Antimicrobial and nanoparticle penetration and killing in infectious biofilms

Rozenbaum, René Theodoor

Chapter 7

General discussion

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Since the onset of human existence bacterial infections have contributed to diseases and death. The discovery of antibiotics has limited death rates due to infections for the first couple of decades. Still, bacterial infections remain a major cause of death worldwide, especially in immunocompromised patients[1,2]. Bacteria in biofilms are more tolerant to antimicrobials than planktonic bacteria[3] and therefore are more difficult to treat with antimicrobials. In addition, genetic resistance of bacteria against antibiotics has dramatically increased the last decade[4,5]. Continuous development of new antimicrobials and targeted delivery strategies of antibiotics are necessary to combat bacterial infections. Therefore, in this thesis we have explored methods to improve the penetration and killing of infectious biofilms.

Biofilm penetration

Altering the zeta potential of engineered nanocarriers has been done in several studies to optimize penetration in biofilms[6–11]. Positively charged particles or antimicrobials are thought to interact with the negative charges of extracellular polymeric substances (EPS) in the biofilm, and it is therefore often assumed that they will remain in the top layers of the biofilm[12–14]. In chapter 5, we show that neutrally, negatively and positively charged dendrons all penetrate into *Pseudomonas aeruginosa* biofilms. Positively charged dendrons accumulated faster and in high concentrations in the top layers of the biofilm, but also reached the deeper layers, while neutrally and negatively charged dendrons accumulated in deeper layers in higher concentrations into the biofilm than positively charged dendrons. One critical drawback is that we only tested the penetration of dendrons into one *P. aeruginosa* strain. Bacterial species and strains differ in the composition and production of EPS[15], which is probably the reason why contradictory results with respect to nanocarrier zeta potentials and biofilm penetration are reported[6–11]. For example, positively charged quantum dots were shown to penetrate into *Escherichia coli* biofilms, while negatively and neutrally charged quantum dots did not penetrate[6]. In contrast, negatively charged polystyrene particles were accumulating faster in *Alteromonas macleodii* biofilms than positively charged polystyrene particles[8]. More research, preferably using different species, will be needed on the effect of zeta potentials of nanocarriers and the penetration in biofilms. An important aspect to keep in mind for penetration of nanocarriers in biofilms is the biofilm thickness and the visualization of penetration. Often studies use thin biofilms of 10-40 µm[6–11], while *in vivo* biofilms can easily be thicker than 50 µm[16]. For visualization often confocal laser scanning microscopy is used and sometimes depending on the bacterial strain it only can visualizes the top 20-40 µm of the biofilm[17,18], and also images are made while the biofilm is submerged in a buffer. We show in chapter 5 that after the penetration of dendrons, submersion in buffer can cause a wash-out of the dendrons depending on the charge. If we would have used confocal laser scanning microscopy to visualize the

penetration, we would have measured lower concentrations for neutrally and negatively charged dendrons in the biofilm.

An interesting idea to battle biofilms is the dissolution of EPS[19]. Recombinant human DNase is for example used daily in the treatment of cystic fibrosis patients to dissolve the DNA present in the mucus layer in the lungs[20]. DNA or normally called eDNA is a part of EPS and is functioning as the glue in the biofilm matrix[21]. In the treatment of cystic fibrosis patients, inhalation of DNase is given before the inhalation of antibiotics, resulting in enhanced efficacy of the treatment[22] due to a better penetration of the antibiotics in the mucus layer. Changing the charge of EPS dissolving agents from a negative to a positive charge may result in higher concentrations of the agent at the surface of biofilms (see also Figure 3 in Chapter 5), and thereby accelerating the dissolvement of EPS. Therefore, combination therapy of dissolving the EPS of a biofilm and target the infectious biofilm will be a promising method for the future. An important aspect to keep in mind is that when administered intravenously or orally, nanocarriers with adsorbed or encapsulated antimicrobials should also be made optimal for systemic longevity in the blood circulation, which may need other characteristics than a nanocarrier must possess for optimal biofilm penetration.

