SURFACE MODIFICATION OF ORTHODONTIC MINI-IMPLANTS WITH H_2O_2 GEL FOR ITS ANTIADHERENT AND ANTIBACTERIAL PROPERITES IN THE PRESENCE OF *STREPTOCOCCUS MUTANS*

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ABSTRACT

Widespread applications have made the use of mini-implants common throughout orthodontic treatment. The mini-implant provides an osseous anchor that is easily inserted, minimally invasive, and easily removed. The failure rate of mini-implants is estimated around 15%; this is high when juxtaposed with an endosseous implant. The majority of failures occur early; this results from a weakened bone-implant interface usually accompanied by soft tissue inflammation. This inflammation is exacerbated by poor oral hygiene and in areas of non-keratinized tissue, which provides an inadequate soft tissue barrier to bacterial challenges. *Streptococcus mutans* is involved in initial biofilm formation and has been implicated in endosseous peri-implantitis in the absence of periodontal disease. *S. mutans* is acidogenic, and has been shown to negatively affect the topography of titanium surfaces. Mini-implants are mainly comprised of titanium alloys due to their biocompatibility and mechanical strength. Literature suggests that enhancing the native TiO_2 on titanium surfaces in the presence of UV light improves osseointegration, increases antibacterial effects, and increases soft tissue adhesion. The goal of this study was to enhance the TiO_2 surface of mini-implants and evaluate the resultant anti-adherent, antibacterial, and topographical changes in the presence of *S. mutans*.

Orthodontic mini-implants compromised of Ti6I4V, (Wrought Titanium-6 Aluminum 4 Vanadium ELI), were modified according to the parameters set by Unosson *et al*, 2015. These samples were then exposed to *Streptococcus mutans* and incubated at 37° C. Initial contact of viable bacteria was evaluated after 4 hours using resazurin dye that was measured on a fluorescence plate reader. Bacterial growth was evaluated at 4, 8,

and 24 hours using a spectrometer to assess turbidity. Four samples were also plated to evaluate growth. SEM images were taken prior to and after treatment to assess topographical changes. ANOVA and pair-wise post-hoc tests were used to analyze the data.

The amount of viable bacteria on modified mini-implant surfaces after 4 hours was significantly decreased when compared to controls ($p< 0.004$). Growth of S. Mutans on the modified surface after 24 hours was significantly less when assessed by spectroscopy ($p<0.00083$).

Preliminary results show that modifying the surface of orthodontic mini-implants with H_2O_2 gel increases antibacterial properties against *Streptococcus mutans*, despite the lack of UV radiation.

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CHAPTER 1
INTRODUCTION

Technological advancements have streamlined orthodontic treatment, providing more proficient patient-centered care. Yet, the guidelines and principles employed have changed little since their conception. Two long withstanding goals of orthodontic therapy include maximizing anchorage potential and preventing unwanted tooth movement. The concept of skeletal anchorage was first explored in conjunction with endosseous dental implants. However, both time and spatial constraints limited the applicability of this treatment. The advent of mini-implants provided clinicians with an osseous anchor that was comparatively smaller, easier to place, minimally invasive, and less expensive. Additionally, these implants enhanced treatment capabilities when addressing open bites, molar extrusion, and space closure all the while eliminating the need for patient compliance (Costello et al, 2010). Widespread applications and increasing use warrant investigation into optimizing treatment with mini-implants. The failure rate of mini-implants has been estimated between 6.6% -16.1% (Antoszewska et al, 2009; Schatzle et al, 2009), which is significantly higher than endosseous dental implants. More current research lends to an overall success rate of 85.8% and a 1-year survival rate of 81.6% (Tsai et al, 2016).

The etiology of this high failure rate is unclear and aims to rectify it are limited. Individual variation likely plays a role in mini-implant success, which is multifactorial in nature. From a chronological perspective, early failures result form a lack of primary stability; this implies an inadequate mechanical interdigitation between the implant surface and the osseous tissue. Later staged failures are attributed to insufficient

maintenance of this interface, most likely the result of a host driven response (Suk Lee et al, 2007). Numerous variables have been implicated in affecting failure rates and include: implant diameter, length, thread design, site of placement, tissue consistency, and force applied (Lee et al., 2007). However, one consistent observation is inflammation of the soft tissue surrounding failed mini-implants (Chaddad, et al, 2008; Chen, et al, 2008, Cheng, et al, 2004; Miyawaki, et al, 2003). The source of this inflammation likely has a bacterial component, which may be exacerbated by poor oral hygiene and an inadequate soft tissue barrier (Park, et al, 2006).

Endosseous dental implants provide a platform from which principles can be applied to mini-implants. The presence of periodontal pathogens has been conclusively linked to peri-implantitis, which results in destruction of osseous tissue, loss of osseointegration, and eventual failure in endosseous implants (Lee, et al, 2010). However, studies examining the presence of periodontal pathogens on mini-implant surfaces found no conclusive data or associations (Apel et al, 2009; Tortamano et al, 2012). This is not unexpected given the following two facts: peri-implantitis develops slowly often years after implant placement and mini-implants are usually only utilized for several months. Recent studies have demonstrated partial osseointegration around mini-implants, evidenced by deposition of new bone, despite their short use (Vannet et al, 2007). A more prudent approach is to consider early endosseous implant failures; they provide a more comparable treatment time. These early losses are associated with a failure to osseointegrate; this has been linked to the initial bacterial biofilm formation and release of metabolic byproducts (Sridhar et al, 2015). Of particular interest, is *Streptococcus mutans* an early colonizer and a species that has been implicated in peri-implantitis.

Studies have shown that *S. mutans* is critical in initial biofilm formation and has been found around implants with peri-implantitis and is not found on periodontally involved teeth (Kumar et al, 2011). This sheds light on the potential interaction between titanium implants and *S. mutans*. Bacterial biofilm formation has been demonstrated on mini-implants, with *S. mutans* being a major contributor to initial formation. Exploration of host responses to this bacterial challenge and eliminating it are credible research endeavors. Alterations of the mini-implant surface have promising potential to do just that.

The majority of mini-implants are comprised of a titanium alloy due to its biocompatibility and mechanical strength. These properties are attributed to a TiO_2 layer that forms spontaneously in the presence of air. However this film is relatively thin (Unosson et al, 2015). Enhancing this protective layer is the subject of numerous surface modification studies. TiO_2 nanotubules have been incorporated on mini-implant surfaces and haven shown increased ossteointegrative potential, namely amplified bone-implant contact (Jang et al, 2015). *In vitro* studies with TiO_2 modified implants revealed the following: an enhanced soft tissue seal due to greater oral mucosa-implant contact without any clinical adverse reactions (Wennerberg et al, 2011). Applications are not limited to dental materials, as anatase TiO_2 coatings have shown reductions in bacterial growth in joint athroplasty implants (Haenle et al, 2011).

Limiting bacterial adherence is critical, especially when titanium surface interactions with *S. mutans* are considered. *Streptococcus mutans* is acidogenic, mostly due to the production of lactic acid as a byproduct of glycolysis. Sridhar *et al* (2015) found this acidity to negatively affect the surface topography of titanium implants. The

formation of pits and surface irregularities lead to the accumulation of bacterial proteins and an enhanced structural site for biofilm advancement. Additionally, this acidity released metal ions from the titanium surface; metallic ions are triggers of a cytotoxic inflammatory response. Additionally, the generation of an acidic environment may also be negatively modulated by the presence of F- containing products. Barrak et al (2015), demonstrated titanium surface pitting, indicative of corrosion when titanium was exposed to F- gel and *S. mutans*.

Generating a cost effective and standardized method for generating a TiO_2 surface on mini-implants has numerous potential benefits. By utilizing a method developed by Unosson *et al* (2015), mini implants were submersed in H_2O_2, aged in H_2O, and an anatase TiO_2 coating was formed. Immersion of these modified mini-implants in *S. mutans* allowed for both antiadherent and antibacterial testing. Microscopic images taken prior to and after the addition of *S. mutans*, provides insight on topographical titanium changes. Until this point, no such methods were used in combination with mini-implants. Given the high failure rate, improvements in mini-implant design are merited. The aim of this study was to test the antibacterial properties of a TiO_2 generated surface, and to examine microscopic surface changes on these modified mini-implants in the presence of *S. mutans*.

CHAPTER 2
REVIEW OF THE LITERATURE

2.1 Identification and Etiologies of Mini-Implant Failures

Prior to identifying the etiologies of failed orthodontic mini-implants, it is imperative to define what constitutes said failure. Generalized parameters for implant success include lack of soft tissue inflammation, absence of measurable mobility, anchorage resistance of immobilized segments, and maintenance throughout the duration of treatment (Antoszeweksa et al., 2009). However, a range of clinical acceptability exists for each. Soft tissue inflammation and proliferation that can be managed by excision or debridement do not necessarily constitute a failure, but rather a consequence of treatment (Reynders, et al., 2009). Similarly, mini-implants may initially display or develop detectable mobility but still provide the intended anchorage (Reynders, et al., 2009; Pickard, et al, 2010). Unlike endosseous dental implants, mini implants do not usually osseointegrate and typically rely on mechanical retention for stability (Reynders, et al., 2009; Seon, et al., 2008). Computed tomography suggests that this retention is not absolute and that a negligible amount of tipping and extrusion of the mini- implant does occur during loading (Garg, et al., 2015). These movements may contribute to clinically observed implant mobility. Irrefutable failures include fracture of the mini-implant upon placement and unintentional removal of the mini-implant during treatment. Collectively, these aforementioned failures contribute to the relatively low success rate of mini implants when compared to conventional dental implants. Literature estimates the success rate to be between 93.4% -83.9% (Antoszewska et al, 2009; Schatzle et al, 2009), while endosseous implant success is reported between 98.1- 96.4 (Manor et al, 2009).

Numerous potential causes of mini-implant failure have been suggested, however further investigation is still warranted. Proposed etiologies can be broadly categorized into the following four categories: implant-related, patient- related, location-related, and orthodontic related (Antoszeweksa et al., 2009). Patient- related factors are subdivided to include two environmental- management factors: oral hygiene and inflammation (Park, et al, 2006). Additionally, failure of the mini-implant can be cataloged chronologically as either an early (short-term) or later (long-term) failure (Lee et al., 2007).

Orthodontic mini-implants vary by the following features: material, design, length, thread shape, diameter, and placement method (Lee et al., 2007). Variants of each factor have been shown to both improve and impair mini-implant success (Pickard, et al, 2010; Pithon, et al 2012; Zhang et al, 2011,). The importance of primary stability cannot be understated, as the majority of mini-implant failures occur after initial placement (Miyawaki, et al, 2003; Wu, T. et al, 2009). Primary stability is best assessed, by quantifying the force necessary to extract mini-implants from osseous tissue (Pithon, et al, 2012). Cylindrical shaped implants showed better primary stability than conical shaped implants, due to greater osseous surface area contact. Primary stability was also shown to be greater in implants with increasing screw length and in areas with increasing cortical bone thickness. This supports studies that have shown greater long- term stability in the maxilla in comparison to the mandible (Zhang, et al, 2011). Although primary stability may be initially greater in the mandible, due to increased cortical bone thickness, healing is enhanced in the maxilla and contributes to superior long- term stability (Lim, et al, 2009; Zhang, et al, 2011).

Additionally, inflammation surrounding the mini-implant adversely affects primary stability (Chaddad, et al, 2008; Chen, et al, 2008, Cheng, et al, 2004; Miyawaki, et al, 2003). After quantifying the success of 134 titanium mini-screws, inflammation of the peri-implant tissue, was correlated with mobility and eventual failure (Miyawaki, et al, 2003). All other factors (screw length, placement surgery, immediate loading, location of implantation, age, gender, crowding of teeth, anteroposterior relationship, controlled periodontitis, and TMJ disorders) except a high mandibular plane angle were not found to be associated with increased failure. Similarly, in a retrospective evaluation of 492 temporary anchorage devices (miniplates, pre-drilling miniscrews, and self-drilling miniscrews), an increased failure rate was statistically associated with local inflammation of the surrounding tissue and immediate loading of the implanted device (Chen, et al, 2008). This correlation suggests, that inflammation may contribute to the destruction of osseous tissue and weaken the mechanical interdigitation of the mini-screw.

The cause of peri-implant tissue inflammation has been debated extensively. Proposed sources of the inflammation include the following: bacterial (Cheng, et al, 2004; Sato, et al, 2006; Tseng, et al, 2006), mobility associated irritation from lack of keratinized tissue (Cheng, et, al, 2004; Lim, et all, 2009), and poor oral hygiene in combination with a bacterial infection (Park, et al, 2006; Wu, et al, 2009).

In a prospective study, the success of 140 mini-implants used for intrusion, uprighting, and protraction of teeth were assessed by various parameters (Cheng, et al, 2004). Peri-implant infection, defined as persistent pain and swelling of the surrounding tissue, was found in 7 cases. Of these failures, 71% were associated with mobility and

were unresponsive to increased oral hygiene measures. The inability to control the inflammation by removal of plaque, suggests that it was bacterial in origin.

In a similar study, forty-five mini-implants were evaluated for the duration of orthodontic treatment (Tseng, et al, 2006). In total, there were four mini-implant failures, categorized by mobility or persistent infection. Half of the failed implants presented with persistent inflammation that did not subside with debridement of the area.

In a slightly different approach, the microflora found in crevices surrounding titanium orthodontic anchor plates was analyzed (Sato, et al, 2006). Gingival crevices that displayed inflammation were found to have predominately gram-negative anaerobic bacterial strains. Inflammation was identified as one or more of the following: swelling, pus, discharge, and or spontaneous pain. In comparison, healthy gingival crevices supported mainly aerobic bacterial growth. This stark comparison, suggested that the microflora present in the transmucosal pocket was comparable to a periodontal pocket and responsible for the observed inflammation. However, the inherent design of an anchor plate does differ comparably to that of a single mini screw (Costello, et al, 2010). The initial incision and placement of a flap create a soft tissue environment that differs from a single mini-implant (Costello, et al, 2010; Sato, et al, 2006).

Inflammation surrounding the mini-implant has also been attributed to the tissue type in which the mini-implant is placed (Lim, et al, 2009). In a retrospective study, 378 mini-implants were evaluated for the following variables: patient factors, mini-screw factors, and doctor factors. It was found that firm keratinized tissue had a 2.7% higher success rate when compared to mini-implants placed in movable mucosa. This compliments other studies that report a decreased success rate in the mandibular movable

mucosa and an increased success rate in the keratinized midpalatal area (Lim, et al, 2009; Kim, et al, 2010).

As mentioned previously, oral hygiene or lack thereof has been linked to increased mini-implant inflammation and failure (Park, et al, 2006; Wu, et al, 2009). In a study of 414 mini-implants, those placed in the left side of the mouth had a 7.2% lower failure rate when compared to the right side (Wu, et al, 2009). All clinicians placing the mini-implants were right handed and thus this disparity was attributed to patient error. Namely, it was assumed that the majority of the 166 patients sampled, was right handed and thus exhibited superior hygiene measures on the left side. This concept is not new and studies comparing right and left-handedness have demonstrated similar results in terms of oral hygiene (Tezel, et al, 2001).

Although the precise cause of mini-implant failure remains unclear, it is quite apparent that multiple factors and individual variation affect success rates. A more plausible theory threads the aforesaid variables into a cohesive and interrelated unit, instead of treating them as distinct entities. Failure rates are increased in areas of inflammation, which is attributed to damage of the osseous and soft tissues surrounding the implant (Park, et al, 2006). It has been suggested that this inflammation is exacerbated and enhanced by poor oral hygiene measures and by weak nonkeratinized tissue. The firmer keratinized tissues may provide a more secure barrier around the mini-implant and thwart infection as well as inflammation.

