

Adhesion of *Streptococcus sanguinis* to Dental Implant and Restorative Materials *in vitro*

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Bacterial adhesion to tooth surfaces or dental materials starts immediately upon exposure to the oral environment. The aim of this study, therefore, was to compare the adhesion of *Streptococcus sanguinis* to saliva-coated human enamel and dental materials during a one-hour period using an *in vitro* flow chamber system which mimicked the oral cavity. After fluorescent staining, the number of adhered cells and their vitality were recorded. The dental materials used were: titanium (Rematitan[®] M), gold (Neocast 3), ceramic (Vita Omega 900), and composite (Tetric Ceram).

The number of adherent bacterial cells was higher on titanium, gold, and ceramic surfaces and lower on composite as compared to enamel. As for the percentage of adherent vital cells, it was higher on enamel than on the restorative materials tested. These results suggested that variations in the number and vitality of the adherent pioneer oral bacteria, *S. sanguinis*, in the *in vitro* system depended on the surface characteristics of the substratum and the acquired salivary pellicle.

The *in vitro* adhesion model used herein provided a simple and reproducible approach to investigate the impact of surface-modified dental materials on bacterial adhesion and vitality.

Keywords: *Streptococcus sanguinis*, Adhesion, Implant and prosthetic materials

INTRODUCTION

Dental plaque, as an oral biofilm, is recognized as a key etiologic factor for caries and periodontal diseases in humans. Bacterial colonization of tooth surfaces or dental materials like filling materials, dental implants, or prostheses starts immediately upon exposure to the oral environment. In the process of plaque formation, early colonizers, including *Streptococcus sanguinis*, adhere to the salivary coating covering the tooth surfaces¹. This initial adhesion is an important step in biofilm formation as it may influence the composition of mature dental plaque².

Different strategies to investigate *in vitro* the development and structure of the biofilm on oral hard tissues and dental materials have been used as tools in dental research. Attachment and growth of various plaque bacteria on substrata has been assessed using different models^{2,3}. However, salivary pellicle-coated enamel as a substratum was found in only a few reports^{4,5}.

Presently, commonly used materials for dental restorations include metals, ceramics, and resin composites. Although these materials are safe and effective for their intended use⁶, there is also an increasing interest in dental materials which minimize plaque formation⁷.

Adhesion and vitality of *S. sanguinis* have been investigated *in vitro* in a flow chamber system using human enamel as a substratum in a salivary milieu⁸. The aim of the present study, therefore, was to compare the adhesion of *S. sanguinis* to commonly used implant and restorative materials with that to human enamel, and also determine the vitality of the initial adhered bacteria. We hypothesized that the number and vitality of adherent bacterial cells would vary with the nature of dental restoratives.

MATERIALS AND METHODS

Bacteria, culture conditions, and hydrophobicity measurement

A 10- μ l inoculum of *S. sanguinis* DSM 20068 (German Collection of Microorganisms and Tissue Culture Cells, Braunschweig, Germany) preserved in skim milk solution at -20 °C was suspended in 5 ml of Schaedler broth (BBL[™], Becton Dickinson, Basel, Switzerland) and incubated aerobically at 37 °C for eight hours. An inoculum was then transferred to fresh Schaedler broth (1:50) and grown at 37 °C for 16 hours. This culture was sonicated for one minute (30 W, Sonifier Ultraschall-Desintegrator, Branson Sonic Power Co., Berlin, Germany), washed with physiological saline, harvested by centrifugation at

8000 g for five minutes, and resuspended in saliva to a density of 10^8 - 10^9 cells/ml.

Density of bacteria per ml in the bacteria-saliva mixtures at the beginning and end of each experiment were determined by phase contrast microscopy (Provis AX70, Olympus AG, Volketswil, Switzerland) using a standard Neubauer chamber.

Hydrophobicity measurement of *S. sanguinis* cells was done as described by Grivet *et al.*⁸⁾ using partitioning into hexadecane (Sigma-Aldrich GmbH, Buchs, Switzerland).

