In Vitro Investigation of the Effect of Oral Bacteria in the Surface Oxidation of Dental Implants

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ABSTRACT

Background: Bacteria are major contributors to the rising number of dental implant failures. Inflammation secondary to bacterial colonization and bacterial biofilm is a major etiological factor associated with early and late implant failure (peri-implantitis). Even though there is a strong association between bacteria and bacterial biofilm and failure of dental implants, their effect on the surface of implants is yet not clear.

Purpose: To develop and establish an in vitro testing methodology to investigate the effect of early planktonic bacterial colonization on the surface of dental implants for a period of 60 days.

Materials and Methods: Commercial dental implants were immersed in bacterial (*Streptococcus mutans* in brain-heart infusion broth) and control (broth only) media. Immersion testing was performed for a period of 60 days. During testing, optical density and pH of immersion media were monitored. The implant surface was surveyed with different microscopy techniques post-immersion. Metal ion release in solution was detected with an electrochemical impedance spectroscopy sensor platform called metal ion electrochemical biosensor (MIEB).

Results: Bacteria grew in the implant-containing medium and provided a sustained acidic environment. Implants immersed in bacterial culture displayed various corrosion features, including surface discoloration, deformation of rough and smooth interfaces, pitting attack, and severe surface rusting. The surface features were confirmed by microscopic techniques, and metal particle generation was detected by the MIEB.

Conclusion: Implant surface oxidation occurred in bacteria-containing medium even at early stages of immersion (2 days). The incremental corrosion resulted in dissolution of metal ions and debris into the testing solution. Dissolution of metal ions and particles in the oral environment can trigger or contribute to the development of peri-implantitis at later stages.

KEY WORDS: bacterial adhesion, biomaterials, implant surface, peri-implantitis

INTRODUCTION

Dental implants are highly successful devices with a 95% success rate and around 500,000 surgeries performed every year.[1,2] Even with the high percentage of success, a large number of implants still fail. Reasons for failure have been generally associated with microbial colonization, peri-implant inflammation, corrosion of materials, implant-abutment design, and implant overload.[3] A large number of failures have been associated with a clinical condition known as peri-implantitis, which is characterized by continuing bone loss around a dental implant in the presence of inflammation.[4] Bacteria are currently assumed to be the primary etiological factor triggering this inflammation.[5]

Previous studies have established a connection between implant failure and presence of bacteria in peri-implant tissues.[6–8] There is evidence from the literature

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showing that the presence of bacterial biofilm is the cause for peri-implantitis, and therefore removing such biofilm systematically should result in the resolution of the condition.[4,9,10] Studies from Mombelli and colleagues (1995) and Quirynen and colleagues (2002) have demonstrated the ability of bacteria to get trapped in the microgaps in the connecting regions of the implant and abutment.[11,12] Both microorganisms and their metabolic products on the surface of dental implants have been shown to induce inflammation.[13,14] Steinbrunner and colleagues (2005) illustrated the leakage of microorganisms through the implant-abutment microgaps and the ability of functional loading to increase microgap leakage.[15] Also, dental implants placed in patients with teeth affected by periodontal disease or with previous history of periodontitis are considered at risk for bacterial biofilm accumulation and peri-implantitis.[16–18] One of the reasons is that the periodontal microflora closely resembles the peri-implantitis microflora.[19] However, there are some marked differences. It has been shown that there is an accentuated increase in other bacteria, specifically *Streptococcus mutans*, around implants with peri-implantitis, not typically seen around teeth with periodontitis.[20] All these studies illustrate the various modalities by which bacteria and bacterial biofilm can accumulate in the peri-implant tissue and the possible role of bacteria in early and late stages of implant failure. So far, it is the inflammatory reaction associated with bacteria that has been most widely discussed. In contrast, the role of bacteria in creating an acidic oral environment and its subsequent effect on the surface of dental implants has not yet been fully investigated.

A relationship between early-/primary-colonizing bacteria, bacterial biofilm, and the ability of bacteria to generate acidic environments, triggering surface oxidation, has been hypothesized in this study. The rationale is based on two different scenarios: (1) contact and adhesion of primary-/early-colonizing planktonic bacteria on surfaces that release lactic acid during glycolysis, thereby decreasing the pH of the oral environment; and (2) biofilm formation on implant surfaces. Bacterial biofilm is suggested to lead to the development of localized crevice environments, which due to differential aeration and oxygen depletion will induce a significant decrease in pH and consequent metal bulk attack. Besides leading to metal particle generation, this condition is hypothesized to disrupt implant osseointegration.[21] Our recent studies involving analysis of implants retrieved after their loss due to peri-implantitis demonstrated the possibility of corrosion and subsequent dissolution of metal ions being triggered by the presence of bacteria.[22] Whether the corrosion products triggered peri-implantitis or the bacteria associated with the inflammatory process triggered corrosion (chemical) and wear (mechanical) is still under investigation.

Considering the clinical importance and the lack of understanding about the effects of bacterial colonization on the corrosion susceptibility of dental implants, the main goal of this study is to develop an in vitro methodology to enable evaluation of the effect of dental implant surface exposure to planktonic bacteria for an extended period of time. The methodology developed enabled the evaluation of the effect of the first scenario: the role of planktonic bacteria on the surface of dental implants. A subsequent study will investigate the effects of a combination of bacterial strains (bacterial biofilm) on the surface of implants subjected to mechanical loading simulating occlusal forces. Furthermore, electrochemical experiments will be performed to evaluate the changes in the surface potential under these conditions. These studies will better elucidate corrosion mechanisms and variation in corrosion kinetics in the oral environment of the patient with dental implants and potentially assist in clinical management of biological complications.

MATERIALS AND METHODS

Routine Bacterial Culturing and Selection of Bacterial Species for Implant Immersion Testing

Bacteria were struck from −80°C freezer stock onto tryptic soy agar plates (BD, Franklin Lakes, NJ, USA) and incubated at 37°C under CO_2-rich microaerophilic conditions (BD GasPak). For pH and implant immersion studies, individual colonies from agar plates were used to inoculate brain-heart infusion (BHI) broth. Uninoculated BHI broth served as a negative control.

