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Microstructural characteristics of thin biofilms through optical and scanning electron microscopy

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Summary

The combination of a conventional *optical microscope* with a specially designed glass flow cell was used to visualize '*in situ*' *biofilms* formed on opaque *thin* biomaterials through a simple non-invasive way (optical microscopy of thin biofilms, OMTB). Comparisons of OMTB with scanning electron microscopy (SEM) images were made. Thin metallic dental biomaterials were used as substrata. They were immersed in a synthetic saliva and in a modified Mitis–Salivarius medium inoculated with a consortium of oral microorganisms. To study the effect of bacterial motility, *Pseudomonas fluorescens* cultures were also used. The processes which give rise to the formation of the biofilm were monitored through OMTB. Biofilm microstructures like pores, water channels, streamers and chains of Streptococci, attached to the surface or floating in the viscous interfacial environment, could be distinguished. Thickness and roughness of the biofilms formed on thin substrata could also be evaluated. Distortions introduced by pretreatments carried out to prepare biological materials for SEM observations could be detected by comparing OMTB and SEM images. SEM images (obtained at high magnification but *ex situ*, not in real time and with pretreatment of the samples) and OMTB images (obtained *in situ*, without pretreatments, in real time but at low magnification) in combination provided complementary information to study biofilm processes on thin substrata.

Introduction

Biofilms are highly structured matrix-enclosed communities whose cells express genes in a pattern that differs profoundly from that of their planktonic counterparts (Stoodley *et al.* 2002). The complexity and sophistication of biofilms are only now being appreciated. Essentially, the most remarkable and dangerous attributes of biofilms are their ubiquity and their notorious resistance to being killed by antimicrobial agents (Costerton *et al.* 1995, 1999; Stewart *et al.* 2000). An adequate understanding of the processes involved in biofilm development is needed to guarantee effective countermeasures.

Microstructural characteristics of the biofilm have been studied during the last decades. For several years biofilms were thought of as uniform layers of cells embedded in an extracellular polymeric matrix (EPM) without heterogeneities inside them. Confocal scanning laser microscopy (CSLM) (Lewandowski 1998; Stoodley *et al.* 2002), however, has revealed a complex biofilm architecture in which microcolonies are enclosed in EPM separated by water-filled channels. 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. Only direct microscopic observations without invasive pretreatments allow the visualization of the actual microstructure. Unfortunately, many research groups cannot use CSLM as a routine tool. Despite the distortions of some high-resolution techniques like scanning electron microscopy (SEM) and the requirement of extensive sample preparation *ex situ* (Beech *et al.* 1996) they are still used (Werner *et al.* 1999; Schmidt *et al.* 2002).

The use of optical microscopy together with a specially designed glass flow cell to observe biofilms formed on thin substrata (optical microscopy of thin biofilms, OMTB) provided a simple non-invasive way to follow the early stages of thin biofilm formation at low magnification. Pretreatments like freezing and staining the samples (Huang *et al.* 1995) are not necessary.

The aim of this work is to show that OMTB is useful to characterize microstructures, roughness and thickness of biofilms formed on thin substrata both *in vitro* and *in vivo* and to detect distortions in SEM images. The advantages of using OMTB and SEM microscopy, in combination, to facilitate the proper interpretation of the real situation are analysed.