Saliva

Whole saliva was collected by paraffin stimulation from a healthy volunteer (after not drinking or eating for two hours). Saliva samples were sonicated (1 minute, 30 W), filtered through a 70- μ m filter (Cell Strainer, Becton Dickinson, Basel, Switzerland), and centrifuged at 22,000 g for 60 minutes at 4 °C. The supernatant was filtered through two low-protein-binding filters (pore sizes of 0.45 μ m and 0.22 μ m; Millex-HV and Millex-GV respectively, Millipore, Switzerland) connected in series. Sterilized saliva was stored at 6 °C and processed within two days. Before use, the pH was adjusted to 7.1-7.3 with phosphate buffer (0.067 mol/l Na_2HPO_4 , 0.067 mol/l KH_2PO_4).

Adhesion substrata

Table 1 lists the materials tested in this study: titanium, gold, ceramic, and composite. Rectangular test specimens ($14.4 \times 14.4 \times 0.2$ mm³) were prepared and polished to a defined surface roughness. Surface roughness was measured by a Hommel tester (T 1000, Hommelwerke GmbH, VS-Schwenningen, Germany). Tooth slices were prepared as previously

described⁹⁾ and mounted with epoxy glue on glass plates (Fig. 1). Only the enamel portion was used for analysis. All the slides were decontaminated with ethanol. Before the adhesion experiments, the slides were exposed to the sterile human saliva used in the flow chamber system at room temperature for 15 minutes.

Measurement of contact angles as indices of hydrophobicity was carried out using a K100 Processor Tensiometer (Krüss GmbH, Hamburg, Germany).

Adhesion of *S. sanguinis* to substrata in the flow chamber

The *in vitro* model used herein was as that previously described by Weiger *et al.*¹⁰⁾. Briefly, the bacteria-saliva suspension circulated from a Teflon dispenser (Multimed GmbH, Kirchheim unter Teck, Germany) to the flow chamber (No. 1301, Minucells, Bad Abbach, Germany) in which the test specimens were mounted in

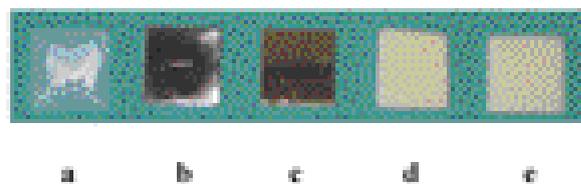


Fig. 1 Slice of human tooth mounted to a glass slide for analysis of enamel adhesion (a); rectangular specimens ($14.4 \times 14.4 \times 0.2$ mm³) of titanium (b), gold (c), ceramic (d), and composite (e).

Table 1 Restorative materials used in this study

Type of material	Chemical composition (%)	Commercial name	Manufacturer
Titanium	Ti (min. 99.5), Fe, O, H, N, C	Tritan, Rematitan [®] M	Dentaurum, Ispringen, Germany
Gold	Au (71.6), Pt (3.7), Ag (12.7), Cu (10.8), Zn (1.1)	Neocast 3	Cendres & Métaux SA, Biel-Bienne, Switzerland
Ceramic	*SiO ₂ (58-62), Na ₂ O (5-6), K ₂ O (9-11), Al ₂ O ₃ (14-16), BaO (3-4), MgO (0.3-0.8), CaO (1-2), ZrO ₂ (1-1.5), SnO ₂ (1-1.5), B ₂ O ₃ (0.3-0.8)	Vita Omega 900	Vita, Bad Säckingen, Germany
Composite	**Bis-GMA (8.3), UDMA (7.6), TEGDMA (4.3), BG (50.6), YbF ₃ (17), O (5), Ba-Al-FG (5), SiO ₂ (1)	Tetric Ceram	Ivoclar Vivadent AG, Schaan, Liechtenstein

*Reference: Rose, DR. Bond strength between a non-precious bonding alloy and ceramic after sol-gel-oxide coating. Thesis, FU Berlin, 2000.

