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Use of a Titanium Nitride for Electrochemical Inactivation of Marine Bacteria

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A titanium nitride (TiN) electrode with very low resistance and good electrochemical stability was constructed and used for electrochemical inactivation of the marine Gram-negative bacterium *Vibrio alginolyticus*. Specific resistance of the TiN electrode, which was formed by reactive sputtering, was 1.1×10^{-4} Ω·cm. When cyclic voltammetry of the TiN electrode-attached *V. alginolyticus* cells of 4.2×10^5 cells/cm^2 was carried out at a scan rate of 20 mV/s in seawater, an anodic peak current appeared around 0.68 V vs Ag/AgCl. In all, 98.7% of *V. alginolyticus* cells attached onto the electrode were inactivated by applying a potential of 0.8 V vs Ag/AgCl in seawater for 30 min. Changes in pH and chlorine concentration were not observed at 0.8 V vs Ag/AgCl. The TiN electrode was oxidized by applying potential of a 0.8 V vs Ag/AgCl and passivated by formation of TiO_2 onto the electrode surface. The TiO_2 thin layer formed on the TiN electrode surface did not impede electrochemical inactivation of marine bacteria. These results show that the TiN electrode can be used as an electrode for electrochemical inactivation of marine bacteria.

Introduction

Biofouling on marine structures such as ship hulls or water-cooling pipes used for ships or thermal power plants is undesirable. For example, biofouling increases fluid frictional resistance and decreases the efficiency of heat transfer (*1*). Marine biofouling has so far been prevented by chemical inactivation reagents, such as copper and organotin coatings (*2*), and by injection of chlorine at higher concentrations. However, these methods are becoming increasingly unsuitable for prevention of biofouling due to the residual toxicity causing pollution of the marine environment (*3*). Attachment of microbial cells to the substratum, subsequent formation of biofilms, and consequent attraction of other microorganisms induce the biofouling of structure surfaces (*4*−*6*). It has been reported that the formation of biofilms are important for subsequent attachment and development of invertebrate larvae (*7*, *8*). These microbial generated biofilms are known to affect the settlement and metamorphosis of oyster larvae and mussel (*9*, *10*). Biofouling has so far been prevented by biocidal chemicals, but the bacteria-grown biofilms are less susceptible to antibacterial agents than those grown in cultures (*11*, *12*). Therefore, inactivation of microbial cells attached on the initial stage may be an effective method to prevent biofoulings due to the formation of biofilms.

Recently, we have reported an electrochemical inactivation method that is based on direct electron transfer between the microbial cell and the electrode without generation of toxic substances (*13*, *14*). This method has been used for inactivation of marine bacteria using an electrically conductive plastic electrode (*15*) and a paint-type electrode made from graphite and silicone (*16*). A carbon−chloroprene sheet (CCS) electrode has been used for the electrochemical prevention of biofouling in seawater cooling pipes (*17*). Furthermore, conductive paint that can be coated as a thin film on the substratum was used as an electrode for electrochemical prevention of biofouling in seawater (*18*). However, these described electroconductive polymer electrodes have some disadvantages in actual use. The major problems are physical degradation during application of a potential in seawater, such as the formation of cracks or reduction of electrical conductivity (*17*), and high electrical resistance. Therefore, an effective material that has a low electrical conductivity and is not degraded during use is still required for electrochemical prevention of biofouling.

Thin TiN films are widely used in several technological applications due to their gold color, great hardness, high melting, and corrosion resistance (*19*−*23*). In the integrated circuit (IC) industry, a TiN layer is an important material and is used as a diffusion barrier between metal and silicon because of its thermal stability and low electrical resistivity (*19*, *22*, *23*). Furthermore, TiN is a biocompatible material (*24*−*26*) and does not release toxic substances (*27*). In this paper, electrochemical properties of the TiN electrode formed by reactive sputtering (*28*) and electrochemical inactivation of the marine Gram-negative bacterium *Vibrio alginolyticus* using this electrode were studied.

