Penetrating the bacterial biofilm: challenges for antimicrobial treatment

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1. Introduction

1.1 Antimicrobial treatment: in desperate need of a wind of change

"Without urgent, coordinated action by many stakeholders, the world is headed for a post-antibiotic era, in which common infections and minor injuries which have been treatable for decades can once again kill" (1)

Dr Keiji Fukuda, WHO’s Assistant Director-General, Health Security.

The increasing antibiotic resistance against commonly used antimicrobial agents is a worldwide threat, and has reached alarming levels. Multidrug resistant bacteria cause the death of 25,000 Europeans each year, with an estimated burden on the healthcare system of € 1.5 billion annually (2). According to a recent study published by the Centre for Disease Control (CDC) nearly half a million people in the United States are infected annually with the life-threatening bacterium Clostridium difficile. Among these, at least 29,000 people died as a consequence of the infection (3). Despite the obvious urgent need for new antibiotics, the number of approvals is low (4). As the impact on public health and economics is tremendous, the World Health Organization (WHO) has made bacterial resistance one of its central focuses. Also, the WHO warned in its last global report that the 21\textsuperscript{th} century can truly be the beginning of the ‘post-antibiotic era’, a period in which people worldwide can die from common infections, because the available antibiotic treatments become ineffective, including the so-called “last resort” antibiotics (1). Obtaining a better understanding of the resistance mechanisms and the concomitant development of improved treatments are key for the future, as acknowledged in the May 2015 World Health Assembly action plan that attempts to manage the crisis globally (Box 1.1) (5).

<table>
<thead>
<tr>
<th>Box 1.1: The WHO’s action plan to combat antimicrobial resistance (5)</th>
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<td>The goal of the WHO’s global action plan is on the one hand to prolong antibiotic treatments that are still successful in eradicating resistant bacteria, and on the other hand to decrease the number of emerging/existing resistant infections worldwide. Their strategy is essentially based on 5 cornerstones:</td>
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<tr>
<td><strong>1. More communication with different sectors (including healthcare, animal science, political authorities...) in order to increase awareness of the resistance problem</strong></td>
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<td><strong>2. Increase academic, industrial and economic research to maximize the knowledge about the incidence of antimicrobial resistance, related microorganisms, mechanisms of resistance development, effectiveness of existing and newly approaching treatments, cost of antimicrobial resistance...</strong></td>
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<td><strong>3. Reduce the amount of infections</strong></td>
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<td><strong>4. Limit misuse of antimicrobial agents</strong></td>
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<td><strong>5. Maximize the economic support to drug development, health care institutions, vaccine supply...</strong></td>
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1.2 The biofilm lifestyle

Intensive research has been conducted to obtain a better understanding of how bacteria become multiresistant to a range of antibiotics, and how this results in chronic, difficult to eradicate infections. *In vitro* and *in vivo* observations of persistent infections showed that bacteria are often organized as bacterial aggregates or biofilms, which contributes to a great extent to the problem of persistent infections (6). In these biofilms, the bacterial cells are packed together into dense large clusters, surrounded by a self-produced matrix of extracellular polymeric substances (EPS), including proteins, DNA and polysaccharides (7). These so-called sessile bacteria can communicate with each other in a highly organized fashion, which enables them to respond and adapt instantly to changing environmental conditions (8). Furthermore, the organized architecture of biofilms also acts as a protective barrier that protects biofilm bacteria from the host immune response (9).

One of the typical properties of biofilms is their increased tolerance to antibiotic agents. They can withstand 10-1000 times higher antibiotic concentrations compared to their free-floating planktonic counterparts (10). This raises the question on how to combat these bacterial biofilm-related infections. In order to find a solution to this pressing problem, one must unravel the precise mechanisms behind the increased biofilm tolerance. Distinction can be made between 4 distinct, but intertwined processes that lead to increased tolerance (10–12):

1. The existence of different microenvironments within a biofilm, such as pockets of anaerobic regions or low pH due to local accumulation of acidic waste products, that may antagonize the action of antibiotics (13).
2. The presence of a subpopulation of difficult to eradicate persister cells.
3. The ability to counteract oxidative stress.
4. The slow or incomplete penetration of antibiotics through biofilms.

In this chapter we will focus on the last tolerance mechanism. Limited mass transfer through biofilms is a recognized mechanism of tolerance and is caused by two main reasons. First, due to the fact that the bacterial cells are packed together in large dense cell clusters composed of hundreds or thousands of bacteria, they are more difficult to reach by antibiotics as compared to free-floating planktonic cells (14),(15). Second, the cells and cell clusters are surrounded by a sticky matrix, sometimes referred to as the "house of biofilm cells" (16). The biofilm matrix is a conglomeration of polysaccharides, proteins and extracellular DNA, providing strength and protection to the biofilm (17). Improving antibiotics penetration through biofilms is an important strategy to achieve a better treatment efficacy of both existing as well as potentially new antibiotics (Box 1.1).