**Bis-GMA: bis-A(di)glycidyl methacrylate; UDMA: urethane dimethacrylate; TEGDMA: triethyleneglycol dimethacrylate; BG: silanized barium glass; Ba-Al-FG: barium-aluminum-fluorosilicate glass

parallel. The dispenser and flow chamber were connected by tubes to a peristaltic pump (Spetec GmbH, Erding, Germany) with an integrated speed controller. Flow rate of the suspension was 0.8 ml/min, which corresponded roughly to physiological conditions of low shear in the oral cavity¹¹. The system was placed on a shaker adjusted at 260 impulses/min to maintain the homogeneity of the bacterial suspension at room temperature for one hour (Fig. 2). Thereafter, test specimens were removed and analyzed microscopically. Each material was tested in at least five independent experiments.

Determination of microbial vitality on substrata and in suspension

The vitality of adhered bacteria was evaluated by applying a dual fluorescent staining method (Live/Dead BacLight Bacterial Viability Kit, MoBiTec, Luzern, Switzerland) according to Decker¹², which allows differentiation between vital and dead bacterial cells. To stain bacteria attached to enamel and restorative dental materials, the test specimens were removed from the flow chamber, carefully dipped into distilled water to eliminate planktonic and loosely attached cells, covered with 7.5 μ l of staining solution for 15 minutes at room temperature in the dark, and subsequently placed on a slide. The cells were analyzed by epifluorescence microscopy (Provis AX70, Olympus AG, Volketswil, Switzerland) using two filters: blue excitation at 450–490 nm (FITC) and green excitation at 546 nm (rhodamine). The number of adherent vital cells and dead cells at eight randomly selected sites on

each substratum were counted and then calculated in per mm^2 (Fig. 3). Results were mean values of at least five independent experiments.

In addition, colony-forming units per ml of the bacteria-saliva suspensions were determined before and after the adhesion experiment. Appropriate dilutions of 100 μ l were plated onto Schaedler agar plates (BBL™, Becton Dickinson, Basel, Switzerland) in duplicate and incubated anaerobically (AnaeroGen™ Compact, Oxoid AG, Basel, Switzerland) at 37 °C for 48 hours.

Statistical analysis

Statistical analysis was performed using an open source programming language, R Version 1.6.1. Mann-Whitney test was used to compare data of each material with those of enamel. Level of significance was set at $\alpha=0.05$.

RESULTS

The tooth slices showed a surface roughness of $R_a=0.24 \mu\text{m}$, corresponding to the average roughness of enamel surfaces. As shown in Table 2, the surface roughness values of the materials tested were close to that of enamel.

Tooth slices showed a moderate hydrophobic surface with contact angles of 64.4° for the uncoated slide and 63.5° for the coated slide. Contact angle values of dental materials were in the range of 59.8° to 82.7° for the uncoated slides and 39.1° to 46.3° for the saliva-coated slides, thus indicating higher hydrophobicity of the uncoated metal surfaces (Table 2). In particular, the highest reduction in contact angle value by saliva coating was observed for the composite slide. Compared to the saliva-coated dental materials, the saliva-coated tooth slide was more hydrophobic.

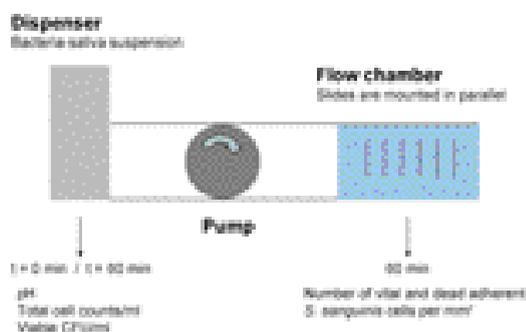


Fig. 2 Schematic diagram of study design. *S. sanguinis* saliva suspension circulated from the dispenser to the flow chamber containing the slides mounted in parallel. Total cell counts per ml, viable CFU per ml, and pH of bacteria-saliva mixture were determined at the beginning and the end of the experimental period. Analysis of slides was conducted after 60 minutes.

