Initial adhesion and surface growth of *Pseudomonas aeruginosa* on negatively and positively charged poly(methacrylates)

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The infection risk of biomaterial implants is determined by an interplay of bacterial adhesion and surface growth of the adhering organisms. In this study, we compared initial adhesion and surface growth of *Pseudomonas aeruginosa* AK1 (zeta potential -7 mV) on negatively charged (PMMA/MAA, zeta potential -18 mV) and positively charged (PMMA/TMAEMA-Cl, zeta potential $+12$ mV) methacrylate copolymers *in situ* in a parallel plate flow chamber. Initial adhesion was measured using phosphate-buffered saline and subsequent surface growth of the adhering bacteria using nutrient broth as growth medium. Initial adhesion was twice as fast on the positively charged methacrylate than on the negatively charged copolymer. Surface growth, however, was absent on the positively charged copolymer, while on the negatively charged methacrylate the number of bacteria increased exponentially during surface growth with a generation time of 32 min. From the results of this study it can be concluded that positively charged biomaterial surfaces might show reduced risks of biomaterials-centered infections, despite being more adhesive.

1. Introduction

Adhesion and subsequent surface growth of adhering bacteria on biomedical implants and devices cause biomaterials-centered infections that usually result in reoperation, amputation, osteomyelitis or death [1]. As most bacteria carry a net negative surface charge, adhesion of bacteria is discouraged on negatively charged surfaces, while it is generally promoted on positively charged surfaces [2–4]. Surface growth of the initially adhering bacteria, on the other hand, was found by Harkes *et al.* [5] to be absent on positively charged methacrylates for *Escherichia coli* O2K2. Barton *et al.* [6] 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 [7] that the generation time of *P. aeruginosa* AK1 growing on biomaterials surfaces decreased with an increasing strength of bacterial adhesion to the surface.

The aim of this study was to compare initial adhesion and subsequent surface growth of *P. aeruginosa* AK1 on a negatively and positively charged methacrylate copolymer.

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2. Materials and methods

2.1. Bacterial strain and growth conditions

P. aeruginosa AK1, a uropathogenic isolate, was first streaked and grown overnight at 37 °C from a frozen stock on a blood agar plate. The plate was then kept at 4 °C, never longer than a week. Several plate colonies were used to inoculate 5 ml of nutrient broth (NB) (Oxoid, Basingstoke, UK) in phosphate-buffered saline (PBS) that was incubated at 37 °C in ambient air for 24 h. This ''preculture'' was used to inoculate a second culture (100 ml NB in PBS) that was grown for 18 h. The bacteria from the second culture were harvested by centrifugation and washed twice with sterile Millipore-Q water. Subsequently, the bacteria were suspended in sterile PBS to a concentration of 3×10^8 cells ml^{-1}.

2.2. Polymer synthesis

Copolymers of methyl methacrylate with either 15% methacrylic acid (PMMA/MAA) or 15% trimethylaminoethyl methacrylate chloride (PMMA/TMAEMA-Cl) were compared. Polymers were synthesized as described previously [3, 8] by radical polymerization of the monomers using 2,2′-azobis(methyl isobutyrate) as an initiator.

2.3. Preparation and characterization of polymer films

Polymer films were spin-coated on microscope glass slides ($25 \times 76 \times 1$ mm) and coverslips ($18 \times 18 \times 0.1$ mm), as described previously [3, 8]. Zeta potentials of the polymer films were derived from the pressure dependence of the streaming potentials, which were measured in PBS (pH 7.0) employing rectangular platinum electrodes (5.0×25.0 mm) located at both ends of a parallel plate flow chamber [9]. Two coated microscope slides, separated by a 0.2 mm Teflon gasket, constituted the top and bottom plate of the chamber. Streaming potentials were measured at ten different pressures ranging from 4.99 to 19.95 MPa and each pressure was applied for 10 s in both directions.

2.4. Bacterial zeta potentials

For zeta potential measurements [10], three independently grown cultures were harvested and washed as described above. Bacteria were re-suspended (5×10^7 cells ml^{-1}) in sterile PBS (pH 7.0) and their electrophoretic mobility was measured immediately after resuspending at 150 V using a Lazer Zee Meter 501 (PenKem, USA) and converted into zeta potentials assuming that the Helmholtz–Smoluchowski equation holds. An automated version of the Lazer Zee Meter was employed [11] allowing the determination of the zeta potential distribution of individual cells.

2.5. The parallel plate flow chamber, image analysis, initial adhesion and surface growth

The flow chamber (dimensions; $l \times w \times h = 76 \times 38 \times 0.6$ mm), image analysis system and the assay for initial adhesion and surface-growth measurements have all been described in detail [12, 13]. Images were taken from the bottom plate (58×38 mm) of a parallel plate flow chamber, consisting of the spin-coated microscope coverslip affixed centrally with double-sided tape (0.06 mm thickness) in a groove ($18 \times 18 \times 0.16$ mm) made in a thicker (2.0 mm) perspex bottom plate. The top plate of the chamber was made of glass. The system was heat sterilized as a whole, except for the perspex plate, which was sterilized with 70% ethanol. The coated coverslip was used without sterilization. The flow chamber was equipped with heating elements and kept at 37 °C throughout the experiment. Initial adhesion and surface growth was observed with a charge-coupled device (CCD)-MXRi camera (High Technology, Eindhoven, The Netherlands) mounted on a phase contrast microscope (Olympus BH-2) equipped with a 40 × ultra-long working distance objective (Olympus ULWD-CD Plan 40 PL). The camera was coupled to an image analyzer (TEA, Difa, Breda, The Netherlands).