In preliminary experiments, several bacterial strains associated with early colonization and peri-implantitis were incubated in BHI medium at 37°C for four days. The pH of the bacterial cultures and uninoculated BHI medium were determined using Hydrion pH strips (Fisher Scientific, Waltham, MA). Table 1 lists the bacterial species tested, along with pH measurement results. The goal of this experiment was to assess the ability of

TABLE 1 List of Bacteria Strains Investigated and pH in BHI Medium Incubated at 37°C

Bacteria	pH levels
Control	7
Escherichia coli	6
Streptococcus mutans UA159	5
Enterococcus faecalis OGIRF	5
Streptococcus sanguinis	5
Streptococcus gordonii DL1.1	5–4
Streptococcus uberis	5
Streptococcus salivarius	5–4

each species to acidify its growth environment. It is evident that almost all bacteria tested were able to produce acidic media. *Streptococcus mutans* was chosen as the bacterial species of interest for immersion studies because it is considered an early colonizer of dental implant bacterial biofilms. Furthermore, increased proportions of *S. mutans* have been found in peri-implantitis compared with healthy implants and to periodontitis.[23]

Preparation of Dental Implants for Immersion

Two sandblasted, large-grit, acid-etched (SLA) dental implants (Straumann USA, Andover, MA, USA) were received for immersion testing. Implant #1 (4.1 × 10 mm) was used for bacterial immersion. Another Straumann dental implant of 4.1 × 16 mm (Implant #2) with the same surface treatment (SLA) was used as the control implant. Both dental implants were cleaned and sterilized according to the following procedure: the implants were (1) immersed in ethanol, (2) ultrasonicated for 1 hour, and (3) air-dried; the samples were subsequently (4) immersed in distilled water, (5) ultrasonicated for another hour, and (6) air-dried. The implants were then stored in separate plastic bags to avoid contamination post-cleaning.

Bacterial Immersion Testing

For immersion testing, a *S. mutans* UA159 colony from tryptic soy agar was inoculated into BHI broth. Two immersion media, *S. mutans* in BHI broth medium and a negative control of uninnoculated BHI (referred to as control), were prepared in two separate test tubes. The total volume of BHI medium in each test tube was 10 mL. The tubes were incubated at 37°C with 100 rpm shaking. Optical density at 600 nm (OD_{600}) was monitored with a spectrophotometer (GENESYS 20, Thermo Scientific, Waltham, MA), and pH was monitored with Hydrion pH strips (Fisher Scientific).

To initiate the implant immersion experiment, the OD_{600} values of culture media were checked after 24 hours' incubation to confirm the bacterial proliferation in the medium inoculated with individual colonies. Dental implants were immersed in ethanol, followed by flame sterilization and cooling. Flame-sterilized dental implants were introduced into the respective bacterial and control BHI media. Figure 1A shows the bacterial immersion setup with implants immersed in bacterial and control media. The length of the immersion testing was set at 60 days. The period of immersion was chosen to simulate exposure of dental implants to early colonizers and to simulate the extent of the healing period. The following procedures were followed at intervals of 2 days throughout the testing period (60 days):

1. Five milliliters of fresh BHI was aliquoted into two separate test tubes. They were marked as "dilution glass tube" and "addition glass tube."
2. BHI aliquoted in the "dilution glass tube" was used as a control (spectrophotometric blank) for that particular day's OD measurement. It was also used as a dilution solvent for OD measurements of the bacterial culture.
3. BHI aliquoted in the "addition glass tube" was used to introduce fresh BHI into bacterial and control media. This was necessary to maintain the media at a constant volume.

For OD_{600} measurements, 1,000 µL of BHI was micropipetted from the dilution glass tube into a cuvette

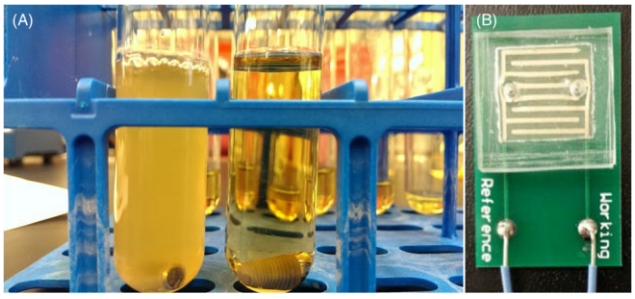

Figure 1 *A*, Dental implants immersed in bacteria (left, Implant #1) and control media (right, Implant #2). *B*, Biosensor used for metal ion detection. The sensor was fabricated at the University of Texas at Dallas in the Biomedical Micro-Devices and Nano-Technology Laboratory.

and used as a blank. Then, 1,000 μL of BHI was micropipetted from the control medium into a cuvette, and the OD was subsequently measured. For the bacterial culture, 750 μL of BHI was micropipetted from a dilution glass tube into a cuvette and 250 μL of bacterial medium was added to produce a 1:4 dilution. The OD was measured subsequently.

For pH monitoring, a pH strip was immersed in a cuvette containing the control medium. For the bacterial culture, 50 μL of bacterial medium was drawn from the test tube, and a few drops were placed on the strip. pH measurements were based on color coding designed by the strip manufacturer.

Aliquots of 1,000 μL of control and bacterial media were collected for metal elution analysis. This was also repeated once in two days, as in previous analyses. Aliquots of 2,000 μL and 1,300 μL of BHI were added into the control and bacterial media, respectively. This step ensured constant volume of bacterial and control media throughout the testing period of 60 days and provided fresh nutrients for bacterial growth. After aliquot media extraction, implant immersion tubes were returned to 37°C incubation with 100 rpm shaking.

Surface Analysis of Dental Implants Post-Immersion Testing

Dental implants were removed from the control and bacteria-containing immersion media, respectively. Implants were then immersed in ethanol, ultrasonicated for 1 hour, and air-dried. The samples were subsequently immersed in distilled water, ultrasonicated for 1 hour, air-dried, and autoclaved.

The surfaces of the dental implant specimens immersed in bacteria and control media were analyzed using different microscopy techniques. Optical microscopy (Keyence VHX-5000, Keyence, Itasca, IL, USA) provided evidence of morphological features on the surfaces of the immersed implants. The microscope employed has a "depth up" feature that can capture the 3D geometry of the implant surface, revealing features such as pits, cracks, and defects on the rough implant surfaces, as well as features on the smooth implant surfaces. Magnifications used ranged from 5× to 1,000×.

Areas of interest identified with digital microscopy were marked and further analyzed using scanning electron microscopy (SEM, JEOL JSM-6010, JEOL, Peabody, MA, USA). The instrument used was equipped with energy-dispersive x-ray spectroscopy (EDS), which enabled analysis of the elemental composition of the surfaces in study. The areas of interest were the junction of the smooth collar and rough surface and the rough surface itself. The smooth/rough junction was a target area because it is the first region to be exposed to bacteria. The rough surface is the region of highest affinity for bacterial cell attachment. These particular areas were surveyed for surface changes that might indicate corrosion, such as discoloration, surface deformation, pitting, scratches, and surface etching.