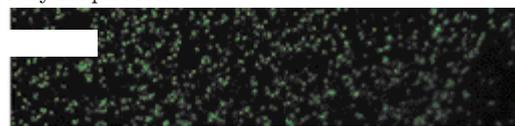


Fig. 3 Representative section of the gold surface after staining. Live attached bacteria are green, while dead ones are red.

Table 2 Surface roughness Ra (μm) and contact angles CA (degrees) of the tested substrata. Shown are means and standard deviations of Ra ($n=10$ for tooth slides, $n=5$ for dental materials), and for CA mean and quality of fit (=linear equation to experimental dataset)

	Enamel	Titanium	Gold	Ceramic	Composite
Ra (μm)	0.24 \pm 0.09	0.25 \pm 0.09	0.15 \pm 0.08	0.23 \pm 0.05	0.21 \pm 0.12
CA (degrees)					
Uncoated	64.4 (98.2%)	62.2 (99.1%)	65.3 (99.5%)	59.8 (98.4%)	82.7 (99.2%)
Saliva-coated	63.5 (99.5%)	42.8 (99.3%)	40.3 (98.9%)	46.3 (99.6%)	39.1 (99.2%)

Table 3 Parameters in the bacteria-saliva suspension at the beginning t_0 and at the end t_{60} of the experimental period. Shown are the means and standard deviations ($n=10$)

	t_0	t_{60}
pH	7.26 \pm 0.07	7.47 \pm 0.06
Total cell counts/ml (log)	8.99 \pm 0.09	8.89 \pm 0.09
Viable CFU/ml (log)	8.42 \pm 0.27	8.36 \pm 0.25

S. sanguinis suspended in PBS was found to be highly hydrophobic since this bacterial strain showed 89.9% partitioning to hexadecane. However, suspension of *S. sanguinis* cells in human saliva resulted in a low (<1%) partitioning to hexadecane, meaning that the bacterial cells exhibited hydrophilic behavior.

During the one-hour experimental period, bacterial density and vitality in the bacteria-saliva suspension of the flow chamber system remained fairly constant. Notwithstanding the slight increase in pH at the end of the test period, the bacteria-saliva suspension could be considered as a resting cell suspension (Table 3).

Figure 4 summarizes the results of the adhesion experiments. The number of adherent *S. sanguinis* cells per mm^2 was significantly higher on titanium, gold, and ceramic surfaces ($p<0.001$) than on enamel, whereas significantly less bacteria adhered to the composite material ($p<0.001$) (Fig. 4a). The percentage of vital adherent *S. sanguinis* was highest on enamel (92.5%), whereas it was significantly lower on the restorative materials tested, ranging from 41.5% to 69.1% ($p<0.001$) (Fig. 4b).

DISCUSSION

In vitro approaches to studying plaque formation yield a two-fold benefit: they ensure strictly standardized test conditions and assure a high reproducibility.

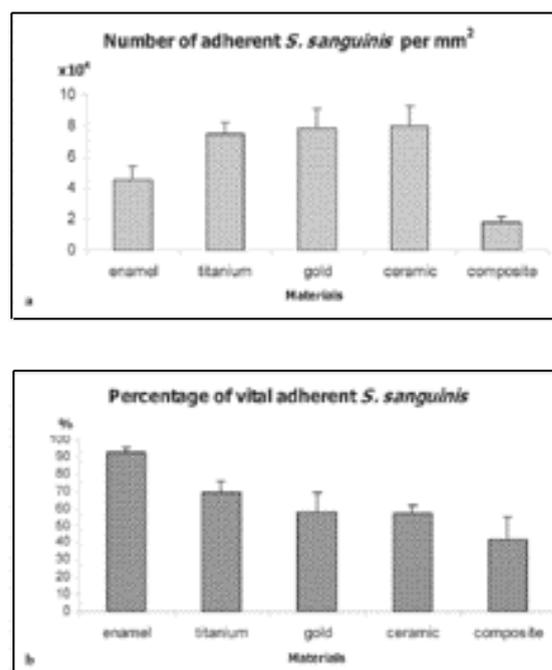


Fig. 4 *S. sanguinis* adhered to human enamel and different dental restorative materials. (a) Total number of cells per mm^2 ; (b) Percentage of vital *streptococci*. Shown are the means and standard deviations ($n=5/10$).

