Survival of adhering staphylococci during exposure to a quaternary ammonium compound evaluated by using atomic force microscopy imaging
Abstract

Effects of a quaternary ammonium compound (QAC) on the survival of adhering staphylococci on a surface were investigated 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. Addition of Ca\textsuperscript{2+}-ions to a QAC solution yielded longer survival of intact, adhering staphylococci, suggesting that Ca\textsuperscript{2+}-ions can impede exchange of membrane Ca\textsuperscript{2+}-ions required for QAC incorporation.
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Introduction

Biomaterial-associated infections, not only of totally internal implants but also of external devices as contact lenses or urinary catheters remain a serious concern in modern health care. The most common causative strains for biomaterial-associated infections are Gram-positive *Staphylococcus aureus*, *Staphylococcus epidermidis* and Gram-negative *Pseudomonas aeruginosa*. Bacteria tend to adhere strongly to biomaterial implants and devices, leading to the formation of a biofilm. In a biofilm, microorganisms embed themselves in a matrix of extracellular polymeric substances (EPS), also-called ‘glycocalyx’ or ‘slime’, offering protection against the host immune system and antimicrobial treatment, to which planktonic organisms are usually much more susceptible. In staphylococcal strains, EPS formation depends in part on the presence and expression of the *icaADBC* gene cluster, which is involved in the production of a polysaccharide intracellular adhesin (PIA). PIA is known to mediate bacterial contact with each other and embeds adhering bacteria in a slimy PIA matrix during biofilm formation. The *ica* operon is widespread in staphylococcal multi-resistant isolates and represents one of the most important virulence factors causing biomaterial-associated infections.

One of the strategies to prevent biofilm formation on the surfaces of biomaterials implants and devices is the application of antimicrobial coatings comprising quaternary ammonium compounds (QAC). Alternatively, water-soluble low molecular weight QACs are often used in contact lens care solutions to convey antimicrobial properties to the solution to enhance lens cleaning. QACs are potent cationic antimicrobials, known to affect the viability of Gram-positive and Gram-negative bacteria in planktonic cultures as well as in biofilms. QACs interact with bacterial cell surfaces to become integrated in the bacterial cell membrane and affect the cytoplasmic
membrane integrity by creating holes, followed by leakage of intercellular constituents, ultimately leading to cell death \textsuperscript{10,24}. Bacterial susceptibility and resistance to QACs has been related with bacterial cell surface hydrophobicity and charge \textsuperscript{4}. The role of bacterial membrane charge may have been underestimated in explaining the mechanisms underlying the antimicrobial efficacy of QACs, as integration of a QAC molecule in the cell membrane requires removal of a Ca\textsuperscript{2+}-ion from the membrane in order to maintain a neutral membrane charge. Therewith it can be expected that the efficacy of QACs will depend on the presence or absence of Ca\textsuperscript{2+} in the surrounding fluid. Effects of Ca\textsuperscript{2+}-ions on QAC antimicrobial efficacy however, have never been thoroughly demonstrated. Surfactant properties of QACs have also been mentioned to contribute to cell surface damage\textsuperscript{12}.

Over the past, different approaches have been taken to visualize the effect of antimicrobial compounds on bacteria. The most common technique used is high-resolution electron microscopy, which requires however extensive sample preparation \textsuperscript{13}. Confocal Laser Scanning Microscopy (CLSM) and Atomic Force Microscopy (AFM) can both be applied to operate under physiological conditions. CLSM imaging is at a more macroscopic level, whereas AFM can assess the topography of a bacterial cell surface \textsuperscript{14,25} at a nanoscopic level. Unlike most other imaging tools, AFM requires neither a vacuum environment nor any special sample preparation. Consequently, AFM has become a well-established technique for producing high resolution images of bacterial cell surfaces \textsuperscript{19,34}. An alternative use of AFM is to measure the ease of bacterial removal from a surface under the influence of a force exerted by the scanning tip. Progressive removal of \textit{P. aeruginosa} and \textit{S. aureus} from titanium oxide substrata was demonstrated with increasing number of scans after air-drying of bacteria to the substratum surface \textsuperscript{33}.

