Differential adhesion of *Streptococcus gordonii* to anatase and rutile titanium dioxide surfaces with and without functionalization with chlorhexidine

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Abstract: The majority of dental implants are composed primarily of titanium and have an outer layer of titanium dioxide. Crystalline titanium dioxide most commonly exists in one of the two structures, anatase and rutile, and both of these have been observed on commercially available dental implants. Early implant failure can be associated with postoperative infection due to implant contamination during or immediately after surgery. The impetus of this study was to investigate whether functionalization of anatase and rutile titanium dioxide surfaces with chlorhexidine-reduced subsequent colonization of the surface by *Streptococcus gordonii*. Exposure to 100 $mg \cdot L^{-1}$ chlorhexidine for 60 s resulted in a fivefold reduction in *S. gordonii* coverage on anatase and a twofold reduction on rutile. This may be related to a preferential adsorption of chlorhexidine to anatase compared with rutile. The reduction in bacterial coverage was not due to desorption of chlorhexidine into solution. More bacteria were observed on anatase than rutile surfaces without chlorhexidine functionalization, indicating that crystal structure may have a significant effect on bacterial colonization. In conclusion, functionalization with chlorhexidine reduced bacterial coverage on titanium dioxide surfaces, and anatase surfaces may be more amenable to such treatment than rutile. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 90A: 993–998, 2009

Key words: anatase; chlorhexidine; implant; *Streptococcus gordonii*; rutile

INTRODUCTION

Most dental implants are fabricated from commercially pure titanium,[1] the outer surface of which is composed of titanium dioxide. Two polymorphic crystal structures of TiO_2 (anatase and rutile) are commonly found on the surfaces of commercially available dental implants.[2,3]

There are a number of differences between the properties of anatase and rutile, including point of zero charge, photocatalytic and photoelectrochemical properties, and unit cell structure.[4,5] This has led some authors to postulate that the two polymorphs might elicit different responses in biological systems. There is evidence that the polymorph significantly affects biological processes such as cell proliferation[6] and activity[7] and *in vivo* calcium phosphate precipitation.[8]

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The rationale for the investigations presented in this manuscript arose from a recent investigation of the adsorption of chlorhexidine (CHX), a common cationic antimicrobial, to anatase and rutile TiO_2. In that study, we reported that CHX adsorbs rapidly (<60 s) to TiO_2 surfaces, and desorbs gradually over a period of several days. Furthermore, in MES buffer at pH 6, the equilibrium amount of CHX which adsorbs to anatase is approximately twice that which adsorbs to rutile, after normalizing for surface area.[9]

CHX has been used preoperatively as an oral rinse in order to reduce the likelihood of postoperative infection and early implant failure.[10,11] CHX has also been used in the treatment of peri-mucositis and peri-implantitis,[12,13] inflammatory processes affecting the tissues around an osseointegrated implant which, if progressive, can result in implant failure. Although it is difficult to obtain quantitative information regarding its prevalence, it was suggested in one study that peri-implantitis was responsible for between 8 and 50% of all implant failures,[14] although a systematic review of the earlier Brånemark implants drew rather a more conservative conclusion of around 3%.[15] Peri-implantitis is associated

with pathogenic microorganisms[16]; greater numbers, and different species, of microorganisms are found in implant sites affected by peri-implantitis than in healthy implant sites.[17]

The aims of the study presented in this manuscript were to compare bacterial colonization of anatase and rutile TiO_2 surfaces, and to investigate whether functionalization with CHX reduced bacterial colonization. These two polymorphs were selected because they have been observed on the surfaces of modern, commercially available implants.[2] The microbe under investigation was *Streptococcus gordonii*, a common oral microbe and recognized primary colonizer.[18] Growth of bacteria on CHX-treated and untreated TiO_2 surfaces was compared with growth in solution as a function of CHX concentration.

MATERIALS AND METHODS

Preparation of anatase and rutile TiO_2 specimens

Commercially pure, grade 2 titanium in 1 mm thick sheet was cut into 10 × 10 mm pieces using a metal guillotine. Specimens were polished on a Tegrapol 15 polishing machine using 500 grit SiC paper, Struers Largo 9 μm polishing liquid, and Struers DAC 3 μm diamond suspension to give a mirror finish and were cleaned by ultrasonication in 70% ethanol.

