

DESIGNING NOVEL ORTHOPEDIC IMPLANTS
BASED ON AN ANODIZATION PLATFORM

BY

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Publications

1. C. Yao and T.J. Webster, Anodization: A promising nano-modification technique of titanium-based implants for orthopedic applications, in Surface Engineering Surgical Tools and Devices (M. Jackson, ed.), Springer, 2007.
2. C. Yao, V. Perla, J.L. McKenzie, E.B. Slamovich, and T.J. Webster, Anodized Ti and Ti6Al4V Possessing Nanometer Surface Features Enhances Osteoblast Adhesion, *J. Biomedical Nanotechnology*, 1(1), 68-73 (2005)
3. C. Yao and T. J. Webster, Anodization: A promising nano-modification technique of titanium implants for orthopedic applications, *Journal of Nanoscience and Nanotechnology*, 6, 2682-2692 (2006).

4. C. Yao and T. J. Webster, Nanostructured metal coatings on polymers increase osteoblast attachment, *International Journal of Nanomedicine*, 2(3), 487-92 (2007).
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2. C. Yao, V. Perla, J.L. McKenzie, E.B. Slamovich, and T.J. Webster, Anodized Ti and Ti6Al4V Possessing Nanometer Surface Features Enhances Osteoblast Adhesion, *J. Biomedical Nanotechnology*, 1(1), 68-73 (2005)
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CHAPTER 1. INTRODUCTION

1.1. Titanium-based Orthopedic Implants: Advantages and Disadvantages

Currently, there are three main types of metals being used in orthopedic applications: stainless steel, cobalt-chromium alloys, and titanium-based alloys. Among them, titanium and its alloys are the most popular due to many reasons to be discussed in detail below. However, if one reviews the current statistics concerning hip and knee replacement failure rates, it is clear that these titanium-based bone implants are facing great challenges to meet high patient demands.

1.1.1. Demands for Implants of High Efficacy

The number of hip and knee replacements performed in the US is on the rise (Table 1-1). Pushing this demand for new, better performing implants is an aging active baby boomer generation whose lifestyles necessitate properly functioning joints as they live longer. In fact, hip replacement surgeries are expected to increase 174% over the next 20 years with knee replacements rising even more (approximately 673%) according to a recent study by the American Academy of Orthopedic Surgeons [1].

A remarkably high number of revision surgeries are performed annually, which indicates a large number of currently-used orthopedic implant failures (Table 1-1).

The poor longevity of total hip replacements has been documented in several studies. Specifically, Moran et al. reported that primary hip and knee joint replacements only have a limited successful lifetime of 10 to 15 years [2]. This lifespan is probably acceptable for patients 65 or older (at the time of orthopedic implant insertion [3]). However, the elderly who receive an implant after bone fracture have a high chance of dying within a short time if entering a nursing home. Moreover, it is well-known that orthopedic implant longevity is significantly shortened (i.e., an increased failure rate) in patients who are younger and, thus, have more physical activity. Last but not least, the average hospitalization cost for a hip revision is \$ 45,000, which is noticeably higher than primary implant surgery which is about \$ 35,000 (all data from 2003); moreover, the average length of stay for a hip revision surgery is one day longer than primary implant surgery (data from 2004). This situation gives rise to a high demand for implants with improved efficacy and prolonged longevity.

Table 1-1 Hip and knee replacements performed in the US from 2001 to 2005 [4].

Description	2001	2002	2003	2004	2005
Total Hip Replacement	165,000	193,000	220,000	234,000	235,000
Partial Hip Replacement	119,000	109,000	108,000	240,000	234,000
Revision of a Hip Replacement	109,000	43,000	36,000	46,000	35,000
Total Knee Replacement	326,000	381,000	418,000	478,000	534,000
Revision of a Knee Replacement	29,000	25,000	33,000	40,000	37,000

1.1.2. Titanium for Orthopedic Implants

To understand why current implants fail, it is important to first discuss what materials are used. Titanium was first introduced as an implant material in the 1960s. The advantageous properties of titanium and titanium-based alloys as bone implants will be reviewed in this section.

1.1.2.1. Titanium Chemistry and Structures

Titanium is an abundant element in earth minerals. There are two titanium allotropes: alpha (α) and beta (β). The alpha phase titanium has a hexagonal closed-packed (hcp) crystal structure and exists at room temperatures. The beta phase titanium is a body-centered cubic (bcc) structure and appears when titanium solidifies to equilibrium from liquid or titanium is heated to over 883 °C. Titanium metals can be alloyed with other elements to achieve stable crystal structures at room temperature. Common alpha-stabilizing elements include aluminum, tin and oxygen while beta-stabilizing elements include niobium, molybdenum, chromium, iron and vanadium; such kinds of alloys are called “alpha” and “beta” titanium alloys. Many alloys contain a combination of the above elements and are classified as “alpha-beta” alloys.

Titanium is a very reactive metal. A passivating oxide layer spontaneously forms on the titanium surface when it is exposed to air, water and other media. Therefore, the pure titanium surface seldom exists and the surface properties of the resulting titanium oxide layer have been the focus of numerous investigators.

In most cases, the oxide layer is composed of titanium dioxide (or titania), which has three different crystalline structures: rutile, anatase and brookite. The first two are more common and both rutile and anatase have a tetragonal crystal structure. Rutile is more thermodynamically stable than anatase and anatase-rutile transformation occurs at high temperatures (700 to 900 °C) [5].

1.1.2.2. Titanium Mechanical Properties

Titanium is a light but strong metal. The density of titanium is about 4.51g/cm^3 , which is over 40 % less than steel (7.9 g/cm^3) and cast CoCrMo (8.3 g/cm^3). The mechanical properties of titanium alloys depend on their crystalline phase (shown in Table 1-2). Unalloyed and alpha titanium alloys exhibit high strength (200 to 600 MPa) and high modulus (over 100 GPa). Beta alloys are more ductile and have a lower elastic modulus (e.g., 80 GPa for as-rolled Ti-30Ta [6], which is closer to that of bone which ranges from 7-28 GPa [7]) but are higher in strength. Typically, alpha-beta alloys have intermediate properties by balancing the amounts of alpha and beta stabilizers. The alpha-beta Ti6Al4V ELI (ultra low interstitial) has been the most widely used type of titanium alloy for orthopedic and dental applications.

