Controlled electrophoretic deposition of bacteria to surfaces for the design of biofilms

Abstract

In this chapter the formation of ordered clusters of both spherical and rod-shaped bacteria on an electrode during electrophoretic deposition is described. Inside clusters, adhering bacteria are regularly spaced with an inter-bacterial distance that can be controlled by adjusting the ionic strength of the suspending solution and the DC current density used. Formed clusters can be immobilised on the surface by applying a sufficiently high current density. This method enables the design of bacterial biofilms for biotechnological and biomedical applications. When AC fields were used, rod-shaped bacteria adhering on the electrode were seen to align parallel to the applied field.

Introduction

Biological systems frequently exhibit features superior to what can be achieved by man-made systems. Nowadays these features can be more and more exploited through incorporation of biological systems in technology. Important examples are biosensors [8, 9, 10] and bioreactors [1, 7, 12] in which bacteria are used to efficiently detect or process, respectively, chemical substances. Construction of these systems often involves adhesion and immobilisation of bacteria on a surface [5, 6, 18], such as an integrated circuit in the case of recently developed biosensors [13]. Designed bacterial biofilms might also be used in biomedical applications. For example, probiotic bacteria could be made to adhere on biomaterial implants and serve as protective coatings on catheters [11] or voice prostheses [4] in order to inhibit the adhesion of pathogenic microorganisms. Given these applications, it would be of great importance if one could actually design bacterial biofilms on surfaces. In this work we describe controlled clustering and immobilisation of bacteria on a surface during electrophoretic deposition. This method could allow the controlled formation of bacterial biofilms.

Materials and Methods

Our experiments were performed in a parallel plate flow chamber [3] consisting of two parallel indium tin oxide (ITO) coated glass plates used as electrodes and separated by Teflon spacers of 0.6 mm thickness. Electrophoretic deposition of bacteria to the bottom electrode,
Electrophoretic deposition constituting the anode, was monitored by a phase-contrast optical microscope (Olympus BH-2) with a 40 x objective. Experiments were done using spherical Streptococcus salivarius HB, Streptococcus salivarius HB-C12 and rod-shaped Actinomyces naeslundii 147 suspended in either Millipore filtered, deionized water or in water with up to $10^{-3}$ M KNO$_3$ at various current densities supplied by a DC current source. Streptococci were cultured in Todd Hewitt Broth, actinomyces were cultured in Scheafler's broth supplemented with 0.01 g l$^{-1}$ hemin. Streptococci were grown in ambient air at 37 °C, while actinomyces were grown in an anaerobic cabinet in an atmosphere of 10% H$_2$, 85% N$_2$ and 5% CO$_2$ at 37°C. For each experiment strains were inoculated from blood agar in a batch culture which was used to inoculate a second culture which was grown for 16 hours prior to harvesting. Bacteria were harvested by centrifugation, washed twice with Millipore water and intermittently sonicated to obtain single cells before being resuspended in Millipore water with variable molarities of KNO$_3$. S. salivarius HB has various surface appendages with lengths between 72 and 178 nm, whereas S. salivarius HB-C12, a spontaneous variant of the latter, is devoid of surface appendages [17]. The microscopic radii of S. salivarius HB-C12 and S. salivarius HB are 570 nm and 580 nm, respectively. The dimensions of A. naeslundii 147 are 700 nm x 3000 nm. All organisms carry a negative surface charge under the experimental conditions.

