Evaluation of early stages of oral Streptococci biofilm growth by optical microscopy. Effect of antimicrobial agents.

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The aim of this chapter was to describe a simple and non invasive methodology and assemblage to assess the initial stages of Streptococci biofilm formation and the influence of antimicrobial agents (AA) and substratum roughness on biofilm distribution, microstructure and compactness. The experimental set up consisted in thin stainless steel orthodontic bands which were immersed in the medium inoculated with microorganisms. The growth of the biofilm was monitored at real time through optical microscopy made *in situ* using a simple assemblage specially designed to observe thin biofilms (Optical Microscopy of Thin Biofilms OMTB). Examples where planktonic cells, sessile cells and cells released from the biofilms were observed before, during and after the AA treatments.

Keywords biofilm, antimicrobial agents, optical microscopy, sessile bacteria, plaque, oral microorganisms.

1. Introduction

Biofilms are composed of microorganisms embedded in a matrix of extracellular polymeric substances (EPS). Biofilm mode of microbial growth protects the microbes from adverse conditions.

The development of a biofilm involves several processes in which different types of cells are included: planktonic cells, sessile cells and released cells. At the beginning, pioneer planktonic microorganisms attach reversibly (Figure 1a). Then, EPS is produced, the cells attach irreversible and become sessile cells (Figure 1b). Afterwards the proliferation of these microbes (Figure 1c) and the development of the microstructure and maturation of the biofilm (Figure 1d) takes place. Finally, the release of cells and groups of cells from the biofilm completes a cycle (Figure 1e) [1].

Although biofilm formation processes are reasonably studied, very little is known about the detachment process. The importance of the released cells has been demonstrated recently [1,2]. These released cells may attach in a remote place and initiate a new biofilm [,1,3].

Confocal Scanning Laser Microscope (CSLM) [2,4] has revealed a complex biofilm architecture in which microcolonies are enclosed in EPS separated by water-filled channels (Figure 1d). The main consequence of this microstructure is that water from the bulk phase can enter into channels and deliver nutrients deep within the complex community.

Sessile cells exhibit an altered phenotype with respect to growth rate and gene transcription of planktonic cells [3]. Importantly, the biofilm cells are more resistant to killing by antimicrobial agents (AA) than planktonic microorganism. However microorganisms seem to loose progressively their resistance to AA when they are released from the biofilm. Thus, released cells are frequently thought as intermediates between sessile and planktonic cells.

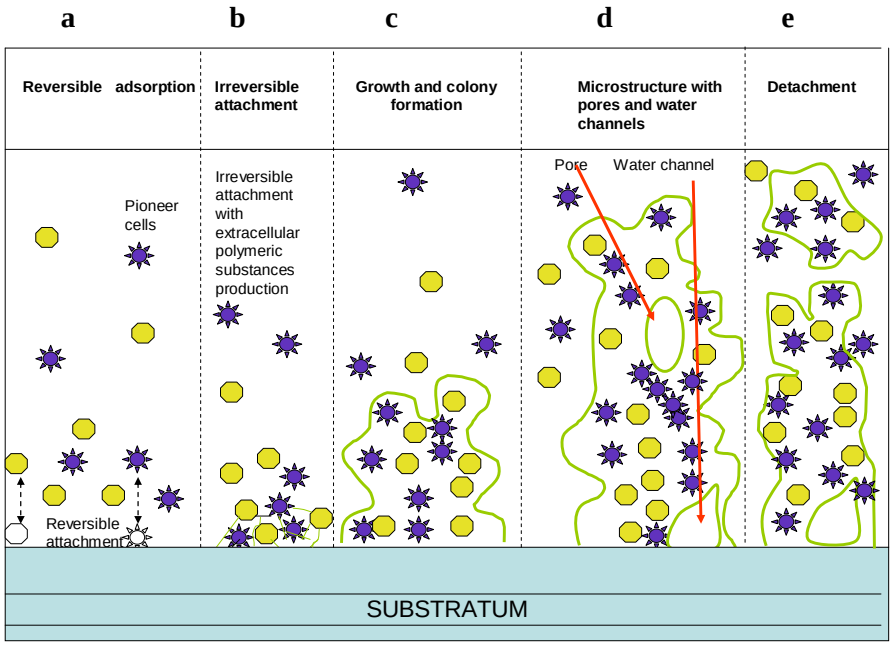

Figure 1. Schema of the different stages of the biofilm development.