Table 1-2 Mechanical properties of titanium alloys and human cortical bone [7].

Material and Designation (or condition)	Young's modulus (GPa)	Tensile strength (MPa)	Yield strength (MPa)
Ti ASTM Grade 1 (alpha)	102.7	240	170
Ti ASTM Grade 2 (alpha)	102.7	345	275
Ti ASTM Grade 3 (alpha)	103.4	450	380
Ti ASTM Grade 4 (alpha)	104.1	550	485
Ti-6Al-4V (alpha/beta; annealed)	110-114	895-930	825-869
Ti6Al4V ELI (alpha/beta)	101-110	860-965	795-875
Ti-13Nb-13Zr (beta; aged)	79-84	973-1073	836-908
Ti-12Mo-6Zr-2Fe (beta; annealed)	74-85	1060-1100	1000-1060
Cortical bone (human femur)	17-28	130	----

The properties of titanium materials are also determined by their microstructure, which can be manipulated through appropriate mechanical processing and thermal treatments. Mechanical processes like cold deformation can result in elongated grains in the working direction while the final heat treatment can recrystallize the elongated structure into an equiaxed structure. The as-worked elongated structures exhibit higher anisotropic tensile strength and lower ductility while the recrystallized equiaxed structures exhibit lower non-directional strength but have higher ductility. For example, the ultimate tensile strength of commercially pure titanium (grade 1) increased from 410 MPa to 560 MPa after 40% cold deformation while at the same time its elongation at fracture decreased from 30% to 16% [6]. These processing conditions signify the ability to tailor the mechanical properties of titanium alloys for desirable orthopedic applications.

Compared to stainless steel and cobalt based alloys, titanium and its alloys have comparable strength and, thus, a much higher specific strength (strength/density).

Moreover, it is important to note that typical titanium based implants have a modulus (~110 GPa) that is about half of stainless steel (210 GPa) and cobalt-based implants (~230 GPa). This is meaningful for bone implants because a mismatch of modulus between the orthopedic implant and bone may lead to stress and strain imbalances that cause implant loosening and eventual failure. Titanium based implants decrease this gap in modulus although it is still a concern for future products. One disadvantage of titanium, however, is poor shear strength and, thus, it is limited in use in wear applications. CoCr alloys are often used for knee applications due to better wear resistance than titanium.

1.1.2.3. Titanium Biological Properties

Of course, the choice of orthopedic implants is not limited to mechanical properties alone, biological properties are also important. It has been found that elements like vanadium (often alloyed with titanium) have corrosion and cytotoxicity risks [8]. As a result, toxic-free titanium alloys such as Ti-13Nb-13Zr and Ti-15Mo have been developed to achieve higher biocompatibility properties since the 1980s [9].

Besides the composition of titanium alloys, the outermost oxide layer of titanium-based orthopedic implants is a main factor influencing biocompatibility properties. Titanium dioxide is the most stable titanium oxide and is resistant to most chemical events. The excellent corrosion resistance of titanium is to a large extent due to this oxide layer. For this reason, titanium is usually considered

biocompatible when used as an orthopedic implant [10]. However, titania surfaces are not completely chemically inert. For example, they can react with organic molecules like butanol [11]. Numerous in vitro and in vivo studies confirmed the biocompatibility of titanium and titanium alloys [10]. With regard to the crystalline phase effects of anatase and rutile titania, some studies have indicated that bone cell adhesion is increased with increased crystallinity; bone cell differentiation [12] and apatite deposition [13] are promoted on anatase but inhibited on anatase/rutile titania crystalline phases. This could be the result of a reduced energy barrier for apatite crystal nucleation on anatase compared to rutile [13]. Much smaller contact angles of various liquids on anatase surfaces than on rutile/anatase surfaces indicate that anatase phase is more hydrophilic and more favorable for cell attachment than rutile [13].

The problem with current titanium-based implants (regardless of chemistry and crystallinity) is that their surfaces lack enough bioactivity to promote and sustain osteoblast functions necessary for quick and strong osseointegration, i.e., there is a lack of direct structural and functional connections between living bone and the surface of load-bearing titanium-based implants. As a result, numerous researchers are focusing on improving titanium implant surface properties to prevent interposition of non-bone (fibrous) tissue and to support better new bone growth.

1.2. Methods to Improve Titanium Implants

1.2.1. Improving Bulk Properties

As mentioned above, to achieve optimal mechanical properties and especially alleviate the modulus mismatch between bone and the implant, beta titanium alloys like Ti-13Nb-13Zr, Ti-15Mo, Ti-12Mo-6Zr-2Fe (TMZF), Ti-15Mo-5Zr-3Al and Ti-30Nb have been developed and show great potential for orthopedic implant applications [14-19]. This is because these alloys combine a low elastic modulus with superior corrosion resistance compared to Ti-6Al-4V.

Another manufacturing process that can be used to create better titanium-based implants involves powder processing including techniques like severe plastic deformation which can create ultrafine grained titanium [20, 21]. This method involves plastic deformation of a metallic material up to the highest amounts of plastic strain (more than 600 to 800%) at low temperatures (typically below 30% of the melting temperature). In this way, initial coarse grains are subdivided into submicron to nanometer grains. Such titanium substrates have comparable mechanical integrity to conventional wrought titanium alloys but have improved bone cell adhesion compared to respective non-plastically deformed counterparts [22].