Titanium Elution Study Post-Immersion Testing

Bacteria-containing and control media aliquots were collected from each immersion testing performed for assessment of titanium elution over time. Media aliquots were collected in microcentrifuge tubes and were sterilized by dry autoclave. The aliquots (of approximately 200 μL) collected during immersion testing were then analyzed using a metal ion electrochemical biosensor (MIEB) platform that detects metal ions based on electrochemical impedance spectroscopy (EIS).

The MIEB measured the electrical impedance in AC steady state; this was accomplished by imposing a small sinusoidal voltage at a particular frequency and measuring the resulting current. The current–voltage ratio gives the impedance. This approach, known as electrochemical impedance spectroscopy (EIS), is used to study a variety of electrochemical phenomena over a wide frequency range.[24] If the impedance of the electrode-solution interface changes when the metal ion is interacting with the sensor surface, EIS is used to detect that impedance change. The impedance of the interface was measured at a single frequency. Impedance measurement does not require special reagents and is amenable to label-free operation.

An illustration of the sensing platform is shown in Figure 1B. The MIEB platform consists of a PCB (printed circuit board) chip with square, interdigitated fabricated gold electrodes. Above the gold electrodes, a layer of poly(dimethysiloxane) (PDMS) was placed to serve as a reservoir for sample analytes. The sensor was connected to the potentiostat (Reference 600 potentiostat, Gamry, Warminster, PA) through wire leads extending from working and reference electrodes as marked in the chip (Figure 1B), and EIS experiments were conducted. The EIS test was performed at a constant peak-to-peak voltage of 10 mV and a frequency of

250 Hz. The impedance of the sample of interest varies with respect to the conductivity of the sample, which is dependent on the concentration of metal ions in the electrolyte (i.e., a decrease in impedance indicates a more conductive medium).

At every chosen experimental time point, the aliquot samples from the control medium were compared to the aliquots obtained from the bacterial medium. The time points chosen for titanium elution analysis were day 0, day 10, and day 22. It should be noted that samples collected within a maximum time window of 22 days were chosen for elution analysis. This was due to the fact that it was the longest time window in which the implants were subjected to immersion without contamination. The bacterial and control immersion media collected were subjected to metal ion detection using the sensor illustrated in Figure 1B. The ratio of change in impedance of the sample medium analyte from baseline was calculated to generate a percentage change in impedance (ΔZ). The percentage change in impedance was calculated according to Equation 1:

$$\%\Delta Z = \left(\frac{Z_{to} - Z_{medium\ aliquot\ at\ time\ t}}{Z_{baseline}} \right) * 100 \qquad (1)$$

where $\%\Delta Z$ is the percentage change in impedance, Z_{to} is the impedance of aliquot medium at day 0, and $Z_{medium\ aliquot\ at\ time\ t}$ is the impedance of the medium aliquot collected at time t.

RESULTS

Optical Density and pH of Bacterial and Control Media

OD_{600} was monitored to confirm bacterial growth in the test culture and the sterility of the control medium throughout the course of the immersion period. OD measurements were performed once every 2 days. The OD_{600} of the bacterial culture was found to be maintained in the range between 0.17(0.68) – 0.26(1.04) for a dilution factor of 1:4, while the OD_{600nm} of the control medium (BHI broth) was maintained close to 0, which indicated no contamination.

In order to understand the type of medium that *S. mutans* generates in its growth environment, the pH of the bacterial medium was monitored through the immersion period and compared with the measurements for the control group. The results revealed striking differences in pH between bacterial and control specimens. Bacterial medium was acidic with a pH value around 5. The control medium was neutral with a pH of approximately 7. The pH values of the immersion solutions were constant through the period of immersion.

Microscopic Analysis of Dental Implants Surface Post-Immersion

When contamination of the control medium was observed during testing, the implants were retrieved from the respective media and resterilized. Such instances served as an opportunity to understand the progressive corrosion caused due to bacteria. Contaminations occurred on day 22, day 44, and day 60. The results provided here reflect the analysis performed on the surface of the implants when they were withdrawn from their respective media after day 22 and day 60. Optical images of the surfaces of the dental implants were taken before immersion. Before immersion, the surfaces of both implant specimens were free of defects or any visible deformation.

Figure 2 shows the surface characterization results after 22 days of immersion. Figure 2A shows the condition of the smooth/rough junction of the implant immersed in control medium (Implant #2). The surface was observed to be pristine, showing the typical manufacturing marks on the smooth collar surface. The border of the smooth collar demonstrated no deformation. Figure 2C shows surface discoloration of the smooth collar area of the implant immersed in bacterial culture (Implant #1). The discoloration was more pronounced in comparison with the appearance of the implant immersed in the control medium (Figure 2A). Another interesting feature of the implant immersed in bacterial medium was the deformation observed at the junction between the smooth collar and rough surface (indicated by arrows). Besides deformation, tiny blue spots were present inside the deformed surface feature. Figure 2B shows 1,000× magnification of the surface of the implant immersed in control medium. The height profile feature of the digital optical microscope was utilized to create a 3D surface map. This feature can provide an approximate measurement of the surface depth and indicate features such as pitting, surface etching, and delamination of the top layers of the surface. The color distribution of the height profile was very uniform (no valleys or peaks), which indicated no regions of deformation. However, the implant immersed