The aim of this study is to determine the efficacy of a commercially available quaternary ammonium compound, manufactured from coconut oil,
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(Ethoquad C/25 (Cocoalkyl methyl (polyoxyethylene) ammonium chloride)) against a number of staphylococcal strains with different ica-status and slime-production. First, minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC) will be determined after which adhering staphylococci are exposed to QAC solutions and their cell surfaces imaged using AFM. While scanning the AFM tip over the adhering bacteria, survival of adhering bacteria on a substratum surface during exposure to solutions with different QAC concentrations will be taken as an indication of cell surface damage. Experiments will be performed in the presence and absence of Ca$^{2+}$-ions in the surrounding fluid in order to rule out surfactant effects and to provide evidence in support of the role of membrane charge exchange in the integration of QAC molecules in the bacterial cell membrane.

Materials and methods

Bacterial strains and growth conditions

Three clinically-isolated staphylococcal strains were used, originating from different infection sites (S. aureus 7232, from an infected total hip arthroplasty, S. aureus 835 from a patient with microbial keratitis and S. epidermidis 3081 from a urinary tract infection) and one ATCC strain (S. epidermidis ATCC 12228). All strains were first grown overnight at 37°C on a blood agar plate from a frozen stock. One colony was inoculated in 10 ml tryptone soya broth (TSB, Oxoid, Basingstoke, UK), and incubated at 37°C for 24 h and used to inoculate the main culture (200 ml), which was incubated for 16 h. Bacteria were harvested by centrifugation for 5 min at 5000 g and 10°C and subsequently washed three times with ultrapure water. Next, bacteria were suspended in 10 ml ultrapure water for bacterial surface
characterisation, fluorescence microscopy, and atomic force microscopy analyses.

Figure 1. Chemical structure of Ethoquad C/25 (Cocoaalkyl methyl (polyoxyethylene) ammonium chloride).

Phenotypic characterization of the staphylococcal strains

All strains were cultured on Congo Red Agar (CRA) plates, prepared by adding 0.8 g of Congo Red (Sigma-Aldrich, Steinheim, Germany), 12 g bacto agar (Becton, Dickinson and Co, Sparks, MD, USA) and 36 g saccharose (Merck, Darmstadt, Germany) to 1 l of brain heart infusion (Oxoid). The inoculated plates were subsequently incubated for 24 h at 37°C and additionally incubated overnight at room temperature. Black coloured colonies were considered indicative of slime production, while strains producing red to pink coloured colonies were classified as non-slime producing. The CRA plate assay was done in triplicate for all strains.

IcaA expression

Expression of icaA in the staphylococcal strains was determined using total RNA isolation and real-time RT-PCR analysis. The sequence of S. aureus ATCC 12600 was used to design two primer sets for real-time RT-PCR analysis of the S. aureus strains: gyrB and icaA. Real-time RT-PCR was performed using 100 nM of each primer under a two-step protocol with an annealing temperature of 61°C. Under these PCR conditions, both the gyrB and icaA
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primer set (gyrB3; 5'-GGAATCGGTGGCGACTTTGATCTAGCGAAA-3', gyrB4; 5'-CGCTCCATCCACATCGGATCACTATAAT-3', icaA1; 5'-CTTGATGATAAATCGTGCCA-3', icaA2; 5'-GACCTCCCAATGTTTCTGGAACCAATCC-3'), 9 yielded specific PCR products with a PCR efficiency of 100%.

For *S. epidermidis* strains, two reference strains were used: a positive control *S. epidermidis* ATCC 35984, which is known to express the icaA gene and a negative control *S. epidermidis* ATCC 12228, which does not express the icaA gene. For real-time RT-PCR analysis two primer sets were designed for *gyrB* and icaA (*gyrB* forward; 5'-GGAGGTAAATTCGGA-3', *gyrB* reverse; 5'-CTTGATGATAAATCGTGCCA-3', *icaA* forward; 5'-GGAAGTTCTGATAATACTGCTG-3', *icaA* reverse; 5'-GATGCTTGTTTGAATTCCCTC-3') 27. RT-PCR analyses were done at an annealing temperature of 58°C.