Dental plaque is a biofilm that grows in the oral cavity. It is a good example to study initial stages of biofilm formation and the effect of antimicrobial agents (AA). The insertion of orthodontic appliances tends to create new surfaces available for plaque formation and to increase the level of microorganisms in the oral cavity. Gaps between synthetic and natural elements are dangerous sites which may be colonized by bacteria [5]. Orthodontic patients with fixed appliances frequently have prevalence of *Streptococcus mutans* in plaque compared with untreated orthodontic patients [6]. These microorganisms have an important role in cariogenesis processes and consequently the study of the growth of this particular biofilm is relevant in the control of plaque formation.

The aim of this chapter was to describe a simple and non invasive methodology developed in our laboratory to evaluate the early stages of the growth of a biofilm of oral microorganisms and the influence of substratum roughness and AA on biofilm microstructure and compactness. The use of optical microscopy together with a specially designed glass flow cell to observe biofilms formed on thin substrata (OMTB) provided a suitable way to follow the early stages of thin biofilm formation at low magnification with low equipment cost.

2. Experimental methodology to observe biofilms in situ at real time and ex situ.

A diagram showing the apparatus designed to follow biofilm growth (OMTB) [5] including glass flow cell adapted to the microscope and the light path is shown in Figure 2. It was developed on the basis of the procedure followed to study the growth of thin crystal layers on a metal substratum [7]. Thin sheets of the different substrata were attached to cylinder-shaped stainless steel holders separated by 3 mm from the bottom of the glass cell. The glass cell was filled with the culture which inlet and outlet velocity was varied between 0 and 4 mL/min. The medium flowed with a laminar regimen (0>Re>20) so that the sheets remained covered by the solution within the cell (volume=25 mL, wall thickness=3mm, hydraulic radius=flow area/wetted perimeter=0.25 cm, equivalent diameter=4 x hydraulic radius=1cm). At appropriate time intervals (15 minutes) the biofilm development on the thin lateral area of the sheets and wires was observed.

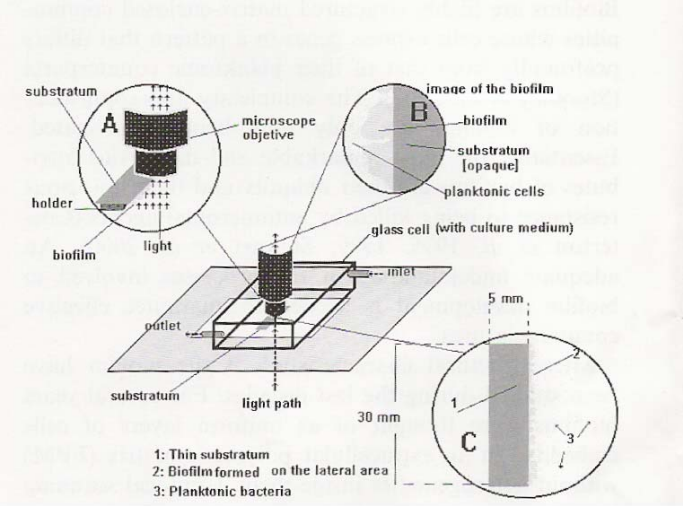

Figure 2. Schema of the assemblage used for microscopic observations of the biofilms including the light path and the flow cell (A,B,C= details).

The substratum was visualized as an opaque surface and the biofilm and the bathing solution as a bright area using a X40 objective (total magnification X400). An inverted microscope with a X40 water-immersion lens and dark-field illumination may also be used with similar purposes.

