In this study, titanium (Ti) surface was modified by anodizing with a mixture of β-glycerophosphate sodium and calcium (Ca) acetate, and the anodized surfaces were characterized by scanning electron microscopy, X-ray diffraction, and electron probe microanalysis. In vitro osteoblast response to anodized oxide was also evaluated. The anodic oxide produced was observed to have interconnected pores (0.5–2 μm in diameter) and intermediate roughness (0.60–1.00 μm). In addition, anodic oxide was observed to have amorphous and anatase oxide. Calcium and phosphorus ions were deposited on the Ti oxide during anodization. Osteoblast differentiation, as indicated by alkaline phosphatase production, was enhanced on anodized surfaces. It was thus concluded from this study that Ca phosphate can be deposited on Ti surfaces by anodization. It was also concluded that the phenotypic expression of osteoblast was enhanced by the presence of Ca phosphate and higher roughness on anodized Ti surfaces.

**INTRODUCTION**

Titanium (Ti) is the implant material of choice for use in dental and orthopedic applications. The stable oxide that forms readily on Ti surfaces was reported to attribute to its excellent biocompatibility. However, it was also reported that bone response to implant surfaces was dependent on the chemical and physical properties of Ti surfaces, thereby affecting implant success. As such, attention has been focused on the surface preparation of the Ti implant.

Several techniques such as plasma spraying, laser deposition, ion beam dynamic mixing, ion beam deposition, magnetic sputtering, hot isostatic pressing, electrophoretic deposition, solgel, ion implantation, sodium hydroxide (NaOH) treatment, and electrochemical methods have been used to deposit hydroxyapatite (HA) or calcium (Ca) phosphate coatings on Ti surfaces. Among the different processes described, plasma spraying of HA and Ti has been the most common method for modifying implant surfaces. However, numerous problems with the plasma-sprayed coatings have also been cited, including variation in bond strength between the coatings and the metallic substrates, nonuniformity in coating density as a result of the process, poor adhesion between the coatings and the metallic substrates, and...
microcracks on the coating surface.\textsuperscript{19–22}

Recently, anodic oxidation has been considered to be an effective technique to modify the thickness, structure, composition, and topography of the Ti oxide. In this study, the effect of electrolyte concentration and voltage on the anodic oxide was investigated. Composition of anodic films formed has been reported to be dependent on the composition of the electrolyte. Electrolyte containing Ca and phosphorus (P) has been explored as means for the formation of anodic films.\textsuperscript{23–26} The use of electrolyte containing a mixture of Ca glycerophosphate (Ca-GP) and CA acetate has been reported to result in the formation of an adhesive porous anodic oxide whose composition includes Ca and P.\textsuperscript{25} Hydrothermal treatments after anodization have also been reported to result in the crystallization of HA on the oxide.\textsuperscript{25} Despite numerous chemical and structural characterizations, cellular responses to these anodized surfaces have yet to be evaluated. As such, using a mixture of Ca-GP and CA as electrolyte, this study evaluated the in vitro osteoblast response to anodized oxide and the hydrothermally treated oxide after anodization.

**Materials and Methods**

**Sample preparation**

Samples (30 \( \times \) 10 \( \times \) 1 mm) were cut from grade 2 commercially pure Ti plate (Metal Samples, Munford, Ala) and were abraded to 1500 grits with SiC sandpaper (Buehler, Ill). The samples were then ultrasonically rinsed in acetone for 180 seconds and pickled with a mixture of aqueous hydrofluoric acid (HF) and nitric acid (HNO\(_3\)), with the mole ratio HF/HNO\(_3\) equal to 1:3. After they were rinsed with distilled water, the samples were dried and then left as controls (Figure 1) or anodized as test groups. Anodization was performed on a regulated direct current power supply with a constant current mode at 70 A/m\(^2\) and a voltage of 350 V for 30 minutes. The electrolyte used for anodization was 0.02 to 0.05 M \( \beta \)-glycerophosphate (\( \beta \)-GP) (Sigma, St Louis, Mo) and 0.1 M CA (Sigma). Anodization was performed at a constant temperature of 20\(^\circ\)C maintained by an Isotemp 215 water bath (Fisher Scientific, Houston, Tex). After anodization, the samples were rinsed with deionized water and air dried. All samples for cell culture study were sterilized under ultraviolet light for 48 hours before the experiment.

**Materials characterization**

Anodized surfaces were uniformly coated with a gold layer for electric conductivity, and their surface morphology was observed with a scanning electron microscope (Model S-2300, Hitachi, Japan). The distribution of elements on the oxide was measured with a Shimadzu 1600 electron probe microanalysis (Shimadzu, Japan).

