General discussion:
Mechanisms of antimicrobial action of quaternary ammonium compounds in solution and immobilized on a surface

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Quaternary ammonium compounds (QAC) are potent cationic antimicrobials used in everyday consumer products like contact lens solutions and mouthrinses as well as in numerous industrial processes, like water purification and antifungal treatment in horticulture. Unlike the case for many antibiotics, the development of bacterial resistance against QACs is considered unlikely, although Pseudomonas aeruginosa strains isolated from contact lens cases have been shown to possess resistance against QACs. The first step in the antimicrobial action of QACs is the approach of the QAC molecule towards the bacterial cell surface. This is mediated by hydrophobic and electrostatic attractions between positively charged QAC molecules and the bacterial cell surface, which is nearly always negatively charged under physiological conditions. Upon their subsequent adsorption, QAC molecules replace Ca\(^{2+}\) or Mg\(^{2+}\) ions from the cytoplasmic membrane in order to maintain charge neutrality in the membrane. The replacement of Ca\(^{2+}\) and Mg\(^{2+}\) ions by QACs destabilizes the intracellular matrix of a bacterium, as the hydrophobic tail interdigitates into the hydrophobic bacterial membrane causing leakage of intracellular fluid and loss of turgor pressure.

Interestingly, several studies have shown that the antimicrobial efficacy of QACs remains preserved when QAC molecules are immobilized on a substratum surface. Such contact-killing coatings have potential in a large number of widely varying applications, including but not limited to surgical equipment and protective apparel in hospitals, medical implants and wound dressings, water purification systems, food packaging and storage materials, and industrial equipment. The use of QACs has been popular since they are easy to manufacture in large quantities and can be used to coat large surface areas. Also QACs have been found to be very stable, especially in the human body. QACs are poorly metabolized and will be mainly
excreted in non-metabolized form, although QACs can be hemolytic when not immobilized on a surface.

Despite the large body of evidence of antimicrobial activity of immobilized QAC molecules, it is difficult to envisage how immobilized QAC molecules can exert the same mechanism of antimicrobial activity as do QACs in solution. Most convincingly to this point, a Gram-negative P. aeruginosa strain, not susceptible to QACs in solution, was killed upon adhesion to a coating of immobilized QACs. Intriguingly, coatings of immobilized QACs even remain antimicrobially active after adsorption of proteins in vitro and under in vivo conditions. Immobilized QACs, especially after adsorption of a protein film as developing rapidly in the human body, are strongly hindered in their search for heterogeneously distributed negative charges on bacterial cell surfaces, which is crucial for their efficacy in solution. Hence, it has been often hypothesized that QAC molecules immobilized to a substratum surface possess other generic mechanisms of action than QACs in solution, but these have never been elucidated.

Whereas there is ample evidence to support the known mechanism of antimicrobial activity of QACs in solution, we here propose a more generic mechanism of antimicrobial activity for immobilized QACs on a substratum or for that matter, positively charged surfaces in general. Gottenbos et al. demonstrated that bacteria showing little desorption from surfaces had more difficulty to divide and grow than bacteria adhering more reversibly, which suggests that the strength of adhesion may be determinant for their growth. In addition, Liu et al. coined the term "stress de-activation" for bacteria adhering strongly to positively charged surfaces to indicate reduced resistance to antimicrobials when stress was applied on the cell membrane due to strong adhesion forces arising from a substratum surface. Opposite to surfaces that attract bacteria strongly, are highly hydrated, polymer-brush coated surfaces, to which bacteria adhere very weakly and remain susceptible
to antimicrobials \textsuperscript{21,36}. These different responses of bacteria upon adhesion to a substratum surface \textsuperscript{7} led us to distinguish three hypothetical regimes of bacterial adhesion forces with a surface:

1. A "planktonic" regime, in which bacteria do not experience that they are adhering weakly on a surface and remain in their planktonic phase, rendering them susceptible to antimicrobial and other environmental attacks;

2. An "interaction" regime, where a battery of defensive mechanisms is switched on by the bacteria in response to their adhering state, as sensed by membrane deformation due to adhesion forces exerted upon them;

3. A "lethal regime" in which strong adhesion forces de-activate the adhering bacteria to impede growth and cause cell death.

