Clin Oral Invest (2004) 8:97–101
DOI 10.1007/s00784-004-0255-6

ORIGINAL ARTICLE

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Impact of the intraoral location on the rate of biofilm growth

Received: 6 August 2003 / Accepted: 13 January 2004 / Published online: 19 February 2004

Abstract The aims of the present study were: a) to assess the impact of the intraoral location on the rate of biofilm growth, and b) to establish an in vivo biofilm model to examine intraoral biofilm growth. Eight healthy volunteers wore acrylic splints with 15 glass slabs each in the upper and lower jaws to build up plaque. After 48 h, the specimens were removed and stained using the vital fluorescence technique. Biofilm thickness was evaluated by confocal laser scanning microscopy (CLSM). The mean plaque thickness amounted to 77.6±29.1 μm on the buccal sites of the upper jaw and 71.9±26.3 μm on the buccal sites of lower jaw. On the palatal site a biofilm of 52.1±26.2 μm thickness was grown, which was significantly less compared with the other locations evaluated ($p<0.001$). The results demonstrate that the in situ biofilm thickness on the buccal sites was similar irrespective of the location in the oral cavity. The new splint system described may be a useful tool for further standardised experimental studies regarding influences on growth and structure of intraoral biofilms.

Keywords Biofilm model · Biofilm thickness · Confocal laser scanning microscopy · Dental plaque

Introduction

The importance of the dental plaque biofilm as an etiological factor of caries and gingivitis has been demonstrated in numerous studies [28]. Bacterial adherence to tooth surfaces and the subsequent plaque maturation are key precursors to these diseases. Consequently, there is a need to obtain information on natural biofilm growth and its undisturbed structure in order to develop new strategies to prevent caries and gingivitis. It is well known that the dental plaque biofilm consists of bacteria cells embedded in an extracellular matrix. It is perforated with channels and adheres to surfaces [6, 24]. The understanding of the precise structure of the biofilm is still a challenge and may lead to the possibility of manipulating plaque growth by antimicrobial agents or other strategies.

Bacterial adherence on oral surfaces has been investigated in many in vitro studies mimicking the clinical situation [10, 23]. However, the conditions in the oral cavity with its diversity of the plaque microflora are different from those of in vitro models. Since it is still not possible to adequately disperse and cultivate the natural plaque biofilm, there is an increasing interest in developing in vivo plaque biofilms models which can be examined ex vivo without any disruption. Thus, for a more detailed investigation, an in situ model seems to be more reliable. It has been proposed to assess biofilm growth by image analysis and to monitor by measuring the surface area covered and biofilm thickness [20]. Reports of in vivo studies investigating plaque formation and biofilm thickness are rare, although the importance of plaque thickness in metabolic processes involved in dental disease has often been emphasized [7, 27].

Several investigations have reported on the evaluation of in situ biofilm growth and thickness using the confocal laser scanning microscopy (CLSM) in combination with the vital fluorescence technique [1, 2, 15, 29, 32]. However, to the best of our knowledge, until now there are no data examining in situ biofilm thickness at different locations.

Therefore, the purpose of the present study was: a) to examine the impact of the intraoral location on biofilm thickness, and b) to establish an in situ biofilm model which allows the standardised formation of dental plaque.