Prior to 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 at a flow rate of 0.025 ml s^{-1} corresponding with a shear rate of 10 s^{-1} for 60 min, while the flow chamber was heated to 37 °C. Subsequently, flow was switched to a bacterial suspension at the same flow rate. The bacterial suspension was perfused through the system for 1 h without recirculation. Following 1 h perfusion of the flow chamber with bacterial suspension, flow was switched to buffer without bacteria to remove unbound bacteria from the tubes and the flow chamber at the same flow rate for 15 min. Finally, flow was switched to full NB in PBS. Growth medium was then perfused through the system at the same flow rate for 6 h without recirculation.

During the experiment, images were recorded and automatically analyzed yielding the number of adhering bacteria as a function of time. The initial increase in the number of adhering bacteria over time was expressed in a so-called initial deposition rate, i.e. the increase in the number of adhering bacteria per unit area and time. The time in which individually adhering bacteria divided was monitored yielding their generation time.

3. Results

3.1. Zeta potentials of the polymer films and bacteria

The PMMA/MAA polymer film had a zeta potential of -18 mV, while PMMA/TMAEMA-Cl had a zeta potential of $+12$ mV. The zeta potential of *P. aeruginosa* AK1 was -7 ± 4 mV.

3.2. Adhesion and surface growth assay

The number of adhering bacteria during adhesion and surface growth on the charged methacrylates are shown in Fig. 1. Numbers of bacteria during deposition were highest on PMMA/TMAEMA-Cl. Corresponding initial deposition rates were 352 cm^{-2} s^{-1} to the negatively charged PMMA/MAA and 803 cm^{-2} s^{-1} to positively charged PMMA/TMAEMA-Cl. When the bacterial suspension was replaced by growth medium, the numbers of bacteria increased exponentially on the PMMA/MAA surface, exceeding the numbers on positively charged PMMA/TMAEMA-Cl already after 5 h of growth. On PMMA-TMAEMA-Cl growth of the adhering bacteria was absent, and a minor desorption of the adhering bacteria was observed. The generation time of *P. aeruginosa* AK1 on PMMA/MAA was 32 ± 6 min (average $\pm$ standard deviation over 56 bacteria).

4. Discussion

Initial adhesion of coagulase-negative staphylococci [2, 4] and *E. coli* [3] has been described to be faster on positively charged PMMA/TMAEMA-Cl than on negatively charged PMMA/MAA copolymers. Our experiments with *P. aeruginosa* AK1 confirm that bacterial adhesion is promoted on positively charged surfaces, due to attractive electrostatic forces [14]. Electrostatic interactions are repulsive between negatively charged *P. aeruginosa* and negatively charged PMMA/MAA.

In addition, Harkes *et al*. [5] found that surface growth of adhering *E. coli* was absent on positively charged PMMA/TMAEMA-Cl, while bacteria adhering on negatively charged PMMA/MAA did grow. Tentatively,

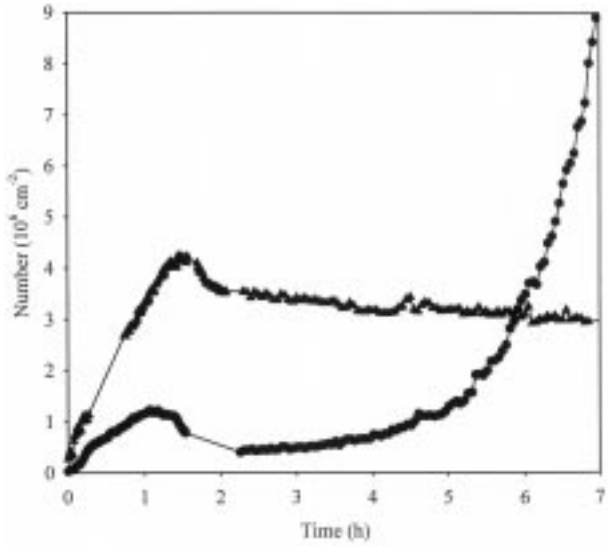

Figure 1 Number of adhering *P. aeruginosa* AK1 during initial adhesion and surface growth on negatively charged PMMA/MAA (circles) and on positively charged PMMA/TMAEMA-Cl (triangles) as a function of time.

it was suggested that on PMMA/TMAEMA-Cl the electrostatic attraction caused an extremely strong binding of the bacteria to the surface, that prevented elongation and subsequent division of the bacteria. More likely, however, adhering bacteria were killed in contact with TMAEMA-Cl groups, as antimicrobial activity of these groups has been reported [15].

Our experiments further quantified differences in initial adhesion and surface growth of *P. aeruginosa* AK1 to positively and negatively charged surfaces and demonstrate that increased adhesion of bacteria on positively charged surfaces is counterbalanced by reduced surface growth, as compared with negatively charged surfaces.

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