In this chapter, we will first look more closely into the structural organization of the bacterial biofilm in relation to hindered antibiotics penetration (Figure 1.1). Special attention will be given to the role of dense cell clusters, the sticky biofilm matrix and the presence of degrading enzymes (11,12,18). Next, we will explain different therapeutic approaches to improve antibiotics penetration through bacterial biofilms. Both physical methods to destruct the biofilm's organization as the use of antibiotic-enclosing nanoparticles will be discussed. At the end of the chapter, the reader will have gained more insight into the different challenges that
antibiotics encounter while diffusing through bacterial biofilms, and will have become more acquainted with different strategies that are currently explored to overcome this problem.

Insert Figure 1.1 here
2. How biofilms pose a challenge to the diffusion of antibiotics

2.1 Mass transport phenomena in biofilms

At first, biofilms were considered as simple systems of 4 planar layers lying on top of each other. These 4 layers were, from the bottom to the top, the substratum, the biofilm, the bulk liquid and a possible gas space (19). It was accepted that passive diffusion was the predominant mode of transport within biofilms. Only in the liquid bulk layer residing on top of the biofilm, convective flow could happen (20,21). With the introduction of confocal laser scanning microscopy (CLSM), this view on the structural organization of biofilms changed radically (22,23). Confocal microscopy enabled 3-D high-resolution imaging of biofilms (24), revealing their complex heterogeneous architectures (25). The bacterial cells were found to be organized in dense clusters of hundreds to even thousands of cells surrounded by a matrix of polymeric material that holds the cell aggregates together and contributes to the strength of the entire biofilm (9). CLSM also revealed that biofilms contain voids - open channels that run through the biofilm and are connected to the bulk liquid (26). Microscopic evaluation confirmed the possibility of liquid flow through these channels (27). In cell clusters, on the other hand, liquid flow was hindered, and became stagnant (27). Hence, two modes of mass transport are active inside biofilms. Inside the voids, both convection and diffusion are possible, whereas in the cell clusters diffusion is the principal mass transport mechanism (28). This was an important discovery, as the voids can transfer solutes quickly throughout the biofilm, but do not give direct access to the inner parts of the cell clusters (29).

Because of the heterogeneity of biofilms and different penetration pathways (via voids and dense cell clusters), contradicting findings were published in literature regarding the existence of a diffusion barrier. Indeed, many studies state that antibiotics move fairly rapidly through biofilms (30–32), however, at the same time, also hindered penetration of antibiotics has been frequently observed, as described above (33–39). Confusion about the existence of a diffusion barrier is confounded by the way it is experimentally investigated. If the arrival of antibiotics at the base of the biofilm is taken as a measure for diffusion (29), often one will conclude that there is no diffusion barrier since antibiotics can reach those parts fairly easily through the large voids. However, this does not necessarily mean that the antibiotics have efficiently penetrated deep into the cell clusters (10). Another important issue is that it may not be sufficient to measure the arrival of antibiotics at a certain location in the biofilm. Also the rate of diffusion is an important aspect. It is possible that an antibiotic molecule is indeed transported through the biofilm, but that its rate of penetration is significantly reduced. When the sessile cells experience gradually increasing antibiotic concentrations instead of an immediate full antibiotic dose, they can have the time to mount a defensive response (40,41). Increased production of alginate has been reported by treating Pseudomonas aeruginosa biofilms with sub-inhibitory imipenem concentrations, hence resulting in a more viscous biofilm matrix (42). Sub-inhibitory concentrations of β-lactams increased colonic acid production in Escherichia coli biofilms, resulting in a more matured biofilm matrix (43).

In conclusion, although limitation of mass transfer remains controversial, the controversy is inspired by differences in measurements, the complex structure of biofilms and the fact that it depends on the specific combination of biofilms and antibiotics (44). One can
safely conclude that a diffusion barrier can exist as evidenced by various studies for many antibiotics.

2.2 Dense cell clusters

To reach the cells in the deeper layers of the clusters, the antibiotics have to gradually diffuse through the narrow spaces in between the cells. A first question is whether there is a size limit for diffusion into the dense cell clusters.

De Beer et al. examined the diffusion of three fluorescent dyes with different molecular weights in mixed biofilms of Klebsiella pneumoniae, Pseudomonas aeruginosa and Pseudomonas fluorescens (39,45). After microinjecting the dyes in the interstitial voids and cell clusters separately, fluorophore diffusion was analyzed. By evaluating how fast fluorophores can diffuse out of the initial spot, the local diffusion coefficient of the molecules could be measured in selected areas – rather than reporting an average diffusion coefficient for the entire biofilm. Fluorescein (MW 332) exhibited similar diffusion in the voids and in the cell clusters, whereas dyes of higher molecular weight such as TRITC-IgG (MW 150 000) and phycoerythrin (MW 240 000) did not diffuse or were slowed down by 40% in the cell clusters, respectively. However, likely these findings are not caused by steric hindrance, but rather by binding interactions with biofilm constituents as penetration of TRITC-IgG was improved by adding bovine serum albumin (39,45). Indeed, in a recent study it was confirmed that nanoparticles up to around 100 nm can penetrate into dense biofilm clusters of Burkholderia multivorans and Pseudomonas aeruginosa virtually as efficient as small dextrans of 4 kDa (33). The authors used inert PEGylated polystyrene nanoparticles and liposomes ranging from 40 to 550 nm, which were visualized in their journey towards the biofilm cell clusters by confocal microscopy (Figure 2.1). A general consensus about the optimal size of nanoparticles was obtained: particles smaller than 100-130 nm are able to diffuse in the cell clusters, while penetration into the clusters was gradually less efficient for larger particles.