Substrata

Orthodontic bands (Ponce bands of 0.2 mm thickness) were mainly used as substrata. Additionally nitinol and stainless steel orthodontic wires of rectangular (0.19 x 0.25 mm) and circular (0.2 mm diameter) section, and Ti sheets were also used. The thickness of the samples varied between 0.05 and 0.25 mm. The formation of the biofilm on the lateral thin areas was observed by OMBT. Previous to each experiment the biomaterials were polished with emery papers of different grades and with alumna (1 μm), and successively rinsed in ethyl alcohol and sterile water. Rough lateral surfaces (polished with 600 grade emery paper) and micro-thorny tips (used in dentistry to remove the nerve of the root canal) were also employed to study the effect of surface unevenness.

Bacterial Culture Conditions and Harvesting

A consortium collected from the oral cavity of several patients with a normal periodontal status was used in the experiments. It was obtained by scraping the gingival area of buccal and lingual tooth surfaces and along the entire fissure or margin of restorations on occlusal surfaces of the patients. Each sample was dispersed by sonication for 10 s in sterile culture medium. Oral micro-organisms were cultured in Mitis-Salivarius Agar medium to isolate *Streptococcus Mitis (S. Mitis) and S. Salivarius.* The isolated microorganisms were maintained under anaerobic conditions in modified Mitis-Salivarius liquid (MMS) medium (tryptose 10 gl^{-1}, proteose peptone No 3, 5 gl^{-1}, proteose peptone 5 gl^{-1}, dextrose 1 gl^{-1}, saccharose 50 gl^{-1}, dipotassium phosphate 4 gl^{-1}, trypan blue 0.075 gl^{-1}, crystal violet 0.0008 gl^{-1} , tellurite 0.004 gl^{-1}, which favours streptococcal growth). Solutions were prepared from analytical grade chemicals. The estimation of the planktonic cell number was made using a Neubauer camera. Direct plate counting method were also employed. A 10 ml inoculum was poured into an Erlenmeyer flask containing 150 ml of the MMS. The initial number of cells was adjusted to ca.10^5 cells/ml.

Pseudomonas fluorescens (*P fluorescens*) were also used in some cases with the aim of comparison. Bacterial culture conditions and harvesting were described elsewhere [8].

Biofilm formation in vitro and in vivo

Stainless steel orthodontic bands were immersed in a *Streptococci* or *P fluorescens* culture to form biofilms on their surfaces. The culture medium was transported to the flow cell described bellow from a batch culture at the stationary phase of growth. Biofilms were formed at 32 ºC in the flow cell both, under static conditions and under flow (continuous culture, inlet and outlet flow up to 4 mL/min).

The biofilms formed *in vivo* in the oral cavity of patients and those formed *in vitro* in the culture media on stainless steel wires used in orthodontic treatments were compared, the *in vivo* biofilms were obtained from patients with orthodontic appliances after 7 days of exposure to the oral environment.

Ex situ observations

Scanning electron microscopy (SEM) observations of the biofilms were also made for comparison. After exposure, biofilmed metal specimens were fixed in 2% glutaraldehyde in sterile synthetic saliva, dehydrated through an acetone series to 100% and critical point dried.

Live and dead kit was used to detected surviving planktonic cells after the AA treatments.

Biofilm Treated with antimicrobial agents

To study the effect of AA stainless steel bands were immersed in the culture media inoculated with Streptococci consortia for seven days (Treatment I) and the growth of biofilms was followed in real time by OMTB, made *in situ*, and SEM made *ex situ*. Subsequent to the preset period the biofilmed bands were removed from the culture medium and were transferred to three different flasks (Treatment II B, F + X_and_CH + X) containing: (B)= a phosphate buffer solution (B: NaCl 8 g/L, KH_2PO_4 0.34 g/L, K_2HPO_4 1.21 g/L) (control); (F + X)= a mouth rinse containing NaF (0.05 %) + X (xylitol) (10%);(CH + X)= a mouth rinse containing Clorhexidine (0.12 %) + X (10 %). 24 h later the samples were removed from these flasks. One of the samples was observed using OMTB and then by epifluorescence microscopy after staining it with acridine orange and other was scrapped to remove sessile cells. The removed cells were suspended in a B and then they were enumerated in a Neubauer camera using the appropriate dilution. Additionally, the released cells suspended in the media were enumerated. A third sample was used to assess the re-growth of the biofilm and subsequently was transferred to a fresh MMS culture medium (Treatment III)

3. Biofilm development observed “in situ” at real time by OMTB and “ex situ” by SEM.

Biofilm growth

The transport of the pioneer cells from the bulk towards the substratum/solution interface, followed by the attachment (first reversible and then irreversible, Figure 1a) of the pioneer cells could be observed by OMTB in real time A preferential approximation of the planktonic cells to the attached cells was observed.