Total RNA was isolated from 16 h cultures grown at 37°C. Bacteria were harvested by centrifugation and frozen at -80°C. Samples were thawed slowly on ice and re-suspended in 100 µl diethylpyrocarbonate treated water, after which the bacterial suspension was frozen in liquid nitrogen. The frozen bacteria were then ground using a mortar and pestle. Total RNA was isolated using the Invisorb Spin Cell RNA Mini Kit (Invitek, Freiburg, Germany). DNA was removed using the DNA free kit from Ambion and absence of genomic DNA was verified by RT-PCR prior to reverse transcription. For all samples, 35 cycles of PCR using the *gyrB* primer set did not result in any detectable signal. For cDNA synthesis, 250 ng of total RNA was used (Iscript, Bio-rad, Veenendaal, The Netherlands). Reactions were prepared in duplicate using the CAS-1200TM pipetting robot (Corbett Life Science, Sydney, Australia). Expression levels of icaA in staphylococci were analysed using the 2^{-∆ΔC_T} method 21 with *gyrB* as reference gene, with respect to ica-positive and ica-negative strains.
Relative expression normalized with respect to *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 35984 (*icaA* positive reference strains) and *gyrB* as a reference gene. The $2^{-\Delta\Delta C_T}$ was calculated from the average $C_T$ values of two reactions and standard deviations are given.

**Zeta potential measurements**

Zeta potentials were determined as a function of pH by particulate microelectrophoresis, assuming the Helmholtz-Smoluchowski equation holds. Bacteria were suspended in 10 mM potassium phosphate buffer (pH 7) to a concentration of $10^7$ bacteria/ml. Readings were taken at an applied voltage of 150 V in a Lazer Zee meter model 501 (PenKem, Bedford Hills, N.Y.). This instrument uses scattering of incident laser light to allow detection of bacteria and was equipped with image analysis options for zeta sizing. Zeta potentials were determined in triplicate with separately cultured bacteria.
Contact angle measurements

Water contact angles were measured on bacterial lawns employing the sessile drop technique as a measure of the bacterial cell surface hydrophobicity. Staphylococci suspended in ultrapure water were deposited on a filter (pore diameter 0.45 µm) using negative pressure. The filter was attached to a sample holder with double-sided sticky tape and dried at room temperature until a stable, so-called plateau contact angle was reached, which was established after 40 min for the non-slime producing and after 50 min for the slime producing strains. Contact angle measurements were determined in triplicate with separately cultured bacteria.

Minimal inhibitory (MIC) and bactericidal (MBC) concentrations

The MIC of (Ethoquad C/25 (Cocoalkyl methyl (polyoxyethylene) ammonium chloride)) (AKZONobel, Amsterdam, The Netherlands; see Figure 1) dissolved in 10 mM potassium phosphate buffer, pH 7.0 was determined using a series of two-fold dilutions in a 96 wells plate under planktonic conditions. The wells with 195 µl TSB and QAC were inoculated with 5 µl of a 10-fold diluted pre-culture and left to incubate at 37°C for 24 h. The well containing the lowest concentration of the QAC that completely inhibited visual bacterial growth was taken as the MIC. The MBC was subsequently determined by adding a droplet of 5 µl from each well showing no visible growth on a TSB agar plate. The MBC was then taken as the lowest concentration of the QAC that prevented further growth of the strain.

MIC and MBC values were also determined in the presence and absence of 0.1 M CaCl\(_2\) in Nutrient Broth (NB, Oxoid). These experiments could not be carried out in TSB because of its high phosphate content, causing the formation of insoluble calcium phosphate.
Table 1. MIC and MBC values (μg/ml) of Ethoquad C/25 in 10 mM potassium phosphate buffer for two ica-positive and two ica-negative staphylococcal strains in TSB or NB, as well as in NB supplemented with 0.1 M CaCl₂. Data for individual strains were obtained in triplicate, yielding identical results.

