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Models for studying initial adhesion and surface growth in biofilm formation on surfaces

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Microbial biofilms cause various problems in industry, waterworks, dentistry and medicine. In this chapter a parallel plate flow chamber system is described, with which different processes in bacterial biofilm formation, i.e. conditioning film formation, initial bacterial adhesion, bacterial surface growth and bacterial detachment can be modeled and monitored in situ. Examples are given of studies concerning the influence of a plasma conditioning film on initial adhesion, the influence of biomaterial surface properties on surface growth and the influence of surface active substances on detachment of biofilm bacteria.

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Introduction

Biofilms can be considered as micro-ecosystems in which different microbial strains and species efficiently cooperate in order to protect themselves against environmental stresses, and to facilitate more efficient nutrient uptake. Most often, biofilms are unwanted, and related to diverse problems as microbially induced corrosion of oil-rigs and pipelines [1], food and drinking water contamination [2,3], dental caries and periodontal diseases [4] and a variety of biomaterial-centered infections in man [5]. Biomaterial-centered infections in man are especially troublesome, since biofilm organisms are protected against the host immune system and cannot be easily eradicated with antibiotics. Consequently, infection of a biomaterial implant will usually result in reoperation, osteomyelitis, amputation or death [6]. Not all biofilms are unwanted, however, and in sewage treatment, biofilms are needed for efficient degradation of xenobiotics [7], while lactobacillus biofilms form part of the normal indigenous microflora in man and their maintenance is essential in the prevention of disease [8].

Mechanism of biofilm formation

The formation of a biofilm in an aqueous environment is generally pictured to proceed in the following sequence [9] (see Figure 1):

1. When organic matter is present, a conditioning film of adsorbed components is formed on the surface prior to the arrival of the first organisms
2. Microorganisms are transported to the surface through diffusion, convection, sedimentation or active movement
3. Initial microbial adhesion occurs
4. Attachment of adhering microorganisms is strengthened through exopolymer production and unfolding of cell surface structures
5. Surface growth of attached microorganisms and continued secretion of exopolymers
6. Localized detachment of biofilm organisms caused by occasionally high fluid shear or other detachment forces operative

Localized detachment of biofilm organisms starts after initial adhesion, although adhesion of individual microorganisms is frequently considered irreversible (whether justified or not), and increases with time as it is related to the number of microorganisms present in the biofilm [10].
Detachment of parts of a biofilm can occur by failure inside the bulk of the biofilm, or by failure in the so-called linking film, involving either detachment of the initially adhering organisms, cohesive failure in conditioning film or interfacial rupture. Furthermore, as the
number of biofilm organisms increases, growth rates will decrease due to nutrient and oxygen limitations and accumulation of organic acids, eventually leading to a stationary biofilm thickness, where adhesion and growth counterbalance detachment.

**Prevention, control and eradication of biofilms**

An obvious approach in the prevention of biofilm formation is the prevention of initial microbial adhesion. Microbial adhesion is mediated by specific interactions between cell surface structures and specific molecular groups on the substratum surface [11], or when viewed from an overall, physico-chemical viewpoint by non-specific interaction forces, including Lifshitz-Van der Waals forces, electrostatic forces, acid-base interactions and Brownian motion forces [12]. Specific interactions are in fact non-specific forces acting on highly localized regions of the interacting surfaces over distances smaller than 5 nm, while non-specific interaction forces have a long range character and originate from the entire body of the interacting surfaces. Upon approach of a surface, organisms will be attracted or repelled by the surface, depending on the resultant of the different non-specific interaction forces. Lifshitz-Van der Waals forces and Brownian motion usually promote adhesion, while electrostatic interactions can be either attractive or repulsive. Most organisms are negatively charged [5] and consequently a negatively charged substratum exerts a repulsive electrostatic force on the organisms. Control of the charge and hydrophobic properties of substratum surfaces is likewise a pathway to influence biofilm interaction with a substratum surface.