In Figure 1 we present atomic force microscopic (AFM) and fluorescence images of \textit{Staphylococcus epidermidis} ATCC 12228 adhering to surfaces exerting different adhesion forces. On a negatively charged glass surface, staphylococci experience a very small adhesion force of around 1 nN (Figure 1-panel IA) in the above defined planktonic regime. As a consequence of weak Lifshitz-Van der Waals attraction, all adhering bacteria were displaced during AFM imaging (Figure 1-panels IA and I-B). Fluorescence imaging however, showed that staphylococci adhering to glass were all alive during exposure to buffer (Figure 1-panel IIA), whereas at the same time they were highly susceptible to QACs in solution (Figure 1-panel IIB), confirming that the adhering staphylococci remained in their susceptible, planktonic state. Imaging of staphylococci adhering to a moderately positively charged poly-L-lysine coated glass surface yielded stronger adhesion forces as a result of electrostatic attraction in addition to Lifshitz-Van der Waals attraction (4 nN, see Figure 1-panel IC) in the interactive regime, enabling imaging (panel IC) and showing live bacteria during exposure to buffer (panel IIC). During
imaging while being exposed to QACs in solution however, wrinkling of the bacterial cell surface occurred $^{10}$, eventually leading to detachment of entire bacteria leaving only minor remnants (panel ID). Fluorescence imaging showed bacterial death during exposure to QACs in solution within 60 min (panel IID). More interestingly, we here repeated these experiments for staphylococci adhering to a hyperbranched coating $^{50}$, comprising a high positive charge density due to immobilized QACs, while being exposed in a phosphate buffer and noticed that despite cell death (panel IIE), no indications of cell surface wrinkling and bacterial detachment could be observed (Figure 1, panel IE). Adhesion forces between the staphylococci and these hyperbranched coatings were extremely high around 100 nN in the lethal regime, as a result of the binding of multiple QAC molecules to the bacterial cell surface through electrostatic attraction.

These observations indicate that immobilized QACs do not cause directly visual membrane damage. Instead, the data point out that the strong adhesion forces arising from immobilized QACs enter bacterial adhesion forces into the lethal regime, i.e. where the stress exerted on the bacterial cell membrane is causing killing. This is a new and generic mechanism for the antimicrobial activity of immobilized QACs that explains many poorly-understood phenomena with respect to the antimicrobial activity of immobilized QAC molecules on a generic basis, including the persistence of antimicrobial activity despite the presence of an adsorbed protein film. Whereas initially bacterial adhesion forces may be attenuated through the presence of an adsorbed protein film, it is known that adsorbed protein films deform during bacterial adhesion $^{37}$ to become squeezed away from underneath an adhering bacterium, which restores lethally strong adhesion forces exerted by the coating of immobilized QACs. This new and generic physico-chemical mechanism of bacterial killing by immobilized QACs supports a recent hypothesis by Bieser and Tiller $^5$ that a positive charged
surface may exert strong forces upon vital anionic lipids in the bacterial cell membrane to yield their removal through the outermost cell surface of an adhering bacterium. This then creates membrane damage and causes cell death. Evidence in support of this mechanism of action can also be taken from observations that immobilized QACs are only antimicrobially active provided that the positive charge density is above a threshold of about $1.6 \times 10^{-4}\text{C/cm}^2$\textsuperscript{26,35}. Additional support is provided by experiments on conducting materials\textsuperscript{46}, showing that an external voltage on the material also yields killing of adhering bacteria, which we can now attribute to increased adhesion forces between the organisms and the material due to the charge applied, yielding removal of charged membrane lipids.

In summary, we propose 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 is based on adsorption, ion-exchange and membrane damage, immobilized QAC molecules enhance the adhesion forces between a bacterium and a substratum surface to a lethally strong attraction, causing reduced growth, stress de-activation and removal of membrane lipids, eventually leading to cell death.

