) and phosphorodiamidate morpholino oligomers (PMO), as well as the negatively charged NAMs locked nucleic acids (LNA) and 2'-OMethyl RNA (2'OMe) are promising to target bacteria (Figure 4) [90-98]. These can be further modified on the backbone by including phosphorothioate internucleotide linkages (PS), instead of the normal phosphodiester linkages (PO), further improving the stability and affinity (Figure 4) [99, 100].

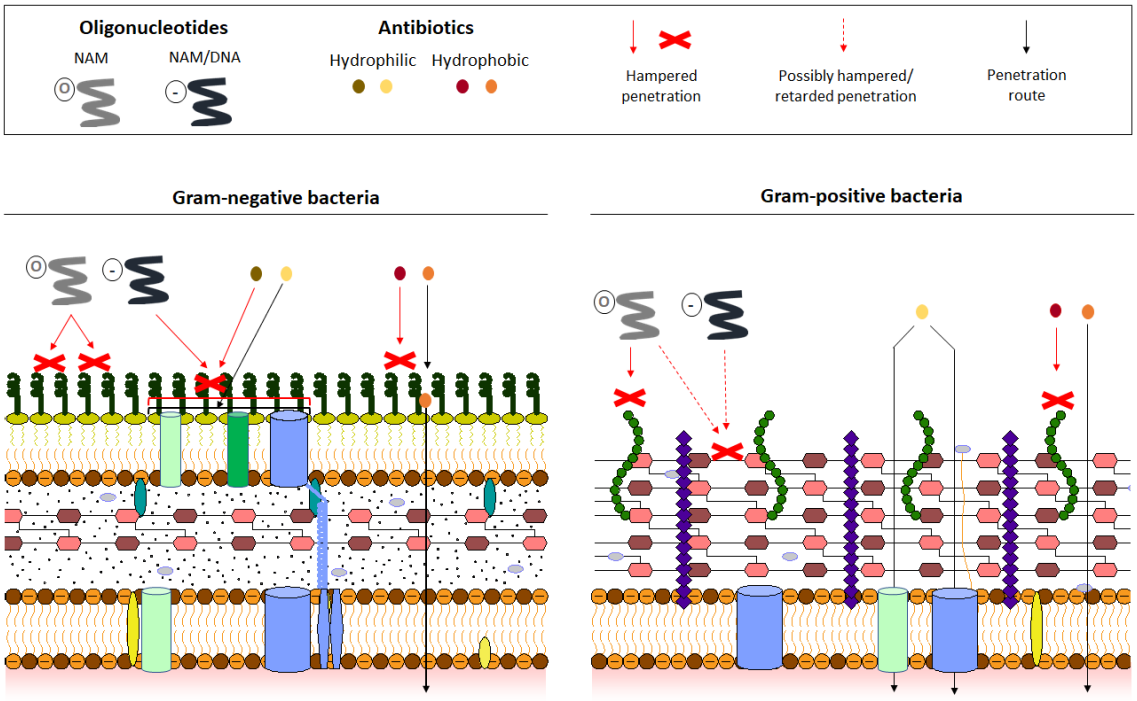
The improved affinity of NAMs towards the target RNA, compared to traditional oligonucleotides, allows the design of shorter NAMs sequences [99, 101] which in turn may

lower their penetration's restraint into bacteria. Actually, decamers PS-LNA/2'OMe were seen to be able to target *H. pylori* cells adhered on a slide [97] and undecamers bactericidal PMO could reduce the growth of *E. coli* in pure culture and in an infected mice, although growth recovery was observed after 4h *in vitro* and 12h *in vivo* with multiple doses necessary to sustain the growth reduction [96]. Also, heptadecamers LNA/DNA were found to penetrate *E. coli*, but a very long incubation of 18h was performed and still only 14% of the bacteria showed association with the LNA/DNA [92]. Clearly, internalization of NAMs in bacteria may happen, depending on the specific bacteria and on the NAM's structure, through currently unresolved mechanisms. However, internalization of oligonucleotides into bacteria is generally poor and insufficient to eradicate bacteria. Thus, although NAMs may solve the stability and affinity issues of natural oligonucleotides, penetration into bacteria remains a major bottleneck of antisense based antibacterial therapy [10, 95, 102, 103].

Very few studies investigated so far the role of the individual bacterial envelope structures in the restricted internalization of nucleic acids in bacterial cells. Studies using the neutrally charged PNA in *E. coli* showed that the OM is the rate limiting layer in the kinetics of PNA penetration [90]. The LPS, in particular, was suggested to be a major barrier for PNA penetration into *E. coli* [84], probably due to the relatively high hydrophobicity of PNA, compared to charged oligonucleotides (Figure 3a). After the OM, PNA needs to cross the peptidoglycan. Good et al. observed that antibiotics, which block peptidoglycan formation, did not improve PNA potency against *E. coli* and thus inferred that the peptidoglycan is not a barrier for PNA penetration [84]. However, this might not be the case for all bacteria. Studies using PNA to hybridize in different bacteria suggested that thick peptidoglycan layers from Gram-positive bacteria, such as *Bacillus cereus* (which has a peptidoglycan 6-7 times thicker than that of *E. coli* [67]), may significantly retard PNA penetration (Figure 3a) [104, 105].

For negatively charged NAMs (Figure 4) to cross the OM, they need to overcome the presumable electrostatic repulsion to the negatively charged LPS and the size filtering effect of OMPs (Figure 2). Most used NAMs have a molecular weight of about 2-4 kDa [10, 91]. Therefore, passive diffusion of the NAMs through the OM is highly unlikely, considering the size exclusion of porins to be 0.7-0.8 nm in diameter and 600 Da in molecular weight (Figure 3a, left pannel) [106]. Even if specific passive channels and active TBDT channels could accommodate the NAMs despite being structurally very different to their substrates, these channels would still be most of the times too narrow for NAMs translocation (Figure 2 and 3a). Therefore, the potential of oligonucleotides in general, and NAMs in particular, as novel antibacterial drugs to solve the antibiotic crisis can only be fulfilled if oligonucleotides become "carried" across the bacterial envelope.

a



b

Internalization of antibiotics in gram-negative bacteria

Hydrophobic antibiotics		Hydrophilic antibiotics		
Diffusion through lipid bilayers	Diffusion hampered by LPS	Diffusion through OMP channels: Passive transport	Diffusion through OMP channels: Active transport	Diffusion hampered by OMP channels
• macrolides • uncharged tetracyclines • uncharged quinolones	• rifamycins • novobiocin • fusidic acid • macrolides	• chloramphenicol (323 Da) • ampicillin (350 Da) • amoxicillin (365 Da) • carbapenems (200-500 Da) • charged quinolones (150-400 Da) • charged tetracyclines (400-500 Da)	• tobramycin (470 Da) • rifamycin (800 Da) • albomycin (1000 Da)	• vancomycin (1450Da) • chloramphenicol in *P. aeruginosa* • charged tetracyclines in *P. aeruginosa* • ampicillin in *P. aeruginosa* • *several antibiotics in bacteria with modified expression of OMP channels*

Figure 3. a) Limited permeability of the bacterial cell envelope of Gram-negative and -positive bacteria to oligonucleotides and common antibiotics. The depicted oligonucleotides include neutral oligonucleotides (NAMs such as PNA and PMO) and negatively charged oligonucleotides (DNA and NAMs such as LNA and 2'OMe). The antibiotics are represented as circles: yellow and orange circles – antibiotics that can be internalized; brown and red circles – antibiotics that cannot penetrate. The antibiotics identified as hydrophilic (brown and yellow circles) are too hydrophilic for diffusion through the lipid bilayers, contrary to the hydrophobic antibiotics (red and orange circles). On the

Gram-positive bacteria, the cross on top of the wall teichoic acids should be considered as hampered penetration by teichoic acids in general (lipoteichoic acids and wall teichoic acids). b) Summary of the main internalization routes in Gram-negative bacteria that allow or hamper the influx of relevant antibiotics, based on the antibiotic's lipophilicity and size (as indicated by the approximate molecular weight of the exemplified antibiotics) [35, 72, 77, 78]. The internalization of hydrophilic antibiotics can be further hampered if the expression of OMP channels in bacteria is modified or suppressed [35, 83]. The envelope structures depicted are identified in Figure 1.

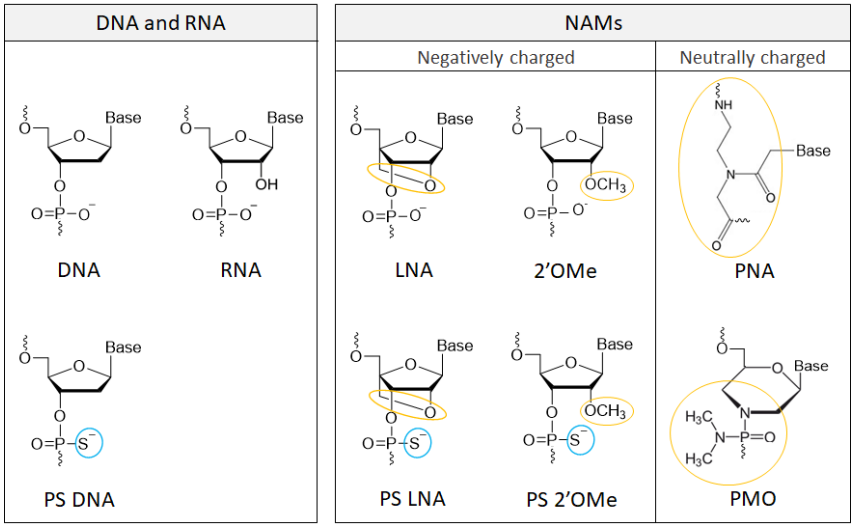


Figure 4. Chemical structure of the most studied nucleic acid mimics (NAMs). LNA: locked nucleic acids; 2'OMe: 2'-OMethyl RNA; PNA: peptide nucleic acid; PMO: phosphorodiamidate morpholino oligomer. The sugar modifications are circled in yellow and the internucleotide linkage modification (phosphorothioate instead of the natural phosphodiester linkage) is circled in blue.

4. Behaviour of nanoparticles and molecular transporters at the bacterial cell envelope

There is a growing interest in nanoparticles (NPs) and molecular transporters to mediate the delivery of antibiotics/oligonucleotides in bacteria [20]. Also, certain NPs and peptides have bactericidal potential *per se* and have thus gained attention to replace antibiotics [12]. However, so far the exploration of NPs and molecular transporters (or carriers) to these ends has mostly relied on a trial and error approach. Clearly, to better understand the potential of NPs and carriers to overcome the bacterial envelope a ´dialogue´ between nanotechnologists and microbiologists is highly needed.

4.1 Siderophores as 'trojan horses' for antibacterial drugs

As mentioned in the section 2, siderophores are low molecular weight compounds, secreted by bacteria under iron-limited conditions, which have a high affinity for Fe(III) ions and are actively transported as a complex into the bacterial cytosol [107, 108]. Thus, conjugation of antibacterial drugs to siderophores holds the potential to allow active uptake by bacteria, so that siderophores can be used as 'trojan horses' for antibacterial drugs (Figure 5). This idea arose from sideromycins, a group of antibiotics formed by analogues of bacterial siderophores linked to low molecular weight metabolic inhibitors, which are taken up by the iron transport system and release the antibacterial inhibitor upon internalization [107]. Therefore, several compounds containing a catechol/hydroxamate siderophore analogue linked via a stable linker to β-lactams or vancomycin or via a cleavable linker to fluoroquinolones or macrolides have been tested against Gram-negative and -positive bacteria [107, 108]. Synthesized catecholate-type siderophores linked to β-lactams (ampicillin, amoxicillin, cephalexin and cefaclor) demonstrated high activity against the Gram-negative *P. aeruginosa*, *Stenotrophomonas maltophilia*, *E. coli*, *K. pneumoniae* and *Serratia marcescens* [109, 110]. For instance, the

siderophore conjugated ampicillin increased the potency of free ampicillin up to 2000-fold in *P. aeruginosa* and *Stenotrophomonas maltophilia*, 500-fold in *Serratia marcescens*, 62-fold in *K. pneumoniae* and 15-fold in *E. coli* [110]. BAL30072, a β-lactam with a siderophore mimicking moiety (for the structure the reader is referred to [111]), also showed interesting results against several Gram-negative bacteria, including multi-resistant and impermeable bacteria as *Burkholderia pseudomallei*, *P. aeruginosa* and *Acinetobacter baumannii in vitro* [111-114]. The *in vitro* potency of BAL30072 compared to free β-lactam antibiotic comparators was found to be up to 30-375 times, 47 times and 8 times higher respectively against strains of *B. pseudomallei*, *Acinetobacter Baumannii* and *P. aeruginosa* [111-114]. These encouraging results led BAL30072 to enter clinical trials (Basilea Pharmaceutica Ltd.) [115, 116].

Although to the best of our knowledge it has never been tested, siderophores may not be an efficient 'trojan horse' for the uptake of antibacterial oligonucleotides, considering that oligonucleotides are around 2-5 times bigger than sideromycins and also larger than the pore size of iron transporters in *E. coli* OM (as discussed in section 3.2). Nevertheless, since our knowledge on the OM permeability is limited it may be worth to investigate.

4.2 Cyclodextrins as 'trojan horses' for antibacterial drugs

As described in section 2, bulk cyclodextrins (CD) are able to diffuse through the CymA channel in *Klebsiella oxytoca*; orthologues of CymA were also found in Enterobacteriaceae and Vibrionaceae [47]. Therefore, cyclodextrins may hold potential as 'trojan horses' for antibacterial drugs (Figure 5). Cyclodextrins (typically 1.4 to 1.8 nm of outer diameter [117]) are investigated for nearly 70 years as drug carriers to improve the bioavailability, stability and solubility of drugs targeted to mammalian cells [117, 118]. They form water soluble cyclic oligosaccharides with an hydrophobic cavity that can enclose hydrophobic antibiotics via noncovalent interactions [119]. β-CD and its derivatives have been used for the inclusion or

association of several antibiotics, such as macrolides, ryfamycins, quinolones, β-lactams, cephalosporins and tetracyclines, improving the antibiotic potency against Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, *Citrobacter* spp, *Enterobacter* spp, *Klebsiella* spp and *A. baumannii*, and Gram-positive bacteria such as *S. aureus* [119-124]. For instance, when tested in *Staphylococcus* spp, *Klebsiella* spp, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter* spp and *Citrobacter* spp, β-CD carriers decreased the minimum inhibitory concentration (MIC) of ampicillin and amoxicillin up to 4 times and the MIC of cefadroxil up to 16 times [121]. Besides improving the antibiotic's stability and solubility, β-CD (and its derivatives) were considered to mediate enhanced permeation of the antibiotics [119-123]. In particular, it has been hypothesized that β-CD may drive internalization of the β-CD-antibiotic complex possibly via (i) CymA orthologue channels, (ii) enhaced adhesion to the bacterial surface (including pore channels) with potential local release of the antibiotic, and (iii) via destabilization of the bacterial envelope [120-123]. Also, β-CD capping silver NPs (AgNPs) improved their interaction at the bacterial envelope of *E. coli*, *P. aeruginosa* and *S. aureus* and enhanced the intracellular delivery of antibacterial silver ions [125].

The potential of CDs as drug carriers into bacterial cells can be restricted by poor colloidal stability of CDs which may result in the formation of aggregates of tens to hundreds of nanometers [119, 122, 123, 126]. Despite being less colloidaly stable than α-CD and γ-CD, β-CD is by far the most used CD carrier in drug delivery [127]. β-CD's can be modified to become more stable, as seen for instance for (2-Hydroxypropyl)-β-CD and for PEGylated CDs [25, 127]. Nevertheless, theoretical studies on the CymA channel (section 2.2.1) suggested that even colloidally stable β-CD may be too large to be efficiently internalized in bacteria via CymA [48]. Differently, the smaller α-CD can better flow through CymA [48]. Therefore, α-CD would theoretically be a better 'trojan horse' for bacteria than β-CD. However, the narrow 0.47–0.53 nm cavity of α-CD limits the enclosure of most antibiotics [117, 119]. Further studies on the

structure of CymA's orthologue channels and the transport of different CDs in various bacteria may help elucidate the chances of CDs to penetrate bacteria with associated antibiotics.

