EFFECT OF ELECTROLYTE CONCENTRATION ON OSTEOBLAST RESPONSE TO ANODIZED TITANIUM

A Thesis

Presented to the Faculty of
The University of Texas Health Science Center at San Antonio
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
MASTER OF SCIENCE

By
Francisco X. Veray D.D.S

San Antonio, Texas

May 2004
EFFECT OF ELECTROLYTE CONCENTRATION ON OSTEONECROBLAST RESPONSE TO ANODIZED TITANIUM

Francisco X. Veray

APPROVED:

Supervising Professor

Date

APPROVED:

Merle S. Olson, PH.D.
Dean
EFFECT OF ELECTROLYTE CONCENTRATION ON OSTEOSBLAST RESPONSE TO ANODIZED TITANIUM

Francisco X. Veray

APPROVED:

[Signature]
Supervising Professor

[Signature]
[Signature]

Date

APPROVED:

[Signature]
Merle S. Olson, PH.D.
Dean
DEDICATION

This manuscript is dedicated to my wife, and kids. Without their support, and understanding it would have been impossible to finish. Thank you for always supporting me. Gracias y los quiero mucho.
ACKNOWLEDGEMENTS

I would like to declared my appreciation to DR. Joo L Ong (Department of Restorative Dentistry, UTHSCSA) for giving me the opportunity to work with him. I’m humble to have worked with a person of such extensive knowledge in the field of Biomaterials. Thank you for showing me with patience what research is all about.

I will like to also thank DR. Hyohan Kim (Department of Dental Biomaterials, Kyungpook National University, Korea) for providing me with the financial support needed to finish this project.

Finally to the rest of my supervising committee DR. Thomas Schneid, and DR Patrick Mattie (Department of Prosthodontics, Wilford Hall Medical Center) thank you for depositing your confidence on me.
EFFECT OF ELECTROLYTE CONCENTRATION ON OSTEOBLAST RESPONSE TO ANODIZED TITANIUM

Publication No. _________________________

Francisco X. Veray, D.D.S. (MS)

The University of Texas Health Science Center at San Antonio
Graduate School of Biomedical Sciences

Supervising Professor: Joo L. Ong

The overall objective of this study was to evaluate the effect of anodized titanium (Ti) surfaces on early osteoblast response in vitro. The specific aims for this study were to 1) determine the effects of varying electrolyte concentration on the properties of anodized Ti surfaces, and 2) determine cell response to anodized surfaces produced by varying the electrolyte concentration. Working hypotheses in this study were 1) properties of anodized Ti surfaces will be altered when produced using various electrolyte concentration, and 2) osteoblast differentiation on anodized Ti surfaces can be enhanced by varying the electrolyte concentration. In order to test these hypotheses, Ti surfaces (15 x 10 x 1 mm) were ground to 1200 grit, ultrasonically cleaned with acetone and a mixture of HF and HNO₃, and followed by rinsing with distilled water.
Surfaces were then anodized at 70A/m² using a DC power supply at a temperature of 25°C, maintained using a water bath. The electrolyte used was 0.2 M calcium acetate and either a 0.02, 0.03, or 0.04 M β- glycerophosphate (β-GP). Controls used were non-anodized Ti surfaces. Surfaces were UV sterilized for 48 hours. Surface roughness, SEM and x-ray diffraction analyses was done in all anodized surfaces. 250,000 cells /ml of an osteoblast precursor cell line was used to measure protein production and ALP over 12 days incubation. All data were statistically analyzed using ANOVA. In this study, the surface roughness of anodized Ti disks was observed to be significantly greater than control Ti surfaces (0.27 ± 0.02 μm). Surface roughness was significantly increased as β-GP concentration was increased from 0.02 M (0.7 ± 0.01 μm) to 0.04 M (1.10 ± 0.01 μm). The anodic oxide film was highly crystalline, consisting primarily of anatase. Crystallinity was observed to decrease with increasing β-GP concentration. Using albumin for protein adsorption study, significantly higher concentration of albumin was adsorbed on 0.03 and 0.04 β-GP concentration surfaces compared to other surfaces. From the cell culture study, no significant difference in protein production was observed. However, ALP specific activity on 0.02 and 0.04 β-GP surfaces were significantly higher compared to other surfaces after 12 days. In summary, differences in surface roughness, and crystallinity between anodized and control surfaces were observed. Albumin adsorption was affected by the concentration of β-GP. It was thus concluded that osteoblast differentiation, as indicated by ALP specific activity, was enhanced on anodized surfaces using electrolyte containing 0.02 and 0.04 β-GP.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>i</td>
</tr>
<tr>
<td>Approval</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Abstract</td>
<td>v</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>A. BACKGROUND</td>
<td>1</td>
</tr>
<tr>
<td>1. Use of Titanium in Implantology</td>
<td>1</td>
</tr>
<tr>
<td>2. Biocompatibility</td>
<td>2</td>
</tr>
<tr>
<td>3. Different Implant Modification</td>
<td>3</td>
</tr>
<tr>
<td>a. Machining</td>
<td>5</td>
</tr>
<tr>
<td>b. Grinding/polishing</td>
<td>6</td>
</tr>
<tr>
<td>c. Blasting</td>
<td>7</td>
</tr>
<tr>
<td>d. Wet chemical etching</td>
<td>8</td>
</tr>
<tr>
<td>i. Acid etching (pickling)</td>
<td>9</td>
</tr>
<tr>
<td>ii. Alkaline etching</td>
<td>10</td>
</tr>
<tr>
<td>e. Passivation treatment</td>
<td>10</td>
</tr>
</tbody>
</table>
f. Heat treatment .................................................. 11

g. Plasma spraying .................................................. 12

h. Electrochemical methods ....................................... 15
   i. Electropolishing .............................................. 15
   ii. Anodization .................................................. 16

4. Research problem .............................................. 19

B. OBJECTIVE ..................................................... 20
   1. Purpose ..................................................... 20
   2. Specific Aims ............................................... 20
   3. Working Hypothesis ....................................... 20

C. MATERIALS AND METHODS ..................................... 21
   1. Materials ................................................... 21
   2. Anodization Process ....................................... 21
   3. X-ray Diffraction ......................................... 22
   4. Roughness .................................................. 22
   5. Protein Adsorption Study .................................. 22
   6. Cell Attachment .......................................... 23
   7. Cell Culture ............................................... 24
      a. Cell surface and associated protein synthesis ....... 25
      b. Alkaline phosphate specific assay .................... 25
LIST OF TABLES

Table 1  Surface roughness of non-anodized and anodized
Ti surfaces as represented by its \( R_a \) values................................. 31

Table 2  Protein adsorption on non-anodized and anodized
Ti surfaces after 15 minutes incubation...................................... 33

Table 3  Cell attachment on non-anodized and anodized
Ti surfaces after 180 minutes incubation.................................. 35
LIST OF FIGURES

Figure 1  X-ray diffraction of anodized Ti at 70 A/m² in β-GP and 0.2 M CA (a) 0.02 M β-GP (b) 0.03 M β-GP (c) 0.04 M β-GP................................................................. 28

Figure 2  X-ray diffraction of non-anodized Ti surface showing the metal Ti peaks..... 29

Figure 3  Alkaline phosphatase specific activity of osteoblast precursor cells after culturing on-anodized Ti surfaces and Ti surfaces anodized at 70 A/m² in β-GP and 0.2 M CA (a) 0.02 M β-GP (b) 0.03 M β-GP (c) 0.04 M β-GP................................. ............ 37
A. BACKGROUND

1. Use of Titanium in Implantology

Titanium and titanium alloys have been a material of choice for used as implant devices in medicine and dentistry for several years now. Its discovery was attributed to Wilhein Gregor, a clergyman and amateur mineralogist. Gregor investigated black magnetic sand located at Menachran in Cornalwall in 1791. He named the sand mechanite and its oxide Menachin (Williams, David 1981). In 1795, Klaproth, a German scientist, named the new mineral titanium after the Titans, sons of the mother Earth. A manufacturing process was developed in 1910 that produced 98-99% pure titanium.