This study confirms that the size of antibiotics is not likely to play a significant role in the diffusion into dense cell clusters (33). What does restrict net antibiotics influx into dense clusters is the fact that most of the cluster volume is occupied by the bacterial cells, while the space in between the cells through which the antibiotics have to diffuse constitutes only a minor volume fraction (Figure 2.2). This evidently limits the amount of antibiotics present within the clusters at any time, potentially leading to a suboptimal dose and ineffective treatment. In addition, as the antibiotics have to diffuse in between densely packed cells, the effective path length towards the cluster center is increased (29), further contributing to a delayed exposure of the deeper cell layers, which have more time to mount a defensive response.
2.3 Binding interactions between antibiotics and matrix constituents

The second important characteristic of biofilms that contributes to their decreased diffusivity is their heterogeneous matrix that surrounds the cells and cell clusters. Antibiotic penetration in the biofilm can be substantially delayed or even inhibited by (transient) binding to matrix constituents (46). In addition, antibiotics may become inactivated by enzymatic degradation or modification.

2.3.1 Physicochemical binding to matrix polymers

It has been demonstrated in many studies that hindered diffusion can be the result of electrostatic interaction between antibiotic molecules and matrix polymers of opposite surface charge (46). For example, aminoglycosides such as tobramycin and gentamicin penetrate biofilms of mucoid Pseudomonas aeruginosa slower than β-lactam antibiotics (Figure 2.2). This is caused by the fact that the aminoglycosides, unlike β-lactams, can bind to extracellular polymers, such as alginate (34–37). Alginate is produced by mucoid Pseudomonas aeruginosa strains and is implicated in bacterial adhesion to substrates such as the respiratory epithelium (47). It is a copolymer consisting of (1,4)-linked-β-D-mannuronate and α-L-guluronate monomers which confers a negative charge to the polysaccharides (Figure 2.3) (48). Positively charged antibiotics, such as aminoglycosides, can bind to negatively charged alginate polymers by means of an electrostatic interaction.

Insert Figure 2.3 here

Recently, it has been found that not only mucoid strains offer protection against antibiotic penetration, but also the matrix in non-mucoid strains contains elements that retard antibiotic diffusion. Tseng et al. clearly demonstrated that, unlike the neutral fluoroquinolone ciprofloxacin, positively charged tobramycin was sequestered at the outer surface of non-mucoid Pseudomonas aeruginosa biofilms. Therefore, it could not fully penetrate the biofilm anymore. To acquire information about which component of the matrix accounts for the tobramycin sequestration, different mutants of known EPS matrix components were examined. However, no polymers could be assigned as sequestering agents for tobramycin, because the penetration of tobramycin was similar in all biofilms. Further experiments showed that the tobramycin binding sites in the biofilm matrix could be saturated by adding high concentrations of either tobramycin or divalent cations. In both cases, the penetration of tobramycin was substantially increased, because ionic interactions with the matrix components were no longer possible (49). Billing et al. found that Psl, a known matrix component of non-mucoid Pseudomonas aeruginosa biofilms, was responsible for sequestering tobramycin. Psl was able to interact electrostatically with tobramycin, in this way contributing to the matrix barrier function of Pseudomonas aeruginosa biofilms (50,51). Finally, also Pel, the third known extracellular polysaccharide involved in Pseudomonas aeruginosa biofilm development - besides alginate and Psl –, has been reported to play a role in protecting Pseudomonas aeruginosa biofilms against aminoglycosides (52).

Hindered penetration in biofilms has also been described for biocides (53). Chlorine, a biocide, is known to suffer from impaired penetration in biofilms (38). This was confirmed by
direct measurement of chlorine concentrations with microelectrodes in biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*; the chlorine concentration inside the clusters was not more than 20% of the incubation solution (38). A follow-up study revealed that biofilm constituents could trap chlorine in the periphery of the biofilm before it could diffuse inside the biofilm (54). Davison *et al.* measured that the time chlorine needed to fully penetrate a *Staphylococcus epidermidis* cluster was about 30 min, while it would take ± 3 s to diffuse if sorption did not occur (600x slower) (55).

2.3.2 Inactivation of antibiotics by enzymes present in the biofilm matrix

Degradative or modifying enzymes that are present in the biofilm matrix constitute yet another barrier to antibiotics in biofilms (12,46,56).