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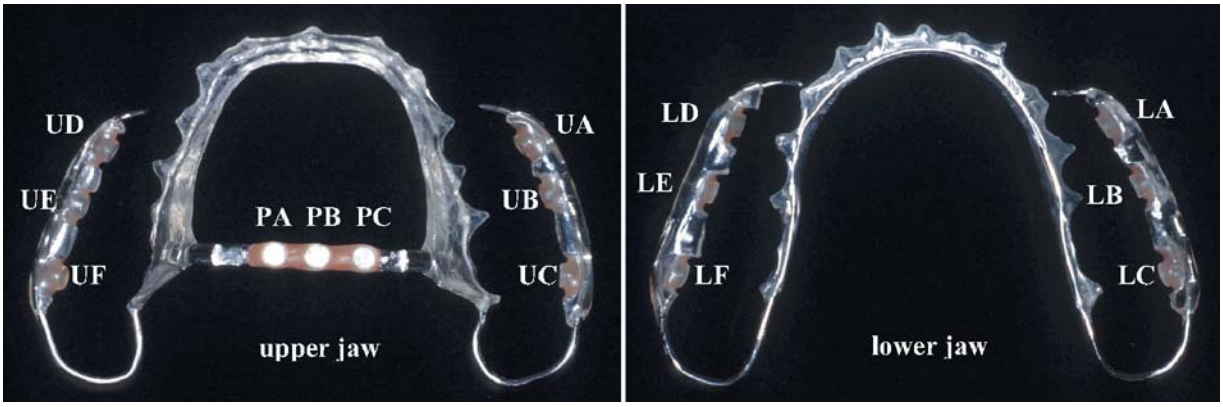

Fig. 1 Removable appliances showing the location of the specimens (*UA–UF* buccal sites in the upper jaw, *LA–LF* buccal sites in the lower jaw, *PA–PC* palatal sites in the upper jaw), the exposed surface being fixed towards the tooth surfaces or the palate, respectively

Material and methods

Eight healthy volunteers (aged 23–30 years; mean 26.5) were selected for this study. All were in good health and none of them had used any mouthrinses or undergone any antibiotic therapy for the 6-month period preceding the start of the present experiment. None of the volunteers showed signs of destructive periodontitis or any other inflammatory conditions of the surrounding soft tissues. All volunteers signed an informed consent form.

Biofilm growth

Prior to the investigation, the subjects received a professional toothcleaning. The volunteers obtained acrylic appliances for each jaw with glass slabs (Menzel, Braunschweig, Germany) to collect a plaque biofilm. The glass slabs were industrially manufactured (3 mm in diameter, 2 mm in height) and were already polished in the same way (4,000 grid).

Three glass discs were inserted into depressions with sticky wax towards the natural teeth on each site of the upper jaw, the lower jaw and on the palatal site in such a way that plaque could grow undisturbed by the tongue or the cheek (Fig. 1). This should imitate an approximal plaque biofilm which is only minimally influenced by shear forces of the oral soft tissues (Fig. 2). The width of the space between the glass and the tooth surfaces was ≈2 mm. This special design guaranteed that removing and replacing of the appliances did not manipulate or disturb the biofilm regrowth. The different locations and their abbreviations are explained in Fig. 1. The splints were worn by the volunteers for 48 h. The subjects were allowed to maintain their regular diet and retained the splints intraorally throughout the whole experimental period, except during their daily mechanical toothbrushing (only with tap water; no toothpaste or mouthrinse was allowed).

Confocal laser scanning microscopy analysis

The plaque covered glass-slabs were carefully removed from the splints, washed with physiological saline (room temperature) and processed without delay. The adhering biofilm was stained with two fluorescent dyes [15] to visualise the highest cell clusters of the biofilms. Immediately after the staining procedure, the biofilms were analysed using a confocal laser scanning microscope (CLSM; LSM 410 invert, Zeiss, Germany). The specimens were inverted onto a drop of saline buffer which was placed onto a chambered cover slip (Lab-Tek II, Nalge Nunc International, USA) in order not to disturb the spatial structure of the biofilm and to hydrate it throughout the observation. Additionally any flattening of the biofilm samples was avoided by fitting the discs to the chambered coverslips. Then, confocal images were obtained from below using a 40× water immersion objective. The biofilm thickness was measured microscopically by focusing on the substratum and moving until the upper biofilm cells were in focus. This was repeated at several sites per single sample until the thickest point of the uneven biofilm surface was found. Then optical sections of 1 μm were scanned through the biofilm from the highest cell clusters. Biofilm thickness was determined by summing up the number of sections.