With respect other metallic implant materials such as stainless steel and cobalt-chrome alloys, titanium (Ti) has been reported to exhibit excellent corrosion resistance. In general, the excellent corrosion resistance of Ti and Ti alloys, with respect to other metallic implant materials, has been attributed to its ability to readily form a stable oxide. It has been reported that the Ti oxide of 3 to 5 nm thick is formed, (Ong 1993) thereby attributing to its excellent biocompatibility with host tissues (Branemark). In addition, the readily formed Ti oxide has been suggested to be amorphous using Raman spectroscopy (Ong 1995). Using high resolution x-ray photoelectron spectroscopy, the peak positions for Ti 2p_{1/2} and Ti 2p_{3/2} peaks and a 5.8 eV peak separation between the two Ti peaks indicated the presence of TiO_{2}, with an oxidation state of 4+ (Ong JL, 1995) In concurrence with Raman spectroscopy, differences in the full width half maximum value of Ti 2p_{3/2} peak for the Ti metals (1.3) and TiO_{2} single crystal (1.1), suggested an amorphous oxide layer. This has been confirmed by other investigators who have shown
FWHM values of 1.3 for Ti 2p$_{3/2}$ for TiO$_2$ films produced by reactive ion plating (Bange, 1991)

2. Biocompatibility

The stability of Ti oxide is known to render Ti metals excellent biocompatibility with host tissues. The official definition for biocompatibility comes from a consensus conference on definitions in biomaterials. Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific application (Williams, DF ed 1987). Never the less, most surgeons agree that if the implant is walled off in a tough, thin avascular capsule, and the reaction site is relatively stable after a month, the implant is considered biocompatible.

The biocompatibility of implants depended on the surface chemical composition and the ability of its surface to adsorb molecules and incorporate elements (Letic-Gavrilovic A. 2000). If the implanted material was incompatible, a thick connective tissue capsule would be formed around the implant and rapid rejection would occur (Linder 1975). However, with less toxic materials such as stainless steel, a thinner connective tissue developed around the implant. Utilization of vitallium and gold was reported to promote bone formation disordered characteristics in the border zone of the implant, and the ability of these implants to osseointegrate under this condition was not clear. However, it has been reported that the absence of well-oriented bone indicated that the implant had not fully accepted and that rejection of the implant may occur with time due to corrosion or release of toxic ions (Albrektsson).

In 1940, Bothe et al observed that Ti was fully tolerated by bone and had a tendency to grow into contact with it. The presence of a stable TiO$_2$ on Ti surfaces had
been reported to prevent cell-mediated rejection reactions (Kasemo 1983). Other investigators reported that the stable Ti oxide layer presumably aided in the formation of an osteogenic extracellular matrix at the implant–bone tissue interface, which was important for osseointegration (Kasemo B 1988, Stanford CM 1991). Per-Ingvar Branemark first defined osseointegration as direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant. Interestingly, it was reported that contact between the host tissues and the Ti implant metal was not established. Bone was reported to be in close apposition to the Ti implant surfaces, but not adhere to it. At an ultrastructural level, a biological layer of 5-10 nm thick was observed, separating Ti implants and bone (Thosen 1997). In reality, it was the tissue and the surface oxide of the metal that formed an intimate contact (Kasemo 1983), suggesting that the interface chemistry is determined by the oxide layer and not by the metal.

3. **Different Implant Modifications**

Aside from implant biocompatibility, surgical techniques, state of host, biomechanical status, and time, Lyndon F. Cooper also described that implant fixture design and surface characteristic affect bone cell reaction. Kasemo (1983) reported that the surface characteristic of implants, include surface composition and structure, surface energy, oxide thickness, and topography, play important roles in the formation of bone and maintenance of bone at implant surfaces. As such, to improve osseointegration, most implant manufacturers often made modifications to implant designs and surface characteristics. It had been documented that certain features of the implant such as
surfaces topographies would influence different bone cell reactions to the surfaces (Jansen 1991).

According to Cooper, implant modifications such as an increase surface roughness increased implant area adjacent to bone. This increase in implant area resulted in improved cell attachment to the implant surface, increased bone at the bone-implant interface, increased biomechanical interaction of the implant to bone. Other investigators such as Buser et al also concluded that implant roughness influenced the amount of bone at the bone-implant interface. Aside from varying implant roughness, other modifications included modification of surface chemistries and structures using physical process such as the use of plasma spraying and metallic oxide enhancement using passivation and anodization. Other surface modifications included the attachment of polyethylene glycol as means of inhibiting bacterial growth (Olsson et al., 1990; Olsson et al., 1991). Radio-frequency glow-discharge (RFGD) treatments have been used to produce clean, surface-activated surface, with a thinner and more stable oxide film (Mattox 1982; Baier 1991; Kawahara et al., 1996). The RFGD treatments were shown to elevate the surface to a higher energy state with the possibility of having superior interface layers for greater cellular-adhesion potential in implant applications (Baier 1991; Baier and Meyer 1988; Baier et al.1982). By altering the surface chemistry of Ti, different rates of cellular attachment have been observed (Keller et al., 1989; Jansen et al., 1991; Ellingsen, 1991; Ong et al., 1992; Sukenik et al., 1990; Keller et al., 1990; Michaels et al., 1991). Listed below are examples of commercially available implant surfaces and the process used for modifying the surface.
a. **Machining**: Medical devices are often manufactured by machining metallic Ti (lathing, milling, threading). The properties of machined surfaces are determined by variables such as work-piece speed, tool pressure and choice of lubricant.