Figure 3. OMTB of early stages of Streptococci biofilm formation. Magnification: X400.

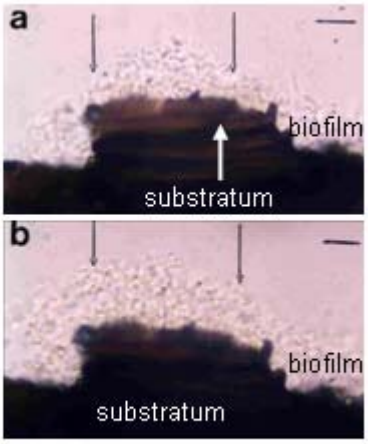

Figure 4. OMTB of biofilm growth on a rough substratum after 24 h (a) and 48 h (b) of immersion in the culture medium inoculated wih *P. fluorescens Adapted from ref.14*

Figure 5. SEM microphotograph of the initial stage of Streptococci colony formation. The distance between white bars indicate 10 μm.

Figure 6. SEM microphotograph of an oral bacterial colony formed in a valley of a rough surface. The distance between white bars indicate 10 μm.

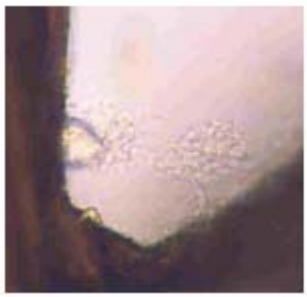

Figure 7. OMTB of *P. fluorescens* biofilm formed on a microthorny tip. Magnification X400.

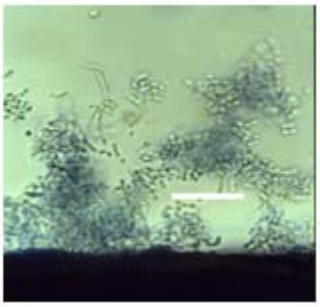

Figure 8. OMTB of Streptococci biofilm (7 days of immersion). Magnification X400.

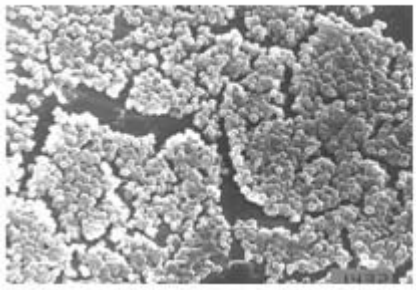

Figure 9. SEM microphotograph of Streptococci biofilm formed on an orthodontic band. The white bar indicates 10 μm.

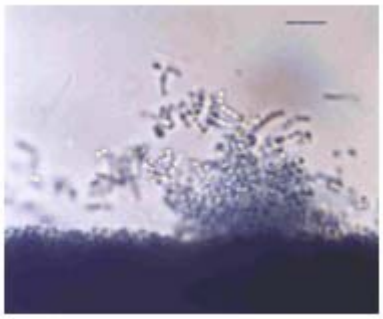

Figure 10. OMTB of a colony showing a colour gradient that could be attributed to different biofilm density. Magnification X400

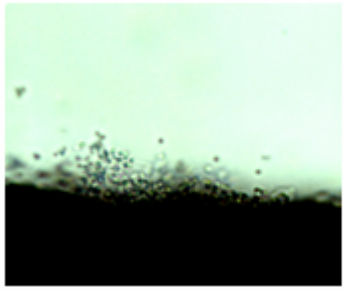

Figure 11. OMTB of biofilm of oral microorganisms after immersion in AA. Magnification X400.

