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In situ analysis of multispecies biofilm formation on customized titanium surfaces

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SUMMARY

Many studies to identify surfaces that enhance the incorporation of dental implants into bone and soft-tissue have been undertaken previously. However, to succeed in the clinical situation, an implant surface must not support development of microbial biofilms with a pathogenic potential. As a first step in investigating this, we used two-species and three-species biofilm models with 16S ribosomal RNA fluorescence *in situ* hybridization and confocal laser scanning microscopy to examine the effect of surface characteristics on biofilm formation by species that can colonize titanium implants *in vivo*: *Streptococcus sanguinis*, *Actinomyces naeslundii* and *Lactobacillus salivarius*. Surfaces blasted with Al_2O_3 (S_a = 1.0–2.0 μm) showed a seven-fold higher bacterial adhesion after 2 h than turned surfaces (S_a = 0.18 μm) whereas porous surfaces, generated by anodic oxidation (S_a = 0.4 μm), showed four-fold greater adhesion than turned surfaces. Hence, increased roughness promoted adhesion, most likely through protection of bacteria from shear forces. Chemical modification of the blasted and oxidized surfaces by incorporation of Ca^{2+} ions reduced adhesion compared with the corresponding non-modified surfaces. After 14 h, biofilm growth occurred in the three-species model but not in the two-species consortium (containing *S. sanguinis* and *A. naeslundii* only). The biofilm biovolume on all surfaces was similar, suggesting that the influence of surface characteristics on adhesion was compensated for by biofilm development.

INTRODUCTION

Osseointegrated titanium implants are commonly used to replace missing teeth and these generally have high long-term survival rates (Ekelund *et al.*, 2003; Lekholm *et al.*, 2006). Most currently available implants have surfaces that are minimally or moderately rough, with an average height deviation of 0.5–2 μm, and these have been shown to give good incorporation of the material into the bone tissue (Albrektsson & Wennerberg, 2004). In addition to modifications of surface topography, the physicochemical properties of titanium implant surfaces have been adapted to achieve better osseointegration. For example, in rats, surface modifications using anodic oxidation result in high implant stability, probably through recruitment of mesenchymal cells and the expression of genes for, for example, alkaline phosphatase and osteocalcin involved in bone remodeling (Omar *et al.*, 2010). Surface modifications including

calcium ions have been found to promote osteoblast adhesion *in vitro* (Ergun *et al.*, 2007) and bone integration in a rabbit model (Frojd *et al.*, 2008). However, when titanium surfaces modified to promote osseointegration become exposed to the oral environment as the result of, for example, a poor soft-tissue seal or bone destruction, they represent surfaces on which commensal oral microorganisms will adhere and form three-dimensional structured bacterial communities known as biofilms.

The microbiota around healthy titanium implants, and to some extent around diseased sites, has been proposed to be similar to that of teeth in similar clinical states (Tanner *et al.*, 1997; Leonhardt *et al.*, 1999). Microbial colonization is initiated by adhesion of pioneer species, such as *Streptococcus oralis*, *Streptococcus sanguinis* and *Actinomyces naeslundii*, through interactions with the salivary pellicle (Kolenbrander *et al.*, 2010). The early colonizers promote the adhesion of secondary colonizers by co-adhesion and biofilm formation proceeds through growth of surface-associated microorganisms. As for dental plaque, microbial biofilms on the surfaces of dental implants are microbial communities where multiple species live in close physical contact, which increases the probability of microbial interactions, both synergistic and antagonistic, between microbial cells (Marsh & Bowden, 2000). With time, a multispecies biofilm is established and the microbial interactions eventually give stability to the community. It has become evident that this mode of colonization enhances the ability of the microorganisms to survive under environmentally stressful conditions as well as in the presence of antimicrobial agents (Marsh, 2003).

Several *in vitro* studies have shown that increasing the roughness of titanium surfaces promotes biofilm formation. For instance, adhesion of *S. sanguinis*, as assessed by culturing, increased stepwise from smooth to moderately rough and rough surfaces (Pereira da Silva *et al.*, 2005) and an investigation using fluorescence microscopy has shown adhesion of this species to be greater on minimally rough surfaces than on smooth surfaces in the presence of saliva (Burgers *et al.*, 2010). Using an eight-species consortium of oral bacteria, more bacteria were found on moderately rough titanium surfaces than on smooth surfaces, in both the presence and absence of saliva (Almaguer-Flores *et al.*, 2010). However, for non-oral bacteria such as *Staphylococcus aureus*, the opposite is true, with no significant differences in the level of adhesion between smooth or minimally rough titanium (Harris *et al.*, 2007). The impact of surface roughness on oral bacterial adhesion has also been investigated regarding other dental materials, e.g. acrylics and resins/composites. Using a single-species model with *S. oralis* or a multispecies model, increased surface roughness was shown to result in greater initial bacterial adhesion (Morgan & Wilson, 2001; Dezelic *et al.*, 2009). However, in both studies the impact of surface roughness diminished with time and with maturation of the biofilm. Incorporation of calcium ions has been shown to increase the adhesion of later colonizers such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in an *in vitro* model using single-species cultures (Yoshinari *et al.*, 2000). The impact of calcium modification on the adhesion of other oral bacteria has not been widely investigated.