Surface spectroscopic analyses of machined titanium implant surfaces indicated a typical oxide layer with thickness of 3-6 nm. Surface properties are often dependent in the type of sterilization used. Different sterilization techniques were shown to produce different surface alterations (Zisman 1964; Young 1988; Keller et al., 1990; Doundoulakis 1987; Lacefield, 1988). A change in the contact angle behavior was reported when metallic specimens were sterilized by conventional steam autoclave (Zisman 1964). A shift in the contact angle to a higher value was consistent with the deposition of hydrophobic organic contaminants over the surface. The deposition of hydrophobic organic contaminants over the surface also resulted in increased oxide thickness of about 250-700 Å, with critical surface tension in the mid 30s dyne/cm (Zisman 1964; Young 1988; Keller et al., 1990). This critical surface tension was reported to decrease with the deposition of hygroscopic salt residues in the contaminant layer (Zisman 1964). With dry heat sterilization, microashed organic contaminations were reported. These contaminations did not alter the surface energy of the specimen (Doundoulakis 1987; Lacefield, 1988). Furthermore, conventional sterilization of Ti devices compromised the surface properties by depositing organic contaminants on the Ti surfaces (Doundoulakis 1987). Fibroblast attachment to the conventionally sterilized Ti surfaces was affected, whereas steam autoclaving of Ti surfaces inhibited cell attachment (Keller et al., 1990). Compared to acid passivation, a significant reduction in fibroblast
attachment was reported when Ti was sterilized using either ethylene oxide gas or ethyl alcohol immersion (Keller et al., 1990).

The Ti surface consisted mainly of TiO$_2$ (Ayrs 1998, Henry 1987, Lausmaa J. 1991), with the presence of small amounts of Ti$_2$O$_3$, TiO, and Ti nitride (Lausmaa J, Kasemo B. 1990). Various types of organic contaminants such as hydrocarbons, fatty acids, silicones, and inorganic contaminants were often detected. Machining grooves were often oriented along the machining direction. Several investigators have reported that tightly spaced grooves have been shown to be more effective than widely spaced grooves in orienting cells through contact guidance (Brunette 1987; Chehroudi et al., 1989; Chehroudi et al., 1990; Ohara and Buck 1979). In addition, since no visible grain was detected, it was indicated that the surface layer was plastically deformed (Buser 1999, Wennerberg 1996, Keller 1994). However, depending on the parameters used for machining, the surface roughness values were reported to be within the range 0.3-0.6 μm when measured by optical or stylus profilometry (Buser 1999, Wennerberg 1996, Keller 1994).

b. **Grinding and polishing:** According to Hignet (1987), these two methods are identical based on the removal of material using a hard abrasive medium. With polishing, the abrasive medium is attached to a soft backing, and the polishing process involves the use of successively finer abrasive grades, applied in different directions and often in combination with lubrication to produce smooth surface finishes. Commonly used polishing media included silicon carbide, alumina and diamond. The finest polishing grades can be used to produce extremely smooth, mirror-like surface finish on flat
specimens, with $R_a$ values of 0.1 $\mu$m or less. Grinding is the preferred term when the medium is coarse and attached to a firm backing, leading to a faster removal and relatively rough surface topographies. Surfaces ground with an abrasive grade 60 was reported to result in a $R_a$ value of approximately 1 $\mu$m (Bowers 1992). Using the coarsest grades can result in $R_a$ value of 5-5 $\mu$m and topography with preferential orientation (Hignet 1987).

It should be mention that this method will lead to plastic deformation and stresses in the surface region of the material. Some of the particles could be embedded into the surface. This is particularly true during grinding of pure Ti. The chemical composition of the surface and the exact nature of the oxide layer could be influenced, for example, by elevated temperatures or lubricants. It should be noted that grinding and polishing are, however in general used as an intermediate step prior to chemical, electrochemical or other methods which will ultimately determine the properties of the finished surface (Brunette 2001)

c. **Blasting:** Another method for modifying surfaces is grit blasting. This process is based on the bombardment of the surface by hard particles at high velocity. These particles can be either dry or suspended in a liquid. Various types of ceramic particles (alumina, silica, titania) of different sizes can be used for modifying titanium surfaces. In biomedical application, blasting techniques are commonly used for cleaning (descaling) and surfaces roughening of commercial implants (e.g. dental implants, pacemakers, femoral stems).
It has been reported that particles are likely to become embedded in the titanium surface during blasting usually alumina and silica particles (Darvell BW 1995, Kern 1994, Wennerberg 1996). For this reason, it is recommended that blasted surfaces be chemically treated in order to remove particulate contamination (ASTM Standard B600 1997). In addition, blasting particles can be used to intentionally modify the surface composition. For example, it has been reported that blasting with hydroxyapatite (HA) particles resulted in the formation of adherent apatite coatings on titanium implant surfaces (Ishikawa 1997). It is also known that controlling particle size resulted in varying surface topography. In general, the more abrasive blasting produces surfaces with irregular topography and sharp features. In contrast, gentle shot peening process produces a much smoother and more rounded surface topographies. In spite of the frequent use of blasting to modify implant surfaces, studies on the composition and thickness of oxide layers on blasted titanium surfaces are lacking. It can be assumed that since blasting process does not involve elevated temperatures and is normally carried out in air or water, the oxide layer on Ti surfaces will be TiO₂.

d. **Wet chemical etching**: The process of etching resulted in the dissolution of the native oxide surface and parts of the underlying metal. In addition, surface roughness is increased with chemical etching. Etching treatments for Ti are based on the use of solution, which react with the oxide and Ti, thereby producing soluble reaction products. However, the choice of chemicals to etch Ti is limited to a few acids and alkaline due to the chemical stability of its oxides. The following is a description of etching using acid and alkaline:
i. **Acid etching (pickling):** Etching, or technically referred to as pickling, is the process whereby the oxide scales are removed to obtain a clean and uniform surface finish. Formation of a thin surface oxide layer of less than 10 nm thick was reported after acid etching (Sitting 1999). In addition, these formed oxide layers have been suggested to grow logarithmically in air, from \( \sim 3 \) nm to \( \sim 6 \) nm during a 400 day period (Sitting 1999). The oxide composition was reported to be predominantly TiO\(_2\), with residues from the etching solution frequently observed.

The recommended and most commonly used solution for acid pickling of Ti and Ti alloys consists of 10-30% (volume) of nitric acid (HNO\(_3\)) and 1-3% (volume) of hydrofluoric acids (HF) in distilled water (ASTM Standard B600, 1997). It is known that HF readily attacks the TiO\(_2\) and reacts with Ti to form soluble titanium fluorides and hydrogen. It was also reported that pickling treatments led to the incorporation of hydrogen ions in the region below the oxide (Taborelli 1997). Such absorption of hydrogen into the Ti has been suggested to cause embitterment of the surface layer (Brunette 2001). As such, the ratio of HNO\(_3\) to HF should be maintained at 10:1 to minimize free hydrogen formation (ASTM Standard B600 1997).

