Antimicrobial effects of positively charged surfaces on adhering Gram-positive and Gram-negative bacteria

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The infection of biomaterials is determined by an interplay of adhesion and surface growth of the infecting organisms. In this study, the antimicrobial effects on adhering bacteria of a positively charged poly(methacrylate) surface (§ potential +12 mV) were compared with those of negatively charged poly(methyl methacrylate) (−12 mV) and a highly negatively charged poly(methacrylate) (−18 mV) surface. Initial adhesion of Staphylococcus aureus ATCC 12600, Staphylococcus epidermidis HBH2 102, Escherichia coli O2K2 and Pseudomonas aeruginosa AK1 to these surfaces was measured in a parallel plate flow chamber in phosphate-buffered saline. Adhering bacteria were allowed to multiply by perfusing the flow chamber with growth medium. All bacteria adhered most rapidly to the positively charged surface, but there was no subsequent surface growth of the Gram-negative strains. On the negatively charged surfaces, despite a slower initial adhesion, surface growth of the adhering bacteria was exponential for both Gram-positive and Gram-negative strains. These results suggest that positively charged biomaterial surfaces exert an antimicrobial effect on adhering Gram-negative bacteria, but not on Gram-positive ones.

Introduction

Infection is still the most common cause of biomaterial implant failure in modern medicine. Despite the advances in the design of, for example, the total artificial heart, mammmary prostheses, different orthopaedic implants and voice prostheses, there is at present no solution to the problem of infection other than removing the implant. Adhesion and subsequent surface growth of bacteria on biomedical implants and devices causes the formation of a biofilm in which the so-called ‘glycocalix’ embeds the infecting bacteria, offering protection against the host immune system and antibiotics. As most bacteria carry a net negative surface charge, adhesion of bacteria is discouraged on negatively charged surfaces, while it is promoted on positively charged surfaces. Adhesion, however, is only one of the first steps in the formation of a biofilm and in order for a biofilm to develop fully, the adhering bacteria have to grow. Surface growth of the initially adhering bacteria was found by Harkes et al. to be absent on positively charged poly(methacrylates) for Escherichia coli. Barton et al. found that surface growth of Pseudomonas aeruginosa correlated with the free energy of adhesion, while no such correlation was found for Staphylococcus epidermidis and E. coli. Recently, we reported that growth of P. aeruginosa on biomaterial surfaces decreased with the increasing strength of adhesion to the surface.

The aim of this study was to determine possible antimicrobial effects on different Gram-positive and Gram-negative bacteria of a homologous series of three methacrylate polymers and copolymers varying in surface charge. To this end, initial adhesion and subsequent surface growth of Staphylococcus aureus, S. epidermidis, E. coli and P. aeruginosa were measured in a parallel plate flow chamber.

Materials and methods

Bacterial strains and growth conditions

S. aureus ATCC 12600, S. epidermidis HBH2 102, P. aeruginosa AK1 and E. coli O2K2 were used in this study. Each strain was grown up from a frozen stock by incubation...
Slides and coverslips were silanized with 1% (v/v) hydrochloric acid (37%, p.a., Merck) and nitric acid (65%, p.a., Merck), ratio 3:1 (v/v) for 20 h. After extensive rinsing with double deionized water and ethanol, the slides and coverslips were cleaned by immersion in a mixture of toluene and dimethylformamide for the copolymers was dispensed on the slides and coverslips to cover the entire surface. Slides and coverslips were then spun at 2000 rpm for 20 s for PMMA and for 60 s for the copolymers. This procedure was repeated twice to obtain a uniform film, assuring that all polymer films had a similar surface topography. Finally, the polymer films were dried for 18 h under vacuum at 60°C to remove remaining solvent. The coverslips were stored in a sterile container to be used for bacterial adhesion and growth experiments. The glass microscope slides were used for surface characterization as follows.