Materials and methods

Substrata

Orthodontic bands (thin stainless steel bands), nitinol (a nickel–titanium alloy) and stainless steel orthodontic wires of rectangular (0.19×0.25 mm) and circular (0.2 mm diameter) section, and titanium (Ti) and brass sheets were used as substrata. The thickness of the samples varied between 0.05 and 0.25 mm. The samples were stuck to two stainless steel holders (5 mm diameter, 3 mm height, distance between them = 20 mm, distance to the upper surface of the cell = 5 mm). The formation of the biofilm on the lateral thin areas was observed by OMTB. Previous to each experiment the biomaterials were polished with emery papers of different grades and with alumina (1 μm), flamed and successively rinsed in ethanol and sterile water. Rough lateral surfaces (polished with 600 grade emery paper) 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. Every 2 months they were completely replaced by new samples obtained from the same patients. Oral microorganisms were culture in Mitis–Salivarius agar medium to isolate *Streptococcus mitis* and *S. salivarius*. The isolated microorganisms were maintained in TVLS (thioglycollate–meat, liver-serum broth, Merck™) under anaerobic conditions, modified Mitis–Salivarius liquid medium (tryptone 10 g l^{-1}, proteose peptone No. 3, 5 g l^{-1}, proteose peptone 5 g l^{-1}, dextrose 1 g l^{-1}, saccharose 50 g l^{-1}, dipotassium phosphate 4 g l^{-1}, trypan blue 0.075 g l^{-1}, crystal violet 0.0008 g l^{-1}, tellurite 0.004 g l^{-1}, which favours Streptococcal growth) and in synthetic saliva B (Baron *et al.* 1978) (NaCl: 6 g l^{-1}, KCl: 0.3 g l^{-1}, $CaCl_2$: 0.2 g l^{-1}, lactic acid: 3.1 g l^{-1}, with the addition of sodium sulphite 0.1 g l^{-1}, potassium thioglycollate 0.5 g l^{-1}, 0.5 g l^{-1} yeast extract and 1 or 4 g l^{-1} glucose, pH = 6.5). All solutions were prepared from analytical grade chemicals. The estimation of the planktonic cell number was made using a Petroff-Hauser camera. A 10 ml inoculum was poured into an Erlenmeyer flask containing 150 ml of the culture medium (saliva B or modified Mitis–Salivarius liquid medium). The initial number of cells was adjusted to ca.10^5 cells/ml.

With the aim of studying the effect of bacterial motility on biofilm formation, a strain of motile *P. fluorescens* (a wild strain isolated from the industrial environment) was also employed in this work. It was maintained in cetrimide agar at 28 °C and its purity was periodically checked by means of Gram stains and oxidase test. The inoculum of *P. fluorescens* was prepared by suspending growth from a cetrimide agar (Bacto Cetrimide Agar Base, dehydrated, Difco™) slant (24 h old) in 2 ml of sterile culture medium. The culture medium consisted of synthetic saliva B.

Biofilm formation in vitro *and* in vivo

The culture medium was transported to the cell of the OMTB assemblage from a batch culture at the stationary phase of growth. Biofilms were formed at 30 °C in the flow cell described below both, in static conditions and under flow (continuous culture, inlet and outlet flow up to 4 ml/min).

With the aim of comparison of the biofilms formed *in vivo* in the oral cavity of the patients with those formed *in vitro* in the culture media on stainless steel wires (0.2 mm diameter) used in orthodontic treatments, observations were made by OMTB and SEM. The *in vivo* biofilms were obtained from five patients with orthodontic appliances after 7 days of exposure to the oral environment.

Microscopic observations of the biofilms

A diagram showing the apparatus designed to follow biofilm growth (OMTB) (Cortizo & Mele 2000) including a glass flow cell adapted to the microscope and the light path is shown in Figure 1. It was developed on the basis of the procedure followed to study the growth of thin crystal layers on a metal substratum (Schilardi *et al.* 1999; Azzaroni *et al.* 1999). Thin sheets of the different substrata were attached to cylinder-shaped stainless steel holders separated by 3 mm from the bottom of the glass