Surface topography of acid etched Ti surfaces was suggested to be dependent on the previous surface condition and the extent of material removed (Brunette D.M 2001). “Mild” treatments were reported to preserve the main features of the previous topography. Microstructures of the bulk material, with clear visible grain and grain boundaries, were observed when significant amounts of material were removed (Niger H 1997, Sitting 1999). When Ti was strongly etched in HF, a faceted structure with clear
visible grains and grain boundaries was observed (Brunette D.M 2001). The faceted surface indicated differential etching rates of various crystalline directions, thereby exposing crystal surfaces oriented in low-index directions. For Ti alloys containing α and β phases, differences in etching rates for the different phases were observed. The β phase was suggested to etch faster compared to the α phase, thereby allowing the β phase to protrude from the α phase (Brunette D.M 2001). When compared to etched Ti surfaces without blasting, a more irregular surface topography was observed when Ti surfaces were blasted prior to acid etching (Buser 1999, Martin 1995). Depending on the pre-treatment and the process conditions, surface roughness of etched surfaces was reported to be in the range of a few 0.1 μm to several μm (Buser 1999, Kinwomen 1992, Urengibock 1994, Niger H 1997 Sitting 1999).

ii. Alkaline etching: Alkaline etching of Ti surfaces are reported primarily as a pre-treatment for coating gel-derived apatite (Nishigushis1999, Wen 1998, Kim 1999, 1997, 1996). Treatment of Ti 4-5 M NaOH at 60 °C for 24 hours had been reported to produce a surface layer consisting of a sodium titanate gel (Kim 1999, 1997, 1996). The surface layers were reported to be in the order of 1 μm thick, with an irregular topography and a high degree of open porosity on the submicron scale. Heat treatments were also reported to be used for modifying the composition and structure of the surface layers if desired (Brunette DM 2001).

d. Passivation treatments: Passivation is used to obtain a uniform oxidized state, with an ultimate goal to obtain a dense, stable oxide film. Two major passivation
treatments for Ti are a) immersion in strongly oxidizing acid, and b) heat treatments in air or immersion in boiling water.

Nitric acid passivation of Ti is most commonly used and a recommended ASTM procedure for medical devices (ASTM Standard F-86 1996). Using this procedure, the Ti is immersed for a minimum of 30 minutes in a 20-40% (volume) solution of HNO₃ at room temperature. Thorough rinsing and drying thereafter neutralize the surface. Passivation treatment is often performed prior to sterilization. However, nitric acid passivation has been reported to have no major influence on the overall surface topography of Ti surfaces (Sitting C, 1999). Thin (2-6 nm) surface oxides, composed mainly of TiO₂ and oxides of alloying elements and some suboxides, were reported at the metal oxide interface (Poullieau 1997). However, due to experimental difficulties involved in evaluating the structure of thin oxide films, there exists no reliable evidence regarding the microstructural properties of passivated oxide films on Ti. With regard to surface contaminants, passivated Ti surfaces show similar characteristic to those of most Ti surfaces oxidized at room temperature (Ong et al., 1993).

e. **Heat treatments:** Heating in air at 400°C to 600°C or ageing in boiling deionized water for several hours have been used as alternative passivation treatments for Ti-6Al-4V alloy (Oji 1999, Lee TM 2000, Browne M 1996, Browne M 1994, Kipaldi DV 2000, Hazan R 1993). No major changes in the overall surface topography were reported with heat treatments when compared to nitric acid passivation (Brunette DM 2001). However, passivation at 400°C was reported to increase oxide thickness to 30 nm (Radegran G, 1991).
f. **Plasma spraying:** Plasma spraying is one of the commercially accepted coating methods for modifying Ti implant surfaces. In the plasma-spraying process, a direct current (DC) electric arc is struck between two electrodes, while a stream of gases passes through this arc. This results in an ionized high temperature gas of up to 30,000°C. A large gaseous expansion occurs as a result of the increase in gaseous temperature, thereby causing the carrier gas stream to pass through the arc at a speed approaching the speed of sound. Coating powder is suspended in the carrier gas stream, which is fed into the plasma flame. The heat content of the plasma flame, and thus the ability to increase the temperature of a particle, depends strongly on the gas used. In addition, the longer a particle resides in the plasma flame, the higher will be the plasma temperature. Ideally, only a thin outer layer of each powder particle gets into the molten plastic state, which unavoidably undergoes some phase transitions. This plastic state, however, is necessary to ensure dense and adhesive coatings. As such, properties of plasma sprayed coatings depends on the optimum relation between particle size, gas used, speed of the plasma, the distance between plasma nozzle and substrates, and the cooling process for the desired composition and crystallinity.

Operation of DC plasma guns used in plasma spraying is characterized by arc instabilities, which affect the plasma jet outside the nozzle. These instabilities are governed by the electrode design, the gas injection mode, the spraying parameters and the condition of the anode wall (Janisson et al., 1999). Among the above factors, the electrode design, the gas injection mode, and the condition of the anode wall have been fixed as soon as a plasma spraying equipment is chosen. In the plasma spraying process,
some basic parameters include power, current, distance between nozzle and substrates, plasma work gas rate, carrier gas rate and powder feed rate. Regulating the above plasma spraying parameters controls the properties of coatings, whereas spraying time was used to regulate the coating thickness. Among the various coating parameters, plasma work gas composition is an important parameter that can be regulated to obtain coatings with the desired properties. First, plasma work gas composition influences the crystallinity of the coating. Argon mixed with hydrogen gives a higher degree of crystallinity. However, without hydrogen, the powder particles cannot enter the gas. This is because the high velocity and viscosity of the argon gas cause the particles to bounce back from the flame, instead of entering it. Thus, it is obvious that the plasma-spraying technology allows for the production of either amorphous or partially crystalline coatings of HA or titanium plasma-sprayed (TPS) on implants surfaces. Second, plasma work gas composition also influences the thickness. For example, the use of nitrogen as the carrier gas resulted in a thicker coating layer as compared to the use of argon as the carrier gas.

It is well known that both Ti and apatite are thermodynamically unstable at plasma spray temperatures. The TPS-coated dental implants are designed for the mandibular symphysis anterior to the mental foramen. For most dental implants, the plasma sprayed layer begins at the implant shoulder. It is from this point that the implant will be completely embedded in jawbone. During deposition, TPS coatings are usually performed in a vacuum atmosphere in order to avoid the oxidation of metal particles. Babbush et al. provides a sample coating parameter for TPS coatings (Babbush et al., 1986). The coating was produced using an argon gas flame spraying powdered titanium at 15,000°C onto a core not heated above 220°C. This process gives a porous coating,
0.04 to 0.05 mm thick, on the threaded area of the implant with bond strength of 0.5 kp/mm² and no impairment of fatigue resistance. Overgaard et al. demonstrated that porosity of plasma-coated surface led to more rapid healing and substantially lesser foreign body reaction (Overgaard et al., 1997). Scanning electron microscopy shows that bone is able to grow into these pores without a connective tissue membrane. Additionally, plasma coating results in a six fold surface enlargement of the anchored implant portion, there by substantially improving the micro anchoring characteristic of the bone and lowering the specific pressure per unit area. However, a very thin TPS coating is not desirable because it cannot provide enough rough surfaces for bone anchoring.

Calcium oxide is usually formed when HA deposition is performed in air. The integrity of the coating produced in air will depend on the amount of calcium oxide formed. The reaction of high concentration of calcium oxide with water usually resulted in poor coating integrity. An increase in calcium oxide formation also occurs when a higher concentration of hydrogen is used as a carrier gas as compared to the use of pure nitrogen as a carrier gas. The use of hydrogen resulted in more flame enthalpy and a lower flame velocity. As such, the particle undergoes more melting and decomposing when a higher concentration of hydrogen is used as a carrier gas.

