Mechanisms of antimicrobial actions of quaternary ammonium compounds
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Chapter 1 gives an overview of the mechanism involved in biomaterial-centred infections (BAI) and the possible strategies to prevent these infections. This chapter introduces the reader into the subject by offering theoretical knowledge concerning BAI as one of the main causes of implant failure in an era in which the number of patients requiring biomaterials implant surgery is steadily increasing. BAI begins with the initial adhesion of infectious organisms that subsequently grow to form a biofilm. Biofilms exhibit high levels of resistance to antibiotics, disinfectants, and detergents. Treatment of infected implants frequently includes long-term antibiotic use, often without success, so that finally an infected implant has to be removed. Quaternary ammonium compounds (QAC) are currently promising compounds which proved to possess strong antimicrobial properties when applied in solution and also when immobilized on a surface. However, although there have been hypotheses regarding the mechanism of action of QACs on bacterial cell surfaces, there is no clear evidence what supports these hypotheses. For practical application of QACs, the mechanism of action for controlling bacterial adhesion and subsequent biofilm formation should be better understood. Therefore, the main aim of this thesis is to explore and understand the bactericidal mechanism of QACs on staphylococci implicated in biomaterial-associated infections.

The study presented in Chapter 2 was designed to investigate the effects of a QAC (Ethoquad C/25 (Cocoalkyl-methyl-(polyoxyethylene)-ammonium-chloride)) on the survival of adhering staphylococci on a surface using atomic force microscopy (AFM). Four strains with different minimal inhibitory (MIC) and bactericidal (MBC) concentrations for the QAC were exposed to three different concentrations of the QAC in potassium phosphate buffer (0.5, 1 and 2 x MBC), while adhering to glass. Adhering staphylococci were repeatedly imaged with AFM in the contact mode and the cell surface was found to wrinkle upon progressive exposure to the QAC until bacteria
disappeared from the substratum. Higher concentrations of QAC yielded faster wrinkling and disappearance of bacteria during imaging. Two slime-producing staphylococcal strains survived longer on the surface than two non-slime-producing strains, despite similar MIC and MBC values. All staphylococci adhering in unscanned areas remained adhering during exposure to QAC. Since MIC and MBC values did not relate with bacterial cell surface hydrophobicities and zeta potentials, survival on the surface is probably not determined by direct interaction of QAC molecules with the cell surface. Instead, it is suggested that the pressure of the AFM tip assists incorporation of QAC molecules in the membrane and enhances their bactericidal efficacy. In addition, the prolonged survival under pressure of slime-producing strains on a surface may point to a new protective role of slime as a stress-absorber, impeding incorporation of QAC molecules. Simultaneously, this study demonstrated that the presence of 0.1 M Ca^{2+}-ions reduced the bactericidal efficacy of QAC molecules. Addition of Ca^{2+}-ions to a QAC solution yielded longer survival of intact, adhering staphylococci, suggesting that Ca^{2+}-ions can impede exchange of membrane Ca^{2+}-ions required for QAC incorporation.

In Chapter 3 the influence of Ethoquad C/25 compound on growth and metabolic activity of staphylococcal biofilms was determined while possible cell wall damage to biofilm organisms was visualized using Focused Ion Beam - Scanning Electron Microscopy (FIB-SEM) 3D-tomographic imaging. Three strains (a non-slime and two slime-producing staphylococcal strains) with different minimal inhibitory and bactericidal concentrations were used in this study. Zeta potentials, water contact angles and elemental compositions of the cell surfaces were measured. Metabolic activities of biofilms exposed to 1 x MBC and 3 x MBC QAC solutions were quantitated using the tetrazolium salt (MTT) reduction assay expressed per unit live biovolume, derived from confocal laser scanning microscopy images. FIB-SEM was utilized for 3D-visualization of the staphylococcal biofilm after QAC exposure. Slime-
producing staphylococci were less negatively charged, more hydrophobic and contained more cell surface nitrogen-rich groups than the non-slime producing strain. QAC solutions at 1 x MBC for planktonic organisms did not kill staphylococci in biofilms and only reduced the metabolic activity of the non-slime producing strain. Slime-producing strains were not affected in their metabolic activity despite cell wall damage shown by FIB-SEM. At 3 x MBC, the amount of biofilm, as well as their metabolic activities, were strongly reduced and the few organisms that could be visualized in FIB-SEM had multiple holes in their cell wall. Summarizing, staphylococcal biofilms can remain metabolically active during exposure to 1 x MBC of a QAC solution, despite cell wall damage. At a higher (3 x MBC) concentration, more severe damage was found, and a small metabolic activity remained even for a slime-producing strain known to be better able to resist antimicrobial attack.