The relatively low success rate of mini-implants and the ambiguity surrounding the etiologies is not unwarranted. More research and elucidation on the topic and clarification in methodology will come with increasing research efforts.

2.2 Oral Microbiological Interactions on Implant Surfaces

Any device placed in the oral cavity, be it an implant, fixed crown, or orthodontic appliance is subject to a diverse microbiological environment. Furthermore, these devices provide novel substrates for bacterial adherence and growth.

Endosseous dental implants have a well-studied and defined interaction with oral tissues and oral flora. Given the inherent structural and material similarities, endosseous implants provide a microbiological model for mini-implants. Upon insertion in the oral cavity, a dental pellicle comprised of salivary proteins and bacteria forms on dental implants (Lee, et al, 2010). This pellicle provides a platform for future bacterial growth. Initial colonizers of this pellicle are heavily compromised of facultative anaerobes mainly of the *Streptococci* species (Fürst, et al, 2006). These primary colonizers provide the framework necessary to develop a biofilm (Lee, et al, 2010). The biofilm refers to a bacterial community that allows metabolic exchange and provides compartmentalized ecological niches for numerous bacterial strains.

The principles of biofilm formation and bacterial aggregation are applicable to all dental materials and mini-implants are no exception. An in vitro study simulated the oral microbial environment in an attempt to replicate intra-oral biofilm formation on titanium and stainless steel orthodontic mini-implants (Chin, et al, 2007). Mini-implants were immersed in human whole saliva for 20 hours in an aerobic incubator before being assessed for the following: surface composition, surface roughness, and biofilm viability. Surface physiological and chemical properties of both the biomaterial and bacterial cell wall influence the initial biofilm formation. The study found oral biofilm on mini-implants to have a bacterial viability of 60% after 20 hours of salivary exposure.

The initial establishment of a biofilm provides a network rich with nutrients and fortification from competing organisms; it is an oasis for future pathogenic bacteria to thrive (Lee, et al, 2010). These pathogenic bacteria are dubbed putative periodontal pathogens and are classified as secondary colonizers. Compromised of mainly gram-negative anaerobic strains, periodontal pathogens trigger peri-implant inflammation (Sridhar, et al, 2015). Destruction of soft tissue (peri-implant mucositis) and osseous tissue (peri-implantitits) via bacterial induced inflammation is the main mechanism of implant failure. Bacteriological sampling of failing dental implants verses stable implants has shown a strong association with the following periodontal pathogens: *Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens,* and *Actinobacilus actinomycetemocmitans* (Leonhardt, et al, 1999).

The proven association between periodontal pathogens and dental implant failure lead to studies that examined its applicability with mini-implants. In a prospective case control study, executed by Apel et al (2009), mini-implants were subject to bacterial analysis. In total, 76 mini-implants were placed either between the maxillary or mandibular second premolar and first molar by the same orthodontist. Mini-implants were used for retraction of the anterior segment, after 3 weeks of healing and deemed successful if they did not exhibit the following: mobility, inflammation, or peri-implant tissue pain. Ultimately, eight mini-implants were classified as failures, which translated into an overall failure rate of 10.5%. Paper points were placed in the sulcus of the eight failed mini-implants and in four stable controls. DNA was extracted from these points, purified, amplified, and then hybridized on a microarray containing 20 bacterial species There was no significant difference between the amount or type of bacteria found

between failed and control mini-implants. However, two bacterial species (*A. viscosus* and *C. gracilis*) associated with periodontal health were absent in seven of the eight failed cases. This suggested a possible shift in the microbial flora present in failed implants. It is important to note that the sample size was small and only twenty bacterial strains, excluding *Streptococcus mutans* were tested.

Aiming to identify a more specific bacterial pattern, a prospective case-control study evaluated mini-implants for periodontal pathogens exclusively (Tortamano, et al, 2012). All mini-implants were placed by the same clinician between the second premolars and first molars and used to retract the anterior segment in first premolar extraction cases. Patients were given 0.12% chlorhexidine gluconate and instructed to rinse three times daily for a week. This hygiene regimen as well as patient exclusion of smokers and diabetics aimed to limit the etiologies of the failures. Of the 31 mini-implants extracted, fifteen were removed due to mobility or clinical signs of inflammation and severed as the experimental group. The control group consisted of sixteen mini-implants without mobility and inflammation. PCR amplification of the DNA purified from the surface of the mini-implants was tested against positive controls of *P. intermedia* (Pi), *P. gingivalis* (Pg), and *A. actinomycetemcomitans* (Aa). No statistical association was found between the presence of any of the three periodontal pathogens tested and the number of failed mini-implants. Unlike the study done by Apel et al, (2009) the entire surface of the mini-implant and not solely the sulcus was sampled. This provides a more sensitive detection method, because the paper cones used by Apel et al (2009) may not have been representative of the entire bacterial biofilm.

When the timing of peri-implantitis is considered and then compared to mini-implant treatment time, the absence of periodontal pathogens is not surprising. First, it is important to denote that endosseous dental implant failure is classified as either early or late (Manor et al, 2009). Early dental implant failure occurs prior to abutment insertion and is attributed to failed osseointegration at the bone-implant interface. Late implant failure ensues after occlusal force is placed and results from a failure to maintain the osseointegrated contact. The latter type of failure is most frequently associated with eventual peri-implantitis, which is responsible for destruction of the osseointegrated tissues supporting the implant (Schaller et al, 2016). In a longitudinal study, 596 dental implants were evaluated radiographically over a nine -year span to assess onset and progression of peri-implantitis. Moderate/severe peri-implantitis was defined by bleeding on probing and greater than 2mm bone loss. Of the 14.5% of patients who exhibited peri-implantitis, 81% showed radiographic evidence 3 years post insertion. This timeline is critical to note, because the presence of anaerobic periodontal pathogens responsible for peri-implantitis is delayed. The average orthodontic treatment time is around two years, and most mini-implant applications only require maintenance of the mini-implant for a fraction of that time. Thus, the absence of periodontal pathogens, which frequently appear much later in cases of peri-implantitis, is almost expected in mini-implant failures.

Evaluation of early dental implant failures is more applicable to mini-implants given the duration of use. Early dental implant failure is more frequent and varies from 1.2-3%, in comparison to late failure which is ranges from 0-1.8% (Palma-Carriò et al, 2011). Initial biofilm formation, and subsequent bacterial metabolic interactions on the surface of dental implants, is reasoned to affect the initial bone-implant interface (Sridhar

et al, 2015). Initial colonizers, although not directly associated with osseous tissue breakdown are able to induce inflammation by their presence and metabolic byproducts. Of particular interest are the *Streptococcal* species, which are directly involved in initial biofilm formation (Al-Ahmad et al 2013). Within 30-120 minutes of insertion, initial biofilm formation commences and is heavily comprised of *Streptococcus* spp. Specifically; *Streptococcus mutans* has been implicated in both initial biofilm formation and with peri-implantitis (Sridhar et al, 2015).

Advances in molecular biology and the ability to sequence genes, has allowed more specific identification and classification of microbiomes. In a retrospective clinic study, samples from the gingival crevices of failed endosseous implants, healthy endosseous implants, periodontal involved teeth, and healthy teeth were compared for differences in microbial communities (Kumar et al, 2012). Pyrosequencing of the 16s rRNA genes allowed for identification of all involved bacterial species, as opposed to more traditional culturing techniques that only allow for identification of targeted organisms. The microbial community of peri-implantitis affected teeth and periodontally involved teeth was similar, except for the presence of *Streptococcus mutans* in the peri-implantitis microbiomes. The presence of *S. mutans* on dental implants initially after placement and during peri-implantitis, but not in periodontal disease suggests a unique interplay between titanium surfaces and this bacterial species.

2.3 Biocompatibility of the Implant at the Bone/Soft Tissue Interface

The success of any implanted medical device depends on biocompatibility; this is determined by physical surface properties, which contact host tissues (Suk Lee et al, 2007). The implant surface interacts with host tissue and dictates what type of interface is formed between the two. This interaction can be classified as bioinert, biotolerant, or bioactive if chemical bonds between the bone and implant are formed. Bond strength of the interface is determined by the type of bond formed, whether it be Van der Waals bonding, physisorption bonding, hydrobonding, or chemical bonding at the interface.

Primary stability of mini-implants is entirely dependent on the bone tissue-implant interface that is established upon insertion (Suk Lee et al, 2007). The biological response elicited from the surface of the implant, and the healing process that ensues determines later staged failures. The type and quality of the bone along with the magnitude and duration of the force applied, also affect this interface. Tight interdigitation between the implant threads and cortical bone provides primary mechanical stability. Endosseous implants require a three-month healing period to allow for deposition of lamellar bone around the implant and to limit the formation of fibrous connective tissue. Immediate loading of mini-implants is a controversial topic, as some studies advocate for a healing period prior to force application (Crismani et al, 2010). Also a topic of debate is osseointegration. For endosseous implants, complete osseointegration is the standard treatment objective. However, the extent of osseointegration of mini-implants given their limited treatment time is contended. The concept of osseointegrated mini-implants also raises the question of impedance upon removal.

A more intermediary concept is partial osseointegration of mini-implants, which can be beneficial for both insertion and removal (Vannet et al, 2007). Studies prior to this have shown that mini-implants display half the osseointegration potential when compared to endosseous dental implants. In a prospective study, mini-implants placed in the lower jaws of beagle dogs were assessed histomorphometrically after an orthodontic load was placed. Variations in the amount of force placed, and the time the force was placed were analyzed. By utilizing vitality staining post mortem, it was shown that all mini-implants had deposits of new bone adjacent to the mini-screw surface. This partial osseointegration was observed in all instances, regardless of when the force was placed. This, in contrast to previous studies found no differences in ossteointegrative potential when the site of placement was accounted for. More importantly, after six months of orthodontic force application, no resistance to removal was observed (Favero et al, 2007; Vannet et al, 2007). Prior studies had demonstrated a positive correlation between orthodontic loading and rate of remodeling and bone density at the peri-implant bone site (Fritz et al, 2004). This increase in bone turnover was hypothesized to increase stability; however, more current studies demonstrate bone deposition regardless of force application (Vannet et al, 2007).

An interface between the mini-implant and the surrounding soft tissue is also established upon insertion. Plaque accumulation and mechanical irritation surrounding this interface presents as acute or chronic inflammation and infection (Suk Lee et al, 2007). The host inflammatory response may lead to epithelial hyperplasia or the formation of an abscess. When placed in movable mucosa this interface is weakened and the likelihood of infection is increased. Unlike osseous tissue, soft tissue does not attach

to the surface of mini-implant. Thus, a soft tissue seal encircling the mini-implant is imperative for cleaning and prevention of infection (Suk Lee et al, 2007). Promotion of peri-implant soft tissue attachment by modifying the implant surface is a widely researched topic and will be covered in a later section.

2.4 Titanium Surface Characteristics and Modifications

The use of titanium as a biomaterial is very popular. Titanium implants are utilized in orthopedic joint reconstruction, bone pins, plates and screws, as well as numerous dental applications. Several physical properties make titanium a biomaterial of choice, especially when the site of interest is osseous. Titanium and titanium-based alloys naturally form a native titanium dioxide TiO_2 layer in the presence of air, which accounts for its biocompatibility and corrosion resistance (Unosson, E. 2015). However this film is usually thin and unevenly distributed. Current research aims seek to achieve the following: enhance biocompatibility and increase ossteointegrative potential, increase bactericidal properties and reduce biofilm formation, and increase soft tissue adhesion without compromising either of the first two objectives.

Surface properties of a mini-implant can be categorized as either structural, which delineates the surface or chemical, which refers to the TiO_2 layer formed (Violant et al, 2014). In terms of topography, mini-implants are made of titanium alloy instead of pure titanium to increase strength, despite the compromise in biocompatibility (Suk Lee et al, 2007). To compensate for a smaller surface area and a less osseointegrated surface, mini implants often have roughened titanium surfaces. A rough titanium surface has been shown to increase the contact area between bone and the implant, which increases

primary stability (Favero et al, 2007). Pure titanium with a rough surface has the greatest biocompatibility. A decrease in biocompatibility is observed when a titanium alloy or machined surface is substituted (Suk Lee et al, 2007). However, with a roughened surface comes a compromise in bacterial adherence. In an *in vitro* evaluation of different implant surfaces, a rough titanium surface was found to have the greatest bacterial attachment (Violant et al, 2014). Furthermore, this adhesion was especially relevant with early bacterial colonizers and the severity of biofilm that was observed. A more ideal surface modification would enhance implant-bone contact while maintaining antibacterial properties.

Several titanium surface modifications seek to monopolize on and enhance the TiO_2 surface layer to reduce bacterial adhesion and increase protein adhesion (Unosson, 2015). TiO_2 provides a non-toxic semiconductor surface that when irradiated allows for the formation of hydroxyl radicals (Visai et al, 2011). Several methods of deposition and structuring of TiO_2 films exist. One simplistic approach is to create a sol-gel either by dipping the targeted substance or via spray coating. Chun *et al* (2007), demonstrated decreases in *S. mutans* and *Porphyromonas gingivalis* adherence by creating TiO_2 coated orthodontic archwires. The sol-gel technique is simple in execution but limited in its ability to uniformly coat complex 3D shapes (Visai et al, 2011). TiO_2 films are also produced via electrochemical means, most popularly by anodic oxidation.

TiO_2 nanotube arrays have been produced on titanium mini-screws via a two-step anodic oxidation process (Jang et al, 2015). Previous studies with TiO_2 nanotubes displayed an ordered nanostructure that was both stable and biocompatible. Anodic oxidation is also known to increase surface roughness and to positively influence

adhesion and differentiation of cells. These previous findings lead to the application of nanotube arrays on mini-implants with the goal of increased osseointegration. In the only documented study, eight mini-implants underwent this modification prior to being implanted in the legs of New Zealand rabbits for 8 weeks (Jang et al, 2015). The bone volume ratio and the bone-to-implant contact was assessed by Micro CT images and compared to unmodified machined titanium mini-implants. Increased formation of bone at three and six weeks was observed in the experimental group. Both the bone volume ratio and bone to implant contact ratio were increased in the modified implants (Jang et al, 2015). However, the study failed to recognize any potential drawbacks to increasing surface roughness, such as increased bacterial adherence. Although no failures were reported, it is important to note that the sample size was small (8 implants).

Explanations for these observations can be hypothesized on a molecular level. Oxidized titanium alloys have shown greater alkaline phosphatase activity when compared to untreated controls (Medina et al, 2015). The surfaces of titanium discs were treated by either thermal oxidation or micro arc oxidation and compared by Medina *et al* (2015). The micro arc oxidized surfaces exhibited higher alkaline phosphatase activity, which is associated with increased differentiation of osteoblasts.

The lack of soft tissue adhesion around an implant is theorized to provide a gateway for microbial organisms to colonize implant surfaces. In addition to limiting fibrous connective tissue formation, TiO_2 films have shown increases in soft tissue attachment in rats (Wennerberg et al, 2011). In the first documented *in vivo* study, the top portion (heads) of mini implants were treated via sol-gel to produce nanoporous TiO_2 films. The threaded core was left untreated, as were the control mini-implants. To limit

individual variation, each patient received two mini-implants: one surface treated and one control. Radiographs were taken upon insertion and after fourteen weeks, when the mini-implants were removed and prepared for histological analysis. It is important to note that no signs of inflammation or infection were reported at any of the implanted sites. The importance of this finding cannot be understated, as all previous trials had been conducted in animal models. The histological analysis revealed that the oral mucosa (epithelium and connective tissue) had 72% contact with the upper portion of the mini implant in the surface treated group, while the controls had 48% contact. Radiographs demonstrated less marginal bone resorption in the surface treated implants, which is interpreted as a byproduct of increased soft tissue adhesion, as the threaded portions were not modified. The study conducted by Wennerberg et al (2011) demonstrated the safe clinical use as well as potential advantages of TiO_2 treated titanium surfaces.