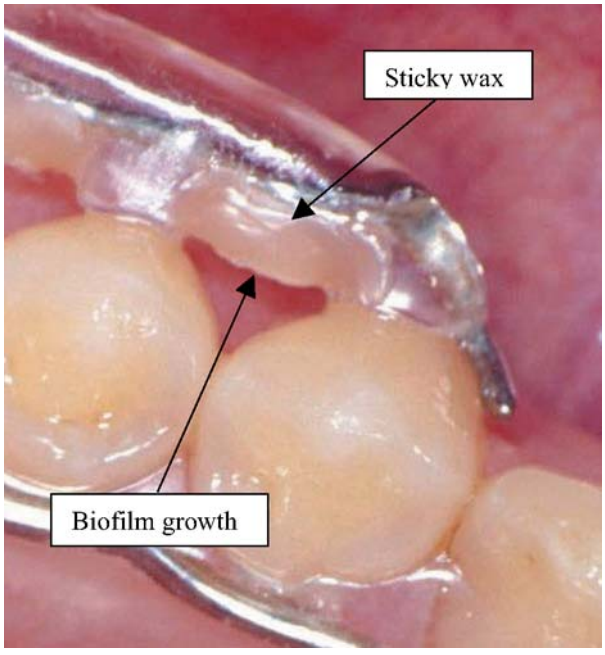

Fig. 2 Higher magnification of location LD in situ

Statistical analysis

All statistics were performed by SPSS (11.0 for windows). For each location (UA-UF; LA-LF, PA-PC), mean values of the biofilm thickness were calculated. ANOVA was applied to search for significant differences between the locations within the upper jaw

(UJ), within the lower jaw (LJ) and within the palatal site (P). Then, the data of the upper jaw, the lower jaw and the palatal site were integrated. Since these mean values (UJ, LJ, P) showed significant differences ($p<0.05$ by ANOVA) and were normally distributed (by Kolmogorov-Smirnov test), comparisons were conducted using paired t-tests.

Results

All specimens of the eight volunteers (15 each) could be used for analysing biofilm thickness. The adhering biofilms on all discs displayed an uneven surface. The maximum thickness of all examined biofilms ranged between 14 and 150 µm. The mean biofilm thickness of all eight volunteers depending on the location is depicted in Fig. 3 and Table 1.

First, the thickness of biofilm of the upper jaw, the lower jaw and the palatal site was compared. The sites of the upper jaw revealed a mean thickness of 77.6±29.1 µm, whereas the mean thickness for the lower jaw was 71.9±26.3 µm. These mean values were not statistically significantly different. The palatal site revealed a thickness of 52.1±26.2 µm, which was significantly less than in either the upper or the lower jaw (Fig. 3). Looking at the different locations at the buccal sites of the upper and lower jaw, the thickness was quite similar (Table 1). The biofilm thickness at the buccal sites of the upper and lower jaws showed no statistically significant differences by ANOVA ($p>0.05$). When comparing the right and the left side, no statistically significant differences were found ($p>0.05$).

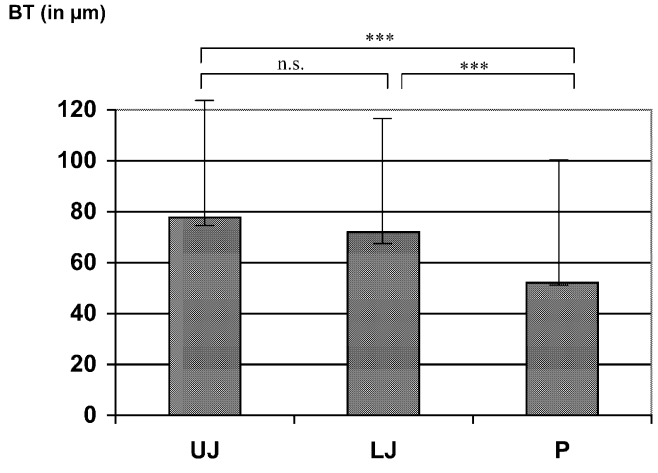

Fig. 3 Mean biofilm thickness (*BT*; in micrometres) after 48 h in the upper jaw (*UJ*, buccal sites), lower jaw (*LJ*) and on the palatal site (*P*). *n.s.* non significant. *** $p\leq0.001$, by paired t-tests