Concerning antibacterial oligonucleotides, they cannot be carried into the CD's cavity due to its hydrophobicity. CDs can instead be associated with cationic polymers to electrostatically complex the negativaly charged oligonucleotides [117], but this would probably hamper CD's mediated uptake. Hence, similarly to siderophores, CDs may be promising to carry low molecular weight antibiotics, but not oligonucleotides.

4.3 Metal NPs to disrupt and penetrate the bacterial envelope

Metals NPs (1-100 nm) like silver, gold, zinc oxide and titanium dioxide NP can have antibacterial properties against Gram-negative and -positive bacteria [20, 25]. The antibacterial effect of metal NPs depends on their ability to interact with the bacterial envelopes. For most antibacterial metal NPs this interaction results in letal disruption of the bacterial cell wall and/or the cytoplasmic membrane [6, 12, 20, 25]. For some antibacterial metal NPs, this interaction allows the internalization of metal NPs, so that these are letal by acting on the bacterial cytosol, targeting RNA, DNA and proteins [6, 12, 20, 25]. The antibacterial properties of metal NPs are long known, but how they interact with the bacterial envelope remains rather unclear [1, 128, 129]. Their action depends on the NP's size, concentration, shape, surface, charge and the capping agents used for stabilisation (which may affect the NP's surface charge), as well as on the type of bacteria [20, 129, 130].

4.3.1 Interactions of metal nanoparticles with the bacterial cell envelope

The interaction of metal NPs with the bacterial **cell wall** has been mostly studied for AgNPs and it is frequently suggested to be driven by electrostatic attractions between the positively

charged NPs and the negatively charged bacterial envelope surface – probably the LPS in Gram-negative bacteria and the teichoic acids in Gram-positive bacteria (Figure 5) [25, 131-133]. However, this does not seem to be a prerequisite since negatively charged AgNPs (diameter from 5 to 20 nm) were also shown to interact at the cell envelope of Gram-negative and -positive bacteria [134-136]. Negatively charged NPs were proposed to interact with metals bound to the LPS in the Gram-negative cell wall (see Figure 1), causing metal depletion and thus perturbing the assembly of LPS and compromising the OM's permeability [135, 137]. Likewise, it is conceivable that anionic metal NPs could also interact with metals bound to teichoic acids in the Gram-positive cell wall. Still, anionic AgNPs were considerably less toxic than cationic AgNPs against Gram-positive *Bacillus* species [136].

Besides electrostatic effects, binding of NPs to certain groups at the bacterial envelope surface has been suggested as well; for instance AgNP have a high affinity towards thiol groups and their antibacterial action was blocked by thiol containing agents [128, 138].

Cell wall's disturbance may be further aided by the formation of pores, called 'pits', by metal NPs (Figure 5), as proposed for AgNP [132, 139, 140]. Such ´pits´ may be breaks in the OM, including LPS – as reported for 5 nm AgNPs against the Gram-negative *E. coli* [139] – or on the peptidoglycan bonds between N-acetylglucosamine and N-acetyl-muramic acid – as suggested for 18 nm AgNP against the Gram-positive *S. aureus* [140].

Some authors discuss that the extent of metal NP interaction at the cell wall may depend on the bacteria Gram type. Several studies report that the thicker peptidoglycan layer in Gram-positive bacteria may render them more resistant to metal NPs [135, 140, 141]. However, the opposite has also been reported [130, 131, 133, 142]. Thus, the different Gram cell wall does not solely explain the interaction of metal NP on bacteria, but clearly the effect is strain dependent as well [142].

Overcoming the cell wall, metal NPs that reach the **cytoplasmic membrane** may disturb it directly by the formation of 'pits' similar to the ones formed in the cell wall (Figure 5). Indeed, AuNPs (2-29 nm of diameter) and PtNPs (2-19 nm of diameter) caused substantial cell wall and cytoplasmic membrane disruption in *Salmonella enteritidis* and *Listeria monocytogenes* [143]. Catechin-CuNPs (5.3 nm of average diameter) were also seen to cause separation of the cytoplasmic membrane from the cell wall, in *S. aureus* [133].

In addition, metal NPs can disturb the CM by interfering with the ATP balance and pmf gradient, eventually causing cell leakage (Figure 5) [6, 12, 132, 133, 144]. Vardanyan et al. verified that 3-15 nm anionic AgNPs increased ion flux across *E. coli* and *Enterococcus hirae* (*E. hirae*) [130]. Morever, the CM's ATPase activity in *E. hirae* was decreased and the coupled H^+ flux, as well as the associated H^+-K^+ exchange, were affected [130]. Upregulation of several OMPs (as OmpA, OmpC, OmpF) with accumulation of their precursors in the cytoplasm was also found as a consequence of pmf/ATP gradient dissipation in *E. coli* exposed to 9.3 nm AgNPs – without energy, OMP precursors cannot be translocated to the cytoplasmic membrane, therefore remaining in the cytoplasm [138].

4.3.2 Evidence for internalization of metal nanoparticles in bacteria

There are several reports on internalized AgNPs, CuNPs, PtNPs, ZnO NPs, TiO_2 NPs, MgO NPs [128, 133, 134, 143, 145-149]. It is frequently difficult to evaluate how NPs penetrated the bacterial envelope, because several reports use bactericidal concentrations [128, 133, 143] and so NP's penetration may be a consequence of their toxicity. Therefore, we only focus on published results obtained at a non-bactericidal concentration of metal NPs. It was found that 1-1.5 mM ZnO NP (14 nm of diameter) were able to be internalized in *E. coli* and even increase the number of *E. coli* colonies, probably because bacteria can metabolize Zn^{2+} as an oligoelement [147]. Anionic ZnO and TiO_2 NPs up to 70 nm were also suggested to penetrate

664 the Gram-negative *Salmonella* Typhimurium (as tested by flow cytometry and TEM) and be
665 transmitted to daughter bacteria, while bigger NP agglomerates adhered to the bacterial
666 envelope [148]. Similarly, AgNPs (normally anionic, as produced by citrate and borohydride
667 reduction [146, 150]) up to 80 nm were found to penetrate live Gram-negative *P. aeruginosa*
668 with bigger NPs remaining mostly adhered on the bacterial surface [145, 151]. In live Gram-
669 positive *B. subtilis*, AgNPs from 8 to 33 nm [146] and even from 84 to 100 nm were reported
670 to be internalized as well [152]. The majority of these NPs are clearly much larger than the
671 reported pore sizes of the bacterial envelopes (as shown in Figure 1, 2 and 5). The penetration
672 of these NPs on live bacteria may be aided by non-specific mild envelope perturbation including
673 transient 'pit' formation [147], while keeping bacteria viable (Figure 5). Alternatively,
674 passive diffusion has been considered for the influx of the ZnO and TiO_2 NPs up to 70 nm (in
675 Gram-negative bacteria) [148] and of AgNPs up to 80 nm (in Gram-negative and -positive
676 bacteria) [146, 152]. However, it was reported that not all internalized AgNPs remained inside,
677 but some were actively exported from *P. aeruginosa* and *B. subtilis* by active transporters
678 (typically involved in antibiotics efflux); bigger AgNPs were more quickly exported while
679 smaller AgNPs remained longer inside bacterial cells [145, 146, 152]. These studies on AgNPs
680 transport were performed in live bacteria to assess real-time transport of AgNPs in and out of
681 bacteria, using a method based on the size-dependent localized surface plasmon resonance
682 (LSPR) spectral colors of single AgNPs by dark-field optical microscopy and spectroscopy
683 [151]. The authors used this LSPR-based microscopy to distinguish internalized AgNPs from
684 AgNPs extracellularly adhered to the bacterial envelope based on the scattering intensity of the
685 AgNPs – intracellular NPs appear blurry and with lower scattered intensity, while extracellular
686 NPs look sharper and brighter (due to the additive scattering of the bacterial membrane) [145,
687 146, 152]. Looking only at the microscopy images, it is not straightforward to visually
688 discriminate envelope adhered and internalized AgNPs [146]. Nevertheless, the presented

method was initially validated by transmission electron microscope (TEM) [145, 151]. Still, it is intriguing that such NPs could diffuse through bacterial active and passive channels despite being up to 10-50 times larger than the respective channel's pores (Figure 2 and 5). The authors argue that this should be due to an enormous ability of the channel's pores to adapt their permeability in live conditions, something that can be overlooked in the crystallography analysis of the protein channels normally used to establish the pore sizes of the bacterial channels [145]. Clearly this is an unresolved issue that needs to be further investigated; we anticipate that the development of microscopy techniques with improved resolution will help to directly visualize in real-time NPs internalization in live bacteria, in the future.

4.4 Carbon nanostructures, dendrimers and chitosan nanoparticles to disrupt and penetrate the bacterial envelope

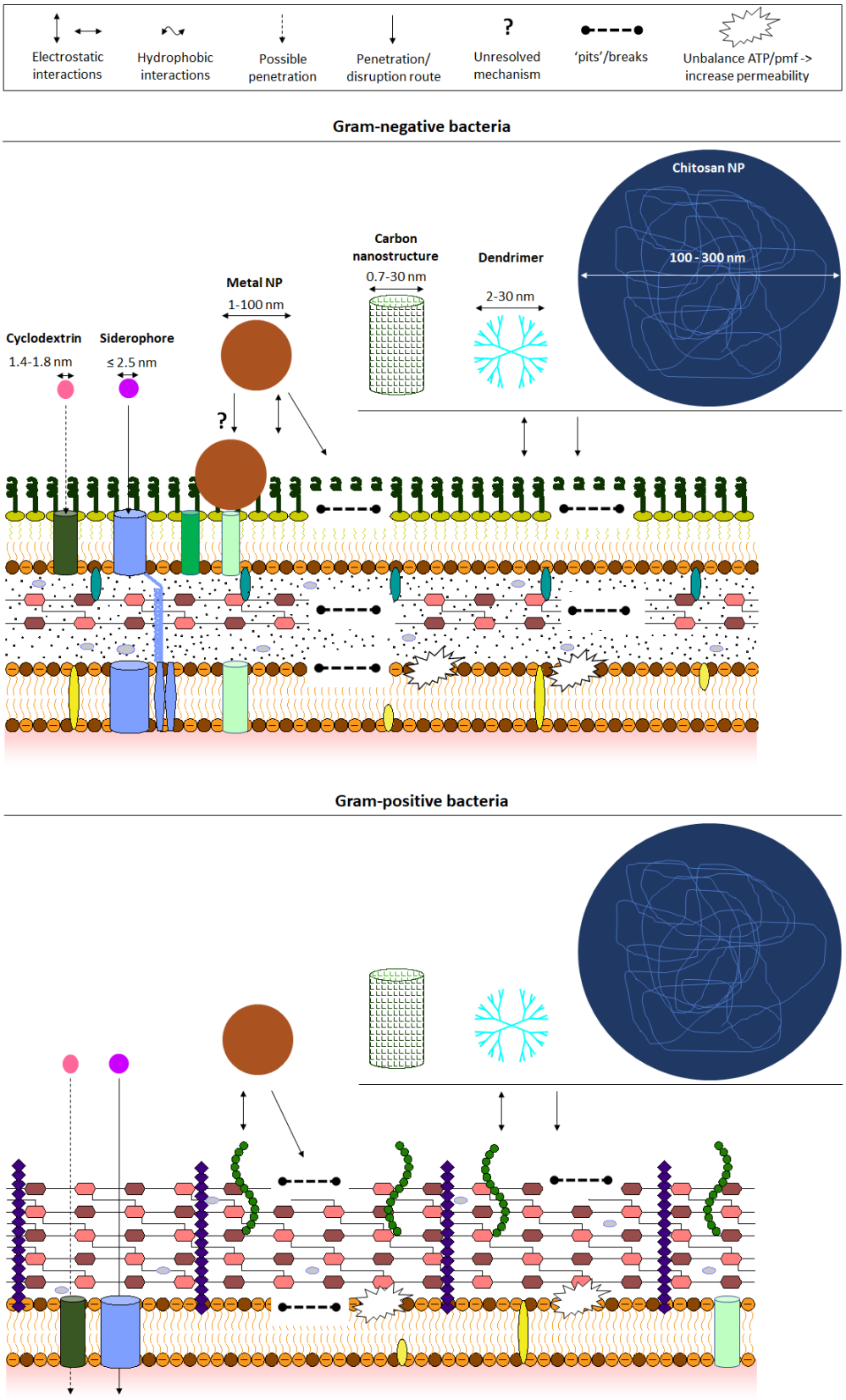
Although their interactions with the bacterial envelope have been less studied, NPs other than metal NPs like carbon nanostructures (as carbon nanotubes and graphene oxide nanoparticles), chitosan NPs and dendrimers may overcome the bacterial envelope as well [153]). However, their internalization in bacteria without disruption of the bacterial envelope has never been reported to our knowledge. Differently, they all have potential to overcome the bacterial envelope by disrupting it [12, 20] (Figure 5). Therefore, they have been used against Gram-positive and -negative bacterial infections; while carbon nanostructures (CNS) have been mostly used as antimicrobials *per se* [154-157], dendrimers and chitosan NPs have been used both alone and as carriers of antibiotics [158-164].

When used in lower concentrations, dendrimers (mostly polyamidoamine (PAMAM) dendrimers), and chitosan NPs, may serve as non-bactericidal reservoirs which can improve antibiotic's half-life, bioavailability and biodistribution [158, 159, 161, 162]. In addition, they

may also perturb the bacterial envelope making it somewhat more permeable which eventually improves the delivery of the antibiotic once it has been released from the NPs [160, 165].

At higher concentrations, the perturbation caused by dendrimers and chitosan NPs can be irreversible and lead to bacteria's disruption [163-165]. This bactericidal effect arises from the NPs highly positive surface charge which promotes electrostatic adsorption of the NPs to the negatively charged bacterial surface, with possible displacement of the metal cations bound to LPS/teichoic acids, and induces permeabilization of outer and cytoplasmic membrane, leakage of cytosolic contents and bacteria disruption (Figure 5) [6, 11, 12, 165].

Carbon nanostructures (CNS), such as carbon nanotubes (CNTs) and graphene nanosheets, have recently gained increased attention as antimicrobials [1]. Their needle-like shape, small size and electronic properties offer interesting bactericidal potential against Gram-positive and negative bacteria [166-168]. The direct disruption of the bacterial envelope by CNS appears to mainly occur at the cytoplasmic membrane [154]. The sharp edges of graphene insert/partially penetrate into the cytoplasmic membrane, leading to the extraction of large amounts of phospholipids (as shown for graphene nanosheets on *E. coli*) and consequent degradation of the membranes with release of intracellular RNA, electrolytes and proteins [155-157] (Figure 5). In addition, the oxidative nature of graphene may also induce lipid peroxidation [154, 169]. These antimicrobial effects of CNS seem to be generally favored by their small size. A decrease in the area of graphene nanosheets from 0.65 to 0.01 μm^2 increased its bactericidal activity against *E. coli* up to 4-fold [154]; the decreased diameter of single-walled CNTs (0.9 - 1 nm) compared to multi-walled CNTs (15- 30 nm) allowed improved penetration into *E. coli* [157] and a much higher bactericidal effect against Gram-positive and Gram-negative bacteria [157, 166].



738

739 Figure 5. Most relevant mechanisms for the direct penetration/disruption of the bacterial envelope

740 by nanosized materials like cyclodextrins, siderophores, metal nanoparticles, carbon nanostructures,

chitosan nanoparticles and dendrimers in Gram-negative (top) and -positive (bottom) bacteria. The double arrow vectors represent electrostatic interactions with the LPS, in Gram-negative bacteria (top), and the teichoic acids (wall teichoic acids and lipoteichoic acids) in Gram-positive bacteria (bottom). The envelope structures depicted are identified in Figure 1.