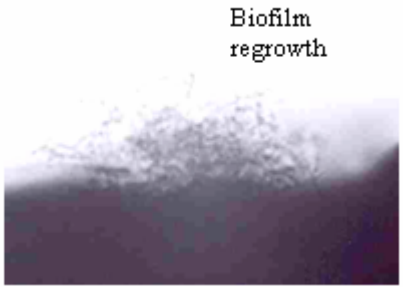

Figure 12. OMTB of a biofilm treated with antimicrobial agents and then immersed in a sterile culture medium. Regrowth of the biofilm can be detected. Magnification X400.

Biofilm regrowth

There was a higher number of planktonic cells near the interface than in the bulk and their residence time was higher in this enviroment (Figure 3). A particular motion of planktonic bacteria around the border of the colonies was detected. At the early stages, chains of bacteria could also be observed vibrating on the border on the stainless steel coupon

In contrast, P. fluorescence biofilms were more compact and formed layers which became thicker at longer exposure periods (Figures 4 a and b). Some “streamers” that oscilate with the flow were also observed.

Extracellular polymeric matrix production

A careful examination of the field through OMTB showed that there was an environmental transformation of the metal/solution interface when the biofilm was growing. The liquid medium became more viscous and the cells displaced at a lower rate in this region [5]. The higher viscosity is probably produced by the EPS exuded by the bacteria towards the liquid surroundings. The dehydration and fixation pretreatments and the higher magnification of the SEM observations resulted in a better image of bacteria, free of the EPS cover (Figures 5 and 6).However, the soft structure of Streptococci immersed in EPS and the spatial distribution of the cells at the interface that can be seen through OMTB can not be distinguished by SEM. Consequently, OMTB and SEM images provide complementary information to achieve a better interpretation of the real biofilm growth process.

Biofilms formed on rough surfaces

The experiments of [9] showed that firstly the biofilms develop within the valleys of the uneven surfaces, smoothing the rough regions. Besides, recent studies [10-12] demonstrated that the irreversible attachment was easier on the valleys of the rough areas and that cells were most readily removed from smoothest surfaces. Grooves and cavities, rather than smooth regions, appeared to be preferentially occupied by pioneer bacteria (Figure 6) and OMTB shows that the apparent roughness of the substrate is reduced (Figure 4). The motility of P. fluorescens cells seems to facilitate the attachment of the cells inside the grooves [13]. Figure 7 shows the attachment of bacteria in a valley of a micro-thorny tip for dental applications. Consequently, it is also useful to employ OMTB to study the effect of roughness on bacterial distribution. The roughness of thin substrata could be easily evaluated by OMTB (Figure 4).

Microstructural characteristics of the biofilms

Microstructural characteristics like pores, interstitial channels and bridges of thin Streptococci biofilms could be distinguished using OMTB (Figure 8). Water channels and bridges are frequently formed when neighbouring fungous-like colonies coalesced at the upper side, close to the solution while the inner side, close to the substratum, remained separated [14]. Conversely, SEM pretreatments impede the visualisation of these microstructural characteristics because the EPS of the biofilm constricts and channels and pores are blocked or altered during dehydratation (Figures 9).

Biofilm thickness and density

The actual thickness of the thin biofilms could also be evaluated at real time by OMTB. Figure 4 shows that the biofilm thickness can be easily measured between the arrows. In the case of rough surfaces the biofilm thickness varied to obliterate the unevenness of the surface, reducing the apparent roughness. A reduction up to a half of the original thickness of the biofilm was observed in SEM microphotographs because of the dehydration of EPS occurring during pretreatments. Additionally, during SEM pretreatments some of the biological material was detached. Thus, comparison of OMTB and SEM images revealed some of the SEM distortions such as detachment of some biofilm patches, reduction of biofilm thickness and discontinuities in the EPS layer.

A colour gradient, which may be attributed to a change in the bacterial and/or EPS density, is observed in Figure 10. A darker and less transparency inner older layer than the outer younger layer were detected in Streptococci colonies at stationary phase of growth. The darker region of the colonies seem to contain higher numbers of cells per unit area and more compact EPS.

