FULL PAPER

A Shape-Adaptive, Antibacterial-Coating of Immobilized Quaternary-Ammonium Compounds Tethered on Hyperbranched Polyurea and its Mechanism of Action

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Quaternary-ammonium-compounds are potent cationic antimicrobials used in everyday consumer products. Surface-immobilized, quaternary-ammonium-compounds create an antimicrobial contact-killing coating. We describe the preparation of a shape-adaptive, contact-killing coating by tethering quaternary-ammonium-compounds onto hyperbranched polyurea coatings, able to kill adhering bacteria by partially enveloping them. Even after extensive washing, coatings caused high contact-killing of *Staphylococcus epidermidis*, both in culture-based assays and through confocal-laser-scanning-microscopic examination of the membrane-damage of adhering bacteria. In culture-based assays, at a challenge of 1600 CFU/cm^2, contact-killing was >99.99%. The working-mechanism of dissolved quaternary-ammonium-compounds is based on their interdigitation in bacterial membranes, but it is difficult to envisage how immobilized quaternary-ammonium-molecules can exert such a mechanism of action. Staphylococcal adhesion forces to hyperbranched quaternary-ammonium coatings were extremely high, indicating that quaternary-ammonium-molecules on hyperbranched polyurea partially envelope adhering bacteria upon contact. These lethally strong adhesion forces upon adhering bacteria then cause removal of membrane lipids and eventually lead to bacterial death.

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1. Introduction

Quaternary-ammonium-compounds (QACs) are potent cationic antimicrobials used in everyday consumer products, like deodorants, contact lens solutions and mouth- and hair-rinse products, as well as in numerous industrial processes.[1] Antimicrobial action of QACs starts when they approach bacterial cell surfaces close enough to interact through hydrophobic and electrostatic attractions[2–5] between positively-charged QAC-molecules and negatively-charged bacterial cell surfaces.[6] Upon their adsorption, QAC-molecules replace Ca^{2+} and Mg^{2+} ions from the cytoplasmic membrane to maintain charge neutrality in the membrane. This ion exchange destabilizes the intracellular matrix of a bacterium,[7] as the hydrophobic tail interdigitates into the hydrophobic bacterial membrane over the entire surface area of a bacterium,[8–10] causing dissolution of the proton motive force and leakage of intracellular fluid containing essential molecules. Both processes result in bacterial cell death.[11]

Antimicrobial efficacy of QACs remains preserved when QAC-molecules are immobilized on a surface.[4,12–15] Such contact-killing coatings have potential in widely-varying applications, including but not limited to surgical equipment and protective apparel in hospitals,[16] medical implants and wound dressings,[17] water purification,[18] food packaging materials[19,20] and industrial equipment.[21] Use of QACs has been popular since they are easily produced and can be conveniently incorporated in coating systems. QACs are also stable in the human body, poorly metabolized and mainly excreted in non-metabolized form.[22] QACs can be hemolytic when not immobilized on a surface and environmentally toxic.[23]

Despite evidence of antimicrobial activity of immobilized QAC-molecules, it is difficult to envisage how immobilized QAC-molecules can exert antimicrobial activity through the same mechanism as QACs in solution. To this point, a Gram-negative *Pseudomonas aeruginosa* strain, not susceptible to QACs in solution, was killed upon adhesion to a coating of

immobilized QACs.[24] Intriguingly, coatings of immobilized QACs remain antimicrobially active after adsorption of proteins *in vitro*[4,25,26] and under *in vivo* conditions.[17,27] Immobilized QACs are hampered in their search for heterogeneously distributed negative charges on bacterial surfaces,[28] as is crucial for their efficacy in solution. Hence, it has been hypothesized[24] but never experimentally demonstrated that QAC-molecules immobilized to a surface must possess another mechanism of action than QACs in solution.

QAC-molecules immobilized on a surface only exert contact-killing when the positive charge density is above a well-defined threshold.[29,30] A limitation of current contact-killing coatings is that the contact area between an adhering organism and a coating is generally small and mainly depending on the deformability of the bacterial cell surface. Therefore, we here describe the preparation of a shape-adaptive coating to enhance the contact area with adhering bacteria, which was characterized in detail by Fourier transformed infrared spectroscopy (FTIR), X-ray photoelectron-spectroscopy (XPS) and water contact angles. This new, contact-killing, multi-layered coating is based on novel hyperbranched polymers, comprising blocked isocyanates,[31] on which multiple QAC-molecules can be covalently tethered. Contact-killing by these coatings was determined in a culture-based assay and by confocal laser scanning microscopic (CLSM) examination of adhering staphylococci after live-dead staining. Leaching of antibacterial compounds was ruled out. Moreover, by comparing effects of QAC-molecules in solution on adhering bacteria with those of QACs immobilized in our hyperbranched coating using Atomic-Force-Microscopy (AFM), we provide evidence to support a new, generic working mechanism for bacterial contact-killing. The study was conducted with *Staphylococcus epidermidis*, as this is the most important colonizer of the human skin and the main causative organism in infections associated with the use of biomaterial implants and devices.[32]

2. Results

2.1. Preparation and Characterization of the Immobilized QAC-tethered Hyperbranched Polyurea

An enabling technology has been developed to make hyperbranched polyurea coatings based on polycondensation of AB_2 monomers.[31] The AB_2 monomers comprised a secondary amine as the A-groups and blocked isocyanates as the B-groups (**Figure 1**).[33] Resulting hyperbranched polyurea groups were provided with one secondary amino group in the focal point and numerous blocked isocyanates at the end of the polymer branches. To anchor these hyperbranched polyurea covalently, a siloxane comprising blocked isocyanate compound was first