Experimental Section

Materials. Marine broth 2216 was purchased from Difco Laboratories (Detroit, MI). Fluorescent dyes propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Molecular Probes, Inc. (Pitchford, OR) and Merck (Darmstadt, Germany), respectively. Other reagents were commercially available analytical reagents or laboratory-grade materials. Seawater from Miura Peninsula (Japan) was treated by autoclaving for 1 min and filtering with a 0.2-μm pore size membrane filter.

Preparation of the TiN Electrode. The TiN layers were formed onto titanium or glass plates by reactive sputtering from elemental titanium target in a mixture of argon and nitrogen. Pure titanium (99.999%) and very pure nitrogen gas (99.9999%) were used for TiN deposition. After loading titanium or glass plates into the sputtering device (Model SPF-312H, Nichiden ANELVA Co., Tokyo, Japan), the system was evacuated to 3×10^{-4} Pa prior to backfilling with argon. Argon gas was introduced into the system to a pressure of 3×10^{-3} Pa. The titanium or glass surface was presputtered for 10 min by argon sputtering before TiN deposition. After nitrogen gas was introduced into the system until 0.8×10^{-3} Pa, TiN was deposited onto titanium or glass plates by sputtering for 60 min. The TiN deposited titanium plate was used as a working electrode. A freshly prepared TiN electrode was used for each experiment. The TiN deposited glass plate was used for assessment of other properties.

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Properties of the TiN Electrode. Composition of the TiN electrode was determined by X-ray photoelectron spectroscopy (XPS) (Model ESCA 5600Ci; ULVAC Phi Co., Tokyo, Japan). Thickness of the TiN electrode was measured by scanning electron microscopy (SEM) (Model JSM-5310LV; Nihondenshi Co., Tokyo, Japan). The amount of dissolved titanium in seawater with an applied potential was determined by inductively coupled plasma (ICP) atomic emission spectrometry (Model P-4000; HITACHI Co., Tokyo, Japan). Surface resistivity was determined by a four-point in-line probe. At least five measurements were taken for each sample, and their average was recorded. Specific resistance (σ) was obtained by multiplying the surface resistivity by the average film thickness. The specific resistance was defined as follows: specific resistance = surface resistivity × film thickness.

Electrochemical Inactivation of *Vibrio alginolyticus* Using the TiN Electrode. *Vibrio alginolyticus* ATCC 17749 was cultured aerobically at 27 °C for 2 h in 10 mL of marine broth, following preculture at 22 °C for 12 h in 15 mL of marine broth. The cells were centrifuged at 1600*g* at room temperature for 10 min, washed, and then suspended in sterilized seawater. The cell concentration was determined using a hemacytometer. The TiN electrode was immersed in 200 mL of suspended *V. alginolyticus* cell suspension (10^8 cells/mL) and incubated for 90 min at room temperature with continuous magnetic agitation (350 rpm) to allow *V. alginolyticus* cells to be attached to the surface of the electrode. The treated electrode was then rinsed by immersion in fresh sterile seawater to remove weakly attached cells. The electrode was subsequently immersed in fresh sterile seawater, and a constant potential was applied with a potentiostat (Model HA-151; Hokuto Denko Co., Japan). A silver−silver chloride reference electrode and platinum wire counter electrode was employed.

Determination of Viable Cell Number. The number of viable and inactive cells attached onto the electrode was determined by direct counting of bacteria using 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (*29*). After *V. alginolyticus* cells attached on the electrode, they were stained first with PI (250 μg/mL) and then with DAPI (20 μg/mL), and the cells stained with PI and DAPI were observed with an epifluorescence microscope (Model IMT-2; Olympus Optical Co., Tokyo, Japan) under UV excitation (wavelength, 300−400 nm). Four positions were photographed for each condition. Viable and inactive cells could be distinguished as blue and red fluorescence cells, respectively. More than 100 fluorescence cells were then enumerated, and the survival ratio was defined as follows: survival ratio (%) = (blue fluorescence cell number/blue fluorescence cell and red fluorescence cell number) × 100.