In this study, we have used two-species and three-species biofilm models as well as fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM), to examine the effect of titanium surface characteristics on biofilm formation by commensal oral bacteria. The bacterial species used were *S. sanguinis* and *A. naeslundii*, two early colonizers on teeth (Kolenbrander & London, 1993), and *Lactobacillus salivarius*, a bacterium frequently isolated from dental plaque (van Houte *et al.*, 1996). The surfaces investigated for biofilm formation were those found to be favorable for osseointegration *in vivo* (blasted, or blasted and subsequently oxidized in the presence of calcium acetate) (Wennerberg *et al.*, 1996; Frojd *et al.*, 2008, 2010) and these were compared with commercially pure titanium turned surfaces or titanium surfaces oxidized in the presence or absence of calcium acetate. Data presented here suggest that increased topographical height deviation enhanced bacterial adhesion whereas incorporation of calcium ions reduced this effect.

METHODS

Preparation and characterization of the titanium surfaces

The original 8-mm discs of commercially pure (grade 4) titanium had a turned surface (turned). This surface was subsequently modified in four different ways: anodization with an electrolyte (sodium

glycerophosphate; hereafter referred to as Ox), anodization with sodium glycerophosphate hydrate and calcium acetate (i.e. OxCa), blasting with 120-μm Al_2O_3 particles (3 cm distance, 3.2 kg pressure; i.e. Bl), or blasting with 120-μm Al_2O_3 particles (3 cm distance, 3.2 kg pressure) followed by anodization with sodium glycerophosphate hydrate and calcium acetate (i.e. BlOxCa). The anodic oxidation process was performed in a galvanistic mode as previously described, by using platinum as counter electrodes and a voltage of 20–130 V (Sul *et al.*, 2001a, 2004).

Three discs of each surface type were characterized. Topographic characterization was carried out with a Zeiss DSM 982 Gemini scanning electron microscope and an optical interferometer (MicroXam™; PhaseShift, Tucson, AZ). To distinguish roughness from errors of form and waviness, a high-pass Gaussian filter (size 50 × 50 μm) was used, and the surface parameters: average height deviation (S_a, μm), density of summits (S_{ds}, μm^{-2}), and surface enlargement (S_{dr}, %), were computed using the SURFASCAN software (Wennerberg & Albrektsson, 2000). Chemical analysis of surfaces was performed using an X-ray photoelectron spectroscope (PHI 5000 ESCA system; Perkin Elmer, Wellesley, MA) with an operating angle of 45° at 150 W and Al as the excitation source.

Immediately before inoculation of bacteria for biofilm assays, discs were cleaned with diluted (1 : 40) Extran MA01®; (Merck, Darmstadt, Germany) in an ultrasonic bath, treated with ethanol, and placed in polystyrene six-well, flat-bottom titer plates (MULTIWELL™; Becton Dickinson, Franklin Lakes, NJ).

Bacterial strains and culture

The oral strains used for biofilm assays were *S. sanguinis* ATCC 10556 (a strain known to interact with saliva), *A. naeslundii* isolated from dental plaque (Wickstrom & Svensater, 2008), and *L. salivarius* isolated from a root canal (Chavez de Paz *et al.*, 2008). All strains were routinely maintained on blood agar or Bacto Todd–Hewitt broth (30 g l^{-1}; Difco Laboratories, Becton Dickinson & Co, Sparks, MD) at 37°C in 5% CO_2.

Biofilm formation assays

Overnight broth cultures were transferred by 1 : 50 dilution into fresh, prewarmed Bacto Todd–Hewitt broth and incubated at 37°C in 5% CO_2 to the mid-exponential growth phase (optical density at 600 nm ≈ 0.6). Cultures were adjusted to give final concentrations of approximately 1×10^8 cells ml^{-1} for *S. sanguinis* or 1×10^7 cells ml^{-1} for *A. naeslundii* and *L. salivarius*. For two-species biofilms, 1.5 ml *S. sanguinis* suspension and 4.5 ml *A. naeslundii* suspension (i.e. approx. 2.5 times more *S. sanguinis* than *A. naeslundii* cells) was inoculated into each well containing the titanium discs. For three-species biofilms, 1 ml *S. sanguinis* suspension mixed with 3 ml *A. naeslundii* and 3 ml *L. salivarius* suspension (i.e. approx. three times more *S. sanguinis* cells than *A. naeslundii* and *L. salivarius* cells) was used as the inoculum. A higher proportion of *S. sanguinis* cells was used to reflect the greater numbers of streptococci present in the oral cavity. The microtiter plate was sealed with paraffin tape and incubated at 37°C on a rotary shaker at 300 cycles per minute in 5% CO_2. Following incubation for 2 or 14 h, the surfaces were rinsed three times with 10 mM potassium phosphate buffer, pH 7.5 (PBS) to remove loosely bound cells. The adherent bacteria were then fixed in 4% paraformaldehyde (PFA) in PBS, overnight at 4°C for 16S ribosomal RNA (rRNA) hybridization. All experiments were carried out three times for each surface using independent bacterial cultures.