Samples were structurally analyzed with an X-ray diffractometer (X’pert-APD, Philips, Netherlands). By using Cu K\(_x\) radiation, data were collected from 10 degrees (20) to 60 degrees (20) with an energy of 40 kV and 30 mA.

Roughness from each surface was measured with SurfTest SV-402 (Mitu-toyo Instruments, Tokyo, Japan) over a length of 10 mm, and the results of measured roughness were presented as Ra values (arithmetic mean deviation of the roughness profile). A total of 3 specimens from each group were analyzed.

**Cell culture study**

The cell culture study was conducted with the American Type Culture Collection (Manassas, Va) CRL-1486 human embryonic palatal mesenchyme cells, an osteoblast precursor cell line, over a 4-day period. Surfaces were seeded with 97 500 cells per milliliter in Dulbecco Modified Eagle’s Medium (DMEM) containing 7% fetal bovine serum, 1% antibiotic-antimycotic solution, and 50 \( \mu \)g/mL ascorbic acid. One milliliter of the cell suspension was plated per well of a 24-well plate containing a control Ti, an anodized surface, or a hydrothermally treated surface. The cells were incubated at 37\(^\circ\)C in a humidified atmosphere of 95% air and 5% carbon dioxide. After 7 days of incubation, the medium was removed and replaced with DMEM containing 2% fetal bovine serum, 1% antibiotic-antimycotic solution, 50 \( \mu \)g/mL ascorbic acid, and 4 mM \( \beta \)-GP. The culture medium was changed every 2 days with DMEM media containing 2% fetal bovine serum, 1% antibiotic-antimycotic solution, 50 \( \mu \)g/mL ascorbic acid, and 4 mM \( \beta \)-GP. On the fourth day, media were decanted and surfaces were washed twice with
a phosphate-buffered solution. The cells were then lysed with 0.2% Triton-X-100 solution (Fisher Scientific) and stored at −20°C until assay.

**Total protein assay**

Triplicate samples from each group were analyzed for total protein synthesis, which was performed by using the Pierce bicinchoninic acid (BCA) protein assay (Pierce, Ill). The cell layer suspension (30 µL) was added to 200 µL of working reagent (sodium carbonate, sodium bicarbonate, BCA detection reagent, sodium tartrate in 0.1 M NaOH, and 4% copper sulfate). The samples were incubated for 30 minutes at 37°C and read with a microplate reader at 600 nm. The absorbance for the cell layer suspension was correlated to a standard protein curve and normalized to nanogram of double-stranded DNA (dsDNA) with a Molecule Probes PicoGreen dsDNA kit (Eugene, Ore).

**Alkaline phosphatase activity**

Triplicate samples from each treatment were used for measuring the alkaline phosphatase (ALP) activity. The cell layer suspension (50 µL) was added to 50 µL of working reagent (1.5 M 2-amino-2 methyl-1-propanol, 20 mM p-nitrophenol phosphate, and 1 mM magnesium chloride). The samples were then incubated for 3 hours at 37°C. Afterward, the reaction was stopped with the addition of 100 µL of 1 N NaOH and read with a TiterTek Multiscan Plus MK II microplate reader (MTX Labsystems Inc, Vienna, Va) at 410 nm. The absorbance for the cell layer suspension was correlated to a standard ALP activity curve prepared with p-nitrophenol stock standard and normalized to nanogram of dsDNA with a Molecule Probes PicoGreen dsDNA kit.

**Statistical analysis**

Differences in surface composition, roughness, and osteoblast responses were statistically compared by the analysis of variance test at an α value of 0.05, with differences assessed by the Student Newman-Keuls post hoc test.

**RESULTS**

Figure 2 shows representative scanning electron micrographs of anodized surfaces. Compared with the dense Ti control surface (Figure 1), the surfaces of anodized specimens indicated the presence of white-gray oxide films with interconnected pores (0.5–2 µm in diameter). The pore diameter increased with an increase in β-GP content. Some microcracks were observed in the oxide film formed with increased electrolyte concentration. The size of pores, which originate from a spark on the interface of the oxide and electrolyte, is related to the nature and concentration of ions in the electrolyte.

**TABLE 1**

<table>
<thead>
<tr>
<th>β-GP Concentration (M)</th>
<th>Titanium</th>
<th>Oxygen</th>
<th>Calcium</th>
<th>Phosphorous</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>37.11 ± 0.56</td>
<td>52.43 ± 0.47</td>
<td>5.12 ± 0.38</td>
<td>6.24 ± 0.47</td>
</tr>
<tr>
<td>0.03</td>
<td>36.01 ± 0.12</td>
<td>53.34 ± 0.39</td>
<td>4.86 ± 0.43</td>
<td>6.21 ± 0.87</td>
</tr>
<tr>
<td>0.04</td>
<td>25.86 ± 0.53</td>
<td>60.78 ± 0.47</td>
<td>6.08 ± 0.26</td>
<td>8.19 ± 0.61</td>
</tr>
<tr>
<td>0.05</td>
<td>25.34 ± 0.34</td>
<td>60.24 ± 0.66</td>
<td>6.43 ± 0.31</td>
<td>9.02 ± 0.52</td>
</tr>
</tbody>
</table>
In addition, electron probe microanalysis (EPMA) showed an increased concentration of oxygen, P, and Ca with the increase of the β-GP concentration (Table 1).

