ABSTRACT

Background: Bacterial colonization of dental implant surfaces is considered the main aetiological factor in peri-implant diseases.

Aim: To devise a protocol that will yield a consistent and viable biofilm on titanium specimens for the purpose of in vitro experimental investigation of microbially-induced surface deterioration and potential peri-implant therapy efficacy.

Materials and Methods: Twelve Southern Implants grade 4 titanium discs, six with machined and six with moderately roughened surfaces were used in this study. Six discs were inoculated with Streptococcus oralis and incubated in brain-heart infusion under anaerobic conditions at 37°C 1, 2 and 3-days. Specimen surface characteristics were evaluated by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Energy dispersive x-ray analysis quantified the surface elemental composition of the discs. Qualitative and quantitative SEM analyses evaluated colonisation and consistency of biofilm formation. The pH of the medium was determined following each incubation period.

Results: There were significant differences in surface roughness between machined (Ra = 0.27µm, Sa = 1.01µm) and moderately roughened (Ra = 2.14µm, Sa = 1.34µm) surfaces. SEM revealed widespread surface irregularities (spikes, valleys and grooves) on the moderately roughened surfaces compared to the machined surfaces. pH measurements of the medium indicated an acidic level (pH = 4.4) after an initially neutral value (pH = 7.0). Colonisation of the disc surface was evident after 24 hours as a multilayered biofilm for both titanium surfaces.

Conclusions: A protocol capable of yielding a consistent viable biofilm on titanium specimens has been devised. This can be used in future studies to investigate the effect of bacterial accumulation and the efficacy of peri-implant therapy on bacterial colonization on implant surfaces.

Descriptors: Dental implants; Titanium; Biofilm; in vitro; infection, corrosion

Running Head: Biofilm formation on titanium surfaces


INTRODUCTION

Long-term prospective studies confirm the high clinical success of implant-supported rehabilitation for fully and partially edentulous subjects (Ma et al., 2010; Simonis et al., 2010). Although this treatment option demonstrates predictable long-term outcomes, biological complications, particularly peri-implant mucositis and peri-implantitis, have been reported (Brägger et al., 2005; Zitzmann & Berglundh, 2008). Bacterial adhesion and consequent colonisation of implant surfaces are the main aetiologic factors in the pathogenesis of peri-implant diseases (Teughels et al., 2006) by inducing an inflammatory response that affects the stability of the peri-implant tissues and the implant surface biocompatibility (Mouhyi et al., 2009).

The oral environment exposes implants to constant electrochemical attack from bacterial acidic by-products (Bjursten et al., 1990; Videla & Herrera, 2005; Hiromoto,
This can lead to implant surface deterioration, termed microbial-induced corrosion, which may affect osseointegration (Guglielmotti & Cabrini, 1997; Olmedo et al., 2003, 2008, 2009; Chaturvedi, 2009). Thus biofilm accumulation, especially with roughened implant surfaces, may affect long term implant success (Quiroynen & Bolten, 1995; Teughels et al., 2006).

Currently there is a critical need to evaluate biofilm accumulation on implants. Several in vivo and in vitro models are used to evaluate bacterial colonisation and consequent biofilm formation (Block et al., 1992; Haas et al., 1997; Kato et al., 1998; Kreisler et al., 2002, 2003; Giannini et al., 2006; Quaranta et al., 2009; Sennhenn-Krichner et al., 2009; Tosun et al., 2012; Goncalves et al., 2010; Mouhyi et al., 1998, 2000; Matsuyama et al., 2003; Shibli et al., 2004; Schwarz et al., 2005, 2006). The in vitro model involves exposure of titanium surfaces to known bacterial species in a test-tube environment for variable lengths of time (Block et al., 1992; Haas et al., 1997; Kato et al., 1998; Kreisler et al., 2002, 2003; Giannini et al., 2006; Quaranta et al., 2009; Sennhenn-Krichner et al., 2009; Tosun et al., 2012; Goncalves et al., 2010); whereas, the in vivo model, involves the use of titanium specimens placed in participants’ oral cavities in the form of either splints or implants to evaluate bacterial colonisation (Mouhyi et al., 1998, 2000; Matsuyama et al., 2003; Shibli et al., 2004; Schwarz et al., 2005, 2006). The main objective of both protocols is to yield a consistent viable biofilm in order to provide a standardised baseline to evaluate microbial-induced implant surface deterioration and peri-implant therapy efficacy.