Another pathway to influence biofilm formation is through inhibition of growth. A possible approach is the design of (antibiotic)slow-release materials mediating direct kill upon contact [13], but such approaches are always temporarily and bear the risk of inducing resistant strains. Alternatively, also physico-chemical surface properties affect growth and it has been found that *Escherichia coli* growth is inhibited on positively charged surfaces through strong attachment [14].

To control beneficial biofilms, conditions have to be adapted to obtain the optimal equilibrium of growth and detachment of biofilm organisms. Growth rate can be controlled by nutrient conditions, reactor design, oxygen household, removal of metabolic end-products and temperature. Detachment can be influenced by substratum surface properties, fluid flow rate [15], mechanical stress and surface active substances [16].

Eradication of unwanted biofilms with disinfectants or antibiotics is hampered, because of the resistance of biofilm organisms against anti-microbial penetration through the
biofilm [17]. Total removal of biofilms is only possible, when biofilms are directly accessible, as e.g. on exterior parts of the human body. Dental biofilms can be removed by mechanical cleansing, in combination with the use of surface active substances, like in dentifrices and mouth washes. Bacteria adhering on contact lenses can be removed by rubbing the lenses between the fingers in combination with cleansing solutions. The strength of biofilm adhesion to a substratum surface, i.e. the ease with which it can be removed, is greatly dependent on the strength with which the initially adhering organisms bind the substratum surface and cohesiveness of the conditioning film [18], which makes initial microbial adhesion and surface growth an important issue of research.

This chapter summarizes the use of a parallel plate flow chamber model to study initial microbial adhesion to surfaces and extends the use of flow chamber devices and data analysis to include surface growth of the initially adhering organisms.

![Figure 2. Detailed view of the parallel plate flow chamber.](image)

**Experimental design**

The parallel plate flow chamber and image analysis used in our laboratory is accurately described in an earlier volume of this series [19], but will be briefly repeated for completeness. Figure 2 shows the chamber (external dimensions 16 x 8 x 2 cm, length by width by height) which is made of nickel-coated brass to allow sterilization. The internal dimensions of the chamber are 7.6 x 3.8 x 0.06 cm, although the height can be varied by using different spacers. Microbial adhesion, surface growth and detachment is directly observed
usually on the bottom plate using phase-contrast microscopy, thus biofilm formation can be followed in situ without any additional shear forces acting on the deposited microorganisms. The bottom plate can be of various materials but can be conveniently made of poly(methyl methacrylate) (PMMA) with a groove in the middle in which a substratum material of interest can be fixed. The top plate is made of glass. The application of phase-contrast microscopy requires transparent substratum materials. Non-transparent, reflective substrata can also be used when an ordinary metallurgical microscope, based on incident reflected light, is available [20]. To allow the detection of microorganisms on opaque materials incandescent dark-field illumination is directed under a low angle onto the bottom plate of the channel by means of a lens supported on a slide [21]. Furthermore, it is possible to use fluorescence microscopy, provided it is ascertained that the required fluorescent dyes do not affect the adhesive properties of the microorganisms.

The microscope images can be recorded using a CCD camera and processed by an image analyzer (TEA, Image-Manager, Difa Breda, the Netherlands) in combination with dedicated image analysis programs [22].

Flow is created by a roller pump and can be pulse-free or controlled pulsatile [23]. Optional is temperature control using heating elements mounted on the sides of the chamber. By means of a valve system it is possible to connect flasks containing, for example, buffer, reconstituted human whole saliva, bacterial suspension, growth medium or detergent solutions, with the flow chamber without passing an air-liquid interface over the adsorbed conditioning film and/or adhering organisms [24].