Rutile TiO_2 was created on the surface of 20 polished titanium squares by heating to 750°C for 1 h and allowing the specimens to cool to room temperature. Anatase TiO_2 was created on the surface of 20 polished titanium squares using electrochemical anodization. The electrolyte was 0.5*M* H_2SO_4, the cathode was a stainless steel wire and was separated from the anode by 20 mm. Specimens were anodized at room temperature for 60 s at 90 V using a DC power supply, without agitation of the electrolyte. After preparation specimens were rinsed in distilled water and ultrasonicated in 70% ethanol for 15 min at room temperature, then allowed to air-dry.

Characterization of TiO_2 specimens

Raman spectroscopy was used to determine the identity and purity of the TiO_2 polymorphs using a Ramascope 2000 spectrometer with a 633 nm HeNe source which delivers ~3 mW of laser into a ~4 μm area, focused using objective magnification 50×. Before the analysis, the spectrometer was calibrated using a monocrystalline silicon standard specimen. Peak fitting and deconvolution of Raman spectra were performed using GRAMS32 software. Surface roughness (R_a, R_m) was analyzed using a Proscan 2000 white light profilometer. This instrument utilizes a beam of white light, which is incident on the surface to be investigated. The specimen is scanned in a raster movement beneath the beam, and the vertical displacement of the surface with respect to the probe is calculated using a chromatic technique. Thus a map of the surface is generated. For these experiments, areas of 1 × 1 mm were measured on each specimen with a step size (point separation) of 2 μm.

Functionalization with CHX

Ten anatase and 10 rutile specimens were functionalized with CHX prior to immersion in bacterial culture. Specimens were immersed in 100 mg L^{-1} CHX in aqueous solution (pH 5.6) for 60 s, after which time CHX adsorption is believed to be saturated.[9] Specimens were then rinsed by immersing briefly in distilled water and touching to absorbent paper to remove excess liquid. Ten anatase and 10 rutile specimens were not functionalized with CHX, to act as controls.

Preparation of *S. gordonii*

S. gordonii DL1 were grown in screw cap bottles containing BHY medium supplemented with 0.5% Yeast Extract at 37°C, overnight. The bacterial culture was centrifuged and the bacteria suspended in sterile saline and adjusted to $OD_{600} = 0.1$ (~8×10^7 CFU mL^{-1}).

Growth of *S. gordonii* in solutions containing CHX

A total of 50 μL portions of culture were inoculated into 1 mL citrate growth medium containing 0, 0.1, and 10 mg L^{-1} CHX. Citrate growth medium contained 2.86 g L^{-1} sodium citrate dihydrate and 0.06 g L^{-1} citric acid monohydrate and was adjusted to pH 6.8 using KOH.

The cultures were incubated for 6 h at 36°C, and 10 μL aliquots were removed after 0, 1, 2, and 6 h. Each aliquot was serially diluted to 10×, 100×, and 1000× dilutions, and 20 μL portions were spotted onto dry BHY plates and incubated overnight. Colony-forming units were counted to ascertain the effect of 0, 0.1, and 10 mg L^{-1} CHX on bacterial growth in solution.

Growth of *S. gordonii* on titanium specimens

Titanium specimens were placed into the wells of 24-well microtitre plates. About 1-mL citrate growth medium (as described earlier) was added to the wells, followed by 50-μL portions of culture. The specimens were incubated for 6 h at 36°C, then removed from the culture, rinsed briefly in PBS, and immediately prepared for analysis.

Preparation of specimens for scanning electron microscopy

Titanium specimens were immersed in 1% glutaraldehyde solution in phosphate buffered saline at pH 7.4 for