Achieving consistency with in vivo models is difficult because of the extremely variable environments between participants (Mouhyi et al., 1998, 2000; Matsuyama et al., 2003; Shibli et al., 2004; Schwarz et al., 2005, 2006). Greater control is exercised in in vitro models by selecting the contaminating species, media and incubation time. However, no clear consensus has emerged for the selection of a reliable and consistent protocol. Thus, a wide variety of fungal (Sennhenn-Krichner et al., 2009) and bacterial species have been involved, most commonly Porphyromonas gingivalis (Haas et al., 1997; Kato et al., 1998; Quaranta et al., 2009; Goncalves et al., 2010), Streptococcus sanguinis (Kato et al., 1998; Kreisler et al., 2002, 2003), Strepotrccocus mutans (Gröbner-Schreiber et al., 2001), Aggregatibacter actinomycetemcomitans (Haas et al., 1997; Giannini et al., 2006), Staphylococcus aureus (Tosun et al., 2012), Enterococcus faecalis (Goncalves et al., 2010), Prevotella intermedia (Haas et al., 1997), Escherichia coli (Giannini et al., 2006) and Bacillus subtilis (Block et al., 1992). In addition, the test species have been incubated in various media including brain-heart infusion (BHI) (Kato et al., 1998; Giannini et al., 2006; Quaranta et al., 2009; Sennhenn-Krichner et al., 2009; Tosun et al., 2012; Goncalves et al., 2010), Columbia blood agar (Kreisler et al., 2002, 2003), nutrient broth (Block et al., 1992; Haas et al., 1997), and Luria-Bertani medium (Matsuyama et al., 2003). Furthermore, several incubation periods have been investigated. Some researchers allowed only 10 minutes (Tosun et al., 2012) for biofilm formation, whereas others allowed several days (Haas et al., 1997). Such variation will influence the resultant biofilm in a number of ways with variable effects on the implant surface. Shorter incubation durations may be sufficient for stable biofilm formation, whereas longer durations may be required for the degeneration of the surface.

This variation between the incubation protocols prevents comparability across studies. The aim of this investigation was to devise a protocol yielding consistent biofilm formation on titanium specimens in order to standardise in vitro experiments investigating microbial-induced surface deterioration and peri-implant therapy efficacy.

**METHODS**

**Titanium discs**

A total of 12 manufactured grade 4 machined (Ra = 0.27µm) and moderately roughened (Ra = 2.14µm) titanium discs (9.0mm diameter; 2mm thickness) comparable to current commercially available implants (Southern Implants (Pty) Ltd, Irene, South Africa) were used to simulate implant surfaces. Four discs were used to evaluate surface characteristics and the remaining eight were used in the biofilm experiments.

**Titanium specimen surface characteristics**

To assess surface morphology, scanning electron microscopy (SEM) images were acquired from all disc surfaces. An accelerating voltage of 5kV was selected for examination using a Field Emission SEM (JEOL 6700F Field Emission SEM, Tokyo, Japan). Surface topographical data were collected using a confocal laser-scanning microscope (Zeiss LSM 510, Carl Zeiss Microscopy GmbH, Jena, Germany). Using a PlanApochromat x20 / NA 0.6 objective, the confocal pinhole was adjusted to give an optical section depth of 3.0µm, operated in reflection mode with the gain adjusted to avoid pixel saturation. The offset was adjusted to ensure that out-of-focus planes had pixel values of zero. The upper and lower z-axis limits were determined empirically, focusing to the highest and lowest point on the surface. The resultant z sequence of images were analysed using plugins available for ImageJ (Rasband, W.S., U.S. National Institutes of Health, Bethesda, Maryland,

Selected areas of the disc were analysed using energy dispersive spectroscopy (EDS) at an accelerating voltage of 25kV. Surface composition was analysed in the backscattered electron mode (BSE). Deadtime was below 15% and counts per second were between 1500 – 2500. Spot analysis of 100s per area was used to identify the elements in the disc.

**Bacterial culture and growth conditions**

*Streptococcus oralis* strain 34 was obtained from Dr. J.O. Cisar (National Institute of Dental and Craniofacial Research, Bethesda, MD.) and cultivated on Columbia sheep blood agar (Fort Richard Laboratories Ltd., Auckland, New Zealand) under anaerobic conditions for 24 hours at 37°C. The culture was re-plated at two week intervals. *S. oralis* was also grown by overnight cultivation in brain-heart infusion (BHI; BactoTm, Becton, Dickinson and Co., Sparks, MD.) incubated at 37°C. For some experiments, BHI was supplemented with filter-sterilised sucrose solution to give a final concentration of 2% (w/v). Biofilms were developed on titanium surfaces by incubating pre-sterilise titanium discs in sucrose-supplemented BHI inoculated with *S. oralis* (100µl from an overnight BHI culture) and incubating at 37°C. The discs were transferred to fresh medium after 24, 48 and 72 hours incubation using plastic forceps to prevent introduction of metal ions. Control discs were similarly incubated and transferred in the uninoculated medium.