The chemical composition of the films was determined by X-ray photoelectron spectroscopy (XPS) using a S-Probe spectrometer (Surface Science Instruments, Mountain View, CA, USA). The elemental surface compositions were expressed in atomic %, setting %C + %O + %N + %Si + %Cl to 100%. Zeta (ζ) potentials of the film surfaces were derived from the pressure dependence of the streaming potentials using rectangular platinum electrodes (5.0 × 25.0 mm) located at both ends of a parallel plate flow chamber, which was made up of the glass slides separated by a 0.2 mm Teflon gasket. Streaming potentials were measured over 7 h in PBS (pH 7.0) at 10 different pressures ranging from 37.5 to 150 Torr. Each pressure was applied for 10 s in both directions. Water contact angles were measured at room temperature with an image analysing system, using the sessile drop technique. Each value was obtained by averaging results of at least three droplets on one sample.

**Bacterial ξ potentials**

Bacterial ξ potentials were derived from particulate microelectrophoresis. Three separate cultures of each strain were harvested and washed as described above. Immediately after the bacteria were resuspended (5 × 10⁷ cells/mL) in sterile PBS (pH 7.0), measurements were taken at 150 V using a Lazer Zee Meter 501 (PenKem, Bedford Hills, NY, USA). These were converted into apparent potentials assuming the Helmholtz–Smoluchowski equation.

**The parallel plate flow chamber, image analysis, adhesion and surface growth assay**

The flow chamber (dimensions: 1 × w × h = 76 × 38 × 0.6 mm), image analysis system and adhesion and surface-growth assays have all been described in detail. Images

![Figure 1. Structural formulae of the monomers used to synthesize the differently charged polymers used in this study.](image-url)
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were taken from the bottom plate (58 × 38 mm) of the parallel plate flow chamber, which consisted of the spin-coated microscope coverslip affixed centrally with double-sided tape (0.06 mm thick) in a groove (18 × 18 × 0.16 mm) made in a thicker (2.0 mm) PMMA plate. The top plate of the chamber was made of glass. The chamber was heat sterilized as a whole, except for the PMMA plate, which was disinfected in 70% ethanol. The coated coverslip had been kept sterile ready for use. The flow chamber was equipped with heating elements and kept at 37°C throughout an experiment. Deposition and surface growth was observed with a CCD-MXRi camera (High Technology, Eindhoven, The Netherlands) mounted on a 40× ultra-long working distance lens (Olympus ULWD-CD Plan 40 PL). The camera was coupled to an image analyser (TEA; Difa, Breda, The Netherlands).

Before each experiment, all tubes and the flow chamber were filled with sterile PBS, taking care to remove all air bubbles from the system. PBS was allowed to flow through the system for 1 h at a flow rate of 0.025 mL/s (corresponding to a shear rate of 10/s), while the flow chamber was heated to 37°C. Flow was then switched back to buffer alone for 15 min at the same flow rate to remove unbound organisms from the tubes and the flow chamber. Finally, flow was switched to a growth medium, TSB for the staphylococci, NB for tubes and the flow chamber. Finally, flow was switched to a shear rate of 10/s), while the flow chamber was equipped with heating elements and kept at 37°C throughout an experiment. Deposition and surface growth was observed with a CCD-MXRi camera (High Technology, Eindhoven, The Netherlands) mounted on a 40× ultra-long working distance lens (Olympus ULWD-CD Plan 40 PL). The camera was coupled to an image analyser (TEA; Difa, Breda, The Netherlands).