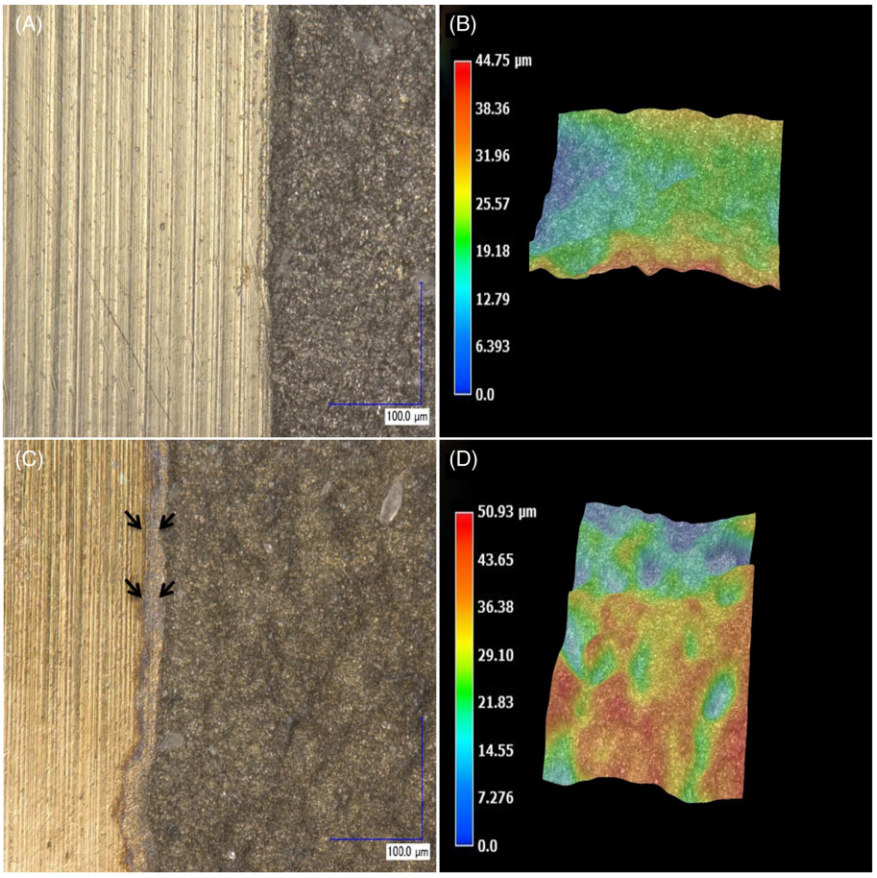

Figure 2 Implant features after 22 days of immersion. (A–B) Control implant: (A) pristine collar-screw transition region of the implant (smooth/rough junction) immersed in control medium; (B) 1,000× 3D height profile of control implant showing uniform profile with no significant deformation. (C–D) Implant immersed in bacterial medium: (C) smooth/rough junction of screw showing yellowish discoloration and deformation of the transition area with blue discoloration; (D) 1,000× 3D height profile of implant immersed in bacterial medium showing pitlike deformations with depth ranging from 25 to 50 µm and an average diameter of 15 µm.

in bacterial medium showed surface deformation, as indicated by the more pronounced changes in the height profile in Figure 2D. The regions with evidence of valleys, or areas with depth, indicate the presence of pitting attack. These features (pits) had an average diameter of 15 µm, and the depth varied between 25 µm and 40 µm.

SEM analysis of the implants retrieved after 22 days of immersion showed characteristic depositions on the surface of the implant immersed in bacterial culture. In some cases, such deposits (in the form of thick black fragments) were seen entrapped in the deformed regions. In Figure 3, A–C, SEM images of the implant immersed in control medium are shown, and in Figure 3, D–F, the surface characteristics of the implant immersed in bacterial culture are shown. The surface of the implant immersed in the control did not experience any significant deformation, as corroborated by the optical microscopy analysis illustrated in Figures 2, A and B. Figure 3B shows small deposits (black dots) on the surface of the implant, which could be a result of dust deposition while fixing the implant in the microscope stage. Figure 3C shows that the bulk of the material is free of defects, deformation, or color change. Figure 3D shows the smooth surface of the implant immersed in bacterial medium, with evidence of deposits on its surface. These deposits could be biological metabolic products from bacteria. There were unsual structures observed on the surface of the implant immersed in bacterial medium. For example, in Figure 3E, these structures are seen as stellate, chainlike features (indicated by arrows). These characteristics may be linked with the response of the bacteria. As shown in Figure 3F, deposition was also found on the rough surface of the implant, which could be related to bacterial metabolic products. EDS analysis of

