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The development of antimicrobial biomaterial surfaces

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General discussion

In this thesis antimicrobial biomaterial surfaces and methods to study the antimicrobial activity of these surfaces were developed. Antimicrobial biomaterial surfaces were defined as surfaces on which the surface growth of adherent bacteria is inhibited without the use of antimicrobial agents leaking from the surface. Inhibition of surface growth should be achieved by changing the physico-chemical properties of the biomaterial surface, or by binding antimicrobial agents covalently to the biomaterial surface.

Changing surface properties and covalent binding of antimicrobial agents

One approach to change the surface properties of biomaterials is to synthesize polymers with the desired surface properties, and subsequently coat the biomaterial surface with this polymer. Adhesive Van der Waals forces and, ideally, adhesive electrostatic forces should keep the polymer chains attached to the surface. The positively charged poly(methacrylate), synthesized as described in Chapters 4 and 5, can thus be coated on most negatively charged surfaces, which are virtually all commonly used biomaterial surfaces. Also, biomaterial implants with complex geometry can be easily coated with these polymers by dip coating [1]. Poly(methacrylates) can be synthesized with a large variety of functional groups, as many different methacrylate monomers are readily available. However, the percentage of ionic or hydrophilic functional groups in the polymers must not be too high, as these increase the solubility of the polymer in water, which results in a lower durability of the coatings. In addition the mechanical properties of the polymer coating must be suitable for the application. Our positively charged poly(methacrylate) has a glass transition temperature of 60°C, meaning that at room temperature the coating might be too stiff to be coated e.g. on silicone rubber catheters.

A second approach was to bind antimicrobial functional groups covalently to the biomaterial surface with silane coupling agents. Also silane coupling reagents are readily available with a large variety of functional groups. Silane coupling reagents react with hydroxyl groups, which are present on e.g. glass and cotton, to form a silicium ether bond [2]. Hydroxyl groups are also introduced on silicone rubber by oxidizing the surface with argon plasma etching [3]. Silanes can also react with itself, forming a crosslinked polymer layer on the biomaterial surface. In *in vitro* experiments as described in Chapter 7, this layer proved to be durable for at least some hours. The durability for prolonged implantation remains to be tested. *In vivo* sterile pus formation was found around the coated silicone rubber, indicating fragmentation of the coating, which can induce a strong foreign body reaction. In the warm, wet, oxygenated *in vivo* environment silicium ether bonds might not be stable towards hydrolysis.

In a pilot study that was not reported in this thesis, another approach to introduce covalently bonded functional groups to biomaterials was tested, so-called graft co-polymerization. In this technique, first peroxide groups are introduced on the biomaterial surface by gas plasma treatment, followed by exposure to oxygen. Subsequently, these peroxide groups are used to initiate polymerization of acrylate monomers, resulting in poly(acrylate) chains covalently bound to the biomaterial surface. We succeeded in graft co-polymerizing a small amount of polyacrylamide onto polyethylene. However, as optimization of the process is very time consuming due to the many different parameters involved, it was chosen to continue first with the methods described above, as faster results could be expected, although eventually graft co-polymerization can result in more durable coatings.

In Table 1 the respective advantages and disadvantages of the three described methods are summarized.

Table 1. Characteristics of the various approaches to introduce functional groups on biomaterial surfaces. + = good, +/- = intermediate and - = bad.

Property	Polymer coating	Silanization	Graft co-polymerization
Durability	+/-	+/-	+
Applicable biomaterial range	+	-	+/-
Difficult implant geometry	+	+/-	+/-
Functionality range	+	+	+
Mechanical properties	+/-	+	+

Methods to study the antimicrobial effects of biomaterial surfaces *in vitro*

In this thesis we used three different *in vitro* methods to study the antimicrobial activities of our antimicrobial biomaterial surfaces. Table 2 shows the various parameters that can be determined, and some characteristics of the respective methods. The parallel plate flow chamber system described in Chapter 2 can be used to study bacterial surface growth *in situ* on translucent or reflecting biomaterials. This method is suitable for evaluating antimicrobial surfaces that decrease surface growth, but can also determine the killing efficacy of surfaces, covalently linked with antimicrobial agents, although non-growing adherent cells are not by definition non-viable. They can also be incapable of growing on the surface for another reason.