Results

Properties of the TiN Electrode. The TiN electrode was formed by reactive sputtering, was a gold color, and was very hard (hardness 1090 Hv). The thickness of the TiN electrode was ca. 1.3 μm, and the growth rate of the TiN film was ca. 0.2 μm/min. The TiN electrode had a greatly reduced resistance. Average specific resistance of the TiN electrode was 1.1×10^{-4} Ω·cm. After the surface of the electrode was etched by argon sputtering for 6 min, composition of the TiN electrode was determined by XPS. N/Ti ratio of the TiN electrode was 0.6, and this TiN electrode was a nonstoichiometric film.

Cyclic Voltammetry of the TiN Electrode with Attached *V. alginolyticus* Cell. Figure 1 shows the cyclic voltammogram obtained at a scan rate of 20 mV/s with the TiN electrode (electrode area 12.5 cm^2) attachment to *V. alginolyticus* of 4.2×10^5 cells/cm^2. Potential in the range of 0−1.0 V vs Ag/AgCl was applied to the TiN electrode in sterilized seawater.

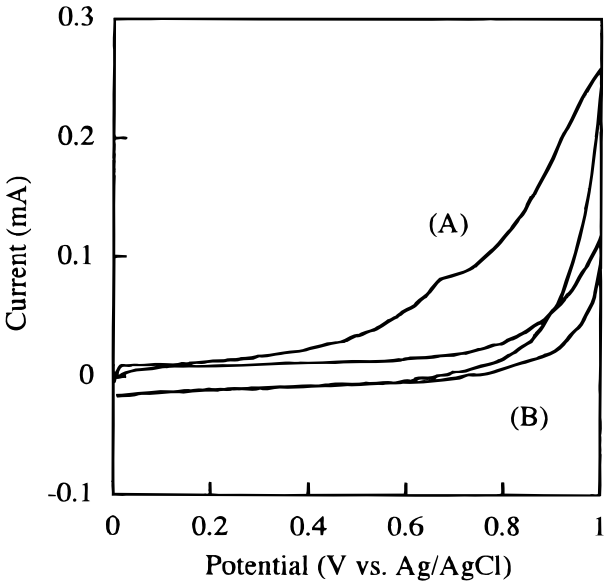

FIGURE 1. Cyclic voltammogram of marine bacteria *V. alginolyticus* on the TiN electrode obtained in sterile seawater at a scan rate of 20 mV/s: (A) *V. alginolyticus*; (B) seawater.

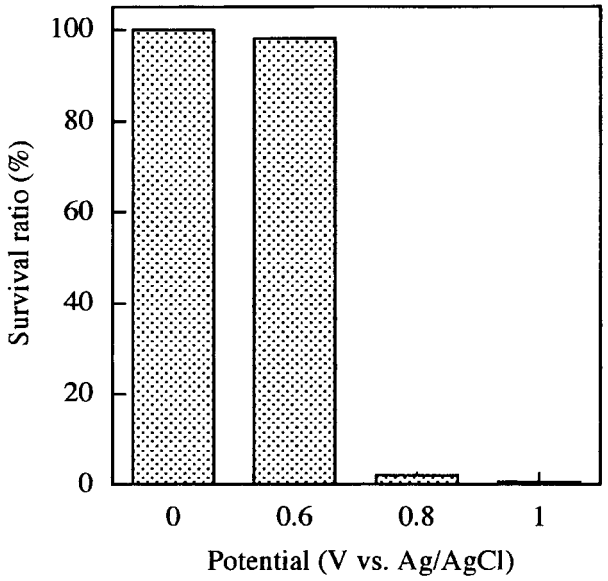

FIGURE 2. Effect of applied potential on survival ratio of *V. alginolyticus* cells attached on the TiN electrode. The potential was applied for 10 min in sterile seawater.

An anodic peak current appeared around 0.68 V vs Ag/AgCl in the first scan in the positive direction (Figure 1A). Oxidative and reductive peak current were not observed (Figure 1B).