**Biofilm quality and quantity**

Analyses of the disc surfaces at baseline and following periods of incubation were undertaken by field emission SEM with EDS to evaluate the quality and consistency of biofilm formation. Following bacterial inoculation, one disc was removed at each observation period and placed in a vial containing 9mL of 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for one hour. The discs were post-fixed in 1% osmium tetroxide. After washing in buffer and then distilled water, the discs were dehydrated through graded ethanol (25%, 50%, 70%, 85%, 95%) for 10 minutes per step, then in 100% ethanol for three washes of 20 minutes. The titanium specimens were then subjected to critical point drying. Discs were placed on aluminium stubs and coated with 10 nm of gold palladium.

ImageJ was used to quantify the number of bacteria on disc surfaces visualised by SEM. Three predetermined areas of 250 µm² imaged at 5,000x, were selected.

**pH measurement**

The pH of the media in which each of the discs was incubated, was measured [Eutech pH 510 pH/mV/Temperature bench meter] after the disc had been transferred to fresh medium. The probe was rinsed thoroughly using ultra-pure water and ethanol to avoid cross-contamination and was calibrated against pH 4.01 and pH 7.01 buffers prior to pH determinations.

**Statistical analysis**

Comparative statistics were undertaken with the Mann-Whitney and the Kruskal-Wallis test. A statistical software package (SPSS 17.0, SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis.

**RESULTS**

SEM examination revealed typical parallel milling grooves on the surfaces of machined discs with isolated areas of surface irregularity whereas the moderately roughened discs showed characteristics of surfaces exposed to an ablative process with widespread irregularities. Surface asperities of the roughened discs appeared randomly oriented in contrast to those of the milled discs which were isolated and concentric (Fig 1). This was further supported by three-dimensional representations generated from CLSM analysis (Fig 1) and topographical analysis revealed that both the profile roughness (Ra) and surface area roughness (Sa) of the moderately roughened surfaces were significantly greater than those of the machined discs (Table 1).

<p>| TABLE1. COMPARISON OF SURFACE ROUGHNESS OF MACHINED AND MODERATELY ROUGHENED TITANIUM DISCS DETERMINED BY CLSM. |</p>
<table>
<thead>
<tr>
<th>Surfaces Characteristics (µm)</th>
<th>Mean (±SD)</th>
<th>Machined</th>
<th>Moderately rough</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra</td>
<td>0.27 (0.12)</td>
<td>2.14 (0.34) *</td>
<td></td>
</tr>
<tr>
<td>Sa</td>
<td>1.01 (0.008)</td>
<td>1.34 (0.08) *</td>
<td></td>
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</tbody>
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* p <0.001 (Mann-Whitney test)

Elemental analysis by EDS indicated that the moderately roughened surfaces had traces of aluminium residue from the ablation process that was completely absent from the machined discs (Fig 2).

Evidence of bacterial colonization of disc surfaces was evident as isolated biofilm mass 24 hours after inoculation with *S. oralis* (Fig 3 and 4). A confluent biofilm had formed over the entire disc surface after 48 hours (Fig 3 and 4) and after 72 hours a very dense biofilm had formed (Fig 3 and 4). There was no apparent difference in biofilm development between machined and roughened discs at any stage of
development when imaged by SEM (Figs 3 and 4). This observation was supported by measurement of the biofilm cell density, which revealed no significant differences between the milled and roughened discs (Table 2). The biofilms appeared to have reached maturity after 48 hours as the mass was not significantly greater after 72 hours (Table 2).

The pH of uninoculated BHI was around neutrality following incubation for up to 72 hours with titanium discs alone, whereas, following incubation with bacteria, the pH of the spent medium was below pH 4.5 at all measured time points (Table 3).

The pH of titanium discs incubated with bacteria was significantly lower than the pH of uninoculated discs. The pH of the spent medium was below pH 4.5 at all measured time points (Table 3). The pH of titanium discs incubated with bacteria was significantly lower than the pH of uninoculated discs. The pH of the spent medium was below pH 4.5 at all measured time points (Table 3).

**p = 0.04 (Kruskal-Wallis test)**

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**DISCUSSION**

It is important to standardise variables that can influence the bacterial colonization on implant surfaces in order to evaluate its effects on surface characteristics. The implant surface material and characteristics, the bacterial strain, incubation period (inoculation protocol), among other factors, are variables that can influence the outcome of in vitro studies.

For this in vitro pilot trial, we used titanium discs surfaces equivalent to current clinically available Southern Implants. Two implant surfaces, represented as machined and moderately roughened discs were evaluated. The surface roughness measurements of the machined surface were within the range of those used in other reports (Al-Nawas et al., 2003; Wennerberg & Albrektsson, 2000). The differences between surface roughness are associated with the manufacturing process used by various implant companies, and the metrical surface analysis settings used by other researchers. Al-Nawas et al., 2003 reported using CLSM, Sa values between 0.37 and 0.97µm for various machined surfaces. For the moderately roughened surfaces, our value of (2.14µm) was within the range of other moderately roughened surface characteristics. Although statistically significant differences exist between the tested surfaces in our study, these differences did not influence the bacterial growth.