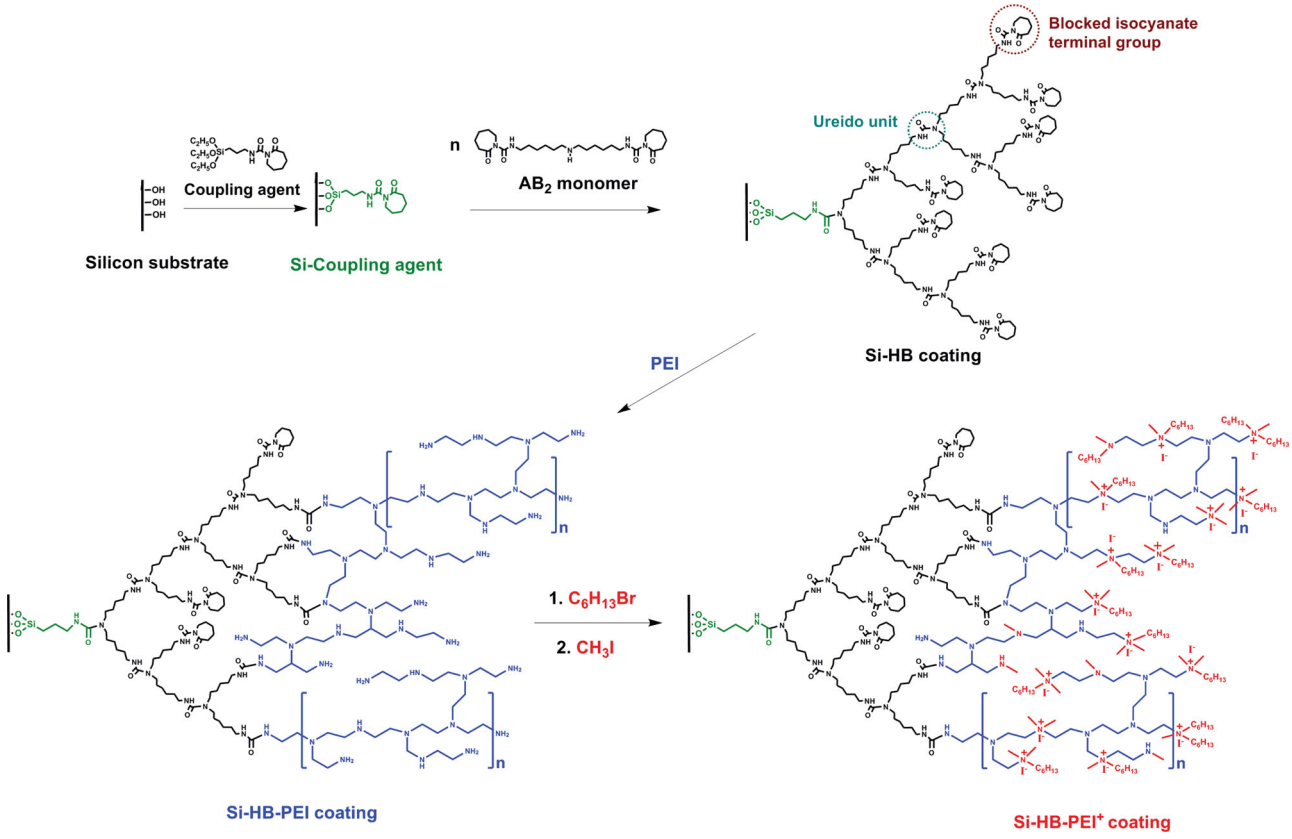

Figure 1. Schematics of the preparation of covalently attached hyperbranched polyurea coatings, tethering of polyethyleneimine and two-step alkylation of PEI that was covalently coupled onto hyperbranched polyurea coatings

covalently grafted onto the silanol groups of the glass slides, allowing the exposed blocked isocyanate groups to react with the focal amino group of the AB_2 monomers or of the hyperbranched polyurea (Figure 1). Thickness of the hyperbranched polyurea coatings (Si-HB coatings) depended on the concentration of AB_2 monomers in the spin-coated solution and coatings with thicknesses ranging from a few nm up to several µm could be realized. After polymerization, non-covalently attached compounds were removed by sequential sonication in ethanol for 20 min, extraction in DMF at 115 °C overnight and a second sonication in ethanol for 20 min. Ethanol and DMF were selected because of the good solubility of the polymers employed in these solvents.

Versatility of this coating concept stems from the presence of numerous blocked isocyanate groups at the periphery of the hyperbranched polyurea, allowing for fixation of a variety of amine-comprising compounds. This hyperbranched polyurea coating however,[31] does not contain any antimicrobial functionality and therefore we here coupled an antimicrobial functionality to the hyperbranched polyurea. To maximize the number of quaternizable amino groups, polyethyleneimine (PEI) was selected as amine-comprising compound. Due to the high concentration of PEI, only a few of the amino groups can react with the blocked isocyanate moieties of the flexible hyperbranched coatings. Because of the limited space for the anchoring points, the flexibility of the coupled PEI was preserved. Unreacted PEI was removed by sonication in methanol for 20 min, extraction in methanol at 65 °C overnight and sonication in methanol for 20 min again.

In this case, 10 wt% and 20 wt% concentrations of PEI drop-casted solutions were used to investigate the effect of the amount of PEI on contact-killing of adhering bacteria. Progress of the surface reaction between Si-HB coating and PEI, resulting in a Si-HB-PEI coating, was followed by transmission FTIR (Figure S1). The reduction of the carbonyl peak of caprolactam at 1701 cm^{-1} and the increase of the ureido carbonyl peak at 1668 cm^{-1} demonstrated reaction progress. The C–N absorption band of PEI was found in the range 1000–1300 cm^{-1}. After two consecutive alkylation steps, amino groups of the Si-HB-PEI coating were converted into hydrophobic, polycationic species (Si-HB-PEI$^+$ coating, see Figure 1). To minimize the possibility that components leached out and influenced the contact-killing of adhering bacteria, samples were sonicated in methanol for 20 min, extracted for 1 day in methanol at 65 °C and again sonicated in methanol for 20 min.

To determine the composition of the outermost layer of the coatings, XPS was performed after each functionalization step. The final coating should contain, besides quaternary-ammonium-ions, iodide as counter ions. Indeed, survey spectra (Figure S2A) reveal the overall composition of the film and show the iodide counter ion peak in the Si-HB-PEI$^+$ coating, indicating the presence of cationic species. Analysis of the N1*s* core level region showed that Si-HB coatings contained one amine-related peak at a binding energy of 399.7 eV with a full width at half maximum of 1.7 eV, accounting for 7.9 at% of the surface composition; this peak originates from nitrogen of the caprolactam and the ureido moieties (Figure S2B).[34–36] After modification of Si-HB coating with PEI, the N1*s* photoemission line became broader with a full width at half maximum of 2.2 eV due to small uncompensated charging in the thicker film despite optimization of the flood-gun parameters, confirming the coupling of PEI. After the alkylation steps, the N1*s* core level spectra of the Si-HB-PEI$^+$ coating could be deconvoluted into two components. The main component at 400.3 eV corresponds to secondary and tertiary nitrogen of the PEI backbone. A shoulder at a higher binding energy was found at 402.2 eV, indicative of cationic quaternary-ammonium-species[35] and accounting for 11% of the total N1*s* peak intensity. This implies that the QAC-nitrogen makes up 1 at% of the surface composition of the outermost layer of the coating, which is comparable to the surface concentration of anionic iodide (0.8 at%). Surface charge densities (measured by fluorescein complexation)[4] of both 10 wt% and 20 wt% Si-HB-PEI$^+$ coatings were comparable in the order of 10^{15} N^+ cm^{-2} (**Table 1**), meaning that 10 wt% is sufficient for a complete coverage of the surface with PEI. Importantly, these surface charge densities are above the threshold of 10^{15} N^+ cm^{-2}, reported to be required to yield effective contact-killing.[29,30]

Table 1. Charge density of different coatings and contact-killing, expressed in percentages with respect to the bacterial challenge applied, of *S. epidermidis* ATCC 12228 in the Petrifilm assay. Numbers of colony forming units (CFUs) for the lowest challenge were verified in a Petrifilm assay without a sample, while higher challenges were derived from a dilution series. Data are presented as means over three experiments with separately prepared bacterial cultures and coatings.