<table>
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<tr>
<th>Strains</th>
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<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
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<td>Ica-positive strains</td>
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<td>30</td>
<td>220</td>
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<td>S. aureus 7232</td>
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<tr>
<td>S. epidermidis ATCC 12228</td>
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<td>150</td>
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Atomic force microscopy

AFM experiments were conducted at room temperature in potassium phosphate buffer as a control, and in potassium phosphate buffer with different concentrations of the QAC (0.5, 1 and 2 x MBC) added. A Dimension 3100 with a Nanoscope IV Digital Instrument from Veeco (Woodbury, USA), was used for imaging staphylococci. Bacterial samples were prepared as follows: Glass slides were sonicated for 3 min in 2% RBS 35 (Omnilabo International BV, The Netherlands) followed by thorough rinsing with tap water, demineralised water, methanol, tap water, and finally demineralised water and dried in air. Bacteria were attached to the glass slide through electrostatic interaction with positively charged poly-L-lysine. A drop of poly-L-lysine 0.01% (wt/vol) (Sigma, Poole, UK) was added on the glass slide and after drying, the slide was rinsed with ultrapure water. A droplet of bacterial suspension (10^10 bacteria/ml) was added to the poly-L-lysine coated glass slide and left for 30 min. Subsequently, the bacterially coated glass slide was rinsed with ultrapure water to remove free-floating bacteria. This glass slide was immediately used for AFM measurements without drying.

The adhering bacteria on the glass slide were scanned with the either AFM, while remaining immersed in a QAC solution (0.5, 1 and 2 x MBC) or potassium phosphate buffer. Deflection images with a scan size of 35 μm x 35 μm were taken at the beginning and end of the experiment. In the centre of these images, an area with a scan size of 8 μm x 8 μm was repetitively scanned during 300 min. The scans were made in the contact mode under the lowest possible applied force (1 to 2 nN) at a scan rate of 1 Hz using DNP probes from Veeco (Woodbury, USA). In each scan, 40-60 adhering bacteria were monitored with respect to possible surface damage and their survival on the surface during exposure to different concentrations of QAC and potassium phosphate buffer. The percentage survival of adhering bacteria on the surface
was plotted as a function of time using Kaplan-Meier curves. The experiments were performed in triplicate with different bacterial cultures to include approximately 100 bacteria for each Kaplan-Meier curve presented.

In addition, these experiments were done in demineralised water, demineralised water supplemented with 0.1 M CaCl$_2$ or 1 x MBC QAC and demineralised water with 0.1 M CaCl$_2$ and 1 x MBC QAC added. Experiments in the presence of CaCl$_2$ could not be conducted in potassium phosphate buffer due to the formation of insoluble calcium phosphate.

**Figure 3.** Fluorescence images of cell surface damaged *S. epidermidis* ATCC 12228 after 45 min exposure to the QAC in a 10 mM potassium phosphate buffer: (A) no QAC, (B) 0.5 x MBC, (C) 1 x MBC, and (D) 2 x MBC. The bar denotes 25 μm.

**Fluorescence microscopy**

Possible cell surface-damaged of bacteria adhering on the glass slides immediately after preparation as described above and after 45 min exposure to a QAC solution or potassium phosphate buffer (control), were visualized by fluorescence microscopy. The samples were stained for 15 min in the dark.
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with 250 μl LIVE/DEAD BacLight viability stain (Molecular probes, Leiden, The Netherlands) containing SYTO 9 dye (green fluorescent) and propidium iodide (red fluorescent) to differentiate between undamaged and cell surface-damaged bacteria. Although the viability stain is mostly advocated to distinguish between live and dead bacteria, in essence this stain allows evaluation of membrane integrity. A functional cytoplasmic membrane does not allow accumulation of propidium into a bacterium to replace SYTO9, that readily penetrates functional cytoplasmic membranes from the DNA. Fluorescent images were collected with a fluorescence microscope (Leica DM4000 B, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

Statistical analyses

Survival of adhering bacteria exposed to the different QAC solutions was compared to the control (potassium phosphate buffer or demineralised water only), using Log-rank (Mantel-Cox) of the Windows package SPSS 12.0.1. Differences were considered statistically significant for p < 0.05.

Results

The genotype of the staphylococcal strains was determined by the icaA expression using real-time PCR relative to the reference gene gyrB and including two icaA positive controls (S. epidermidis ATCC 35984 and S. aureus ATCC 12600). S. aureus 835 and S. aureus 7232 were both found to be icaA-positive, whereas S. epidermidis 3081 and S. epidermidis ATCC 12228 were icaA-negative (see Figure 2). Both ica-positive strains (S. aureus 835 and S. aureus 7232) showed black colonies on CRA agar plates, while the two ica-negative strains (S. epidermidis ATCC 12228 and S. epidermidis 3081) displayed red-pink colonies. In addition, the ica-positive strains had less negative zeta
potentials (-24 and -28 mV) and more hydrophobic water contact angles (32 and 43 degrees) than the ica-negative ones (zeta potentials -35 and -34 mV and water contact angles 12 and 17 degrees).