4.5 'Membrane active' peptides to disrupt and penetrate the bacterial envelope

'Membrane active' peptides refer to antimicrobial peptides (AMPs) and cell-penetrating peptides (CPPs), both acting on the cytoplasmic membrane [170]. This 'membrane activity' allows AMPs and CPPs to respectively kill bacteria and transport drugs across the bacterial envelope. There is some confusion in the literature on the denominations; while AMPs are used to kill bacteria *per se*, CPPs are molecular transporters used to carry attached drugs into the cytosol of mammalian or bacterial cells (without cell lysis) [171, 172].

AMPs naturally exist to protect higher organisms against pathogenic microorganisms [173]. AMPs selectively act against bacteria and not mammalian cells, because the higher fluidity and anionic character of bacterial CM, compared to that of mammalian cells CM, favour the peptide's binding and insertion [26, 172, 174]. The higher fluidity of bacterial membranes derives from the absence of cholesterol and the presence of the fluid lipid PE. The higher anionic character, on the other hand, results from the presence of the anionic PG at the bacterial CM's surface (in mammalian cells the anionic lipids are sequestered in the inner leaflet of the membrane and the outer leaflet is mostly decorated with zwitterionic phosphatidylcholine (PC) and sphingomyelin) [26, 55, 172, 174, 175]. Most AMPs are bactericidal by disrupting the bacterial CM [26], but some AMPs exist, such as buforin II, indolicin and pleurocidin, that cross the CM without disruption and lethally inhibit the synthesis/activity of DNA, RNA or proteins in the bacterial cytosol [172, 176].

CPPs, on the other hand, started to be earlier applied in mammalian cells and only more recently became also investigated to carry drugs into bacterial cells [171]. Hence, AMPs and CPPs have been mostly discussed separately, although similar mechanisms of 'membrane activity' have been proposed in the literature for CPPs and AMPs [171, 176]. Actually, despite the diversity of AMPs and CPPs (for a comprehensive description of different AMPs and CPPs the reader is referred to [176] and [177], respectively), most of them share general features: (i) small size, with less than 50 (AMPs) and 30 (CPPs) amino acids, (ii) net positive charge, and (iii) a significant amount of hydrophobic amino acids [175, 178, 179]. Therefore, we will discuss the interactions of AMPs and CPPs with the bacterial envelope together, as 'membrane active' peptides.

4.5.1 Interactions of 'membrane active' peptides with the bacterial envelope

As referred above, 'membrane active' peptides are active at the cytoplasmic membrane. Therefore, to reach the cytoplasmic membrane they first need to interact with the **cell wall**, through a rarely studied mechanism. Nevertheless, it is believed that the initial interaction is mediated by non-specific electrostatic interactions of the cationic peptides with the anionic LPS and teichoic acids, respectively in Gram-negative and -positive bacteria (Figure 6a) [172, 176]. Although a minority, anionic AMPs and CPPs also exist [176, 180]; we can thus envision that similarly to metal NPs, anionic AMPs and CPPs may be electrostatically attracted to the divalent cations bound to LPS/teichoic acids. Electrostatic interactions together with hydrophobic interactions may help the peptides to further translocate the OM in Gram-negative bacteria (Figure 6a) via a "self-promoted uptake" [90, 181-183]. Alternatively, it has been suggested that specific interactions at the cell wall surface, for instance with LPS, PE, or lipid II (peptidoglycan precursor), can promote the initial interaction of peptides with the bacterial envelope [26, 172, 174].

Reaching the peptidoglycan, it has been assumed in the literature that the peptides (with a molecular weight typically below 5 kDa) may freely diffuse through the peptidoglycan mesh (considering the size exclusion for globular hydrophilic molecules of 50 kDa) [55, 182]. However, the role of peptidoglycan in the interaction with the peptides is not clear [55]. Still, the peptides should pass the cell wall very fast, as dissipation of the electrochemical gradient across the CMs is seen within a few seconds of bacteria exposure to AMPs [174, 184].

'Membrane active' peptides normally cross the cell wall without disrupting the OM in Gram-negative bacteria [183]. An exception was recently found for the newly developed 7.7 nm large "star-shaped nanoengineered AMP polymer" (SNAPP), composed of cationic lysine and hydrophobic valine monomers polymerised from a polyamidoamine (PAMAM) dendritic core [183]. Due to its particular architecture, SNAPPs cannot transverse the OM via a "self-promoted uptake" like general AMPs do [183]. Differently, after electrostatically binding to LPS, the SNAPPs (at high concentration) cause lethal OM's destabilization and fragmentation [183]. SNAPPs can also further mildly perturb the CM and unbalance its ion movement [183]. This explains that the SNAPPs were not only active against Gram-negative bacteria, but also showed a moderate activity against Gram-positive bacteria [183].

Reaching the **cytoplasmic membrane**, where typical AMPs and CPPs are active, the peptides first bind to the membrane by electrostatic interactions (Figure 6a) [26, 79, 176, 185]. When the peptides reach a threshold concentration on the membrane, which for AMPs is about full membrane coverage, the peptides insert themselves into the membrane bilayer via hydrophobic interactions (Figure 6a) [26, 79, 176, 185].

The modes of membrane insertion/translocation depend on the peptide and the membrane [26]. For mammalian cells, CPPs have been proposed to translocate the mammalian membrane either by endocytosis or by direct penetration [170, 171]. Endocytosis is not applicable in bacteria. Differently, the direct penetration of CPPs in mammalian cells is proposed to occur

via three possible models which are the same exact models proposed for the insertion of AMPs into bacterial CMs [171, 176]. Thus, carrier CPPs and antimicrobial AMPs should use the same mechanisms to force their translocation/insertion into the bacterial CM [186]. The three typically proposed models are: the barrel-stave pore, the toroidal pore and the carpet mechanism (Figure 6b) [171, 172, 174, 175]. In the **barrel-stave pore** the peptides insert perpendicularly to the membrane surface and pack together parallel to the hydrocarbon chains, forming an aqueous pore (Figure 6b) [172, 174, 175]. The cytoplasmic diameter of the barrel-stave pore formed by the AMP alamethicin has been estimated by structural studies to be around 1.8 nm [187]. In the **toroidal pore** model, the peptides also insert perpendicularly in the bilayer but they induce a toroid-like curvature in the membrane, so that lipid inner and outer leaflets are forced to bend towards one another stablishing a continuity [172, 174-176]. The pore is thus formed by both the inserted peptides and the phospholipid head groups (Figure 6b) [172, 174-176]. The AMPs melittin and magainin form toroidal pores in lipid vesicles of 2.5-3 nm and 3.0-5 nm, respectively [188, 189]. The (barrel-stave/toroidal) pores may allow the passage of molecules as big as 40 kDa, according to studies performed using the AMP maculatin that forms pores of 1.4 and 4.5 nm in diameter on lipid vesicles mimicking *S. aureus* membrane [190]. The formation of barrel-stave/toroidal pores requires that the peptide is long enough to span the hydrophobic core of the bilayer [190]. Differently, this is not needed in the **carpet model**, since the peptides absorb parallel to the bilayer surface and produce a detergent-like effect that eventually results in membrane disintegration into micelles (Figure 6b) [172, 174-176, 178]. Therefore, smaller peptides, can act via the carpet model [191]; this is the case for the AMPs aurein and cecropin, the later shown to form pores on *E. coli* of 4.2 nm in diameter [192].

Instead of (or in addition to) self-assembling to form pores, adsorption of the peptides onto the membrane may dissipate the transmembrane potential, pH gradient and osmotic balance (Figure 6a) [178, 182, 190]. Different modes of action can be related, depending on the peptide

concentration. For instance, at low concentrations the AMP cecropin was bactericidal to *E. coli* by dissipation of transmembrane electrochemical ion gradients (as judged from ion gradients dissipation in lipid vesicles), while higher concentrations were needed to release cytoplasmic contents [193]. The extent and duration of the membrane action of the peptides will dictate the viability of the cytoplasmic membrane. It may depend, besides peptide's concentration, on the the peptide's charge, hydrophobicity, sequence, structure and size [172, 176, 186, 194].

Peptides able to remain inserted into the bacterial CM long enough to form irreversible pores or lesions will kill bacteria. This is the case for most AMPs. However, after insertion into the cytoplasmic membrane, CPPs (with its attached cargo) and the minority of AMPs that have their target in the bacterial cytosol (nucleic acids and proteins) still need to desorb from the CM to reach the cytosol. How exactly it happens is still a matter of debate. For the AMPs (with a cytosolic target) the peptides forming the pore might be randomly internalized by disintegration of the pore [186, 195]. The same mechanism may be reasoned to occur for CPPs. In addition, we can reason that peptides free in solution (which are expected to be in a relative higher number for CPPs than AMPs, owing to the specifically high affinity of AMPs to bacterial CMs) might flow into the bacterial cytosol via the formed pore. This may be possible if the formed pore is as large as 2-5 nm with a size exclusion of 40 kDa, as reported for AMPs [187-189, 196].

4.5.2 CPPs as molecular transporters of antibacterial drugs

CPPs have been explored to carry covalently conjugated antibiotics and, most commonly, antisense oligonucleotides across the bacterial envelope into the cytosol.

The highly cationic 12 amino acids (a.a.) Pen peptide (RQIKIWFQNRRW, where R is arginine, Q is glutamine, I is isoleucine, K is lysine, W is tryptophan, F is phenylalanine and N is asparagine) was designed based on the 16 a.a penetratin (a well-known CPP for mammalian

cells) and conjugated to the antibiotic tobramycin (lethal by ribosome inhibition), in order to increase the antibiotic's uptake in persister *E. coli* and *S. aureus* [79]. Persister bacteria have decreased active transport and thus do not take up tobramycin. The Pen peptide alone caused extensive permeabilization which was *per se* bactericidal [79]. The conjugation of the antibiotic to the peptide, forming the conjugate Pentobra, decreased the permeabilization potential of the peptide [79]. Nevertheless, Pentobra killed more 4-6 logs of persister bacteria than the free tobramycin, showing the importance of the combined effect of tobramycin and the Pen peptide [79].

An earlier report prepared the cationic peptide $(KFF)_3K$ to permeabilize the Gram-negative OM to hydrophobic antibiotics (which penetrate intact OM very poorly, Figure 3) [197]. Pre-treatment of bacteria with this peptide was found to sensitize enteric bacteria, such as *E. coli*, *E. cloacae*, *K. pneumoniae*, and *S. typhimurium*, to the hydrophobic antibiotic rifampin [197]. The peptide $(KFF)_3K$ was also found to permeabilize the OM of *E. coli* to antisense PNA [84, 90, 93]. Since then, the peptide $(KFF)_3K$ has been widely covalently conjugated to antisense oligonucleotides to transport them into the bacterial cytosol. However, this has been nearly restricted to neutrally charged oligonucleotides (PNA and PMO) [90, 91, 198, 199], since the covalent conjugation to negatively charged is technically difficult [101]. Besides *E. coli*, $(KFF)_3K$ was also shown to improve the potency of PNAs and PMOs into the Gram-negative *Salmonella* Typhimurium, *Klebsiella pneumoniae* and Gram-positive *Staphylococcus aureus*, *Bacillus subtilis* and *Corynebacterium efficiens* [9, 200, 201]. This improved potency was ascribed to $(KFF)_3K$ mediated improved penetration [90]. Depending on the concentration, the $(KFF)_3K$ could also contribute to the killing efficacy of the antisense PNA by causing bactericidal cell leakage [90]. $(KFF)_3K$ could act not only against the cytoplasmic membrane, as expected, but it also disrupted the OM of *E. coli* [90, 183].

Other CPPs have been tested as well, which – compared to the peptide $(KFF)_3K$ – had a lower toxicity towards bacteria and when conjugated to an antisense PNA still allowed improved bactericidal effects. This was the case for the CPPs Tat (which is derived from the transactivator of transcription (TAT) of HIV and has the sequence GRKKRRQRRRPQ) [185, 202, 203], $(RXR)_4XB$ and $(RFR)_4XB$ (G is glycine, P is proline and X is 6-aminohexanoic acid) [103, 199]. The Tat conjugates were tested against the Gram-positive *Streptococcus pyogenes* [185]. The $(RXR)_4XB$ and $(RFR)_4XB$ conjugates were tested against the Gram-positive *Listeria monocytogenes in vitro* and in a *Caenorhabditis elegans* infection model [103]. Conjugates of $(RXR)_4XB$ were also tested *in vitro* against the Gram-negative *Salmonella* Typhimurium, *Klebsiella pneumoniae*, *Escherichia coli*, and *Shigella flexneri* and also *in vivo* in a mice model infected with *E. coli* or *S. flexneri* [199].

Besides the peptide sequence, the extent of CPP permeabilization has been observed to depend on the bacteria. Vaara et al. showed that $(KFF)_3K$ was bactericidal *per se* against the Gram-positive Micrococcus tested, while it did not affect the viability of the tested Gram-negative (*E. coli*, *E. cloacae*, *K. pneumoniae*, and *S. typhimurium*) [197]. Hatamoto et al. also found that the Gram-positive bacteria *Bacillus subtilis* and *Corynebacterium efficiens* exhibited increased susceptibility to $(KFF)_3K$ conjugated to antibacterial PNA than the Gram-negative *E. coli*; however, the Gram-negative bacterium *Ralstonia eutropha* was not affected by the conjugate [201]. Hence, it is not evident that a Gram-dependent susceptibility exists.

The cargo transported by the peptide can also affect the conjugate's translocation in different ways. For instance, in the aforementioned example of Pentobra, the conjugation of the cationic tobramycin to the Pen peptide decreased the permeabilization of *E. coli* and *S. aureus* compared to the Pen peptide alone [79]. In contrast, the conjugation of PNA to the $(KFF)_3K$ peptide made it more membrane-active towards *E. coli* than the $(KFF)_3K$ peptide alone [90]. The authors

hypothesize that PNA, being neutrally charged, may increase the amphipathic character of the conjugate and thus benefit the conjugate's interaction with the bacterial membranes [90].