**Results and Discussion**

At low current densities (< 50 µA/cm$^2$) bacteria arriving at the anode from water and low ionic strength (< $10^{-3}$ M KNO$_3$) solutions, adhered but kept moving over the surface through Brownian motion, i.e. adhering bacteria were not immobilised. For current densities in excess of 50 µA/cm$^2$, bacteria were seen to become immobilised. When ionic strengths in the order of $10^{-3}$ M KNO$_3$ were used, bacteria were always immobilised regardless of the current density. This indicates that although a positive potential is applied to the bottom electrode, at low ionic strengths there is still electrostatic repulsion between the bacteria and the ITO surface strong enough to prevent immobilisation of adhering bacteria. Similar experiments with polystyrene particles [2,15] needed to be conducted using surfactants to prevent immobilisation of the particles on the ITO, probably because these particles were larger than our bacteria and thus experience a relatively stronger force originating from the electric field.
Also, formation of bacterial clusters could be observed on the anode. Bacteria were seen to interact with each other and with already formed clusters over a distance of several bacterial diameters. Clustered bacteria remained to demonstrate Brownian motion and occasionally escaped from the clusters. In time, clusters grew until eventually a stationary degree of clustering was reached. The average cluster size increased with increasing current density and decreasing ionic strength. For example, at a bacterial density of $6 \times 10^6$ cm$^{-2}$ after applying a current for 5 minutes the average cluster size was $150 \pm 31$ bacteria at a current density of 25 µA/cm$^2$ and $102 \pm 20$ bacteria at 15 µA/cm$^2$ in water whereas in $2 \times 10^{-5}$ M KNO$_3$ the average cluster size was $61 \pm 14$ bacteria at 25 µA/cm$^2$ (averages of 5 clusters). Fig. 1 gives examples of clusters formed using different current densities and ionic strengths. When the current was

![Figure 1](image-url)

**Figure 1.** Dependence of the degree of clustering in aqueous solutions of *S. salivarius* HB-C12 on ionic strength and current density. Images were taken after 5 minutes. (A) Current density 25 µA/cm$^2$, suspending solution: $4 \times 10^{-3}$ M KNO$_3$ (conductivity: 4.8 µS/cm). (B) Current density 25 µA/cm$^2$, suspending solution: $2 \times 10^{-3}$ M KNO$_3$ (conductivity: 2.8 µS/cm). (C) Current density 25 µA/cm$^2$, suspending solution: water (conductivity: 0.8 µS/cm). (D) Current density 40 µA/cm$^2$, suspending solution: water.
switched off, clusters rapidly broke up and part of the deposited bacteria diffused away from the surface. The formation of clusters occurred faster around immobilised bacteria, indicating that the force responsible for clustering is stronger when a bacterium is in closer contact with the surface. Inside clusters, bacteria were rather regularly spaced as can be seen from the sharp maximum in the radial pair distribution function $g(r)$ in Fig. 2, indicating the relative density of bacteria adhering on a surface as a function of the distance between them. The location of the maximum of $g(r)$ corresponds to the average nearest-neighbour distance between deposited spherical bacteria and is a measure for the magnitude of the forces responsible for clustering. When the current increases to 40 $\mu$A/cm², the distance for which $g(r)$ is maximal decreases to 1.3 $\mu$m which approximately corresponds to contact between bacteria (Fig. 1d).

![Figure 2. Radial pair distribution function $g(r)$ as a function of the centre-to-centre distance $r$ of deposited *S. salivarius* HB-C12 calculated by computer image analysis. 2000 bacteria were included in the analysis. Measuring conditions were as in Fig. 1c. Average bacterial density was $3 \times 10^6$ cm⁻².]

In addition to the experiments with DC currents described above, we also applied AC voltages with frequencies up to 2 MHz and amplitudes up to 13 V. No bacterial clustering was observed then. However, when the amplitude of the AC voltage exceeded 3 V, the rod-shaped *A. naeslundii* 147 almost instantaneously aligned parallel to the field whereas it otherwise
adheres flat on the surface (compare Fig. 3a and Fig. 3b). This effect disappeared for frequencies above 1 MHz.

**Figure 3.** Images of rod-shaped *A. naeslundii* 147 adhering on an electrode. Dimensions of the depicted images are 57 µm x 82 µm. (A) Clustering of *A. naeslundii* 147 adhering flat on an ITO electrode in water. Current density 25 µA/cm². Image was taken after 5 minutes. (B) Alignment of *A. naeslundii* 147 parallel to an applied AC field on an ITO electrode in water. Amplitude of the applied field was 3.3 V, frequency was 1 kHz.

