Polymer brush-coatings to prevent biomaterials associated infection
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Biofilms on polymer brush-coatings remain susceptible for antibiotic treatment
Abstract

Biomaterial associated infection (BAI) is the most important cause of implant failure. BAI begins with adhesion of bacteria to the biomaterial surface and their subsequent growth into a biofilm. Polymer brush-coatings are known for their superior anti-adhesive properties. Yet a polymer brush-coating does not completely prevent bacterial adhesion and the few adhering bacteria on a brush-coated surface have shown the ability to grow into a biofilm. However, these biofilms develop slowly and form scattered colonies which are penetrable to nutrients. Biofilm formation on brush-coatings may also be more susceptible to antibiotics. Therefore, we here investigate the growth of *Staphylococcus aureus* ATCC 12600 biofilms, a common cause of BAI, on pristine and polymer brush-coated silicone rubber in the absence and presence of three concentrations of gentamicin (0.5, 5 and 50 µg ml$^{-1}$). Biofilms grew on silicone rubber and maintained their viability in the presence of gentamicin, regardless of its concentrations. On polymer brush-coatings, however, the presence of gentamicin in the growth medium suppressed biofilm formation and decreased the number of viable bacteria. Biofilm growth on brush-coatings was almost completely prevented in the presence of 50 µg ml$^{-1}$ gentamicin. We conclude that, contrary to biofilms on biomaterial surfaces in the absence of a polymer brush-coating, biofilms on polymer brush-coatings remain susceptible to antibiotics, which prevents biofilm formation.
Introduction

Biomaterial associated infection (BAI) remains the number one cause of prosthetic implant failure, despite the development of various state-of-the-art strategies to control BAI after implantation. The occurrence of BAI for primary implants varies from 1 to 30% with an associated mortality of up to 25%, depending on the type of implant [1]. Common treatment procedures for patients suffering from BAI include long-term application of high doses of antibiotics, but frequently the fate of an infected biomaterial is removal [2]. Microbial adhesion is considered to be the onset of BAI, after which the adhering organisms grow to form a biofilm. In a biofilm, microorganisms are embedded in a complex extracellular polymeric matrix and are resistant against antibiotic treatment and the host immune system [3]. Surface modifications can significantly reduce microbial adhesion to biomaterial surfaces [4]. Polymer brush-coatings are currently the most promising non-adhesive coatings as they reduce the adhesion of various bacterial strains and yeasts by orders of magnitude [5]. A polymer brush is formed when hydrophilic polymer chains are end-grafted to a surface in a high density, forcing the polymer chains to stretch away from the surface into the adjacent medium [6]. Compression of such a structure upon microbial approach gives rise to an osmotic pressure and decreased mobility (conformational entropy) of the polymer chains in the brush, which causes repulsion of the approaching organisms.

Polymer brush-coatings, however, do not reduce microbial adhesion to zero and in case of staphylococci the few bacteria adhering to a polymer brush have been demonstrated to be able to form a biofilm [7], albeit at a reduced rate when compared with uncoated substrata. In addition, we observed alteration of the biofilm structure from a contiguous layer on pristine silicone rubber to scattered microcolonies on a polymer brush-coating. Furthermore, the biofilm on the brush-coated surface appeared to be more viable, probably due to better penetration of nutrients into the
microcolonies. We therefore speculate that antimicrobial agents may also be more effective towards the bacteria in biofilms formed on a polymer brush. Hence, the aim of this study is to compare the influence of antibiotic treatment on biofilm formation on silicone rubber with and without a polymer brush-coating. To this end, we grew biofilms of *Staphylococcus aureus* ATCC 12600, a common cause of BAI, on pristine and polymer brush-coated silicone rubber in media containing different concentrations of gentamicin, a commonly used antibiotic against staphylococci, and monitor the development of the biofilms in a parallel plate flow chamber (PPFC).

**Materials and Methods**

**Preparation of silicone rubber surfaces and brush-coatings.** Implant grade silicone rubber sheets (thickness 0.5 mm, Medin, Groningen, The Netherlands) were rinsed with ethanol (Merck, Darmstadt, Germany) and demineralised water. Subsequently, sheets were sonicated for three min in 2% RBS 35 detergent (Omnilabo International BV, Breda, The Netherlands) and rinsed thoroughly with demineralised water, washed in methanol (Merck) and rinsed with demineralised water again. A silicone rubber sheet was fixed in the bottom plate of a PPFC, after which the entire chamber was sterilized by rinsing with 70% ethanol and sterile demineralised water. Subsequently, the silicone rubber was exposed to a filter-sterilized solution of 0.5 g l\(^{-1}\) Pluronic F-127 (a copolymer of polyethylene oxide (PEO) and polypropylene oxide (PPO) with structure PEO\(_{99}\)PPO\(_{65}\)PEO\(_{99}\), molecular weight 12600; Sigma-Aldrich, USA) in phosphate buffered saline (PBS: 10 mM potassium phosphate, 150 mM NaCl, pH 6.8) for 20 min at room temperature. Non-attached polymer was removed from the chamber by flow with an excess amount of PBS. NaCl, K2HPO4 and KH2PO4 were of analytical grade, as purchased from Merck.
Susceptibility of biofilms on polymer brush to antibiotic