Table 2. Comparison of three *in vitro* methods to study the antimicrobial activity of biomaterial surfaces, i.e. surface growth measurements of adherent bacteria in the parallel plate flow chamber (PPFC), viability staining of adherent bacteria in the PPFC and colony forming units (CFU) determination. Shown are the various parameters that can be determined, the applicability and the reliability.

Parameter	Growth PPFC	Viability stain PPFC	CFU determination
Initial adhesion rates	Yes	Yes	No
Surface growth rates	Yes	No	No
Desorption rates	Yes	No	No
Viable bacteria	Yes	Yes	Yes
Non-viable bacteria	Yes/No*	Yes	No
Viable but non-growing bacteria	Yes/No*	No	No
Possible with all materials	No	Yes	Yes
Clear indisputable method	Yes	Yes	No

* No distinction can be made between these two states of adherent bacteria.

A faster way to measure the viability of adhering bacteria in the parallel plate flow chamber is to add a viability stain in stead of growth medium after the initial adhesion phase. The live/dead *bac*light™ bacterial viability stain (Chapter 7) can be used, where viable bacteria appeared green fluorescent, whereas dead bacteria with disrupted cell membranes colored red fluorescent. Also staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is possible, as used by Habash *et al.* [4]. Here, only the actively respiring bacteria are stained red fluorescent. We also used this staining in a few experiments (not reported) and found for *S. epidermidis* HBH_2 102 on silicone rubber that after 1 h of initial adhesion in buffer only few

adhering bacteria were actively respiring, usually those that were present in groups. This corresponded to what we found in Chapter 4, where only 7% of *S. epidermidis* HBH_2 102 cells on PMMA/MAA was growing. However the CTC stains did not correspond at all with the live/dead viability stains in Chapter 7 where this bacterial strain showed a viability of 94% on silicone rubber after 1 h of initial adhesion. This indicates that *S. epidermidis* with intact cell membranes are not necessarily actively respiring or capable of surface growth within the first hours after administration of growth medium. These bacteria might have been in the so-called viable but non-culturable state. However, for *P. aeruginosa* AK1 the percentage of actively respiring bacteria on silicone rubber as determined with CTC was found to be much higher, corresponding to the high percentage of growing cells on PMMA/MAA (Chapter 4) and the cells with intact cell membranes on silicone rubber (Chapter 7).

The third method is to let the bacteria adhere to the biomaterial surface and subsequently detach them from the surface by ultrasonic treatment of the biomaterial and determine the number of detached colony forming units (CFU). This method is extensively used in the literature, but only reveals the number of viable detachable adhering bacteria (see Table 2). Another disadvantage is that some operations are debatable. The biomaterial passes an air/liquid interface, which is known to exert a high shear force on the bacteria, which could push the bacteria from the surface before the ultrasonic treatment. Also, not all the adhering bacteria may be removed, especially on extremely adhesive positively charged surfaces.

In conclusion, determination of surface growth combined with a stain to test the viability of non-growing cells in a parallel plate flow chamber is the most suitable method to evaluate antimicrobial biomaterial surfaces.

Animal models to study biomaterial-centered infections

Direct/indirect immediate local infections

In immediate local infection models the bacteria are implanted together with the biomaterial. A direct infection model was applied in Chapters 5 and 7, where the bacteria were seeded directly on the biomaterial surface before subcutaneous implantation in the rats. In this way no conditioning film was present between bacteria and biomaterial, as was the case in the *in vitro* experiments. This model resembles in the clinic the cases where bacteria are seeded before or during insertion of the implant from the air, the surgeon or the patient’s skin. Our results showed that only *P. aeruginosa* AK1 was capable of growing on the implant surface.