The deposition of HA coatings on medical devices are between 50-to100 µm thick. Coatings with thickness greater than 80 µm become brittle, whereas very thin HA coatings may resorb too fast. Bond strength of the HA coating to the metal is difficult to measure, but generally is higher than that of porous-bead coatings. Significant porosity of the HA material lowers the mechanical strength and increases the resorption rate.
Good coatings are completely dense and consist of pure HA. The Ca/P ratio in the coating should be as close to that of natural HA (10/6=1.67). Combinations of HA with tricalcium phosphate have been studied but clinical application of these coatings is rare. This is because tricalcium phosphate has been known to have a much faster degradation rate than HA, and may lead to uncontrolled degradation of the entire coating. Other calcium phosphates such as fluorapatite (Clemens et al., 1998; Gineste et al., 1999), whitlockites (Dhert et al., 1993), or brushite (Kumar et al., 1999), have been studied as coating material, but no advantages were established to justify their clinical applications (Overgaard et al., 1997; Overgaard et al., 1998; Overgaard et al., 1997).

g. **Electrochemical methods:** Electrochemical surface treatments methods are based on different chemical reactions occurring at an electrically energized surface (electrode) placed in an electrolyte. The two most commonly used electrochemical methods are electropolishing and anodic oxidation. For both methods, the specimen to be treated is made the anode in an electrochemical circuit. When the electrical power is connected, a current flows through the circuit due to reduction and oxidation reactions at the electrodes and ion conduction through the electrolyte. Depending on the choice of electrolyte and the other processing parameters such as electrode potential, temperature, and current the process will have different effects on the sample (anode) surface (Brunette DM, 2001).

i. **Electropolishing:** This process is controlled electrochemical dissolution of the surface. The electrolyte usually consists of a mixture of an acid and alcohols. The
standard electrolyte used for electropolishing Ti is composed of 60 ml perchloric acid, 350 ml n-butanol, and 540 ml methanol held at 25°C or lower. The electrolyte serves two purposes a) electrode reaction that yields soluble products, and b) the formation of a viscous layer that does not conform to the microtopography and which provides a mass transport – controlled reaction (dissolution rate). The polishing action of the process is due to the fact that the viscous layer is thinner (and the electropolishing current higher) at protruding parts, which therefore dissolve faster than other parts of the surface. A successful result requires that, in addition to the right choice of electrolyte, the hydrodynamic conditions be controlled to achieve a proper viscous layer at the surface. Depending on the exact process parameters, the rate of material removal (dimensional change) will be in the range 1-10 μm per minute for Ti. In addition, electropolishing leads to a crystalline termination of the metal underlying the oxide as a result of the non-mechanical removal of material. However, electropolishing process does not add any mechanical stresses to the surface. The thickness of surface oxide produced by electropolishing method approximately 5nm and is composed mainly of TiO₂. It had also been reported that electropolished Ti surfaces produced a mirror finish, with a typical surface roughness value (Rₐ) of 10 nm. (Lausmaa J 1990). It was also observed that electropolished Ti surfaces were almost smooth using SEM, with the exception of occasional pits that were preferentially located at grain boundaries (Lausmaa J 1990).

ii. Anodization (anodic oxidation): Anodic oxidation electrode reactions, in combination with electrical-field driven metal and oxygen ion diffusion, leads to the formation of an oxide film at the anode surface. The main technological use of anodizing
Ti is to produce different types of protective oxide films on metallic Ti surfaces. Another use of the anodization process is to obtain an increased oxide thickness, thereby resulting in enhanced corrosion protection and decreased ion release. In addition to the increased oxide thickness, porous oxide coatings are usually formed. Controlling the process parameters, such as anode potential, electrolyte composition, temperature and current, could vary the structural and chemical properties of anodic oxides on Ti. Different diluted acids such as H$_2$SO$_4$, H$_3$PO$_4$, and acetic acid are commonly used as electrolytes for anodic oxidation of Ti. The Ti and oxygen ions formed in these redox reactions are driven through the oxide by the externally applied electric field, thereby leading to the growth of the oxide. Since anodic Ti oxides have a high resistivity relative to the electrolyte and the metallic parts of the electrical circuit, the applied voltage drop would occur over the oxide film of the anode. As long as the electric field is strong enough to drive the ions through the oxide, a current will flow and the oxide will continue to grow. As a result, the final oxide thickness, $d$, during anodic oxidation is almost linearly dependent on the applied voltage, $U$:

$$d = \alpha U$$

where $\alpha$ is a growth constant, which is usually within the range 1.5 nm V$^{-1}$ to 3 nm V$^{-1}$. This linear relationship holds below the dielectric breakdown limit of the oxide, which is around 100 V, depending on electrolyte and other process conditions.

The anodizing process can be done either at constant current (galvanostatic process) or constant voltage control. If the anodizing is carried out at voltages above the breakdown limit, the oxide will no longer be resistive enough to prevent further current flow and oxide growth. At such high voltages the process will lead to increased gas
evolution and frequently also sparking. This type of anodizing is often referred to as spark anodizing. This usually happens when using voltages of 150-200 V and thereby resulting in a less uniform and a more porous oxide as compared to samples anodized below the dielectric breakdown limit. Spark anodizing can be used for producing firmly adherent oxide films up to 20 μm in thickness. On Ti metal, spark deposited anodic films was observed to result in porous and relatively rough topography, but lacking sharp edges. It had been reported that the open porosity opened up possibilities with regard to drug incorporation and release around Ti implants (Dunn 1993, 1994). In addition, the precipitation of apatite on oxide surfaces after hydrothermal treatment provided an alternative mean for preparing “bioactive” surfaces (Suh et al., 2003; Zhu et al., in press; Rodriguez et al., in press). It was reported that the occurrence of spark discharge at a high electrolytic voltage during anodic oxidation was a superior method for forming rough and porous oxide surfaces (Ito 1988).

For oxide films prepared below the breakdown voltage, these films contain anions from the electrolyte. Oxide composition of these films can be varied by carrying out the process in electrolytes containing calcium (Ca) and/or phosphorus (P) ions, such as glycerophosphate/calcium acetate mixtures (Ishizawa H, Ogino M, 1995). It had also been reported that this anodization method could be adapted to any shaped Ti, with the resulting Ti oxide to be greater than 1 μm thick (S. Ito 1998). The incorporation of P and Ca into Ti oxide in vitro had been observed to result in the formation of a calcium phosphate layer similar to apatite (Hanawa T. 1991). Other studies had observed interconnected pores (1-2 μm in diameter) on anodic oxide, with intermediate roughness of 0.60-1.50 μm (Zhu et al., 2002). In addition, the anodic oxide was observed to contain
a mixture of amorphous, anatase, and rutile oxides (Zhu et al., 2002). With an increase in the anodizing voltage and/or concentration of calcium incorporated into the oxide, the degree of oxide crystallinity was observed to increased (Zhu et al., 2002). However, with an increase in the concentration of β- GP, the degree of oxide crystallinity decreased (Zhu et al., 2002).