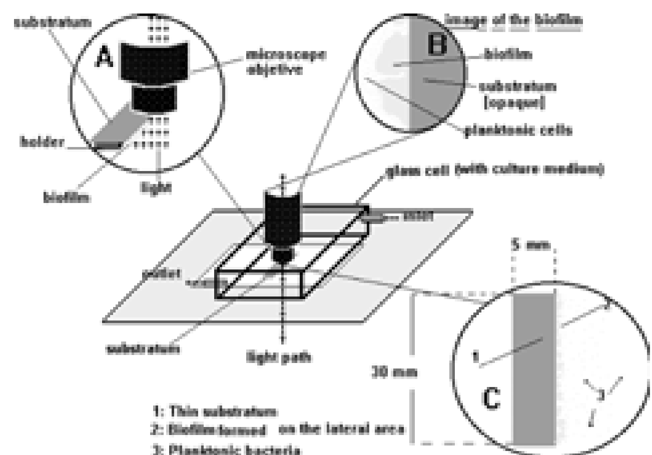

Figure 1. Schema of the assemblage used for microscopic observations of the biofilms including the light path and the flow cell (A = a higher magnification of the objective lens and the light path, B = image of the biofilm detected by the observer, C = the substratum (1) with the biofilm (2) and planktonic cells (3) at a higher magnification).

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 within the cell (volume = 25 ml, wall thickness = 3 mm, hydraulic radius = flow area/wetted perimeter = 0.25 cm, equivalent diameter = 4 × hydraulic radius = 1 cm). The flow rate, perpendicular to the sample, varied, in the plane of focus, between 0 and 0.20 cm/s.

At appropriate time intervals (15 min) the biofilm development on the thin lateral area of the sheets and wires was observed. The microscope objective was placed so that the biofilm/substratum interface was in the middle of the field (see Figure 1, insets A and B). The immersed less were removed from the culture medium immediately after the observation. The cell has an elliptic hole (longer diameter = 6 cm, shorter diameter = 3 cm) at the top so that the lens can get into the cell and move from one extreme of the band or wire to the other. This hole is sealed with a Teflon cover.

The substratum was visualized as an opaque surface and the biofilm and the bathing solution as a bright area using a ×40 objective (total magnification ×400). An inverted microscope with a ×40 water-immersion lens and dark-field illumination may also be used with similar purposes.

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

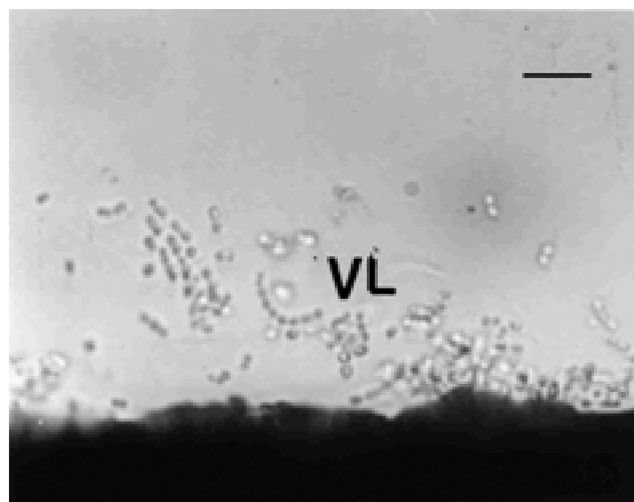

Figure 2. OMTB microphotograph of the consortium of oral microorganisms growing on a stainless steel orthodontic band. Bacterial chains are floating in the viscous liquid layer (VL) close to the liquid/metal interface. Bar = 9.1 μm.