During the experiment, images were recorded and analysed automatically to give the number of adhering bacteria as a function of time. The initial deposition rate was expressed as the increase in the number of adhering bacteria per unit area and time. The division time of adhering bacteria was also monitored to measure a generation time. The numbers of growing and non-growing bacteria were determined, starting with the image taken after 2 h of flow with growth medium, and following the individual bacteria for the next 4 h. Subsequently, a percentage of growing bacteria relative to the number of adhering bacteria at 2 h was calculated. A desorption rate constant \((k_{\text{des}})\) was obtained by performing a non-linear least squares fit of the increasing part of the growth curve using the model of Barton et al. \(^{19}\)

\[
n_i = n_{i0} + n_{i\infty} (2^{\Delta t / \theta} - k_{\text{des}} \Delta t)^t
\]

In this equation, \(n_i\) is the number of bacteria after the \(i\)th time increment \((\Delta t)\), \(n_{i0}\) is the determined number of non-growing bacteria, \(n_{i\infty}\) is the number of growing bacteria at the beginning of the logarithmic growth phase and \(\theta\) is the generation time. Desorption of non-growing bacteria was negligible compared with desorption of growing bacteria. When no growing bacteria were present, \(k_{\text{des}}\) was determined by dividing the number of desorbed bacteria by the number of adhering bacteria for each time increment after 2 h of flow with growth medium and calculating the average.

Results

Characterization of polymer films

Table I gives \(\xi\) potentials of the polymer films in PBS. \(\xi\) potentials ranged from –18 mV (PMMA/MMMA) to +12 mV (PMMA/TMAEMA-Cl) and were stable over 7 h. Water contact angles on the polymer films were typical of an intermediately hydrophobic surface and showed no major variation with \(\xi\) potential. This indicated that all results could be interpreted without the interfering influences of substratum hydrophobicity. XPS analyses indicated that the positive charge originated from nitrogen-containing groups, while the increased negative charge was caused by oxygen-containing groups on the modified acrylate surfaces.

Bacterial \(\xi\) potentials

All bacterial strains studied here were negatively charged in PBS and their \(\xi\) potentials were –10 mV for \(S. aureus\) ATCC 12600, –8 mV for \(S. epidermidis\) HBH 102, –16 mV for \(E. coli\) O2K2 and –7 mV for \(P. aeruginosa\) AK1.

Adhesion and surface growth

Figures 2 and 3 show examples of images taken during surface growth of \(S. aureus\) and \(P. aeruginosa\), respectively. Note that more bacteria adhered to the positively charged PMMA/TMAEMA-Cl surface after 2 h, but after additional growth (4 h) the most \(S. aureus\) microcolonies were found on the PMMA (–) surface. Proliferating \(P. aeruginosa\) were seen only on the negatively charged surfaces and growth appears absent by comparison with the images taken after 2 and 4 h on the positively charged PMMA/TMAEMA-Cl surface. The numbers of adhering bacteria

Table I. \(\xi\) Potentials in PBS, water contact angles and chemical composition of various PMMA-based polymer films employed in this study

<table>
<thead>
<tr>
<th>PMMA/ MAA</th>
<th>PMMA</th>
<th>PMMA/ TMAEMA-Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\xi) Potential (mV)</td>
<td>–18</td>
<td>–12</td>
</tr>
<tr>
<td>Water contact angle (°)</td>
<td>70</td>
<td>71</td>
</tr>
<tr>
<td>C (%)</td>
<td>69.3</td>
<td>70.6</td>
</tr>
<tr>
<td>O (%)</td>
<td>29.4</td>
<td>28.6</td>
</tr>
<tr>
<td>N (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Si (%)</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Cl (%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
during adhesion and surface growth on the charged methacrylates are shown graphically in Figure 4. During the growth phase, proliferating staphylococci were present on all surfaces from 1 h after the introduction of growth medium. On the negatively charged surfaces, most of the \textit{E. coli} and \textit{P. aeruginosa} cells were proliferating within 30 min. The numbers of \textit{E. coli} increased slowly, however, because most newly formed bacteria desorbed directly from these surfaces. Table II gives initial deposition rates \((j_0)\), percentages of growing bacteria after 2 h, generation times \((g)\) and desorption rate constants \((k_{\text{des}})\) of adhering bacteria. Initial deposition rates were highest for the staphylococci and generally increased as the substrata became less negatively charged. Under conditions of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{A representative example of images of surface-growing \textit{S. aureus} ATCC 12600 on negatively charged PMMA/MAA (–), PMMA (–) and on positively charged PMMA/TMAEMA-Cl (+). The top series, taken after 2 h, shows only adhesion, while the bottom series, taken 4 h after the introduction of the growth medium, shows surface growth. The bar represents 10 \textmu m.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{A representative example of images of surface-growing \textit{P. aeruginosa} AK1 on negatively charged PMMA/MAA (–), PMMA (–) and on positively charged PMMA/TMAEMA-Cl (+). The top series, taken after 2 h, shows only adhesion, while the bottom series, taken 4 h after the introduction of the growth medium, shows surface growth. The bar represents 10 \textmu m.}
\end{figure}
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Figure 4. Example of the number of adhering bacteria on negatively charged PMMA/MMMA (– –) and PMMA (–) and on positively charged PMMA/TMAEMA-Cl (+) in a parallel plate flow chamber. The dashed lines indicate the time period during which PBS was perfused through the flow chamber before the introduction of growth medium. (a) S. aureus ATCC 12600; (b) S. epidermidis HBH2 102; (c) E. coli O2K2; (d) P. aeruginosa AK1.