The elemental analysis of the machined and roughened surfaces indicates that the roughened surfaces were grit-blasted with aluminium oxide because of the presence of Al in the spectra.

For the contamination protocol, S. oralis was used because of its acidogenic metabolism, which was reflected in the pH measurements for each incubation period. This characteristic is relevant when there is need to evaluate the corrosive effect of acid-producing bacteria on implant surfaces. The importance of the peri-implant environment has been addressed in several studies that exposed different types of titanium specimens to a variety of oral microorganisms, artificial saliva, sulfuric and lactic acid, and dietary products in an attempt to understand the factors contributing to implant surface deterioration (Björsten & Lekholm, 1993; Chang et al., 2003; Hjalmarsson et al., 2010). One-way understanding may facilitate the development of more complex biofilm, including the so-called microbial-induced corrosion organisms (Videla & Herrera, 2005; Hiromoto, 2008; Chaturvedi, 2009). Therefore, it is essential to preserve a stable titanium dioxide layer in order to maintain a corrosion-resistant surface; however, this layer is not exempt from corrosive attack (Chaturvedi, 2009).

The three-day incubation period used in this study has also demonstrated that S. oralis grows rapidly, achieving a dense and mature biofilm after two days of incubation. Furst et al., 2007, demonstrated a rapid bacterial colonization on oral Ti implants 30 minutes after implant placement. Among the evaluated bacteria, S. oralis became prominent after one week. This was demonstrated in our pilot trial in which bacterial colonization...
Figure 1. SEM and CLSM 3-D surface representation for machined (A, B) and moderately roughened (C, D) titanium surfaces.

Colonization was observed on both machined and moderately roughened surfaces from the first day of the incubation. The SEM examination of the biofilm showed minimal differences between surface types during the three days of incubation. Both machined and moderately roughened surfaces supported a dense cell culture at the second and third day of incubation, in which bacteria were numerous and clustered in a multi-layered disposition. Although statistically significant differences in Ra values were measured between machined and moderately roughened surfaces, indistinguishable bacterial growth occurred at one day. This differs from other reports where surface characteristics such as rough titanium surfaces supported greater bacterial attachment compared to smoother surfaces. Almaguer-Flores et al (2010), reported that the incubation medium influences the adhesion of certain species to amorphous carbon and titanium films. Furthermore, of the nine reference strains evaluated, the surface roughness and chemistry influenced to different levels the bacterial colonization. In contrast, Quirynen et al (1996) reported, in a short-term clinical study, that after one month of intraoral exposure, the effect of titanium abutment surface roughness had no major effect on either the quantitative or qualitative microbiologic adhesion and colonization. However, Ra values for the Ti abutments ranged between 0.05 and 0.21μm,
which are considered “fine machined” surfaces. Due to the rapid growth characterised by clusters of cells, multilayered disposition and polysaccharides production, a manual count methodology was selected. The limitations of evaluating SEM microphotographs with this methodology have to be considered.

Another potential application of this method is in the evaluating of efficacy of treatment modalities for peri-implant diseases. Different levels of photodynamic therapy efficacy have been reported in the literature (Mouhyi et al., 2000; Giannini et al., 2006; Quaranta et al., 2009; Sennhenn-Kirchner et al., 2009; Tosun et al., 2012). This variation in laser decontamination is associated with different surface types, microbiological contamination and laser irradiation protocols. In the present study, both titanium discs and the bacterial inoculation protocol were controlled. It is important to take into consideration the rapid growth and thick multilayered biofilm seen with *S. oralis*. These characteristics may impair SEM observations if we need to evaluate the effect of photodynamic therapy on titanium implant surfaces. Based on the SEM observations a short incubation period will permit both evaluation of the efficacy of the photodynamic therapy and the effect of the irradiation on the implant surface.

Figure 2. Photograph and surface elemental analysis of machined (A, B) and moderately roughened (C, D) titanium discs.
**CONCLUSIONS**

Within the limitations of this in vitro study, it can be concluded that Ti disc surfaces differ in terms of surface topography and roughness. These surface differences did not influence the bacterial colonization during the three-day incubation period. *S. oralis* consistently produces an acidic medium, which will permit further investigations of the effect of a corrosive environment on implant surfaces.

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**Figure 4.** SEM of uninoculated moderately roughened disc surface (A) and the bacterial growth after incubation with *S. oralis* for 1 day (B), 2 days (C) and 3 days (D).

### REFERENCES


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