Antimicrobial peptides

Antimicrobial peptides (AMPs) have long been mentioned as a potential candidate for the treatment of bacterial infections, for the reason that development of bacterial resistance against AMPs seemed improbable[23]. However, recent studies have shown that AMP resistance can occur, for example by modifying the cell membrane[24]. In Gram-positive bacteria like *Staphylococcus aureus*, positively charged molecules such as L-lysine can be incorporated into the cell wall teichoic acids[25]. This reduces the negative charge in the bacterial cell wall, and so decreases electrostatic interaction with the cationic AMPs[26]. In Gram-negative bacteria, the charge of the lipopolysaccharide (LPS) can be increased by addition of 4-aminoarabinose to lipid A, which decreases the affinity between AMPs and LPS. Furthermore, bacteria can produce proteases and activate efflux pumps to frustrate AMP activity[26].

Antimicrobial activity of AMPs is often tested in solutions with a low salt concentration (10 mM) in which bacteria are not metabolically active[27–29]. In Chapter 6 we argue whether this is relevant for the *in vivo* situation, in which in addition to proteins also high salt concentrations (around 140 mM) are present. The stability of AMPs is depending on the salt concentration and decreases when the salt concentration is too high[30]. The antimicrobial activity of the AMPs magainin 1, cecropin P1[31], and human β-defensin-1[32] for example almost disappeared when tested in 100 mM NaCl in comparison to 0 mM NaCl. Another drawback of AMPs is their sensitivity to proteolytic degradation[33]. Both *P. aeruginosa* and *S. aureus* can secrete peptidases (elastase and ureolysin) which inactivates

LL-37[34,35]. These proteases hydrolyze positions involving hydrophobic side chains of the AMP, resulting in loss of AMP effectivity[36]. This sensitivity of AMPs to salts and proteolytic degradation is probably the reason that *in vitro* and *in vivo* biofilm studies only prevention or inhibition of biofilm growth is observed, instead of biofilm killing[28,37]. Considering both the development of resistance and the sensitivity towards salts and proteolytic degradation are probably reasons that AMPs are less effective as thought for combatting bacterial infections. AMPs might still be considered as effective antimicrobials in the future, since there are still several AMPs tested in clinical trials[38].

Future perspectives

Within the coming decades, antimicrobial resistance will increase and therewith increase the need for continuous development of new antimicrobials and targeted delivery strategies. The world health organization launched in 2015 a 'global action plan on antimicrobial resistance'[39]. Herein, five objectives are mentioned to sustain future treatment of infections: '(1) to improve awareness and understanding of antimicrobial resistance, (2) to strengthen knowledge through surveillance and research, (3) to reduce the incidence of infection, (4) to optimize the use of antimicrobial agents, and (5) to ensure sustainable investment in countering antimicrobial resistance'[39]. This must result in prevention of further development of antimicrobial resistance, so that infectious diseases can still be treated with safe and effective antimicrobials. Exploring synergistic interactions of antimicrobials might be one of the options to optimize the use of antimicrobial agents, also because treatment with synergistic antimicrobials can slow down the development of antimicrobial resistance[40]. So far, little is known about the underlying mechanisms of synergistic interactions of antimicrobials with antibiotics or other antimicrobial compounds as monolaurin. Therefore, combinational therapies need to be investigated in order to battle future antimicrobial resistance.

Engineered nanocarriers releasing antimicrobials are heavily investigated, with liposomal nanoparticles the most popular one, from which a few are already used in the clinic[41]. Targeted therapies in which nanocarriers with antimicrobials engineered in a way that biofilm penetration is achieved, longtime circulation in blood is guaranteed and release of antimicrobials only occurs in the biofilms would be ideal to kill the bacteria in the biofilm and to prevent development of antimicrobial resistance.

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