Samples	Charge density [N^+ cm^{-2}]	Bacterial challenge per cm^2		
		16 CFU	160 CFU	1600 CFU
Bare glass	0	<1%	<1%	TMTC[a)]
Si-HB coating	0	<1%	<1%	TMTC
Si-HB-PEI$^+$ coating (10 wt%)	6×10^{15}	87%	98%	>99.9%
Si-HB-PEI$^+$ coating (20 wt%)	4×10^{15}	>99%	>99.9%	>99.99%

[a)]TMTC: too many to count, indicating absence of quantifiable bacterial killing.

Water contact angles on the Si-HB-PEI$^+$ coated glass were 51 ± 3 degrees, which is somewhat lower than the contact angles measured on glass slides coated with only the coupling agent (65 degrees) or with the Si-HB coating (66 degrees). The decrease in hydrophobicity can be explained by the presence of charged groups. Nonetheless, despite these charges, the coatings were not in the hydrophilic regime because of the shielding of the positive charge of the quaternary-ammonium-ions by hydrophobic alkyl chains, important to create good contact-killing of adhering bacteria.[37]

2.2. Bacterial Contact-Killing

Contact-killing of *Staphylococcus epidermidis* ATCC 12228 was evaluated in a Petrifilm assay (Table 1) and using CLSM (**Figure 2**). No contact-killing was observed on bare glass slides and Si-HB coatings in the nutrient-rich environment of the Petrifilm assay. The Si-HB-PEI$^+$ coating (10 wt%) showed 87% reduction in the number of colony forming units (CFU) at the lowest bacterial challenge, increasing to >99.9 at the

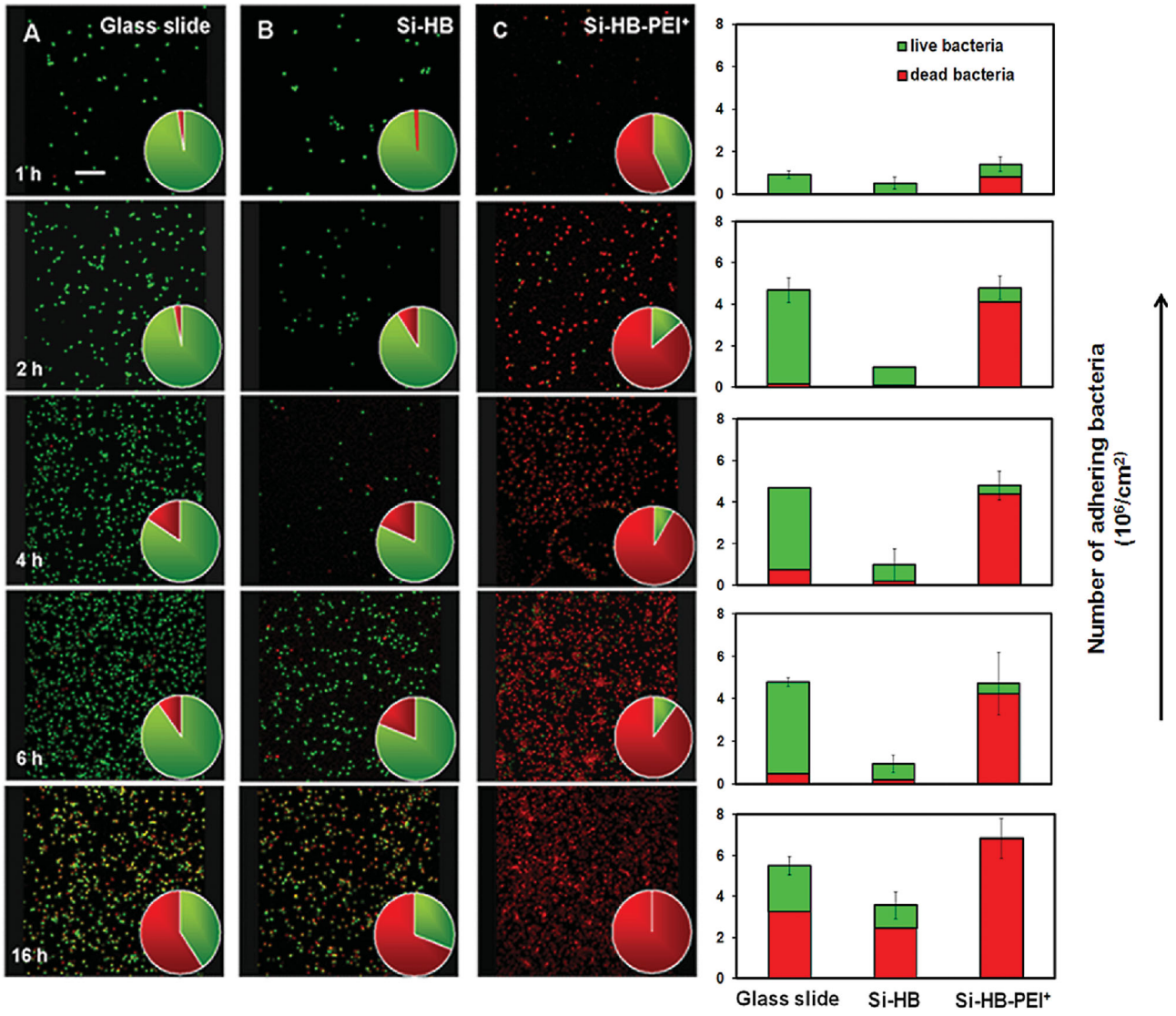

Figure 2. Contact-killing of *S. epidermidis* ATCC 12228 by quaternary-ammonium-compounds tethered onto hyperbranched polyurea in potassium-phosphate-buffer. CLSM overlay projections of live (green fluorescent) and dead (red fluorescent) *S. epidermidis* ATCC 12228 after incubation for 1, 2, 4, 6 and 16 h at 37 °C on different substrata together with the number of adhering live and dead *S. epidermidis* ATCC 12228 per unit area; corresponding percentages of live and dead bacteria are plotted in the images in the right corner and the numbers of live and dead bacteria are plotted in the panels on the right. (A) Glass (note that after 16 h dead bacteria appear due to nutrient-deprivation in potassium-phosphate-buffer); (B) Si-HB (note that after 16 h dead bacteria appear due to nutrient-deprivation in potassium-phosphate-buffer); (C) Si-HB-PEI$^+$ (20 wt%). Errors bars denote standard deviations over three separate experiments with different bacterial cultures and coatings, while percentages live and dead bacteria were obtained from a total of 6 images taken over the different experiments and comprising a minimum of 100 bacteria.

highest challenge of 1600 CFU/cm^2 (see Table 1). The 20 wt% Si-HB-PEI$^+$ coating displayed >99% and >99.9% contact-killing at bacterial challenges of 16 and 160 CFU/cm^2, respectively increasing to >99.99% contact-killing at the highest challenge. Contact-killing by the Si-HB-PEI$^+$ (10 wt%) coating was slightly less than that by the Si-HB-PEI$^+$ (20 wt%) coating, despite the fact that both comprised comparable numbers of immobilized cationic quaternary-ammonium-species and surface charge densities above the required threshold for contact-killing. This suggests that the Si-HB-PEI$^+$ (20 wt%) coating is more flexible, allowing positively charged groups to better envelope and shape-adapt to an adhering bacterium.