2.5 Oxidation of Titanium Surfaces by *Streptococcus mutans*

The majority of endosseous dental implant failures are early, yet the mechanism of such a failure is poorly understood. Late endosseous implant failures, although less prevalent, are known to be caused by pathogenic bacterial tissue destruction. The formation of a biofilm on the implant surface has been proven and theorized to be involved in implant failure (Sridhar al, 2015). Specifically, the involvement of *S. mutans* an early colonizer and inhibitor of peri-implantitis biofilms is of interest. Sridhar *et al* (2015) postulated that the acidogenic properties of *S. mutans* would create an acidic environment for the implant. This acidity could trigger a surface oxidation reaction on the implants. Sridhar *et al* (2015) gave the following two possible causes: the lactic acid

formed by the bacteria during glycolysis causes the drop in pH, or the inner layers of the biofilm, which lack oxygen induce a decrease in pH. Implications of this surface oxidation potentially include the following: imperfections on the implant surface, which promote bacterial adherence, release of metal ions, and impeded osseointegration.

Based on these conjectures, Sridhar et al (2015) developed a method to test the effects of early colonizers on implant surfaces. One implant was immersed in *Streptococcus mutans,* while the other was immersed in un-inoculated BHI medium, as a control. Both implants were immersed for 60 days during which the pH was monitored. After immersion, the surfaces of the both implants were evaluated using an optical microscope with 3D capabilities, and several areas were also evaluated with scanning electron microscopy. Throughout the 60-day immersion period, samples were taken from each implant vial and replenished. These samples were subject to metal ion detection via a metal ion electrochemical biosensor.

In terms of pH, the sample immersed in *S. mutans* had a notably acidic pH of 5, compared to the control, which had a pH of 7. When evaluating the surface of the experimental implant, noteworthy differences were found including: deformation between the collar, roughened surface, pits, valleys, and deposits. These deposits were theorized to be metabolic byproducts of *S. mutans*. Interestingly, a change in color was observed only after two days, with a progressive worsening until rust was evident on the 22nd day. Also of significance was the increase in metallic particles detected in the experimental medium. This study provides a substantial basis in investigating the electrochemical interaction between bacteria and the implant surface. *S. mutans* produces an acidic environment which potentially corrodes the oxide layer, revealing titanium that

can dissolute into the surrounding area. These leached ions have been shown to elicit cytotoxic responses (Sridhar et al, 2015).

2.6 Interaction of Fluoride and *Streptococcus mutans* on Titanium

Contributory to titanium implant biocompatibility is the TiO_2 layer, which forms spontaneously when exposed to air. This layer helps prevent corrosion of the metal surface in the mouth. It has been shown that F- containing products diminish this protective layer and allow for corrosion and ion release (Strietzel et al, 1998). Furthermore, this response is exacerbated in acidic environments (Barrak et al, 2015). This builds off the aforementioned study, which demonstrated the production of an acidic environment by *S. mutans* on dental implants. Orthodontic patients are routinely instructed to brush at least three to four times a day and to utilize fluoride- containing products. Additionally, hygiene protocol for mini-implants includes meticulous brushing and rinsing.

Barrak *et al* (2015) compared titanium surfaces exposed to *S. mutans* and F- for bacterial biofilm quantity and surface changes. Three different fluoride containing products were used (Elmex mouthwash, 1% NaF, and Elmex gel) and one non-treated control. Bacterial protein quantity was assessed after 5, 10, and 21 days of incubation and final titanium surfaces were subject to scanning electron microscopy evaluation. By the 10^{th} day, a slight difference in biofilm formation was observed; the gel and rinse both had decreased amounts. After 21 days of incubation, both the gel and rinse had considerably less bacterial content when compared to both the 1% NaF and control. However the surface tomography of the titanium treated with the gel differed from the surface treated

with the rinse. Evident on the gel treated surface were areas of pitting, indicative of corrosion. Ultimately, the rinse was found to be the most suitable hygiene aid.

2.7 H_2O_2 Gel Deposition and Biochemistry

A review of the literature has provided the following potential benefits of an enhanced TiO_2 layer on mini-implants: improved osseointegration, antibacterial effects, and improved soft tissue adhesion. The aim of modifications is improved efficacy without detrimental compromises. Unosson *et al* (2012) developed a cost effect method for producing an anatase TiO_2 coating on titanium discs. This wet chemical treatment is ideal for mini-implants, because of its ability to coat the entire surface. The original method also uses a UV light to produce a photocatlaytic reaction, resulting in the formation of hydroxyl radicals. These radicals produce lipid peroxidation resulting in bacterial cellular death. Surfaces treated with H_2O_2 and not exposed to UV light still had increased antibacterial activity. This was attributed to surface bound Ti-peroxy radicals which resulted from the oxidation process. This method is also ideal for catalase negative bacteria, like *S. mutans* because they are more sensitive to H_2O_2.

CHAPTER 3
AIMS OF THE INVESTIGATION

This study will address three specific aims:

(1) Utilize the parameters set by Unosson *et al*, 2015 to generate a H_2O_2 gel surface on orthodontic mini-implants. These parameters were previously tested on uniform discs comprised of titanium alloy. The three dimensional irregularity of the mini-implant surface will add additional complexities to the technique.

(2) To determine if the proposed surface modification of orthodontic mini-implants significantly reduces adherence and viability of two relevant bacteria. This will be assessed by exposing the modified mini-implants and unmodified controls to *Streptococcus mutans* and *Streptococcus sanguis* for different time intervals. Evaluation of bacterial adherence between the two groups will be accomplished via spectroscopy of the samples turbidity. The amount of viable bacteria will be analyzed by fluorescent plate reading of samples after the addition of a metabolic dye. Several samples will be plated to ascertain a more quantifiable difference in growth of the bacteria.

(3) Evaluate surface characteristics and micromorphology of modified and unmodified mini-implants with SEM images and EDS on the implant surface.

CHAPTER 4
MATERIALS AND METHODS

4.1 Experiment Overview

Throughout the duration of the experiment, 47 sterile mini-implants were modified and exposed to S. mutans, 16 were exposed to S. sanguis, 31 unmodified sterile implants were exposed to S. Mutans and 16 unmodified sterile implants were exposed to S. sanguis. In total 27 mini-implants served as controls in one of the following protocols: (1) unmodified mini-implant with no bacterial exposure, or (2) modified mini-implant with bacterial exposure. These served to monitor potential contamination throughout the experiment. No controls and thus no experimental mini-implants were deemed contaminated in any of the experiments. The exact breakdown of the number of implants allocated to each category is summarized (**Table 1**). From Trial 1 with *S. mutans*, four SEM images were taken for four samples at varying magnifications. The surface composition of these four samples was also evaluated. In brief, bacterial adherence and growth differed significantly between the untreated and treated samples. The following sections give a more detailed and specific account of experiment results.

Table 1

Summary of Experiment Specifics

Experiment Type and Test	***Streptococcus mutans* Trial 1**	***Streptococcus mutans* Trial 2**	***Streptococcus sanguis* Trial 1**
Number of Implants Evaluated at Initial Contact by Spectrophometer	2 modified 2 controls	11 modified 6 unmodified 4 controls	4 modified 4 unmodified 2 controls
Number of Implants Evaluated at Initial contact by Fluorescent Plate Reader	Not Tested	8 modified 6 unmodified 7 controls	Not Tested
Number of Implants Tested for Growth by Spectrophometer	2 modified 2 unmodified 2 controls	12 modified 6 unmodified 3 controls	16 modified 16 unmodified 3 controls
Number of Implants tested for Growth by Plating	Not Tested	2 modified 2 unmodified	Not Tested
Number of Implants used to take SEM Images	1 modified 1 unmodified 2 controls	Not Tested	Not Tested
Number of Implants used to take EDS	1 modified 1 unmodified 2 controls	Not Tested	Not Tested

4.2 Mini Implant Specifications

The Aarhus system of mini-implants manufactured by American Orthodontics was used. These mini- implants are self -drilling and threaded in design. All mini-implants had the following specifications: button head or through-hole head, overall length- 9.8mm, tissue collar length- 1.5mm, thread length- 6.0mm, and thread diameter- 1.5mm (**Figure 1**). They are compromised of Ti6I4V, a titanium alloy (Wrought

Titanium-6 Aluminum-4 Vanadium ELI). Prior to use, mini- implants were sealed in individual sterilization pouches (3.5”x 9”). Sealed implants were then placed in sterilization (STATIM 5000) on the heavy duty unwrapped cycle (132°C holding time 6 minutes).

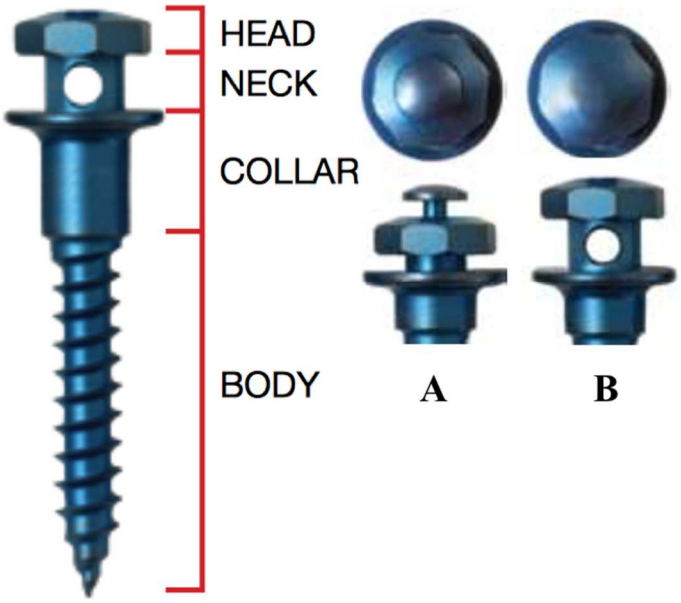

Figure 1: Images adapted from American Orthodontics, “A Clinical Guide to Aarhus Mini-implants and Skeletal Anchorage.” A, Button Head; B, Through-Hole Head

4.3 Scanning Electron Microscope Images and EDS

The Scanning Electron Microscope (FEI Quanta 450FEG SEM) at the College of Engineering laboratory at Temple University was utilized. Several pre-treatment and post treatment images at varying magnifications were taken. The following specifications were employed: 30.00 kv (HV), 3.0 (spot), LFD (det), SE (mode), 6.91 mm (HFW), and 0° tilt. Samples were not coated or hydrated prior to taking images. This microscope was equipped with an Energy Dispersive Spectrometer (Oxford Aztec Energy Advanced EDS

System), which allowed limited analysis of the elemental composition of selected sample surfaces.

4.4 H_2O_2 Surface Modification

The following method is adapted from a technique first described by Unosson *et al*, (2012). The following steps were carried out via the aseptic technique to reduce contamination risks. In addition, untreated controls were allocated at each step to assess possible contamination. Sterilized mini-implants were placed in sterilized 6 ml test tubes containing 2 ml of 30mass% H_2O_2 solution and held at 80° C for 24 hours in a water bath. Following this oxidation, the remaining H_2O_2 was emptied and 6ml of double distilled and double ionized H_2O was added to the test tubes **(Figure 2)**. The first set of samples (*S. mutans* Trial 1) was held at 80°C for 72 hours. The remaining samples were held at 80°C for 1 hour, to better maintain the Ti- peroxy gel layer. Implants were then removed with forceps from the test tubes and placed in sterile wells to dry for 20 minutes.

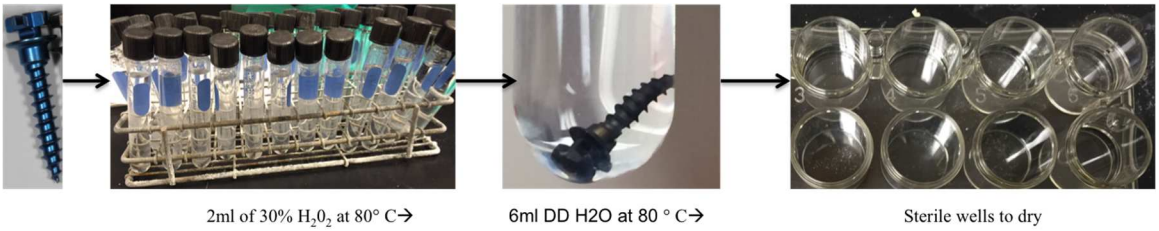

Figure 2: Schematic of Adapted H_2O_2 Surface Modification

4.5 Bacterial Preparation

The strains *Streptococcus mutans* strain 25175 (Lot #60629082 and *Streptococcus sanguis* strain 10556 (Lot #62071803) used in this study were obtained from American Type Culture Collection, Manassas, Va. The frozen preculture (-60 °C)

was incubated at 37°C in Brain Heart Infusion (BHI), until an optical density equivalent to 0.5 McFarland standard was achieved at 600nm.

4.6 Initial Bacteria Adhesion

Modified samples were placed in 2ml of fresh BHI broth and 30µl of *Streptococcus mutans* was added. Samples were incubated at 37°C and removed at 1 hour or at 4 hours. Unmodified and modified controls with bacteria and without bacteria were subject to the same protocol. Following incubation, samples were rinsed with 6ml of double distilled/deionized water and put in eppendorf tubes (**Figure 3**). Samples were vortexed for 10s in 2ml of DD H_2O prior to evaluation, to dislodge any non -adherent bacteria. Samples were then placed in 2ml of fresh BHI broth, and incubated at 37°C for 24 hours. Aliquots of the solution in these wells was then removed and evaluated on the spectrophometer, as described in the protocol below.

Figure 3: Transferred Mini-implants to Eppendorf Tubes Prior to Vortexing

4.7 Viability of Initial Bacteria

To estimate the number of viable bacteria at initial contact, samples were treated as follows: 2ml of fresh BHI broth and 30µl of *Streptococcus mutans* were added to mini-implants and incubated at 37°C for 15 hours. Mini-implants were then rinsed with 2ml of DD H_2O, placed in eppendorf tubes, vortexed for 10s, placed in 2ml fresh BHI broth and incubated at 37°C for 4 hours. After incubation, 2µl of Resazurin dye was added to each well on the plate, prior to evaluation on the fluorescent microplate reader.

4.8 Fluorescent Microplate Analysis

The fluorescent microplate reader located at the Pharmacogenmoics lab at The Temple University School of Pharmacy was utilized. Resazurin is a dye that is metabolically broken down into Resofrin, a compound with a different relative fluorescent intensity. Thus, the relative fluorescent intensities of different solutions can be used as an indicator of bacterial viability. The machine was first equilibrated using 2ml of Broth and 2µ of Resazurin dye that was serially diluted six times with H_2O at different excitation and emission spectrums. Samples were then evaluated at the appropriately calibrated settings.