The MIC and MBC values for the QAC of the slime producing and non-slime producing staphylococcal strains were similar (see Table 1). Growth in TSB yielded similar MIC values for all strains as did growth in NB, but higher MBC values were obtained. Upon supplementing NB medium with 0.1 M CaCl₂, both MIC and MBC values of the strains increased. This increase was much stronger for the non-slime producing strains than for the slime-producing ones.

**Figure 4.** AFM deflection images of staphylococcal strains during exposure to 10 mM potassium phosphate buffer and QAC solutions in buffer after a single scan (35 μm X 35 μm) and after multiple scans in the centre region (8 μm X 8 μm, inserts) at a rate of 1 Hz during 300 min. (A, B) *S. aureus* 7232 (slime producer) exposed to buffer (A) and a 2 x MBC QAC solution (B), (C, D) *S. epidermidis* ATCC 12228 (non-slime producer) exposed to buffer (C) and a 2 x MBC QAC solution (D). The bars denote 2.5 μm for the upper pictures and 1 μm lower pictures.

In Figure 3, fluorescence images are shown of *S. epidermidis* ATCC 12228 (non-slime producer) adhering to a glass slide as prepared for AFM
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measurements and exposed to the QAC during 45 min. In potassium phosphate buffer, all bacteria showed green fluorescence, attesting of an undamaged cytoplasmic membrane, but addition of 0.5 x MBC of the QAC already resulted in red fluorescence of 90% of all bacteria, indicating loss of membrane integrity. Addition of QAC at 1 x and 2 x MBC caused loss of membrane integrity for 94% and 99% of all bacteria, respectively.

AFM deflection images of *S. epidermidis* ATCC 12228 (non-slime producer) and *S. aureus* 7232 (slime producer) exposed to potassium phosphate buffer and a QAC solution (2 x MBC) are shown in Figure 4. For both strains, almost all bacteria disappear from the scanned area after multiple scans when exposed to a QAC solution. Adhering bacteria outside the repetitively scanned area mostly survive on the surface. No detachment was observed when the staphylococci were exposed to potassium phosphate buffer only, while bacteria also appeared larger than when exposed to QAC. Closer inspection of adhering staphylococci exposed to a 1 x MBC solution (Figure 5) shows that detachment occurs progressively with time and that the bacterial cell wall loses integrity within 30 min after exposure to the QAC and consequently disappears from the scanned area. In Figure 6B, an enlarged image of *S. epidermidis* ATCC 12228 after 60 min exposure to a 1 x MBC QAC solution is presented. Surface wrinkling can be clearly seen after exposure to a QAC solution, as opposite to the smooth surface (Figure 6A) expressed by bacteria exposed to potassium phosphate buffer only.

Staphylococcal detachment during exposure to QAC was monitored for 300 min and the bacteria remaining on the surface were plotted in a Kaplan-Meier survival curve, as presented in Figure 7. Exposure to buffer did not cause any bacterial removal, but exposure to a QAC solution yielded significant (p < 0.05) staphylococcal removal. *S. epidermidis* ATCC 12228 and *S. epidermidis* 3081, the non-slime producing strains, were more readily removed from the surface than *S. aureus* 835 and *S. aureus* 7232, the slime
producing strains. Removal was easier after exposure to higher concentrations of QAC.

Figure 8 summarizes the effects of staphylococcal exposure to QAC in the presence and absence of 0.1 M CaCl$_2$. Both for the slime producing and non-slime producing strains, staphylococcal removal was virtually absent in demineralised water and demineralised water supplemented with 0.1 M CaCl$_2$. The non-slime producing strain, S. epidermidis ATCC 12228 was almost fully removed after 300 min when exposed to 1 x MBC of the QAC, but addition of 0.1 M CaCl$_2$ to the solution significantly ($p < 0.05$) impeded removal to about 40% (Figure 8 A). Similarly, the slime producing strain, S. aureus 835 also showed a large removal after exposure to a QAC solution, while here addition of 0.1 M CaCl$_2$ fully reduced removal to the control levels. These observations indicate that the presence of Ca$^{2+}$-ions reduces the effects of QAC.