CLSM analysis of the contact-killing of adhering staphylococci on the different surfaces (Figure 2) in potassium-phosphate-buffer, i.e. in absence of nutrients, also indicated a strong contact-killing ability of our coatings and a significant number of dead (or technically more correct "membrane-damaged"), adhering bacteria was observed already after 1 h of incubation to a Si-HB-PEI$^+$ (20 wt%) coating (57% ± 14%, $p < 0.005$, Student's t-test), while glass slides and Si-HB coatings showed no significant contact-killing upon incubation. Interestingly, while the number of adhering bacteria on Si-HB-PEI$^+$ (20 wt%) coatings increased with incubation time up to 7×10^6 bacteria/cm^2, the percentage bacterial killing reached a steady state of around

95% between 2 and 6 h. After 16 h, 100% bacterial killing was achieved, but this includes effects of bacterial starvation due to nutrient-deprivation in potassium-phosphate-buffer.

During the preparation of our coatings, we paid considerable attention to remove all leachable compounds that may interfere with the contact-killing mechanism of the coatings. Absence of leachable compounds from thin coatings is hard to demonstrate analytically, but bacteria are extremely sensitive to possible antibacterial compounds leaching out of the coatings. Zones of inhibition around Si-HB-PEI$^+$ coated samples on bacterially inoculated agars were found to be completely absent (Figure S3), ruling out the possibility that antibacterial compounds leached out of the coatings.

2.3. Mechanism of Bacterial Killing of Dissolved *versus* Immobilized Quaternary-Ammonium-Compounds

In order to derive a mechanism for bacterial contact-killing by immobilized QACs *versus* killing by dissolved QACs, we applied AFM. To this end, we repeatedly imaged (**Figure 3**) *S. epidermidis* ATCC 12228 adhering to i) negatively-charged glass, ii) glass coated with α-poly-L-lysine as common in AFM imaging of microorganisms[39–41] and iii) a Si-HB-PEI$^+$ coating (20 wt%) while being exposed to 10 mM potassium-phosphate-buffer or a QAC solution at 1x MBC (minimal bactericidal concentration). In addition, bacterial probe AFM was performed to measure the forces with which staphylococci adhered to the different surfaces and CLSM images of the adhering staphylococci were taken after live-dead staining to visualize possible bacterial membrane-damage. On a negatively-charged glass surface, staphylococci experience small adhesion forces of around 1 nN (Figure 3 – panel 1a). As a consequence of these weak adhesion forces, all adhering bacteria were displaced during AFM imaging (Figure 3 – panels 1a and 1b). Fluorescence imaging showed that staphylococci adhering to glass were all alive, possessing intact membranes, during exposure to buffer (Figure 3 – panel 2a), whereas they were susceptible to QACs in solution (Figure 3 – panel 2b). Imaging of staphylococci adhering to positively-charged α-poly-L-lysine coated glass yielded stronger adhesion forces as a result of electrostatic attraction in addition to Lifshitz-Van der Waals attraction (4 nN, Figure 3 – panel 1c) and showing live bacteria during exposure to buffer (Figure 3 – panel 2c). During imaging while being exposed to QACs in solution however, the bacterial surfaces wrinkled and disintegrated, leading to detachment of entire bacteria with only minor remnants left behind (Figure 3 – panel 1d). CLSM imaging showed bacterial death during exposure to dissolved QACs within 60 min (Figure 3 – panel 2d). This attests to membrane-damage over the entire cell surface. These observations hold QACs, but also for cationic antimicrobial peptides (Figure S4), suggesting that QACs might be regarded as "synthetic antimicrobial peptides". More interestingly, for staphylococci adhering to the Si-HB-PEI$^+$ hyperbranched coating (20 wt%), cell death was observed while being exposed to phosphate-buffer (Figure 3 – panel 2e). However, neither cell surface wrinkling nor increases in bacterial surface roughness, as occurring during exposure to dissolved cationic antimicrobials (Figure S4), could be observed. Bacterial detachment was not detected either (Figure 3 – panel 1e). From this, we conclude that membrane-damage is confined to the enveloping region between a bacterium and the hyperbranched coating. Adhesion forces between adhering staphylococci and hyperbranched coatings were extremely high around 100 nN. This is above all previous measurements of bacterial adhesion forces to surfaces,[39,41] demonstrating envelopment of the bacterial surface by multiple QAC-molecules through electrostatic attraction. As a result of these high adhesion forces and localized membrane-damage, staphylococci appeared more pancake-shaped than spherical.

3. Discussion

We have prepared a new, highly effective contact-killing coating consisting of hyperbranched, cationic QAC-molecules comprising polymers. Opposite to other positively charged PEI$^+$ coatings using a short chain coupling agent to tether PEI to a surface,[13,42,43] we applied a flexible, hyperbranched coating on a surface and tether PEI to it in a bulk reaction, i.e. in high concentrations. This implies that the number of amino groups of PEI involved in the anchoring will be limited, again yielding a more flexible surface, able to envelope adhering bacteria. The ability of our coating to kill adhering bacteria upon contact was confirmed both under nutrient-rich conditions, i.e. in culture-based Petrifilm® assays and under nutrient-deprivation, i.e. using CLSM examination after live-dead staining of adhering bacteria. Therewith we used two methods with opposite nutrient-conditions, whose relevance depends on the application aimed for. Technically, live-dead staining only yields an indication of possible membrane-damage[44] but not necessarily of bacterial cell death as can only be concluded from culture-based assays. In a completely different, airborne-assay to quantify bacterial contact-killing,[4,45] a bacterial suspension is sprayed onto a contact-killing surface and spray-coated bacteria are allowed to dry onto the coating after which the material is placed in a Petridish and covered by nutritious agar for CFU evaluation. Drying of bacteria onto the coating reflects conditions of nutrient-deprivation with possible effects on bacterial viability (see our Figure 2) and at the same time creates an intimate contact between bacterial cell surfaces and immobilized QACs, enhancing their contact-killing efficacy with respect to killing in a liquid phase. Under the experimental conditions as applied with our hyperbranched coatings, there is always a water layer present. However, again depending on the applications aimed for (i.e. airborne or waterborne bacterial contamination of a surface), bacterial adhesion from an aqueous phase may or may not be more appropriate for evaluation. Most, if not all papers describing contact-killing of adhering bacteria by positively-charged coatings neglect the possibility of bacterial killing due to leaching of antibacterial compounds. In order to conclude that our hyperbranched Si-HB-PEI$^+$ coating kills adhering bacteria solely through a contact-killing mechanism, we thoroughly washed our coatings after the various steps in the coating procedure. As a consequence, no growth inhibition was observed around Si-HB-PEI$^+$ coated glass slides on bacterially inoculated agar plates (see Figure S3) indicating absence of leachable antibacterial compounds. Also in this respect, the use of multiple methods to establish contact-killing is important,