Earlier works using polystyrene [2, 15, 19] and silica [15] particles also describe clustering phenomena at electrodes. Trau *et al.* [15] used DC and AC fields and in both cases observed clustering which was attributed to electrohydrodynamic flow [16]. The passage of a current leads to an accumulation of ions in the vicinity of the electrodes. The presence of particles near the electrode causes lateral variations in this ion-accumulation leading to electrohydrodynamic fluid flow directed towards particles causing them to move toward one another. Böhmer [2] observed clustering of particles at the positive electrode in DC fields and explained this in terms of electro-osmotic flow of the liquid around the particles [14]. However, clustering and alignment phenomena have never been observed for rod-shaped colloidal particles, as for *A. naeslundii* 147.

We have shown that bacteria can be forced to form ordered clusters at interfaces during electrophoresis and that the inter-bacterial spacing inside these clusters can be controlled by adjusting the ionic strength of the suspending solution and the current density used. Clusters formed can be 'frozen' by applying a current density high enough to immobilise the adhering bacteria. This enables design of bacterial biofilms for biotechnological applications (for
example biosensors and bioreactors) or as protective coatings of probiotic bacteria on e.g. silver-impregnated urinary catheters or voice prostheses in biomedical applications.

References

Electric field induced desorption of bacteria from a conditioning film covered substratum

Chapter 5b

Abstract

Desorption of three oral bacterial strains from a salivary conditioning film on an indium tin oxide (ITO) electrode during application of a positive (bacterial adhesion to the anode) or a negative electric current has been studied in a parallel plate flow chamber. Bacterial adhesion was from a flowing suspension of high ionic strength, after which the bacterial suspension was replaced by a low ionic strength solution without bacteria and currents ranging from –800 to +800 µA were applied. *S. oralis* J22 desorbed during application of a positive and negative electric current with a desorption probability that increased with increasing (absolutely seen) electric current. Two actinomyces strains, however, could not be stimulated to desorpb by the electric currents applied. The desorption forces acting on adhering bacteria are electroosmotic in origin and working parallel to the electrode surface in case of a positive current, while they are electrophoretic and electrostatic in origin and working perpendicular to the surface in case of a negative current. By comparison of the effect of positive and negative electric currents, it can be concluded that parallel forces are more effective in stimulating bacterial desorption than perpendicular forces. The results of this study point to a new pathway of cleaning industrial and biomedical surfaces without the use of detergents or biocides.

Introduction

Formation of biofilms on industrial equipment surfaces imposes the need for frequent cleaning and in food industry encompasses the danger of food poisoning [1]. Bacterial biofilms also develop at biomaterials implant surfaces inside the human body, leading to infection and ultimately removal of the implant [2]. On tooth surfaces, dental biofilms cause caries and periodontal diseases [3]. As many microbial strains have become resistant to antibiotics [4] and other biocides [5], new strategies to fight biofilms are needed.

The development of a biofilm starts with the formation of a conditioning film consisting of adsorbed proteins and other macromolecules from the environment and is followed by adhesion of bacteria and their growth. Current strategies to prevent biofilm formation include the use of anti-fouling surfaces [6], growth-inhibitory surfaces [7, 8] and the application of antimicrobials [9] and detergents [10]. Mechanically-based cleaning methods exist as well, such as brushing [10], the use of ultrasound [11], or the passage of liquid-air interfaces over
adhering micron-sized particles and bacteria [12, 13]. Most of these methods, however, apply removal forces thinning down a biofilm from its outer surface, while it has been argued that the weakest link in the chain connecting a biofilm to a substratum surface is often constituted by the conditioning film and the initially adhering organisms [14]. A more effective cleaning method would be to break this weakest link and therewith remove the biofilm as a whole.