Figure 5. AFM deflection images of S. epidermidis ATCC 12228 (non-slime producer) during exposure to 10 mM potassium phosphate buffer (A) and 1 x MBC QAC solution (B) at different time points (0, 30, 45 and 60 min), while scanning continuously at a rate of 1 Hz. The scan area equals 8 μm x 8 μm. The bar denotes 1μm.
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Discussion

For practical application of QACs, the mechanism of action for controlling bacterial adhesion and subsequent biofilm formation should be better understood. In this paper, cell surface damage of adhering staphylococci under the influence of Ethoquad C/25 (Cocoalkyl methyl (polyoxyethylene) ammonium chloride), followed by removal from a substratum by an AFM tip, was demonstrated. Two slime-producing staphylococcal strains remained adhering longer on a substratum surface than two non-slime-producing strains, although their MIC and MBC values were similar. Addition of Ca²⁺-ions to the QAC solution yielded longer survival of intact, adhering staphylococci on the substratum surface, suggesting that positively charged Ca²⁺-ions can impede exchange of membrane Ca²⁺-ions by QAC. These results support one of the mechanisms for the bactericidal properties of QACs.

AFM and fluorescent images show that removal of adhering staphylococci starts with membrane damage followed by wrinkling of the cell surface, after which entire bacteria or their remnants detach. Gram-positive bacteria have a phospholipid cytoplasmic membrane surrounded by a peptidoglycan outer layer. Phospholipids consist of two long fatty acids connected via glycerol to phosphoric acid. The negative rest charge of the phosphoric acid is neutralized by calcium or magnesium ions. The replacement of these divalent cations by QAC molecules destabilizes the membrane, which results membrane damage (see fluorescent micrographs in Figure 3) followed by leakage of the intracellular matrix of a bacterium and loss of turgor pressure. Loss of turgor pressure is evidenced by the wrinkling of the staphylococcal cell surfaces and a decrease in bacterial volume (see AFM images in Figure 4), similar as observed after bacterial exposure to antimicrobial peptides. Upon addition of Ca²⁺-ions to a QAC solution, these
Ca^{2+}-ions compete with cationic QAC molecules for a place in the membrane, therewith reducing the antimicrobial efficacy of the QACs. We clearly demonstrate here for the first time that this exchange mechanism is valid for slime-producing and non-slime producing staphylococci. It is interesting that addition of Ca^{2+}-ions to a QAC solution reduced the efficacy of the QAC stronger in case of a slime-producing strain than in case of a non-slime producing strain.

Since MIC and MBC values did not relate with bacterial cell surface hydrophobicities and zeta potentials, survival of bacteria on the surface is probably not only determined by direct interaction of QAC molecules with the cell surface. Rather the loss of turgor pressure results in shrinkage of the adhering bacterium and therewith disruption of adhesive bonds that weakens the adhesion forces. As a result, the minimal forces, as exerted by the AFM tip, are now sufficient to detach a bacterium from the surface.

Adhering staphylococci are neither as heavily surface damaged nor removed from the substratum surface during exposure to the QAC outside of the extensively scanned area (Figures 4B and C) than inside the scanned area. This may indicate that the incorporation of QACs in the membrane, leakage associated loss of turgor pressure and subsequent removal from the surface are accelerated by external stress, as applied here through the force exerted by the AFM tip and arising from the substratum. This is supported by the observation that strong adhesion forces of staphylococci to surfaces as a cause of external stress caused a higher percentage of dead bacteria in the absence but particularly in the presence of bactericidal silver ions^{20}.

The current study suggests that this type of stress deactivation can be caused by external forces\textsuperscript{20}, exerted here by an AFM tip and accelerates incorporation of QAC molecules in the cell membrane and enhance the bactericidal efficacy of the QAC.
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Figure 6. High resolution AFM deflection images of *S. epidermidis* ATCC 12228 (non-slime producer) during exposure to 10 mM potassium phosphate buffer (A) and 1 x MBC QAC solution (B, C) at 60 min, while scanning continuously at a rate of 0.3 Hz. The scan area equals 2 µm x 2 µm. The bar denotes 0.3 µm.