On silicone rubber the numbers of *S. aureus* ATCC 12600 had decreased 8 fold in 3 days and 16 fold in 1 week, while 1 out of 4 implants was sterile after 1 week. The numbers of *E. coli* O2K2 decreased fast, a 100 fold in 2 days. The same was found for *S. epidermidis* HBH_2 102, where, after seeding of 10^6 CFU cm^{-2} on silicone rubber, 6 out of 7 implants (in 7 rats) were sterile after 3 days, while only 10^3 CFU cm^{-2} were harvested from the infected implant (non-reported experiments).

The observed differences between *P. aeruginosa* and the other bacterial strains were probably due to *P. aeruginosa*'s capability to form slime and fast biofilm formation. *In vitro* (Chapters 3, 4) *P. aeruginosa* AK1 formed a monolayer biofilm within 7 h, while this took longer for the staphylococci and especially for *E. coli* O2K2 because of its high desorption rates from the surface. *S. epidermidis* HBH_2 102 is also a good slime producer, but this is much more dependent on the nutrient conditions, as was observed in Chapter 3. Besides slime formation and fast surface growth, other virulence factors of *P. aeruginosa* AK1, like endotoxin formation and motility can be important. Advantages for causing infection of *S. aureus* over *S. epidermidis* are faster surface growth rates (Chapter 4), production of exotoxins and circumventing phagocytosis by elimination of antibodies and phagocytes.

An indirect local infection model was also applied in Chapter 7. Here, 10^9 CFU *S. aureus* were added to the implant site, after inserting the implants, to study the effect of a conditioning film of plasma proteins on the antimicrobial biomaterial surface before adhesion of the bacteria. This animal model is most often chosen in literature to study biomaterial-centered infections. It resembles the clinical cases where the bacteria are seeded from the operation wound after the insertion of the implant, which is generally believed to be the most common cause of biomaterial-centered infections. In our study all implant sites showed clinical signs of infection, and bacteria were recovered from 7 out of 8 silicone rubber implants, which was equal to the direct infection model. Thus, from these results, the direct and indirect model have a similar likelihood of representing the pathogenesis of biomaterial-centered infections.

In non-reported experiments the addition of 2 x 10^8 CFU *S. aureus* led to sterile implants after 1 week (two rats). This corresponds to the literature, where addition of 10^9 CFU *S. aureus* to polyethylene implants in rats resulted in infection of most of the implants at 7 days, while addition of 10^8 CFU showed no evidence of infection [5]. However, it was shown that seeding biomaterials with as low as 100 to 1000 CFU *S. aureus* could produce clinical biomaterial-centered infections in mice and rabbits [6, 7]. Also Zimmerli *et al.* [8] found that

an avirulent strain, *S. aureus* Wood 46, could produce a biomaterial-centered infection with only 100 CFU in >95% of their so-called tissue cages implanted subcutaneously in guinea pigs. This corresponds to an experiment in man where 100 CFU of *S. aureus* were enough to cause infections in the presence of a suture [9]. These data indicate that rats might react differently to the induced biomaterial-centered infections than other animals, including man. While it was shown in man and guinea pigs that the surroundings of biomaterials are in fact immuno-compromized, rats may have other immunologic pathways to eradicate bacteria from inert surfaces. Also pointing to this direction are the reported major differences between the foreign body reaction of rats and mice [10].

Late hematogenous infections

An advantage of antimicrobial biomaterial surfaces over antibiotic releasing materials should be the prolonged duration of the protection against infections. The use of such biomaterial surfaces could protect implants against postulated late hematogenous seeding of microorganisms, if at all occurring. In Chapter 6 was studied if subcutaneously implanted biomaterials in rats could be infected by hematogenous seeding after 4 weeks of implantation. It was found that none of the implants were infected by intravenously injected bacteria or translocated intestinal bacteria. These results can be explained by the fact that the bacterial concentration near the subcutaneous implants was much lower than in the local infection models, due to dilution effects, filtering of the blood by organs (injected staphylococci did infect all kidneys) and tissues and the action of the immune system. Moreover, at this late stage the subcutaneous biomaterials were covered with host cells, therewith reducing the chances of bacterial adhesion.