**Initial microbial adhesion**

Initial microbial adhesion experiments are typically done in buffer solutions without any additional nutrients, to avoid complications caused by conditioning films or growth of microorganisms during deposition. Before each experiment all air bubbles are removed from the tubing and flow chamber and the buffer solution is perfused through the system for a predetermined time. Subsequently, flow is switched to a suspension of microorganisms in buffer. During deposition, images of the bottom plate are recorded and the organisms present on the surface are counted using the image analysis program. From the number of microorganisms plotted versus time the initial deposition rate ($j_0$) is determined and through an iterative procedures the number of bacteria adhering at a stationary end-point time ($n_\infty$) is found.
As natural biofilms are not formed in plain buffer, it is relevant to study adhesion also in the fluids present in nature. For example in studies on biofilm formation on teeth [25], urinary catheters [26], contact lenses [16] or body implants [27], buffer can be replaced by saliva, urine, tear fluid or blood plasma. Here initial adhesion can be influenced by the composition of the conditioning film. In blood serum, for example, albumin reduces staphylococcal adhesion [27], while fibronectin can promote adhesion of certain staphylococcal strains [28,29]. The contribution of surface growth to the number of attached bacteria cannot easily be separated from that of adhesion, which complicates these types of experiments.

Surface growth
The use of the parallel plate flow chamber can be extended to study surface growth of sessile microorganisms [30,31]. For this purpose, the entire system is sterilized at 120°C, except for the PMMA bottom plate and the substratum material of interest, which are sterilized by 70% ethanol. During the experiment the flow chamber can be heated to 37°C to get more relevant results for surface growth on biomaterials. The experiment starts with initial adhesion of the microorganisms during a short duration of time, as microorganisms tend to lose their viability in buffer. This can be avoided, however, by supplementing the buffer with growth medium. For Pseudomonas aeruginosa, for example, it was found that only 2% of the adherent bacteria was metabolically active after 4 h deposition in phosphate buffered saline (PBS), while in a minimal (2%) growth medium 67% appeared metabolically active [32]. In the above case, addition of a minor amount of growth medium did not seriously complicate interpretation of the results.

After microorganisms are seeded on the substratum surface, the flow chamber is washed with adhesion buffer at the same flow rate as during deposition, to remove the planktonic and loosely adhering organisms. Subsequently, flow is switched to growth medium at the same flow rate and continued for a predetermined time. Images are recorded during surface growth, from which the number of organisms present is determined. With appropriate analysis, it is possible to follow individual microorganisms and determine their generation time.
Eradication of biofilms

After microorganisms are deposited or grown in the flow chamber, various effects of environmental stress on the biofilm can be studied. To study the influence of high shear stress on the adhering organisms, we usually pass an air bubble over the substratum surface [33]. The passing of a liquid-air interface results in a detachment force on the adhering organisms of around $10^{-7}$ N [19], which is much higher than the shear resulting from the flowing liquid. The number of organisms present before, and after the passage of the liquid-air interface can be determined as a measure of the strength of microbial adhesion.

To determine detergent-stimulated detachment of biofilm organisms, a surfactant solution can be led over the adhering microorganisms. Images are recorded before, during and after the treatment, and the microorganisms are enumerated. In this way, the activity of, for example, mouth washes and contact lens cleansing solutions can be evaluated.

Examples of the use of parallel plate flow chamber for studying biofilm formation and eradication

Influence of a blood plasma conditioning film on initial staphylococcal adhesion

For six staphylococcal strains, staphylococcal adhesion was studied on silicone rubber with and without pre-adsorbed plasma proteins [27]. First, when appropriate, flow was switched to plasma for 1.5 h to create a conditioning film. Thereafter, flow was switched for 30 min to buffer for removal of all remnants of plasma from the tubes and the chamber, and then to the bacterial suspension which was circulated through the system for 4 h.

Figure 3 is an example of the deposition kinetics of one strain, *S. epidermidis* 242. The presence of a conditioning film on silicone rubber had a reducing effect on the adhesion of all strains studied. The reduction of the initial deposition rate, $j_0$, and the adhering numbers in a stationary end-point varied between 77-97% and 55-98%, respectively, depending on the strain used.
Figure 3. Deposition kinetics of *S. epidermidis* 242 to silicone rubber with (triangles) and without (circles) a plasma coating. For details, see Van der Mei *et al.* [27].