In the present study, the *in vitro* system used integrated host components such as enamel, saliva, and *S. sanguinis* to mimic some *in vivo* features of the oral cavity. The enamel analyzed herein represented the enamel portion of longitudinally cut tooth slices, which most likely had a different structure from the surface enamel to which bacteria adhere *in vivo*. Notwithstanding the difference, this set-up allowed comparison with other studies^{5,9}.

Results obtained with the model system revealed differences in cell adhesion and vitality of the adherent cells, thereby pointing to the different material characteristics of the substrata. It has been reported that the physicochemical properties of

a material like surface free energy, hydrophobicity, and surface roughness, as well as material composition, affect initial bacterial adhesion¹³⁻¹⁵.

Quirynen and Bollen¹⁵ suggested that surface roughness and surface free energy were the main material-linked factors influencing bacterial adhesion. They further showed that the influence of surface roughness was stronger than that of surface free energy and surface hydrophobicity. Generally, rough surfaces promote bacterial adhesion whereas smooth surfaces minimize it¹³⁻¹⁵. According to Bollen *et al.*¹⁶, surface roughness below Ra=0.2 μm had no further quantitative and qualitative effects on bacterial adhesion. Moreover, variations around this value had only a negligible impact on bacterial adhesion. In this study, the roughness of all test specimens was about 0.2 μm hence differences in bacterial adhesion could not be explained in terms of surface roughness. This would mean that any observed differences in bacterial adhesion most likely resulted from other surface properties and the composition of the materials used.

Several studies reported that initial adhesion was promoted if both bacteria and the surfaces involved had similar hydrophobic properties^{8,17,18}. In this study, the *S. sanguinis* cells suspended in saliva showed a hydrophilic nature. In other words, it was expected that their hydrophilic nature would favourably facilitate their adhesion to more hydrophilic surfaces, like the pellicle-coated restorative materials used herein. Indeed, more bacterial cells adhered to three of the dental materials than to enamel which had a moderately hydrophobic surface. These results were in agreement with other studies^{4,8,13}. However, it should also be highlighted that fewer bacteria attached to the composite, despite its hydrophilic surface property which was similar to the other materials tested.

In the oral environment, all solid surfaces are covered by an acquired salivary pellicle. The pellicle on tooth enamel and restorative materials is formed by a selective adsorption of salivary macromolecules. In this light, the physicochemical properties of a material influences microbial adhesion either directly or through adsorption of salivary proteins^{19,20}.

In the present study, salivary coating changed the surface hydrophobicity of the dental materials in a similar way. This was because no major differences in contact angle were registered between the materials. Amongst which, the composite material registered the highest reduction in contact angle value. At this juncture, it should be highlighted that pellicle composition may vary between different restorative materials. Consequently, different pellicle components would act as binding receptors for *S. sanguinis* cells, thus leading to differences in cell adhesion²¹.

Bacterial vitality during adhesion and biofilm formation is an important factor in the pathogenicity

of dental plaque²². The ability of bacteria to grow and produce acids is an essential process in dental caries development. In the present study, the percentage of adherent vital *S. sanguinis* was highest on the enamel surface and lower on restorative materials. Weiger *et al.*¹⁰ observed that during the initial events of microbial attachment, dead rather than vital *S. sanguinis* cells preferably attached to solid surfaces. Based on the data obtained, it could be said that this process was even more pronounced on the implant and restorative materials tested herein. Besides, some dental restoratives release metallic or fluoride ions into the environment with a possible influence on the vitality of adherent bacteria²³⁻²⁵. This may augment the explanation for the lower percentage of adherent vital cells on the restorative materials used in this study.