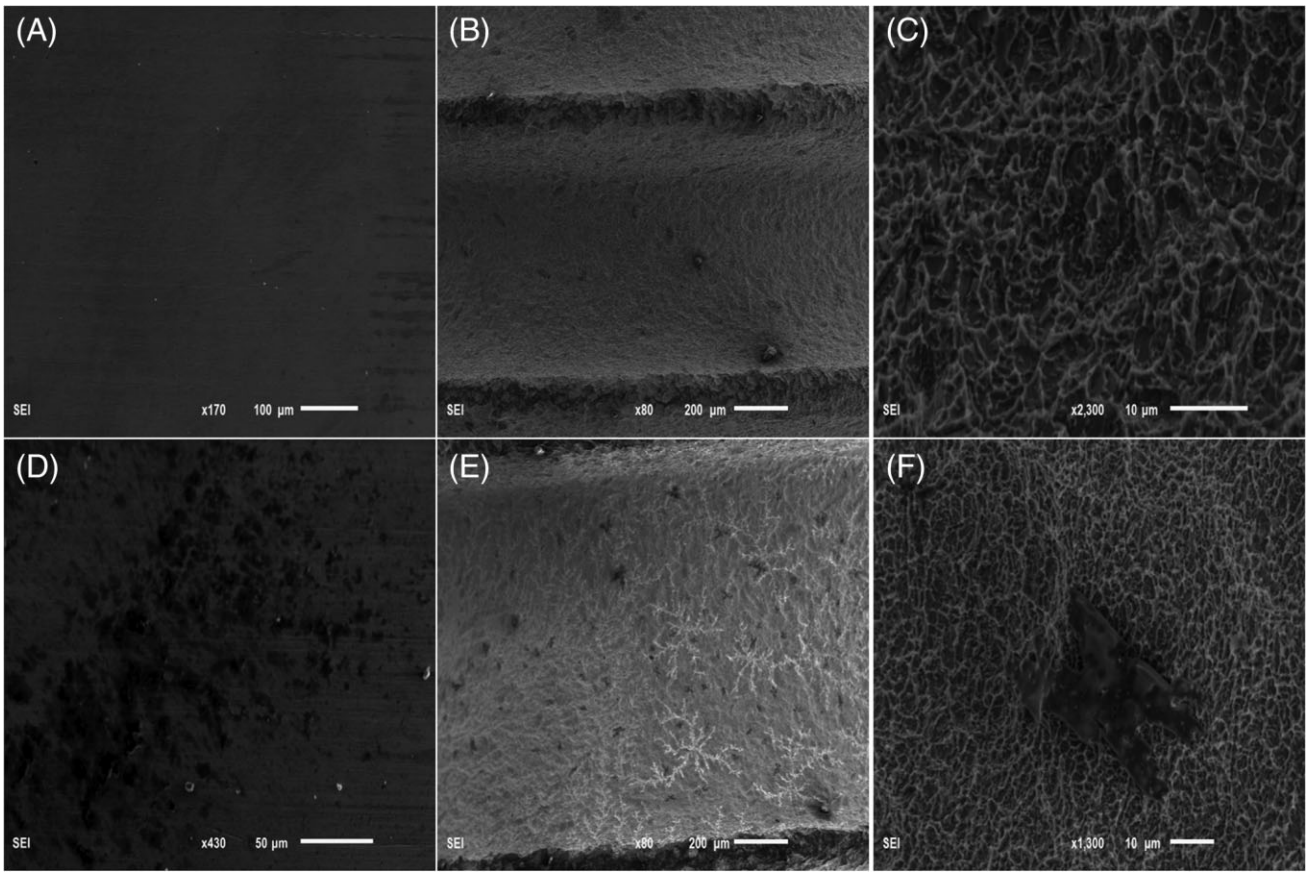

Figure 3 Surface features after 22 days of immersion. (A–C) Control implant: (A) smooth surface of control implant showing no deformation; (B) low magnification (200 μm) of rough surface showing scattered black dots; (C) high magnification of the rough surface showing no areas of apparent deformation. (D–F) Implant immersed in bacteria medium: (D) a clump of black deposits observed on the smooth surface; (E) stellar structures observed on the rough surface, which were only observed when using lower magnifications; (F) high magnification (10 μm) showing black fragment on the rough surface.

implant #1 and implant #2 after 22 days of immersion predominantly showed the presence of three elements in significant quantity. They were Ti, O, and C. Implant #1 showed 65–94% Ti, 8–20% O, and 5–17% C. Implant #2 had 43–75% Ti, 8–24% O, and 2–17% C.

Figure 4 shows the features for the control and bacterial immersion implants after 60 days of immersion. After 60 days of immersion in bacterial medium, the implant revealed more discoloration than when surveyed after 22 days of immersion (Figure 2C). Figure 4A shows the unaffected surface of Implant #2, immersed for 60 days in control. Also, the 3D height profile of the surface at 1,000× did not change significantly in comparison to the profile obtained after immersion of the implant for 22 days in the control medium (Figure 4B). On the other hand, the implant immersed in bacteria showed severe discoloration of the smooth collar and smooth-rough interface, as seen in Figure 4C. A purple-red discoloration feature was prominently present on the surface of the implant. 3D height profile at 1,000× magnification (Figure 4D) showed pits of similar dimensions to those visible on the surface of the same implant after 22 days of immersion. There were instances when the diameter of surface pits became wider (>15 μm) in comparison to the diameter of the pits observed after 22 days of immersion. However, the average depth of these pit-like features remained the same.

Figure 5 illustrates the surface features observed with SEM analysis of the implants after 60 days of immersion in bacteria and control media. In Figure 5, A–C, it is shown that the smooth collar and rough surfaces of the implant had no significant surface deformation even after 60 days of immersion in control solution. On the other hand, scratches could be observed on the smooth surfaces (Figure 5D) of the implant immersed in bacteria. Deposition of dark pigmented byproducts

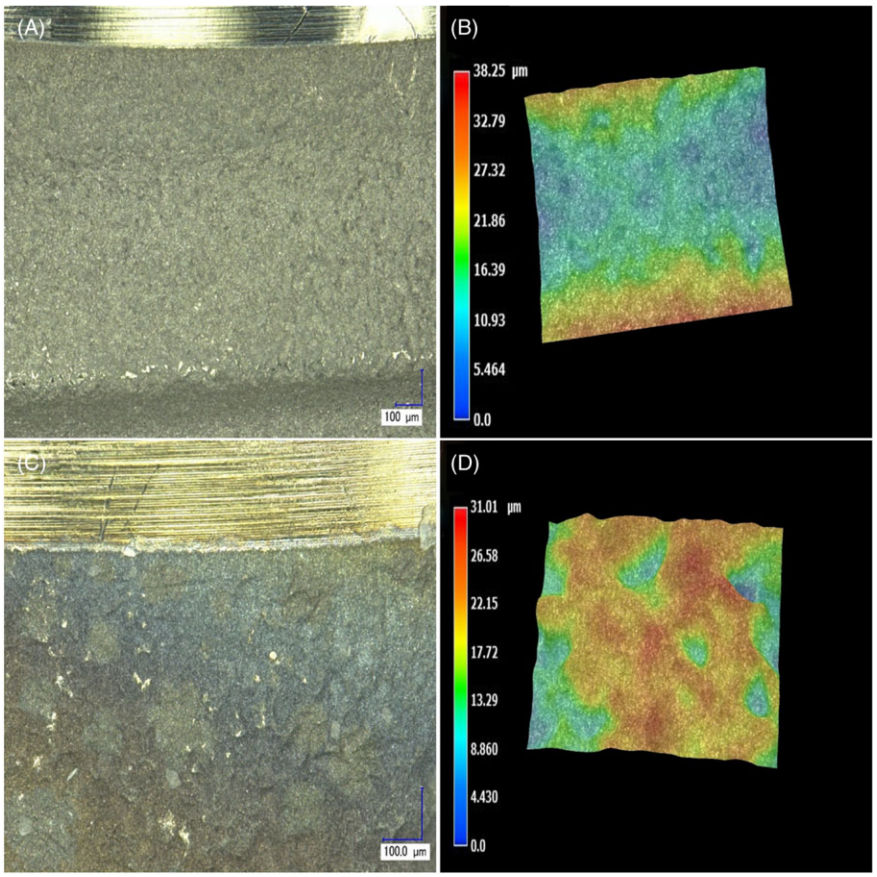

Figure 4 Implant features after 60 days of immersion. (A–B) Control implant: (A) smooth/rough junction of control implant showing more pronounced discoloration compared to control implant imaged after 22 days; (B) 1,000× 3D profile of control implant provided uniform profile distribution. (C–D) Implant immersed in bacteria: (C) smooth/rough junction of implant immersed in bacteria showed a high surface discoloration (blue discoloration); (D) 1,000× 3D profile showing the presence of pitlike deformations on the rough surface.

assumed to be bacterial metabolic secretions were prominent on the rough surface of the sample after 60 days (Figure 5E). A pit-like feature (Figure 5F) was also observed on the rough surface of this implant. Further EDS analysis of both implants showed almost identical results with the analysis performed with the implant immersed for 22 days. Implant #1 had 57–80% Ti, 8–34% O, 4–19% C. Implant #2 consisted of 85–95% Ti, 4–9.53% O, 2–5% C.