In a previous paper [15], we have shown that approximately $10^4$ adhering streptococci per cm$^2$ per second can be removed from a conducting surface by the application of an electric current of about 10 μA/cm$^2$, while using the substratum surface as a cathode. This new cleaning methodology has not yet been applied to bacteria adhering to a conducting surface in the presence of a conditioning film. Hence, the aim of this paper is to determine if streptococci and actinomyces strains adhering to a salivary conditioning film on a conducting surface can be removed by the application an electric current.

Materials and methods

Bacterial strains

Three bacterial strains were used throughout this study: Streptococcus oralis J22 and the rod-shaped Actinomyces naeslundii T14V-J1 and A. naeslundii 147. S. oralis J22 was cultured in Todd Hewitt broth at 37 °C in ambient air. A. naeslundii T14V-J1 and A. naeslundii 147 were cultured in Schaedler’s broth supplemented with 0.01 g/l hemin in an anaerobic cabinet (DW scientific, West Yorkshire, UK) in an atmosphere of 10% H$_2$, 85 % N$_2$ and 5 % CO$_2$ at 37 °C. For each experiment strains were inoculated from blood agar plates in a batch culture for 24 h. This culture was used to inoculate a second culture which was grown for 16 h prior to harvesting.

Bacteria were harvested by centrifugation (5 min at 10,000 g), washed twice with demineralized water and resuspended to a concentration of $3 \times 10^8$ bacteria/ml in adhesion buffer (2 mM potassium phosphate, 1 mM CaCl$_2$ and 50 mM KCl, pH 7). Before being resuspended, streptococci and actinomyces were sonicated at 30 W for 30 s while cooling on an ice/water bath, to obtain single cells (Vibra Cell model 375, Sonics and Materials Inc., Danbury, Connecticut, USA).
Saliva

Human whole saliva from twenty healthy volunteers of both sexes was collected into ice-chilled cups after stimulation of the salivary flow by chewing Parafilm®. After the saliva was pooled and centrifuged at 12,000g for 15 min at 4°C, phenylsulfonyl fluoride was added to a final concentration of 1 mM as a protease inhibitor. The solution was again centrifuged, dialyzed for 48 h at 4°C against demineralized water, and freeze-dried for storage. Reconstituted, human whole saliva was prepared from the lyophilized stock by dissolution of 1.5 mg/ml in adhesion buffer.

Parallel plate flow chamber

Bacterial adhesion and subsequent desorption was studied in a parallel plate flow chamber [16]. Top and bottom plates of the flow chamber consisted of transparent, conducting indium tin oxide (ITO) coated glass plates (29 cm² surface area) with the coated side in contact with the solution. ITO-coated glass plates were obtained from Philips Components, Heerlen, The Netherlands, and were produced by DC sputtering. Adhering bacteria were observed with a CCD-MXR camera (High Technology, Eindhoven, The Netherlands) mounted on a phase-contrast microscope (Olympus BH-2) equipped with a 40 x ultra long working distance objective (Olympus ULWD-CD Plan 40 PL). All fluids used circulated through the chamber under the influence of hydrostatic pressure at a flow rate of 0.02 ml/s (shear rate of 10 s⁻¹), while fluids were recirculated using a peristaltic pump.

ITO-coated glass plates were cleaned by 1 min sonication in methanol, followed by thorough rinsing with tap water and 1 min sonication in Millipore filtered, deionized water. Thus cleaned ITO surfaces had water contact angle of 85 degrees.

Experimental protocol

Experiments were done at room temperature. First, saliva was flowed through the chamber for 2 h to create a salivary conditioning film. Subsequently, a bacterial suspension was flowed through the chamber until 3.5 x 10⁶ cm⁻² bacteria were found adhering to the bottom. Then, the chamber was perfused with cell-free 1 mM potassium phosphate buffer (pH 7) and as soon as the flow system was free of bacterial cells electric currents incrementing from −800 to +800 μA were applied. Each current was applied for 10 min. A negative current indicates that the bottom plate was used as the cathode, while positive currents indicate that the bottom plate was the anode. During application of the electric current, images of the bacteria adhering to
the bottom plate were grabbed and stored in the computer. In order to distinguish between adhering bacteria and in focus flowing desorbed bacteria, fifteen successively (approximately 1 s time interval) images were grabbed, averaged and thresholded to eliminate in focus flowing bacteria from the averaged image.