Surprisingly, in these experiments, 5% of the implants were infected, possibly due to perioperative contamination, as the bacterial strains did not correspond with the injected or translocated strains. It might be that these low virulent (no clinical infection signs) bacteria, probably originating from the commensal skin flora of the rats, are immunotolerated by the rats, thus being able to colonize the biomaterial surface without being eradicated, a hypothesis proposed in Chapter 1.

From these results can be concluded that late hematogenous infections are much less likely to occur than immediate local infections. Also in the literature little evidence can be found for the occurrence of late hematogenous infections. This indicates that most

biomaterial-centered infections can be prevented with antimicrobial biomaterial surfaces that are active for only a short period after implantation.

Mechanisms of antimicrobial activity of biomaterial surfaces

As with all antimicrobial agents, the various types of bacteria studied in this thesis reacted differently towards the respective antimicrobial and non-antimicrobial biomaterial surfaces. It was observed that growth of *P. aeruginosa* AK1, a Gram-negative rod, was hindered on surfaces onto which it attached strongly, resulting in slower surface growth rates (Chapter 3) (see Figure 1).

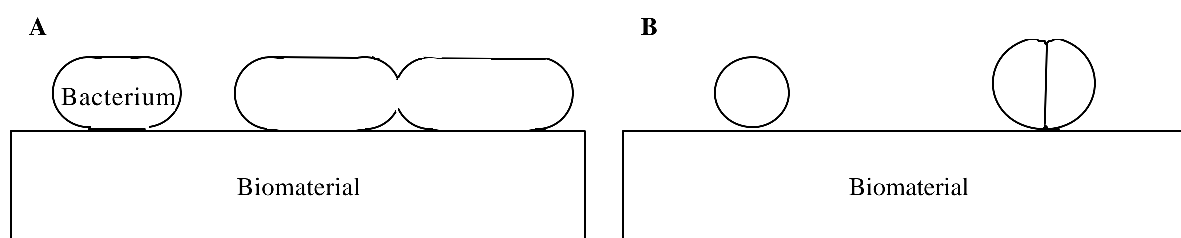

Figure 1. Schematic presentation of surface growth of rod shaped *P. aeruginosa* and *E. coli* (A) and round shaped *S. aureus* and *S. epidermidis* (B). Note that the rod shaped bacteria have to overcome the physical attraction to the biomaterial surface in order to elongate and divide.

Ultimately on positively charged poly(methacrylate) the attraction to the surface was so strong, that none of the adhering *P. aeruginosa* AK1 could grow (Chapter 4). It became clear in Chapter 5 that attraction to the surface was not the only growth inhibition mechanism. When the adhering bacteria were detached from the positively charged surface only half of the bacteria were able to form a colony on agar, i.e. were viable. The positive charge in the polymers was induced by quaternary ammonium groups, which can be toxic for bacteria and probably killed half of the adhering bacteria. Also the quaternary ammonium containing silane coating was shown to be toxic for 75% of the adhering *P. aeruginosa* AK1 cells (Chapter 7). Like on the positively and negatively charged poly(methacrylates) (Chapter 5), the number of viable bacteria were the same on the positively charged silane coated silicone rubber and the negatively charged plain silicone rubber, as initial adhesion was much higher to positively charged surfaces. In a non-reported pilot experiment, surface growth of adhering *P. aeruginosa* AK1 on the same quaternary ammonium silane coated glass was measured in a parallel plate flow chamber, exactly as it was done on the positively charged

poly(methacrylates) (Chapter 4). We saw that none of the adhering bacteria were able to grow on this surface, indicating that the surface growth of adhering viable bacteria was probably also inhibited by the strong attraction of a positively charged surface. However, this physical surface growth inhibition can be overcome by *P. aeruginosa* AK1 *in vivo*, as was shown in Chapter 5.