Anderl *et al.* showed that the inability of ampicillin to eradicate wild-type *K. pneumoniae* biofilms was caused by enzymes in the biofilm surface layer degrading ampicillin before reaching the cells (Figure 2.4) (57). In β-lactamase deficient strains, ampicillin readily penetrated the biofilms, which supports the hypothesis that the penetration limitation is due to a reaction-diffusion barrier.

*Insert Figure 2.4 here*

Besides antibiotics, also biocides can be enzymatically inactivated in biofilms (53,58,59). Studies concerning the penetration of hydrogen peroxide into *Pseudomonas aeruginosa* biofilms revealed that penetration was largely retarded, likely by catalases (60). Catalases are enzymes that defend cells against oxidative stress by converting hydrogen peroxide into water and oxygen molecules ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$) (61). To test this assumption, $\text{H}_2\text{O}_2$ penetration was assessed in wild-type and catalase-deficient *Pseudomonas aeruginosa* biofilms. In the wild-type biofilms, $\text{H}_2\text{O}_2$ failed to fully penetrate the biofilm mass, as opposed to the catalase-deficient mutant biofilms, where $\text{H}_2\text{O}_2$ succeeded in penetrating the biofilm (60). Hence, catalase plays a role in protecting biofilm bacteria by decomposing $\text{H}_2\text{O}_2$ into its degradation products $\text{H}_2\text{O}$ and $\text{O}_2$ in a reaction-diffusion mechanism (62).
In the previous section we have discussed the two factors that contribute to hindered diffusion of antibiotics in biofilms, i.e. reduced penetration in dense cell clusters and binding to matrix constituents. Consequently, improved antibiotics penetration could be achieved by either releasing the cells from their protective environment (63,64), or by shielding the antibiotics from interacting with matrix constituents (65,66). In the next sections we will discuss the various approaches that have been developed to this end.

3. How to improve antibiotic delivery to biofilm cells?

3.1 Interference with the biofilm structure

In order for antibiotics to more easily reach all cells in biofilms, cells could be released from their protective environment by interfering with the biofilm structure (35,67–72). Indeed, it has been observed in many studies that by degrading the biofilm structure, bacteria become more susceptible to antimicrobial treatment (71). An additional benefit is that releasing bacteria from their protective environment can also enhance recognition by polymorphonuclear leukocytes, aiding the immune system to clear the bacterial biofilm infection (73). Furthermore, degradation of the biofilm does not only increase antibiotic diffusion, but also the diffusion of nutrients, which activates the bacterial cells and renders them more susceptible for antibiotics (74).

Interference with the biofilm structure can be achieved by specific compounds that degrade the biofilm matrix, for instance matrix dispersants that destabilize EPS or specific biofilm components such as eDNA (63,72). Messiaen et al. investigated the potential of NaClO and dispersin B to degrade the EPS matrix and the specific matrix component poly-β-1,6-N-acetylglucosamine (PNAG) of Burkholderia cepacia biofilms, respectively (75). Destabilization of both compounds resulted in increased tobramycin susceptibility, confirming the fact that the EPS matrix plays a role in the tobramycin susceptibility of Burkholderia cepacia biofilms (75). Hatch et al. demonstrated the potential of alginate lyase in reducing the diffusion barrier of mucoid Pseudomonas aeruginosa biofilms to aminoglycosides (47). Alginate lyase enzymatically cleaves the glycosidic bounds of alginate by β-elimination (76). Degradation of the biofilm matrix by alginate lyase increased the diffusion of aminoglycosides into mucoid Pseudomonas aeruginosa biofilms and improved the aminoglycoside activity (47).

Another way to interfere with biofilm formation is to target the bacterial communication system, called quorum sensing (QS). QS is the process in which bacteria release signaling molecules in a cell-density dependent manner, leading to altered gene expression. QS regulates different bacterial processes such as virulence, motility, biofilm formation, etc… (77,78). Although more research needs to be performed to elucidate the precise role of QS in biofilm formation (79,80), many studies reported that quorum sensing inhibitors (QSI) can be a promising new class of antimicrobial agents that affect the biofilm’s structural organization (81,82). Davies et al. demonstrated that lasI mutants of Pseudomonas aeruginosa formed flat biofilms instead of the complex bacterial communities in case of wild-type biofilms. Moreover, the quorum-sensing mutants were also more susceptible to sodium dodecyl sulfate, demonstrating the potential of QSI’s in increasing the susceptibility of biofilms (83). Similar results were reported for Burkholderia cenocepacia (84), where QS-deficient mutants showed an
altered biofilm formation and increased sensitivity to sodium dodecyl sulfate (84). Brackman et al. investigated the potential of combining different antibiotics and QSI’s to affect the susceptibility of medically relevant biofilms. Both QSI’s targeting the acylhomoserine lactone-based QS-system of Pseudomonas aeruginosa and Burkholderia cepacia, as the peptide-based system of Staphylococcus aureus, were investigated. They concluded that treating biofilms with QSI’s is promising for the eradication of bacterial biofilm infections, as the combined use of QSI’s and antibiotics resulted in increased killing efficiency (85).