1.2.2. Improving Surface Properties

The surface of an implant plays a critical role in determining a device's performance because only it is in direct contact with tissues and bodily fluids [23]. Although it has been well established that the titanium dioxide layer on titanium has the ability to generate an appropriate host response, and protects the underlying metal from corrosion, conventional titanium implants do not proactively support new bone growth and usually require bioactive coatings to increase their chance of binding to surrounding bone, e.g. a ceramic-like hydroxyapatite (HA) coating to enhance osseointegration [24]. Because HA coatings are not stable for long time periods (such as over 10 years), there are additional problems associated with titanium-based coatings (such as delamination, wear debris generation, etc.). A much better approach to improving the success of titanium is to alter its surface to proactively promote new bone growth and sustain such bone growth over long time periods.

The goal of titanium implant surface modification is to produce a desired chemical composition and topography to help bone growth. Different classes of surface treatments have been used for titanium in medical applications. They can be mainly divided into three categories: a) cleaning and/or removal of the native surface layer, b) modification of the surface structure and topography, and c) modification of the composition and structure of the oxide layer or controlled formation of a new surface layer [25]. The above can be achieved through mechanical, chemical, or electrochemical methods as will be described.

1.2.2.1. Mechanical Methods

These methods include machining (such as turning and milling), grinding, polishing, and blasting. Machining techniques are usually not intentional means to modify surface properties. In turning, a cutting tool is used to remove materials from a rotating workpiece. In milling, a rotating tool with multiple cutting edges is moved slowly to the material to generate a straight surface. However, machining does form a specific surface topography and composition. The surface topography created by machining is characterized by grooves along the machining direction. Surface roughness values are usually within the micro-scale as a result of machining [26]. Such surface structures dictate cellular responses such as the orientation, migration and activation of bone forming cells [27]. For example, titanium implants with machined surfaces (grooves of 1 μm and lower) demonstrated better implant-bone contact than electropolished implants with extremely smooth surfaces [28]. Studies have shown the surface oxide layer after machining consists of titania as well as other oxide states, which may contribute to the observed promoted bone growth. Also, various organic contaminants and inorganic impurities usually appear in the oxide film. Grinding and polishing are similar methods which produce additional desired micron surface topographies [25]. Compared to grinding, polishing can achieve a much more smooth surface [25]. Blasting is also a commonly used method to clean and finish a surface. These methods may have some undesirable effects on the titanium surface, for example, the polishing particles (such as alumina) may

become embedded into a highly pure titanium implant during processing. To date, the effect of such contamination on bone formation has not been clearly studied.

1.2.2.2. Chemical Methods

The chemical methods utilized to modify titanium mainly include chemical etching (acid etching or alkaline etching) and passivation treatments. Acid etching (or pickling) is a useful tool to produce clean and uniform surface finishes. Due to the stability of titanium oxides, the choice of chemicals is limited to a few acids like HCl, HNO₃, H₂SO₄, and HF. The extent of etching depends on the processing conditions such as the acid concentration, temperature and time. Some researchers have studied the bioperformance of etched titanium surfaces [25, 29]. For example, Oliveira et al. found that titanium-based surfaces treated by H₂SO₄/H₂O₂ had a texture characterized by nanopitting with a honeycomb-like appearance and such surfaces upregulated the early expression of bone sialoprotein and osteopontin by osteogenic cells [29].

Surfaces of pure titanium and many titanium alloys can also be treated with NaOH and then heated to form a sodium titanate layer that facilitates the formation of apatite [30]. The bone-like apatite layer can tightly bond the metal implant to the surrounding living bone. This technique will be discussed in more detail in section 1.4.2.3.

Though chemical methods can produce nanotopography on an implant surface, it has numerous drawbacks including the formation of uncontrollable, random, heterogeneous surface topographies. Techniques that provide surface feature symmetry or regularity are also desirable. Moreover, chemical treatment may lead to residues like hydrogen and fluorine from the etching solution [25].

Lastly, passivation treatments are used for obtaining a uniformly oxidized surface, including nitric acid passivation and heat treatment. No major influence on the overall surface topography of titanium has been shown by nitric acid passivation [31]. Heating in air at 400-600 °C or aging in boiling water has been reported as an alternate passivation method for Ti-6Al-4V alloys [32]. These methods are recommended for the purpose of improving corrosion resistance. Moreover, some researchers reported that heat treated (oxygen/air) Ti-6Al-4V promoted fibronectin (a key protein that mediates osteoblast adhesion) adsorption and osteoblast-like cell adhesion mainly because of the chemical changes (increase Al and/or V) in the oxide layer [33].

1.2.2.3. Electrochemical Methods

In contrast to the above mentioned techniques, electrochemical methods can create highly controllable nano surface features. The two most commonly used electrochemical methods are electropolishing and anodization. Electropolishing is usually used to create a fine finishing surface for metals and the details can be

found in [34]. Due to its importance in the present research, the details of anodization will be discussed in section 1.3.

1.2.2.4. Other Methods

Plasma deposition is another common method used to deposit a bioactive coating (e.g., HA) on a titanium implant surface. Plasma surface modification, another plasma treatment based on the exposure of the sample surface to a glow discharge, can be used for surface cleaning by noble gases or changing chemical composition by reactive gases. For example, plasma nitriding of Ti-6Al-4V in NH_3 at 850 °C for 20 hours will result in a nitrogen reaction layer and, thus, a change in the ultimate tensile strength from 894 MPa to 984 MPa [6]. Some studies reported that osteoblast proliferation and viability appeared to be significantly reduced after nitriding but osteoblast phenotype expression and protein synthesis capabilities were not affected [35].

Ion implantation is another method that has been used to modify the surface of titanium. It is a method that adds dopants onto the substrates and produces a chemical composition that otherwise would be impossible due to thermodynamic reasons. For titanium applications, ion implantation is mainly used to improve the wear resistance of titanium by doping nitrogen onto titanium based implants [36, 37]. Some researchers also reported that attached bone cells spread more and occupy larger surfaces on ion implanted titanium alloy surfaces than untreated alloys [38].