**Adhesion and surface growth of Staphylococcus epidermidis on materials with various wettabilities**

Figure 4. The number of *S. epidermidis* HBH2 102 during deposition and surface growth on silicone rubber (triangles) and glass (circles) [30].
The adhesion and surface growth of *Staphylococcus epidermidis* HBH$_2$ 102 was determined on different materials with varying water contact angles between 15° and 110° [30]. The flow chamber temperature was kept at 36°C during the experiment. The initial adhesion rate was determined in PBS for 1 h, the flow chamber was washed with PBS for 15 min and subsequently was switched to 20 times diluted tryptone soya broth (TSB) in PBS. During 24 h surface growth was followed. Due to its grape forming mode of growth, individual staphylococci are not easily counted and the numbers of adhering bacteria were derived from the measurement of the surface coverage of the biofilm. Figure 4 shows the numbers of bacteria on silicone rubber and glass. The desorption of biofilm bacteria was determined and expressed as the fraction of the biofilm that detaches per minute (k$_{des}$). The generation time (g) can be calculated using a modification of the mathematical model for microbial biofilm growth by Barton *et al.* [31]:

\[
    n_i = n_0 \left( 2^{\frac{g}{k_{des} \Delta t}} - k_{des} \Delta t \right)^i
\]

in which $n_i$ is the number of adhering bacteria after time $i \times \Delta t$ and $n_0$ is the bacterial number at the start of the growth phase.

Figure 5 gives the generation time of this staphylococcal strain on the different materials as a function of their water contact angle, revealing a relationship between substratum wettability and surface growth.
**Detachment of Pseudomonas aeruginosa from contact lenses by ophthalmic solutions**

To study the efficacies of two contact lens cleansing solutions, first a biofilm was formed on a contact lens quarter and mounted in the parallel plate flow chamber [16]. For this purpose, a suspension of *P. aeruginosa* no. 3 in saline supplemented with 2 % TSB was circulated through the system for 20 h to allow adhesion and surface growth. Then flow was switched for 1 h to saline to remove non-adhering bacteria from the system and subsequently 8 ml of an ophthalmic solution or saline (control), followed by 16 ml saline to clean the chamber of the solution, was perfused through the flow chamber. To study renewed bacterial adhesion, flow with saline was continued for 30 min, after which a new suspension of freshly cultured bacteria was circulated through the system for 4 h. After removal of non-adhering bacteria with saline for 1 h, another dose of ophthalmic solution was applied. Finally a liquid-air interface was led over the surface. Figure 6 illustrates the number of adhering *P. aeruginosa* on a contact lens with a tear film during one complete experiment. The ophthalmic solution clearly decreases the number of bacteria while the liquid-air interface does not yield a significant effect.

![Figure 6](image-url)  
**Figure 6.** Number of adhering *P. aeruginosa* no. 3 to a contact lens with an adsorbed tear film. After the adhesion and surface growth, the surface was rinsed by a detergent mixture (R I), followed by a second adhesion phase and a second rinse (R II). Finally an air bubble was passed over the surface. For details see Landa et.al. [16].
Advantages and disadvantages of the system

The major advantages of the system outlined are controlled shear and mass transport; a high data density in time; and the avoidance of air-liquid interface passages over the adhering microorganisms. Furthermore, the *in situ* observation offers the great advantage that all events in initial biofilm formation, including adhesion and growth can be followed in time and that the fate of an individual microorganism in the biofilm can be studied.

A disadvantage of the system is that biofilm formation is only viewed in two dimensions, and consequently only initial biofilm formation can be studied. As the biofilm thickness extends to above one layer, the events are not clearly visible anymore. To study more mature biofilms, a three-dimensional viewing system is needed, as provided by scanning confocal laser microscopy combined with three-dimensional image analysis software [34]. Unfortunately this method is invasive due to the fluorescent staining, and biofilm processes cannot be followed *in situ*.

References

Models for studying initial adhesion