Incubation with whole saliva

Unstimulated whole saliva was collected from a healthy volunteer with good oral health, and shown to contain 10^7 bacteria per ml by culturing on blood agar. Aliquots (6 ml) were added to the six-well plates containing the titanium surfaces and incubated at 37°C in 5% CO_2 for 14 h with gentle shaking (300 r.p.m.). The surfaces were rinsed three times with PBS and fixed overnight at 4°C in 4% PFA.

Mechanical removal of microbial biofilms

Three discs of each surface type that had been incubated for 14 h in three-species biofilm assays were mechanically cleaned using 20 strokes with a soft toothbrush. The discs were then washed twice with ultra-pure H_2O and the adherent bacteria were fixed overnight at 4°C in 4% PFA for 16S rRNA hybridization.

16S rRNA FISH

Surfaces with fixed bacteria were washed with cold sterile PBS and subjected to cell membrane permeabilization using 100 μl lysozyme (Sigma, St Louis, MO) [(70 U μl^{-1}) in 100 mM Tris–HCl, pH 7.5 (Sigma), 5 mM EDTA (Merck)] for 9 min at 37°C. The surfaces were then rinsed with ultra-pure H_2O and dehydrated with 50, 80 and 99% ethanol for 3 min and left to dry at room temperature. A total of 30 μl hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl buffer, pH 7.5, with 0.01% sodium dodecyl sulfate and 25% formamide) containing 20 ng ml^{-1} of labeled oligonucleotide probes was pipetted onto the titanium discs.

For two-species biofilms, the probe cocktail consisted of the streptococcal probe STR493 (5′-GTTAGCCGTCCCTTTCTGG-3′) (Franks *et al.*, 1998), fluorescently labeled green with ATTO-488, and a red-labeled ATTO-565 probe EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′) (Amann *et al.*, 1990). For three-species biofilms, the probe cocktail consisted of probe STR493 (green), a red-labeled LAC722 probe (5′-YCACCGCTACACATGRAGTTCCACT-3′) targeting the lactobacilli group (Sakai *et al.*, 2004), and a probe IF201 (5′-GCTACCGTCAACCCACCC-3′) labeled with Pacific blue for identification of *A. naeslundii* (Foster & Kolenbrander, 2004). After incubation with saliva, biofilms on the titanium surfaces were labeled using the EUB388 probe to give a measure of the total biovolume and the proportion of streptococci was assessed using the STR493 probe.

Oligonucleotides were hybridized at 47°C in a humid chamber for 90 min and the surfaces were then washed three times with 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.01% sodium dodecyl sulfate, and twice with 159 mM NaCl, for 30 and 15 min, at 47°C under gentle shaking. Finally, the titanium surfaces were washed with 1 ml ice-cold ultra-pure H_2O, mounted and glued to glass slides for analysis with inverted CLSM.

CLSM and image analysis

Titanium discs subjected to FISH were examined with an Eclipse TE2000 inverted CLSM (Nikon Corporation, Tokyo, Japan). Green fluorescence was provided by an argon laser (488-nm laser excitation), red fluorescence was given by a helium-neon laser (543 nm laser excitation), and blue fluorescence was provided by a UV laser (390-nm laser excitation). The CLSM images were acquired with an oil immersion objective (×60) and images were obtained with a zoom factor of 1.0, a pixel resolution of 0.42 μm $pixel^{-1}$, and a field resolution of 512 × 512 pixels. Each stack had a substratum coverage field area of 215 × 215 μm. In three-dimensional section analyses, the *z*-step was 2 μm and the number of steps was adjusted to take into account differences in surface roughness.

The CLSM images were obtained from a total of 15 randomly selected sites per disc. The image stacks were converted into TIFF format and processed through the general-user interface of the image analysis software BIOIMAGE_L (Chavez de Paz, 2009), which calculated the biofilm biovolume. The software also identified color tonalities, which in the two-species and three-species biofilm experiments represented different bacterial species and analysed the proportion of bacterial species independently. In the saliva experiments, the software identified the proportion of streptococci relative to the total biofilm biovolume.

Statistics and treatment of data

The biofilm biovolume is presented in the text and figures as biovolume per actual surface area. The actual surface area has been correlated with the additional surface area because of the roughness (derived from the S_{dr} value). To compare biofilms within one experiment, the non-parametric Friedman test was used. For paired investigations between the surfaces within one experiment, Wilcoxon signed rank test was used. A confidence interval of 95% was chosen and *P*-values below 0.05 were considered significant.