4.9 Bacterial Growth

Modified and unmodified samples were placed in 2ml of fresh BHI broth and 30µl of *Streptococcus mutans* was added. For the second sample set, 30µl of *Streptococcus salivarius* was added. Samples were incubated at 37°C for 24 hours. Implants were then rinsed with 6ml of double distilled/deionized water and placed in eppendorf tubes. Samples were vortexed for 10s in 2ml of DD H2O. Samples were then placed in fresh BHI, incubated at 37°C and assessed at 8 and 24 hours. 30µl aliquots were taken from two modified and exposed 24 hour samples, serially diluted (10^{-10}) plated on BHI agar, and incubated for 24 hours at 37°C. Two unmodified and exposed samples from the 24 hour well were also serially diluted and plated. Total growth was assessed by CFU. Grid paper was used to help quantify the number of colonies, when counting of individual colonies was not possible.

4.10 Spectrophotometer Analysis

Both initial contact and growth was assessed at the specified times by measuring relative absorbance on a spectrophotometer. The baseline absorbance was calculated at 500nm, with a control solution of 1µl of fresh BHI in 9µl of DD H2O. At the specified times, 1µl from the modified and unmodified samples was diluted in 9µl of DD H2O and analyzed on the spectrophotometer. Differences in the turbidity of the solution represented differences in bacterial accumulation.

4.11 Statistical Analysis

ANOVA and pairwise post hoc tests (paired and unpaired student t test's) were used to compare differences between initial adherence and growth of S. Mutans.

CHAPTER 5
RESULTS

5.1 Initial Adherence Via Spectrometer Analysis

Initial adherence of the bacteria to the implant surface was measured after 1 and 4 hours of incubation. The relative turbidity of the solution in comparison to the control solution was measured. Visual differences in the turbidity of the solutions were evident, and correlated with data collected from the spectrophometer measured at 400nm (**Figure 4**). After 1 hour, 4/6 modified samples with *S. mutans* had an absorbance value of 0 while the other two samples had values of 0.001nm. The unmodified samples had absorbance values ranging from 0.002-0.003nm. After 4 hours, the modified samples ranged from 0.001- 0.006 with an average value of 0.0028nm. The average value of the unmodified samples was .013 nm at 4 hours. The unmodified samples had a relative absorbance value that was 4.6 times larger than the modified samples, after 4 hours of incubation (**Figure 5**). When exposed to *S. sanguis*, both initial values were greater for the modified and unmodified samples. Values for both groups decreased slightly at 4 hours, with the unmodified group being slightly larger. All results are listed (**Tables 4-6 [Appendix A**].

Figure 4: Visual Differences in Turbidity of Solutions Sampled

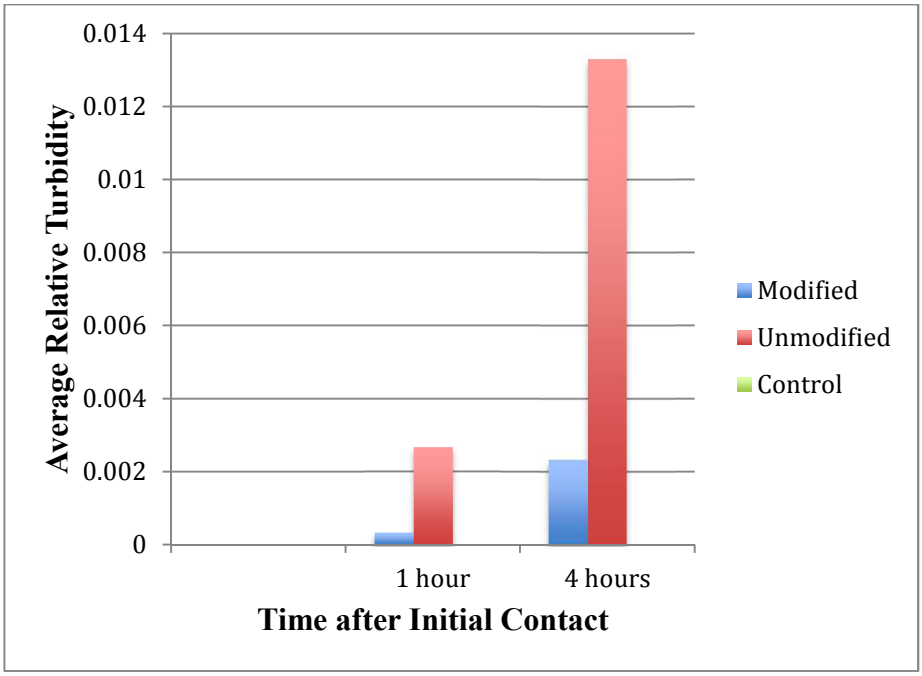

Figure 5. Absorbance values from Trial 2 with S. mutans initial adherence evaluated on the spectrophotometer at 400nm

5.2 Assessment of Viable Bacteria Via Fluorescent Microplate

Several excitation and spectrum settings were tested to calibrate the machine with the sample material. After serially diluting 2ml of BHI broth and 2µl of resazurin dye the optimum settings were found to be: excitation spectrum 500nm, emission spectrum

585nm. This was determined because the diluted values decreased accordingly at these settings. Both the modified and unmodified controls had similar values without exposure to bacteria (**Table 2).** Mean values for the control, untreated, and treated groups were 2243.4 $\pm$ 1782.2 , 9458.4 $\pm$ 432.3 , & 8839.8 $\pm$ 308.3 ; respectively. ANOVA indicated significant differences between the mean values of groups ($p< 5.2E-10$). The difference between the modified and unmodified mini-implants was statistically significant ($p<0.004$).

Table 2

Results of Vialbe Bacteria at Initial Contact by Fluorescent Plate Reader

Sample	N	Average Value
Modified Unexposed	2	8809.597
Unmodified Unexposed	2	8555.0105
Broth + Dye Control	2	7956.5695
Unmodified Exposed	6	9458.373
Modified Exposed	8	8839.758

5.3 Growth assessed with Spectrophometer and Plating

Growth of the bacteria on the implant surface was assessed at 8 and 24 hours by evaluating aliquots from sample broth on the spectrometer at 400nm. Visual color changes on the mini-implant surface were evident upon removal from the broth (**Figure 6**). In Trial 2, the average absorbance value of the unmodified sample aliquot at 8 hours was 0.385nm and the average value of the modified samples at 8 hours was 0.0985nm. After 24 hours, the average absorbance value of the modified samples was 0.15833nm and the average value of the unmodified samples was 0.442nm; this was statistically significant ($p<0.0000414$). After 8 hours of incubation with *S. sanguis,* the average value

of the unmodified samples was 0.2190nm and the average value of the modified samples was 0.01035nm. These averaged values were statistically significant ($p<0.00083$). After 24 hours of incubation with *S. sanguis*, the average value of the modified samples was 0.199nm and the average value of the unmodified samples was 0.404nm. In Trial 1 with *S. mutans*, the sample size at 24 hours was too small for statistical evaluation, but the values are similar to the results of Trial 2. The relative turbidity values from Trial 2 with *S. mutans* and Trial 1 with *S. sanguis* have been plotted on growth curves (**Figure 7 and Figure 8**). Two unmodified and two modified samples were taken from Trial 2 with *S. mutans* after 24 hours and serially diluted and plated. One of the modified 24-hour samples was contaminated, as evidenced by a suspected fungus growth on the perimeter of the plate (**Figure 9**). The other uncontaminated modified sample had 3 CFU after ten dilutions. Both unmodified samples were uncontaminated, and the number of CFU was estimated with the use of grid paper. The boxes in the grid paper used measured 1.5cm x1.5cm. In sample 3 the average diameter of one CFU was 0.3cm and the average diameter was 0.2cm in sample 4. Estimates were made based on the amount of box filled for boxes that contained conjoined colonies unable to be counted individually. For sample 3 a full box was estimated to contain 25 CFU and in sample 4 a full box was estimated to contain 45 CFU. Based on these specifications sample three was estimated to have 250 CFU and sample four was estimated to have 560 CFU (**Figure 10**). A single examiner did the counting twice.

Figure 6: Visual Differences of Mini-implant Surfaces; A, example of a sterile mini-implant from Aarhus system with a different length and head then used in the experiment, however the material and thus the color are the same; B, modified/exposed for 24 hours; C, unmodified/exposed for 24 hours; D, modified/unexposed

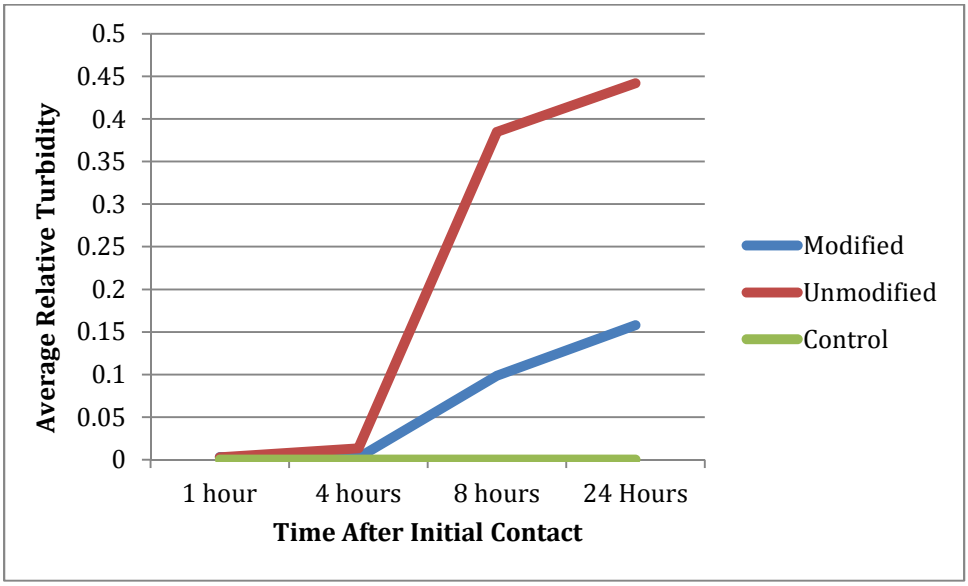

Figure 7: Growth Curve from *S. Mutans* Trial 2

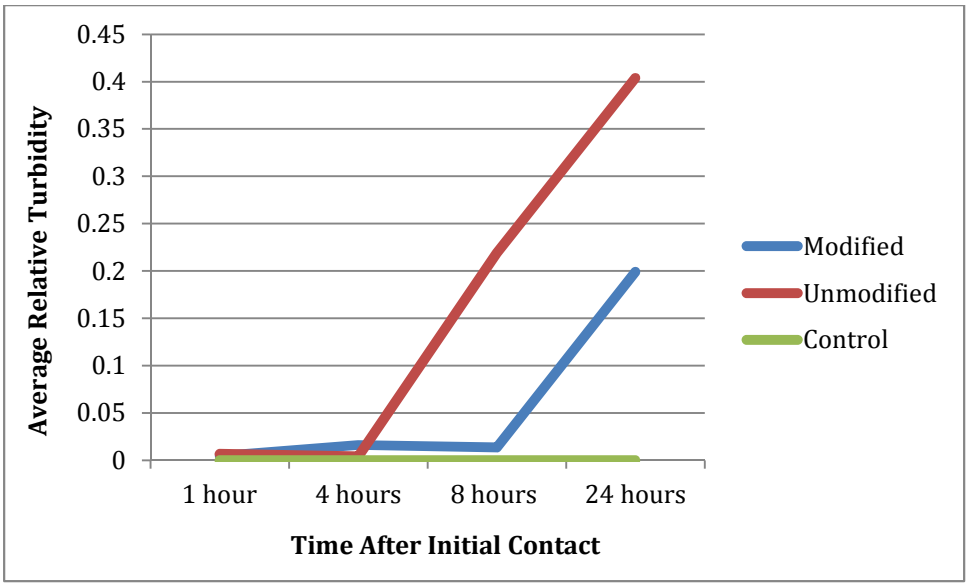

Figure 8: Growth Curve form *S. Sanguis* Trial 1

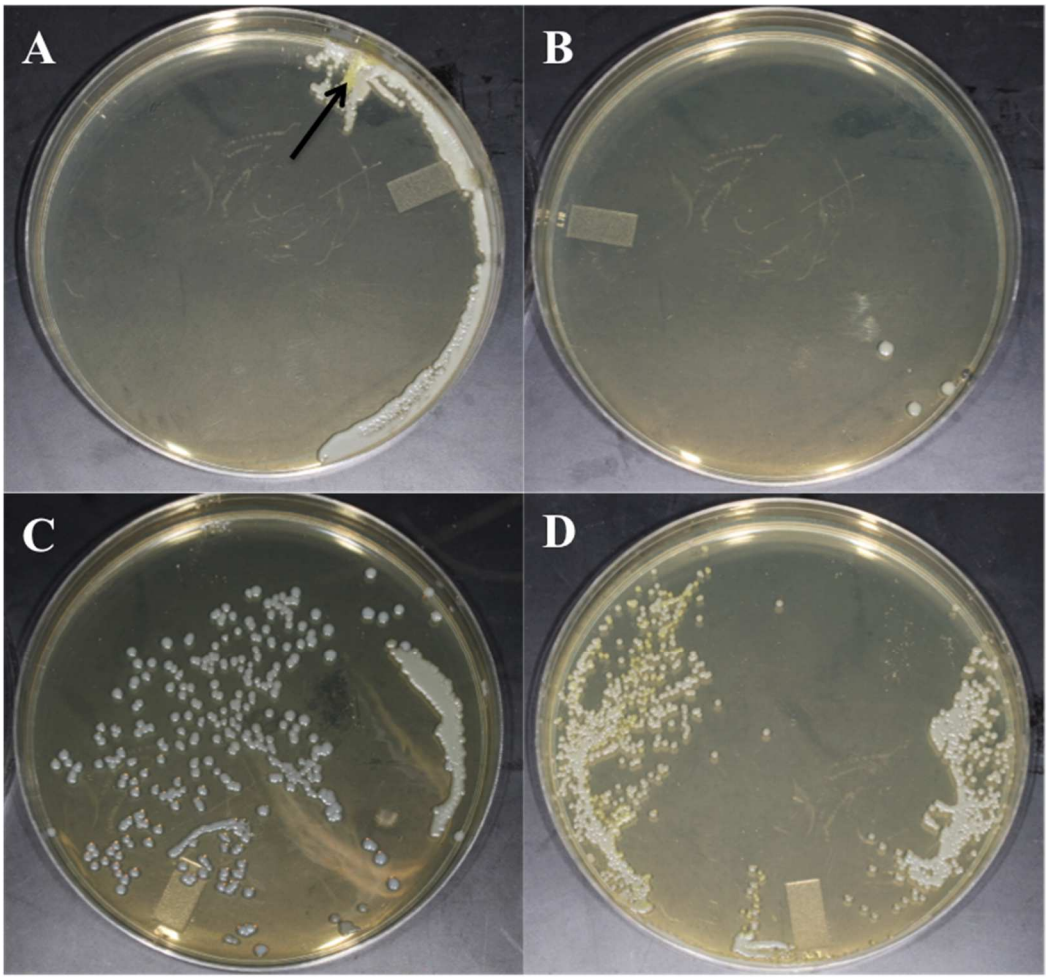

Figure 9: Plating from serial dilution 10^{-10} of *S. Mutans* Trial 2. A, modified sample 1 with suspected fungal contamination; B, modified sample 2 with 3 CFU present; C, unmodified sample 3; D, unmodified sample 4.