The disadvantage of using pharmacological compounds is that their specificity restricts broad applicability (86). Indeed, the biofilm matrix composition differs between various bacterial species and strains (87,88), and the presence of polymicrobial biofilms further increases the matrix heterogeneity (86,89). Physical approaches, on the other hand, that interfere with the biofilm structure could be more generally applicable.

Many studies investigated the potential of ultrasonic waves to mechanically disrupt biofilms and consequently enhance antibiotic efficacy (90–94). Williams et al. showed that ultrasonic treatment of mucoid strains of Pseudomonas aeruginosa resulted in a significant decrease in alginate viscosity. Degradation of the mucoid polymer markedly increased the diffusion of tobramycin and piperacillin and consequently their efficacy (95). Another example is the use of low-frequency ultrasound to disrupt biofilms of Escherichia coli, which consequently enhances the efficacy of gentamycin in the eradication of the biofilm (96). Also, Dong et al. demonstrated the advantage of applying ultrasound (and ultrasound mediated microbubbles) in the treatment of Staphylococcus epidermidis biofilms, as ultrasound improved the penetration of vancomycin significantly (91). Furthermore, promising in vivo data were reported regarding the use of ultrasound for disrupting biofilm structures, such as in the treatment for chronic rhinosinusitis (97). Also, different in vivo studies reported that ultrasound could be used to disrupt biofilms of Escherichia coli, resulting in enhanced efficacy of aminoglycosides (98–100). Carmen et al. assessed the capability of low-frequency ultrasound to enhance the activity of vancomycin on the gram-positive bacterium Staphylococcus epidermidis. Biofilms were formed on polyethylene disks and implanted subcutaneously in rabbits. The combination of low-frequency ultrasound and systemic vancomycin treatment reduced the number of viable bacteria, without causing detrimental tissue damage (101).

Another example is the use of laser-generated shockwaves to physically disrupt biofilms. Efficient Staphylococcus epidermidis biofilm disruption was reported with the use of a Q-switched ND:YAG laser (70). Nigri et al. demonstrated that the combination of laser-generated shockwaves with tobramycin could be used for efficient eradication of biofilms related to vascular prosthetic grafts. Shockwaves were generated in biofilms consisting of Staphylococcus epidermidis and Staphylococcus aureus, both isolated from vascular grafts. Subsequent tobramycin treatment resulted in a significant decrease in colony forming units, pointing out the potential of shockwaves to disrupt biofilm structures and to enhance the antibiotic’s efficiency (102).

In conclusion, physical methods such as ultrasonic treatment have been successfully used to disrupt biofilms and release bacteria from their protective environment. Hence, the combination of physical biofilm disruption and concurrent antibiotic treatment is a promising route to explore in order to eradicate persistent biofilm infections (73,103).
3.2 Nanocarriers for improved delivery of antibiotics in biofilms

3.2.1 Nanocarriers protect antibiotics from interactions with biofilm components

Encapsulation of antibiotics inside nanomaterials reduces physicochemical interactions between biofilm matrix components and antimicrobial molecules (18,66,104–107). Hence, nanosized carriers can protect antibiotics from detrimental interactions with biofilm components (33). Meers et al. investigated if encapsulation of amikacin in DPPC:Chol liposomes could be beneficial in the treatment of chronic *Pseudomonas aeruginosa* biofilm infections. By CLSM it could be shown that those liposomes were able to penetrate *Pseudomonas aeruginosa* biofilms, resulting in a higher antibiotics concentration near the center of the biofilm as compared to the bulk fluid. Consequently, liposomal amikacin could reduce the bacterial count of *Pseudomonas aeruginosa* infections more than free amikacin (108,109). Mugabe et al. compared the activity of liposome-encapsulated gentamicin and free gentamicin against resistant strains of *Pseudomonas aeruginosa* isolates. DMPC:Chol, DPPC:Chol and DSPC:Chol liposomes encapsulating gentamicin exhibited a higher antimicrobial activity against *Pseudomonas aeruginosa* biofilms than gentamicin by itself. The authors attributed this effect to either an enhanced diffusion of the enclosed antibiotics through the biofilms, or protection provided by the liposomes against enzymatic degradation (110).

Nanocarriers also offer protection against antibiotic degradation by detrimental enzymes that can be present in the biofilm matrix (65). Nacucchio et al. investigated if shielding piperacillin inside liposomes could offer protection against degradation by β-lactamases leading to improved bacterial efficacy. Therefore, both piperacillin containing phosphatidylcholine:cholesterol (1:1) liposomes and exogenous β-lactamase were added to *Staphylococcus aureus* biofilms and staphylococcal growth was examined. Non-encapsulated piperacillin was hydrolyzed by exogenous β-lactamases and resulted in similar *Staphylococcus aureus* growth as the control. Attaching piperacillin to the outside of (empty) liposomes provided protection against β-lactamases, as lower viability of *Staphylococcus aureus* was observed. This is thought to be due to steric hindrance for the enzymes. However, most successful protection was provided when piperacillin was encapsulated in the interior of the liposomes, resulting in the lowest staphylococcal growth of all conditions (111).