Electric field induced desorption probabilities $\beta$ are then determined from the decrease in the number of adhering bacteria per unit area and time divided by the number of adhering bacteria per unit area at the start of the application of an electric current.

**Results**

Fig. 1a gives an example of the number of adhering *S. oralis* J22 to the ITO electrode during application of the incremental electric currents. At the on-set of the electric current, about $3.2 \times 10^6$ bacteria/cm$^2$ adhere. Figs. 1b and 1c show the effects of applying $-800$ and $+400$ µA, respectively, with the time scale adjusted to make $t=0$ correspond with the onset of the electric current. Note that initially the density of adhering bacteria decreases linearly during application of an electric current, as a result of which the number of bacteria adhering at the on-set of the different electric currents are not the same. This was accounted for in analyzing the data by calculating desorption probabilities, defined as the decrease in the number of adhering bacteria per unit time and area relatively to the number of bacteria adhering per unit area at the on-set of an electric current. Desorption probabilities thus obtained are summarized for the bacterial strains used in Table 1.

Table 1 includes the density of adhering bacteria at the start of the application of the electric current. For *S. oralis* J22, desorption probabilities increase with increasing (absolutely seen) currents, regardless of whether a positive or a negative electric current was applied. The actinomyces strains adhered significantly stronger than the streptococcal strain used and desorption probabilities did not vary systematically with electric current, although desorption did occur. Note that in the absence of an electric current, none of the three strains desorbed under the present experimental conditions.
Figure 1.

(a) Example of the number of *S. oralis* J22 adhering to an ITO electrode surface as a function of time during application of electric currents. Applied currents during the different time periods were: 0 s-954 s: -400 µA; 1249 s-1900 s: -600 µA; 2259 s-2865 s: -700 µA; 3508 s-4121 s: -800 µA; 4492 s-4823 s: +200 µA; 4851 s-5175 s: +400 µA, 5200 s-6050 s: +600 µA.

(b) As in (a), for the application of −800 µA. Note that the time scale is adjusted to make t=0 coincide with the start of the application of the electric current. Line indicates a linear fit.

(c) As (b), for the application of +400 µA.
**Table 1.** Desorption probabilities $\beta$ for different electric currents applied, together with the numbers of adhering bacteria $N$ at the start of an electric current. All experiments were done in triplicate with separately cultured bacteria and new ITO electrode surfaces, yielding the standard deviation indicated.

<table>
<thead>
<tr>
<th>Current (µA)</th>
<th>N ($10^6$ cm$^{-2}$)</th>
<th>$\beta$ ($10^{-5}$ s$^{-1}$)</th>
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<tr>
<td><strong>S. oralis J22</strong></td>
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<tr>
<td>-200</td>
<td>3.6 ± 0.5</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>-400</td>
<td>3.7 ± 0.3</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td>-600</td>
<td>3.6 ± 0.1</td>
<td>15.2 ± 15.2</td>
</tr>
<tr>
<td>-800</td>
<td>3.5 ± 0.4</td>
<td>23.1 ± 3.8</td>
</tr>
<tr>
<td>+200</td>
<td>2.9 ± 0.6</td>
<td>35.6 ± 26.8</td>
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<tr>
<td>+400</td>
<td>2.6 ± 0.8</td>
<td>50.9 ± 20.5</td>
</tr>
<tr>
<td>+600</td>
<td>1.7</td>
<td>125.1</td>
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<tr>
<td><strong>A. naeslundii T14V-J1</strong></td>
<td></td>
<td></td>
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<tr>
<td>-400</td>
<td>4.6</td>
<td>5.9</td>
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<tr>
<td>-600</td>
<td>4.5</td>
<td>0</td>
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<tr>
<td>-800</td>
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<tr>
<td>+800</td>
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<td>0</td>
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<tr>
<td><strong>A. naeslundii 147</strong></td>
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<td></td>
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<tr>
<td>-400</td>
<td>2.1 ± 0.1</td>
<td>0.3 ± 0.3</td>
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<tr>
<td>-600</td>
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<tr>
<td>-800</td>
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Discussion