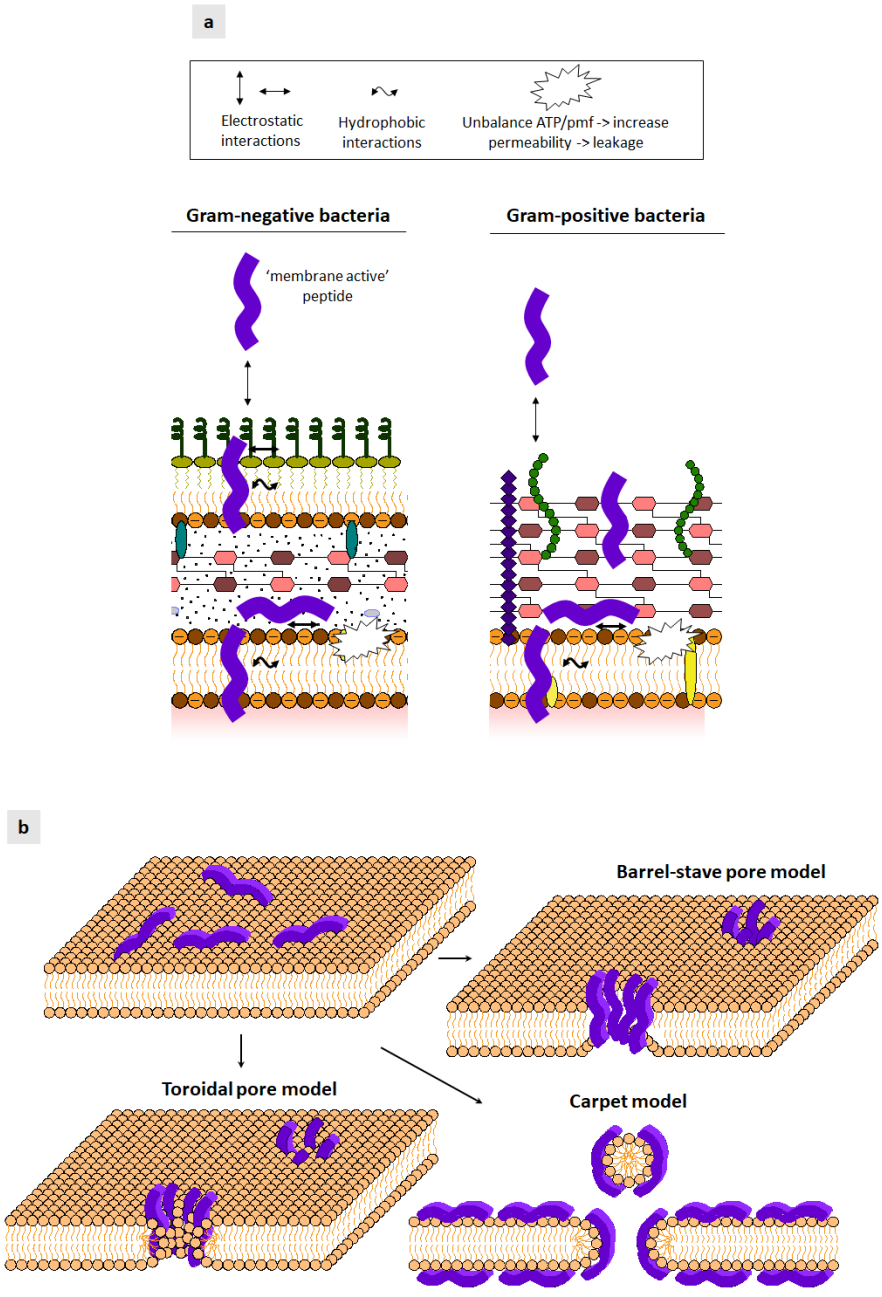


Figure 6. a) Interactions of 'membrane active' peptides with the bacterial cell envelope of Gram-negative and -positive bacteria, mainly determined by i) electrostatic interactions between the charged residues of the peptide and the LPS (in Gram-negative bacteria), the teichoic acids (in Gram-positive bacteria) and the phospholipids (in both Gram types), and ii)

hydrophobic interactions between the hydrophobic residues of the peptide and the lipid layers [55, 183, 196]. b) Different models for the peptide insertion into the bacterial cytoplasmic membrane, similar for Gram-negative and -positive bacteria (thus the represented membrane phospholipids are not differentiated) [26, 175]. The envelope structures depicted are identified in Figure 1.

4.6 Fusogenic liposomes to deliver drugs in bacteria

Liposomes started to be extensively investigated already in the 1970's for drug delivery into mammalian cells [204, 205], but only recently liposomes attracted interest to carry antibacterial drugs. Liposomes, together with poly(lactic-co-glycolic acid) (PLGA) NPs, are the most popular NPs used in antimicrobial studies. However, they are mainly used to improve the pharmacokinetics and the tolerability of antibiotics; in particular they are used to protect antibiotics from degradation in the body and/or to increase the local antibiotic concentration by sustained release [206-211]. Far less explored is the ability of some liposomes to overcome the bacterial envelope barrier by fusing with bacterial membranes, which may help to intracellularly deliver their antibacterial cargo [209]. Besides classic antibiotics, liposomes may offer an interesting solution for the delivery into bacteria of negatively charged oligonucleotides, as these can be easily complexed to cationic liposomes by electrostatic interactions. Fusion between liposomes and bacteria can only occur at the level of the Gram-negative OM lipids and the Gram-positive cytoplasmic membrane, which will be discussed in the sections below.

4.6.1 Interactions of liposomes with the envelope of Gram-negative bacteria

For liposomes to be able to reach the OM lipids for fusion, they first need to overcome the LPS. Cationic liposomes will bind electrostatically to the negatively charged bacteria surface

[212]; in particular, binding to the Gram-negative LPS may lead to LPS chains (O-antigen and core) flattening so that liposomes make contact with the negatively charged lipids of the OM [213] (top panel of Figure 7). For negatively charged liposomes, such as dipalmitoylphosphatidylcholine (DPPC) /dimiristoylphosphatidylglycerol (DMPG) liposomes (Figure 8), Ca^{2+} is typically added to the liposome suspension which can limit the electrostatic repulsion between the anionic liposomes and the LPS; in addition, Ca^{2+} may bridge the liposomes-OM interaction and contribute to dehydrate the OM's PE thereby improving fusion [213]. Apart from Ca^{2+}, other divalent cations can also mediate fusion; nevertheless, cations with larger ionic radius seemed to be less efficient, as they probably increase the liposome-OM distance [213]. Electrostatic attraction of anionic liposomes to the metal cations bound to the LPS may also contribute to the initial interaction with the cell wall [135, 137].

When in contact with the bacterial OM lipids, liposomes may then fuse with them. Fusion occurs when two bilayers, in this case the liposome bilayer and the OM lipid bilayer, merge into a single bilayer [214]. Fusion with the OM lipids is fast and occurs spontaneously by hydrophobic and van der Waals interactions [11]. Fusion critically depends on the lipid composition of the two bilayers involved [214]. The PE moiety is the most commonly referred fusogenic lipid [215, 216]. PE has a low hydration of its polar head group, which may decrease the fluid spacing between bilayers and thereby facilitates energetically favorable interactions between lipid bilayers [215-217]. In addition, PE has a cone-shaped molecular shape (small head cross section and large chain cross section) and ability to promote bilayer-to-hexagonal phase transition which may trigger membrane destabilization [215, 216]. Therefore, PE, in the form of the dioleoylphosphatidylethanolamine (DOPE) lipid (Figure 8), has been incorporated into liposomes to produce fusogenic liposomes. DOPE containing liposomes have successfully improved antibiotics penetration into the Gram-negative *P. aeruginosa*, *E. coli*, *Klebsiella spp.* and *A. Baumannii* [209, 217, 218] and have intracellularly delivered NAMs into the Gram-

971 negative *H. pylori* [219]. In particular, the improved penetration of antibiotics in *P. aeruginosa*
972 allowed a decrease of the minimum inhibitory concentration (MIC) of tobramycin of at least
973 640-fold [218], and of meropenem (a carbapenem) up to 4-fold [209]. Also, the delivery of
974 vancomycin (too large to cross the bacterial envelope of Gram-negative bacteria (Figure 3b)
975 reduced the MIC of vancomycin by up to 85-fold in *E. coli* and *A. Baumannii*, up to 20-fold in
976 *Klebsiella spp.* and up to 10-fold in *P. aeruginosa* [217].

977 Liposomes without DOPE have been also reported to be able to intimately interact and even
978 fuse with bacteria, improving the permeation of antibiotics. A popular formulation is
979 DPPC/DMPG negatively charged liposomes, frequently called "fluidosomes" [213, 218, 220-
980 222]. This designation comes from the ability of DMPG to increase the fluidity of liposomes;
981 indeed, DMPG is a phospholipid with short acyl chains and a high number of unsaturated bonds
982 which results in a relatively low gel-liquid crystalline transition temperature (Tc) [223, 224].
983 Studies report efficient interaction of DPPC/DMPG liposomes with bacteria, improving the
984 permeation of antibiotics [220-222] and even of one antisense PS DNA oligonucleotide [225].
985 However, it has also been reported that encapsulation of antibiotics in DPPC/DMPG liposomes
986 even lowered the antimicrobial efficiency when compared to free antibiotic [209]. It was latter
987 clarified that DPPC/DMPG are not fusogenic *per se* and that their improved antibiotic
988 permeation depends on the presence of divalent cations as Ca^{2+} to reduce the electrostatic
989 repulsion between the negatively charged liposomes and the bacteria surface [213, 218].

990 In addition, intimate interactions were observed between the more rigid liposomal
991 formulations, such as distearoylglycerophosphocholine (DSPC)/cholesterol (Chol),
992 phosphatidylcholine (PC)/Chol/dioleoyltrimethylammoniumpropane (DOTAP) or DPPC/Chol
993 (Figure 8), and Gram-negative bacteria like *P. aeruginosa* and *Burkholderia cenocepacia* [212,
994 226, 227]. Despite the inclusion of lipids with increased Tc and Chol (which should increase
995 liposomes rigidity/stability), fusion/adhesion of these liposomes with the bacterial OM was

reported [212, 226, 227]. However, others have seen that the inclusion of only 10% Chol dramatically lowered DPPC/DMPG fusion in *P.aeruginosa* [213, 218].

The studies using DPPC/DMPG and more rigid liposomes (containing Chol and DSPC) show that fusion with bacterial membranes may occur to some extent using non-DOPE liposomes. Although there is no PE in the liposomes, there is a high amount of PE in the Gram-negative OM that may mediate fusion [55, 228]. Indeed DPPC/DMPG liposomes cannot fuse with the more rigid cytoplasmic membrane of human cells (which contain cholesterol and have PC as the major lipid on their membrane surface and a minor amount of PE sheltered in the inner CM's leaflet) [55, 228]. However, DPPC/DMPG liposomes could fuse with Gram-negative bacteria and the higher the PE content of the bacterial OM, the better fusion occured [213, 228]. When PE (in the form of DOPE) is also included in liposomes, composed for instance of DPPC/DMPG liposomes [218], DPPC/Chol hemisuccinate liposomes [217] or PC/Octadecylamine [209], the tendency for fusion between bacteria and liposomes is further enhanced, as seen by the significant improvement of the antibiotics efficacy [212, 217, 218].

Although the composition of the liposomes affects their fusion ability, a clear effect of the size of liposomes has not been observed [218, 225]. From the bacteria side, beside the PE content, the effect of other bacterial features on fusion is not well understood and may depend on fine details. For instance, various *P. aeruginosa* strains showed different degrees of fusion with PC/Chol/DOTAP liposomes, while the expression of one 18-kDa OMP was found to be positively correlated with fusion [212].

Upon fusion with the Gram-negative OM lipids the incorporated antibiotics/antibacterial oligonucleotides will be delivered into the periplasm (top panel of Figure 7). From here, the molecules will have to cross the viscous periplasmic space and will make contact with the peptidoglycan, where antibiotics that disrupt the peptidoglycan synthesis have their site of action. Other antibiotics and oligonucleotides acting rather on the cytosol will have to cross the

peptidoglycan and the cytoplasmic membrane as well, as discussed in sections 3.1 and 3.2, respectively [36].

4.6.2 Interactions of liposomes with the envelope of Gram-positive bacteria

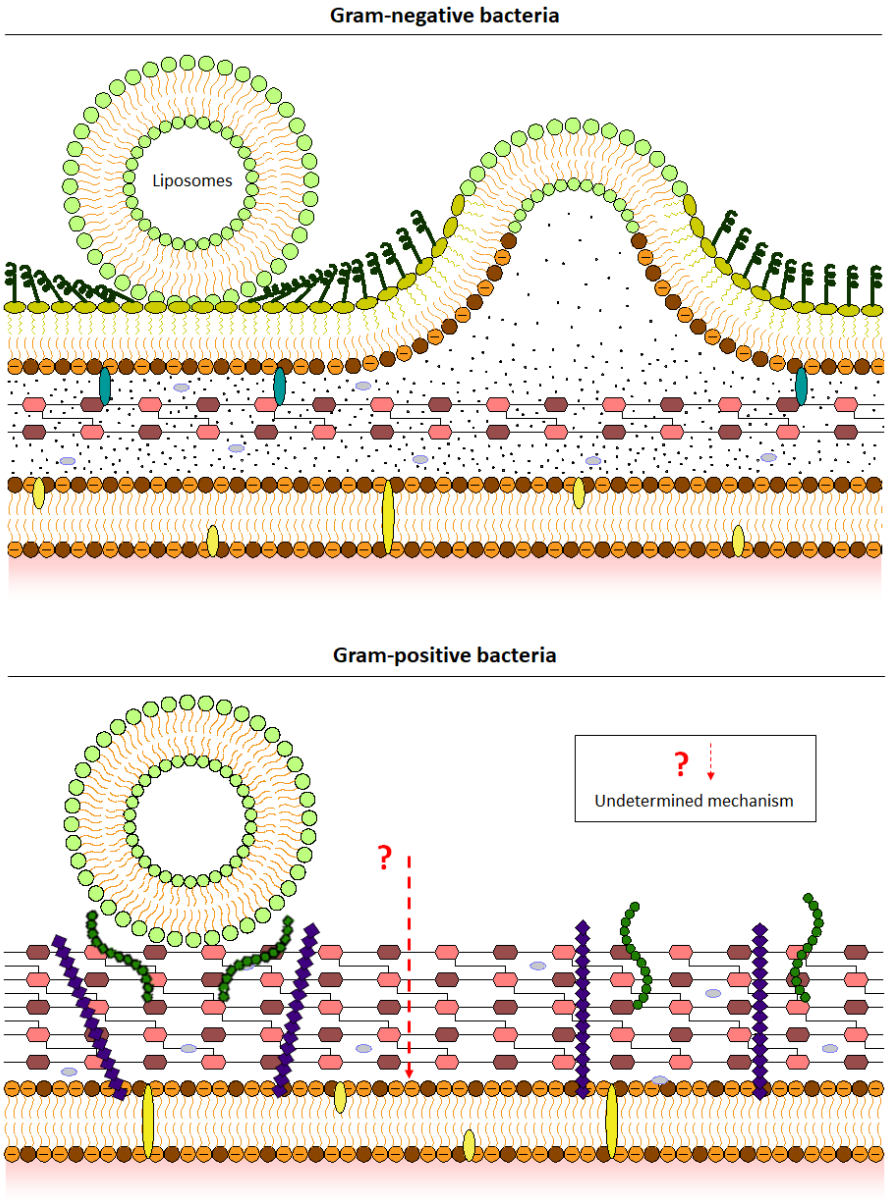
Liposomes for the delivery of antibiotics and oligonucleotides into Gram-positive bacteria will first contact with the teichoic acids. It can be reasoned that liposomes will electrostatically interact with teichoic acids the same way that liposomes interact with LPS (described in the previous section), so that liposomes binding to and flattening of the teichoic acids layer may occur (bottom panel of Figure 7). However, for fusion to occur in Gram-positive bacteria, liposomes do not only have to overcome the teichoic acids but also the thick peptidoglycan, in order to contact and fuse with the cytoplasmic membrane lipids.

It is currently unknown whether or not liposomes could overcome the peptidoglycan of Gram-positive bacteria (bottom panel of Figure 7). The studies on fusion of liposomes with Gram-positive bacteria are few; they were performed with the bacteria *S. aureus*, *Enterococcus faecalis* and *Streptococcus agalactiae* and liposomes composed of DPPC/DMPG (in the presence of Ca^{2+}), egg PC/DMPG/DSPE-PEG, DPPC/DOTAP/DSPE-PEG (with and without wheat germ agglutinin as a targeting moiety), dimyristoylphosphocholine (DMPC)/Chol/DHP (dihexadecylhydrogenphosphate) and DMPC/Chol/ DPPS (dipalmitoylphosphatidylserine), (Figure 8) [213, 229-231]. These studies suggest fusion to explain the improved efficiency of the encapsulated compared to the free antimicrobial drug [213, 229-231]. Also, one study in *S. aureus*, resistant to β-lactam antibiotics, showed that the bacteria susceptibility could be improved by the use of anionic egg PC/DMPG/DSPE-PEG liposomes, carrying a PS DNA to downregulate a gene of resistance to β-lactams [229]. The PS DNA was first complexed with the cationic polymer polyethyleneimine (PEI), resulting in an 80 nm complex that was subsequently encapsulated into the liposomes [229]. The observed downregulation was

interpreted as a result of liposomal fusion and intracellular delivery of the PS DNA [229], but the authors did not report the effect of the complex alone nor the interaction of liposomes with *S. aureus*. Actually only one of the studies on Gram-positive bacteria explicitly demonstrates interaction between liposomes and bacteria, using flow cytometry and fluorescence microscopy [232], and none of them show experimental evidence of direct intracellular delivery by liposomes. Therefore, it may be possible that liposomes only enable sustained local drug release close to the Gram-positive bacterial surface [230], resulting in an increased drug concentration gradient across the bacterial envelope. For drugs with limited (but not impossible) permeation of the bacterial envelope this may be sufficient to improve the drug diffusion across the peptidoglycan and cytoplasmic membrane [230]. Note that local drug release may also contribute to the delivery into Gram-negative bacteria, especially when non-fusogenic liposomes (liposomes without DOPE) are employed.