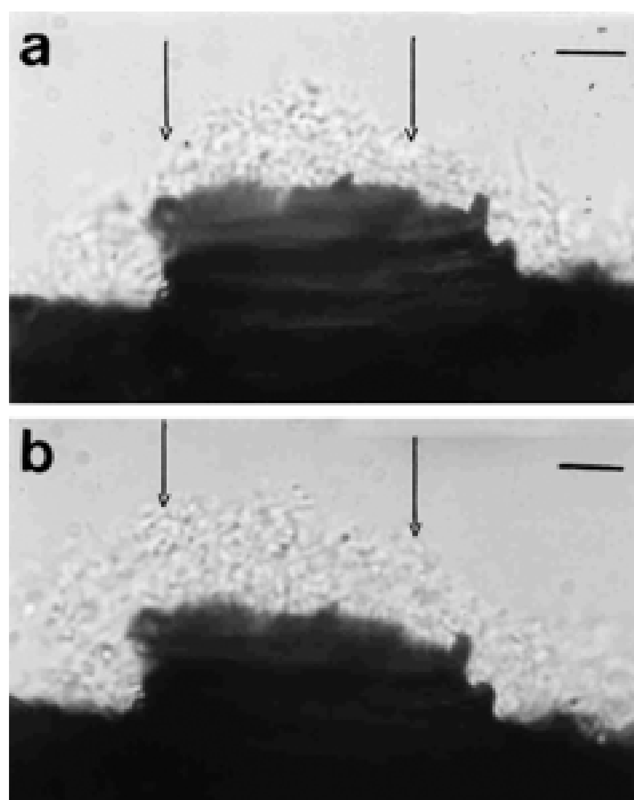

Figure 3. OMTB microphotograph of a *Pseudomonas fluorescens* biofilm growing on a brass sheet after: (a) 24 h and (b) 48 h of immersion in the culture medium. The arrows indicate the change in the thickness. Bar = 9.1 μm.

Results and discussion

Biofilm growth

The different processes which give rise to the formation of a *P. fluorescens* biofilm *in vitro* could be monitored through continuous optical microscopy (OMTB). The transport of cells from the bulk towards the substratum/solution interface could be observed, followed by the attachment of the pioneer cells. A random displacement of motile *P. fluorescens* that were approaching to the interface was expected. However, the number of *P. fluorescens* cells that were near the attached pioneer cells was higher than those observed close to the bare substratum. In the case of the consortium of oral microorganisms, there was also a higher number of planktonic cells near the interface (Figure 2) than in the bulk, and their residence time was higher than in the surroundings. In agreement with previous observations (Davies *et al.* 1998; Singleton *et al.* 1997) there was a particular motion of planktonic bacteria around the border of the colonies and a higher planktonic bacterial density was noticed in this region, compared to that observed in the bulk solution or the uncolonized substratum. Sometimes biofilm layers were formed which became thicker at longer time (Figure 3a and b).

Streamer-like protuberances formed by bacteria embedded in EPM could be seen by OMTB. They were stretching, oscillating and relaxing in agreement with CLSM observations previously reported (Lewandowski 1998; Stoodley *et al.* 1998). The partial detachment of

the biofilm layers also yielded to the formation of streamers.

Biofilms formed *in vivo* in the oral environment showed similar characteristics to those formed *in vitro* with respect to shape, thickness and microstructure. However, a higher number of filamentous bacteria was observed.

Extracellular polymeric matrix

The mobility of the cells was estimated by measuring the time employed by them to move within the microscopic field from one extreme to the other. It could be noticed that the velocity of the cells which were far from the substratum (in the middle of the glass cell) was close to twice the rate corresponding to the cells moving in the vicinity of the substratum (at a distance shorter than 1 mm from it). It has been found (Donlan & Costerton 2002) that communities of attached bacteria in aquatic systems are encased in a exopolymer matrix which is polysaccharide in nature. The intrinsic viscosity ($[\eta] = \lim_{c \to 0}[(\eta_{\text{solution}} - \eta_{\text{solvent}})/\eta_{\text{solvent}}c]$, $[\eta]$ is the intrinsic viscosity, η the viscosity, c the concentration of the polymer) increases strongly in the presence of polysaccharides and is a function of the weight of the polymeric molecule (Rees *et al.* 1982). A suitable measure of the viscosity in the vicinity of the sheet is very difficult to perform. Notwithstanding this, the reduction in the velocity of the microorganisms which are closed to the substratum may be assigned to the increase in the viscosity of the solution due to the presence of exopolysaccharides in the vicinity of the attached cells.