Electrochemical Inactivation Using the TiN Electrode. The TiN electrode was incubated in seawater containing *V. alginolyticus* at the cell concentration of 10^8 cells/mL for 90 min, and the number of attached cells was determined to be 4.2×10^5 cells/cm^2. Viable cells attached onto the surface of the electrode was a monolayer. A potential in the range of 0.0−1.0 V vs Ag/AgCl was subsequently applied for 10 min in sterile seawater, and survival ratio was calculated. Figure 2 shows the effect of applied potential on the survival ratio of *V. alginolyticus* cells attached on the TiN electrode. Application of a 0.6 V vs Ag/AgCl potential did not inactivate cells attached on the electrode. The survival ratio was greater than 98% below 0.6 V vs Ag/AgCl and decreased sharply 2.0% when a potential of 0.8 V vs Ag/AgCl was applied. When a constant potential of 0.8 V vs Ag/AgCl was applied, the survival

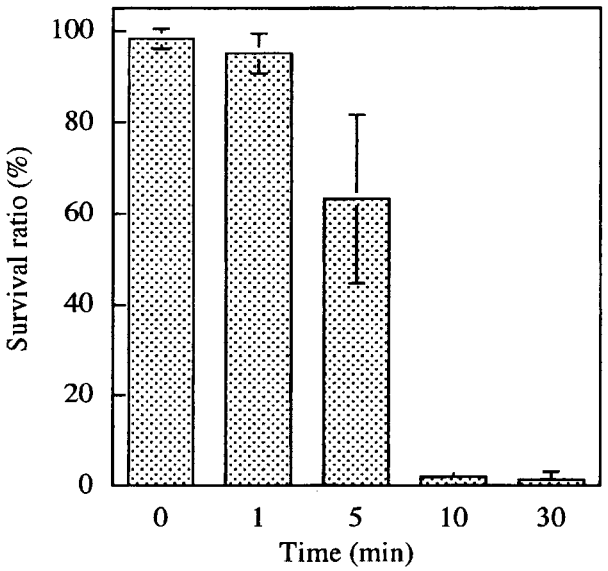

FIGURE 3. Time course of survival ratio of *V. alginolyticus* cells attached on the TiN electrode. A constant potential of 0.8 V vs Ag/AgCl was applied to the TiN electrode in sterile seawater.

ratio decreased with time, and the survival ratio was 63.2% after 5 min and 1.3% after 30 min, respectively, as shown in Figure 3. Change of the number of attached cells on the electrode with application potential was not observed.

Electrochemical Characterization of the TiN Electrode. A constant potential of 0.8 V vs Ag/AgCl was applied to the TiN electrode (electrode area 6 cm^2) in 400 mL of seawater. The amount of titanium dissolved from the TiN electrode was 8.4 $\mu g/cm^2$ at 24 h and 8.7 $\mu g/cm^2$ at 8 days, respectively. Amount of dissolved titanium did not increase with the passage of time. Furthermore, titanium, oxygen, and nitrogen of the TiN electrode surface, which was applied a 0.8 V vs Ag/AgCl potential in seawater for 8 days, were alternately determined by XPS and etching with argon sputtering. Titanium and oxygen were detected from the electrode surface, but nitrogen was not detected. When the TiN electrode surface was etched by argon sputtering, oxygen on the electrode was detected during etching for 2 min. The TiN electrode surface was oxidized by application of a potential, and an oxide layer was formed on the electrode. Change in the XPS spectrum of TiO_2−$Ti_{2_{p3/2}}$, TiN−$Ti_{2_{p1/2}}$, and TiN−$Ti_{2_{p3/2}}$ obtained from the TiN electrode surface with argon sputtering time is shown in Figure 4. The component at a binding energy of 458.8 eV was assigned to TiO_2 (*22*, *23*).