RESULTS

Characteristics of the titanium surfaces

The topographic appearance of the surfaces, as revealed by interferometry and scanning electron microscopy, is presented in Fig. 1 (left and middle panels). The anodized surfaces (Ox, OxCa and BlOxCa) showed a porous outer layer (Fig. 1E,H,N) with the calcium-modified surfaces [OxCa, (Fig. 1H)

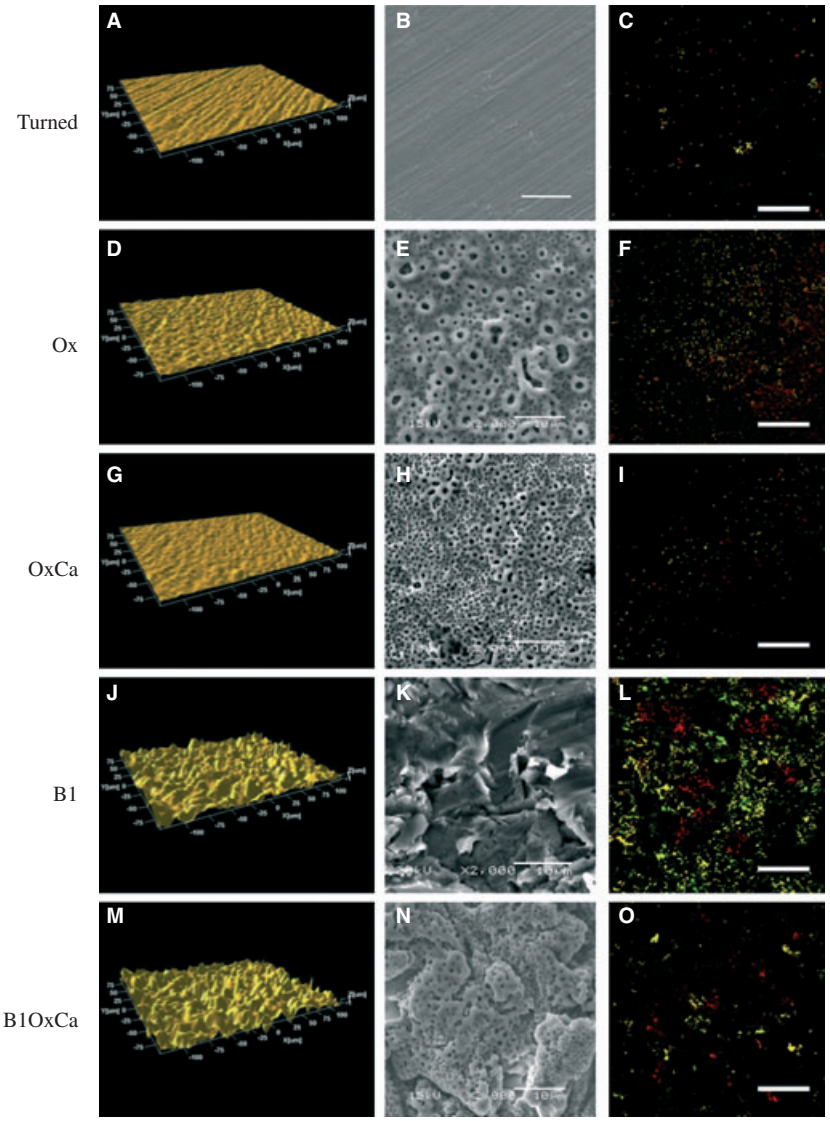

Figure 1 Images of titanium surfaces derived from interferometry produced with SPIP™ (Image Metrology, Lyngby, Denmark; left panel), scanning electron microscopy (SEM) images (middle panel), and confocal laser scanning microscopy (CLSM) images of the different surfaces with two-species 2-h biofilms (right panel). Biofilms were visualized with fluorescence *in situ* hybridization using oligonucleotide probes targeting *Streptococcus sanguinis* (green) and all bacteria, in this case *Actinomyces naeslundii* (red). Scale bars in SEM and CLSM images represent 10 and 25 μm, respectively.

and BlOxCa (Fig. 1N)] showing smaller and more homogeneously distributed porous structures (0.5–0.8 μm) than the Ox surface (Fig. 1E), possibly because of the presence of calcium in the electrolyte during the anodic oxidation process. The topographic measurements are summarized in Table 1. Based on the surface parameter 'average height deviation from the mean plane' (S_a), the turned, Ox and OxCa surfaces were categorized as smooth and the Bl and BlOxCa surfaces were categorized as moderately rough (Albrektsson & Wennerberg, 2004). The turned, Ox and OxCa surfaces had an additional surface area (S_{dr}) of <50% whereas the blasted surfaces had an S_{dr} >50%, suggesting that the area available for bacterial binding was greater on the latter surfaces.

Bacterial adhesion to the different surfaces after 2 h

Confocal laser scanning micrographs showing 16S rRNA FISH of *A. naeslundii* and *S. sanguinis* on the

Table 1 Characteristics of the surfaces

	Smooth surface[1]			Moderately rough surface[1]	
Topography	Turned	Ox	OxCa	Bl	BlOxCa
Average height deviation (S_a, μm)	0.18 ± 0.02	0.4 ± 0.07	0.22 ± 0.01	1.52 ± 0.11	1.54 ± 0.14
Summit density (S_{ds}, summits μm^{-2})	0.13 ± 0.02	0.24 ± 0.01	0.23 ± 0.003	0.16 ± 0.02	0.18 ± 0.003
Developed surface area (S_{dr}, %)	4 ± 1	43 ± 8	15 ± 2	76 ± 9	88 ± 9

[1]Surface characteristics are defined according to Albrektsson & Wennerberg (2004).

different titanium surfaces after 2 h are presented in the right-hand panel of Fig. 1. A conditioning film from the Todd–Hewitt broth medium in which the bacteria were suspended most likely formed on the surfaces and both species adhered to all surfaces although the total level of adhesion differed between them. On all the smooth surfaces, bacteria were sparsely but evenly distributed with no distinct clusters (Fig. 1C,F,I). In contrast, on both the moderately rough surfaces, *A. naeslundii* and *S. sanguinis* were present as distinct aggregates. Although the two surfaces had similar surface topography (Table 1), the level of adhesion to the BlOxCa surface (Fig. 1O) appeared to be much lower than to the Bl surface (Fig. 1L) suggesting a diminishing effect of the calcium modification on bacterial adhesion.