A great number of cells and bacterial chains that were floating at the viscous interface could be seen in the case of both *in vivo* and *in vitro* biofilms (Figure 2). Streptococcal chains were usually attached by one of their extremities, with the other extremity oscillating in the liquid medium. However, Figure 4 shows a distorted SEM image of bacterial chains in which they seem to be attached to the metal surface. In spite of the SEM impediment of visualizing the soft structure of streptococci immersed in EPM and the spatial distribution of the cells at the interface that can show OMTB, the dehydration and fixation pretreatments and the higher magnification of the SEM observations result in a better image of bacteria, free of the EPS cover (Figures 4 and 5). After the dehydration process the distorted EPM is visualized in Figure 5 as particulates between bacteria. Consequently, OMTB and SEM images provide complementary information to achieve a better interpretation of the microphotographs.

Biofilms on rough surfaces

Zelver *et al.* (1985) measured a decrease in the friction factor during the first 30 h of biofilm growth on a rough surface. Their experiments suggest that firstly the biofilm may develop within the valleys of the uneven surface,

Figure 4. SEM microphotograph showing oral microorganisms colonies formed on a stainless steel band after 7 days of immersion. The border of the sample is clean showing that detachment of cells occurred during SEM pretreatments. Bar = 10 μm.

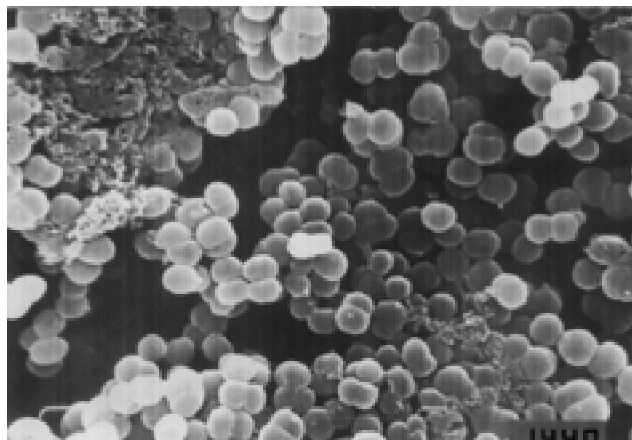

Figure 5. Details of Figure 6. Bar = 10 μm.

smoothing the rough regions. Moreover, recent studies (Mele & Cortizo 2000; Stewart *et al.* 2000; Pasmore *et al.* 2002) have demonstrated that cells were most readily removed from smoothest surfaces and that irreversible attachment was easier on the valleys of the rough areas. In agreement, the present results show that grooves and cavities, rather than smooth surfaces, appeared to be preferentially occupied by bacteria and that the apparent roughness of the substrate is reduced (Figure 3) both in the presence of the *P. fluorescens* (motile bacteria) and the biofilms of the consortium of oral microorganisms (non-motile bacteria). The motility of *P. fluorescens* cells seems to facilitate the attachment of the cells inside the grooves (Scheuerman *et al.* 1998). Consequently, it is also useful to employ SEM and OMTB in combination to study the effect of roughness on bacterial distribution. The roughness of thin substrata could be easily evaluated by both OMTB and SEM (Figures 3 and 4). The height of the peaks and the distance between peaks and valleys could be measured (for example the distance between the roughness peaks pointed with the white bars in Figure 3 are 5.5 and 17 μm respectively).