The epifluorescence microscopy enumerations were in agreement with the results obtained after scrapping the coupons. Released cells were observed after filtration of the medium and staining. an extracellular matrix that protect them from the environment could be detected.

Effect of Antimicrobial Agents on biofilms

To study AA effect three treatments were performed. During biofilm formation, the Ponce bands remained 7 days in the MMS medium inoculated with a Streptococci consortium (Treatment I). The metal samples with the biofilms were then transferred to the B, F + X or CH + X solutions (Treatments II: B, F + X and CH + X respectively). After 24 h immersion period it could be noticed that the surfaces of the orthodontic bands immersed in F + X or CH + X (Figure 11) were cleaner than the control with only few chains attached to the border of the coupons. These chains were immersed in a viscous medium and the colonies were more compact than those of the control B. However, planktonic cells released from the biofilm were found in the solutions which were sterile at the beginning of the experiment. The treated biofilmed samples were then transferred to a sterile culture medium (MMS) to evaluate the possible re-growth (Treatment III). Again, a great number of cells was released from the treated biofilm. The number of planktonic and sessile cells was higher than the number of cells of the biofilms after Treatment II. This indicates that the reproduction of cells occurred (Figure 12). The re-growth was accompanied by the production of a viscous polymeric layer. Released cells were viable but poorly cultivable cells.

Figure 13 shows a schema of the changes of the biofilm microstructure during and after the antimicrobial agents treatments. Individual cells and no chains of Streptococci could be seen in the liquid media.

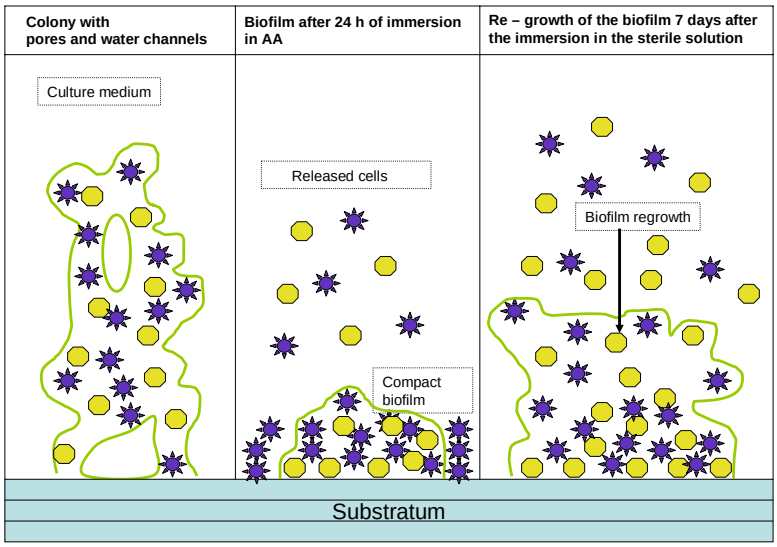

Figure 13. Schema of the changes of the biofilm microstructure before and after the AA treatments.

4. General remarks

The different processes that give rise to the formation of the biofilm on thin substrata could be monitored at real time by OMTB. Changes of the viscosity near the interface, of the apparent roughness of the substrata and of the rate and direction of the planktonic cells displacement towards the interface could be observed. Spatial distribution of cells at the interface and microstructural details were detected, and changes in roughness and thickness of the biofilm formed on thin substrata could be measured. Distortions introduced by pretreatments carried out to prepare biological materials for SEM observations could be noticed by comparing OMBT and SEM images. However, in spite of the SEM alterations, the dehydration and fixation pretreatments and the higher magnification yielded to a good image of bacteria because they were free of the EPS cover. Thus, OMTB and SEM images of biofilms formed “in vivo” or “in vitro” provide complementary information to achieve a better approach to the real biofilm situation [14]).

OMBT revealed that antimicrobial treatments (F + X and CH + X) were able to modify the morphology of bacterial colonies, inhibit the growth of the sessile bacteria and favour the detachment. It was also detected that, a significant number of sessile cells with a more resistant physiological state could remain alive after AA treatments and either re-grow slowly as sessile cells or become planktonic under better environmental conditions.

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