Analysis of the images obtained after 16S rRNA FISH and CLSM revealed significant differences in biofilm biovolume on the different surfaces ($P < 0.05$) (Fig. 2A). As suggested by the right-hand panels in Fig. 1, the blasted (Bl) surface had the largest mean biofilm biovolume (0.125 ± 0.049 $\mu m^3\ \mu m^{-2}$) whereas the OxCa and turned surfaces had the lowest mean biovolumes (0.027 ± 0.026 and 0.044 ± 0.027 $\mu m^3\ \mu m^{-2}$, respectively). Hence, there was a clear difference between the moderately rough and the smooth surfaces with respect to the adherence of *S. sanguinis* and *A. naeslundii* after 2 h. In comparison with the Ox and Bl surfaces, the OxCa and the BlOxCa surfaces showed lower biofilm biovolumes, again suggesting that oxidation in the presence of calcium reduced bacterial adhesion.

In the three-species biofilm system, the pattern of adhesion after 2 h of incubation with *S. sanguinis*, *L. salivarius*, and *A. naeslundii* was similar to that seen for the two-species cultures (Fig. 2B). However, the biofilm biovolumes were lower (turned 0.008 ± 0.001 $\mu m^3\ \mu m^{-2}$; Ox 0.032 ± 0.028 $\mu m^3\ \mu m^{-2}$; OxCa 0.013 ± 0.011 $\mu m^3\ \mu m^{-2}$; Bl 0.034 ± 0.013 $\mu m^3\ \mu m^{-2}$; and BlOxCa 0.009 ± 0.004 $\mu m^3\ \mu m^{-2}$) than those seen for the two-species consortium, suggesting that the presence of *L. salivarius* reduced the adhesion of *S. sanguinis* and *A. naeslundii*. The differences observed between the surfaces were not significant.

Grouped, paired comparisons of the two-species and three-species biofilm biovolumes on the different surfaces showed that there was a significant difference between the turned and Bl surfaces, suggesting that bacterial adhesion increased with increasing surface roughness (Fig. 3A). The same effect of

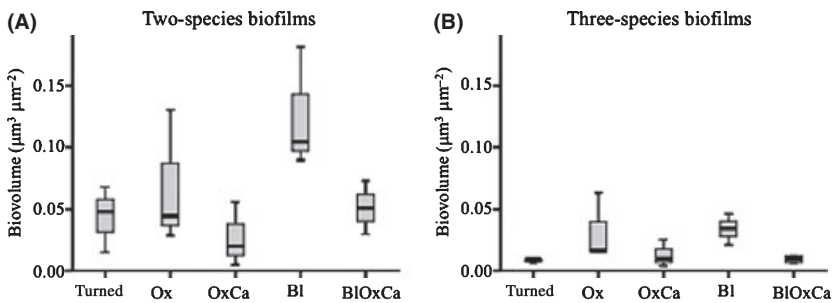

Figure 2 A boxplot showing bacterial adhesion on turned, oxidized (Ox), oxidized with incorporation of calcium (OxCa), blasted (Bl) and BlOxCa surfaces after 2 h from (A) two-species (*Streptococcus sanguinis* and *Actinomyces naeslundii*) and (B) three-species (*S. sanguinis, A. naeslundii* and *Lactobacillus salivarius*) consortia. Biovolume of biofilms was calculated using BIOIMAGEL (Chavez de Paz, 2009). The results are based on a minimum of three independent experiments for each surface.

blasting, leading to an increase in bacterial adhesion, was seen even after calcium modification of the surfaces (Fig. 3A). The increased oxide layer (on the Ox surface) also significantly increased the biofilm formation over that found on the turned surface, possibly because of the larger surface area (Fig. 3B). On both the smooth and moderately rough surfaces, the presence of calcium resulted in significantly less bacterial adhesion than on the corresponding surfaces without calcium modification (Fig. 3C).

Biofilm formation on the different surfaces after 14 h

Biofilm growth on the different titanium surfaces was investigated after 14 h of incubation at 37°C. In two-species cultures, the total biovolumes after 14 h were in the same range as those seen after 2 h except for the blasted surface where the biofilm biovolume had increased three-fold (Fig. 4A). This suggests that little or no bacterial growth had occurred within the 14-h period. The biovolumes on the different surfaces were significantly different after 14 h ($P < 0.05$). The two moderately rough surfaces showed the highest biovolumes: the Bl with a mean biovolume of 0.402 ± 0.247 $\mu m^3\ \mu m^{-2}$ and the BlOxCa surface with a mean biovolume of 0.113 ± 0.028 $\mu m^3\ \mu m^{-2}$ (Fig. 4A). The 14-h biofilm comprised both *S. sanguinis* and *A. naeslundii*, although the amount of *A. naeslundii* was generally lower than that of *S. sanguinis*.