Culturing and harvesting of bacterial cells. *S. aureus* ATCC 12600 was first grown aerobically overnight at 37°C on blood agar plates from frozen stocks. These plates were kept at 4°C, never longer than two weeks. One colony was used to make a pre-culture in 10 ml tryptone soya broth (TSB, OXOID, Basingstoke, England). This pre-culture was incubated at 37°C for 24 h and used to inoculate a second culture of 200 ml which was incubated for 16 h. The culture was harvested by centrifugation for 5 min at 5000 × g and washed twice with sterile demineralised water. To break up bacterial aggregates, bacteria were sonicated intermittently while cooling in an ice/water bath for three times 10 s at 30 W (Vibra Cell model 375; Sonics and Materials, Danbury, CT, USA). This procedure was found not to cause cell lysis. Finally, staphylococci were resuspended in 200 ml sterile PBS to a concentration of 3 × 10^8 per ml. Minimal inhibitory and minimum bactericidal concentrations (MIC and MBC) of the strain against gentamicin were determined in liquid medium supplemented with different concentrations of gentamicin, yielding a MIC and MBC of 20 µg ml^-1 and 40 µg ml^-1, respectively.

Biofilm growth in the absence and presence of gentamicin. The parallel plate flow chamber (PPFC, 175 × 17 × 0.75 mm³) and image analysis system have been previously described [8]. Images were taken from pristine silicone rubber and silicone rubber coated with a PEO brush, affixed to the polymethyl-methacrylate bottom plate of the chamber. The top plate was made of glass. Staphylococcal adhesion and growth were monitored using a Fire wire CCD camera, mounted on a phase contrast microscope equipped with a 40× ultra long working distance objective. The camera was coupled to a PC with proprietary image analysis software. Each image (1392 × 1040 pixels with 8 bit resolution) was obtained from summation of 15 consecutive images (time interval 0.25 s) in order to enhance the signal to noise ratio and to eliminate moving organisms from the analysis. Before each experiment, all tubes and the flow chamber were sterilized and filled with PBS, while care was taken to remove
air bubbles from the system. Flasks, containing bacterial suspension and buffer, were positioned at the same height with respect to the chamber to ensure that all fluids circulate through the chamber at the desired rate immediately after starting the flow. Staphylococci were allowed to adhere at room temperature to the substratum surface during 25 min under a flow-induced shear stress of 0.005 Pa. Subsequently, flow was switched from bacterial suspension to 10% TSB medium for 5 min to flush out unattached bacteria from the chamber, after which the temperature was raised to 37 °C, shear stress was decreased to 0.002 Pa, and bacteria were allowed to grow for 3.5 h. After initial adhesion and growth, flow was switched to 10% TSB medium containing three different concentrations (0.5, 5 and 50 µg ml⁻¹) of gentamicin sulphate (Sigma-Aldrich, USA) for 16 h at 37 °C, i.e. antibiotic concentrations well below or above the MIC (20 µg ml⁻¹) and MBC (40 µg ml⁻¹) values found. From the images taken during initial bacterial adhesion and subsequent growth, numbers of adhering bacteria and the percentage surface coverage by biofilm were determined. All experiments were done in threefold, with separately grown bacteria.

**Bacterial viability assay.** At the end of each flow experiment, 20 h old biofilms were removed from the substratum surfaces using a sterile cotton swab and suspended in 0.5 ml demineralised water. Subsequently, 10 µl of the suspension was transferred onto a glass slide and stained for 20 min in the dark with a live/dead stain (*BacLight*™, Molecular Probes Europe BV). Using fluorescence microscopy (Leica, Wetzlar, Germany), the percentages of live and dead bacteria were evaluated, from which the percentage live bacteria in the biofilms was determined.
Fig. 1. Surface coverage by staphylococcal (S. aureus ATCC 12600) biofilm as a function of time during adhesion and growth on pristine (top) and polymer brush-coated (bottom) silicone rubber. After adhesion (first 30 min) and initial growth (210 min), biofilms were allowed to grow in medium containing different concentrations of gentamicin. Error bars represent the standard deviations over three separate experiments.
Results

Fig. 1 shows the surface coverage by staphylococcal biofilms on pristine and polymer brush-coated silicone rubber during growth in the absence and presence of gentamicin (control data in the absence of antibiotic were taken from Nejadnik et al., 2008b). Inclusion of gentamicin in the growth medium only slightly reduced the rate of biofilm formation on pristine silicone rubber and in the presence of antibiotic in the growth medium, the silicone rubber surface was completely covered with biofilm in less than 12 h. Biofilm growth on polymer brush-coatings was not affected by the lowest gentamicin concentration (0.5 µg ml⁻¹), but higher concentrations of gentamicin, i.e. 5 and 50 µg ml⁻¹, suppressed the biofilm formation by 41 and 95% respectively.