Table II. Initial deposition rates ($j_0$), percentages of growing bacteria, generation times ($g$) and desorption rate constants ($k_{des}$) of Gram-positive and Gram-negative bacteria on PMMA/MMMA (– –), PMMA (–) and PMMA/TMAEMA-Cl (+) polymer films with different charge

<table>
<thead>
<tr>
<th>Strain</th>
<th>Charge</th>
<th>$j_0$ (cm$^2$/s)</th>
<th>% growth</th>
<th>$g$ (min)</th>
<th>$k_{des}$ (/10^5/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus ATCC 12600</td>
<td>– –</td>
<td>1600</td>
<td>8</td>
<td>41</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>1780</td>
<td>34</td>
<td>32</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2700</td>
<td>13</td>
<td>39</td>
<td>23</td>
</tr>
<tr>
<td>S. epidermidis HBH2 102</td>
<td>– –</td>
<td>1900</td>
<td>7</td>
<td>48</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>1360</td>
<td>26</td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3630</td>
<td>15</td>
<td>48</td>
<td>17</td>
</tr>
<tr>
<td>E. coli O2K2</td>
<td>– –</td>
<td>240</td>
<td>91</td>
<td>24</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>720</td>
<td>59</td>
<td>22</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1720</td>
<td>0</td>
<td>no growth</td>
<td>2</td>
</tr>
<tr>
<td>P. aeruginosa AK1</td>
<td>– –</td>
<td>350</td>
<td>75</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>430</td>
<td>70</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>660</td>
<td>0</td>
<td>no growth</td>
<td>1</td>
</tr>
</tbody>
</table>

Values measured for $j_0$, % growth, $g$, and $k_{des}$ in duplicate experiments were similar within 20, 40, 5 and 25%, respectively.
electrostatic attraction, as on PMMA/TMAEMA-Cl, initial adhesion rates were maximal. Initial adhesion rates of *P. aeruginosa* AK1 were the lowest of all four strains, but also increased as the electrostatic repulsion between the bacteria and the substratum surface disappeared on PMMA/TMAEMA-Cl. Staphylococci grew on all substratum surfaces, although the addition of negative and positive charge to PMMA decreased the relative number of growing staphylococci by a factor of 4 and 2, respectively. The generation times of adherent staphylococci were comparable with those measured for planktonic *S. aureus* ATCC 12600 (31 min) and *S. epidermidis* HBH2 102 (46 min). Gram-negative bacilli grew only on negatively charged surfaces and their generation times also compared with the generation times of planktonic bacteria, viz. 23 min for *E. coli* O2K2 and 43 min for *P. aeruginosa* AK1. All strains showed desorption of adhering bacteria. For the staphylococci, desorption rate constants on the positively charged material were similar to those on the negatively charged materials. As noted, Gram-negative bacilli did not grow on the positively charged surface and they had a very low desorption rate. Desorption was higher from the negatively charged surfaces.