Subsequently, the generation of chlorine and a change in pH were measured using a chlorine electrode (Model 97-70; Orion Research Incorporated Boston, MA) and pH electrode (Type 6155; DKK Co., Tokyo, Japan), respectively. When a potential range of 0.0−1.2 V vs Ag/AgCl was applied for 30 min in 50 mL of sterile seawater, pH change or generation of chlorine did not occur. Then the generation potential of chlorine was examined. However, chlorine was not detected below a potential of 2.0 V vs Ag/AgCl.

Furthermore, the effect of the TiO_2 layer on the TiN electrode on the survival ratio of *V. alginolyticus* cells was investigated. The TiO_2 layer was formed on the electrode by application of a constant potential of 0.8 V vs Ag/AgCl for 8 days in seawater. The treated electrode was immersed in 200 mL of *V. alginolyticus* cell suspension (10^8 cells/mL) and incubated for 90 min at room temperature. The electrode was subsequently immersed in fresh sterile seawater, and a constant potential of 0.8 V vs Ag/AgCl was applied for 10 min in sterile seawater. The survival ratio was 2%.

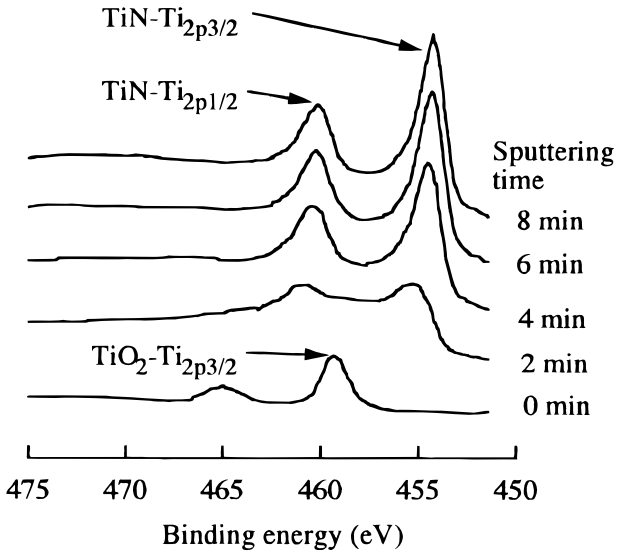

FIGURE 4. Change in XPS spectrum of TiO_2−$Ti_{2_{p3/2}}$, TiN−$Ti_{2_{p1/2}}$, and TiN−$Ti_{2_{p3/2}}$ obtained from the TiN electrode surface with argon sputtering time. A constant potential of 0.8 V vs Ag/AgCl was applied to the TiN electrode for 8 days in seawater.

Discussion

We demonstrate that TiN may be used as an effective material that has a low electrical conductivity and is not degraded during use for electrochemical prevention of biofouling. The TiN is a new type of electrode that can inactivate bacteria attached on it with a much lower potential and in a shorter time than the graphite−silicone electrode (GSE) or carbon−chloroprene sheet (CCS) electrode described previously (*15*, *17*). The specific resistance of the TiN electrode was very low, 1.1×10^{-4} Ω·cm. As shown in Figure 1A, an anodic peak current appeared around 0.68 V vs Ag/AgCl in the first scan, in the positive direction. The peak potential around 0.68 V vs Ag/AgCl from *V. alginolyticus* seems to be related to coenzyme A (CoA) in the cell (*13*, *15*). When a potential of 0.8 V vs Ag/AgCl was applied to the TiN electrode, *V. alginolyticus* attached on the TiN electrode was inactivated electrochemically. Furthermore, survival ratio was also investigated according to the plate count method described previously (*15*). When a constant potential of 0.8 V vs Ag/AgCl was applied to the TiN electrode for 30 min, the survival ratio of *V. alginolyticus* cells attached on the electrode was 2.5%. This also results that the TiN electrode can inactivate *V. alginolyticus* cells attached on it at a much lower potential than the conductive polymer electrodes described previously (*15*, *17*, *18*).