In the present study it is demonstrated that bacteria adhering to a conducting substratum can be stimulated to desorb by the application of an electric current, also when the substratum is covered by a conditioning film. The forces acting on adhering bacteria upon the application of an electric current are schematically indicated in Fig. 2. Application of an electric current implies the existence of an electric field throughout the solution in-between the parallel plates of the flow chamber with a strength equal to the electric current divided by the product of the electrode area and the specific conductivity of the solution (141 µS/cm for a 1 mM potassium phosphate buffer). The electric field causes an electrophoretic force to work on the adhering bacteria, proportional to the electrophoretic mobility of the bacteria and the electric field strength and directed perpendicular to the electrode surface [17]. For the strains involved in this study the electrophoretic force is in the order of \(10^{-13}\) N at –800 µA, which is about two

![Figure 2. Schematic indication of the forces resulting from an applied electric current acting on adhering negatively charged bacteria (indicated by the spheres) during application of a positive or a negative current. Indicated electrostatic forces are relative to when no current is applied. Actually, the electrostatic force may still be repulsive when a positive current is applied, but the repulsion will be reduced as compared to when no current is applied.](image-url)
Electric field induced desorption

Orders of magnitude smaller than bacterial adhesion forces reported [18, 19]. The electrophoretic force resulting from a negative electric current stimulates desorption, while a positive electric current yields an electrophoretic force opposing desorption.

Attractive forces in-between the negatively charged bacteria adhering at the electrode arise during application of a positive electric current, because the electric field associated with the current leads to electroosmotic fluid flow directed towards adhering bacteria [20]. This electroosmotic force strongly depends on the separation distance between bacteria and under the experimental conditions used it is estimated to be in the order of $10^{-14}$ N for bacterial cells at a 1 µm surface-to-surface separation distance [20, 21]. Although the electroosmotic force works parallel to the surface and does not directly stimulate desorption, a force of $10^{-14}$ N may cause adhering bacteria to move over the surface [22], which will stimulate their desorption.

During application of a negative current, the bottom electrode becomes more negatively charged and the electric field associated with these charges leads to enhanced electrostatic repulsion between adhering bacteria and the electrode. Enhanced electrostatic repulsion for the used conditions and strains represents a force in the order of $10^{-11}$ N [15] and is thus an important factor stimulating desorption.

From the above it can be concluded that application of a negative electric current yields greater removal forces than application of a positive current, but yet desorption probabilities were found to be largest upon application of a positive current. This indicates that bacteria can be more effectively removed by applying a force parallel to a substratum surface than perpendicular to it, as has been suggested before for the removal of adhering particles [23].

Not all strains could equally well be stimulated to desorb by an electric current, and in contrast to streptococci, actinomyces could hardly be stimulated to desorb. Also in other studies, A. naeslundii has been described to bind strongly to a salivary conditioning film and adhering actinomyces could withstand detergents and the passage of liquid-air interfaces [24]. This is probably a results of the relatively long surface appendages the actinomyces possess assisting them in their adhesion to a substratum surface.

Summarizing, electric currents stimulating bacterial desorption may constitute a useful, environmentally friendly new method for cleaning industrial and biomedical surfaces. In the present study, electric currents were limited to 800 µA in order to prevent degradation of the ITO electrode, but in practical situations (e.g. stainless steel) higher currents can be used without adverse effects to yield enhanced desorption.
Chapter 5b

References