Even when interaction of liposomes and the OM of Gram-negative bacteria occurs, it remains very challenging to experimentally distinguish between adhesion of the liposomes on the OM with (only) local drug release and fusion of the liposomes with the OM followed by intracellular delivery. This is due to technological limitations. Electron microscopy typically shows generic interaction/adhesion at the bacterial envelope. Immunohistochemistry combined with TEM can be useful to find intracellularly delivered drugs, but it depends on the availability of specific antibodies. Epi-fluorescence microscopy and flow cytometry based on fluorescent constructs (liposomes carrying fluorescent molecules) can hardly distinguish between surface adhered fluorescent constructs and internalized fluorescent molecules, since the optical resolution limit is rather close to the bacteria size (optical resolution being typically not higher than ~0.25 µm while width of most bacteria ~0.5-1 µm). Fillion et al. proposed to distinguish in *E. coli* surface adhesion of DPPC/DPMG liposomes (carrying a fluorescently labelled PS DNA) from intracellular delivery of the fluorescently labelled PS DNA by incubating *E. coli*

with the constructs respectively at 4 °C vs 37 °C and measuring the fluorescence by fluorescence-activated cell sorting (FACS) [225]. However, it is our belief that incubation at 4 °C is not a sufficiently good control, because adhesion at 4 °C may be lower than the adhesion that can occur at 37 °C. Differently, our group showed recently the use of fluorescence microscopy to visualize bacteria with fluorescence coming exclusively from intracellularly delivered fluorescent molecules; fluorescence coming from constructs remaining adhered on the OM could be removed by a mild triton wash [219]. Thus, the fraction of molecules actually delivered intracellularly by the liposomes could the quantified based on the fluorescence intensity [219].



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1082 Figure 7. Illustration of the intracellular delivery mechanism by liposomes able to fuse with bacterial
1083 membranes. In Gram-negative bacteria (top), liposomes fuse with the outer membrane (adapted
1084 from [213]). At the Gram-positive envelope (bottom), liposomes would have to cross the thick
1085 peptidoglycan layer to fuse with the cytoplasmic membrane, via an undetermined mechanism. The
1086 envelope structures depicted are identified in Figure 1.

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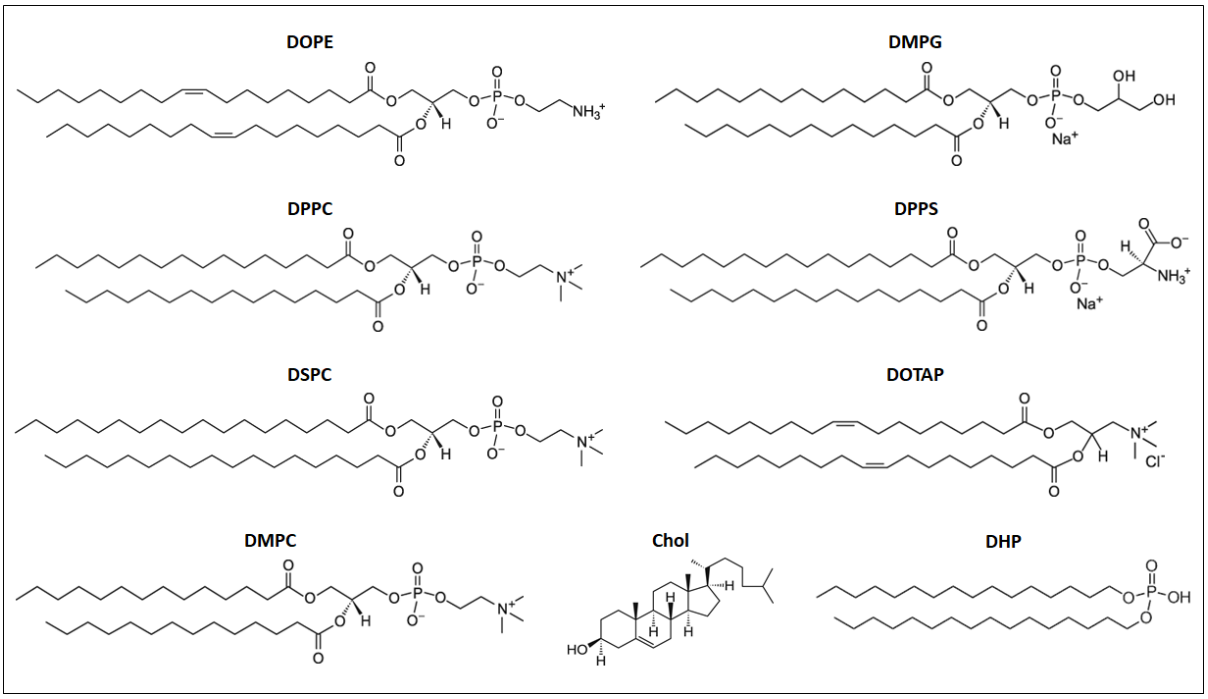

Figure 8. Representative struture of common lipids used in liposomal formulations applied to bacteria. DOPE: dioleoylphosphatidylethanolamine; DMPG: dimiristoylphosphatidylglycerol; DPPC: dipalmitoylphosphatidylcholine; DSPC: distearoylglycerophosphocholine; DOTAP: dioleoyltrimethylammoniumpropane; DMPC: dimyristoylphosphocholine; DHP: dihexadecylhydrogenphosphate; DPPS: dipalmitoylphosphatidylserine; Chol: cholesterol.

5. Conclusions and future perspectives

Nanoparticles and molecular transporters may provide valuable tools to overcome the bacterial cell envelope which limits the internalization of bactericidal drugs. As discussed along this review, such NPs/molecules can be (i) bactericidal *per se*, if they directly penetrate the bacterial envelope causing envelope disruption (metal NPs, CNS, chitosan NPs, dendrimers and AMPs), (ii) carriers of bactericidal drugs that penetrate the bacterial envelope with the attached drugs without necessarily causing disruption (CPPs, siderophores and eventually cyclodextrins), or (iii) delivery vectors that do not penetrate bacteria but intracellularly deliver

the drugs into bacteria (fusogenic liposomes). According to the current knowledge CPPs and liposomes are the best positioned candidates. These are especially interesting to intracellularly transport novel NAM drugs which suffer from very poor internalization, in particular CPPs to transport neutral NAMs and liposomes to transport anionic NAMs. NAMs are promising to be used as antibacterial drugs, by targeting essential bacterial genes, and as drug adjuvants, to restore bacteria susceptibility to antibiotics. NAMs can provide a virtual endless source of drugs, since even if the bacterial target undergoes a mutation the NAM can be easily redesigned to become effective again. Therefore, combining NAMs with a CPP/liposomal carrier holds promise to address the antibiotic crisis and redirect the fight against bacterial infections. Nevertheless, the design of efficient constructs would benefit from a better understanding of the dynamics at the bacterial envelope interface. X-ray crystallography has allowed very useful characterization of some protein channels and more still need to be studied. Still, it would be interesting that these studies could be complemented with investigating the real-time transport in living bacterial cells, so that the eventual adaptability of bacterial envelope's permeability could be evaluated and the chances of the NPs to overcome the bacterial envelopes could be fully understood. The possibility to test transport in live bacteria is challenged by the small size of bacterial cells which limits the direct appreciation of NPs internalization by common techniques as flow cytometry and fluorescence microscopy. Super-resolution microscopy is starting to be used to elucidate the dynamics of some bacterial physiological processes [233-235]. We envision that its continuous advance and availability will position super-resolution microscopy as a critical tool, in the future, to evaluate NPs interaction with bacterial cells and boost the application of nanomedicine towards bacterial cells.

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References

1. Zhu, X., Radovic-Moreno, A.F., Wu, J., Langer, R. and Shi, J., Nanomedicine in the management of microbial infection – Overview and perspectives. Nano Today, (2014). **9**(4): p. 478-498.
2. Tommasi, R., Brown, D.G., Walkup, G.K., Manchester, J.I. and Miller, A.A., ESKAPEing the labyrinth of antibacterial discovery. Nat Rev Drug Discov, (2015). **14**(8): p. 529-542.
3. Huwaitat, R., McCloskey, A.P., Gilmore, B.F. and Laverty, G., Potential strategies for the eradication of multidrug-resistant Gram-negative bacterial infections. Future Microbiol, (2016). **11**: p. 955-72.
4. Frieden, T., Antibiotic resistance threats in the United States, U.S.D.o.H.a.H. Services, Editor. (2013), Centers for Disease Control and Prevention: United States.
5. Masi, M., Réfregiers, M., Pos, K.M. and Pagès, J.-M., Mechanisms of envelope permeability and antibiotic influx and efflux in Gram-negative bacteria. (2017). **2**: p. 17001.
6. Pelgrift, R.Y. and Friedman, A.J., Nanotechnology as a therapeutic tool to combat microbial resistance. Advanced Drug Delivery Reviews, (2013). **65**(13–14): p. 1803-15.

7. Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K.M., Wertheim, H.F.L., Sumpradit, N., *et al.*, Antibiotic resistance—the need for global solutions. The Lancet Infectious Diseases, (2013). **13**(12): p. 1057-98.
8. Wilson, D.N., Ribosome-targeting antibiotics and mechanisms of bacterial resistance. Nat Rev Micro, (2014). **12**(1): p. 35-48.
9. Bai, H., Xue, X., Hou, Z., Zhou, Y., Meng, J. and Luo, X., Antisense antibiotics: a brief review of novel target discovery and delivery. Curr Drug Discov Technol, (2010). **7**(2): p. 76-85.
10. Woodford, N. and Wareham, D.W., Tackling antibiotic resistance: a dose of common antisense? J Antimicrob Chemother, (2009). **63**(2): p. 225-9.
11. Zhang, L., Pornpattananangku, D., Hu, C.M. and Huang, C.M., Development of nanoparticles for antimicrobial drug delivery. Curr Med Chem, (2010). **17**(6): p. 585-94.
12. Huh, A.J. and Kwon, Y.J., "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. J Control Release, (2011). **156**(2): p. 128-45.
13. Piddock, L.J., Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol, (2006). **4**(8): p. 629-36.
14. Poole, K., Efflux-mediated antimicrobial resistance. J Antimicrob Chemother, (2005). **56**(1): p. 20-51.
15. Li, X.Z. and Nikaido, H., Efflux-mediated drug resistance in bacteria. Drugs, (2004). **64**(2): p. 159-204.
16. Zgurskaya, H.I., Löpez, C.A. and Gnanakaran, S., Permeability Barrier of Gram-Negative Cell Envelopes and Approaches To Bypass It. ACS infectious diseases, (2015). **1**(11): p. 512-522.
17. Sahay, G., Alakhova, D.Y. and Kabanov, A.V., Endocytosis of nanomedicines. J controlled release, (2010). **145**(3): p. 182-95.
18. Lonhienne, T.G., Sagulenko, E., Webb, R.I., Lee, K.C., Franke, J., Devos, D.P., *et al.*, Endocytosis-like protein uptake in the bacterium Gemmata obscuriglobus. Proc Natl Acad Sci U S A, (2010). **107**(29): p. 12883-8.
19. Gao, W., Thamphiwatana, S., Angsantikul, P. and Zhang, L., Nanoparticle approaches against bacterial infections. Wiley Interdiscip Rev Nanomed Nanobiotechnol, (2014). **6**(6): p. 532-47.
20. Zazo, H., Colino, C.I. and Lanao, J.M., Current applications of nanoparticles in infectious diseases. J Control Release, (2016). **224**: p. 86-102.
21. Forier, K., Raemdonck, K., De Smedt, S.C., Demeester, J., Coenye, T. and Braeckmans, K., Lipid and polymer nanoparticles for drug delivery to bacterial biofilms. J Control Release, (2014). **190**: p. 607-23.
22. Nikaido, H., Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science, (1994). **264**(5157): p. 382-8.
23. Silhavy, T.J., Kahne, D. and Walker, S., The bacterial cell envelope. Cold Spring Harbor Perspectives in Biology, (2010). **2**(5): p. a000414.
24. Holst, O., Moran, A.P. and Brennan, P.J., Chapter 1 - Overview of the glycosylated components of the bacterial cell envelope, in *Microbial Glycobiology*. (2010), Academic Press: San Diego. p. 1-13.
25. Hajipour, M., Fromm, K., Ashkarran, A., Jimenez de Aberasturi, D., de Larramendi, I.R. and Rojo, T., Antibacterial properties of nanoparticles. Trends Biotechnol., (2012). **30**(10): p. 499-511.
26. Lohner, K., New strategies for novel antibiotics: peptides targeting bacterial cell membranes. Gen Physiol Biophys, (2009). **28**(2): p. 105-16.

27. Denyer, S.P. and Maillard, J.Y., Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. J Appl Microbiol, (2002). **92 Suppl**: p. 35s-45s.
28. Nikaido, H., Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev, (2003). **67**(4): p. 593-656.
29. Raetz, C.R., Enzymology, genetics, and regulation of membrane phospholipid synthesis in *Escherichia coli*. Microbiological Reviews, (1978). **42**(3): p. 614-659.
30. Seltmann, G. and Holst, O., The bacterial cell wall. (2002): Berlin : Springer.
31. Nikaido, H., Transport across the bacterial outer membrane. J Bioenerg Biomembr, (1993). **25**(6): p. 581-9.
32. Plesiat, P. and Nikaido, H., Outer membranes of Gram-negative bacteria are permeable to steroid probes. Mol Microbiol, (1992). **6**(10): p. 1323-33.
33. Konovalova, A. and Silhavy, T.J., Outer membrane lipoprotein biogenesis: Lol is not the end. Philosophical Transactions of the Royal Society B: Biological Sciences, (2015). **370**(1679): p. 20150030.
34. Wiener, M.C. and Horanyi, P.S., How hydrophobic molecules traverse the outer membranes of Gram-negative bacteria. Proceedings of the National Academy of Sciences, (2011). **108**(27): p. 10929-10930.
35. Delcour, A.H., Outer membrane permeability and antibiotic resistance. Biochimica et biophysica acta, (2009). **1794**(5): p. 808-16.
36. Braun, V., Bös, C., Braun, M. and Killmann, H., Outer membrane channels and active transporters for the uptake of antibiotics. The Journal of Infectious Diseases, (2001). **183**(Supplement_1): p. S12-S16.
37. van den Berg, B., Going forward laterally: transmembrane passage of hydrophobic molecules through protein channel walls. Chembiochem : a European journal of chemical biology, (2010). **11**(10): p. 1339-1343.
38. Nestorovich, E.M., Danelon, C., Winterhalter, M. and Bezrukov, S.M., Designed to penetrate: time-resolved interaction of single antibiotic molecules with bacterial pores. Proc Natl Acad Sci U S A, (2002). **99**(15): p. 9789-94.
39. Pongprayoon, P., Beckstein, O., Wee, C.L. and Sansom, M.S., Simulations of anion transport through OprP reveal the molecular basis for high affinity and selectivity for phosphate. Proc Natl Acad Sci U S A, (2009). **106**(51): p. 21614-8.
40. Bajaj, H., Acosta Gutierrez, S., Bodrenko, I., Malloci, G., Scorciapino, M.A., Winterhalter, M., *et al.*, Bacterial outer membrane porins as electrostatic nanosieves: exploring transport rules of small polar molecules. ACS Nano, (2017). **11**(6): p. 5465-73.
41. Ghai, I., Pira, A., Scorciapino, M.A., Bodrenko, I., Benier, L., Ceccarelli, M., *et al.*, General method to determine the flux of charged molecules through nanopores applied to β-Lactamase inhibitors and OmpF. The Journal of Physical Chemistry Letters, (2017). **8**(6): p. 1295-301.
42. Yildiz, Ö., Vinothkumar, K.R., Goswami, P. and Kühlbrandt, W., Structure of the monomeric outer-membrane porin OmpG in the open and closed conformation. The EMBO Journal, (2006). **25**(15): p. 3702-3713.
43. Yoshimura, F. and Nikaido, H., Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. J Bacteriol, (1982). **152**(2): p. 636-42.
44. Lepore, B.W., Indic, M., Pham, H., Hearn, E.M., Patel, D.R. and van den Berg, B., Ligand-gated diffusion across the bacterial outer membrane. Proceedings of the National Academy of Sciences of the United States of America, (2011). **108**(25): p. 10121-26.