4. Research Problem

Although anodization has been used as a mean to modify implant surfaces, the effect of electrolyte concentration on surface properties and cellular responses has not been documented. As such, it is proposed that there is a need to characterize anodized surfaces using available analytical tools and systemically evaluate osteoblast response to the characterized surfaces.
B. OBJECTIVE

1. Purpose

The objective of this study was to examine the effect of electrolyte concentration on anodized Ti surfaces and its effect on the phenotype expression of osteoblast precursor cells.

2. Specific Aims

Aim I: To evaluate the effects of varying electrolyte concentration on the properties of anodized Ti surfaces

Aim II: To evaluate cell responses to well-characterized anodized surfaces produced by varying the electrolyte concentration.

3. Working Hypothesis

Hypothesis I: Properties of anodized Ti surfaces will be altered when produce using various electrolyte concentrations.

Hypothesis II: Varying the electrolyte concentration can enhance osteoblast differentiation on anodized Ti surfaces.
C. MATERIALS AND METHODS

1. Materials

Specimen (size 15 mm x 10 mm x 1 mm) were cut from commercially pure (cp) Ti plate grade 2 using an Isomet 11-1180 Low speed saw (Buehler Ltd, Evanston, Illinois, U.S.). The specimens were abraded using silicon carbide sand paper No. 1200 and followed by ultrasonic cleaning in acetone for 180 seconds. The specimens were subsequently be pickled using a mixture of aqueous hydrofluoric acid (HF) and nitric acid (HNO₃) (mole ratio HF:HNO₃ = 1:3). The specimens were then rinsed with distilled water, air dried, and divided into 5 groups. One of the groups was used as controls (no treatment), whereas the other 4 groups will be anodized using various electrolyte concentrations.

2. Anodization Process

Anodizing was performed on a regulated DC power supply and conducted by employing a constant current at 70 A/m² (EC135-90 Thermo EC) in an electrolyte mixture containing 0.2 M calcium acetate (CA) and varying concentration of β-glycerophosphate (β-GP). The concentrations of β-GP will be 0.02 M, 0.03 M, 0.04 M, and 0.05 M. Temperature was maintained at 25°C by water bath during anodizing. After anodization, specimens were rinse with distilled water several times and air-dried. All samples were sterilized under UV light for 48 hours prior to materials characterization and in vitro cell culture study.
3. **X-ray Diffraction (XRD)**

Crystallographic structure and crystallinity of Ti surfaces were determined using Advance D 8 x-ray diffractometer from Bruker AXS. Three samples from each concentration were analyzed from 25° (2θ) to 95° (2θ) at 0.1° per minute scan rate. The samples were analyzed using Cu Kα radiation having energies of 40 keV and 30 mA was used.

4. **Roughness**

Using a Profilometer (Taylor- Hobson surtronic 3), triplicate specimens were tested. Range of the measuring styli will be 9.99 μm. Machine was calibrated using a reference specimen: 230 u inches= 5.8 mm. Mean surface roughness between the different Ti surfaces were statistically analyzed using the ANOVA test, and differences were considered significant if P < 0.05

5. **Protein Adsorption Study**

Bovine serum albumin, fraction V (Pierce, PERBIO, IL) and bovine plasma fibronectin (Sigma, MO) was used as model proteins in this study. The manufacturer confirmed purity of the proteins. 200μl of each protein solution (1mg/ml protein/saline solution) were pipette onto Ti disks in a 6 well plate (Figure 1). The study was then conducted in a sterile humidified incubator at 37°C for 15 minutes. The non-adherent proteins was removed and washed twice using saline. The removed solution was saved and recorded as total volume. 100 μl aliquot of the initial and removed solution was mixed with 150 μl of Micro (Bicinchoninic Acid) BCA working reagent in a 96 well
plate and incubated at 37°C for 120 minutes. Protein concentrations were analyzed using the micro BCA protein assay (Pierce Chemical Co., Rockford, IL) and measured using an Opsys MR microplate reader (Dynex Technologies, Franklin, MA) at 595nm. Each protein concentration was calibrated using standard curve. The degree of adsorption was determined by subtracting the residual protein from the initial added protein. Measurement was performed in triplicate for each time point. Mean adsorption protein concentrations between the different Ti surfaces were statistically analyzed using the ANOVA test, and differences were considered significant if P < 0.05.

6. Cell Attachment Study

The cell attachment study was conducted using the ATCC CRL 1486 human embryonic palatal mesenchyme (HEPM) cells, an osteoblast precursor cell line. The cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 7% FBS, penicillin (5000 units/ml), and streptomycin (5000 µg/ml), and fungizone (250 µg/ml) in a 5% CO₂ humidified incubator at 37°C and the medium was changed twice a week. Osteoblasts from pre-confluent cultures was harvested with 0.25% trypsin – 1mMEDTA (GibcoBRL, life Technologies, NY), and was centrifuged, and produced to cell suspension with serum-free DMEM. The cells were seeded onto the Ti sample in 6 well culture plates at a density of 10,000 cells each sample (Figure 1) and incubated in a 5% CO₂ humidified incubator at 37°C for 180 minutes. Cell concentration was analyzed using the Vybrant™ cell adhesion assay (Molecular Probes, Eugene, OR). The non-adherent cells were then removed and washed twice using serum-free DMEM. The removed solution was saved and recorded as total volume. Calcein AM (from the
adhesion assay kit) was added into the initial and removed solution at a concentration of 5 μM. Calcein AM is nonfluorescent but once loaded into cells, is cleaved by endogenous esterases to produce highly fluorescent calcein. A 100 μl aliquot of the initial and removed solution containing calcein AM was mixed with 100 μl of phosphate buffer saline (PBS) in a 96 well plate and incubated at 37°C for 120 minutes. Cell adhesion can subsequently be determined fluorometrically using a SPECTRAmax GEMINI XS microplate reader (Molecular Devices Corp, Sunnyvale, CA) at absorbance maximum of 494nm and emission maximum of 517nm. Each cell concentration was calibrated using standard curve. The degree of cell attachment was determined by subtracting the residual cell from the initial cell concentration seeded. Mean attachment cell concentrations between the different Ti surfaces was statistically analyzed using the ANOVA test, and differences was considered significant if P < 0.05.