Besides liposomal entrapment, encapsulation in polyacrylate nanoparticles has also been reported to protect antibiotics against enzymatic degradation. Turos et al. showed that nanoparticles loaded with penicillin exhibited increased antimicrobial activity against MRSA infections compared to free penicillin. Antimicrobial activity was even restored when exogenous β-lactamases were added, proving the fact that polymeric encapsulation can hide antibiotics against enzymatic attack by β-lactamases (112).

3.2.2 Nanocarriers can be designed to increase local antibiotic delivery

Besides size, surface charge and hydrophobicity are important for efficient delivery of NPs inside biofilms (113,114). For instance, Li et al. showed that anionic and neutral quantum dots could not penetrate *E. coli* biofilms, while cationic ones could (115). Furthermore, the authors demonstrated that hydrophobic cationic quantum dots could reach sessile cells, while more hydrophilic cationic quantum dots were bound to EPS matrix components. This shows
that, by rationally designing the NP outer surface through modifications of charge and hydrophobicity, nanoparticle-enclosed drugs can be targeted towards the EPS matrix (e.g., dispersing agents such as dispersin B) or bacterial aggregates (e.g., antimicrobial agents such as vancomycin) (115).

By making use of charge-based attraction of NPs to biofilm constituents, nanometer-sized delivery vehicles can reach parts in biofilms that are inaccessible to free antibiotics (114). Duncan et al. combined encapsulated peppermint oil and cinnamaldehyde with cationic silica NPs to treat bacterial biofilm infections (116). Self-assembly of the silica NPs at the oil-water phase resulted in capsules consisting of a core of peppermint oil and cinnamaldehyde and a shell of cationic NPs. Due to the penetration properties of the positive silica NPs, the capsules were able to diffuse into the entire biofilm, as visualized by confocal microscopy. Antimicrobial activity of the capsules was assessed against full-grown biofilms of Escherichia coli, Pseudomonas aeruginosa, methicillin-resistant Staphylococcus aureus and Enterobacter cloacae. It was found that viability significantly decreased with capsules carrying phytochemicals compared to free phytochemicals. Hence, transporting antimicrobial agents by nanometer-sized delivery vehicles directly to the biofilm-enclosed bacteria, greatly improved their therapeutic efficiency (116).

Inclusion of certain lipids such as phosphatidylinositol (PI) or stearylamine (SA) can also be used to achieve interaction between phospholipid liposomes and specific compounds in the biofilm (117). The targeting potential of PI has been demonstrated to a variety of biofilm-enclosed bacteria such as Streptococcus mutans, Streptococcus gordonii, Streptococcus sanguis, Proteus vulgaris, Staphylococcus epidermidis... (118–120). Attractive interactions are accomplished by forming hydrogen bonds between hydroxyl groups of inositol-molecules and polymers present on the bacterial surface (121). Stearylamine, on the other hand, can be used to achieve electrostatic interactions between positively charged SA-bearing liposomes and negative charges present in the bacterial biofilm (122). Stearylamine is known to guide liposomes to sessile bacteria such as Staphylococcus epidermidis, Staphylococcus aureus, and in a lower extent to Proteus vulgaris and oral bacteria such as Streptococcus mutans and Streptococcus sanguis (123,124). When comparing the affinity of PI:DPPC liposomes and DPPC-cholesterol-SA liposomes to Staphylococcus epidermidis biofilms, SA-liposomes possessed greater affinity for the bacteria (125). A possible explanation could be that hydrogen bonds originating from PI-biofilm interactions are less efficient than the electrostatic interactions between SA-liposomes and negatively charged biofilm constituents (65).

The possible disadvantage of using charge-based or hydrophobic interactions is the lower specificity, as most bacteria have a negative cell wall, accessible for electrostatic interactions, and matrix components, able to interact through hydrogen bonds (126). Indeed, to avoid detrimental side-effects, it is essential that antibiotics are directed to the pathogenic bacterial species (and/or strains), while they do not exert any bacteriostatic/bactericide effect on the commensal bacteria (127). Precise targeting of NPs to specific bacterial species and strains can be accomplished by functionalizing nanocarriers with targeting agents such as antibodies or lectins which interact with EPS and/or sessile bacteria (114). Highly specific targeting to Streptococcus oralis was accomplished by decorating DPPC:PI liposomes with antibodies raised to surface antigens of S. oralis. The immunoliposomes possessed high specificity to S. oralis, as little liposome adsorption was noticed around other oral bacteria like Streptococcus sanguis. For low chlorhexidine concentrations, targeted liposomal delivery
resulted in enhanced growth inhibition of *S. oralis* in comparison with non-encapsulated bactericides (128,129). Other site-directing molecules used to target NPs to bacterial biofilms include carbohydrate-binding lectins such as concanavalin A (con-A) and wheat germ agglutinin (WGA) (130,131). Con-A selectively targets α-D-mannopyranosyl and α-D-glucopyranosyl units, while WGA has a high specificity for N-acetylneuraminic acid and N-acetylg glucosamine, present in peptidoglycan layer of bacterial cell walls and some lipopolysaccharides (132,133). Effective targeting to the carbohydrate receptors of *Helicobacter pylori* could be obtained by functionalizing gliadin nanoparticles with lectins, more specifically fucose-specific (UEA-I) and mannose-specific (Con A) lectins (134). WGA functionalized DPPC:DOTAP:NHS-PEG<sub>2000</sub>-DSPE:mPEG<sub>2000</sub>-DSPE liposomes delivered more temoporfin to MRSA biofilms than unmodified liposomes or free temoporfin and resulted in complete eradication of the MRSA infection (133), highlighting the potential of targeted NPs to increase the antibiotic’s bioavailability and hence efficacy to bacterial biofilms.