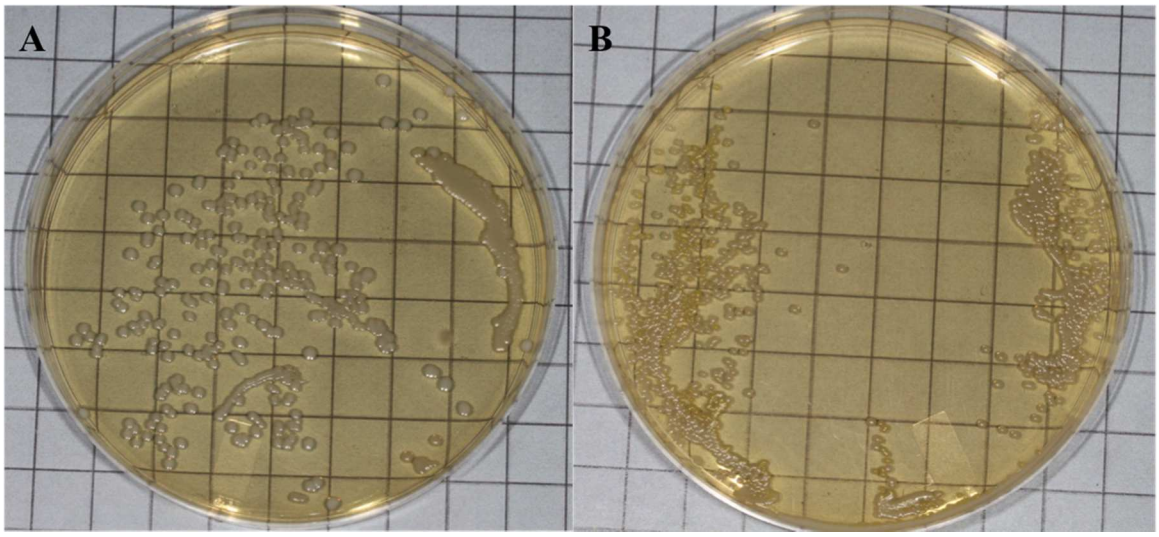

Figure 10: Plating from serial dilution 10^{-10} of unmodified samples 3 and 4 on grid paper.

5.4 Microscopic morphological changes

From *S. mutans* Trial 1, the following four samples were used to take SEM images: a sterile unmodified mini-implant not exposed to *S. mutans* (sample 1), a sterile unmodified mini-implant exposed to *S. mutans* (sample 2), a modified mini-implant not exposed to *S. mutans* (sample 3), and a modified mini-implant exposed to *S. mutans* (sample 4). The first image was taken at a mag of 60X (**Figure 11**). All additional images were taken from the left-most portion superior portion of the collar of the mini-implants. Images at magnifications of 2000X and 4000X were taken for samples 1 and 3 (**Figure 12**). Images of the unmodified/exposed sample and the modified/exposed sample were taken at magnifications of 250X and 500X (**Figure 13**). To evaluate changes in dimension images of sample 1 and sample 4 at a magnification of 1,000X were taken and the height of the collar lip was approximated with a digital ruler tool (**Figure 14**).

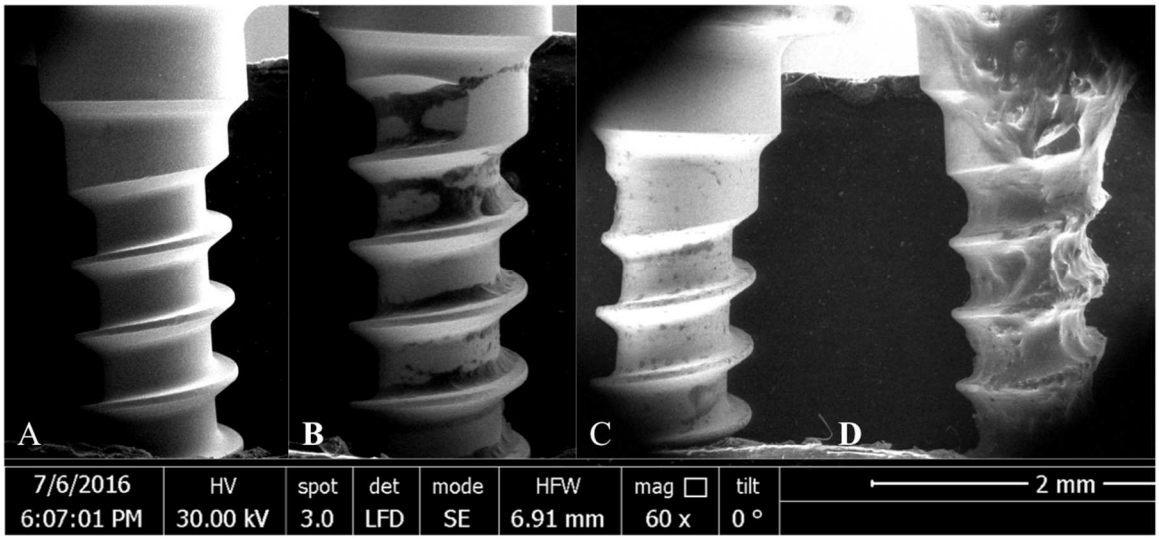

Figure 11: A, unmodified/unexposed; B, modified/unexposed; C, modified/exposed; D, unmodified/exposed.

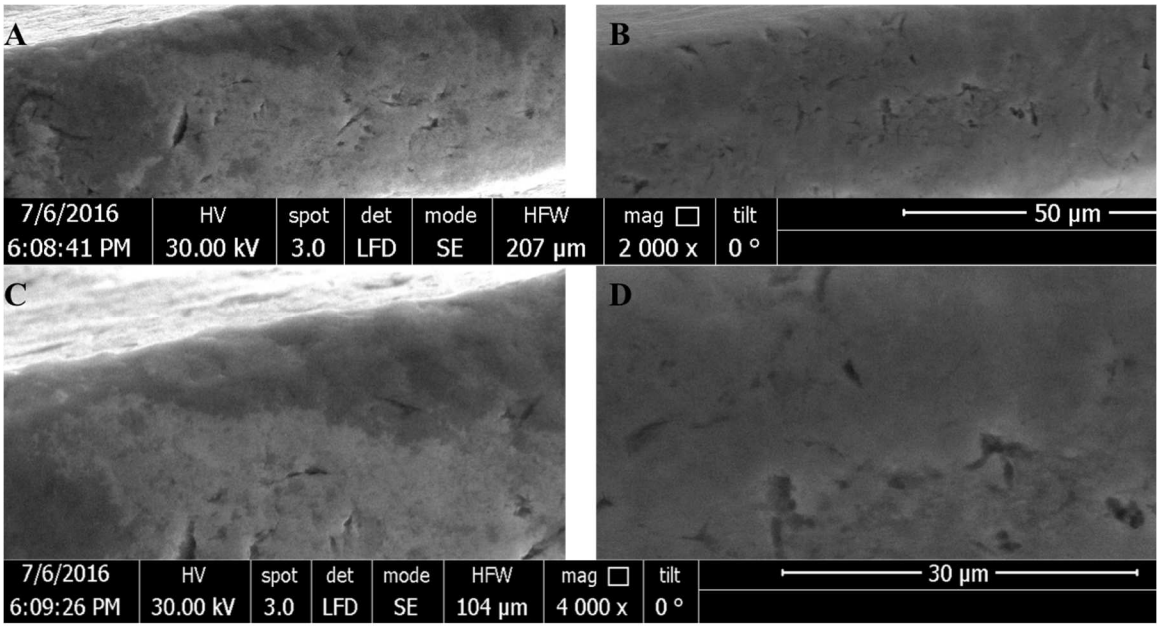

Figure 12: A, unmodified/unexposed 2000x; B, modified/unexposed 2000x; C, Unmodified/unexposed 4000x; D, modified/unexposed 4000x

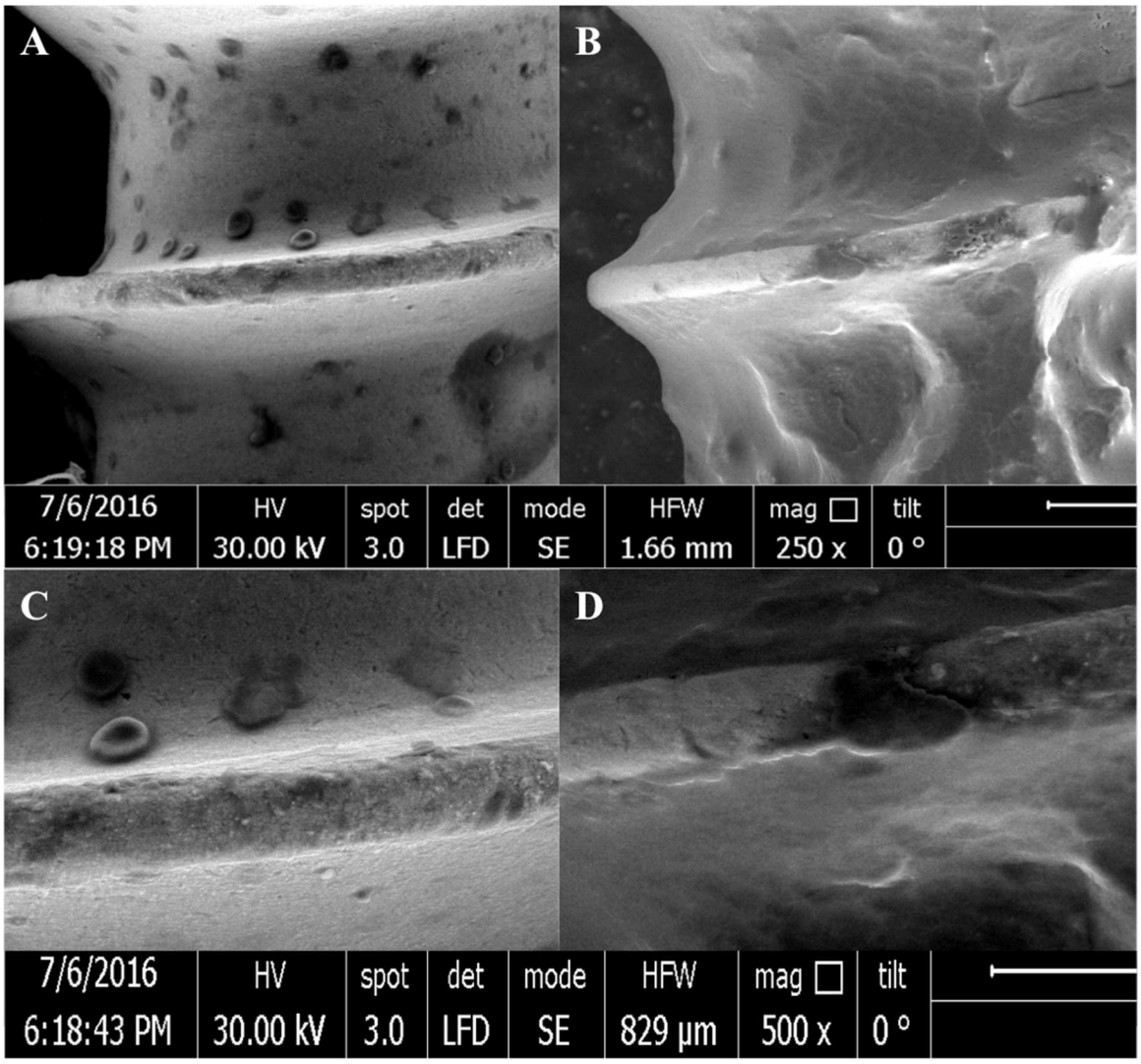

Figure 13: A, modified/exposed 250X; B, unmodified/exposed 250X; C, Modified/exposed 500X; D, unmodified/exposed 250X

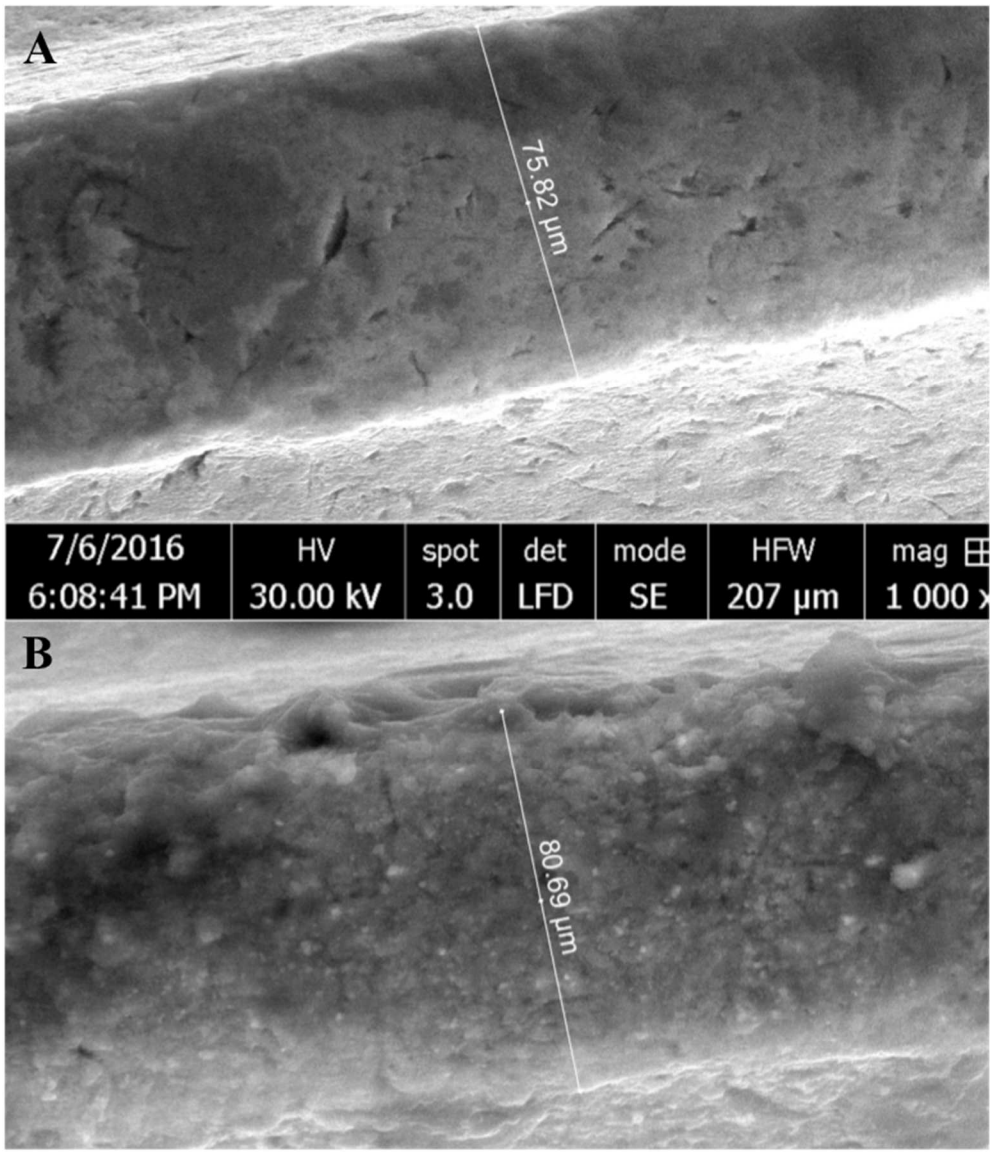

Figure 14: A, unmodified/unexposed 1000X; B, modified/exposed 1000X

5.5 Surface Elemental Composition

From *S. Mutans* Trial 1, the same sample set evaluated by SEM was also subject to EDS (Energy Dispersive X-ray Spectroscopy). The interaction of the X-ray on the surface of the implants reveals the elemental composition in the area radiated. For each sample, two areas were chosen from an image magnification of 1000X. Each area

selected on a sample generated a plot of the elements present and quantified the amount by a percentage (**Figure 15**). The average value for each element on the surfaces tested is summarized (**Table 3**). All images of the areas used and graphs generated for each sample are listed (**Figures 16-19**).