An Experimental Section (including information on the bacterial strain used and its culture conditions, preparation of the (hyperbranched) coatings, imaging and adhesion force measurements and the assessment of bacterial killing) is included in the Supporting Information.
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Figure 1

(I) AFM deflection images of *S. epidermidis* ATCC 12228 adhering on different glass surfaces during exposure to 10 mM potassium phosphate buffer at pH 7.0 (A, C and E) or a 1 x MBC QAC solution in 10 mM potassium phosphate buffer (B and D). (A and B) negatively charged glass surface (note that all adhering bacteria are removed by scanning), (C and D) poly-L-lysine coated glass surface, (E) hyperbranched QAC coating, immobilized on a glass surface. Images were taken at different time points, while scanning continuously at a rate of 1 Hz. The bar denotes 1 µm.

(II) Fluorescence images of *S. epidermidis* ATCC 12228 adhering to the different glass surfaces (see panel I) after exposure to potassium phosphate buffer (A, C and E) or a 1 x MBC QAC solution (B and D). Bacteria have been stained with Baclight ® Live-Dead stain, rendering dead bacteria (or technically more correct according to the working mechanism of the stain: membrane damaged bacteria) red fluorescent, opposed to live bacteria showing green fluorescence. The bar denotes 18 µm.
Reference List


Supporting Information

Bacterial strain and culture condition

*Staphylococcus epidermidis* ATCC 12228, originating from blood of a patient with an intravascular catheter infection was used in this study. The strain was streaked on a blood agar plate from a frozen stock (7 v/v% DMSO) and grown overnight at 37°C. One colony was inoculated in 10 mL tryptone soya broth (TSB, Oxoid, Basingstoke, UK) and incubated for 24 h at 37°C. This culture was used to inoculate a main culture of 200 mL TSB, which was incubated for 16 h. Bacteria were harvested by centrifugation for 5 min at 5000 g and 10°C and subsequently washed two times with 10 mM potassium phosphate buffer, pH 7.0.

Preparation of (hyperbranched) coatings

Materials

Glass slides were obtained from Waldemar Knittel Glasbearbeitungs-GmbH, (Braunschweig, Germany) and cleaned in RBS 35 (Omnilabo International BV, The Netherlands). Poly(ethyleneimine) 750 kDa 50 wt% in water, bishexamethylene triamine, iodomethane, tert-amyl alcohol, poly-L-lysine (all Sigma-Aldrich), 1-bromohexane, potassium hydroxide, dimethylformamide (all Across organic), carbonyl biscaprolactam (DSM, Geleen, The Netherlands), ethanol (Merck, Darmstadt, Germany), methanol and toluene (all Lab-Scan) were used as received. AB₂ monomer (N,N'-(azanediylbis(hexane-6,1-diyl))bis(2-oxoazepane-1-carboxamide) was synthesized from carbonyl bispicaprolactam and bishexamethylene triamine. The coupling agent (2-oxo-N(3-triethoxysilyl)propyl)azepane-1-carboxamide)
was synthesized from carbonyl biscaprolactam and (3-aminopropyl) triethoxysilane.

**Cleaning of the glass surface**

Glass slides (2.5 cm x 2.5 cm) were sonicated 3 min in 2% RBS 35 followed by thorough rinsing with tap water, demineralized water, methanol, tap water, and finally demineralized water and dried in air, yielding a zero degrees water contact angle.

**Preparation of poly-L-lysine coated glass**

The glass slides were immersed in a 0.01% (wt/vol) poly-L-lysine solution for 20 min at room temperature (RT) after which the slides were rinsed with water followed by drying in air.

**Preparation of a hyperbranched QAC coating**

Hydrophilic glass slides were immersed for 10 min in 3 v/v% solution of the coupling agent in ethanol, placed in a vacuum oven and heated at 110°C for 2 h, after which the samples were washed with ethanol. A solution of AB₂ monomers (20 wt% in ethanol, 80 µl) was dropped on the glass slide and spin coated (1 min at 2000 rpm). Polymerization of AB₂ monomers on the surface was carried out at 145°C for 2 h under nitrogen. Unreacted compounds were removed by extraction in DMF overnight at 115°C and sonication in 100 mL ethanol for 20 min at RT. The coated glass slides were dried and stored under nitrogen.