### 3.2.3 Nanocarriers can shuttle antibiotics inside bacterial cells

Apart from a better delivery of antibiotics deep into biofilms, nanomaterials can also aid in improved delivery of antibiotics into cells. Gram-negative (and to a lower extent gram-positive) bacterial cells are protected with multi-component barrier systems, restricting the permeation of antimicrobials (135,136). As the antibiotic targets are often located inside the cell (cytosol or cytoplasmic membrane) (137), cellular impermeability seriously decreases antibiotics efficiency (135,136). Therefore, nanocarriers – especially liposomes - that are designed to interact with cellular membranes, can be of paramount importance to increase antibiotic interaction with bacterial cells (138).

The advantage of using liposomes as carriers for antibiotics, is that they are able to fuse with bacterial membranes, because the liposomal wall consists of amphiphatic phospholipids that resemble the cellular membrane. A particular class of liposomes, the so-called ‘fluidosomes’, deserve extra attention in this regard, as they have an enhanced capability to interact and fuse with phospholipid membranes (138). Fluidosomes consist of lipids, for example 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (139), that enhance the fluidity of nanocarriers. Mugabe *et al.* reported that liposomal entrapment of gentamicin increased the susceptibility of clinical isolates of *Pseudomonas aeruginosa* towards this antibiotic (110). Further investigation revealed that those fluidosomes guided the encapsulated aminoglycoside directly through the bacterial envelope, thereby avoiding the problem of restricted penetration through porin channels (140). Transmission Electron Microscopy images revealed a close interaction between DPPC:Chol liposomes encapsulating aminoglycosides such as amikacin, gentamicin or tobramycin and the cell membrane of *Pseudomonas aeruginosa*. Liposome-bacterial membrane fusion was further evidenced by flow cytometry and lipid mixing studies (138,140).

Halwani *et al.* investigated the potential of fluidosomal encapsulation of different aminoglycosides in the treatment of *Burholderia cenocepacia* infections. Again, the same event was observed: fluidosomes were able to transfer aminoglycosides through the cell membrane of *Burkholderia cenocepacia*, thereby greatly increasing the efficiency of the antibiotics (141).

**Insert Figure 3.1 here**

Beaulac *et al.* investigated the suitability of fluidosomes against a wide range of
pathogens including *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Escherichia coli* and *Staphylococcus aureus* (142). Liposomes consisting of dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylglycerol (DMPG) were loaded with subinhibitory-concentrations of tobramycin and, surprisingly, possessed strong bactericidal activity against all strains; the same but non-encapsulated amount of tobramycin (only 50% of the tobramycin MIC) could not exert this effect (142). It was shown by negative staining that fluidosomes interacted closely with the bacterial outer membrane (Figure 3.1). Immuno-electron microscopy images showed that more tobramycin could penetrate the bacterial cells in case of fluidosomal entrapment than in case of free tobramycin. Integration of fluidosomal phospholipids in bacterial membranes could be confirmed by Fluorescence Activated Cell Sorting. After fusion, the fluorescent lipophilic marker PKH-2GL was detected in bacterial cells, proving that fluidosomal phospholipids were integrated in bacterial walls (143).

*In vivo* experiments confirmed the potential of fluidosomes in the treatment of bacterial infections. DPPC:DMPG liposomes containing tobramycin were tested in an *in vivo* rat model by infecting the rats with a mucoid strain of *Pseudomonas aeruginosa*. The fluidity of the liposomal wall was important, as only fluid DPPC:DMPG liposomes (transition phase temperature around 30°C) eradicated the pulmonary mucoid *Pseudomonas aeruginosa* infection. Both tobramycin loaded rigid liposomes (transition phase temperature around 40°C), as free tobramycin, were not effective in treating the *Pseudomonas aeruginosa* infection, highlighting the importance of the liposomal wall fluidity for optimal fusion with bacterial membranes (144).

### 3.2.4 Responsive nanoparticles: antibiotic release on demand

As mentioned in the previous section, nanocarriers can be designed to shuttle antibiotics directly into the bacterial cells, thus greatly improving their cytosolic delivery. In other cases, it is more favourable to deliver agents in the proximity of the bacterial cell, rather than intracellularly, e.g. in case of DNase that degrades extracellular DNA (145). To this end, nanocarriers can be functionalized with responsive moieties that enable triggered release of the antibiotic, once the carrier reaches the target site (146).