45. van den Berg, B., Prathyusha Bhamidimarri, S., Dahyabhai Prajapati, J., Kleinekathöfer, U. and Winterhalter, M., Outer-membrane translocation of bulky small molecules by passive diffusion. Proc Natl Acad Sci U S A, (2015). **112**(23): p. E2991-9.
46. Ye, J. and van den Berg, B., Crystal structure of the bacterial nucleoside transporter Tsx. The EMBO Journal, (2004). **23**(16): p. 3187-3195.
47. van den Berg, B., Prathyusha Bhamidimarri, S., Dahyabhai Prajapati, J., Kleinekathofer, U. and Winterhalter, M., Outer-membrane translocation of bulky small molecules by passive diffusion. Proc Natl Acad Sci U S A, (2015). **112**(23): p. E2991-9.
48. Bhamidimarri, Satya P., Prajapati, Jigneshkumar D., van den Berg, B., Winterhalter, M. and Kleinekathöfer, U., Role of electroosmosis in the permeation of neutral molecules: CymA and Cyclodextrin as an example. Biophysical Journal, (2016). **110**(3): p. 600-11.
49. Hearn, E.M., Patel, D.R., Lepore, B.W., Indic, M. and van den Berg, B., Transmembrane passage of hydrophobic compounds through a protein channel wall. Nature, (2009). **458**(7236): p. 367-70.
50. Schauer, K., Rodionov, D.A. and de Reuse, H., New substrates for TonB-dependent transport: do we only see the 'tip of the iceberg'? Trends Biochem Sci, (2008). **33**(7): p. 330-8.
51. Krewulak, K.D. and Vogel, H.J., Structural biology of bacterial iron uptake. Biochim Biophys Acta, (2008). **1778**(9): p. 1781-804.
52. Braun, V., FhuA (TonA), the career of a protein. Journal of Bacteriology, (2009). **191**(11): p. 3431-36.
53. Ferguson, A.D., Hofmann, E., Coulton, J.W., Diederichs, K. and Welte, W., Siderophore-Mediated Iron Transport: Crystal Structure of FhuA with Bound Lipopolysaccharide. Science, (1998). **282**(5397): p. 2215-20.
54. Sochacki, K.A., Shkel, I.A., Record, M.T. and Weisshaar, J.C., Protein diffusion in the periplasm of *E. coli* under osmotic stress. Biophysical Journal, (2011). **100**(1): p. 22-31.
55. Malanovic, N. and Lohner, K., Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. Biochimica et Biophysica Acta (BBA) - Biomembranes, (2016). **1858**(5): p. 936-46.
56. Demchick, P. and Koch, A.L., The permeability of the wall fabric of *Escherichia coli* and *Bacillus subtilis*. Journal of Bacteriology, (1996). **178**(3): p. 768-73.
57. Hughes, R.C., Thurman, P.F. and Stokes, E., Estimates of the porosity of *Bacillus licheniformis* and *Bacillus subtilis* cell walls. Z Immunitatsforsch Exp Klin Immunol, (1975). **149**(2-4): p. 126-35.
58. Typas, A., Banzhaf, M., Gross, C.A. and Vollmer, W., From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat Rev Micro, (2012). **10**(2): p. 123-36.
59. Lambert, P.A., Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. J Appl Microbiol, (2002). **92 Suppl**: p. 46s-54s.
60. Sycuro, L.K., Pincus, Z., Gutierrez, K.D., Biboy, J., Stern, C.A., Vollmer, W., *et al.*, Peptidoglycan crosslinking relaxation promotes *Helicobacter pylori*'s helical shape and stomach colonization. Cell, (2010). **141**(5): p. 822-33.
61. Xie, H., Patching, S.G., Gallagher, M.P., Litherland, G.J., Brough, A.R., Venter, H., *et al.*, Purification and properties of the *Escherichia coli* nucleoside transporter NupG, a paradigm for a major facilitator transporter sub-family. Mol Membr Biol, (2004). **21**(5): p. 323-36.
62. Brown, S., Santa Maria, J.P. and Walker, S., Wall teichoic acids of gram-positive bacteria. Annual review of microbiology, (2013). **67**: p. 313-36.

63. Weidenmaier, C. and Peschel, A., Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat Rev Microbiol, (2008). **6**(4): p. 276-87.
64. Navarre, W.W. and Schneewind, O., Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiology and Molecular Biology Reviews : MMBR, (1999). **63**(1): p. 174-229.
65. Epand, R.F., Savage, P.B. and Epand, R.M., Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins). Biochim Biophys Acta, (2007). **1768**(10): p. 2500-9.
66. Matias, V.R.F., Al-Amoudi, A., Dubochet, J. and Beveridge, T.J., Cryo-transmission electron microscopy of frozen-hydrated sections of *Escherichia coli* and *Pseudomonas aeruginosa*. Journal of Bacteriology, (2003). **185**(20): p. 6112-18.
67. Vollmer, W. and Seligman, S.J., Architecture of peptidoglycan: more data and more models. Trends in Microbiology. **18**(2): p. 59-66.
68. Strauss, J., Burnham, N.A. and Camesano, T.A., Atomic force microscopy study of the role of LPS O-antigen on adhesion of *E. coli*. J Mol Recognit, (2009). **22**(5): p. 347-55.
69. Lam, J.S., Graham, L.L., Lightfoot, J., Dasgupta, T. and Beveridge, T.J., Ultrastructural examination of the lipopolysaccharides of *Pseudomonas aeruginosa* strains and their isogenic rough mutants by freeze-substitution. J Bacteriol, (1992). **174**(22): p. 7159-67.
70. Yao, X., Jericho, M., Pink, D. and Beveridge, T., Thickness and elasticity of Gram-Negative murein sacculi measured by atomic force microscopy. Journal of Bacteriology, (1999). **181**(22): p. 6865-6875.
71. Brooks, B.D. and Brooks, A.E., Therapeutic strategies to combat antibiotic resistance. Advanced Drug Delivery Reviews, (2014). **78**: p. 14-27.
72. Silver, L.L., Challenges of antibacterial discovery. Clin Microbiol Rev, (2011). **24**(1): p. 71-109.
73. Coates, A.R.M., Halls, G. and Hu, Y., Novel classes of antibiotics or more of the same? British Journal of Pharmacology, (2011). **163**(1): p. 184-94.
74. Kohanski, M.A., Dwyer, D.J. and Collins, J.J., How antibiotics kill bacteria: from targets to networks. Nature reviews. Microbiology, (2010). **8**(6): p. 423-35.
75. Kahne, D., Leimkuhler, C., Lu, W. and Walsh, C., Glycopeptide and lipoglycopeptide antibiotics. Chem Rev, (2005). **105**(2): p. 425-48.
76. Wright, G.D., Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biology, (2010). **8**: p. 123-123.
77. Nikaido, H., Multidrug efflux pumps of gram-negative bacteria. J Bacteriol, (1996). **178**(20): p. 5853-9.
78. Ofek, I., Cohen, S., Rahmani, R., Kabha, K., Tamarkin, D., Herzig, Y., *et al.*, Antibacterial synergism of polymyxin B nonapeptide and hydrophobic antibiotics in experimental gram-negative infections in mice. Antimicrob Agents Chemother, (1994). **38**(2): p. 374-7.
79. Schmidt, N.W., Deshayes, S., Hawker, S., Blacker, A., Kasko, A.M. and Wong, G.C.L., Engineering persister-specific antibiotics with synergistic antimicrobial functions. ACS Nano, (2014). **8**(9): p. 8786-93.
80. Braun, V., Pramanik, A., Gwinner, T., Köberle, M. and Bohn, E., Sideromycins: tools and antibiotics. Biometals, (2009). **22**(1): p. 3-13.
81. Chopra, I. and Roberts, M., Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and Molecular Biology Reviews, (2001). **65**(2): p. 232-60.

82. Danelon, C., Nestorovich, E.M., Winterhalter, M., Ceccarelli, M. and Bezrukov, S.M., Interaction of zwitterionic penicillins with the OmpF channel facilitates their translocation. Biophys J, (2006). **90**(5): p. 1617-27.
83. Pages, J.M., James, C.E. and Winterhalter, M., The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat Rev Microbiol, (2008). **6**(12): p. 893-903.
84. Good, L., Sandberg, R., Larsson, O., Nielsen, P.E. and Wahlestedt, C., Antisense PNA effects in *Escherichia coli* are limited by the outer-membrane LPS layer. Microbiology, (2000). **146 (Pt 10)**: p. 2665-70.
85. Mellbye, B.L., Weller, D.D., Hassinger, J.N., Reeves, M.D., Lovejoy, C.E., Iversen, P.L., *et al.*, Cationic phosphorodiamidate morpholino oligomers efficiently prevent growth of *Escherichia coli in vitro* and *in vivo.* J Antimicrob Chemother, (2010). **65**(1): p. 98-106.
86. Cerqueira, L., Azevedo, N.F., Almeida, C., Jardim, T., Keevil, C.W. and Vieira, M.J., DNA mimics for the rapid identification of microorganisms by fluorescence *in situ* hybridization (FISH). International Journal of Molecular Sciences, (2008). **9**(10): p. 1944-60.
87. Campbell, M.A. and Wengel, J., Locked vs. unlocked nucleic acids (LNA vs. UNA): contrasting structures work towards common therapeutic goals. Chem Soc Rev, (2011). **40**(12): p. 5680-9.
88. Järver, P., Coursindel, T., Andaloussi, S.E., Godfrey, C., Wood, M.J. and Gait1, M.J., Peptide-mediated cell and *in vivo* delivery of antisense oligonucleotides and siRNA. Molecular Therapy Nucleic acids, (2012). **1**(6): p. 1-17.
89. Ashizawa, A.T. and Cortes, J., Liposomal delivery of nucleic acid-based anticancer therapeutics: BP-100-1.01. Expert Opinion on Drug Delivery, (2015). **12**(7): p. 1107-20.
90. Eriksson, M., Nielsen, P.E. and Good, L., Cell permeabilization and uptake of antisense peptide-peptide nucleic acid (PNA) into *Escherichia coli.* J Biol Chem, (2002). **277**(9): p. 7144-7.
91. Readman, J.B., Dickson, G. and Coldham, N.G., Translational inhibition of CTX-M extended spectrum β-Lactamase in clinical strains of *Escherichia coli* by synthetic antisense oligonucleotides partially restores sensitivity to cefotaxime. Frontiers in Microbiology, (2016). **7**: p. 373.
92. Traglia, G.M., Sala, C.D., Fuxman Bass, J.I., Soler-Bistué, A.J.C., Zorreguieta, A., Ramírez, M.S., *et al.*, Internalization of Locked Nucleic Acids/DNA Hybrid Oligomers into *Escherichia coli.* BioResearch Open Access, (2012). **1**(5): p. 260-263.
93. Good, L., Awasthi, S.K., Dryselius, R., Larsson, O. and Nielsen, P.E., Bactericidal antisense effects of peptide-PNA conjugates. Nat Biotechnol, (2001). **19**(4): p. 360-4.
94. Guo, Q.Y., Xiao, G., Li, R., Guan, S.M., Zhu, X.L. and Wu, J.Z., Treatment of *Streptococcus mutans* with antisense oligodeoxyribonucleotides to gtfB mRNA inhibits GtfB expression and function. FEMS Microbiol Lett, (2006). **264**(1): p. 8-14.
95. Mellbye, B.L., Puckett, S.E., Tilley, L.D., Iversen, P.L. and Geller, B.L., Variations in amino acid composition of antisense peptide-phosphorodiamidate morpholino oligomer affect potency against *Escherichia coli in vitro* and *in vivo.* Antimicrob Agents Chemother, (2009). **53**(2): p. 525-30.
96. Geller, B.L., Deere, J., Tilley, L. and Iversen, P.L., Antisense phosphorodiamidate morpholino oligomer inhibits viability of *Escherichia coli* in pure culture and in mouse peritonitis. J Antimicrob Chemother, (2005). **55**(6): p. 983-8.

97. Santos, R.S., Dakwar, G.R., Xiong, R., Forier, K., Remaut, K., Stremersch, S., *et al.*, Effect of native gastric mucus on *in vivo* hybridization therapies directed at *Helicobacter pylori*. Molecular Therapy - Nucleic Acids, (2015). **4**: p. e269.
98. Fontenete, S., Leite, M., Guimaraes, N., Madureira, P., Ferreira, R.M., Figueiredo, C., *et al.*, Towards fluorescence *in vivo* hybridization (FIVH) detection of *H. pylori* in gastric mucosa using advanced LNA probes. PLoS One, (2015). **10**(4): p. e0125494.
99. Lundin, K.E., Hojland, T., Hansen, B.R., Persson, R., Bramsen, J.B., Kjems, J., *et al.*, Biological activity and biotechnological aspects of locked nucleic acids. Adv Genet, (2013). **82**: p. 47-107.
100. Wojtkowiak-Szlachcic, A., Taylor, K., Stepniak-Konieczna, E., Sznajder, L.J., Mykowska, A., Sroka, J., *et al.*, Short antisense-locked nucleic acids (all-LNAs) correct alternative splicing abnormalities in myotonic dystrophy. Nucleic Acids Research, (2015). **43**(6): p. 3318-31.
101. Järver, P., Coursindel, T., Andaloussi, S.E.L., Godfrey, C., Wood, M.J.A. and Gait, M.J., Peptide-mediated cell and *in vivo* delivery of antisense oligonucleotides and siRNA. Molecular Therapy. Nucleic Acids, (2012). **1**(6): p. e27.
102. Bistue, A.J.S., Martin, F.A., Vozza, N., Ha, H., Joaquin, J.C., Zorreguieta, A., *et al.*, Inhibition of aac(6')-Ib-mediated amikacin resistance by nuclease-resistant external guide sequences in bacteria. Proc Natl Acad Sci U S A, (2009). **106**(32): p. 13230-5.
103. Abushahba, M.F.N., Mohammad, H., Thangamani, S., Hussein, A.A.A. and Seleem, M.N., Impact of different cell penetrating peptides on the efficacy of antisense therapeutics for targeting intracellular pathogens. Scientific Reports, (2016). **6**: p. 20832.
104. Santos, R.S., Guimaraes, N., Madureira, P. and Azevedo, N.F., Optimization of a peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) method for the detection of bacteria and disclosure of a formamide effect. J Biotechnol, (2014). **187**: p. 16-24.
105. Rocha, R., Santos, R.S., Madureira, P., Almeida, C. and Azevedo, N.F., Optimization of peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) for the detection of bacteria: the effect of pH, dextran sulfate and probe concentration. J Biotechnol, (2016). **226**: p. 1-7.
106. Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., *et al.*, Crystal structures explain functional properties of two *E. coli* porins. Nature, (1992). **358**(6389): p. 727-33.
107. Skwarecki, A.S., Milewski, S., Schielmann, M. and Milewska, M.J., Antimicrobial molecular nanocarrier-drug conjugates. Nanomedicine, (2016). **12**(8): p. 2215-40.
108. de Carvalho, C.C.C.R. and Fernandes, P., Siderophores as "Trojan Horses": tackling multidrug resistance? Frontiers in Microbiology, (2014). **5**: p. 290.
109. Heinisch, L., Wittmann, S., Stoiber, T., Berg, A., Ankel-Fuchs, D. and Mollmann, U., Highly antibacterial active aminoacyl penicillin conjugates with acylated bis-catecholate siderophores based on secondary diamino acids and related compounds. J Med Chem, (2002). **45**(14): p. 3032-40.
110. Wittmann, S., Schnabelrauch, M., Scherlitz-Hofmann, I., Mollmann, U., Ankel-Fuchs, D. and Heinisch, L., New synthetic siderophores and their beta-lactam conjugates based on diamino acids and dipeptides. Bioorg Med Chem, (2002). **10**(6): p. 1659-70.
111. Page, M.G.P., Dantier, C. and Desarbre, E., *In vitro* properties of BAL30072, a novel siderophore sulfactam with activity against multiresistant Gram-negative bacilli. Antimicrobial Agents and Chemotherapy, (2010). **54**(6): p. 2291-302.