In three-species biofilm experiments incubation for 14 h gave rise to significantly higher biofilm biovolumes on all surfaces ($P < 0.05$) compared with the values after 2 h (Figs 4B and 5 left panel). This suggests that, in contrast to the two-species biofilms, bacterial growth had occurred in the three-species consortium containing *L. salivarius*. The effect was especially pronounced for the calcium-modified surfaces where the biofilm biovolumes were much greater than those on the corresponding non-calcium modified surfaces despite the lower initial levels of adhesion seen on the former surfaces after 2 h. The major component of the three-species biofilms was *L. salivarius* and *A. naeslundii* was present in only small amounts (Figs 4B and 5) and was predominantly found in the upper levels of the biofilms.

Mechanical abrasion of established biofilms

Fourteen-hour three-species biofilms were subjected to systematic brushing and the remaining bacteria were visualized using 16S rRNA FISH. After brushing, significant differences between the smooth and moderately rough surfaces were found in the amount of bacteria remaining on the surface ($P < 0.05$). On the smooth surfaces, most of the bacteria were removed with the lowest biofilm biovolumes remaining on the OxCa (0.004 ± 0.003 $\mu m^3\ \mu m^{-2}$) and the turned (0.009 ± 0.006 $\mu m^3\ \mu m^{-2}$) surfaces. The Ox surface had a remaining biofilm biovolume of 0.014 ± 0.010 $\mu m^3\ \mu m^{-2}$. On the moderately rough blasted surfaces bacteria were poorly removed as

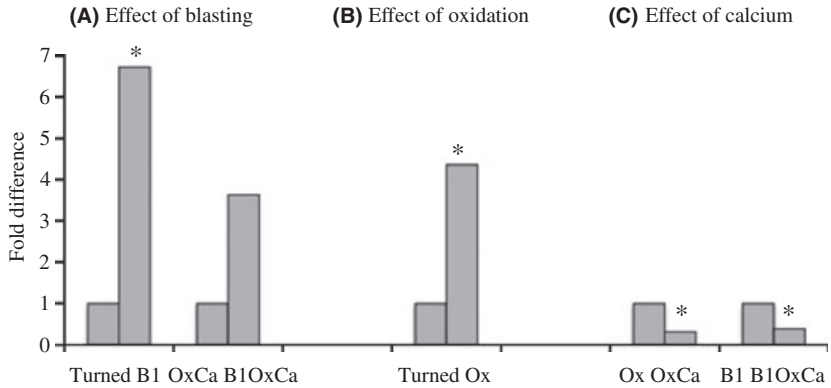

Figure 3 Comparison of biofilm biovolume on different titanium surfaces after 2 h to evaluate the effect of (A) blasting (Bl), (B) oxidation (Ox) and (C) incorporation of calcium(Ca). In each case, the biovolume on a reference surface was normalized to a value of 1 and the comparison was expressed as 'fold difference'. The reference surfaces for effect of blasting were the turned and OxCa surfaces, for the effect of Ox, was the turned surface and for the effect of calcium, were the Ox and Bl surfaces. *$P < 0.05$.

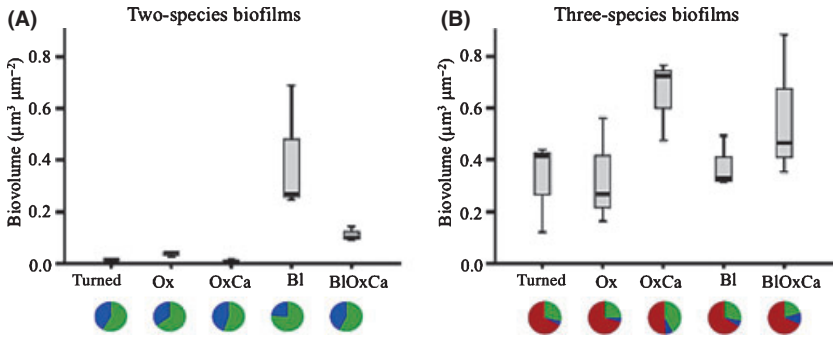

Figure 4 A boxplot showing microbial biofilm biovolume after 14 h in (A) two-species and (B) three-species models. Biovolume of biofilms was calculated using BIOIMAGEL (Chavez de Paz, 2009). The results are based on a minimum of three independent experiments. Proportions of the bacterial species are displayed as pie charts; green, *Streptococcus sanguinis*; blue, *Actinomyces. naeslundii*; and red, *Lactobacillus salivarius*.

shown by the relatively high biofilm biovolumes remaining after brushing (Bl 0.109 ± 0.057 μm^3 μm^{-2} and BlOxCa 0.025 ± 0.001 μm^3 μm^{-2}). After brushing, the biofilms were dominated by *L. salivarius* and *S. sanguinis* whereas *A. naeslundii* was rarely seen, suggesting that *A. naeslundii* does not adhere as strongly as the other two species.