A solution of poly(ethyleneimine) in water (PEI, 50 wt%) was freeze-dried overnight and the residue was dissolved in methanol (20 wt%). Ninety µL PEI (20 wt%) was dropped on the hyperbranched polymer coating and
spin-coated (1 min at 2000 rpm). The coupling reactions were carried out at 125°C for 52 h under nitrogen. Unreacted PEI was removed with methanol in an ultrasonic bath for 20 min at RT followed by extraction in methanol at 65°C overnight and again sonication in methanol for 20 min at RT, followed by drying under nitrogen. Next, the samples were immersed in 50 mL of 1-bromohexane and heated under nitrogen at 90°C overnight. A suspension of 0.2 g KOH in 50 mL tert-amyl alcohol was added and the reaction was continued for another 3 h. The samples were sonicated in 100 mL methanol for 20 min, after which the samples were immersed in a solution of iodomethane (15 v/v% in tert-amyl alcohol). The alkylation reaction was carried out at 42°C overnight, afterwards the samples were washed by extraction in refluxing methanol overnight and sonication in methanol for 20 min at RT, followed by drying under nitrogen.

**Atomic force microscopy**

AFM experiments were conducted at room temperature in potassium phosphate buffer as a control and in potassium phosphate buffer supplemented with QAC at their planktonic minimal bactericidal concentration (MBC, found to be 150 µg/mL for this particular strain). A Dimension 3100 with a Nanoscope IV Digital Instrument from Veeco (Woodbury, USA), was used for imaging staphylococci.

For imaging, bacteria were attached to the differently treated glass slides by placing a droplet of bacterial suspension ($10^{10}$ bacteria/mL) in buffer on the glass slides for 30 min. Subsequently, the bacterially coated glass slide was rinsed with potassium phosphate buffer to remove free floating bacteria and the glass slide was immediately used for AFM measurements without drying. The adhering bacteria on the glass slide were scanned with the AFM, while immersed either in buffer or buffer supplemented with QAC. Deflection
images were taken while repetitively scanning 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). The experiments were performed in triplicate with different bacterial cultures.

For adhesion force measurements using AFM (AFM; BioScope Catalyst AFM with ScanAsyst Veeco, Camarillo, California, USA), NP-0 tipless cantilevers were employed. Before each measurement, cantilevers were calibrated using the thermal tuning method and actual spring constants were always found to be close to the producer's specification of 0.06 N/m. For measurements on hyperbranched, positively charged coatings, a stiffer cantilever (Cantilever A) had to be used with a spring constant of 0.58 N/m. Bacterial probes were prepared by immobilizing single bacteria to a cantilever and always used immediately after preparation. All adhesion force measurements were performed in potassium phosphate buffer under a loading force of 5 nN and at three randomly chosen spots on glass, poly-L-lysine coated glass and hyperbranched QAC coatings. At least 15 retract force-curves were recorded on one spot, requiring a minimum of at least six bacterial probes.

**Evaluation of contact-killing of adhering bacteria**

For evaluation of contact-killing of adhering bacteria by the different coatings, the slides were incubated in a staphylococcal suspension (3 x 10^7 bacteria/mL) in a 6-well polystyrene plate (Greiner Bio-One B.V., Alphen a/d Rijn Leiden, The Netherlands) containing either potassium phosphate buffer or a QAC solution at 1 x MBC. After incubation for 60 min and 300 min at 37°C under rotation (90 rpm), bacterial suspension was removed and the substrata were gently washed with potassium phosphate buffer to remove the free-floating bacteria. Confocal laser scanning microscopy (CLSM) was employed to
differentiate between live and dead bacteria, to which end the samples were stained in the wells with 250 µL LIVE/DEAD BacLight viability stain (Molecular Probes, Leiden, The Netherlands) containing SYTO 9 dye (fluorescent green) and propidium iodide (fluorescent red). Staining was done for 15 min in the dark. Confocal images were collected using a Leica TCS-SP2 CLSM (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) using 488 nm excitation and emission filters of 500 to 550 nm (green, live) and 605 to 720 nm (red, dead). This assay reveals dead bacteria, or technically more correct considering the working mechanism of the stain \(^1\), bacteria with a damaged cell membrane, as red fluorescent, whereas live organisms expressing an intact membrane appear green fluorescent.

### Reference List