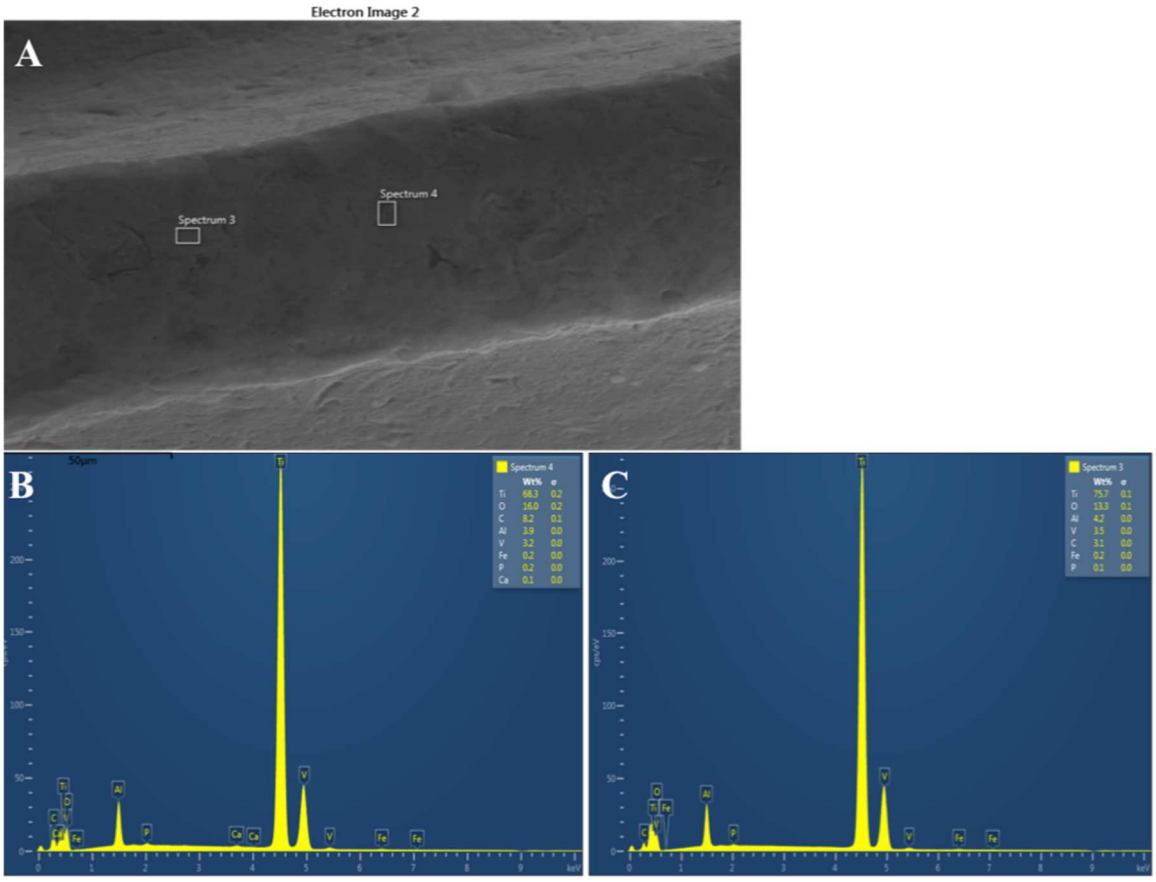

Figure 15: EDS results from the unmodified/unexposed sample *S. Mutans* Trial 1

Table 3
EDS Values from *S. Mutans* Trial 1

Sample	Ti	O	Al	V	C
Unmodified/Unexposed	72	14.65	4.05	3.35	5.65
Modified/Unexposed	62.3	20.25	3.3	2.7	9.95
Unmodified/Exposed	40.15	23.8	1.6	1.7	18.55
Modified/Exposed	35.15	22.15	1.75	0	32.35

CHAPTER 6
DISCUSSION

Advancements in orthodontic treatment seek to decrease reliance on patient compliance and to limit unfavorable tooth movements. Traditional methods to increase anchorage and limit tooth movement include, but are not limited to: increasing tooth mass, opposing posterior segments against the palate or lower anterior teeth, and using external traction. However, mini-implants offer orthodontists an osseous anchor that provides the most rigid control with the least unwanted movement (Antoszewska et al, 2009). Failure rates for mini-implants are varied, but are considerably higher than endosseous dental implants, a well-documented and comparable counterpart (Lee, et al, 2010).

The parameters for a failed mini-implant are not absolute, but rather loosely defined. Fracture of the mini-implant, loosening, or infection of surrounding tissue that warrants premature removal are deemed failures. The etiology of mini-implant failure may be more appropriately dubbed etiologies; multiple factors have plausible contributions. Proposed etiologies can be grouped into four categories: characteristics inherent to the implant such as length, diameter, or thread design; location of the mini-implant; patient related factors that include hygiene and inflammatory response; and orthodontic which includes forces and duration of treatment (Chaddad, et al, 2008). Mini-implants rely on a mechanical interdigitation with bone and better resemble early endosseous dental implant failures (Chaddad, et al, 2008). Although, partial osseointegration has been demonstrated on the mini-implant surface this is likely not accountable for failure, as seen in later stage endosseous implant failures. Periodontal

pathogens have been conclusively linked to late stage endosseous failures, resulting in osseous destruction and weakening of the bone-implant interface (Lee, et al, 2010). However, this microbial-implant interaction is slow and prolonged. A more immediate response is the formation of dental pellicle and the adherence of early colonizing bacterial species. In particular, *Streptococcus mutans* is a known early colonizer and has been linked to endosseous implant failure in the absence of periodontal disease (Sridhar et al, 2015). Previous studies have documented its adhesion to titanium surfaces, including mini-implants (Barrak et al, 2015). Its physical presence provides an infrastructure for additional bacterial colonization; this threatens the integral implant-bone interface. Additionally*, S. mutans* is an acidogenic bacteria; its metabolic byproducts have been shown to compromise the integrity of titanium-alloy surfaces (Sridhar et al, 2015). Titanium alloys are the most frequently used materials for mini-implants.

The most critical consideration for medical devices that will be implanted in the human body is biocompatibility. The material must be inert and avoid triggering an unwanted host immune response that results in rejection of the foreign object. The ideal material must strike a delicate balance between biocompatibility and integration without compromising strength. Aims to increase the bone to surface contact have been demonstrated with several techniques that "roughen" the surface (Favero et al, 2007). Although pure titanium has superior biocompatibility, titanium alloys often comprise mini-implants because they increase strength thereby compensating for diminished dimensions. Without modification, a thin TiO_2 layer forms on the surface of titanium alloys; increasing this dimension can be beneficial. This oxidized surface has increased

levels of phosphatases and when exposed to UV light produces radicals with bactericidal properties (Visai et al, 2011). Previous studies found the TiO_2 UV activated layer to increase antibacterial properties, improve soft tissue seal, and increase the bone to surface contact (Wennerberg et al, 2011). However, the methods employed vary in cost, time, and application. Unosson *et al* (2015) demonstrated a cost-effective method that involved oxidization and aging of the titanium surface prior to UV activation. More interesting, the modified surfaces displayed antibacterial effects even without the UV irradiation (Unosson, et al, 2015).

The aim of this study was to apply the modification technique described by Unosson *et al* (2015) to orthodontic mini-implants. Quantifiable changes in *S. mutans* both in adherence and growth were assessed. Concurrently, the surface of the implants prior to modification and bacterial introduction were evaluated. Initial adherence of bacteria to the modified surface was significantly decreased. Furthermore the proportion of viable bacteria attached to the surface was decreased. Growth of the bacteria assessed at 24 hours was also significantly decreased. When comparing SEM images, the starkest comparison exists at a magnification of 60x. It can be surmised that the darkened areas on the inner threads of the mini-implants represent the oxidized surface. After introduction of the bacteria obvious accumulations exist, despite vortexing and rinsing with sterile H_2O. Without extracting samples and testing the accumulations, the composition cannot be conclusively elicited. However, it is highly likely that they represent increases in bacterial accumulations depicted as a biofilm with materials present in the blood heart infusion medium. When the same portion of collar of the mini-implants was examined at 1000X mag an increase in the vertical dimension was observed. This increase can be

hypothesized to be a result of the TiO_2 layer thickness and bacterial accumulation. A very preliminary analysis of the surface composition was performed via EDS. Confirmation of the manufactures reported metal composition was obtained (TiOAlV). After modifications and bacterial introduction, increases in O and C were expectedly seen.

Apart from the reduction in bacterial adherence and growth, changes seen on the unmodified samples provide insight as well. When unmodified mini-implants were exposed to *S. mutans*, obvious changes in color resulted after 24 hours of incubation. Given the acidogenic nature of *S. mutans* and the relatively harsh environment it creates, compromise of the titanium surface is inevitable. Sridhar *et al* (2015) demonstrated microscopically the damage *S. mutans* has on titanium based surfaces; there was structural damage and leeching of metal ions from the surface. This negative effect was exacerbated in the presence of fluoride (Barrak et al, 2015). Orthodontic patients are routinely instructed in oral hygiene measures, including fluoride rinses and fluoridated toothpaste.

Several limitations exist in this study, most notably the use of an in-vitro environment. Without an in-vivo environment, effects from saliva and additional bacteria cannot be determined. The second limitation was the duration of the experiment. The formation of a biofilm usually coincides with 24 hours. Extending the duration of the experiment would have the following benefits: monitor the durability of the H_2O_2 layer and account for later bacterial growth. Additionally, the uniformity of the H_2O_2 layer should be quantified; preliminary images suggest a prediction for the inner threads. In addition, future studies should take 3D images to better quantify surface deformities and irregularities.

In conclusion, the surface modification decreased bacterial adherence and growth on orthodontic mini-implants. The images obtained demonstrate that the integrity of the modified surfaces was better preserved. The data obtained provides the preliminary framework for future research. Expanding upon this investigation may prove useful in reducing the failure rate of orthodontic mini-implants.

CHAPTER 7
CONCLUSIONS

1. After modifying the surface of orthodontic mini-implants with H_2O_2 gel, initial adherence of *S. mutans* was decreased compared to controls. The number of viable bacteria that adhered was also significantly decreased.
2. When growth of the *S. mutans* and *S. sanguis* were evaluated after 24 hours, the number was significantly decreased compared to controls.
3. Visual changes both macroscopically and microscopically were evident after exposure to *S. mutans* and following the modification. The immediate change in color after addition of *S. mutans* likely reflects the acidic environment produced by the bacteria. The darkening of the surface is a likely byproduct of the oxidation produced by the modification. Microscopically, the unmodified mini-implants had gross accumulations throughout the surface, while the modified surface integrity was better preserved.

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APPENDICES

APPENDIX A
S. Mutans Trial 1 Spectroscopy Data

Sample: S. Mutans Trial 1	1 hour	4 hours	8 hours	24 hours	48 hours	
Treated	0.039	0.050	0.055	0.056	0.113	
Untreated				0.178	0.902	
Control (treated no bacteria)	0.006	0.005	0.006	0.005		

APPENDIX B
S. Mutans Trial 2 Spectroscopy Data

Sample: S. Mutans Trial 2	1 hour	4 hours	8 hours	24 hours
Treated	0.001 0.001 0 0 0 0	0.003 0.001 0.006 0.002 0.002	0.072 0.112 0.141 0.041 0.113 0.112	0.062 0.144 0.192 0.201 0.222 0.129
Untreated	0.003 0.002 0.003	0.013 0.014 0.013	0.356 0.413 0.387	0.439 0.438 0.449

Untreated Control	0	0.001	0.002	0
Treated Control	0	0	0.003	

APPENDIX C

S. Sanguis Trial 1 Spectroscopy Data

Sample S. Sanguis Trial 1	1 hour	4 hours	8 hours	24 hours
Untreated	0.006 0.007	0.003 0.005	0.219 0.212 0.219 0.211 0.245 0.201 0.206 0.225 0.223 0.224 0.212 0.232	0.438 0.326 0.424 0.429
Treated	0.005 0.004	0.002 0.003	0.40 0.133 0.073 0.026 0.007 0.153 0.018 0.012 0.12 0.015 0.173 0.112	0.245 0.109 0.258 0.184
Control	0.002	0.002	0.005 0.003	0.003

APPENDIX D
S. Mutans Fluorescent Plate Data

Sample								
Treated Control	8721.787	8897.316						
Untreated Control	8569.236	8540.785						
Treated	8420.988	8682.32	9074.231	8988.165	9160.316	8993.322	9032.694	8366.03
Untreated	8977.414	10113.882	9588.329	8978.943	9619.383	9472.735		
Broth + Resazurin (Control)	7813.41	8099.729						
Broth (Control)	664.5							
Dilution Calibration	5266.478	3374.64	2073.9	1216.107	821.674	707.827		

APPENDIX E
S. Mutans Trial 2 Statistics

	S Mutans Untreated	*S. Mutans treated*
Mean	0.442	0.158333333
Variance	3.7E-05	0.003470667
Observations	3	6
Pooled Variance	0.002489619	
Hypothesized Mean Difference	0	
df	7	
t Stat	8.040014911	
P(T<=t) one-tail	4.41449E-05	
t Critical one-tail	1.894578605	

P(T<=t) two-tail	8.82899E-05	
t Critical two-tail	2.364624252	
Standard Deviation	(±) 0.0060824	(±) 0.5891237

APPENDIX F
S. Sanguis Trial 1 Statistics

	S. Sanguis Untreated	*S. Sanguis Treated*
Mean	0.219083333	0.076545455
Variance	0.000143356	0.004026673
Observations	12	11
Pooled Variance	0.001992554	
Hypothesized Mean Difference	0	
df	21	
t Stat	7.649767529	
P(T<=t) one-tail	8.37586E-08	
t Critical one-tail	1.720742903	
P(T<=t) two-tail	1.67517E-07	
t Critical two-tail	2.079613845	
Standard Deviation	(±) 0.011973	(±) 0.063456

APPENDIX G
S. Mutans Fluorescent Reader Statistics

	Treated	*untreated*
Mean	8839.75825	9458.372883
Variance	95019.87338	186863.2449
Observations	8	6
Pooled Variance	133287.9449	
Hypothesized Mean Difference	0	
df	12	
t Stat	-3.137482803	
P(T<=t) one-tail	0.004285947	
t Critical one-tail	1.782287556	

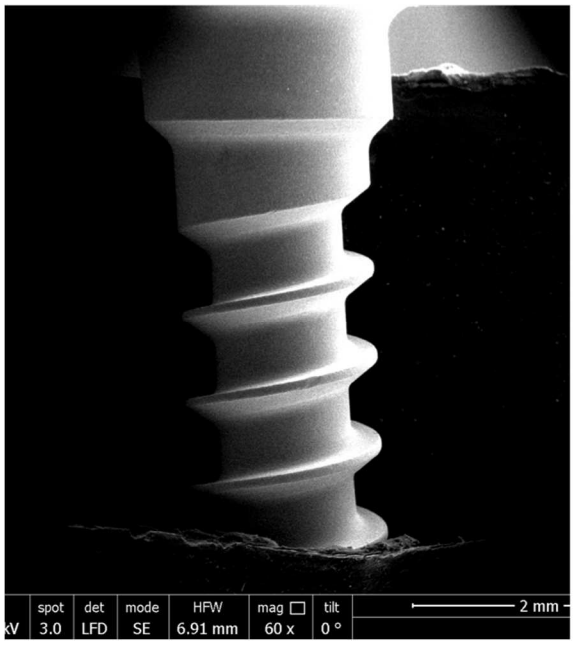
P(T<=t) two-tail	0.008571895	
t Critical two-tail	2.17881283	
Standard Deviation	(±) 308.252294	(±) 432.22682