As mentioned above, plasma surface modification and ion implantation are mainly used to change chemical composition of the implant and can not produce titanium or titania oxide nanostructures on an implant surface.

1.3. Anodization for Nano-modification: Basics and Capabilities

The ability for producing controlled nanostructures was emphasized in the previous section. The importance of this comes from the fact that natural bone is composed of different kinds of nanostructures. In this section, we will first review the natural bone environment and then introduce another technique, anodization, which can be used to biomimic bone more accurately than any other process described thus far.

1.3.1. Biomimicking: Learn From the Natural Bone Environment

1.3.1.1. Bone Structure and Composition

As the primary structural element in the human body, bone consists of an organic matrix and an inorganic mineral phase, 50:50 % by volume and 25:75 % by weight. The fibrous, organic matrix of bone (osteoid) is composed of 90 wt.% collagen and 10 wt.% amorphous ground substance (including glycosaminoglycans and glycoproteins). The main inorganic components of bone minerals are hydroxyapatite (HA; $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) crystals and calcium phosphate. Collagen in bone is Type I (a triple helix protein 300 nm in length, 0.5 nm in width, and periodicity of 67 nm [39]) and has stable intermolecular cross-

links, which provides much toughness to bone. Mineralized collagen fibers are arranged into lamella sheets (3-7 μm) and are assembled into a spiral configuration. Bone apatite crystals are approximately 20 to 40 nm long and are uniquely patterned within the collagen network (shown in Figure 1.1) [40]. Such anisotropically oriented HA and collagen along the direction of principal forces in the body provides enough strength for cortical bone to support physiological loads. One could even call this arrangement nanotubular.

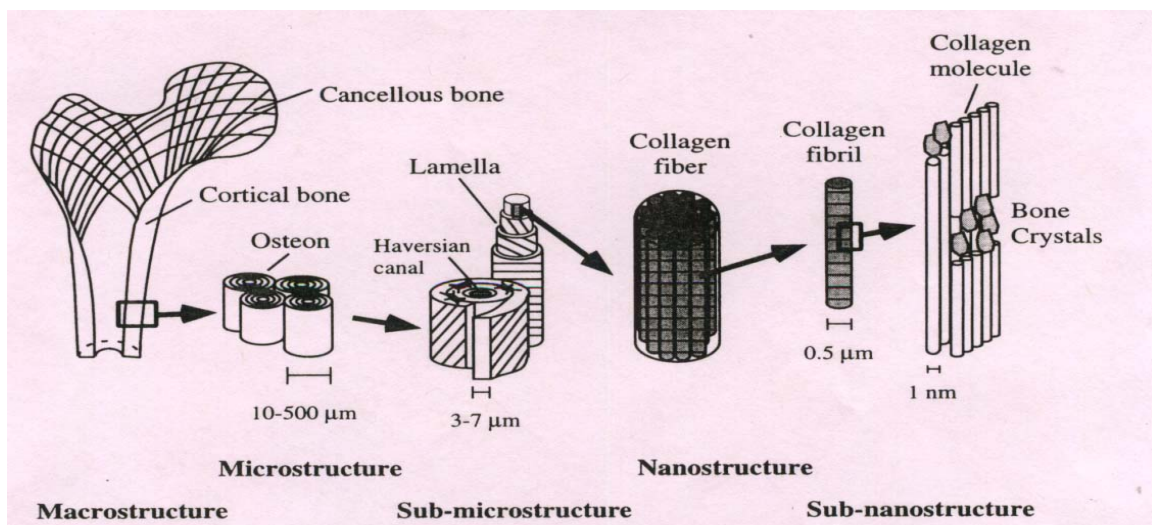


Figure 1.1 The multi-scale structure of natural bone (adapted from [40]).

In short, natural bone is composed of features at multiple scales, especially at the nanoscale which usually does not appear on conventional titanium-based implants. It is important to note that this size scale (the nanoscale) is a favorable environment for bone related proteins and cells.

1.3.1.2. Proteins and Cells Involved in New Bone Growth

As a living organ, bone has the ability to self-repair. This repair process is called "Bone Remodeling" and it involves three types of cells: osteoblasts (bone-forming cells), osteoclasts (bone-resorbing cells) and osteocytes (bone-maintaining cells). Whenever there is a tiny crack inside the bone (for example, after landing from a high jump), the osteoclasts are recruited to this fracture site to resorb bone around the crack by secreting an acidic solution (called tartrate-resistant acid phosphatase) to dissolve calcified materials and hydrolytic enzymes to digest the collagen matrix. Osteoclast function also results in longitudinally orientated tubular channels. Then, osteoblasts on the surface of these channels work in coordination to deposit successive layers of lamellar bone by first secreting collagen to form a surrounding matrix, called an "osteoid", and then controlling calcium and mineral deposition. Differentiated osteoblasts embedded in the mineralized extracellular matrices are called osteocytes. Osteocytes are physically connected via cell-cell binding domains and chemically communicate to each other so that they can sense changes in pressure or fluid flow in the bone to help direct osteoclastic activity to resorb bone and osteoblastic activity to develop new bone. The remodeling of bone is continuous during growth and throughout the lifetime of humans.

When an implanted hip replacement is inserted into the human body, a complex series of events involving instantaneous interactions of the biomaterial surface

with proteins and then subsequently cells ensues. It is this initial interaction of proteins and then cells that will determine eventual biomaterial success or failure.

One aspect that is not involved in bone remodeling is the foreign body response, which means the immune system will treat an inserted biomaterial as a “foreign body”, possibly causing inflammation and implant encapsulation with fibrous tissue. This fibrous tissue (fibrosis) is a result of greater fibroblast (connective tissue forming cells) than osteoblast functions. Considering that fibrous encapsulation may lead to insufficient osseointegration and, thus, poor mechanical fixation of the implant to juxtaposed bone, uncontrollable fibroblast function should be avoided in the design of a successful implant.