Discussion

Although valuable information can be obtained from models that involve the disruption of the biofilm, the non-destructive analysis of biofilms is essential in understanding biofilm processes [14]. Most studies use laboratory model systems such as chemostats or flow cells for analysing the structure of biofilms or examine cariogenicity [e.g. 25, 26]. However, most models use biofilms of selected bacteria strains (single- and mixed-species biofilms) or plaque microcosms and not orally grown biofilms on hard surfaces comprising a rather undefined mixture of microorganisms. These methods have already revealed the complex structure of biofilms [5] and allow for the standardised control and examination of different influences such as mouthrinses on the biofilm [23]. However, they still cannot adequately reflect the physiological intra-oral situation (e.g. bacterial co-aggregation of different species, influence of endogenous factors) and thus, the obtained results need to be interpreted with caution. Therefore, the aim of this study was to establish an in vivo model and to determine the thickness of undisturbed plaque formation.

Different kinds of individual splints have been used to collect plaque [17, 21, 29, 31]. The design of the intraoral splint to collect an in situ biofilm in the present investigation followed, in general, the pattern described in previous studies [1, 2, 16]. However, there were some modifications in splint design in order to allow a better and more comfortable plaque growth and to build up standardised oral biofilms simultaneously in the upper and the lower jaw. The inserted glass slabs were turned towards the teeth to avoid any disturbance by tongue or cheeks, but with some space to provide a nutritious aqueous environment (Fig. 2). On the buccal sites, this should mimic interproximal plaque, which is also largely protected from shearing forces of mastication. To avoid any optical disturbance by the known auto fluorescence of enamel [16], glass slabs were used. As previously shown, there are no differences in plaque regrowth between enamel and glass [16]. Moreover, there is evidence that plaque growth is highly influenced by surface roughness

Table 1 Mean ± SD values of biofilm thickness (micrometres) at the different locations of the upper jaw (*UA-UF*), the lower jaw (*LA-LF*) and the palatal site (*PA-PC*) as explained in Fig. 1

UA	UB	UC	UD	UE	UF	PA	PB	PC
75.25±21.84	76.00±36.82	79.00±26.17	78.25±26.62	84.75±36.92	72.50±26.21	56.25±29.62	54.00±26.53	46.00±22.61
n.s.						n.s.		
LA	LB	LC	LD	LE	LF			
77.00±19.80	70.75±28.69	73.00±27.42	62.25±23.59	73.00±30.27	76.00±28.27			
n.s.								

n.s. no significant differences by ANOVA ($p>0.05$)

and surface free energy [13]. Quirynen and van Steenberghe [18] have demonstrated that surface irregularities were responsible for the different plaque growth patterns on the various teeth. Our findings support this conclusion since all glass slabs were polished in the same way (4,000 grid) and revealed similar biofilm thickness. Another important reason is the standardised, sheltered position of the slabs in the appliance. Differences in biofilm thickness between buccal locations in the oral cavity, e.g. upper and lower jaw, were not found. In contrast, in the studies performed by Quirynen and van Steenberghe [18], Furuichi et al. [8] and Ramberg et al. [19], differences in plaque growth on teeth were explained with their natural irregularities, which were excluded in the present study by using standardised conditions at all locations.

Dental plaque may act as a barrier to diffusion of organic acids, but also of potential neutralizing agents towards the enamel surface [12, 22]. When considering the importance of plaque thickness in metabolic processes involved in dental disease, it has to be pointed out that there are only a very limited number of investigations which have evaluated intraoral plaque thickness and, thus, a comparison with other studies is difficult.

There are methods to measure plaque thickness by electronic probes [11] or by a laser scanning probe [30]. Some other studies examined plaque thickness microscopically by means of CLSM. In this instance biofilm thickness is defined to be the distance between the substratum and the peaks of the highest cell clusters [20].