### Discussion

Current approaches to the development of new biomaterials with a low risk of becoming infected once implanted, are based predominantly on developing non-adhesive surfaces. It is known that initial adhesion of coagulase-negative staphylococci and *E. coli* is faster on positively charged PMMA/TMAEMA-Cl copolymers than on negatively charged PMMA and PMMA/MAA copolymers. This was also found in this study. This is because of the absence of repulsive electrostatic interactions between the negatively charged bacteria and the positively charged PMMA/ TMAEMA-Cl. Our results on the surface growth of the initially adhering bacteria suggest, however, that adhesion and surface growth may be oppositely affected by substratum charge. Positively charged surfaces may attract more bacteria, but this effect is readily counterbalanced by the absence of any growth, at least for the Gram-negative strains used in this study. Positively charged surfaces may therefore be regarded as antimicrobial surfaces for these organisms.

Previously, it has been demonstrated that when the binding strength of adhering *P. aeruginosa* AK1 to substrata increases, the surface growth reduces. Complete inhibition of growth, as found here for Gram-negative bacilli, possibly indicates that elongation of adhering bacteria, necessary for cell division, is impeded by strong binding through attractive electrostatic interactions. Soluble quaternary ammonium salts have been known for a long time to exhibit antimicrobial activity through interaction with the bacterial cell membrane. The quaternary ammonium groups of the positively charged polymer, although insoluble, may disrupt the cell membrane of the Gram-negative organisms. Gram-positive bacterial strains have a comparatively thicker and more rigid peptidoglycan layer and extensive contact of the membrane with the immobilized quaternary ammonium groups is less likely to occur, even under conditions of electrostatic attraction. This might explain why the surface growth of Gram-positive bacteria is less affected by the substratum charge. Several groups have also reported a reduction in viable count, greatest for Gram-negative bacteria, when adding positively charged insoluble powders to bacterial suspensions. From these experiments, however, it is not clear whether this reduction is the result of strong bacterial binding to the particles or of reduced viability of planktonic organisms. Our results clearly show that a positively charged surface can totally inhibit growth of some adhering bacteria.

Initial bacterial adhesion has always been recognized as an essential step in biofilm formation. This study shows that when a biomaterial surface is more negatively charged, this may reduce the chance of bacterial adhesion, and delay the formation of a biofilm. Positively charged surfaces are more adhesive, but the strong electrostatic attraction of the organisms impedes surface growth of Gram-negative bacilli. This study therefore points to a new pathway for the development of biomaterials with a low risk of infection and complements current approaches based on preparing non-adhesive surfaces. This may be important in clinical situations where adsorbing proteins are not abundantly present to mask the initial surface, for example in urinary catheters or voice prostheses for laryngectomized patients with hampered salivary flow due to irradiation. As biomaterials are often infected during implantation surgery, a positively charged surface could prevent bacterial proliferation.

As it has also been argued that adsorbed protein films convey the properties of the underlying surface to the interface with adhering bacteria, the present results pose an interesting dilemma. Adhesion and growth appear to be oppositely affected by the surface characteristics of a biomaterial. This may explain why several biomaterials have been found to be non-adhesive *in vitro*, while showing huge biofilm formation once implanted in the human body. Everaert et al., for instance, found that hydrophilized silicone rubber in laryngectomized patients with reduced salivary flow attracted lower numbers of yeasts and bacteria under laboratory conditions in a parallel plate flow chamber than authentic, hydrophobic silicone rubber, but during use as a voice prosthesis a much thicker biofilm formed on the hydrophilized silicone rubber.

In conclusion, in order to develop biomaterial surfaces with a low risk of infection, *in vitro* studies should not only take into account initial adhesion, but also look at surface growth of the adhering bacteria.
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References


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