Lowest bacterial vitality and adhesion were detected on the Tetric Ceram composite, which releases fluoride ions into the environment. Fluoride is known to have inhibitory antibacterial and anti-adherent effects²⁵. Thus, the fluoride released might have soundly contributed to the low vitality and adhesion of *S. sanguinis* cells. By way of practical application, the extent to which topically applied fluoride penetrates plaque biofilm is an important subject, since even limited fluoride penetration may serve to inhibit growth of plaque bacteria²⁶. Therefore, a constant release of fluoride ions from a substratum like composite, in addition to topically applied fluoride, might result in an improved inhibition of plaque bacteria.

The observation that the vitality of adhered *S. sanguinis* varied between dental materials suggested that different materials exerted different influences during early colonization. It was either dead cells were preferentially attracted, or that bacteria died after initial adhesion due to an antibacterial effect of the dental material, e.g., by leaching compounds. Within the detection range of this *in vitro* model, it was found that two keys factors played a pivotal role during the early stages of *S. sanguinis* colonization: physicochemical surface characteristics of the dental materials and stereo-specific interactions through the pellicle. The results confirmed that hydrophobicity, in the presence of adsorbed salivary components, significantly influenced bacterial adhesion to dental materials. In the case of the composite material, salivary pellicle might have significantly reduced the adhesion of *S. sanguinis*.

Dental plaque is a complex microbial biofilm. It involves many bacterial species which adhere to the tooth surface or restorative materials and which also interact with each other. Inevitably and predictably then, the composition and activity of dental plaque are also influenced by bacterial replication during phases of nutrient supply²⁷. *Streptococcus sanguinis* is just

one of several early colonizers, but is thought to play an important role in the initial stages of plaque formation¹¹.

In the present study, we used an *in vitro* model to quantify bacterial adhesion and vitality to different dental materials by using fluorescence microscopy. Through this model, it was confirmed that the number and vitality of adhering bacterial cells varied with the nature of the dental restoratives. For other future studies, the parameters of the system, bacterial species, dental materials, and liquid environment can be changed and then their influences evaluated individually. In particular, the influence of surface modification of dental materials can be determined directly.