Saliva-derived biofilms

The biofilm biovolumes accumulated on all surfaces from human whole saliva over 14 h were generally lower than those seen after incubation with nutrient-rich growth medium. The 16S rRNA FISH showed that at least one-third of the total biofilm biovolume was made up of streptococci, and the highest proportion of streptococci was found on the blasted surface. In keeping with the results of the two-species and three-species biofilm experiments, the blasted (Bl) surface had a greater biofilm biovolume per unit surface area (0.260 ± 0.101 μm^3 μm^{-2}) than the turned (0.015 ± 0.004 μm^3 μm^{-2}), Ox (0.031 ± 0.006 μm^3 μm^{-2}), BlOxCa (0.031 ± 0.009 μm^3 μm^{-2}) and OxCa (0.059 ± 0.016 μm^3 μm^{-2}) surfaces. There were significant differences between the biovolumes on the different surfaces ($P < 0.05$).

DISCUSSION

Streptococcus sanguinis and *A. naeslundii* are recognized as early components of dental biofilms and as biofilm formation has been proposed to occur in a similar way on tooth and implant surfaces, we have used them as model organisms to study initial biofilm formation on the surfaces used on titanium dental implants. *Lactobacillus salivarius* was also included in the three-species consortium because it is commonly isolated at low levels in dental plaque (van Houte *et al.*, 1996). The model, which is compatible with CLSM and 16S rRNA FISH, allowed investigation of bacterial load as well as distribution of different species, even when bacteria were embedded in a matrix of salivary proteins. The results presented here suggest that modification of titanium surface topography by, for instance, blasting or incorporation of calcium ions affects bacterial adhesion and growth.

One mechanism that is used to increase the bone-to-implant contact and promote rapid osseointegration is to increase the implant surface roughness (Rasmusson *et al.*, 2001). As a consequence, we have investigated the effects on biofilm formation by comparing two titanium surfaces of different roughness. One of the surfaces (Bl) had an S_a value of 1–μm and is therefore classified as moderately rough whereas the turned surface is regarded as smooth (S_a value <0.5 μm) (Albrektsson & Wennerberg, 2004). Developed surface area ratio (S_{dr}) is a parameter that takes into account both the number and height of the peaks on a given surface. This provides information regarding the surface enlargement if a given surface is flattened out and the surface area exposed for potential interactions. The blasted surface had an S_{dr} value of 76 ± 9% and hence a much greater surface area for potential bacterial interactions than the turned one, which had an S_{dr} value of 4 ± 1%. Not surprisingly, bacterial adhesion from

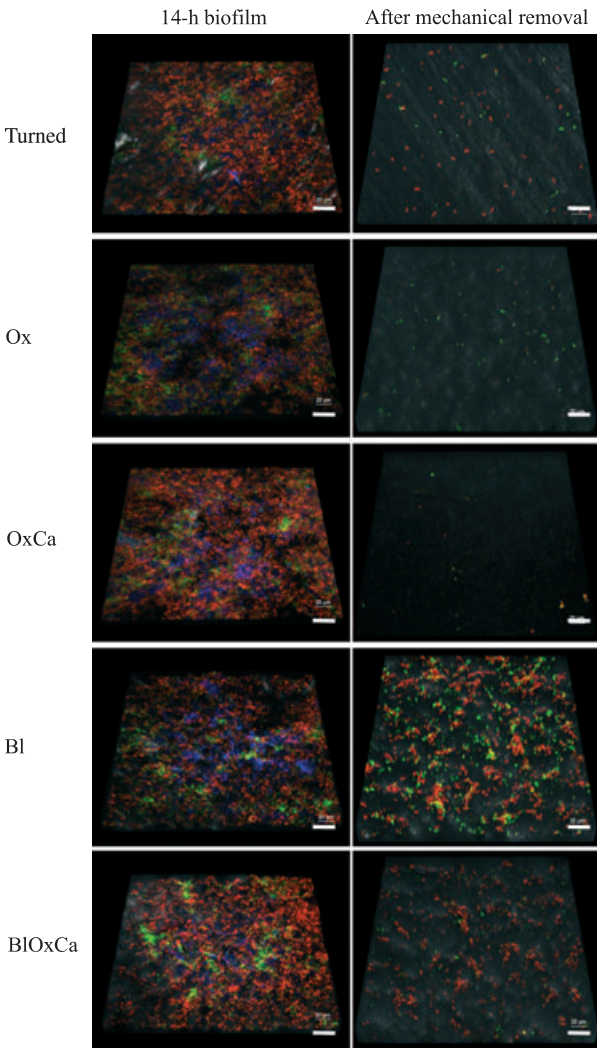

Figure 5 Three-dimensional images of 14-h three-species biofilms (left column) and after mechanical abrasion by brushing (right column). Biofilms were visualized with fluorescence *in situ* hybridization using oligonucleotide probes targeting *Streptococcus sanguinis* (green), *Actinomyces naeslundii* (blue) and *Lactobacillus salivarius* (red). Scale bar 20 μm.