7. **Cell Culture**

Similar to the cell attachment study, HEPM cells were used for the cell differentiation study. The cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 7% FBS, penicillin (5000 units/ml⁻¹), and streptomycin (5000 μg/ml⁻¹), and fungizone (250 μg/ml) in a 5% CO₂ humidified incubator at 37°C and the medium was changed twice a week. Osteoblasts from pre-confluent cultures were harvested with 0.25% trypsin – 1mMEDTA (GibcoBRL, life Technologies, NY), and was centrifuged, and produced to cell suspension with serum-free DMEM. The cells were seeded onto the Ti sample in 6 well culture plates at a density of 10,000 cells each sample and incubated in a 5% CO₂ humidified incubator at 37°C. At day 0, 4, 8, and 12, the cells on different
surfaces was harvested and assayed for protein production and alkaline phosphatase activity.

a. **Cell surface and matrix associated protein synthesis**: Total cell surface and matrix associated protein synthesis was measured using the Pierce Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). At the day of the assay, media was removed from the cell culture and the cell layers lyses with 1 ml of triton X-100 (0.2%). An aliquot of the triton lysate (30: 1) will be added to 200: 1 of BCA working reagent and the samples incubated for 30 minutes at 37°C. The concentration of cell surface and matrix associated protein synthesized was determined from the absorbance read at 570 nm using a microplate reader using a standard protein concentration curve. The concentration of proteins on different surfaces was statistically compared using ANOVA at and α value of 0.05.

b. **Alkaline phosphatase (ALP) specific assay**: On the day of the assay, medium was removed from the cell cultures and the cell layers lyses with 1 ml Triton X-100 (0.2%). An aliquot of the triton lysate (50: 1) will be added to 50: 1 of working reagent containing equal parts (1:1:1) of 1.5 M 2-amino-2-methyl-1-propanol (Sigma), 20 mm p-nitrophenyl phosphate (Sigma) and 1 mm magnesium chloride. The samples were then incubated for 1 hour at 37°C. After incubation, the reaction was stopped with 100: 1 of 1 N sodium hydroxide and the absorbance read at 410 nm using a microplate reader. ALP activity was determined from the absorbance using a standard curve prepared from p-nitrophenol stock standard (Sigma). The ALP specific activity was calculated by
normalizing the ALP activity to protein concentration, and differences in ALP specific activity was statistically compared using ANOVA.

8. Statistics

Statistical analyses was carried out using ANOVA, with the Student Newman-Keuls procedure as the post hoc test for the evaluation differences between the bone cell responses on the different Ti roughness and crystallinity. The statistical analyses for each physical analytical technique are outlined under the appropriate technique above.
D. RESULTS

1. X-ray diffraction (XRD)

Figure 1 shows the representative x-ray diffraction patterns for Ti surfaces after anodization at 70 $A/m^2$ in electrolytes containing 0.2 M CA and various $\beta$-GP concentrations. As observed, all anodized surfaces exhibited crystalline titanium oxide, with reflection peaks corresponding to anatase. These were different from non-anodized Ti surfaces, where no oxide peaks was observed (Figure 2). The peaks on non-anodized Ti surfaces corresponded to metal Ti.
Figure 1

X-ray diffraction of anodized Ti at 70 A/m² in β-GP and 0.2 M CA (a) 0.02 M β-GP (b) 0.03 M β-GP (c) 0.04 M β-GP.
- Ti (Substrate)
- TiO₂ (Anatase)
Figure 1.II

X-ray diffraction of non-anodized Ti surface showing the metal Ti peaks.
2. Roughness

Table 1 shows the surface roughness of non-anodized Ti surfaces and Ti surfaces after anodization at 70 A/m² in electrolytes containing 0.2 M CA and various β-GP concentrations. In general, the $R_a$ values of anodized surfaces were observed to be significantly higher compared to non-anodized surfaces. In addition, the $Ra$ value of anodized Ti surface was observed to significantly increased as the rougher than the control and that as β-GP concentration in the electrolyte was increased.
Table 1

Surface roughness of non-anodized and anodized Ti surfaces as represented by its $R_s$ values.
<table>
<thead>
<tr>
<th>Surface Roughness (µm)</th>
<th>Non-anodized Ti surface</th>
<th>Anodized Ti surface</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.02 β-GP</td>
<td>0.03 β-GP</td>
</tr>
<tr>
<td>0.27 ± 0.02</td>
<td>0.70 ± 0.01</td>
<td>0.97 ± 0.02</td>
</tr>
</tbody>
</table>
3. **Protein adsorption study**

Using albumin as a model protein for the protein adsorption, a significantly higher concentration of proteins was observed to be adsorbed on anodized Ti surfaces as compared to non-anodized Ti surfaces (Table 2). No statistical difference in protein adsorption was observed between different Ti surfaces after anodization at 70 A/m² in electrolytes containing 0.2 M CA and various β-GP concentrations.
Table 2.

Protein adsorption on non-anodized and anodized Ti surfaces after 15 minutes incubation.
<table>
<thead>
<tr>
<th>Non-anodized Ti surface</th>
<th>Anodized Ti surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02 β-GP</td>
<td>0.03 β-GP</td>
</tr>
<tr>
<td>47.5 ± 0.5</td>
<td>50.2 ± 0.4</td>
</tr>
</tbody>
</table>
4. **Cell attachment study**

As shown in Table 3, no statistical difference in initial cell attachment was observed between non-anodized Ti surfaces and Ti surfaces after anodization at 70 A/m² in electrolytes containing 0.2 M CA and various β-GP concentrations.
Table 3.

Cell attachment on non-anodized and anodized Ti surfaces after 180 minutes incubation.
<table>
<thead>
<tr>
<th>Non-anodized Ti surface</th>
<th>Anodized Ti surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02 β-GP</td>
<td>0.03 β-GP</td>
</tr>
<tr>
<td>71.2 ± 1.3</td>
<td>69.8 ± 2.6</td>
</tr>
</tbody>
</table>
5. **Alkaline phosphates (ALP) specific activity**

As shown in Figure 3, no significant difference in ALP specific activity of osteoblast precursor cells were observed after 4 and 8 days of incubation. However, at day 12, the ALP specific activity on Ti surfaces anodized using electrolyte containing 0.02 and 0.04 β-GP was significantly enhanced as compared to the non-anodized Ti surfaces and Ti surfaces anodized using electrolyte containing 0.03 β-GP.
Figure 3

Alkaline phosphatase specific activity of osteoblast precursor cells after culturing on non-anodized Ti surfaces and Ti surfaces anodized at 70 A/m² in β-GP and 0.2 M CA (a) 0.02 M β-GP (b) 0.03 M β-GP (c) 0.04 M β-GP.
E. DISCUSSION

The passivating surface oxide on titanium is one of the elements considered in the explanation for the favorable biologic response of this metal in implant applications (Effaha, et al., 1995). In an attempt to enhance osseointegration, surface implant surfaces are often modified using processes such as plasma spraying and anodization. Plasma spraying of implants surfaces with HA and Ti is commonly performed on dental implant surfaces, and investigations on the effect of these coatings on osseointegration have been extensively documented (Cooley, et al., 1992; Bloebaum, et al., 1991; Rivero et al., 1988; Yang and Ong 2003). Anodization is one of the many experimental surface modification techniques that have experimented. The process of anodization resulted in the formation of thick and porous oxide films on Ti surfaces by spark discharging. It was demonstrated that increasing the oxide thickness through anodization resulted in an increase in corrosion resistance of Ti (Ong, et al., 1993).