Netuschil et al. [16] found biofilms with a thickness of 6–45 μm after 3 days growth (72 h) on enamel and glass slabs which were inserted in acrylic appliances. With a similar splint design, Auschill et al. [1] showed biofilm thickness varying from 15–31 μm after 5 days (120 h) on enamel specimens. Two reasons can explain these low values compared with the present results: in both studies biofilms were dried and embedded prior to CLSM-evaluation and amounted to approximately half of the corresponding wet biofilm. Moreover, the rather sparse thickness reflects the fact that the smooth surface plaque collected in these studies was much more exposed to the salivary environment, since the specimens were inserted towards the cheek, not towards the teeth like in the present study.

Wood et al. [29] bonded an in situ device comprised of a nylon ring attached to an enamel substrate on the first or second molars of eight volunteers. After 96 h they found plaque heights between 75–220 μm at the enamel/ring junction and between 35–215 μm toward the centre of the device. Zaura-Arite et al. [32] found an average thickness of 34.4 μm in dentinal groves. There was obvious no difference in the speed of plaque growth.

It should be mentioned that all these authors emphasised the great variation in plaque thickness between the different volunteers, which is also true for the present investigation, showing relatively high standard deviations. However, for each single volunteer, similar biofilm thickness on the buccal sites of the upper and lower jaw could be followed through the different locations. Plaque thickness on the slabs turned towards the palate was significantly lower than those from the other locations. It is believed that the diversity of the plaque microflora is due primarily to the endogenous nutrients supplied by the host, rather than by exogenous factors in the diet [20]. Moreover, acidic glycoproteins from the saliva, gingival crevicular fluid and already attached bacteria play an important role in forming an organic film and providing attachment sites for the colonisation and growth of bacterial microcolonies [3, 4]. The same is true for nutrients for biofilm growth, which originate from saliva as well as from metabolic products of adjacent bacterial colonies [9]. While all volunteers showed no signs of any inflammatory conditions of soft tissues, gingival crevicular fluid should not have an impact on biofilm growth. Different plaque growth in the palate could only be explained by a position far away from teeth with their microflora and near mucosal surfaces. This should be kept in mind for further use of this model.

In conclusion, this study demonstrates similar biofilm thickness at different locations in the buccal region of the upper and the lower jaw, with a lower growth on the palatal site. The in vivo biofilm model used represents a valuable and standardised tool for in vivo/in situ testing of different influences on oral biofilm growth.

Acknowledgements This study was financially supported by a grant from the Deutsche Forschungsgemeinschaft (DFG; Ar 341/1-1). The authors thank Marie Follo, Ph.D., Department of Haematology and Oncology, Core Facility, Albert-Ludwigs-University, Freiburg, Germany for her help in the image analysis.

References

1. Auschill TM, Arweiler NB, Netuschil L, Brecx M, Reich E, Sculean A (2001) Spatial distribution of vital and dead microorganisms in dental biofilms. Arch Oral Biol 46:471–476
2. Auschill TM, Arweiler NB, Sculean A, Brecx M, Reich E, Netuschil L (2002) The effect of dental restorative materials on dental biofilm. Eur J Oral Sci 110:48–53
3. Bos R, Van der Mei HC, Buscher HJ (1999) Physico-chemistry of initial microbial adhesive interactions—its mechanisms and methods for study. FEMS Mirocbiol Rev 23:179–230
4. Bradshaw DJ, Marsh PD, Watson GK, Allison C (1997) Effect of conditioning films on oral microbial biofilm development. Biofoul 11:217–226
5. Caldwell DE, Korber DR, Lawrence JR (1992) Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. J Microbiol Meth 15:249–261
6. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR (1995) Microbial biofilms. Annu Rev Microbiol 49:711–745
7. Dibdin GH (1981) Diffusion of sugars and carboxylic acids through dental plaque in vitro. Arch Oral Biol 26:515–524
8. Furuichi Y, Lindhe J, Ramberg P, Volpe AR (1992) Patterns of de novo plaque formation in the human dentition. J Clin Periodontol 19:423–433
9. Gilbert P, Das J, Foley I (1997) Biofilm susceptibility to antimicrobials. Adv Dent Res 11:160–167
10. Guggenheim M, Shapiro S, Gmür R, Guggenheim B (2001) Spatial arrangements and associative behaviour of species in an in vitro oral biofilm model. Appl Environ Microbiol 3:1343–1350