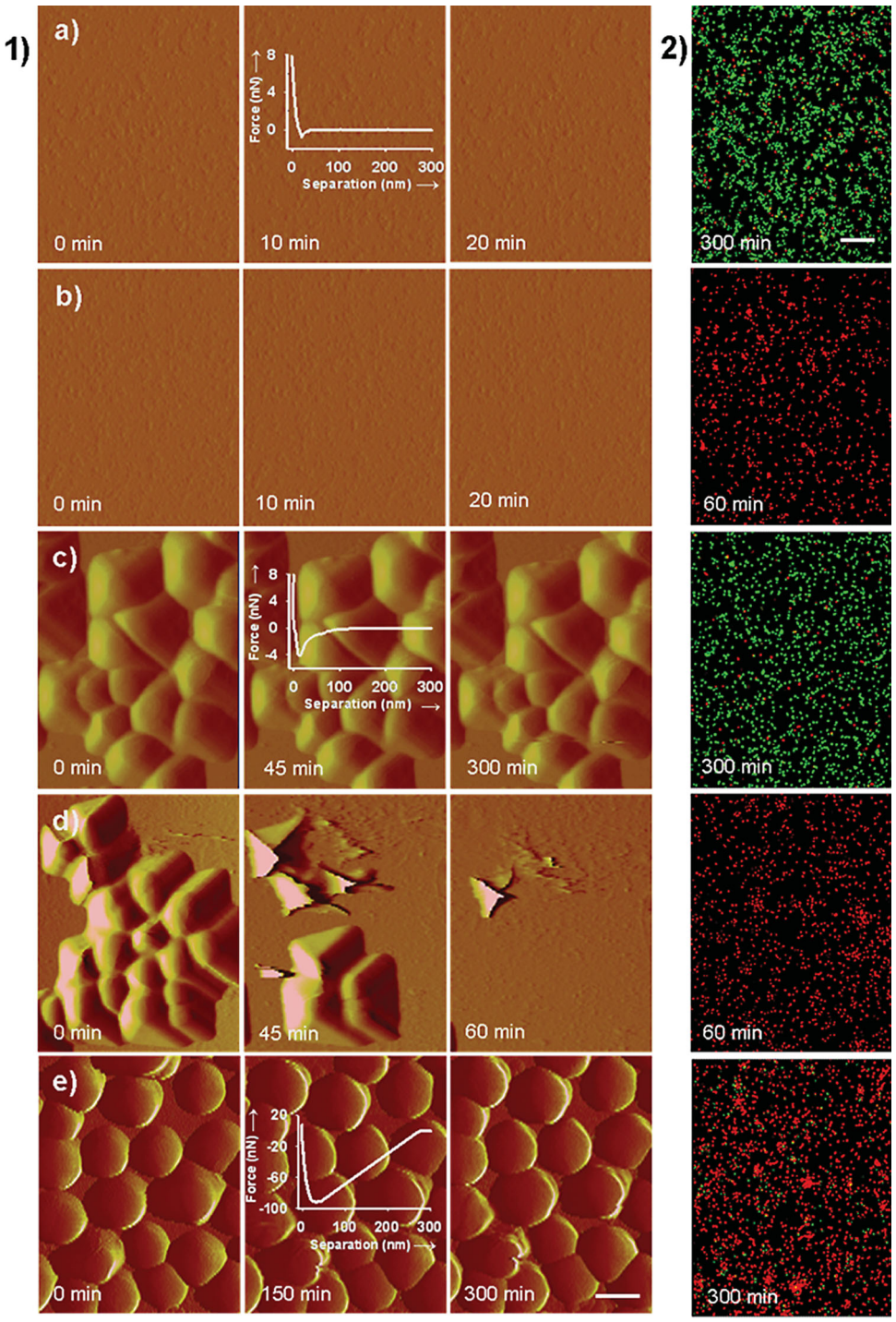

Figure 3. Morphology and viability of adhering *S. epidermidis* ATCC 12228 on different surfaces in absence and presence of dissolved QACs, together with the staphylococcal adhesion forces. Panel 1: AFM deflection images of *S. epidermidis* adhering on different surfaces during exposure to 10 mM potassium-phosphate-buffer at pH 7.0 (a, c and e) or a 1 × MBC QAC solution in 10 mM potassium-phosphate-buffer (b and d). a),b) negatively-charged glass surface (note that all adhering bacteria are removed by scanning) c),d) α-poly-L-lysine coated glass surface (Adapted with permission.[38] Copyright 2011, American Society for Microbiology. e) Si-HB-PEI$^+$ coating (20 wt%), 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. The graphs overlaid on the images represent examples of force-distance curves between *S. epidermidis* ATCC 12228 and the corresponding surface. Panel 2: Fluorescence images of *S. epidermidis* ATCC 12228 adhering to the different surfaces (see panel 1) after exposure to potassium-phosphate-buffer (a, c and e) or a 1 × MBC QAC solution (b and d). Bacteria were stained with *Bac*light live-dead stain, rendering dead bacteria (or technically more correct according to the working mechanism of the stain: severely membrane-damaged bacteria[42] red fluorescent, opposed to live bacteria showing green fluorescence. The bar denotes 18 µm.

because bacterial killing by leaching of antibacterial compounds can easily dominate over the contact-killing ability of a coating, depending on the fluid volume in which the experiments take place.