112. Mushtaq, S., Warner, M. and Livermore, D., Activity of the siderophore monobactam BAL30072 against multiresistant non-fermenters. J Antimicrob Chemother, (2010). **65**(2): p. 266-70.
113. Mima, T., Kvitko, B.H., Rholl, D.A., Page, M.G., Desarbre, E. and Schweizer, H.P., *In vitro* activity of BAL30072 against *Burkholderia pseudomallei*. Int J Antimicrob Agents, (2011). **38**(2): p. 157-9.
114. Higgins, P.G., Stefanik, D., Page, M.G., Hackel, M. and Seifert, H., *In vitro* activity of the siderophore monosulfactam BAL30072 against meropenem-non-susceptible *Acinetobacter baumannii*. J Antimicrob Chemother, (2012). **67**(5): p. 1167-9.
115. Fernandes, P. and Martens, E., Antibiotics in late clinical development. Biochemical Pharmacology, (2017). **133**: p. 152-63.
116. Butler, M.S., Blaskovich, M.A. and Cooper, M.A., Antibiotics in the clinical pipeline in 2013. J Antibiot, (2013). **66**(10): p. 571-91.
117. Davis, M.E. and Brewster, M.E., Cyclodextrin-based pharmaceutics: past, present and future. Nat Rev Drug Discov, (2004). **3**(12): p. 1023-35.
118. Karginov, V.A., Cyclodextrin derivatives as anti-infectives. Current opinion in pharmacology, (2013). **13**(5): p. 1-15.
119. Imperiale, J.C. and Sosnik, A.D., Cyclodextrin complexes for treatment improvement in infectious diseases. Nanomedicine (Lond), (2015). **10**(10): p. 1621-41.
120. Li, M., Neoh, K.G., Xu, L., Yuan, L., Leong, D.T., Kang, E.-T., *et al.*, Sugar-grafted cyclodextrin nanocarrier as a "trojan horse" for potentiating antibiotic activity. Pharmaceutical Research, (2016). **33**(5): p. 1161-74.
121. Athanassiou, G., Michaleas, S., Lada-Chitiroglou, E., Tsitsa, T. and Antoniadou-Vyza, E., Antimicrobial activity of beta-lactam antibiotics against clinical pathogens after molecular inclusion in several cyclodextrins. A novel approach to bacterial resistance. J Pharm Pharmacol, (2003). **55**(3): p. 291-300.
122. Teixeira, K.I., Araujo, P.V., Neves, B.R., Mahecha, G.A., Sinisterra, R.D. and Cortes, M.E., Ultrastructural changes in bacterial membranes induced by nano-assemblies beta-cyclodextrin chlorhexidine: SEM, AFM, and TEM evaluation. Pharm Dev Technol, (2013). **18**(3): p. 600-8.
123. Suarez, D.F., Consuegra, J., Trajano, V.C., Gontijo, S.M., Guimaraes, P.P., Cortes, M.E., *et al.*, Structural and thermodynamic characterization of doxycycline/beta-cyclodextrin supramolecular complex and its bacterial membrane interactions. Colloids Surf B Biointerfaces, (2014). **118**: p. 194-201.
124. Aleem, O., Kuchekar, B., Pore, Y. and Late, S., Effect of beta-cyclodextrin and hydroxypropyl beta-cyclodextrin complexation on physicochemical properties and antimicrobial activity of cefdinir. J Pharm Biomed Anal, (2008). **47**(3): p. 535-40.
125. Jaiswal, S., Duffy, B., Jaiswal, A.K., Stobie, N. and McHale, P., Enhancement of the antibacterial properties of silver nanoparticles using beta-cyclodextrin as a capping agent. Int J Antimicrob Agents, (2010). **36**(3): p. 280-3.
126. He, Y., Fu, P., Shen, X. and Gao, H., Cyclodextrin-based aggregates and characterization by microscopy. Micron, (2008). **39**(5): p. 495-516.
127. González-Gaitano, G., Rodríguez, P., Isasi, J.R., Fuentes, M., Tardajos, G. and Sánchez, M., The aggregation of cyclodextrins as studied by photon correlation spectroscopy. Journal of inclusion phenomena and macrocyclic chemistry, (2002). **44**(1): p. 101-5.
128. Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramirez, J.T., *et al.*, The bactericidal effect of silver nanoparticles. Nanotechnology, (2005). **16**(10): p. 2346-53.

129. Lemire, J.A., Harrison, J.J. and Turner, R.J., Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat Rev Micro, (2013). **11**(6): p. 371-84.
130. Vardanyan, Z., Gevorkyan, V., Ananyan, M., Vardapetyan, H. and Trchounian, A., Effects of various heavy metal nanoparticles on *Enterococcus hirae* and *Escherichia coli* growth and proton-coupled membrane transport. Journal of Nanobiotechnology, (2015). **13**(1): p. 69.
131. Paredes, D., Ortiz, C. and Torres, R., Synthesis, characterization, and evaluation of antibacterial effect of Ag nanoparticles against *Escherichia coli* O157:H7 and methicillin-resistant *Staphylococcus aureus* (MRSA). International Journal of Nanomedicine, (2014). **9**: p. 1717-29.
132. Eckhardt, S., Brunetto, P.S., Gagnon, J., Priebe, M., Giese, B. and Fromm, K.M., Nanobio silver: Its interactions with peptides and bacteria, and its uses in medicine. Chemical Reviews, (2013). **113**(7): p. 4708-54.
133. Li, H., Chen, Q., Zhao, J. and Urmila, K., Enhancing the antimicrobial activity of natural extraction using the synthetic ultrasmall metal nanoparticles. Sci Rep, (2015). **5**: p. 11033.
134. Sondi, I. and Salopek-Sondi, B., Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. Journal of Colloid and Interface Science, (2004). **275**(1): p. 177-82.
135. Fayaz, A.M., Balaji, K., Girilal, M., Yadav, R., Kalaichelvan, P.T. and Venketesan, R., Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. Nanomedicine, (2010). **6**(1): p. 103-9.
136. El Badawy, A.M., Silva, R.G., Morris, B., Scheckel, K.G., Suidan, M.T. and Tolaymat, T.M., Surface charge-dependent toxicity of silver nanoparticles. Environ Sci Technol, (2011). **45**(1): p. 283-7.
137. Amro, N.A., Kotra, L.P., Wadu-Mesthrige, K., Bulychev, A., Mobashery, S. and Liu, G.-y., High-resolution atomic force microscopy studies of the *Escherichia coli* outer membrane: structural basis for permeability. Langmuir, (2000). **16**(6): p. 2789-96.
138. Lok, C.N., Ho, C.M., Chen, R., He, Q.Y., Yu, W.Y., Sun, H., *et al.*, Proteomic analysis of the mode of antibacterial action of silver nanoparticles. J Proteome Res, (2006). **5**(4): p. 916-24.
139. Li, W.R., Xie, X.B., Shi, Q.S., Zeng, H.Y., Ou-Yang, Y.S. and Chen, Y.B., Antibacterial activity and mechanism of silver nanoparticles on *Escherichia coli.* Appl Microbiol Biotechnol, (2010). **85**(4): p. 1115-22.
140. Mirzajani, F., Ghassempour, A., Aliahmadi, A. and Esmaeili, M.A., Antibacterial effect of silver nanoparticles on *Staphylococcus aureus.* Res Microbiol, (2011). **162**(5): p. 542-9.
141. Kim, J.S., Kuk, E., Yu, K.N., Kim, J.H., Park, S.J., Lee, H.J., *et al.*, Antimicrobial effects of silver nanoparticles. Nanomedicine, (2007). **3**(1): p. 95-101.
142. Ruparelia, J.P., Chatterjee, A.K., Duttagupta, S.P. and Mukherji, S., Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomater, (2008). **4**(3): p. 707-16.
143. Sawosz, E., Chwalibog, A., Szeliga, J., Sawosz, F., Grodzik, M., Rupiewicz, M., *et al.*, Visualization of gold and platinum nanoparticles interacting with *Salmonella enteritidis* and *Listeria monocytogenes.* Int J Nanomedicine, (2010). **5**: p. 631-7.
144. Lok, C.N., Ho, C.M., Chen, R., He, Q.Y., Yu, W.Y. and Sun, H., Proteomic analysis of the mode of antibacterial action of silver nanoparticles. J Proteome Res, (2006). **5**.

145. Xu, X.H., Brownlow, W.J., Kyriacou, S.V., Wan, Q. and Viola, J.J., Real-time probing of membrane transport in living microbial cells using single nanoparticle optics and living cell imaging. Biochemistry, (2004). **43**(32): p. 10400-13.
146. Lee, K.J., Browning, L.M., Huang, T., Ding, F., Nallathamby, P.D. and Xu, X.-H.N., Probing of multidrug ABC membrane transporters of single living cells using single plasmonic nanoparticle optical probes. Analytical and bioanalytical chemistry, (2010). **397**(8): p. 3317-28.
147. Brayner, R., Ferrari-Iliou, R., Brivois, N., Djediat, S., Benedetti, M.F. and Fiévet, F., Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano Letters, (2006). **6**(4): p. 866-70.
148. Kumar, A., Pandey, A.K., Singh, S.S., Shanker, R. and Dhawan, A., Cellular uptake and mutagenic potential of metal oxide nanoparticles in bacterial cells. Chemosphere, (2011). **83**(8): p. 1124-32.
149. Stoimenov, P.K., Klinger, R.L., Marchin, G.L. and Klabunde, K.J., Metal oxide nanoparticles as bactericidal agents. Langmuir, (2002). **18**(17): p. 6679-6686.
150. Lee, K.J., Nallathamby, P.D., Browning, L.M., Osgood, C.J. and Xu, X.-H.N., *In vivo* imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. ACS Nano, (2007). **1**(2): p. 133-43.
151. Xu, X.-H.N., Chen, J., Jeffers, R.B. and Kyriacou, S., Direct measurement of sizes and dynamics of single living membrane transporters using nanooptics. Nano Letters, (2002). **2**(3): p. 175-182.
152. Browning, L.M., Lee, K.J., Cherukuri, P.K., Nallathamby, P.D., Warren, S., Jault, J.-M., *et al.*, Single nanoparticle plasmonic spectroscopy for study of the efflux function of multidrug ABC membrane transporters of single live cells. RSC advances, (2016). **6**(43): p. 36794-802.
153. Chen, C.Z. and Cooper, S.L., Recent Advances in Antimicrobial Dendrimers. Adv. Mater., (2000). **12**: p. 843-6.
154. Perreault, F., de Faria, A.F., Nejati, S. and Elimelech, M., Antimicrobial Properties of Graphene Oxide Nanosheets: Why Size Matters. ACS Nano, (2015). **9**(7): p. 7226-36.
155. Tu, Y., Lv, M., Xiu, P., Huynh, T., Zhang, M., Castelli, M., *et al.*, Destructive extraction of phospholipids from *Escherichia coli* membranes by graphene nanosheets. Nat Nano, (2013). **8**(8): p. 594-601.
156. Nanda, S.S., Yi, D.K. and Kim, K., Study of antibacterial mechanism of graphene oxide using Raman spectroscopy. Scientific Reports, (2016). **6**: p. 28443.
157. Kang, S., Herzberg, M., Rodrigues, D.F. and Elimelech, M., Antibacterial effects of carbon nanotubes: size does matter! Langmuir, (2008). **24**(13): p. 6409-13.
158. Cheng, Y., Qu, H., Ma, M., Xu, Z., Xu, P., Fang, Y., *et al.*, Polyamidoamine (PAMAM) dendrimers as biocompatible carriers of quinolone antimicrobials: an *in vitro* study. Eur J Med Chem, (2007). **42**(7): p. 1032-8.
159. Ma, M., Cheng, Y., Xu, Z., Xu, P., Qu, H., Fang, Y., *et al.*, Evaluation of polyamidoamine (PAMAM) dendrimers as drug carriers of anti-bacterial drugs using sulfamethoxazole (SMZ) as a model drug. Eur J Med Chem, (2007). **42**(1): p. 93-8.
160. Mishra, M.K., Kotta, K., Hali, M., Wykes, S., Gerard, H.C., Hudson, A.P., *et al.*, PAMAM dendrimer-azithromycin conjugate nanodevices for the treatment of *Chlamydia trachomatis* infections. Nanomedicine, (2011). **7**(6): p. 935-44.
161. Chakraborty, S.P., Sahu, S.K., Pramanik, P. and Roy, S., Biocompatibility of folate-modified chitosan nanoparticles. Asian Pacific Journal of Tropical Biomedicine, (2012). **2**(3): p. 215-19.

162. Lin, Y.H., Tsai, S.C., Lai, C.H., Lee, C.H., He, Z.S. and Tseng, G.C., Genipin-cross-linked fucose-chitosan/heparin nanoparticles for the eradication of *Helicobacter pylori.* Biomaterials, (2013). **34**(18): p. 4466-79.
163. Qi, L., Xu, Z., Jiang, X., Hu, C. and Zou, X., Preparation and antibacterial activity of chitosan nanoparticles. Carbohydr Res, (2004). **339**(16): p. 2693-700.
164. Friedman, A.J., Phan, J., Schairer, D.O., Champer, J., Qin, M., Pirouz, A., *et al.*, Antimicrobial and anti-inflammatory activity of chitosan-alginate nanoparticles: a targeted therapy for cutaneous pathogens. J Invest Dermatol, (2013). **133**(5): p. 1231-9.
165. Chen, C.Z. and Cooper, S.L., Interactions between dendrimer biocides and bacterial membranes. Biomaterials, (2002). **23**(16): p. 3359-68.
166. Arias, L.R. and Yang, L., Inactivation of bacterial pathogens by carbon nanotubes in suspensions. Langmuir, (2009). **25**(5): p. 3003-12.
167. Carpio, M., Santos, I.E., Wei, C.M., Rodrigues, X. and F, D., Toxicity of a polymer-graphene oxide composite against bacterial planktonic cells, biofilms, and mammalian cells. Nanoscale, (2012). **4**(15): p. 4746-56.
168. Chen, J., Peng, H., Wang, X., Shao, F., Yuan, Z. and Han, H., Graphene oxide exhibits broad-spectrum antimicrobial activity against bacterial phytopathogens and fungal conidia by intertwining and membrane perturbation. Nanoscale, (2014). **6**(3): p. 1879-89.
169. Kotchey, G.P., Allen, B.L., Vedala, H., Yanamala, N., Kapralov, A.A., Tyurina, Y.Y., *et al.*, The enzymatic oxidation of graphene oxide. ACS Nano, (2011). **5**(3): p. 2098-108.
170. Koren, E. and Torchilin, V.P., Cell-penetrating peptides: breaking through to the other side. Trends in Molecular Medicine, (2012). **18**(7): p. 385-93.
171. Wang, F., Wang, Y., Zhang, X., Zhang, W., Guo, S. and Jin, F., Recent progress of cell-penetrating peptides as new carriers for intracellular cargo delivery. J Controlled Release, (2014). **174**: p. 126-136.
172. Andersson, D.I., Hughes, D. and Kubicek-Sutherland, J.Z., Mechanisms and consequences of bacterial resistance to antimicrobial peptides. Drug Resist Updat, (2016). **26**: p. 43-57.
173. Zetterberg, M.M., Reijmar, K., Pranting, M., Engstrom, A., Andersson, D.I. and Edwards, K., PEG-stabilized lipid disks as carriers for amphiphilic antimicrobial peptides. J Control Release, (2011). **156**(3): p. 323-8.
174. Melo, M.N., Ferre, R. and Castanho, M.A.R.B., Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations. Nat Rev Micro, (2009). **7**(3): p. 245-250.
175. Wimley, W.C., Describing the mechanism of antimicrobial peptide action with the interfacial activity model. ACS Chem Biol, (2010). **5**(10): p. 905-17.
176. Brogden, K.A., Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Micro, (2005). **3**(3): p. 238-50.
177. Milletti, F., Cell-penetrating peptides: classes, origin, and current landscape. Drug Discovery Today, (2012). **17**(15–16): p. 850-60.
178. Schmidt, N.W. and Wong, G.C.L., Antimicrobial peptides and induced membrane curvature: geometry, coordination chemistry, and molecular engineering. Current Opinion in Solid State and Materials Science, (2013). **17**(4): p. 151-63.
179. Bahnsen, J.S., Franzyk, H., Sandberg-Schaal, A. and Nielsen, H.M., Antimicrobial and cell-penetrating properties of penetratin analogs: Effect of sequence and secondary structure. Biochimica et Biophysica Acta (BBA) - Biomembranes, (2013). **1828**(2): p. 223-32.