The biofilms are known to affect the settlement and metamorphosis of oyster and mussel larvae (*9*, *10*) and due to attachment and accumulation of microbial cells (*5*, *7*, *8*). Previously, prevention of marine biofouling by electrochemical inactivation has been reported, and monospecies laboratory data used *V. alginolyticus* were supported by field data (*17*, *18*). Under flowing seawater condition, biofouling of pipes inside was inhibited by application of a 1.2 V vs SCE potential (*17*). This result suggested that, even when flowing seawater supplied a continuous source of energy substrate and nutrients, biofouling was inhibited electrochemically. Therefore, the TiN electrode may provide significant advantages over methodologies based on prevention of biofouling by electrochemical inactivation of bacteria. Furthermore, when a constant potential above 2.0 V vs Ag/AgCl was applied to the TiN electrode in seawater, chlorine was not produced whereas oxygen evolved. Oxygen was detected above a potential of 1.4 V vs Ag/AgCl. These results suggest that water was preferentially oxidized over the chlorine ion

in seawater by using the TiN electrode. Thus, the TiN electrode has greatly improved properties for use as an electrode for electrochemical inactivation of bacteria.

When a constant potential of 0.8 V vs Ag/AgCl was applied to the TiN electrode in seawater, the amount of titanium dissolved from the TiN electrode was 8.4 $\mu g/cm^2$ at 24 h, but thereafter, the electrode did not dissolve further. Furthermore, a TiO_2 layer was formed on the electrode surface. These results suggest that the TiN electrode surface is oxidized initially and the electrode is passivated by formation of a TiO_2 onto the electrode surface by application of potential (Figure 4). XPS depth profile of the TiN electrode applied potential indicated that a TiO_2 layer was extremely thin. The argon sputtering time in which oxygen was detected from the TiN electrode surface increased with increasing potential (data not shown). This result shows that the TiO_2 layer on the TiN electrode grows with increasing potential, and it is a similar behavior to anode oxidation of titanium (*32*, *33*). Musher and Gordon have suggested that the TiN surface was oxidized and that oxidation of TiN was due to the N/Ti ratio and surface morphology (*34*). The N/Ti ratio of the TiN electrode formed by reactive sputtering was 0.6, and this electrode was titanium rich. This implies that oxidation of the TiN with an applied potential is due to oxidation of excess titanium into the TiN electrode. Therefore, if the TiN electrode is a stoichiometric film (N/Ti = 1), oxidation of the TiN electrode surface with an applied potential may not occur.

However, the survival ratio of *V. alginolyticus* that attached on the TiN electrode where the TiO_2 layer was formed by application of a potential was the same as compared with the TiN electrode without the TiO_2 layer. XPS depth profile of the TiN electrode at an applied potential of 0.8 V vs Ag/AgCl indicated that the TiO_2 layer was very thin. These results suggest that electron transfer between the microbial cell and the electrode was not impeded by the TiO_2 thin layer.

Bacteria attached on the TiN electrode were inactivated in a short time by application of a low potential without generating toxic substances. Furthermore, the cyclic voltammogram and dissolution behavior of the TiN electrode indicated that the TiN electrode is electrochemically stable. The TiN electrode will be applied to prevent biofouling in the near future.