In order to understand how fibroblast functions may be greater than osteoblast functions, an understanding of initial protein interactions with biomaterial surfaces is necessary. Importantly, before cells adhere to the implant surface, proteins interact with the surface. For oxide-covered titanium surfaces, proteins adsorb within seconds of exposure to body fluids, e.g. blood plasma. The amphoteric oxide surface binds structural water in the outermost atomic layers (forming --O^- , --OH^- and --OH_2^+ sites) and possesses a weak negative charge at physiological pH. After that, a primary strongly-adsorbed protein layer and a secondary loosely-held protein layer are formed at this interface. This is called the electrochemical double layer.

The composition and conformation of the initially adsorbed proteins may be influenced by the underlying properties of the substrates. Some proteins (e.g., vitronectin, fibronectin, and laminin) mediate desirable and undesirable cellular events such as adhesion and differentiation [41]. For example, Howlett et al. found that the attachment of bone-derived cells to steel, titanium and other materials is dependent on the presence of vitronectin [42]. Considering that protein adsorption triggers cell adhesion to the implant surface, the difference in cell responses may be explained through the investigation of initial protein adsorption and conformation.

Cell attachment and adhesion is a critical first step in cellular responses. After that, cells will spread over the surface to form confluent multilayers due to growth and division (called cell proliferation) and begin to synthesize collagen as well as other proteins (such as vitronectin and fibronectin). Osteoblast proliferation decreases as osteoblasts differentiate from non-calcium producing to specialized calcium producing cells. As osteoblasts differentiate, osteoblasts secrete osteocalcin (a calcium-binding protein), bone sialoprotein and other matrix proteins; the extracellular matrix develops and becomes mineralized after a few weeks. Importantly, cells attach to proteins adsorbed on surfaces via integrins [43], which are receptors that recognize specific attachment sites on proteins (like Arg-Gly-Asp (or RGD) sequences in fibronectin) (shown in Figure 1.2).

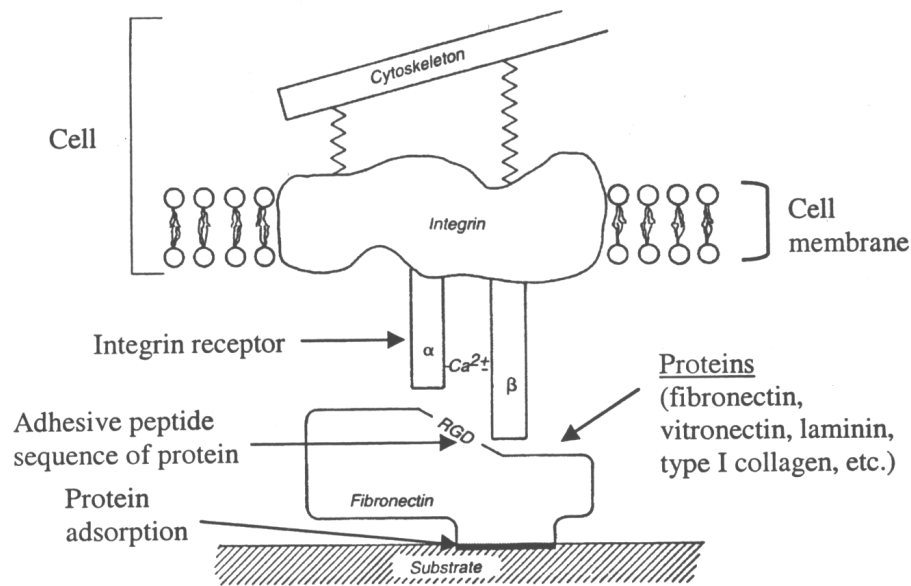


Figure 1.2 Schematic of the interaction of cells to an implanted surface as mediated by proteins (Adapted and redrawn from [44]).

Overall, it is well known that not only do numerous nanofeatures exist in biological systems (e.g., nanoscale bone constituents and organelles of cells), but nanoscale interactions are critical for all biological systems. The question is how the nanotopography of the unliving surface affects an osteoblast response. Some studies have already revealed enhanced cell-implant interactions on nanoporous or nanophase materials [45-48]. Some researchers have also suggested that the adhesion of cells to nanotopographies may be controlled to a considerable extent by the regularity, order as well as scale, of nanofeatures [49]. Thus, topographical patterns on titanium implant surfaces may influence osteoblast responses in a positively controlled manner.

1.3.2. Mechanism of Anodization

As mentioned, anodization or anodic oxidation is a well-established surface modification technique for valve metals that produces protective layers. Typical anodization procedures include alkaline cleaning, acid activation, and electrolyte anodizing. Acid activation is performed in a mixture of nitric acid and hydrofluoric acid (HF) to remove the natural titanium oxide layer and surface contaminants. The electrolyte anodization is carried out in an electrochemical cell which usually has a three-electrode configuration (titanium anode, platinum cathode and Ag/AgCl reference electrode). When a constant voltage or current is applied between the anode and cathode, electrode reactions (oxidation and reduction) in combination with field-driven ion diffusion (see Figure 1.3) lead to the formation of an oxide layer on the anode surface.

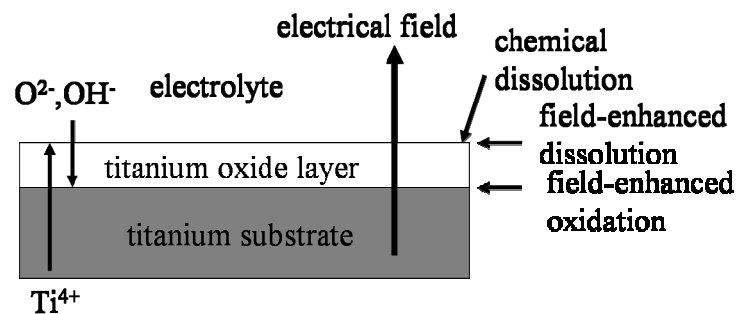
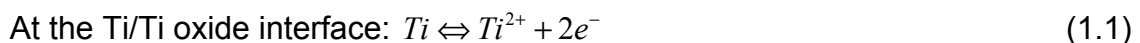
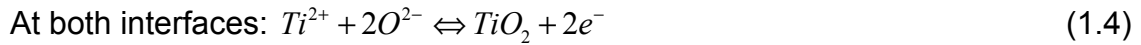
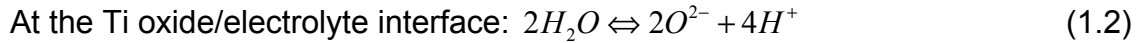


Figure 1.3 Schematic of ion diffusion under an electric field during anodization.