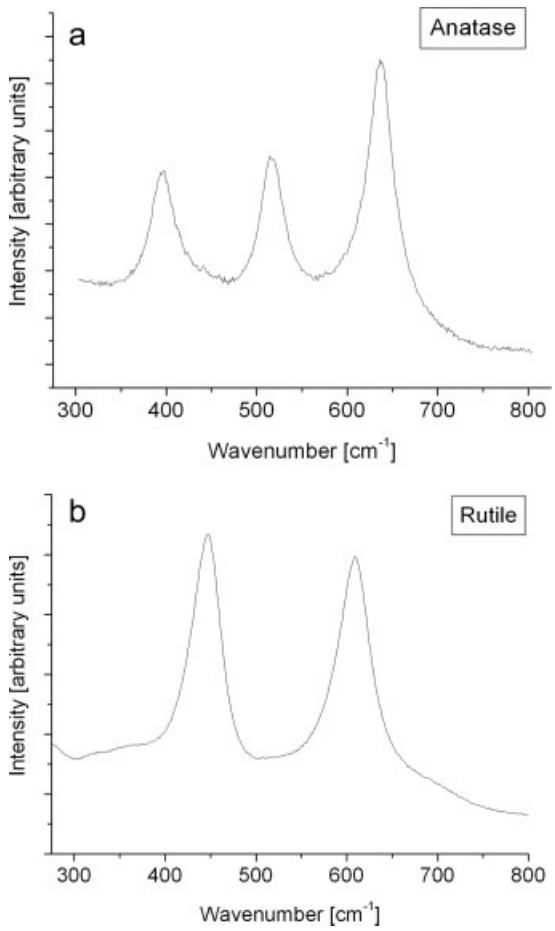

Figure 1. Raman spectra indicating anatase (a) and rutile (b) crystal structures on the TiO_2 surfaces.

1 h, dehydrated by immersing in a graded series of ethanol in water (20, 40, 60, 80, 100% ethanol, 20 min each), and finally immersed in hexamethyldisilazane solution for 45 min and allowed to dry in air overnight.

Scanning electron microscopy and image analysis

A Hitachi S2300 scanning electron microscopy (SEM) was used to obtain images of the titanium specimens and bacteria. Ten images of randomly selected, widely spaced areas were obtained at a magnification of 1500× on each specimen, giving a total of 100 sample areas for each specimen group.

A grid of 20 × 20 squares was overlaid on each SEM image, and each of the 400 squares was scored 0 (less than 50% covered with bacteria) or 1 (more than 50% covered with bacteria) allowing a calculation of the % bacterial coverage to be calculated to an accuracy of 0.25%.

Statistical analysis

The variances of the two sets of roughness (R_a, R_m) data differed by more than a factor of three, and some skewness was observed in the data; thus, a nonparametric Mann-Whitney test was used to compare the medians of the two data sets.

The bacterial coverage data was also not amenable to parametric statistical analysis, since the variances of the different groups varied by more than factor of three and the data were skewed. Therefore, a Kruskal-Wallis test was used to determine whether there were statistically heterogeneous groups, and a box-whisker plot with median notches was used to identify 95% confidence intervals.

RESULTS

The method for creating rutile surfaces was successful and reliable, as indicated by clearly resolved Raman peaks at 446 and 609 cm^{-1} [Fig. 1(b)].[19] That for creating anatase was ~50% successful; only specimens which showed clear, unambiguous peaks at 396, 514, and 636 cm^{-1} characteristic of anatase[19] such as that in Figure 1(a) were accepted and other specimens were discarded. The median values for roughness of the rutile specimens were R_a = 0.137 μm, R_m = 0.672 μm, and that of the anatase specimens was R_a = 0.136 μm, R_m = 0.663 μm. There was no statistically significant difference between the roughness parameters of the two groups at a 95% confidence level (R_a: p = 0.89; R_m: 0.54).

The number of colony-forming units in growth media incubated with 0, 0.1, and 10 mg L^{-1} are shown in Table I. The presence of CHX and either concentration had no clear effect on bacterial growth, with the possible exception of 10 mg L^{-1} CHX after a 6-h incubation.

The median percent bacterial coverage on the four sets of specimens (anatase with/without CHX, rutile with/without CHX) are shown in Figure 2. There were significant differences between all groups ($p < 0.001$) at a 95% confidence level, with bacterial coverage following the pattern: anatase no CHX > rutile no CHX > rutile with CHX > anatase with CHX.

Representative SEM images showing bacteria on the titanium surfaces are shown in Figure 3(a–d). Greater numbers and longer chains of *S. gordonii* can

TABLE I
Colony Forming Units of *S. gordonii* in Solutions Containing Differing Concentrations of Chlorhexidine

	[CHX], mg L^{-1}		
Time, h	0	0.1	10
0	48,100	75,000	49,600
1	2,510	2,090	2,880
2	1,250	670	1,330
6	630	730	40