11. Main C, Geddes DA, McNee SG, Collins WJ, Smith DC, Weetman DA (1984) Instrumentation for measurement of dental plaque thickness in situ. J Biomed Eng 6:151–154
12. Mandel ID (1974) Relation of saliva and plaque to caries. J Dent Res 53:246–266
13. Marshall KC (1992) Biofilms: an overview of bacterial adhesion, activity and control at surfaces. Am Soc Microbiol News 58:202–207
14. McCoy JC, Bryers JD, Robbins J, Costerton JW (1981) Observations in fouling biofilm formation. Can J Microbiol 15:263–276
15. Netuschil L, Reich E, Brecx M (1989) Direct measurement of the bactericidal effect of chlorhexidine on human dental plaque. J Clin Periodontol 16:484–488
16. Netuschil L, Reich E, Unteregger G, Sculean A, Brecx M (1998) A pilot study of confocal laser scanning microscopy for the assessment of undisturbed dental plaque. Arch Oral Biol 43:277–285
17. Nyvad B, Kilian M (1987) Microbiology of early colonisation of human enamel and root surfaces in vivo. Scand J Dent Res 95:369–360
18. Quirynen M, van Steenberghe D (1989) Is early plaque growth rate constant with time? J Clin Periodontol 16:278–283
19. Ramberg P, Furuichi Y, Lindhe J, Gaffar A (1992) A model for studying the effects of mouthrinses on de novo plaque formation. J Clin Periodontol 19:509–520
20. Roberts SK, Bass C, Brading M, Lappin-Scott H, Stoodley P (1999) Biofilm formation and structure: What's new? In: Newman HN, Wilson M (eds) Dental plaque revisited—oral biofilms in health and disease. BioLine, Cardiff; pp 15–36
21. Robinson C, Kirkham J, Percival R, Shore RC, Bonass WA, Brookes SJ, Kusa L, Nakagaki H, Kato K, Natress B (1997) A method for the quantitative site specific study of the biochemistry within dental plaque biofilms formed in vivo. Caries Res 31:194–200
22. Saxton CA (1975) Determination by electron microscope autoradiography of the distribution in plaque of organisms that synthesize intracellular polysaccharide in situ. Caries Res 9:418–437
23. Shapiro S, Giertsen E, Guggenheim B (2002) An in vitro oral biofilm model for comparing the efficacy of antimicrobial mouthrinses. Caries Res 36:93–100
24. Singleton S, Treloar R, Warren P, Watson GK, Hodgson R, Allison C (1997) Methods for microscopic characterization of oral biofilms: analysis of colonization, microstructure, and molecular transport phenomena. Adv Dent Res 11:133–149
25. Sissons CH (1997) Artificial dental plaque biofilm model systems. Adv Dent Res 11:110–126
26. Sissons CH, Cutress TW, Hoffman MP, Wakefield JSJ (1991) A multi-station dental plaque microcosm (artificial mouth) for the study of plaque growth, metabolism, pH and mineralization. J Dent Res 70:1409–1416
27. Tatevossian A (1979) Diffusion of radiotracers in human dental plaque. Caries Res 13:154–162
28. Theilade J (1989) Dental plaque and dental calculus. In Lindhe J (ed): Textbook of clinical periodontology, 2nd edn. Munksgaard, Copenhagen, pp 92–128
29. Wood SR, Kirkham J, Marsh PD, Shore RC, Nattress B, Robinson C (2000) Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. J Dent Res 79:21–27
30. Yeganeh S, Lynch E, Jovanovski V, Zou L (1999) Quantification of root surface plaque using a new 3-D laser scanning method. J Clin Periodontol 26:692–697
31. Zaura-Arite E, ten Cate JM (2000) Effect of fluoride- and chlorhexidine containing varnishes on plaque composition and on demineralisation of dentinal grooves in situ. Eur J Oral Sci 108:154–164
32. Zaura-Arite E, Van Marle J, ten Cate JM (2001) Confocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. J Dent Res 80:1436–1440