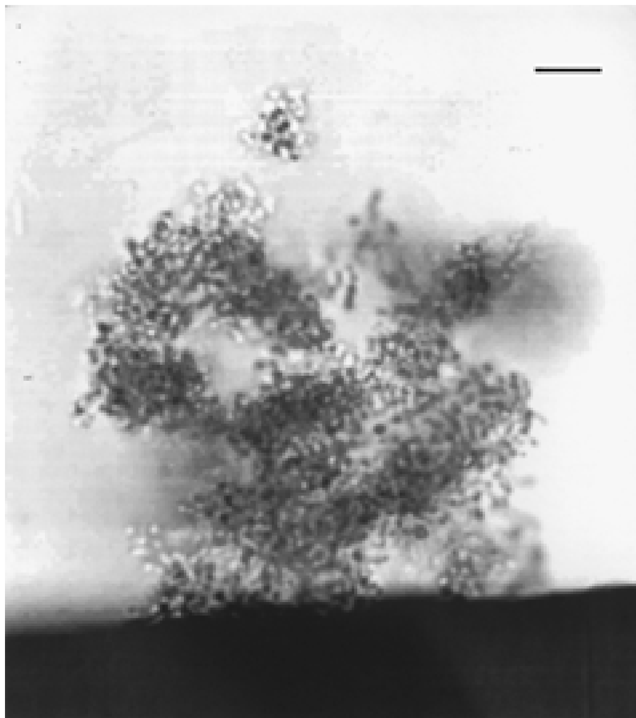

Figure 6. OMTB microphotograph of a colony of the consortium of oral microorganisms growing on an stainless steel orthodontic band. Microstructural details like pores and channels can be distinguished. Bar = 9.1 μm.

Microstructural characteristics of the biofilms

Microstructural characteristics like pores, interstitial channels and bridges of thin biofilms (Figure 6) could be distinguished using OMTB. Water channels and bridges are frequently formed when neighbouring fungus-like colonies coalesced at the upper side, close to the solution, while the inner side, close to the substratum, remained separated (Yang *et al.* 2000). Conversely, SEM pretreatments impede the visualization of these microstructural characteristics because the EPM of the biofilm constricts and channels and pores are blocked or enlarged during dehydration (Figures 4 and 5).

The images of the microstructural details of the biofilms obtained using OMTB resemble those previously reported employing more sophisticated microscopic techniques (de Beer *et al.* 1994; Beyenal *et al.* 1998; Rasmussen & Lewandowski 1998; Wattanakaroon & Stewart 2000). However, it is worth mentioning some limitations of the method: the depth of the field of the microscope objective, the low magnification and the requirement of thin substrata.

Biofilm thickness and density

The actual thickness of the thin biofilms could be evaluated in real time by OMTB. Figure 3 shows that the biofilm thickness can be easily measured between the arrows and the substrata. It can be inferred that the biofilm thickness varied from 7.3 to 13.5 μm (left arrows) and from 6.4 to 14 μm (right arrows) during the growth, and that the thickness of the microbial colony of Figure 6 is 59.2 μm. A reduction up to a half of the original thickness of the biofilm is observed in SEM microphotographs because of the dehydration of EPM occurring during pretreatments. In addition, Figure 4 shows that the border of the biomaterial is clean and there is no colony on the lateral area of the substratum. This indicates that 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 EPM layer.

A colour gradient, which may be attributed to a change in the bacterial and/or EPM density, could be observed in the microscopic examinations and can be detected as a grey gradient in the black and white microphotograph of Figure 6. A darker colour and less transparency in the inner older layer than in the outer younger layer were detected in colonies of oral microorganisms at the stationary phase of growth. It appears that there are higher numbers of cells per unit area and more compact EPM in the darker region of the colonies.

Conclusions

The employment of SEM and OMTB in combination is useful to compare images obtained *in situ*, without invasive pretreatment but at low magnification (OMTB) with those obtained *ex situ*, with pretreatments and at higher magnification (SEM). The different processes that give rise to the formation of the biofilm on thin substrata could be monitored in real time by OMTB. Changes of viscosity near the interface, in the apparent roughness of the substrata and in 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 OMTB and SEM images. However, in spite of the SEM distortions, the dehydration and fixation pretreatments and the higher magnification yielded to a better image of bacteria when 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 situation using conventional microscopic techniques.

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