two-species and three-species consortia over 2 h was seven times greater on the blasted surface than on the turned one (Fig. 3). However, the greater S_{dr} alone cannot wholly account for this difference and one explanation for this is that the topography (peaks and troughs) on the rougher surface provides the bacteria with protection from removal by shear forces. This idea is supported by the fact that on the blasted surfaces much of the biofilm biovolume remained after mechanical removal with a soft toothbrush, whereas almost all the bacteria were removed from the turned surface. Results from a previous *in vivo* study also show that rough plasma-sprayed titanium surfaces had significantly more bacteria remaining after toothbrushing than smooth, turned titanium surfaces (Amarante *et al.*, 2008). After 14 h, the biovolume of *S. sanguinis* and *A. naeslundii* biofilms on the blasted surface had increased more than threefold compared with that present after 2 h, suggesting that bacterial growth had occurred on this surface. However, no significant increase in biofilm biovolume was observed on the turned surface over the same period, suggesting that moderately rough surfaces with a higher S_a value enhanced the growth rate of attached microorganisms over the 14 h but that the smooth surface did not. Such observations would agree with the findings of studies *in vivo*, which report higher plaque accumulation rates with increased surface roughness (Quirynen & Bollen, 1995; Elter *et al.*, 2008; Burgers *et al.*, 2010).

In addition to changing the surface roughness, electro-chemical modifications such as anodic oxidation have been used as a means of enhancing osseointegration of titanium dental implants (Sul *et al.*, 2001b; Ivanoff *et al.*, 2003). Anodic oxidation of titanium produced a surface with porous structures and mainly anatase crystallinity, which although classed as smooth, was clearly rougher than the turned surface (S_a 0.4 ± 0.07 mm) and had a developed surface area intermittent between the turned and blasted surfaces. After 2 h bacterial adhesion was four times greater on the Ox surface than on the turned one and as for the moderately rough blasted surface, the S_{dr} of the Ox surface was not sufficient to completely explain this increased biofilm formation. Hence, the roughness of the Ox surface, although not as great as that of the blasted surface, appears, to some extent, to have prevented biofilm removal by shear forces. At the same time, anatase crystallinity of titanium has been suggested to markedly reduce bacterial adhesion (Del Curto *et al.*, 2005). Furthermore, anodic oxidization resulted in low adhesion of *Aggregatibacter actinomycetemcomitans in vitro* (Yoshinari *et al.*, 2000). In our study, bacterial adhesion to the Ox surface was similar to or higher than to the turned control surface, suggesting either that the titanium oxide crystallinity after anodic oxidation had no antimicrobial effect, or that this was compensated for by the slightly higher surface roughness. After 14 h, in contrast to the blasted surface, no

significant growth of the attached bacteria was seen even if *L. salivarius* was present in the consortium. Possibly, this can be explained by the lower S_a value for the Ox surface compared with the blasted one because a larger average surface height deviation might be expected to provide more microniches that bring bacteria into close association where growth-promoting microbial interactions can occur.

Although the effect of calcium incorporation on bacterial adhesion is still largely unknown (Klinge & Meyle, 2006), such modification has been shown to enhance osseointegration (Sul *et al.*, 2002, 2004; Frojd *et al.*, 2008). In this study, initial bacterial adhesion from both the two-species and three-species consortia to the oxidized and calcium-modified surfaces (OxCa) or oxidized and calcium-modified blasted surfaces (BlOxCa) was reduced by more than 50% after 2 h compared with the Ox and Bl surfaces (Fig. 3). This suggests that the incorporation of calcium ions onto the surface prevents adhesion of *S. sanguinis*, *A. naeslundii* and *L. salivarius*. On the other hand, the presence of calcium on the OxCa, as well as on the BlOxCa, surfaces led to a significantly higher rate of growth over 14 h by the three-species consortium compared with the reference surfaces (Fig. 4B). The average height deviation and developed surface area of the Bl and BlOxCa surfaces were very similar (Table 1), and hence the effect appears unlikely to be the result of surface topography but rather of the incorporation of calcium. As a similar increase in growth on the calcium-containing surfaces was not seen in the two-species consortium, the increase in biofilm biovolume can be attributed to the presence of *L. salivarius* in the consortium. Although the biofilm-forming capacity of lactobacilli has been shown to be dramatically increased in the presence of *Streptococcus mutans* (Filoche *et al.*, 2004; Wen *et al.*, 2010) or *A. naeslundii* (Filoche *et al.*, 2004) the mechanism underlying the increased biofilm growth on calcium surfaces seen in this study is not known. However, these results emphasize the fact that in addition to the characteristics of the surface, the model bacteria used will influence the biofilm formation on titanium surfaces and care should therefore be exercised in extrapolating the results of *in vitro* experiments to the *in vivo* situation.

In conclusion, the results presented here suggest that both the topographical and chemical properties of titanium surfaces can exert differential effects upon the growth of colonizing species and, over time, may influence the composition of the attached biofilm. In the longer term, changes in the delicate balance within these communities, including excessive accumulation and changes in composition, can generate biofilms with pathogenic potential and eventually lead to implant-related disease. Calcium-coated surfaces supported lower initial levels of biofilm formation than non-calcium-containing ones. Based on this observation, in combination with their good osseointegration properties (Frojd *et al.*, 2008), calcium-coated surfaces would appear to be promising candidates for use in dental implants. Further investigations using mixed-species microbial consortia as well as clinical investigations are therefore required to determine how the surface characteristics affect the properties of the attached microbial biofilms.

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