180. Kristensen, M., Birch, D. and Mørck Nielsen, H., Applications and challenges for use of cell-penetrating peptides as delivery vectors for peptide and protein cargos. International Journal of Molecular Sciences, (2016). **17**(2): p. 185.
181. Hancock, R.E.W. and Lehrer, R., Cationic peptides: a new source of antibiotics. Trends in Biotechnology, (1998). **16**(2): p. 82-8.
182. Dathe, M. and Wieprecht, T., Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. Biochimica et Biophysica Acta (BBA) - Biomembranes, (1999). **1462**(1–2): p. 71-87.
183. Lam, S.J., O'Brien-Simpson, N.M., Pantarat, N., Sulistio, A., Wong, E.H.H., Chen, Y.-Y., *et al.*, Combating multidrug-resistant Gram-negative bacteria with structurally nanoengineered antimicrobial peptide polymers. Nature Microbiology, (2016). **1**: p. 16162.
184. Friedrich, C.L., Moyles, D., Beveridge, T.J. and Hancock, R.E., Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria. Antimicrob Agents Chemother, (2000). **44**(8): p. 2086-92.
185. Patenge, N., Pappesch, R., Krawack, F., Walda, C., Mraheil, M.A., Jacob, A., *et al.*, Inhibition of growth and gene expression by PNA-peptide conjugates in *Streptococcus pyogenes.* Mol Ther Nucleic Acids, (2013). **2**: p. e132.
186. Henriques, Sónia T., Melo, Manuel N. and Castanho, Miguel A R B., Cell-penetrating peptides and antimicrobial peptides: how different are they? Biochemical Journal, (2006). **399**(Pt 1): p. 1-7.
187. He, K., Ludtke, S.J., Huang, H.W. and Worcester, D.L., Antimicrobial peptide pores in membranes detected by neutron in-plane scattering. Biochemistry, (1995). **34**(48): p. 15614-15618.
188. Ladokhin, A.S., Selsted, M.E. and White, S.H., Sizing membrane pores in lipid vesicles by leakage of co-encapsulated markers: pore formation by melittin. Biophysical Journal, (1997). **72**(4): p. 1762-66.
189. Matsuzaki, K., Sugishita, K.-i., Ishibe, N., Ueha, M., Nakata, S., Miyajima, K., *et al.*, Relationship of membrane curvature to the formation of pores by magainin 2. Biochemistry, (1998). **37**(34): p. 11856-63.
190. Sani, M.A., Whitwell, T.C., Gehman, J.D., Robins-Browne, R.M., Pantarat, N., Attard, T.J., *et al.*, Maculatin 1.1 disrupts *Staphylococcus aureus* lipid membranes via a pore mechanism. Antimicrobial Agents and Chemotherapy, (2013). **57**(8): p. 3593-600.
191. Fernandez, D.I., Le Brun, A.P., Whitwell, T.C., Sani, M.-A., James, M. and Separovic, F., The antimicrobial peptide aurein 1.2 disrupts model membranes via the carpet mechanism. Physical Chemistry Chemical Physics, (2012). **14**(45): p. 15739-51.
192. Lockey, T.D. and Ourth, D.D., Formation of pores in *Escherichia coli* cell membranes by a cecropin isolated from hemolymph of *heliothis virescens* larvae. European Journal of Biochemistry, (1996). **236**(1): p. 263-71.
193. Silvestro, L., Gupta, K., Weiser, J.N. and Axelsen, P.H., The concentration-dependent membrane activity of cecropin A. Biochemistry, (1997). **36**(38): p. 11452-60.
194. Patrzykat, A., Friedrich, C.L., Zhang, L., Mendoza, V. and Hancock, R.E.W., Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli.* Antimicrobial Agents and Chemotherapy, (2002). **46**(3): p. 605-14.
195. Matsuzaki, K., Murase, O., Fujii, N. and Miyajima, K., Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore. Biochemistry, (1995). **34**(19): p. 6521-26.
196. Sani, M.-A. and Separovic, F., How membrane-active peptides get into lipid membranes. Accounts of Chemical Research, (2016). **49**(6): p. 1130-1138.

197. Vaara, M. and Porro, M., Group of peptides that act synergistically with hydrophobic antibiotics against Gram-negative enteric bacteria. Antimicrobial Agents and Chemotherapy, (1996). **40**(8): p. 1801-5.
198. Soofi, M.A. and Seleem, M.N., Targeting essential genes in Salmonella enterica Serovar Typhimurium with antisense peptide nucleic acid. Antimicrobial Agents and Chemotherapy, (2012). **56**(12): p. 6407-9.
199. Bai, H., Sang, G., You, Y., Xue, X., Zhou, Y., Hou, Z., *et al.*, Targeting RNA polymerase primary σ(70) as a therapeutic strategy against methicillin-resistant *Staphylococcus aureus* by antisense peptide nucleic acid. PLoS ONE, (2012). **7**(1): p. e29886.
200. Nekhotiaeva, N., Elmquist, A., Rajarao, G.K., Hallbrink, M., Langel, U. and Good, L., Cell entry and antimicrobial properties of eukaryotic cell-penetrating peptides. Faseb j, (2004). **18**(2): p. 394-6.
201. Hatamoto, M., Nakai, K., Ohashi, A. and Imachi, H., Sequence-specific bacterial growth inhibition by peptide nucleic acid targeted to the mRNA binding site of 16S rRNA. Appl Microbiol Biotechnol, (2009). **84**(6): p. 1161-8.
202. Green, M. and Loewenstein, P.M., Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell, (1988). **55**(6): p. 1179-88.
203. Frankel, A.D. and Pabo, C.O., Cellular uptake of the tat protein from human immunodeficiency virus. Cell, (1988). **55**(6): p. 1189-93.
204. Gregoriadis, G., The carrier potential of liposomes in biology and medicine (second of two parts). N Engl J Med, (1976). **295**(14): p. 765-70.
205. Gregoriadis, G., The carrier potential of liposomes in biology and medicine (first of two parts). N Engl J Med, (1976). **295**(13): p. 704-10.
206. Omri, A. and Ravaoarinoro, M., Preparation, properties and the effects of amikacin, netilmicin and tobramycin in free and liposomal formulations on Gram-negative and Gram-positive bacteria. Int J Antimicrob Agents, (1996). **7**(1): p. 9-14.
207. Schiffelers, R., Storm, G. and Bakker-Woudenberg, I., Liposome-encapsulated aminoglycosides in pre-clinical and clinical studies. Journal of Antimicrobial Chemotherapy, (2001). **48**(3): p. 333-44.
208. Salem, I.I. and Düzgünes, N., Efficacies of cyclodextrin-complexed and liposome-encapsulated clarithromycin against *Mycobacterium avium* complex infection in human macrophages. International Journal of Pharmaceutics, (2003). **250**(2): p. 403-14.
209. Drulis-Kawa, Z., Gubernator, J., Dorotkiewicz-Jach, A., Doroszkiewicz, W. and Kozubek, A., *In vitro* antimicrobial activity of liposomal meropenem against *Pseudomonas aeruginosa* strains. International Journal of Pharmaceutics, (2006). **315**(1–2): p. 59-66.
210. Mohammadi, G., Nokhodchi, A., Barzegar-Jalali, M., Lotfipour, F., Adibkia, K., Ehyaei, N., *et al.*, Physicochemical and anti-bacterial performance characterization of clarithromycin nanoparticles as colloidal drug delivery system. Colloids and Surfaces B: Biointerfaces, (2011). **88**(1): p. 39-44.
211. Kashi, T.S., Eskandarion, S., Esfandyari-Manesh, M., Marashi, S.M., Samadi, N., Fatemi, S.M., *et al.*, Improved drug loading and antibacterial activity of minocycline-loaded PLGA nanoparticles prepared by solid/oil/water ion pairing method. Int J Nanomedicine, (2012). **7**: p. 221-34.
212. Drulis-Kawa, Z., Dorotkiewicz-Jach, A., Gubernator, J., Gula, G., Bocer, T. and Doroszkiewicz, W., The interaction between *Pseudomonas aeruginosa* cells and cationic PC:Chol:DOTAP liposomal vesicles versus outer-membrane structure and envelope properties of bacterial cell. Int J Pharm, (2009). **367**(1-2): p. 211-9.

213. Wang, Z., Ma, Y., Khalil, H., Wang, R., Lu, T., Zhao, W., *et al.*, Fusion between fluid liposomes and intact bacteria: study of driving parameters and *in vitro* bactericidal efficacy. International Journal of Nanomedicine, (2016). **11**: p. 4025-4036.
214. Jahn, R., Lang, T. and Sudhof, T.C., Membrane fusion. Cell, (2003). **112**(4): p. 519-33.
215. Haque, M.E., McIntosh, T.J. and Lentz, B.R., Influence of lipid composition on physical properties and PEG-mediated fusion of curved and uncurved model membrane vesicles: "Nature's own" fusogenic lipid bilayer. Biochemistry, (2001). **40**(14): p. 4340-8.
216. Simões, S., Slepushkin, V., Düzgünes, N. and Pedroso de Lima, M.C., On the mechanisms of internalization and intracellular delivery mediated by pH-sensitive liposomes. Biochimica et Biophysica Acta (BBA) - Biomembranes, (2001). **1515**(1): p. 23-37.
217. Nicolosi, D., Scalia, M., Nicolosi, V.M. and Pignatello, R., Encapsulation in fusogenic liposomes broadens the spectrum of action of vancomycin against Gram-negative bacteria. Int J Antimicrob Agents, (2010). **35**(6): p. 553-8.
218. Ma, Y., Wang, Z., Zhao, W., Lu, T., Wang, R., Mei, Q., *et al.*, Enhanced bactericidal potency of nanoliposomes by modification of the fusion activity between liposomes and bacterium. Int J Nanomedicine, (2013). **8**: p. 2351-60.
219. Santos, R.S., Dakwar, G.R., Zagato, E., Brans, T., Figueiredo, C., Raemdonck, K., *et al.*, Intracellular delivery of oligonucleotides in *Helicobacter pylori* by fusogenic liposomes in the presence of gastric mucus. Biomaterials, (2017). **138**: p. 1-12.
220. Beaulac, C., Sachetelli, S. and Lagace, J., *In vitro* bactericidal efficacy of sub-MIC concentrations of liposome-encapsulated antibiotic against Gram-negative and Gram-positive bacteria. J Antimicrob Chemother, (1998). **41**(1): p. 35-41.
221. Beaulac, C., Sachetelli, S. and Lagace, J., *In vitro* bactericidal evaluation of a low phase transition temperature liposomal tobramycin formulation as a dry powder preparation against Gram negative and Gram positive bacteria. Journal of Liposome Research, (1999). **9**(3): p. 301-12.
222. Sachetelli, S., Khalil, H., Chen, T., Beaulac, C., Sénéchal, S. and Lagacé, J., Demonstration of a fusion mechanism between a fluid bactericidal liposomal formulation and bacterial cells. Biochimica et Biophysica Acta (BBA) - Biomembranes, (2000). **1463**(2): p. 254-66.
223. Rukavina, Z. and Vanić, Ž., Current trends in development of liposomes for targeting bacterial biofilms. Pharmaceutics, (2016). **8**(2): p. 18.
224. Anderson, M. and Omri, A., The effect of different lipid components on the *in vitro* stability and release kinetics of liposome formulations. Drug Deliv, (2004). **11**(1): p. 33-9.
225. Fillion, P., Desjardins, A., Sayasith, K. and Lagace, J., Encapsulation of DNA in negatively charged liposomes and inhibition of bacterial gene expression with fluid liposome-encapsulated antisense oligonucleotides. Biochim Biophys Acta, (2001). **1515**(1): p. 44-54.
226. Halwani, M., Mugabe, C., Azghani, A.O., Lafrenie, R.M., Kumar, A. and Omri, A., Bactericidal efficacy of liposomal aminoglycosides against *Burkholderia cenocepacia.* Journal of Antimicrobial Chemotherapy, (2007). **60**(4): p. 760-9.
227. Mugabe, C., Halwani, M., Azghani, A.O., Lafrenie, R.M. and Omri, A., Mechanism of enhanced activity of liposome-entrapped aminoglycosides against resistant strains of *Pseudomonas aeruginosa.* Antimicrob Agents Chemother, (2006). **50**(6): p. 2016-22.
228. Desjardins, A., Chen, T., Khalil, H., Sayasith, K. and Lagace, J., Differential behaviour of fluid liposomes toward mammalian epithelial cells and bacteria: restriction of fusion to bacteria. J Drug Target, (2002). **10**(1): p. 47-54.

229. Meng, J., Wang, H., Hou, Z., Chen, T., Fu, J., Ma, X., *et al.*, Novel anion liposome-encapsulated antisense oligonucleotide restores susceptibility of methicillin-resistant *Staphylococcus aureus* and rescues mice from lethal sepsis by targeting mecA. Antimicrob Agents Chemother, (2009). **53**(7): p. 2871-8.
230. Furneri, P.M., Fresta, M., Puglisi, G. and Tempera, G., Ofloxacin-loaded liposomes: *In vitro* activity and drug accumulation in bacteria. Antimicrobial Agents and Chemotherapy, (2000). **44**(9): p. 2458-64.
231. Yang, K., Gitter, B., Ruger, R., Albrecht, V., Wieland, G.D. and Fahr, A., Wheat germ agglutinin modified liposomes for the photodynamic inactivation of bacteria. Photochem Photobiol, (2012). **88**(3): p. 548-56.
232. Yang, L., Harroun, T.A., Weiss, T.M., Ding, L. and Huang, H.W., Barrel-stave model or toroidal model? A case study on melittin pores. Biophysical Journal, (2001). **81**(3): p. 1475-85.
233. Biteen, J.S. and Moerner, W.E., Single-molecule and superresolution imaging in live bacteria cells. Cold Spring Harb Perspect Biol, (2010). **2**(3): p. a000448.
234. Karunatilaka, K.S., Cameron, E.A., Martens, E.C., Koropatkin, N.M. and Biteen, J.S., Superresolution imaging captures carbohydrate utilization dynamics in human gut symbionts. MBio, (2014). **5**(6): p. e02172.
235. Turnbull, L., Toyofuku, M., Hynen, A.L., Kurosawa, M., Pessi, G., Petty, N.K., *et al.*, Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. Nat Commun, (2016). **7**: p. 11220.