In this study, all anodized surfaces were observed to exhibit crystalline titanium oxide, with reflection peaks corresponding to anatase. No oxide peaks was observed on non-anodized Ti surfaces, suggesting the presence of amorphous oxide. In addition to differences in oxide structures, the roughness ($R_a$) of the anodic oxide films in this study was observed to be range from 0.7 to 1.10 μm, whereas the surface roughness of non-anodized Ti surface was 0.27 μm. It is speculated that increasing the roughness of the anodize titanium surface may increase surface area, there by providing for stronger osteoblast adhesion. Groessner – Schreiber and Tuan showed that both the synthesis and mineralization of the bone extracellular matrix are enhanced upon culturing on rough–textured and porous–coated titanium surfaces, indicating further that the physical properties of the titanium surface contribute additionally to the normal functioning of the bone cells (Mc Alarney ME, Oshiro Ma). Other investigators have reported an increased
osteocalcin and ALP production when cells were cultured on rough surfaces (Carlsson L) (Schreckenbach JP). Aside from the changes in oxides and surface roughness, previous studies on anodized Ti surfaces have reported an increase in oxide thickness as the concentration of β-GP was increased. Similarly, an increase in CA concentration from 0.1 to 0.2 M was reported to enhance oxide crystallinity. (Zhu X., Kim KH).

Initial biological responses to anodized surfaces were examined by evaluating protein adsorption and initial cell attachment. Using albumin as a model protein for the protein adsorption, a significantly higher concentration of proteins were observed to be adsorbed on anodized Ti surfaces as compared to non-anodized Ti surfaces. However, no statistical difference in protein adsorption was observed between different Ti surfaces after anodization at 70 A/m² in electrolytes containing 0.2 M CA and various β-GP concentrations. There are several factors that could affect the adsorption of the proteins used in this study. The isoelectric point of titanium oxide was reported to be 4.0–6.2 (Cuypers et al., 1978; Lumbikanonda and Sammons, 2001). At pH of 7.4, the oxide's anionic character was reported to attract a variety of cations, which subsequently enabled the surface to bind electrostatically to a variety of proteins (Cuypers et al., 1978; Sundgren et al., 1986). In addition, Ti surfaces were reported to consist of hydrophilic (polar) and hydrophobic (nonpolar) components, with the average polar/nonpolar ratio being 0.21 ± 0.07 (Grinnell and Feld, 1981). The amount of adsorbed proteins in other studies was reported to be significantly higher on the hydrophobic surfaces as compared to hydrophilic surfaces (Cuypers et al., 1978; Sundgren et al., 1986; MacDonald et al., 2002; Grinnell and Feld, 1981; Grinnell and Feld, 1982).

In this study, no statistical difference in cell attachment was also observed between the non-anodized and anodized surfaces. It has been reported in other studies that cell attachment
was not dependent on the amount proteins adsorbed on the surface (Grinnell and Feld, 1981; Grinnell and Feld, 1982).

In addition, no significant difference in ALP specific activity by cells cultured on all surfaces was observed after the cells were incubated for 4 and 8 days. However, at day 12, the ALP specific activity on Ti surfaces anodized using electrolyte containing 0.02 and 0.04 β-GP was significantly enhanced as compared to the non-anodized Ti surfaces and Ti surfaces anodized using electrolyte containing 0.03 β-GP. ALP activity production is used as biochemical markers for determining osteoblast differentiation and is considered to be important factors in determining bone mineralization (Lian JB, Stein GS 1998). The developmental stages of osteoblast differentiation is indicated by three stages; the first stage is the proliferative period which is often characterized by the production of (H4) histone, growth factors (TGF-β1), adhesion proteins (fibronectin), collagen, and low levels of osteopontin. The second stage is the maturation of matrix, and this is indicative by the peak levels of alkaline phosphatase mRNA (ALP) produced. The final stage is the mineralization period, characterized by an increased in mRNA transcripts of osteocalcin, osteopontin, and bone sialoprotein reflects calcium (Ca^{2+}) deposition. In addition, apoptotic cells are observed during the mineralization stage and are associated with the formation of bone forming nodules. It is known that ALP hydrolyzes phosphate ester, increasing the local phosphate concentration, and enhancing the rate and extent of mineralization.
F. SUMMARY AND CONCLUSION

In this study, surface roughness of anodized Ti disks was observed to be significantly greater than control Ti surfaces, with a significant increase in surface roughness as β-GP concentration in the anodization electrolyte was increased from 0.02 M to 0.04 M. In addition to a rougher surface compared to control Ti surfaces, the anodic oxide film was observed to be highly crystalline, consisting primarily of anatase. Crystallinity was observed to decrease with increasing β-GP concentration. In the protein adsorption study, a higher concentration of albumin was adsorbed on Ti surfaces anodized using electrolytes containing 0.03 and 0.04 β-GP as compared to other surfaces tested. Using osteoblast precursor cells cultured on Ti surfaces, no significant difference in protein production by cells cultured on all tested surfaces. However, ALP specific activity by cells cultured on Ti anodized using 0.02 and 0.04 β-GP surfaces were significantly higher compared to other surfaces after 12-day incubation. It was thus concluded that surface roughness, crystallinity, and protein adsorption were affected by the concentration of β-GP in the electrolyte. It was also concluded that osteoblast differentiation, as indicated by ALP specific activity, was enhanced on anodized surfaces using electrolyte containing 0.02 and 0.04 β-GP.
G. FUTURE STUDIES

Although this study has shown promise for anodized Ti surfaces, additional investigations need to be performed to optimize the anodized Ti surfaces prior to using them in patients. Additional proteins, such as fibronectin and vitronectin need to be investigated, as these are cell adhesion proteins that can affect biological activities. In addition, since the body contains more than one protein, multiple protein studies on these surfaces need to be investigated. The effect of anodization on other extracellular proteins produced by osteoblasts cells is critical for the long success of the implant. As such, the effect of these surfaces on proteins such as osteocalcin needs to be addressed. Aside from in vitro cell culture studies, these surfaces will need to be evaluated in an animal model prior to clinical trials.
H. BIBLIOGRAPHY


VITA

Francisco X. Veray-Mazo, son of Francisco X. Veray-Torregrosa and Maria del Pilar Mazo-Franco, was born in San Juan, Puerto Rico in 1966. He is married to Ada M. Vazquez-Bernier and has three kids Natalia, Diego, and Francisco.

Francisco attended Nuestra Señora del Pilar High School and graduated in May 1985. He was accepted in Sacred Heart University where he pursued a B.S in Biology until 1990. He earned his Doctor Dental Surgery from Marquette University School of Dentistry in May 1995. After graduating from the dental school, Francisco completed an Advanced Education in General Dentistry at Naval Dental Center, San Diego CA. His next assignment was as a division officer for two years onboard the USS Constellation CV-64. In the next three years, he was the branch director at Naval Security Group Activity, Sabana Seca P.R. Currently, he is a LT. Commander in United States NAVY, Dental Corps, and is in an interservice Prosthodontic training program at Willford Hall Medical Center, Lackland AFB San Antonio, TX and Master program with University of Texas Health Science Center San Antonio.