The percentage surface coverage by biofilm including the fraction of viable bacteria in the biofilms is presented in Fig. 2. The fraction of viable bacteria in biofilms on pristine silicone rubber amounts approximately 40% and is hardly influenced by the absence or presence of gentamicin. The fraction of viable bacteria in biofilms on polymer brush-coatings is much higher than in biofilms on pristine silicone rubber (81% versus 40%, see Nejadnik et al., 2008b). Antibiotic presence during growth however, strongly decreases the fraction of viable bacteria in biofilms on the brushes, opposite to the observations on uncoated silicone rubber.

The percentage reduction in viable bacteria in the biofilms on silicone rubber and brush-coated silicone rubber is presented in Table 1, as calculated with respect to the number of viable bacteria in biofilms grown in the absence of gentamicin. It can be seen from Table 1 that antibiotic treatment does not cause a reduction in the number of viable bacteria on silicone rubber, but on polymer brush-coatings the reduction in the number of viable bacteria increased with increasing gentamicin concentration during growth.
**Fig. 2.** The percentage surface coverage by biofilm and the fraction of viable bacteria (dashed part) in these biofilms on pristine (SR) and polymer brush-coated (Brush) silicone rubber after growth in the absence or presence of different concentrations of gentamicin. Error bars represent the standard deviations calculated based on three separate experiments.

**Table 1.** Percentage reduction in number of viable bacteria on silicone rubber and polymer brush-coated silicone rubber during growth in the presence of various concentrations of gentamicin. All percentages are calculated with respect to the number of viable bacteria on the surfaces in the absence of any antibiotics. ± represents the standard deviation of the reduction, calculated based on the standard deviations of values obtained from triplicate experiments in the presence and absence of antibiotic.

<table>
<thead>
<tr>
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<th>0.5 µg ml(^{-1})</th>
<th>5 µg ml(^{-1})</th>
<th>50 µg ml(^{-1})</th>
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<tr>
<td>Silicone rubber</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brush</td>
<td>35 ± 24</td>
<td>65 ± 39</td>
<td>96 ± 27</td>
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**Discussion**

Bacteria in biofilms on silicone rubber and other biomaterial surfaces are protected against antibiotic treatment. This study is the first to demonstrate that staphylococcal biofilms on a polymer brush-coating remain susceptible to antibiotics, in addition to the known reduction in biofilm formation on a polymer brush-coating. Both the strong reduction in biofilm formation on polymer brush-coatings and the susceptibility of these biofilms to antibiotics make polymer brush-coatings ideal for application on biomaterials implants.

The mechanism of resistance of bacteria in biofilms against antibiotics is not fully understood. There is evidence that a biofilm-specific phenotype is formed resulting in activation of sub-microbial components which make bacteria resistant [9]. Changes in gene regulation occur within minutes after bacterial attachment to a solid surface [10], suggesting that adhering bacteria may sense a solid surface leading to a signalling cascade that causes genes to be up- or down-regulated [11]. Possibly, all or at least some of the altered organisms are more resistant to antimicrobial agents [9], allowing a biofilm to form. We here hypothesize that on a highly hydrated polymer brush-coating, allowing only weak interaction between the biofilm and the underlying surface [12], organisms do not sense that they are actually on a surface and continue to behave like planktonic organisms.

Alternatively, there are reports that relate the reduced susceptibility of biofilm to antibiotics, to a reduced accessibility of the antibiotic to the micro-organisms [9]. Although the extracellular polymeric matrix of the biofilm is not impenetrable for antimicrobial agents, it may delay the antibiotics to reach the cells in the biofilm, which is in line with the current finding that it takes longer for bacteria adhering to a polymer brush-coating to form a thick, dense biofilm. *In vivo* this may be advantageous, because antimicrobial mechanisms in the body triggered by the immune system will have a longer time to act against single adhering bacteria on a polymer
brush-coating prior to the formation of a contiguous biofilm. Furthermore, our previous finding that the biofilm on polymer brush-coating is more viable than on pristine silicone rubber suggests better penetration of nutrients and may, likewise, support the idea that penetration is a determining factor in the increased susceptibility to antibiotics. Irrespective of the underlying mechanism, however, our current findings have enormous clinical implications, as they show a pathway toward biomaterial implant coatings that allow antibiotic treatment to prevent biofilm formation and therewith to decrease the risk of BAI.

**Conclusions**

Biofilm growth of staphylococci adhering on silicone rubber is not affected by the presence of gentamicin in the medium, even if the gentamicin concentration is above MBC, but staphylococcal biofilms on a polymer brush-coating remain susceptible to antibiotics during growth and, moreover, growth is strongly retarded. Presence of gentamicin in a concentration above MBC during growth of adhering staphylococci on a polymer brush-coating, prevented biofilm formation. Polymer brush-coatings therefore are promising for biomaterial implant coating, especially since they allow treatment of biofilms by antibiotics, contrary to biofilms on other biomaterial surfaces.
Chapter 5

Reference