The main chemical reactions specifically involved in anodizing titanium are listed below:





Because titanium oxides have higher resistivity than the electrolyte and the metallic substrate, the applied voltage will mainly drop over the oxide film on the anode. As long as the electrical field is strong enough to drive the ion conduction through the oxide, the oxide film will keep growing. This explains why the final oxide thickness, d , is almost linearly dependent on the applied voltage, U :

$$d \approx aU \quad (1.5)$$

where a is usually a constant within the range 1.5-3 nm/V [50].

1.3.3. Capability to Form Microstructures

The anodization technique was discovered in the early 1930's and was widely studied in the 1960's to enhance titanium implant osseointegration. These processes usually adopted a high voltage anodization (called Anodic Spark Deposition, ASD) of titanium in electrolyte solutions whose ions would be embedded into the oxide coating, resulting in a microporous structure (some of them are shown in Table 1-3).

Table 1-3 Experimental conditions of some ASD studies. RT stands for room temperature.

Electrolyte Composition	Molarity	Voltage (Current)	Time (s)	Temperature (°C)	Reference
Sulfuric acid	1	125	-	-	[51]
	0.5, 1, 3	90, 155, 180	-	-	[52]
Acetic acid	1	80	-	RT	[28]
	0.1	40-80	8-67	17	[53]
Phosphoric acid	2	175	-	25	[51]
	0.2	200, 300, 350 (70 A/cm ²)	-	20	[54]
	1	40-80	10-47	17	[53]
Sodium tripolyphosphate	0.15	(210 A/cm ²)	-	-	[55]
Sodium hydroxide	5	10-20	-	25	[56]
	0.1	40-80	22-110	17	[53]
Calcium hydroxide	0.1	40-80	13-53	17	[53]
Calcium glycerophosphate and calcium acetate	0.02/0.1	(70 A/cm ²)	1530	4.1-4.5	[55]
	0.03/0.15	200, 260, 300 (70 A/cm ²)		20	[54]
	0.15/0.02	190-600	180	-	[57]
	0.02/0.15	(70 A/cm ²)	1800	-	[58]
	0.02/0.15	350	1200	20	[59]
β glycerophosphate and sodium acetate	0.06/0.3	250-350 (50 A/cm ²)	-	-	[60]

The mechanism of the ASD is usually described by the avalanche theory. During anodization, the newly-formed oxide layer on the anode is a dielectric barrier to the current flow and it keeps growing until reaching the dielectric breakdown limit. Generally, the anodized layer is not uniform due to the existence of flaws, defects,

local stress, and non-uniform oxide thickness. When the applied voltage increases, the potential drop at the weak points exceeds the dielectric limit so that sparking happens. The local temperature at these points can be up to several thousand Kelvin and lead to a local melting process. Thermal stressing of these anodized titanium leads to the multiplication of weak points, i.e., a cascading process, and consequently breakdown of the dielectric.

1.3.4. Capability to Form Nanostructures

While the generation of micro-structures through titanium anodization is well-established, current research efforts focus on creating biologically-inspired nanometer surface structures. Studies have shown that nanoporous structures can be created by anodizing titanium in chromic acid at 10 to 40 V [61]. Another unique surface morphology obtained through titanium anodization is self-ordered nanotubular structures (some of the specific anodizing parameters are listed in Table 1-4). For these studies, fluorine electrolyte solutions are used and the applied voltage must be much lower than the dielectric breakdown.

Table 1-4 Survey of different fluorine solutions to produce titania nanotubular structures of different size and thickness.

Electrolyte Composition (pH)	Voltage (V)	Time (h)	Temperature (°C)	Thickness (nm)	Pore diameter (nm)	Ref.
CH ₃ COOH and 0.5 M HF	10	4	-	60	500 (inter)	[62]
0.5 or 1.5 % HF	10-40	<1	18	250	25-65	[63]
0.5 % HF	10-23	<1	-	300	22-76	[64]
KF and NaF (4.5)	25	20	-	4400	115	[65]
DMSO and CH ₃ COOH and 4 % HF	20	70	RT	2300	60	[66]
1 M H ₂ SO ₄ and 0.15 % HF	30	24	-	540	140	[67]
CH ₃ COOH and 0.5 % NH ₄ F	20	1	-	200	30	[68]
1 M (NH ₄) ₂ SO ₄ and 0.5 % NH ₄ F	20	-	-	2500	100	[69]
1 M (NH ₄)H ₂ PO ₄ and 1M H ₃ PO ₄ and 0.5 % HF	20	40	-	4070	50	[70]
0.138 M HF or NaF + 0.5 M H ₃ PO ₄	20	-	24	500	100-120 (outer)	[71]