It is interesting to note that discoloration became noticeable after 2 days of immersion. Figure 6A illustrates a side-to-side comparison of implant surface appearance after only 2 days of immersion in control and bacterial media. Surface discoloration increased over time to produce rust after 22 days (Figure 6B). At the end of the experimental period of 60 days, the surface of the dental implant immersed in bacterial medium exhibited significant discoloration and corrosion of the rough interfaces.

Titanium Elution Study

Metal ion elution from the surface of implants immersed in control and bacterial media was detected using the biosensor. Samples collected from the media on days 0, 10, and 22 were used for metal ion analysis using this novel sensor platform. Each set of aliquot media, consisting of a control and bacterial media collected at the mentioned time points, was analyzed and compared twice in two separate chips. Figure 7 shows the percentage change in impedance of the sample collected in the bacterial medium with respect to the sample collected in the control medium. The results show that percentage change in impedance is higher in the bacterial medium in comparison

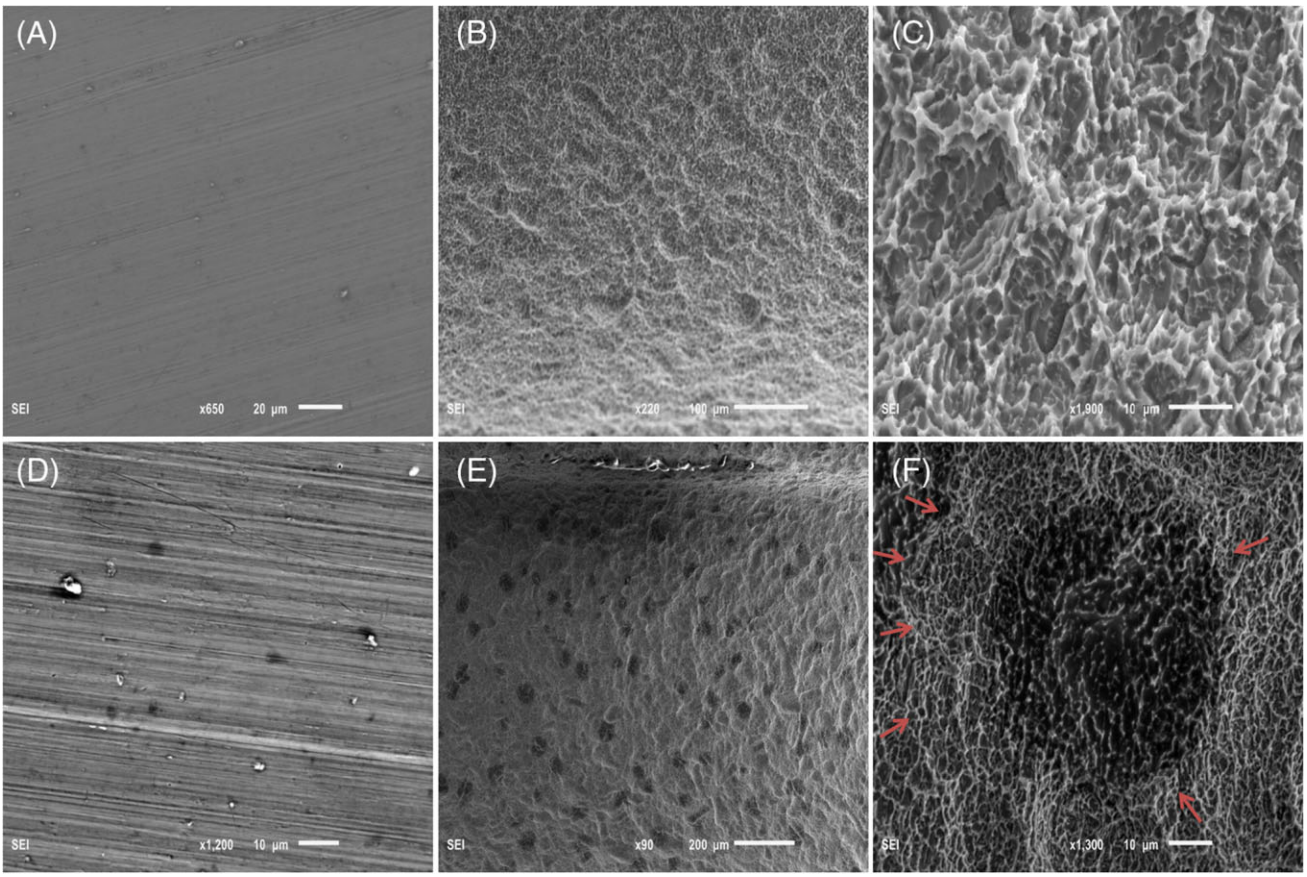

Figure 5 Surface features after 60 days of immersion. (A–C) Control implant: (A) Pristine smooth surface of control implant showing machining marks; (B) Low magnification (100 μm) of rough surface showing no deformation; (C) High magnification of rough surfaces devoid of surface deformations. (D–F) Implant immersed in bacteria medium: (D) smooth surface of bacterial implant with scratches; (E) low magnification (200 μm) of the rough surface showing black spots scattered across the surface; (F) high magnification (10 μm) of the rough surface showing a pitlike feature, which was filled with fluid.

to the control medium at each time point. This shows the increased quantity of metallic particles detected in the bacterial medium in comparison to the control medium. However, the result displayed an inconsistency, as the increase in the percentage change in impedance in comparison with the control did not increase with respect to increasing time.

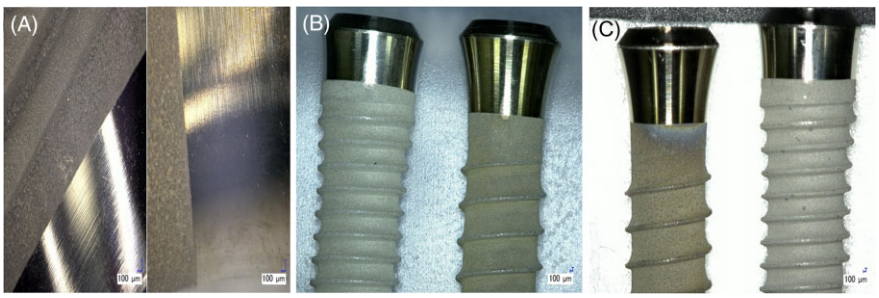

Figure 6 (A–C) Comparison of the morphological features of implants immersed in control and bacterial media over a period of 60 days. (A) surface discoloration evident after immersion in bacteria for 2 days (right); (B) incremental discoloration of implant immersed in bacteria (right) after 22 days; (C) severe discoloration at the smooth/rough junction of the implant immersed in bacteria (left) after 60 days, with rust deposition.