In Addendum to Chapter 3, the effects of QACs on staphylococcal biofilms growth were analysed and compared to gentamicin sulphate, an aminoglycoside antibiotic commonly used in orthopaedic surgery for local treatment of infection. Gentamicin sulphate solutions at their planktonic MBC were insufficient to kill staphylococci in their biofilms mode of growth and only reduced metabolic activity of a non-slime producing strain. However, for the slime-producing strains, exposure of biofilms to gentamicin sulphate at their MBCs lead to increased slime production and metabolic activity pointing that the presence of slime plays a significant role in protecting the organisms against gentamicin sulphate, enabling bacteria to withstand unfavourable environmental conditions, as antibiotic presence. This indicated that the mechanism of action of gentamicin sulphate on growth of staphylococcal biofilms is different as found for QACs in Chapter 3.

Antimicrobial peptides, such as Gramicidin S, possess a tertiary structure in which the cationic groups are spatially located on one side of the peptide and the hydrophobic moieties at the other side of the molecules.
Similarly, cationic compounds as QACs display only antibacterial activity if they possess a hydrophobic unit, besides the quaternary ammonium moiety. The resemblance of these requirements for both classes of compounds, to display antibacterial behaviour, suggests a similar way of action. Therefore, in Chapter 4 we investigated and compared the initial effects of different cationic antimicrobials (QAC, Gramicidin S and gentamicin sulphate) on the cell surface structure of adhering Staphylococcus epidermidis using Peak-Force-Tapping AFM. Adhering S. epidermidis ATCC 14990 was exposed to different antimicrobials at their minimal bactericidal concentration and to potassium phosphate buffer, as a control. During exposure, adhering bacteria were repeatedly imaged applying a force of 3 nN and the percentage of staphylococci that remained adhering was registered as a function of time. Surface roughness of bacteria that remained adhering was calculated from the AFM images. Upon progressive exposure of adhering staphylococci to all three cationic antimicrobials, wrinkling and disappearance of bacteria was observed during scanning with the AFM tip. Exposure to buffer did not yield wrinkling or disappearance of adhering staphylococci, while after 300 min more bacteria had detached when exposed to gentamicin sulphate than to the other two cationic antimicrobials. Bacterial cell surface roughness after exposure to the antimicrobials and scanning increased from 15 nm prior to the experiment to 138 nm for QAC and Gramicidin S and to 145 nm for gentamicin sulphate. The bacterial cell surface roughness was affected upon exposure to the antimicrobial compounds, which confirms that membrane degradation is a significant contributing factor to their bactericidal activities. Antimicrobial effects on the cell surfaces of adhering staphylococci were found to be similar for a QAC, an antimicrobial peptide and an aminoglycoside, suggesting similar membrane interactions.
Finally, in Chapter 5, we hypothesized that QAC molecules immobilized on a surface possess a totally different, generic working mechanism than QAC molecules in solution. Whereas the working mechanism of QACs in solution are based on adsorption, ion-exchange and membrane damage, immobilized QAC molecules enhance the adhesion forces between a bacterium and a substratum surface to cause reduced growth, stress deactivation and eventually cell death.