The need of fluoride ions to form nanoporous titania structures on a titanium surface under relatively low voltages was first reported by Zwilling et al. [72]. In 1999, Grimes and co-workers successfully fabricated self-ordered titania nanotube arrays after anodizing titanium between 10 and 40 V in dilute (0.5-1.5 wt. %) aqueous HF solutions [63]. It was found that the diameters of the nanotubes were defined by the applied voltage while the final length of the titania nanotubes were independent of the anodization time. In that study, the titania nanotube diameter was approximately 60 nm and the nanotube length was 200

nm when anodized at 20 V in 0.5 % HF solution for 20 min. Schmuki and co-workers also observed self-ordered nanotubular titania films anodized in HF/H₂SO₄ or CH₃COOH/NH₄F electrolyte solutions [67]. Moreover, the nanotubular (or nanopore) structures were also achieved on titanium in organic electrolytes. Choi et al. anodized nano-indented titanium in an ethanolic HF solution and produced a pore lattice with a 500 nm inter-pore distance [62]. Schmuki's group also reported titania nanotubular structures when anodizing in non-aqueous mixtures of ethanol and ammonium fluoride without an imprinting treatment [68]. However, in these studies, the depth of titania nanotubes was limited to a few hundred nanometers. Recently, high-aspect-ratio titania nanotubes up to several micrometers deep were reported by both Grime's and Schmuki's research groups. The Grime's group reported the formation of 4.4 μm long titania nanotube arrays by anodizing titanium in NaF or KF of pH 4.5 [65]. They also reported the formation of 2.3 μm thick titania nanotubular structures using DMSO/ethanol/HF electrolyte solutions [66]. Meanwhile, Schmuki's group succeeded in using neutral fluoride solutions to produce nanotubular structures up to 2.5 μm in depth [69]. They achieved this by using a buffered neutral electrolyte (NH₄F) solution and controlling the pH value to enhance acidification at the bottom of tubes.

Chemical dissolution, field-assisted dissolution and oxidation are the three main reactions in fluorine electrolyte anodization. Among these, field-enhanced dissolution has been considered as the predominant mechanism of titania

nanotubular structure formation. Grime and co-workers proposed a mechanism for the formation of anodized titania nanotubes based on a point defect model [64]. They proposed that the initial pore formation was a result of the localized dissolution at weak points. Meanwhile, the unanodized metallic portions exist between the pores. Later, voids were formed in these inter-pore regions by field-enhanced oxidation/dissolution. The growth of voids in equilibrium with the pores would form the final nanotubular structures. This model will be further discussed in the discussion section.

1.3.5. Capability to Mediate Chemistry

The ions contained in the electrolyte solution are usually present in the thick, porous ASD film and the concentration of these elements decreases from the outer layer towards the substrate [54]. For electrolytes containing Ca and P, such as calcium glycerophosphate (Ca-GP) and calcium acetate (CA), both Ca and P were contained in the oxide layer with a Ca/P ratio close to HA (1.67) [73]. After an additional hydrothermal treatment (e.g., high pressure steaming), HA crystals were randomly precipitated on the anodic oxide film. These HA crystals were usually columnar or needle-like. This could be another way to create HA coatings on titanium implants as opposed to plasma spraying.

Another approach recently reported the formation of apatite layers onto the anodized titanium by simply soaking crystalline titania in simulated body fluid (SBF) because anodized titanium with anatase and rutile titania surfaces were

shown to induce apatite formation in vitro [74]. This will be discussed in detail in section 1.4.2.3.

1.3.6. In vitro and in vivo Studies on Anodized Titanium

1.3.6.1. In vitro studies

Most in vitro studies have reported bone cell responses to micro-porous ASD anodized and anodized / hydrothermally treated titanium surfaces (not anodized titanium with nanotubular structures). Rodriguez et al. reported increased osteocalcin production by osteoblasts on anodized and hydrothermally treated titanium surfaces but the highest alkaline phosphatase (ALP) activity on control (unanodized) titanium throughout an 8 day study by precursor osteoblast cell line (ATCC, CRL-1468) [75]. Both osteocalcin and ALP are markers of osteoblast differentiation from non-calcium depositing to calcium depositing cells. They explained that a decrease in ALP activity was in part attributed to the maturation of osteoblast precursor cells and in part attributed to the increased production of a mineralized matrix. Zhu et al. studied the effects of topography and composition of anodized titanium surfaces on osteoblast (SaOS-2 derived from human osteosarcoma) responses [54]. Their cell experiments showed an absence of cytotoxicity and an increase in cell attachment and proliferation after anodization in an electrolyte solution composed of Ca-GP and CA. The cells on the surfaces with micropores showed an irregular and polygonal growth profile and more lamellipodia while cells on the titanium controls showed many thick

stress fibers and intense focal contacts. Suh et al. studied the effects of hydrothermally treated anodic films similar to the Zhu formulations and observed that the hydrothermal treatment had an effect on early osteoblast attachment, resulting in a more well-spread shape compared to the cellular rounded shape observed on anodized and control titanium surfaces after 6 h [58].

Importantly, when this thesis work started, no in vitro studies existed concerning orthopedic applications of anodized titanium with nanotubular structures. Since then, series of in vitro studies on nanotubular titania structures have been conducted and will be elaborated in the results and discussion sections.

1.3.6.2. In vivo studies

While in vitro assays may generate a quick assessment of cytocompatibility properties, in vivo studies are necessary to fully evaluate new bone growth. As with in vitro analysis, the varied anodized titanium oxide properties not only include thickness, but also morphology, chemical composition, crystallinity, and surface roughness. All of the following in vivo studies are on the micro-porous ASD anodized titanium implants but not anodized titanium with titania nanotubes.

Some in vivo studies have been mainly interested in studying the effects of thick, porous oxide coatings on new bone growth. Less than 200 nm thick oxide films on threaded titanium implants from anodization in acetic acid showed no significant difference in new bone growth compared to unanodized samples after

implantation into rabbit proximal tibial bones for 6 weeks [28]. In contrast, a $\text{H}_3\text{PO}_4/\text{H}_2\text{SO}_4$ electrolyte solution has usually been used to form thick anodic films up to tens of microns. Enhanced bone bonding was found for titanium screws with micron-thick porous anodic oxide films compared to unanodized titanium screws in both a rabbit tibia model and a dog mandible model [76-78].