This hypothesis would also explain why slime-producing staphylococci adhere longer on a surface than non-slime producing ones. Apart from the protection of the slime as such, the slime might also absorb some of the stress exerted by the AFM tip or arising from the substratum to decrease stress deactivation of the bacteria. With more stress absorbed by the slime, evidently membrane incorporation of QAC molecules and leakage of intracellular contents required for bactericidal efficacy is more difficult. Moreover, it is well possible that membrane damage has remained sub-critical in slime-producing strains, as here addition of Ca²⁺-ions reduced the efficacy of the QAC to control levels. Stress-absorption would constitute a new, hitherto unknown role of bacterial slime.

Water-soluble low molecular weight QACs are applied in contact lens care solutions to convey bactericidal properties to these solutions \cite{7}, but also when immobilized on a surface, QACs like poly(4-vinyl-N-alkylpyridinium) bromide possess bactericidal efficacy \cite{31}. 

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Figure 7. Kaplan Meier curves expressing the % of staphylococci that remain adhering on glass during exposure to a QAC solution in a 10 mM potassium phosphate buffer, while scanning continuously at a rate of 1 Hz. (A) 0.5 x MBC, (B) 1 x MBC, (C) 2 x MBC. The black line in each graph represents exposure to 10 mM potassium phosphate buffer (control) and is valid for all strains.
Based on the above hypothesis, it can be anticipated that the efficacy of immobilized QAC molecules may be larger than of QAC molecules in solution, because substratum surfaces become positively charged after QAC immobilization and thus exert a strong interaction force on negatively charged bacterial cell surfaces. In the current study, it is difficult to estimate the relative contributions of the stress exerted by the AFM tip on the bacterial cell surface and the one arising from the bacterial interaction with positively charged, poly-L-lysine coated glass surfaces on which AFM experiments were conducted. Figure 3 shows that there is membrane damage under the sole influence of QAC solutions of bacteria adhering to positively charged, poly-L-lysine coated glass surfaces, but no extensive leakage of intracellular content and detachment is observed in the absence of the additional pressure exerted by the tip. A verification of the role of positively charged substratum surfaces in stress de-activation by the use of naturally occurring and mostly negatively charged substratum surfaces is unfortunately impossible. Adhesion forces under these conditions are very weak and yielded complete removal of adhering bacteria within one or two scans (unpublished), which is the reason why Verran et al. artificially air-dried bacteria first to a substratum to irreversibly increase Lifshitz-Van der Waals attraction.

In summary, we have demonstrated that application of an external stress on adhering bacteria may enhance the bactericidal efficacy of QAC molecules to yield not only membrane damage, but also disintegration of the cell membrane and complete disappearance of adhering staphylococci from a surface. Slime-producing staphylococci remain adhering longer on a surface during exposure to QAC molecules and mechanical stress, which may suggest a new protective role of slime as a stress-absorber. Along opposite lines, stress activation may provide an alternative pathway to enhance the efficacy of QACs, but possibly also of other antimicrobials, perhaps even yielding efficacy against otherwise resistant strains. The potential use of stress-activation...
however, needs to be further exploited first and might be related to the so-called bio-acoustic effect, describing ultrasound enhancement of antibiotic efficacy. Ultrasonic pressure waves might then be the source of stress deactivation, as constituted in the current study by the AFM tip 29.

Figure 8. Kaplan Meier curves expressing the % staphylococci that remain adhering on a substratum surface during exposure to demineralised water and 0.1 M CaCl₂ in demineralised water, 1 x MBC QAC in demineralised water with and without addition of 0.1 M CaCl₂, while scanning continuously at a rate of 1 Hz. (A) S. epidermidis ATCC 12228, (B) S. aureus 835.

Simultaneously, this paper demonstrates that the presence of 0.1 M Ca²⁺-ions reduces the bactericidal efficacy of QAC molecules. Whether Ca²⁺ reduction of QAC bactericidal efficacy plays a role with respect to QACs immobilized on implant surfaces in the human body may be doubted, however. The serum level of ionized calcium is closely regulated between 1.1 and 1.4 mM, which is 100-fold lower than the 0.1 M applied here to demonstrate exchange inhibition.

Acknowledgements

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Reference list


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Chapter 2


AFM imaging of adhering staphylococci exposed to QAC