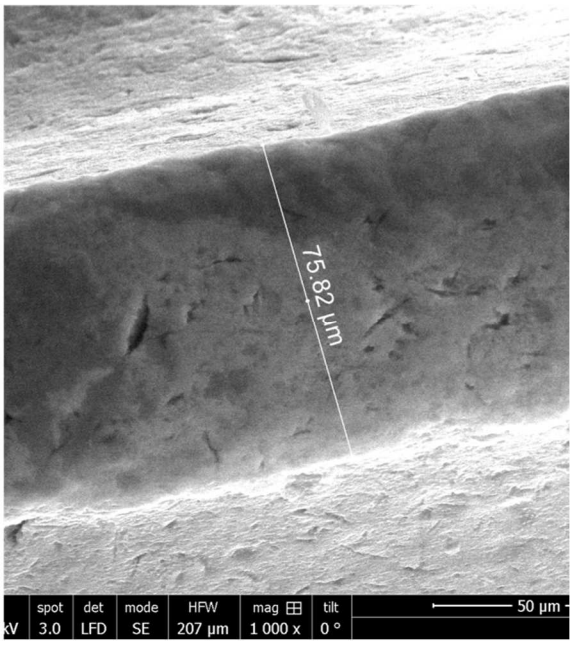
APPENDIX H
S. Mutans Fluorescent Data ANOVA

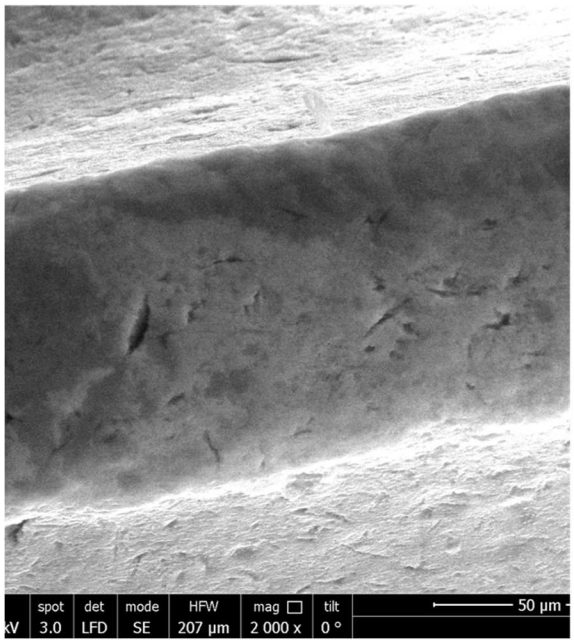
Groups	*Count*	*Sum*	*Average*	*Variance*
Control	6	13460.626	2243.437667	3176410.988
Treated	8	70718.066	8839.75825	95019.87338
Untreated	6	56750.2373	9458.372883	186863.2449

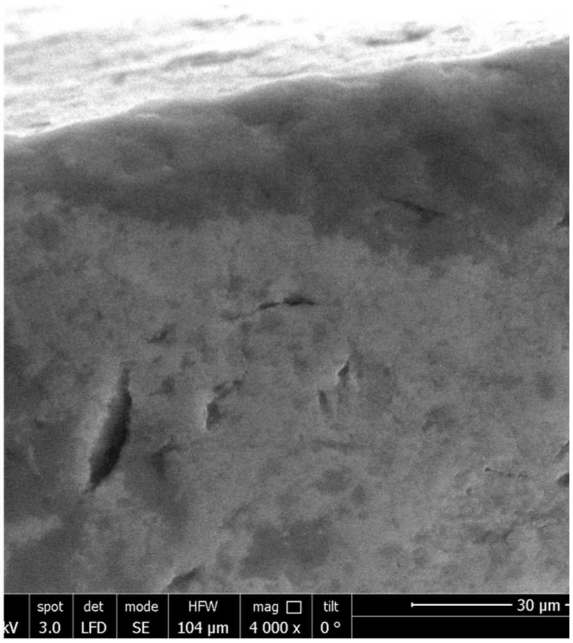
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	199045432.7	2	99522716.33	96.78146515	5.12944E-10	3.591530568
Within Groups	17481510.28	17	1028324.134			
Total	216526942.9	19				

APPENDIX I
SEM Images Unmodified/Unexposed









APPENDIX J
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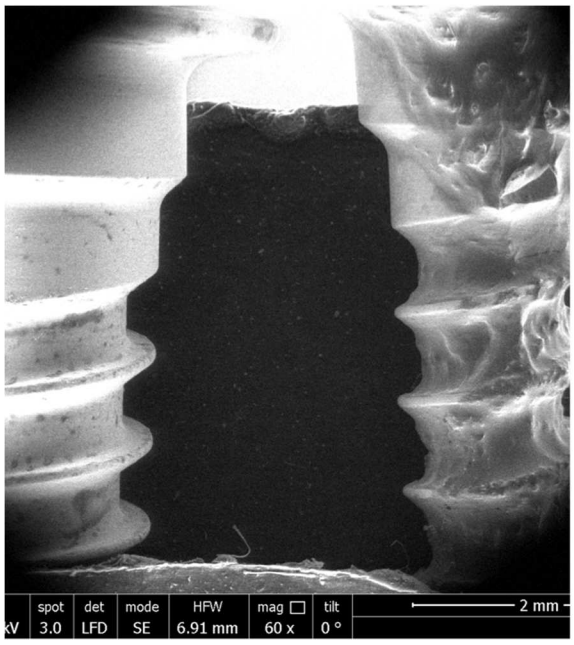
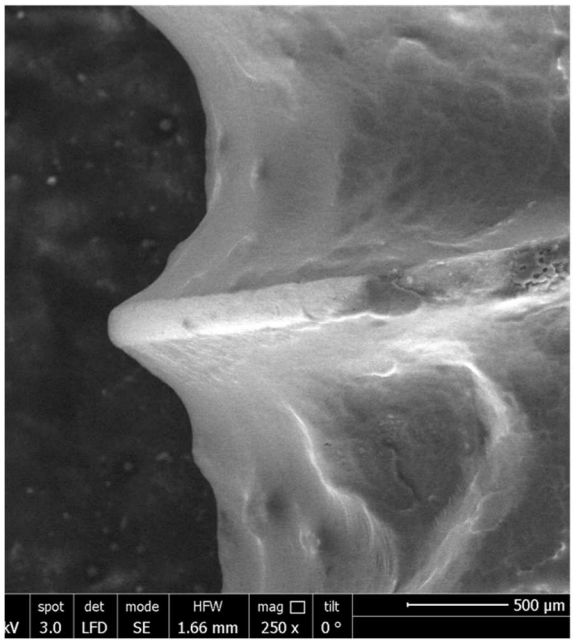
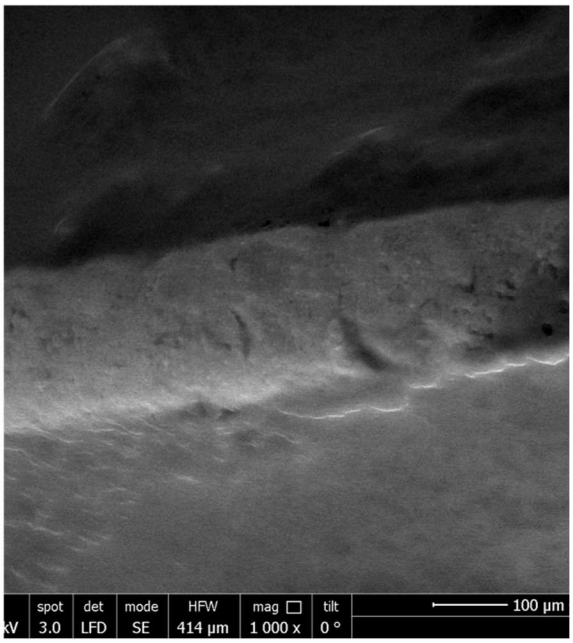
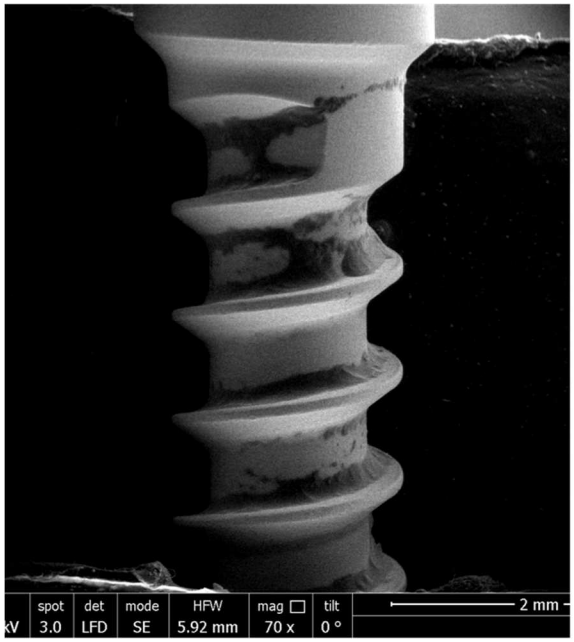
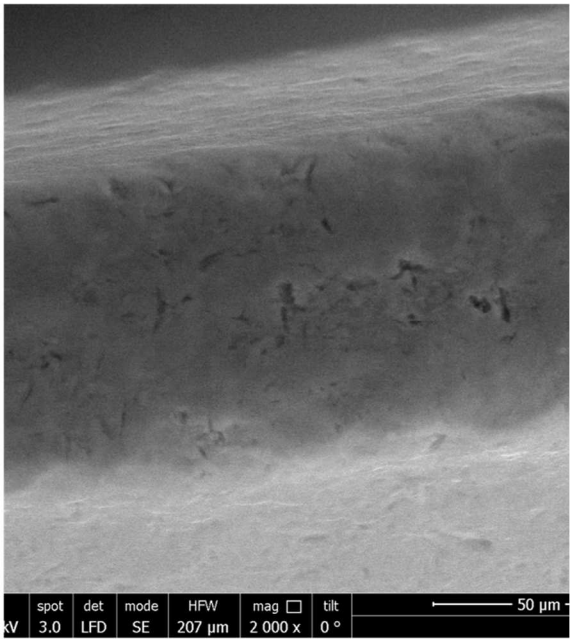


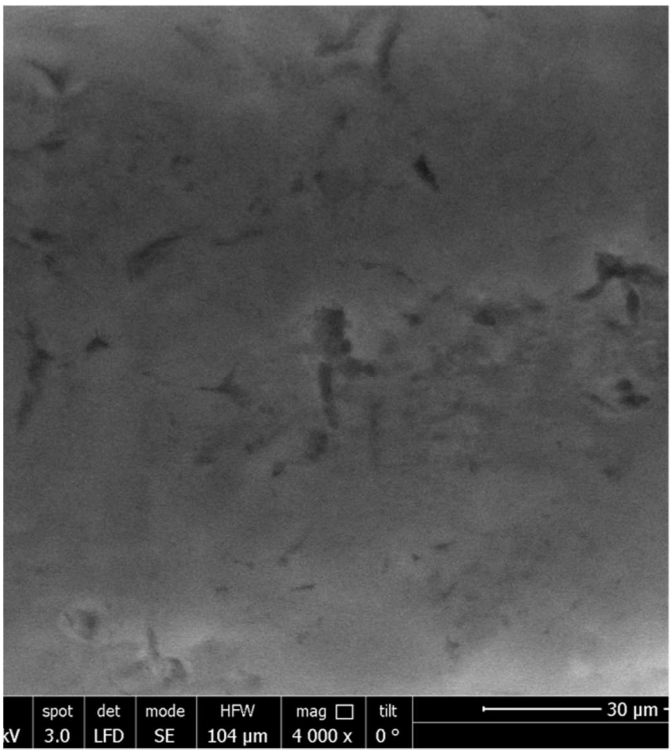




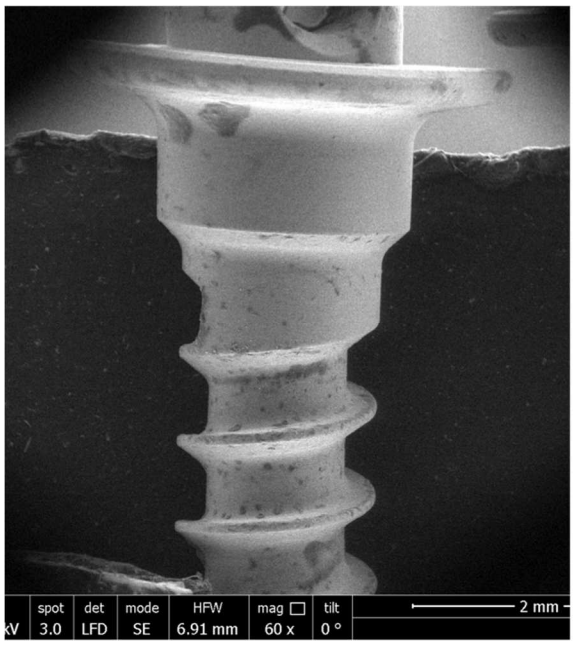
APPENDIX K
SEM Images Modified/Unexposed