The possibility of contact-killing of adhering bacteria has slowly emerged from literature, starting with observations by Gottenbos et al.[27] that bacteria showing little desorption from surfaces had more difficulty to divide and grow than bacteria adhering more reversibly, showing that the strength of adhesion may be decisive for their growth. In addition, Liu et al.[46] coined the term "stress-induced de-activation" for bacteria adhering strongly to positively charged surfaces to indicate reduced resistance to antimicrobials. Staphylococcal adhesion forces on our hyperbranched Si-HB-PEI$^+$ coatings are far too high to allow bacterial desorption, as observed for adhesion forces of less than around 1 nN[47] and in fact exceed all currently known forces by which bacteria adhere to surfaces[39,41] by a factor of around six. We envisage that the branches of the hyperbranched trees with their cationic end-groups envelope the surface of an adhering bacterium to create a larger contact area and correspondingly higher adhesion forces than can be achieved with "regular" positively charged coatings. For this reason, we call our hyperbranched coatings "shape-adaptive".

Opposite to surfaces that attract bacteria strongly, are highly hydrated, polymer-brush coated surfaces, whereon bacteria adhere weakly and remain susceptible to antimicrobials.[47] These different responses of bacteria upon adhesion to a surface[48] have been categorized into three regimes of bacterial adhesion forces with a surface:

1. A "planktonic" regime, in which bacteria do not experience even a weak adhesion to a surface and remain in their planktonic phase, rendering them susceptible to antimicrobials;
2. An "interaction" regime, where membrane deformation due to adhesion forces is sensed, and defensive mechanisms are switched on by a bacterium;
3. A "lethal" regime, in which strong adhesion forces de-activate adhering bacteria to impede growth and cause cell death.

Comparison of the effects of QACs in solution and immobilized on a surface evidences major differences. Dissolved QACs yield clear membrane-damage and cell death, eventually causing detachment of adhering staphylococci or of its remnants by an AFM tip[38] in line with current views on the working mechanisms of dissolved QACs. Although our coating comprising immobilized QACs, yields demonstrable penetration of propidium iodide into the adhering organisms, with consequent localized membrane-damage, shape transformation and cell death, immobilized QACs do not bring forth membrane disintegration over the entire bacterial surface, as observed for dissolved QACs. This indicates a generic mechanism of antimicrobial activity for QACs immobilized on a surface or for that matter, positively-charged surfaces in general, based on adhesion forces according to the lethal regime outlined above. Such a mechanism explains many poorly understood phenomena with respect to the antimicrobial activity of immobilized QAC-molecules, including the persistence of antimicrobial activity in presence of adsorbed proteins. Whereas initially bacterial adhesion forces may be attenuated through the presence of adsorbed proteins, it is known that adsorbed protein films are displaced and deformed during bacterial adhesion[49] to reduce their thickness, restoring lethally strong adhesion forces exerted by immobilized QACs. Similarly, also bacterial surface structures have been demonstrated to slowly collapse during adhesion bringing the membrane closer to a substratum surface[50] to facilitate strong adhesion forces.

This new, physico-chemical mechanism of bacterial-killing by immobilized QACs for the first time experimentally supports a recent hypothesis by Bieser and Tiller,[51] that positively-charged surfaces may exert strong enough forces arising from the electric field accompanying the presence of the positive charges, upon vital anionic-lipids in bacterial membranes to yield their removal through the outermost surface of an adhering bacterium. This then creates localized membrane-damage and causes cell death.[52] Additional support is provided by experiments in which we covered a stainless steel electrode with Parafilm®, and allowed staphylococci to adhere and grow under an applied DC voltage, yielding a strong electric field with a minor current of 10 nA. Here we observed similar killing of adhering staphylococci (data not shown) as observed for our hyperbranched QAC coatings, caused by electrostatic attraction in the applied electric field and extraction of anionic-lipids out of the bacterial membrane, while the virtual absence of an electric current rules out electrochemical effects. Existence of a threshold or lethally strong adhesion force/electric field is in line with the current notion that immobilized QACs are only antibacterially active[29,30] when the positive-charge density is above a critical threshold (10^{15} N^+ cm^{-2} or 1.6×10^{-4} C cm^{-2}). The proposed mechanism disproves the putative requirement of calcium-ion exchange in bacterial-killing by immobilized QACs[29] and confirms that immobilized QACs can kill adhering bacteria despite the presence of surrounding Ca^{2+} ions (as in the human body) that may impede replacement of Ca^{2+} ions.

Although coatings of immobilized QACs on surfaces have many applications, they are increasingly considered for biomaterials implants and devices in order to prevent biomaterial-associated infections. Infection is the number one cause of failure of biomaterial implants and devices.[32] It is assumed by the general public that biomaterial implants and devices are inserted in a sterile way, yet the wound-site and the biomaterial implant surface may become bacterially contaminated during surgery. Estimates are that in a standard operating theater during a surgical procedure of 1 h, the total number of bacteria-laden particles falling into a wound amounts about 270 bacteria/cm^2,[53] which is in the lower range of the bacterial challenges applied (see Table 1). Since the immune system is compromised in the vicinity of a biomaterial, these per-operatively introduced bacteria can remain dormant for a long period of time to yield a clinically manifest infection many years post-implantation.[32] QAC coatings can help to eradicate these low numbers of per-operatively introduced bacteria prior to reaching sufficiently high numbers that allow generic network interactions such as quorum-sensing to occur and facilitate the formation of a mature biofilm.[54] Therewith QAC coatings will help to reduce the incidence of biomaterial-associated infection. For instance, Gottenbos et al.[27] demonstrated that QAC coatings on silicone rubber subcutaneously implanted in rats for 3 or 7 days and pre-operatively contaminated with *Staphylococcus aureus* resulted in infection in only 1 out of 8

animals, whereas on silicone rubber implants without a QAC coating, infection resulted in 7 out of 8 animals. Recently, it was demonstrated in a sheep trauma model, that orthopaedic-fracture-hardware painted with *N,N*-dodecylmethyl-PEI not only prevented implant colonization with biofilm but even promoted bone healing.[17] Based on the working mechanism for immobilized QACs revealed here, tissue damage is unlikely because mammalian cell membranes are comprised mainly of neutral zwitter-ionic lipids (opposite to the bacterial membrane, possessing predominantly anionic lipids).[55] Evidently, zwitter-ionic lipids will not be removed from membranes by electrostatic interactions arising from positively charged QACs. A microscopic evaluation of adhesion and spreading of human skin fibroblasts on our coatings (Figure S5) indicated that fibroblasts spread well within 24 h and reached confluence on bare glass slides and Si-HB coatings within 96 h of growth on Si-HB-PEI$^+$ (10 wt%) coatings. In contrast, fibroblasts remained rounded on the Si-HB-PEI$^+$ (20 wt%) coating. Thus it is concluded, that the Si-HB-PEI$^+$ (10 wt%) coating is preferred for biomedical applications requiring tissue integration, while the Si-HB-PEI$^+$ (20 wt%) coating is preferred for applications requiring only bacterial killing without tissue integration, or other industrial or environmental applications.