The antimicrobial effects of charged surfaces were also tested for another Gram-negative rod shaped bacterium: *E. coli* O2K2 (Chapters 4,5,7). *In vitro* the same effects were seen as those described above for *P. aeruginosa* AK1, i.e. no surface growth, and killing of adhering bacteria on the positively charged surface. The differences in initial adhesion between positively and negatively charged surfaces were larger for *E. coli*, as this strain has a higher negative surface charge. *In vivo*, however, the physical inhibition of surface growth appeared to be effective (Chapter 5). As *E. coli* O2K2 is not a very good slime and biofilm producer, it was proposed that viable adhering *P. aeruginosa* AK1 cells could overcome the physical surface growth inhibition by forming slime between cells and the biomaterial surface, therewith loosening the bond between them, in contrast to *E. coli*.

The staphylococci studied in this thesis reacted in a different pattern towards biomaterial surfaces. Surface growth rates of *S. epidermidis* HBH_2 102 correlated with the surface hydrophobicity, although this effect was only seen under low nutrient conditions, and polypropylene was an exception to this rule (Chapter 3). Interestingly, corresponding to the very slow *in vitro* surface growth on glass and polypropylene, in Chapter 6 only glass and polypropylene showed no perioperatively introduced biomaterial-centered infections, although, due to the low number of infections found, this relation is not statistically significant.

The staphylococci were probably affected by the positively charged poly(methacrylate), as the percentage of growing bacteria was half of that on normal PMMA (Chapter 4). This corresponds with the Gram-negative rod shaped bacteria, that were recovered from the positively charged surface, from which the viability was half of those recovered from PMMA (Chapter 5), indicating that the quaternary ammonium groups were also toxic for the staphylococci. However, surface growth of staphylococci was not inhibited by strong physical attraction, as these round bacteria do not need to elongate much before cell division (see Figure 1). In Chapter 7 almost all adhering staphylococci were killed by the positively charged silane coated silicone rubber, even when the surface was precoated with plasma proteins. *In vivo*, this activity was retained, although here probably plasma proteins

did cover a considerable part of the antimicrobial groups, resulting in a lower killing efficacy. The quaternary ammonium groups in this coating are probably more active, because the long hydrophobic alkyl chain can interfere with the lipids of the cell membrane, thereby disrupting its integrity. The Gram-negative bacteria might be less affected by this compound as these bacteria have double cell membranes.

Future research

It would be interesting to test the hypothesis that perioperatively introduced biomaterial-centered infections in rats are caused by immunotolerated microorganisms. To this end the percentage of perioperative infections could be increased by rubbing the implants over the skin of the rats before implantation. Immunotolerance of the rats for the infecting microorganisms could be determined by the method of Duchmann *et al.* [11].

Positively charged coatings might also be effective in the prevention of urinary tract catheter infections, as *E. coli* is often involved in these infections. The adhesive character of the surface might also inhibit the migration of bacteria along the catheter surface from the outside of the body to the bladder, were bacteriuria is caused. Also the quick migration, so-called swarming, of proteus spp., the second most common pathogen in these infections, might not be possible on these coatings. To test this, adhesion, growth and motility could be studied in urine in the parallel plate flow chamber. In this application the coatings should also be durable for the insertion period, which should also be tested. An approach to make more durable coatings could be the graft co-polymerization of methacrylates functionalized with quaternary ammonium groups.

Another interesting approach to make specific adhesive surfaces for growth inhibition of rod-shaped bacteria, might be coating of biomaterial surfaces with immunoglobulins. When monoclonal immunoglobulin G (IgG) directed against for example *P. aeruginosa* is bound with the Fc side to the biomaterial, in theory this surface should be very adhesive for the bacteria, thus preventing their proliferation on the surface. An indication that this might work can be obtained from the study of Poelstra *et al.* [12] where adsorbed pooled human IgG on the substratum surface decreased the surface growth rates of *P. aeruginosa* by 15%.

Studying the relationship between adhesion and growth can be also very interesting on metals, as their surface charge can be easily varied using an electrical power source [13]. In

this way it might be possible to determine which attraction force is needed to slow down growth rates of for example *P. aeruginosa*.

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