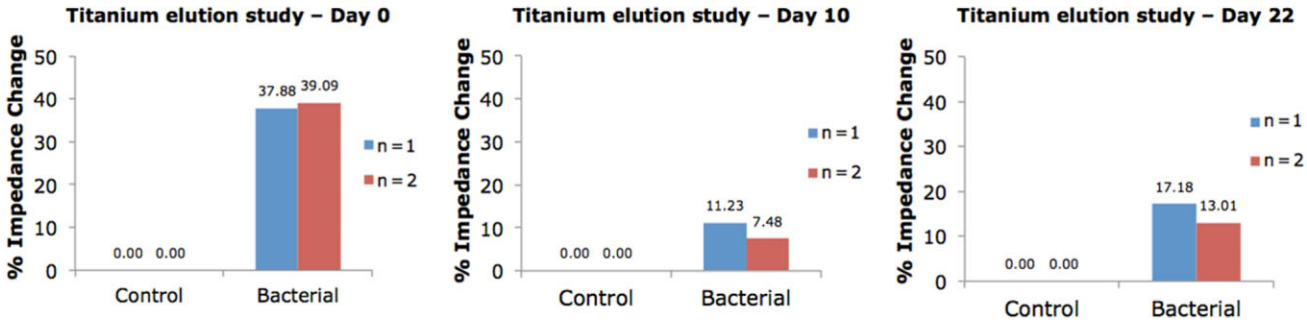

Figure 7 Percentage change in impedance of bacterial medium with respect to control on day 0, day 10, and day 22, where n is the implant number.

DISCUSSION

The main goal of this first study was to develop an in vitro testing methodology to understand and evaluate the role of early-/primary-colonizing planktonic bacteria associated with peri-implantitis on the surface of dental implants using immersion testing in bacteria-containing medium. It was hypothesized that bacteria could create an acidic environment causing breakdown of the Ti oxide layer, which could trigger corrosion and dissolution of metal ions into the oral tissues. Continuous metal ion accumulation in oral tissues at later stages can induce foreign-body reactions, which are speculated to either trigger or accelerate inflammatory processes associated with peri-implantitis and implant failure.[22]

In the first phase of the study, a set of bacteria strains were analysed. Bacteria tested included early-/primary-colonizing bacteria such as *Escherichia coli, Streptococcus mutans, Enterobacterium faecalis, S. sanguinis, S. gordonii, S. uberis*, and *S. salivarius*[25] and bacteria associated wih peri-implantitis such as *Acinetobacter actinomycetemcomitans* and *S. mutans.*[20] All these bacteria are either aerobic or facultative anaerobes that can release organic acids and fit into the first scenario of the hypothesis of this study. All these bacterial strains were investigated for the ability to lower the pH and grow for extended periods of time. It was evident from analyzing the pH of the bacterial medium (Table 1) that most of the strains investigated were able to reduce the pH of their growing medium. This can be attributed to the ability of bacteria to release organic acids during their energy catabolism.[26] The ability of bacteria to grow for an extensive period of time was also taken into consideration. *A. actinomycetecomitans* and *S. gordonii* grew as clumps in the culture medium and were not able to provide a reliable optical density to work with for a long period of time. *S. mutans* was chosen as the strain of interest for the immersion testing. This was due to the fact that *S. mutans* was better able to provide an acidic environment, reliable growth, and viability over a long period of time in relation to the other strains cultured. The testing methodology developed in this initial study will enable investigation of other strains of interest in future studies. The range of OD readings obtained proved that the testing methodology was able to keep bacteria viable and the control sterile throughout the course of the experiment. The bacteria created and sustained an acidic oral environment for the immersed implant during the entire duration of the experiment. There were a few instances when the control medium became contaminated. When contamination was detected, the test was immediately interrupted. Implants immersed in bacteria and control media were withdrawn from the respective media. After resterilization, the implants were reused and introduced into a fresh set of bacteria and control media to repeat the test. In another instance of contamination, the medium was observed to become contaminated at day 22. With this particular sample, the test was resumed from day 22. Other contamination cases were observed to occur at day 44 and day 60. With these last cases, the implants were withdrawn from the respective media, and testing was continued post-resterilization. In all these instances, sterilization did not have any effect on the surface of dental implants. This was quite evident from the microscopic images from the discoloration pattern of the implant immersed in bacteria and the pristine quality of the implant immersed in control medium.

At present, bacteria and bacterial biofilm are assumed to be the vital etiological factors for early implant failures and peri-implantitis.[4,5,11,21] The bacteria

inducing inflammatory processes are hypothesized to attack the surface of the metal by changing the electrochemical environment of the implant. However, the role of the acidic environment that can be created by bacteria has not been widely discussed as one of the factors causing implant corrosion and metal release. This study showed that primary-colonizing planktonic bacteria can effectively create the conditions needed to trigger surface changes and corrosion. Microscopic evaluation of the surface of the implants immersed in both control and bacterial media demonstrated several features that confirmed the initial hypothesis of the study. Discoloration of the surface was observed even after only two days of immersion in bacterial medium (Figure 6A). This shows that surface changes can start at early stages in bacteria-containing medium. In a previous retrospective retrieval analysis of dental implants from patients with peri-implantitis, one of the implants failed just after 3 weeks of implantation. That implant had clear signs of tribocorrosion (combined effect of wear and corrosion) with striking discoloration and delamination of the top surface layers.[22] Pontoreiro and colleagues (1994) showed the ability of bacterial plaques to cause inflammation over a period of 3 months.[5] Changed electrochemistry induced by bacteria may be considered as one of the possible causes behind early failures.