4. Conclusion

We have developed a new enabling technology to covalently tether QACs in a hyperbranched configuration onto surfaces. Hyperbranched Si-HB-PEI$^+$ coatings demonstrated high contact-killing efficacies toward adhering staphylococci, without any demonstrable leaching of antibacterial compounds. Moreover, hyperbranched Si-HB-PEI$^+$ coatings (10 wt%) did not negatively affect adhesion and spreading of fibroblasts. Comparison of effects of dissolved and immobilized QACs on adhering staphylococci demonstrated for the first time that QACs immobilized on a surface possess a different, generic working mechanism than QACs in solution. Whereas the working mechanism of QACs in solution is based on their membrane interdigitation over the entire surface of a bacterium, immobilized QAC-molecules locally enhance the adhesion forces between a bacterium and a surface to a lethally strong attraction, causing reduced growth, stress-induced deactivation and localized removal of membrane lipids, eventually leading to cell death. Hitherto immobilized QAC coatings have not been used in clinical applications, partially because proper understanding of their mechanism of antimicrobial action was lacking. The generic mechanism of antimicrobial action for immobilized QAC coatings proposed here paves the way for an urgently needed, further downward translation of such coatings toward clinical application.

5. Experimental Section

Materials for Coating Preparation: Sources of materials used for coating preparation are given in the Supporting Information.

Synthesis of AB_2 Monomers: In a three neck flask, provided with a reflux condenser, carbonyl biscaprolactam (23.22 g, 92 mmol) and bishexamethylene triamine (9.98 g, 46 mmol) were dissolved in 40 mL toluene and stirred at 80 °C for 20 h in a nitrogen atmosphere. The toluene solution was cooled to room-temperature and extracted five times with an aqueous solution containing 5 wt% $CaCl_2$. After the aqueous layer was removed, the organic layer was concentrated to half of its volume using a rotary evaporator. Finally, the product was purified by using a chromatography column. Toluene was used first as eluent to remove remaining carbonyl biscaprolactam, followed by ethanol to obtain the final product with a yield of 80%. 1H-NMR (400 MHz, $CDCl_3$ δ 1.33–1.47 (m, 16H, $NCH_2(CH_2)_4CH_2$), 1.70 (m, 12H, $CH_2(CH_2)_3CH_2N$, ring), 2.56 (m, 4H, CH_2NH), 2.67 (m, 4H, $NCOCH_2$, ring), 3.25 (q, 4H, $CONHCH_2$), 3.96 (t, 4H, CH_2N, ring), 9.23 (broad s, 1H, CONH).

Synthesis of 2-oxo-N(3-triethoxysilyl)propyl)azepane-1-carboxamide (Coupling Agent): In a three neck flask, provided with a reflux condenser, carbonyl biscaprolactam (11.34 g, 45 mmol) and (3-aminopropyl) triethoxysilane (9.95 g, 45 mmol) were dissolved in 40 mL toluene. The reaction was carried out under nitrogen at 80 °C overnight. After the solution was cooled to room-temperature the remaining toluene was distilled under reduced pressure. The obtained coupling agent was stored under nitrogen. According to the NMR spectrum, the yield was more than 98%. 1H-NMR (400 MHz, $CDCl_3$) δ 0.60 (m, 2H, $SiCH_2$), 1.19 (m, 9H, CH_3CH_2OSi), 1.65–1.74 (m, 8H, $SiCH_2CH_2$ and $CH_2(CH_2)_3CH_2$, ring), 2.67 (t, 2H, $NCOCH_2$, ring), 3.28 (q, 2H, $NHCH_2$), 3.79 (q, 6H, CH_2OSi), 3.97 (m, 2H, CH_2NCO, ring), 9.27 (broad s, 1H, CONH).

Hyperbranched Polymer Coating (Si-HB Coating): Glass slides (dimensions 2.5 cm x 2.5 cm) were cleaned in dichloromethane and in methanol for 10 min at room-temperature in a sonic bath. Subsequently, slides were placed in hot piranha solution (H_2SO_4:H_2O_2 = 7:3) for 2 h at 100–110 °C. Next, glass slides were three times sonicated in water for 15 min, then another 15 min in methanol at room-temperature and dried under nitrogen. The resulting hydrophilic glass slides were immersed in the 3 v/v% solution of coupling agent in ethanol for 10 min at room-temperature, placed in a vacuum oven and heated at 110 °C for 2 h under vacuum. Unreacted coupling agent was removed by washing the glass slides in 100 mL ethanol for 20 min in a sonic bath at room-temperature and again washed for another 10 min in 50 mL ethanol before drying under nitrogen. A solution of AB_2 monomers (20 wt% in ethanol, 80 µL) was spin-coated onto glass slides (2000 rpm, 60 s). Polymerization of AB_2 monomers on the surface was carried out at 145 °C for 2 h under nitrogen. Unreacted compounds were removed by sonication in 100 mL ethanol for 20 min at room-temperature and dried under nitrogen, followed by extraction in dimethylformamide at 115 °C overnight and sonication again in 100 mL ethanol for 20 min at room-temperature. Coated glass slides were dried and stored under nitrogen. Infrared absorption bands of Si-HB coatings on silicon wafers showed vibrations at 3345 cm^{-1} (NH stretching), 2930 cm^{-1} (CH_2 antisymmetric stretch), 2860 cm^{-1} (CH_2 stretch), 1701 cm^{-1} (C=O, caprolactam ring), 1650 cm^{-1} (C=O from urea linkages), 1534 cm^{-1} (C–N and N–H from urea linkages), 1400 cm^{-1} (CCH, NCH, C–N from caprolactam ring), and 950–1280 cm^{-1} (Si–O stretch).

Modification of Hyperbranched Coating with Polyethyleneimine and Alkylation (Si-HB-PEI$^+$ Coating): A solution of PEI in water (50 wt%) was freeze-dried overnight (M_w = 750 kDa) and the residue dissolved in methanol. Two solutions with 10 wt% and 20 wt% PEI were prepared. 90 µL of the PEI solution was dropped on Si-HB and spin-coated (2000 rpm, 60 s). Anchoring 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 room-temperature, followed by extraction in methanol at 65 °C overnight, and again sonication in methanol for 20 min at room-temperature, followed by drying under nitrogen. After reaction with PEI the coatings became slightly yellow.

In a round flask provided with a reflux condenser, Si-HB-PEI coated glass slides were immersed in 50 mL 1-bromohexane and heated under nitrogen at 90 °C overnight. Next, a potassium hydroxide suspension (0.3 g in 50 mL 2-methyl-2-butanol) was added and the reaction was continued for another 3 h at 90 °C. Afterwards, the coated glass slides were sonicated in 100 mL methanol for 20 min at room-temperature. Coatings were dried and stored under nitrogen.