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REFERENCES

- 1) Li J, Helmerhorst EJ, Leone CW, Troxler RF, Yaskell T, Haffajee AD, Socransky SS, Oppenheim FG. Identification of early microbial colonizers in human dental biofilm. *J Appl Microbiol* 2004; 97: 1311-1318.
- 2) Kolenbrander PE. Oral microbial communities: Biofilms, interactions, and genetic systems. *Annu Rev Microbiol* 2000; 54: 413-437.
- 3) Guggenheim B, Guggenheim M, Gmür R, Giertsen E, Thurnheer T. Application of the Zürich biofilm model to problems of cariology. *Caries Res* 2004; 38: 212-222.
- 4) Takatsuka T, Konishi N, Nakabo S, Hashimoto T, Torii Y, Yoshiyama M. Adhesion *in vitro* of oral streptococci to porcelain, composite resin, cement and human enamel. *Dent Mater J* 2000; 19: 363-372.
- 5) Decker EM, Weiger R, Wiech I, Heide PE, Brex M. Comparison of antiadhesive and antibacterial effects of antiseptics on *Streptococcus sanguinis*. *Eur J Oral Sci* 2003; 111: 144-148.
- 6) Schweikl H. Cytotoxic and mutagenic effects of dental composite materials. *Biomaterials* 2005; 26: 1713-1719.
- 7) Mjör IA, Moorhead JE, Dahl JE. Reasons for replacement of restorations in permanent teeth in general dental practice. *Int Dent J* 2000; 50: 361-366.
- 8) Grivet M, Morrier JJ, Benay G, Barsotti O. Effect of hydrophobicity on *in vitro* streptococcal adhesion to dental alloys. *J Materials Sci: Mater Med* 2000; 11: 637-642.
- 9) Decker EM, Weiger R, von Ohle C, Wiech I, Brex M. Susceptibility of planktonic *versus* attached *Streptococcus sanguinis* cells to chlorhexidine. *Clin Oral Invest* 2003; 7: 98-102.
- 10) Weiger R, Decker EM, Krastl G, Brex M. Deposition and retention of vital and dead *Streptococcus sanguinis* cells on glass surfaces in a flow-chamber system. *Arch Oral Biol* 1999; 4: 621-628.
- 11) Dawes C, Watanabe S, Biglow-Lecomte P, Dibdin GH. Estimation of the velocity of the salivary film at some different locations in the mouth. *J Dent Res* 1989; 68: 1479-1482.
- 12) Decker EM. The ability of direct fluorescence-based, two-colour assays to detect different physiological states of oral streptococci. *Lett Appl Microbiol* 2001; 33: 188-192.
- 13) Sardin S, Morrier J-J, Benay G, Barsotti O. *In vitro* streptococcal adherence on prosthetic and implant materials. Interactions with physicochemical surface properties. *J Oral Rehab* 2004; 31: 140-148.
- 14) Bos R, van der Mei HC, Busscher HJ. Physicochemistry of initial microbial adhesive interactions its mechanisms and method for study. *FEMS Microbiol Rev* 1999; 23: 179-229.
- 15) Quirynen M, Bollen CM. The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. *J Clin Periodontol* 1995; 22: 1-14.
- 16) Bollen CM, Papaioannou W, Van Eldere J, Schepers E, Quirynen M, Van Steenberghe D. The influence of abutment surface roughness on plaque accumulation and periimplant mucositis. *Clin Oral Impl Res* 1996; 7: 201-211.
- 17) Satou J, Fukunaga A, Morikawa A, Matsumae I, Satou N, Shintani H. Streptococcal adherence to uncoated and saliva-coated restoratives. *J Oral Rehabil* 1991; 18: 421-429.
- 18) Teughels W, Van Assche N, Sliopen I, Quirynen M. Effect of material characteristics and/or surface topography on biofilm development. *Clin Oral Implant Res* 2006; 17: 68-81.
- 19) Gong K, Mailloux L, Herzberg MC. Salivary film expresses a complex, macromolecular binding site for *Streptococcus sanguis*. *J Biol Chem* 2000; 275: 8970-8974.
- 20) Carlén A, Nikdel K, Wennerberg A, Holmberg K, Olsson J. Surface characteristics and *in vitro* biofilm formation on glass ionomer and composite resin. *Biomaterials* 2001; 22: 481-487.
- 21) Steinberg D, Eyal S. Early formation of *Streptococcus sobrinus* biofilm on various dental restorative materials. *J Dent* 2002; 30: 47-51.
- 22) Bowden GH. Dental plaque revisited: oral biofilms in health and disease, Boline, Cardiff, 1999, pp.211-253.
- 23) Auschill TM, Arweiler NB, Brex M, Reich E, Sculean A, Netuschil L. The effect of dental restorative materials on dental biofilm. *Eur J Oral Sci* 2002; 110: 48-53.
- 24) Boeckh C, Schumacher E, Podbielski A, Haller B. Antibacterial activity of restorative dental biomaterials *in vitro*. *Caries Res* 2002; 36: 101-107.
- 25) Hamilton IR, Bowden GH. Fluoride in dentistry, Munksgaard, Copenhagen, 1988, pp.77-103.
- 26) Watson PS, Pontefract HA, Devine DA, Shore RC, Nattress BR, Kirkham J, Robinson C. Penetration of fluoride into natural plaque biofilms. *J Dent Res* 2005; 85: 451-455.
- 27) Marsh PD. Dental plaque as a microbial biofilm. *Caries Res* 2004; 38: 204-211.