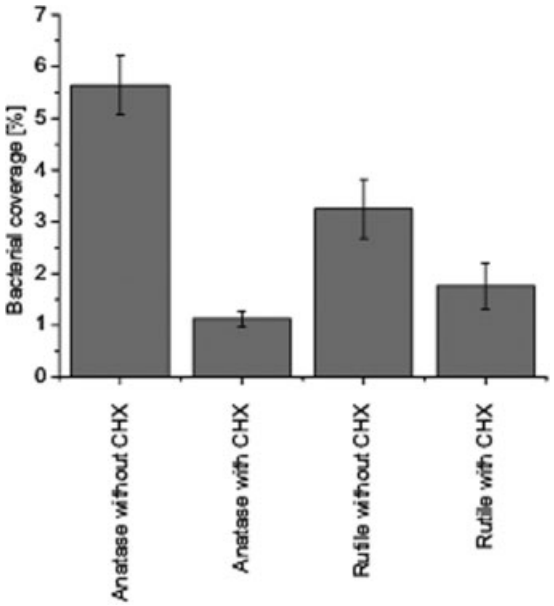

Figure 2. Median % bacterial coverage on anatase and rutile specimens with and without CHX pretreatment. Error bars represent 95% confidence intervals.

be observed on the untreated anatase and rutile specimens compared with the specimens, which were treated with CHX.

DISCUSSION

About 0.1 mg L^{-1} CHX was estimated to be the maximum possible concentration of CHX that would result if all of the CHX adsorbed to the titanium specimen immediately desorbed into the growth medium.

If all of the surface of the titanium specimen is coated with CHX, and if all of the adsorbed CHX immediately desorbs on immersion in aqueous solution (bacterial culture), the maximum concentration of CHX which results in the bacterial culture is given by:

$$[\mathrm{CHX}]_{\max} = (m_{\mathrm{CHX}} \times A_{\mathrm{Ti}})/V_{\mathrm{med}}$$

Where m_{CHX} is the mass CHX adsorbed per unit area TiO_2, A_{Ti} is the surface area of the titanium