The second alkylation step was performed in a closed round flask. Samples were immersed in a solution of 15 mL iodomethane in 100 mL

2-methyl-2-butanol. Reaction was carried out at 42 °C overnight; the samples were sonicated in 100 mL methanol for 20 min at room-temperature and followed by extraction in methanol at 65 °C for 1 day and another sonication in methanol for 20 min at room-temperature. The resulted Si-HB-PEI$^+$ coatings were dried and stored under nitrogen. Infrared absorption bands of Si-HB-PEI$^+$ coating on silicon wafers showed vibrations at 3315 cm^{-1} (NH stretching), 2931 cm^{-1} (CH_2 antisymmetric stretch), 2860 cm^{-1} (CH_2 stretch), 1666 cm^{-1} (C=O from urea linkages), 1534 cm^{-1} (C–N and N–H bending from urea linkages and alkylated PEI), 1400 cm^{-1} (CCH, NCH, C–N stretching), 950–1280 cm^{-1} (Si–O stretch).

Coating Characterization: Methods used to characterize the coatings are described in the Supporting Information.

Bacterial Contact-Killing: *S. epidermidis* ATCC 12228 was first streaked on a blood agar plate from a frozen stock solution (7 v/v% DMSO) and grown overnight at 37 °C on blood agar. One colony was inoculated in 10 mL tryptone soya broth (TSB, Oxoid, Basingstoke, UK) and incubated at 37 °C for 24 h. This culture was used to inoculate a main culture of 200 mL TSB, which was incubated for 16 h at 37 °C. 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.

The ability of the coatings to kill adhering staphylococci was evaluated using two distinctly-different assays. The Petrifilm assay employed is based on culturing of organisms that survive contact with the coatings under nutrient-rich conditions. CLSM was applied to visualize bacteria adhering to the coatings under nutrient-deprivation in a potassium-phosphate-buffer after staining in order to create red and green fluorescent bacteria, representing dead (or technically more correct "membrane-damaged")[44] and live organisms, respectively. Bacterial concentrations of 10^6, 10^5 and 10^4 bacteria mL^{-1} were used to evaluate the contact-killing in the Petrifilm assay and a bacterial concentration of 3×10^7 bacteria mL^{-1} was used for CLSM imaging of dead and live bacteria adhering to the coatings.

The Petrifilm Aerobic Count plate (3M Microbiology, St. Paul, MN, USA) consists of two films: a bottom film containing standard nutrients, a cold-water gelling agent and an indicator dye that facilitates colony counting and a top film enclosing the sample within the system. The bottom film containing the gelling-agent was first swelled with 1 mL sterile demineralized water for 40 min and transferred to the transparent top film before usage. Next, 10 µL bacterial suspensions with the different concentrations were placed on bare, as well as on Si-HB and Si-HB-PEI$^+$ coated glass slides (2.5 cm × 2.5 cm). After closure of the Petrifilms system with a glass slide in between, the staphylococcal suspension spread over the entire surface area of the samples, enabling calculation of the bacterial challenge per cm^2 from the dimensions of the samples and the bacterial concentration in suspension (see also Table 1). Petrifilms were incubated at 37 °C for 48 h after which the numbers of CFU were counted. As a control, 10 µL of the bacterial suspension was inoculated on Petrifilm without a sample in between.

For CLSM evaluation of the number of dead and live bacteria adhering to the coatings, four mL of 3×10^7 bacteria mL^{-1} in potassium-phosphate-buffer were inoculated in a 6-well polystyrene plate (Greiner Bio-One B. V., Alphen a/d Rijn, The Netherlands) containing a bare glass slide, Si-HB and Si-HB-PEI$^+$ coated samples. After incubation for 1, 2, 4, 6 and 16 h at 37 °C under shaking (90 rpm), bacterial suspensions were removed and the samples were gently washed with sterile potassium-phosphate-buffer to remove the free-floating bacteria. CLSM was subsequently employed to differentiate between live and dead adhering bacteria. Samples were stained in the wells with 250 µL live-dead *Bac*light viability stain (Molecular Probes, Leiden, The Netherlands) containing SYTO 9 dye (yielding green fluorescence in live organisms) and propidium iodide (yielding red fluorescence in cell membrane-damaged, dead organisms). Staining was done for 15 min in the dark. Confocal images were collected using a Leica TCS-SP2 Confocal laser scanning microscope (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). Numbers of live and dead bacteria were counted and percentage bacterial viabilities were calculated by dividing the number of live bacteria by the total number of live and dead bacteria adhering to the different coatings.

To demonstrate possible leaching of antibacterial compounds out of Si-HB-PEI$^+$ coating, a glass slide with a Si-HB-PEI$^+$ (20 wt%) coating was placed on an agar plate inoculated with *S. epidermidis* ATCC 12228 and the zone of inhibition around the sample was evaluated after 96 h incubation at 37 °C. The absence of a zone of inhibition was taken as an indication that no antibacterial compounds leached out of the coatings.

Antimicrobial Mechanisms of Dissolved and Immobilized QACs using AFM:AFM experiments were conducted at room-temperature in potassium-phosphate-buffer as a control and in potassium-phosphate-buffer supplemented with QAC (Ethoquad C/25(Cocoalkyl methyl(polyoxyethylene)ammonium chloride)) (AKZONobel, Amsterdam, The Netherlands) at the minimum bactericidal concentration for *S. epidermidis* ATCC 12228 in a planktonic state (MBC, 150 µg mL^{-1}).[38] A BioScope Catalyst AFM with ScanAsyst (Veeco, Camarillo, California, USA) was used for imaging staphylococci. For imaging, staphylococci were allowed to adhere to the different coatings by placing a droplet of a bacterial suspension (10^{10} bacteria mL^{-1}) in buffer on them for 30 min. Subsequently, the bacterially coated surfaces were rinsed with potassium-phosphate-buffer to remove free floating bacteria and immediately used for AFM measurements without drying. The bacteria on the glass slide were scanned with 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, 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 producers' specification of 0.06 N m^{-1}. For measurements on hyperbranched, positively-charged coatings, a stiffer cantilever had to be used with a spring constant of 0.58 N m^{-1}. Bacterial probes were prepared by immobilizing single bacteria on a cantilever,[39] the probes were 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, on α-poly-L-lysine coated glass and on the hyperbranched QAC coatings. At least 15 retract force-curves were recorded on one spot, requiring a minimum of six bacterial probes.

For evaluation of contact-killing of adhering bacteria, slides were incubated in a staphylococcal suspension (3×10^7 bacteria mL^{-1}) in a 6-well polystyrene plate (Greiner Bio-One B. V., Leiden, The Netherlands) containing either potassium-phosphate-buffer or a QAC solution in buffer at 1 x MBC. After incubation for 60 min and 300 min at 37 °C under rotation (90 rpm), the bacterial suspension was removed, the surface gently washed with potassium-phosphate-buffer to remove the free-floating bacteria and stained with 250 µL live-dead *Bac*light viability stain for CLSM enumeration of live and dead bacteria.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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