X-ray diffraction patterns indicated that the anodic oxide films contained anatase (Figure 3). The amount of amorphous structure increased, and the X-ray diffraction peaks corresponding to anatase oxide became lower as the β-GP concentration increased. The integrated intensities of all peaks of anatase were about 8000, 4500, 2800, and 2500 for oxide produced with 0.02, 0.03, 0.04, and 0.05 M β-GP with 0.1 M Ca, respectively. Significant changes in surface roughness were observed for all treated surfaces compared with control Ti surfaces (Table 2). In addition, the roughness of the anodic oxide films was observed to be between 0.60 and 1.00 μm, and the roughness of anodic oxide films increased as the β-GP concentration increased.

From the cell culture study, osteoblasts cultured on controls and anodized surfaces did not exhibit significant differences in protein production (Figure 4). However, osteoblasts cultured on anodized surfaces exhibited significantly higher ALP production when compared with controls (Figure 5). In addition, ALP production increased with the β-GP concentration of electrolyte.

### TABLE 2

<table>
<thead>
<tr>
<th>Surface roughness (mean ± SD; μm) of anodic oxide films of titanium in different β-glycero-phosphate (β-GP) concentrations of electrolyte at 0.1 M calcium acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control titanium</td>
</tr>
<tr>
<td>β-GP concentration (M)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

#### DISCUSSION

Biomaterial interactions with adjacent host tissues and biofluids have been reported to be directly related to the properties of implant surfaces.²⁷ These events were reported to be accompanied by absorption and incorporation of biological molecules and the attachment of surrounding cells.²⁸ In this study, it was observed that the modification Ti by anodization resulted in the formation of porous oxide, with the structure of the oxide observed to be anatase. As observed from the EPMA analysis, an increase in β-GP concentration also resulted in an increase in P and Ca concentrations.²⁹,³⁰ With different concentrations of electrolytes, P and Ca have been reported to be incorporated in the anodic oxides and thus were suggested to have improved the surface reactivity of Ti.³¹–³³ In particular, the incorporation of Ca and P in anodic oxides was suggested to promote the deposition of bonelike apatite so as to enhance osseointegration. The surfaces of anodized specimens observed in this study indicated the presence of white-gray oxide films with interconnected pores (0.5–2 μm in diameter). The pore diameter increased with an increase in the β-GP content (Figure 2). In addition, the presence of porous surfaces on the anodic oxide was suggested to increase the surface roughness and energy and may cause microscopic tissue-cell ingrowth, thereby improving implant fixation.³⁴

From the cell culture study, osteoblasts cultured on controls and anodized surfaces did not exhibit significant differences in protein production (Figure 4). However, osteoblasts cultured on anodized surfaces exhibited significantly higher ALP production when compared with controls (Figure 5). In addition, ALP production increased with the β-GP concentration of electrolyte.
viability, the data suggested that anodized Ti surfaces containing Ca and P possessed similar bio-
compatibility with control Ti. Osteoblasts cultured on anodized surfaces were observed to exhibit
significantly higher ALP production when compared with controls (Figure 5). In addition, ALP
production increased with the β-GP concentration of electrolyte, suggesting β-GP treatment en-
hanced cell differentiation. The enhanced cell proliferation is the result of higher Ca and P
deposition and higher surface roughness. It was suggested that anodized surfaces played a role
in enhancing bone apposition on the implant surface. Despite the knowledge of bone response to
Ca phosphate surfaces, the in vitro and in vivo bone responses to the anodized surface need to
be further evaluated in future studies.

**CONCLUSIONS**

The anodic oxide film formed on Ti with a mixture of β-GP and Ca acetate was observed to have
interconnected pores (0.5–2 μm in diameter) and intermediate roughness (0.60–1.0 μm) and con-
sist of a mixture of amorphous and anatase. In addition, Ca and P ions were deposited on the Ti
oxide during anodization. Osteo-
blast differentiation, as indicated
by ALP production, was en-
hanced on anodized surfaces. It
was thus concluded from this
study that Ca phosphate can be
deposited on Ti surfaces by an-
odization. It was also concluded
that the phenotypic expression of osteoblast was enhanced by the
presence of Ca phosphate and
higher roughness on anodized
Ti surfaces.

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