Many studies were conducted to investigate different triggers for antibiotic release from nanocarriers such as liposomes and polymeric NPs (147–150). Both internal triggers such as enzymes and pH variations, as external factors such as light, heat and ultrasound can be used to release drugs out of carriers. A comprehensive discussion of this subject is beyond the scope of this chapter and therefore we want to refer the reader to recent reviews on this topic (151,152). We would like to highlight one trigger mechanism that selectively activated nanocarriers at the bacterial biofilm site, the so-called ‘microbial responsive nanocarriers’. It has been demonstrated that rhamnolipids, a certain type of virulence factor of *Pseudomonas aeruginosa*, could disturb the liposomal membrane, in this way selectively releasing amikacin out of the liposomes at the microbial infection site (108,153). Rhamnolipids were able to trigger release of amikacin from DPPC:cholesterol liposomes in a very efficient way: one rhamnolipid molecule per 100 lipids present in liposomes was sufficient to lead to amikacin release (108).

Another example of microbial responsive nanocarriers is the release of gentamicin out of polymer nanocarriers in contaminated wounds. It has been shown that fluids from
*Staphylococcus aureus*-infected wounds contained thrombin-like molecules, capable of destabilizing thrombin-sensitive linkers that conjugated gentamicin on polymer nanoparticles (154). A third example is the release of vancomycin out of AuNP functionalized liposomes by α-toxins secreted by *Staphylococcus aureus*. Alpha-toxins are known for their pore-forming abilities in membranes, and can be used to disrupt liposomal membranes for antibiotic release (155).
4. Conclusion

With multiresistant bacteria emerging worldwide and the shortage of novel antibiotics in the development pipeline, novel approaches for antimicrobial treatment are required. Making use of existing antibiotic molecules, but increasing their delivery towards biofilm cells, is a very promising route to follow. Disrupting biofilms, by enzymatic degradation as well as physical methods, has been shown to greatly enhance antibiotic's efficacy. However, more research should be performed in order to translate these concepts into clinical settings. On the other hand, the potential of nanoparticle-assisted antibiotic delivery has been assessed by many studies, and promising results paved the way for intense worldwide investigation. To achieve maximal output, smart engineering of the NP is essential: NP size, surface charge, chemical composition, attachment of site-directing molecules and responsive linkers are crucial in obtaining elevated antibiotic delivery. Further research should take into account those factors and assess the biofilm penetration ability of the carriers with advanced microscopy techniques such as Single Particle Tracking and Fluorescence Correlation Spectroscopy. Furthermore, more research should focus on the construction of NPs that effectively target biofilm-enclosed cells, while at the same time avoiding interactions with tissues distributed throughout the entire human body. Instead of charge-based interactions, which will not exclusively occur at the biofilm, one should screen for specific targeting agents, directed against precise bacterial determinants, while considering that higher specificity is often accompanied with lower affinity. Also, as the majority of published papers include in vitro data only, assessment of the toxicity and applicability in vivo is urgently needed. In conclusion, rationally designed antibiotic-enclosing NPs have potential to increase antibiotic delivery, and could be useful in the future treatment of infectious diseases.
5. References

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**Figure legends**

**Figure 1.1** Schematic representation of the architecture of bacterial biofilms and different approaches to improve antibiotic penetration.
*Left:* Two important features of biofilms contribute to their limited diffusivity to antimicrobial agents. First, cells are packed into dense cell clusters, due to which antibiotics cannot easily reach deep cell layers. Second, sessile cells are surrounded by a biofilm matrix that can hinder antibiotic diffusion, due to physicochemical interactions of antibiotics with matrix constituents. *Right:* Improved antibiotic penetration can essentially be obtained by two therapeutic approaches. One the one hand, the biofilm structure can be degraded in order to release cells from their protective environment. On the other hand, antibiotics can be shielded from interactions with biofilm matrix constituents by encapsulation in nanocarriers.

**Figure 2.1:** The percentage of penetrated polystyrene nanoparticles (open), liposomes (dashed) and control molecules (FITC-dextran 4000 Da, grey) in *Burkholderia multivorans* LMG18825 (left) and *Pseudomonas aeruginosa* LMG27622 biofilms (right) (33).

**Figure 2.2:** Restricted antibiotic diffusion into dense cell clusters of bacterial biofilms.

**Figure 2.3:** Structure of aminoglycosides gentamicin, tobramycin and the polysaccharide alginate.

**Figure 2.4:** Structure of ampicillin, with the characteristic β-lactam ring.

**Figure 3.1:** Electron microscopy image of the interaction between fluidosomes and *Pseudomonas aeruginosa* 429 by negative staining with 1% phosphotungstic acid. Fluidosomes came in close contact with the bacterial cell membrane and eventually fusion took place (enlargement of the outer membrane) (143).