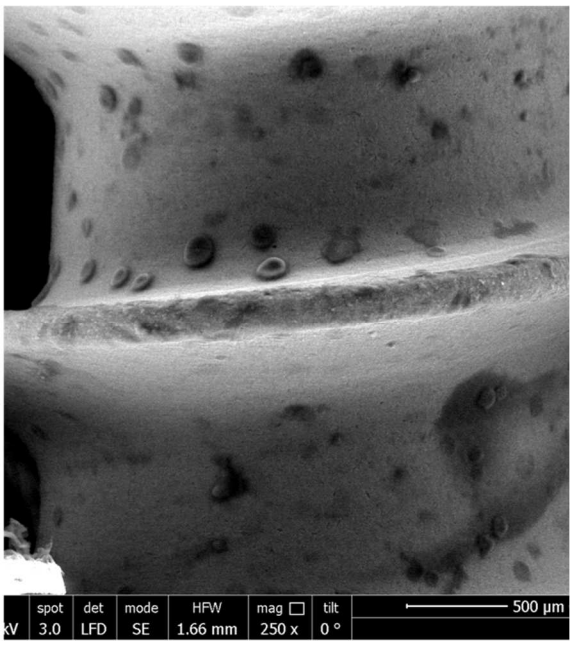


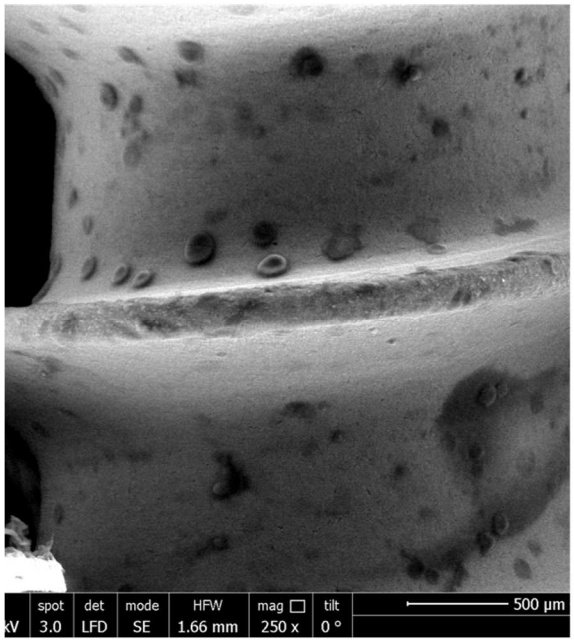


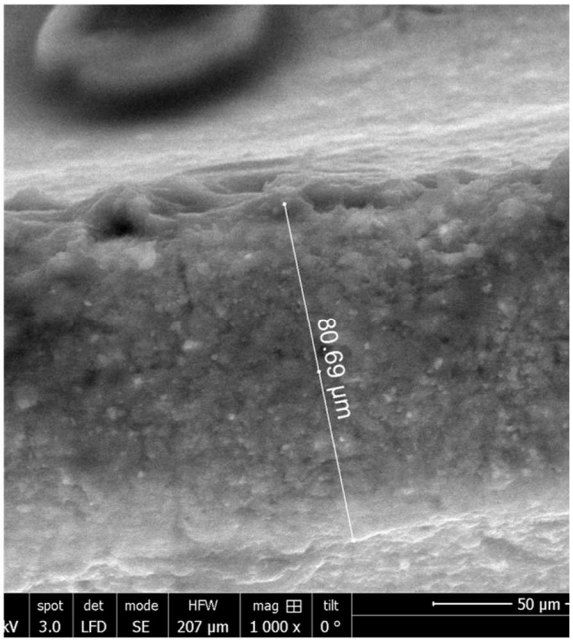


APPENDIX L
SEM Images Modified/Exposed



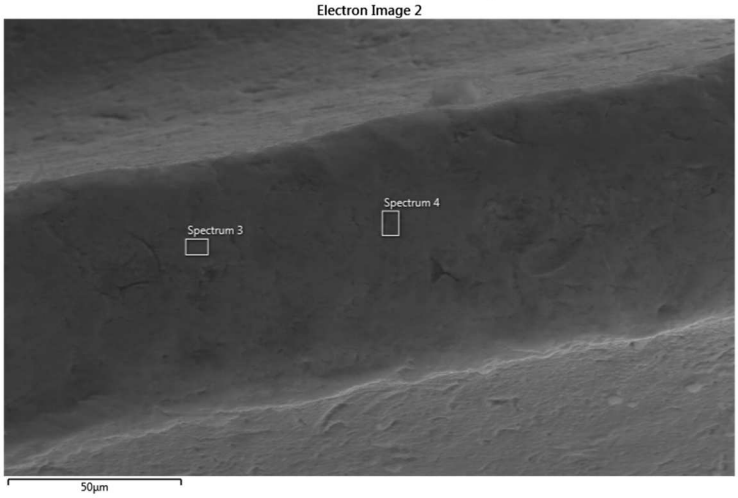


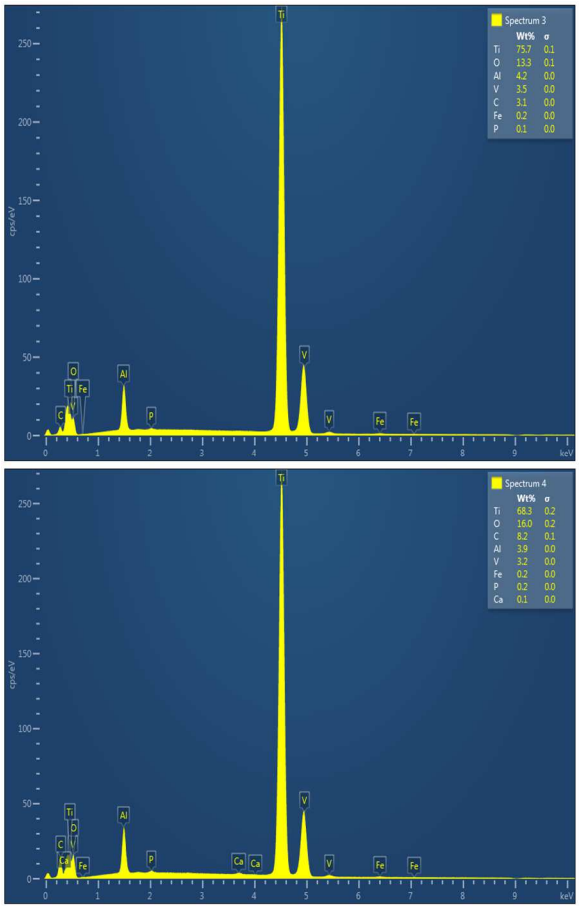




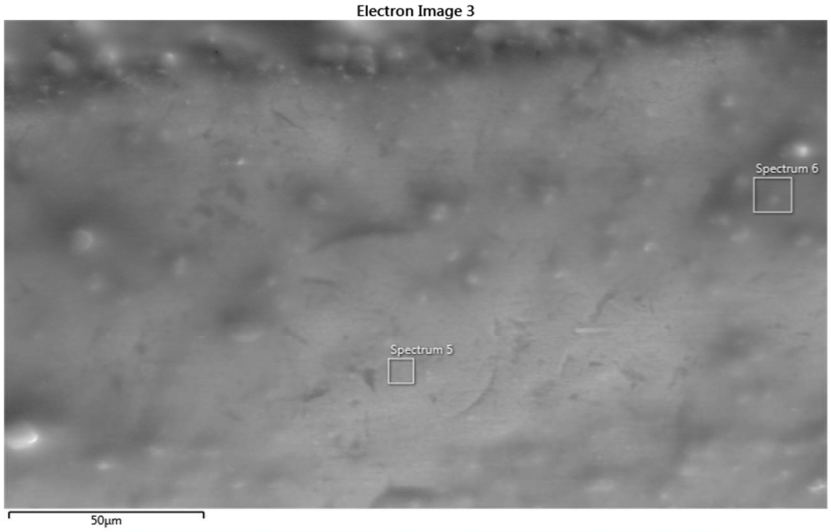
APPENDIX M

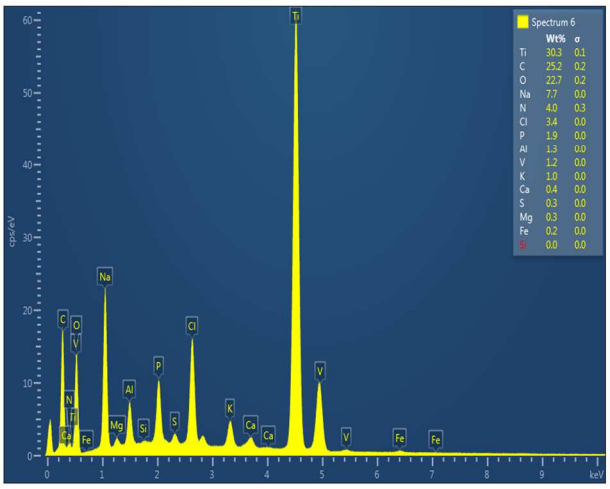
EDS Unmodified/Unexposed

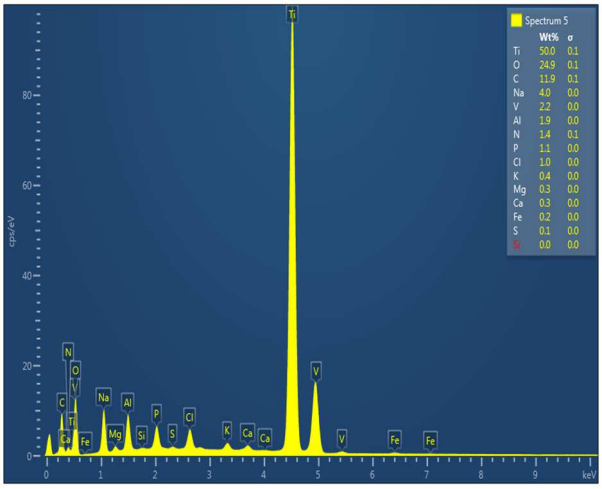




APPENDIX N
EDS Unmodified/Exposed

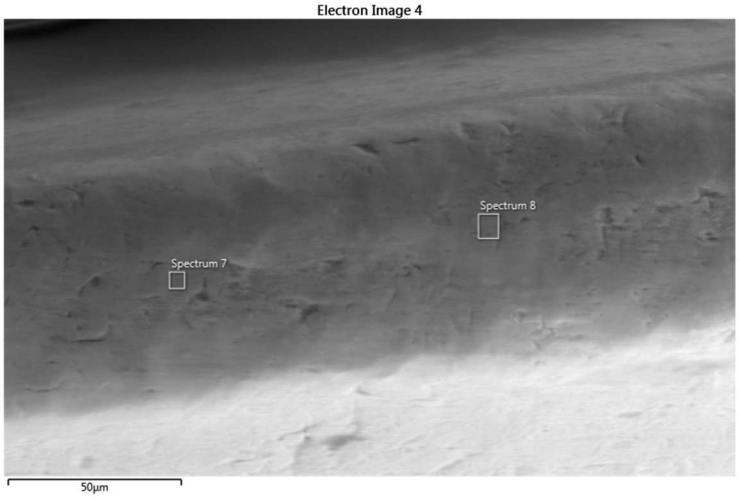

Electron Image 3
Spectrum 6
Spectrum 5
50µm

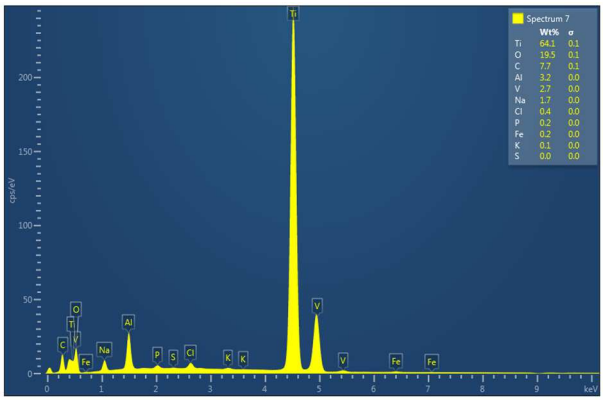

Spectrum 6
Wt% σ
Ti 30.3 0.1
C 25.2 0.2
O 22.7 0.2
Na 7.7 0.0
N 4.0 0.3
Cl 3.4 0.0
P 1.9 0.0
Al 1.3 0.0
V 1.2 0.0
K 1.0 0.0
Ca 0.4 0.0
S 0.3 0.0
Mg 0.3 0.0
Fe 0.2 0.0
Si 0.0 0.0
cps/eV
keV

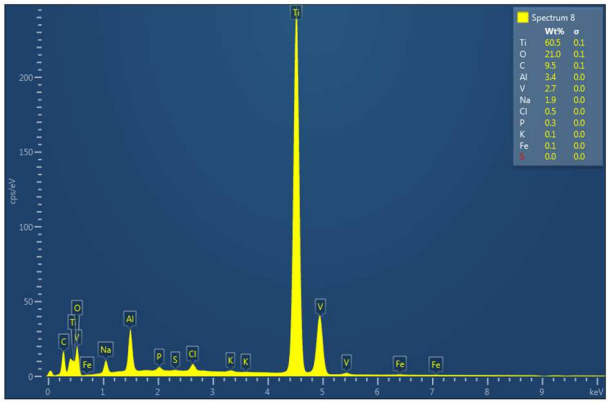

Spectrum 5
Wt% σ
Ti 50.0 0.1
O 24.9 0.1
C 11.9 0.1
Na 4.0 0.0
V 2.2 0.0
Al 1.9 0.0
N 1.4 0.1
P 1.1 0.0
Cl 1.0 0.0
K 0.4 0.0
Mg 0.3 0.0
Ca 0.3 0.0
Fe 0.2 0.0
S 0.1 0.0
Si 0.0 0.0
cps/eV
keV

APPENDIX O

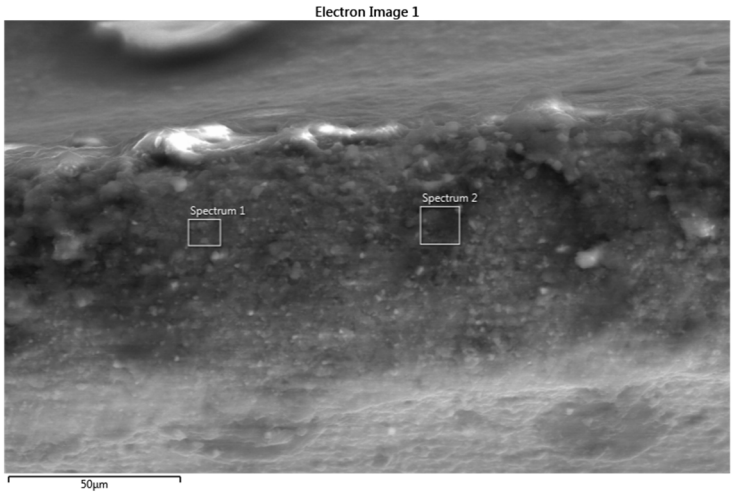
EDS Modified/Unexposed

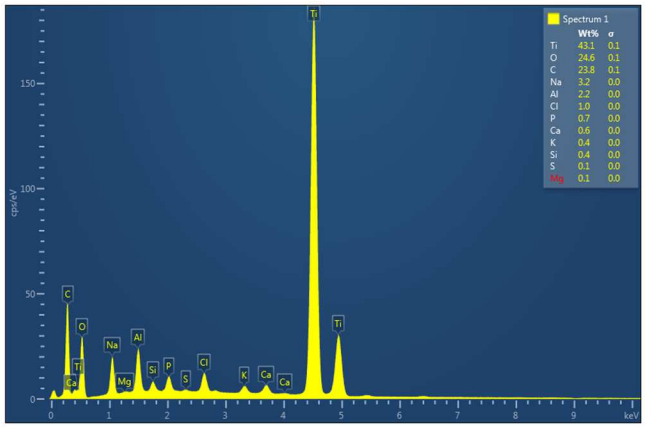


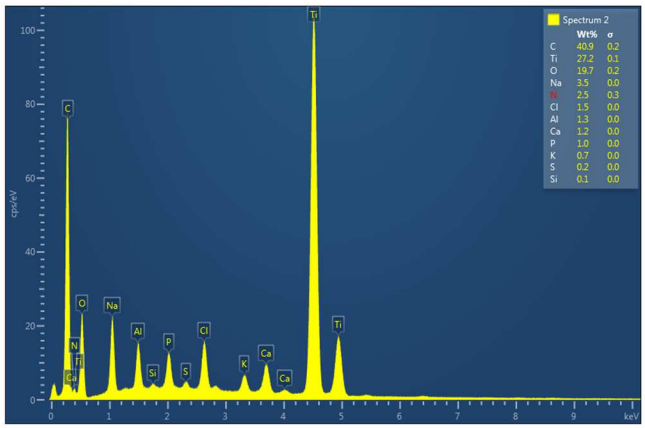




APPENDIX P
EDS Modified/Exposed


Electron Image 1
Spectrum 1
Spectrum 2
50µm


Spectrum 1
Wt% σ
Ti 43.1 0.1
O 24.6 0.1
C 23.8 0.1
Na 3.2 0.0
Al 2.2 0.0
Cl 1.0 0.0
P 0.7 0.0
Ca 0.6 0.0
K 0.4 0.0
Si 0.4 0.0
S 0.1 0.0
Mg 0.1 0.0
cps/eV
keV


Spectrum 2
Wt% σ
C 40.9 0.2
Ti 27.2 0.1
O 19.7 0.2
Na 3.5 0.0
N 2.5 0.3
Cl 1.5 0.0
Al 1.3 0.0
Ca 1.2 0.0
P 1.0 0.0
K 0.7 0.0
S 0.2 0.0
Si 0.1 0.0
cps/eV
keV