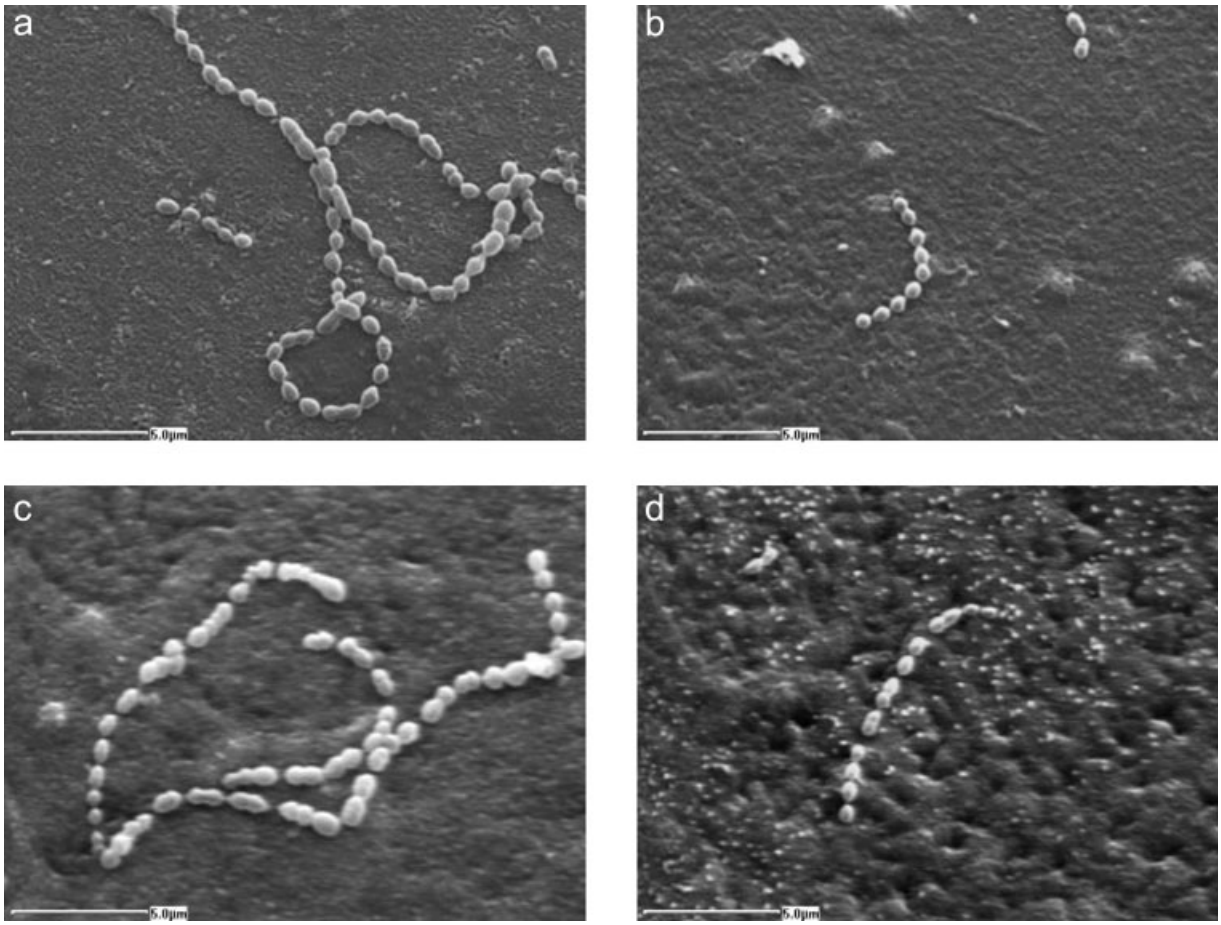

Figure 3. Representative SEM images showing differential coverage of *S. gordonii* on titanium surfaces according to surface crystal structure and CHX functionalization. (a) anatase without CHX; (b) anatase with CHX; (c) rutile without CHX; (d) rutile with CHX.

specimen, and V_{med} is the volume of the bacterial growth medium in which the specimen is immersed. For 100 mg/L CHX, the mass CHX adsorbed to TiO_2 is $\sim$0.4 mg/m^2.[9] The surface area is $2(0.01)^2 + 4(0.001 \times 0.01)$ (the two faces plus the four edges), or 2.4×10^{-4} m^2. The volume of growth medium is 1 mL. Thus $[CHX]_{max} = 0.1$ mg L^{-1}.

The reduced bacterial coverage by *S. gordonii* following treatment of anatase and rutile TiO_2 surfaces with CHX must thus be attributed to the CHX, which is immobilized on the titanium surface, since CHX in solution at a concentration equivalent to that which would result if all the CHX on the surface immediately desorbed, had no effect on bacterial counts.

Functionalization with CHX resulted in a greater proportional reduction in bacterial coverage with anatase specimens (80% reduction) than with rutile specimens (46% reduction). This probably reflects a greater adsorption of CHX to anatase, as observed in the study mentioned earlier, in which it was observed that slightly more than double the CHX adsorbed to anatase compared to rutile.[9] Thus, double the CHX on the surface results in approximately half the bacteria, within experimental errors.

It is important to note that there was a statistically significant difference between bacterial coverage on the two polymorphs without CHX functionalization. Almost twice as many microbes were observed on anatase specimens (5.64% coverage) than on rutile specimens (3.25% coverage). This cannot be simply due to specimen roughness as the two polymorphs had very similar mean roughness values. It may be due to different morphologies of the two surfaces; although R_a values were virtually identical, this does not indicate that the morphologies were necessarily similar. It may, however, indicate that there is an inherent difference between the colonization and growth of microorganisms on these two polymorphs of TiO_2. This may relate to surface energy. It is unlikely to relate to photoactivated antimicrobial activity of the TiO_2, since the specimens were exposed to little UV light during incubation, and furthermore the photoactivity of anatase would be expected to be greater than rutile.

It is interesting to consider the distinction between bacterial colonisation of the surface, and bacterial reproduction on the surface. It can be seen in Figure 3 that on the surfaces which were not functionalized with CHX, the bacteria are predominantly present as long chains of more than 10 cells, whereas on the surfaces, which were functionalized with CHX, the chains are typically much shorter. It is conceivable that bacteria which adhere to the functionalized surface suffer an inhibition of their reproduction resulting in shorter, stunted chains, whereas those which colonies the bare TiO_2 surface are more readily able to form long chains characteristic of streptococcus species.

CONCLUSIONS

Exposing anatase and rutile TiO_2 surfaces to 100 mg/L CHX solution (pH 5.6) for 60 s reduced subsequent coverage by *S. gordonii*. This must be due to CHX immobilized on the surface since an equivalent concentration in solution had no significant effect on bacterial growth. Bacterial coverage was reduced more on anatase surfaces than rutile surfaces; this may reflect a greater adsorption of CHX to anatase than rutile. There was also greater bacterial coverage on untreated anatase than on untreated rutile surfaces; the reasons for this are not currently clear.

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