Literature Cited

(1) Marshall, K. C. *ASM News* **1992**, *58*, 202.
(2) Austin, B. *Marine Microbiology*; Cambridge University Press; Cambridge, 1988.
(3) Jackson, S. M.; Jones, E. B. G. *Int. Biodeterior.* **1988**, *24*, 277.
(4) Crisp, D. J.; Ryland, J. S. *Nature* **1960**, *185*, 119.
(5) Carson, J.; Allsopp, D. In *Biodeterioration 5*; Oxley, T. A.; Barry, S., Eds.; John Wiley and Sons: New York,1981; p 291.
(6) Dempsey, M. J. *Mar. Biol.* **1981**, *61*, 305.
(7) Henschel, J. R.; Cook, P. A. *Biofouling* **1990**, *2*, 1.
(8) Brancato, M. S.; Woollacott, R. M. *Mar. Biol.* **1982**, *71*, 51.
(9) Weiner, R. M.; Walch, M.; Labare, M. P.; Bonar, D. B.; Colwell, R. R. *J. Shellfish Res.* **1989**, *8*, 117.
(10) Satuito, C. G.; Natoyama, K.; Yamazaki, M.; Fusetani, N. *Fish. Sci.* **1995**, *61*, 223.
(11) Anwar, H.; Dasgupta, M. K.; Costerton, J. W. *Antimicrob. Agents Chemother.* **1990**, *34*, 2043.
(12) Beer, D. D.; Srinivasan, R.; Stewart, P. S. *Appl. Environ. Microbiol.* **1994**, *60*, 4339.
(13) Matsunaga, T.; Namba, Y.; Nakajima, T. *Bioelectrochem. Bioenerg.* **1985**, *13*, 393.
(14) Matsunaga, T.; Namba, Y. *Anal. Chem.* **1984**, *56*, 798.
(15) Nakasono, S.; Nakamura, N.; Sode, K.; Matsunaga, T. *Bioelectrochem. Bioenerg.* **1992**, *27*, 191.
(16) Nakasono, S.; Matsunaga, T. *Denki Kagaku* **1993**, *61*, 899.
(17) Nakasono, S.; Burgess, J. G.; Takahashi, K.; Koike, M.; Murayama, C.; Nakamura, S.; Matsunaga, T. *Appl. Environ. Microbiol.* **1993**, *59*, 3757.
(18) Okochi, M.; Nakamura, N.; Matsunaga, T. *Denki Kagaku* **1995**, *63*, 1200.
(19) Schintlmeister, W.; Pacher, O.; Pfaffinger, K.; Raine, T. *J. Electrochem. Soc.* **1976**, *123*, 924.
(20) Cho, J. S.; Nam, S. W.; Chun, J. S. *J. Mater. Sci.* **1982**, *17*, 2495.
(21) Nicolet, M.-A. *Thin Solid Films* **1978**, *52*, 415.
(22) Ting, C. Y. *Thin Solid Films* **1984**, *119*, 11.
(23) Wittmer, M.; Studer, B.; Melchior, H. *J. Appl. Phys.* **1981**, *52*, 5722.
(24) Buchanan, R. A.; Rigney, E. D., Jr.; Williams, J. M. *J. Biomed. Mater. Res.* **1987**, *21*, 355.
(25) Röstlund, T.; Thomsen, P.; Bjursten, L. M.; Ericson, L. E. *J. Biomed. Mater. Res.* **1990**, *24*, 847.
(26) Behrndt, H.; Lunk, A. *Mater. Sci. Eng.* **1991**, *139*, 58.
(27) Tateishi, T.; Ushida, T.; Ito, A.; Aoyagi, J.; Homma, T.; Ise, H. In *Bioceramics, Vol. 1.2*; Heimke, G., Ed.; German Ceramic Society: Cologne, 1990; p 193.
(28) Wittmer, M. *J. Vac. Sci. Technol. A* **1985**, *3*, 1797.
(29) Matsunaga, T.; Okochi, M.; Nakasono, S. *Anal. Chem.* **1995**, *67*, 4487.
(30) Moalder, J. F.; Stickle, W. F.; Sobol, P. E.; Bomben, K. D. *Handbook of X-ray Photoelectron Spectroscopy*; Perkin-Elmer Co., Physical Electronics Division: Eden Prairie, MN, 1992.
(31) Miyagi, M.; Sato, Y.; Mizuno, T.; Sawada, S. *Titanium* **1980**, *4*, 2867.
(32) Ohtsuka, T.; Masuda, M.; Sato, N. *J. Electrochem. Soc.* **1985**, *132*, 787.
(33) Ong, J. L.; Lucas, L. C. *Appl. Surf. Sci.* **1993**, *72*, 7.
(34) Musher, J. N.; Gordon, R. G. *J. Electrochem. Soc.* **1996**, *143*, 736.

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