Summary

Our life expectancy is increasing and with an aging population, the need for biomaterial implants and devices is growing as well. The number one cause of failure of biomaterial implants and devices is biomaterial-associated infections (BAI). BAI is difficult to treat with antibiotics as a result of the protection offered by the biofilm mode of growth and a hampered host immune system around a biomaterial implant or device. Not seldom the outcome of a BAI is revision surgery. **Chapter 1** gives a brief introduction on the impact and development of a BAI. A universal method to create biomaterial implants and devices with intrinsic antimicrobial functionalities is difficult. Immobilizing antimicrobial coatings on existing biomaterials is relatively simple and can be helpful in preventing BAI. Therefore the aim of this thesis is to explore the preparation of a coating that kills bacteria upon contact and to evaluate *in vitro* the resistance of such coatings against bacterial adhesion and biofilm formation. Evaluations will not only involve the ability of the coatings to inhibit biofilm formation, but also encompass a study on phagocytosis of contact-killed bacteria, as the development of a layer of dead bacteria may interfere with the contact-killing abilities of such coatings.

Chapter 2 gives an overview of different methods for preventing biofilm formation on biomaterials, including coatings with and without leachables. Several tethered antimicrobials are discussed, like lysozyme, antimicrobial peptides and quaternary ammonium compounds (QUATs). We focus on coating without leachables and especially on QUATs, as they are synthetically well accessible and used already for a long time in disinfectants. The different mechanisms known in literature by which the QUAT molecules act as an antimicrobial are discussed. One proposed mechanism is the penetration of QUATs through the peptidoglycan layer into the cytoplasmic membrane, while disordering this membrane. In a related mechanism, it is proposed that QUATs destabilize the bacterial cell membrane by displacement of cationic ions which normally stabilize the membrane. Finally, with the focus on cationic polymers, several polymers frequently used in antimicrobial coatings like polyvinylpyridine, polyethyleneimine and poly(meth)acrylates are reviewed.

In **chapter 3** we have developed a method to synthesize AB_2 monomers, the corresponding hyperbranched and the corresponding amphiphilic hyperbranched polymers in a one-pot procedure, starting from two commercial available compounds. Since the B-groups were blocked isocyanates, the end groups of the hyperbranched polyurea were blocked isocyanates as well. Coupling of a range of monomethoxy-poly(ethylene glycol)s onto the blocked isocyanates, yielded a platform of amphiphilic hyperbranched polymers, with controllable hydrophobic cores and hydrophilic shells. After the three consecutive reaction steps, without intermediate purification, the final polymers were purified by precipitation in a non-solvent, in which the polymer precipitated and the excess poly(ethylene)glycol remained dissolved. Pyrene inclusion experiments showed the formation of micelles above a critical concentration. Both cryo-transmission electron microscopy and dynamic light scattering revealed the presence of two distinct particle populations, being the primary micelles and aggregates thereof. All micelles showed a lower critical solution temperature behavior, with transitions close to body temperature. The low cytotoxicity of the micelles make them promising for drug or biocide delivery.

Based on the platform developed in **chapter 3**, we describe in **chapter 4** the preparation of a shape-adaptive, contact-killing coating by tethering QUATs 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 QUATs 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 QUAT-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.

QUAT-coatings uniquely kill adhering bacteria upon contact and therefore require a minimal cationic-charge surface density for effective contact-killing of adhering bacteria. Reports in literature suggest the minimal density is in the order of 10^{14} positive charges per cm^{-2}. The positive charges on a QUAT-coating are created by quaternization of nitrogen which is achieved through alkylation. In **chapter 5** we investigate the contribution of additional alkylation with methyl-iodide to the cationic-charge density of hexyl-bromide alkylated QUAT-coatings by measuring charge density with fluorescein staining. X-ray photoelectron spectroscopy was used to determine the at% alkylated nitrogen. Also streaming potentials, water contact-angles and bacterial contact-killing were measured. Cationic-charge density increased with methyl-iodide alkylation times up to 18 h, accompanied by an increase in the at% alkylated-nitrogen. Zeta potentials became more negative upon alkylation as a result of shielding of cationic charges by hydrophobic alkyl-chains. Contact-killing of Gram-positive staphylococci only occurred when the cationic-charge density exceeded 10^{16} cm^{-2} and was carried by alkylated-nitrogen (electron-binding energy 401.3 eV). Gram-negative *Escherichia coli* was not killed upon contact with the coatings. Therewith this study reveals that cationic-charge density is neither appropriate nor sufficient to determine the ability of QUAT-coatings to kill adhering bacteria. Alternatively, the at% of alkylated-nitrogen at 401.3 eV is proposed, as it reflects both cationic-charge and the type of charged species. The at% $N_{401.3}$ eV should be above 0.45 at% for Gram-positive bacterial contact-killing.

Reliable *in vitro* evaluation methods for bacterial contact-killing surfaces do not yet exist, while more importantly results of different evaluation methods are often conflicting. Therefore, in **chapter 6,** we compare five methods to evaluate bacterial contact-killing surfaces. Our comparison is based on determining the contact-killing efficacy of an established, contact-killing alkylated hyperbranched polyurea-polyethyleneimine coating upon contact with a *S. epidermidis* strain. Depending on the method used, different results were obtained in bacterial contact-killing. We conclude that the Petrifilm® and Japanese Industrial Standards (JIS) methods are preferable: Petrifilm® is most convenient and possibly more reliable. Like all others, these methods need a complementary assay to exclude killing resulting from release of antimicrobial compounds, because even a small release of an antimicrobial compound will have a large influence on bacterial killing in the small fluid volumes of the assays. The modified JIS method is acceptable, but does not contain

balanced amount of nutrients compared to the Petrifilm® method and should only be used with respect to a non-contact killing control. The American Society for Testing and Materials (ASTM) and bacterial spray methods are not reliable, the main reason being the lack of control over the applied bacterial challenge.

Many new biomaterials have been evaluated for their ability to inhibit bacterial colonization and stimulate tissue-cell-integration, but neglect the role of immune cells. In **chapter 7** we compare macrophage phagocytosis of adhering *Staphylococcus aureus* on QUAT-coatings and patterned poly(ethylene)glycol-hydrogels versus common biomaterials and stainless steel in order to identify surface conditions that promote clearance of adhering bacteria. Staphylococci were allowed to adhere and grow on the materials in a parallel-plate-flow-chamber, after which murine macrophages were introduced. From the decrease in number of adhering staphylococci, phagocytosis-rates were calculated, and total macrophage displacements during an experiment determined. Hydrophilic surfaces had the lowest phagocytosis-rates, while common biomaterials had intermediate phagocytosis-rates. Patterning of poly(ethylene)glycol-hydrogel coatings increased phagocytosis-rates to the level of common biomaterials, while on cationic-coatings phagocytosis-rates remained relatively low. Likely, phagocytosis-rates on cationic coatings are hampered relative to common biomaterials through strong electrostatic binding of negatively-charged macrophages and staphylococci. On polymeric biomaterials and glass, phagocytosis-rates increased with macrophage displacement, while both parameters increased with biomaterial surface hydrophobicity. Thus hydrophobicity is a necessary surface condition for effective phagocytosis. Concluding, next-generation biomaterials should account for surface effects on phagocytosis in order to enhance the ability of these materials to resist BAI.

Finally in the general discussion, **chapter 8,** we emphasize the impact of our work on the development of future applications and discuss the limitations of the methods used.