Further microscopic analysis using a combination of SEM and optical microscopy demonstrated other instances and areas of the implants with yellow/blue discoloration and pit-like deformations. The results showed that 22 days of immersion in bacteria-containing medium resulted in surface corrosion features like microscopic deformation and discoloration at the smooth-rough interface (Figure 2C) and mild rusting along the surface of the implant (Figure 6B). Surface profiles demonstrated pitting deformations with peaks and valleys (Figure 2D). Corrosion was demonstrated to be intensified with immersion time. Figure 4C showed macroscopic discoloration features on the surface after 60 days of immersion. The surface profile demonstrated pitlike deformations (Figure 4D) similar to the case shown in Figure 2D. In addition, "peaks and valleys" pit-like features observed on the surface of the implant after 60 days seemed to be filled with biological products (Figure 5F). The presence of biological matter on the surface of the implant after 60 days of immersion was more pronounced, even visible in low-magnification images (Figure 5E), compared with the surface of the implant immersed for 22 days. Detection of carbon content in the EDS analysis confirmed that the materials were biological. However, the incremental surface oxidation of the implant immersed in bacteria over time was clearly explained by surface discoloration. In all these cases, surface discoloration is associated with an oxidized form of titanium ion (Ti^{3+}/Ti^{4+}).[22,27] This surface oxidation results from highly acidic electrochemical attack. Reducing acids have the ability to oxidize bulk Ti to the trivalent Ti^{3+} state, which provides a violet color and, on further exposure to acidic conditions, can be converted to the pale yellow Ti^{4+} ion. Sartori and colleagues (2009) noticed the presence of black spots and stains on SEM images of the surface of Ti subjected to highly acidic solutions.[28] Similar features were evident in the SEM images (Figure 3D and Figure 5D) of the implant immersed in bacteria.

Pitting attack is a localized form of corrosion that nucleates cavities in the bulk of the material.[27] The "peak and valley" pit-like deformation features observed with the immersed implants validated one of the possible corrosion mechanisms hypothesized in the retrieval analysis.[22] Bhola and colleagues (2011) stated that TiO_2 provides the necessary protection of dental implant surfaces against pitting corrosion.[29] However, the EDS results obtained in this study corroborated damage of the oxide layer, with a high percentage of titanium (60–90%) and lower percentage of oxygen (0–20%) in areas with apparent corrosion features. These results reveal that the bulk titanium (high Ti concentration) was exposed to the action of the acidic environment upon corrosion of the protective oxide layer (low oxygen concentration).

As discussed, surface corrosion of dental implants will lead to dissolution of metal ions. In order to quantify metal ion dissolution in the testing solutions, samples from bacterial and control media were collected and analyzed for Ti elution using the EIS sensor chip illustrated in Figure 1B. The sensor was able to detect the presence of more metallic ions in the bacteria-containing medium compared to the control (Figure 7). This is demonstrated by a decrease in impedance with respect to the control at each time point. However, the percentage change in impedance for aliquots from bacteria-containing medium was not consistently increasing with respect to time. This suggests that bacterial colonies might have interfered with the sensor

measurements. Nevertheless, the presence of metal ions detected by the sensor demonstrated the elution of Ti in an acidic medium. The elution of titanium has been previously observed in in vitro and in vivo studies.[30] Kumazawa and colleagues (2002) illustrated the ability of leached Ti particles to create cytotoxic responses in vivo.[31] Retrieval analysis of modular orthopedic implants have also elucidated the role of dissolution of metal ions in osteolysis and subsequent implant failure.[27] Currently, corrosion is considered as one of the secondary triggering factors for peri-implantitis.[26] Bhola and colleagues (2011) and Mohyi and colleagues (2012) questioned the alleged role of Ti particle release in inducing peri-implantitis.[29,32] In a retrieval analysis of dental implants from patients with peri-implantitis, Rodrigues and colleagues (2013) observed tribo-corrosion features such as discoloration, pitting, surface, etching, fatigue cracking, scratches, and surface delamination that suggested dissolution of large amounts of metal ions into the oral environment.[22] It can be noted that similar corrosion features were observed in this study with the exception of fatigue cracking, scratches, and surface delamination, which are associated with mechanically assisted wear. Hence, early surface damage can be accelerated by late-stage functional loading.

In this study, it was demonstrated that early-colonizing bacteria associated with peri-implantitis are able to create the ideal conditions to trigger corrosion of the surface of dental implants, which leads to dissolution of metal ions in the surrounding medium. In this study, the effect of an electrochemical environment alone was evaluated. Nonetheless, in the oral environment, occlusal loading could contribute to the acceleration of corrosion, which would lead to even more severe surface deformation, wear, and accelerated release of Ti metal ions/particles. Whether this condition (oxidation and dissolution of metal ions) is sustained in vivo will depend on the loading environment and stability of an implant. The interplay between the mechanical scenario and the response by the bacteria will be explored in future studies. However, this initial investigation confirmed the hypothesis that early-colonizing planktonic bacteria associated with peri-implantis are able to lower the pH to the levels needed to trigger corrosion. Therefore, it was illustrated in this study that presence of bacteria will change the electrochemical environment surrounding a dental implant.

Some of the limitations of this study included instances of contamination, which required interruption of the experiments. To better prevent contamination and contact of the implants with open environments during extraction of medium aliquots for testing, a chamber is currently being designed that will enable collection of metered amounts of extracts without exposing the testing implant. Also, another limitation was that the study was based on a more qualitative assessment of the surface. However, as the goal was to maintain the implants immersed in bacteria contaning-media for extended periods of time, this study primarely focused on reporting the features observed on the surface. A subsequent study will investigate and report the synergistic effect of bacteria-containing medium and mechanical loads on the implant surface and changes in surface potential.

CONCLUSION

A testing methodology was developed to study the role of oral bacteria on the surface of dental implants. Bacteria are considered primary triggering factors for inflammatory reactions. This study illustrates the role of early-colonizing bacteria associated with peri-implantitis in creating an acidic environment. The acidic environment can propagate a chemical attack on the surface of a dental implant, leading to metal ion dissolution. Metal ion dissolution was enhanced when the implant was immersed in bacteria-containing medium. Metal ion detection was done through a novel electrochemical biosensor. From this study, it was observed that chemical attack can be rapid, with surface changes being experienced as early as day 2 of immersion. These findings demonstrate that bacteria can induce changes of the dental implant surfaces, leading to dissolution of metal debris even during early healing stages. The progressive accumulation of metal wear in the oral environment, accelerated by mechanical factors at later stages, could be related to inflammatory responses or foreign-body reactions, which can lead to loss of osseointegration and implant failure. Future investigation is warranted to elucidate possible mechanisms to prevent and to treat dental implant surface corrosion.

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