More importantly, changes in surface chemistry could play a more important role in inducing new bone growth than oxide thickness. Several in vivo studies have focused on Ca-P enriched anodized titanium with and without hydrothermal treatments [57, 59, 60, 73, 79]. Ishizawa et al. produced 1-2 μm thick HA films on anodic oxide layers of titanium and compared bone growth on them with unanodized titanium [73]. They found stronger bone bonding via push-out tests with anodized titanium compared to unanodized titanium after 8 weeks in a transcortical rabbit femoral model [80]. Following their in vitro work, Fini et al. found the lowest bone contact percentage on anodized titanium while the highest was found on the anodized and hydrothermally treated titanium in a rat femoral condyle model after 8 weeks [60]. The low bone contact on anodized titanium was probably due to the in vivo reduction and degradation of the amorphous titania layer while HA crystals aided bone opposition. Giavaresi et al. also supported the positive role HA produced after hydrothermal treatment in accelerating bone ingrowth and bone mineralization in a sheep femoral diaphysis model [79]. Son et al. reported significantly increased removal torque strength for

anodized titanium implants compared to unanodized implants after 6 weeks of implantation into a rabbit tibia metaphysis [59].

One drawback of most of the above studies is that both chemical composition and surface morphology changed after titanium anodization so that it is hard to verify the role of one material property over the other. However, Sul indirectly verified the chemical change associated with anodization in vivo by maintaining surface morphology/roughness and changing chemical characteristics (specifically, S, P and Ca enriched implants via titanium anodization using different electrolyte compositions) [81]. The removal torque value (RTQ) showed significant differences between Ca-containing anodized titanium implants and unanodized titanium implants as well as S-containing anodized titanium implants and unanodized titanium implants in a rabbit femora and tibiae model after 6 weeks. The bone to metal contact was 186%, 232%, and 272% higher in S, P, and Ca implants, respectively, when compared to the control groups (unanodized titanium). These results confirmed that ions incorporated into the titanium oxide layer during anodization could have important roles in enhancing bone juxtaposition.

1.4. Drug Delivery Applications

Besides the nanoscale dimensional advantages involved in cell-implant interactions, the potential of such nanotubular structures on a titanium implant surface created by anodization lies in another field, that is, controlled local drug

delivery. The use of anodized nanotubular titanium for drug delivery has been well under-studied to date.

1.4.1. Drug Candidates

Although infection is not the most common reason for implant failure, infection of tissue-surrounding-orthopedic implants after implantation still poses a great threat to patients. Infection enters the body through skin (i.e., puncture wounds) and such infection of skin and other soft tissues can subsequently lead to bone infection (osteomyelitis) and joint infection (septic arthritis). Without prompt treatment, infection due to a orthopedic implant can become chronic and depending upon severity and other factors, osteomyelitis can cause irreversible damage (necrosis) to bone and bone cells. As a result, antibiotic (oral) treatment is usually prescribed to patients to prevent infection.

However, current ways to deliver such antibiotics to fight such infection (orally, intravenously, intramuscularly or topically) are not very effective at reaching the infection site. The previous use of antibiotic bone cements helped decrease infection rates [82], but the current trend in orthopedics (especially for younger patients) is to use cementless bone implants. Therefore, a local delivery of antibiotics by the medical device itself would be a novel, more effective way to fight infection without undesirable systematic toxicity. Antibiotics like penicillin/streptomycin (P/S), penicillin G sodium salt (P-G) are common candidates here (their molecular structures are given in Figure 1.4).

From the signal transduction point of view, the introduction of drugs to either minimize inflammation or induce new bone growth will be a direct way to promote implant-bone bonding if the release is local and can be sustained for a proper period of time. Dexamethasone can be used as an anti-inflammatory and immunosuppressant. Importantly, dexamethasone has been shown to induce osteoblast differentiation in several cell culture systems, including osteoblastic differentiation of vascular smooth muscle cells in vascular calcification and similarly differentiation of human bone marrow stromal cells in bone mineralization [83, 84]. In short, dexamethasone will decrease fibrous tissue formation resulting from an excessive foreign body response, promote osteoblast differentiation, and potentially increase new bone formation.

Last but not least, shorter regions of bone growth factors (like bone morphogenetic protein 2, BMP-2) can be used as a bone growing drug. Comparing the size of BMP molecules to anodized titania nanotubular structures, it is only feasible to use some smaller active fragments of the protein instead of the whole molecule. A peptide sequence (such as KIPKASSVPTELSAISTLYL) containing 20 amino acids (the 73–92 peptide of BMP-2) has been observed to be one of the cell receptor-binding sites on BMP-2 and may stimulate the differentiation of bone precursor cells to become osteoblasts to deposit bone [85, 86]; it is the so-called knuckle region of BMP-2.

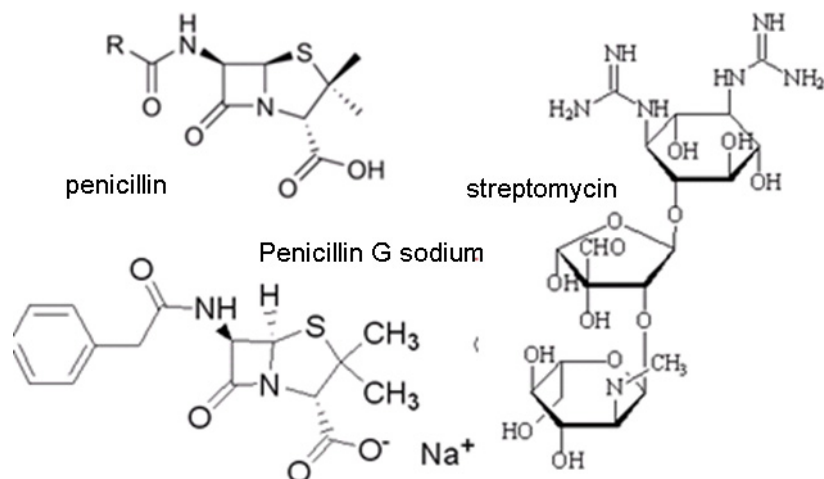


Figure 1.4 Chemical structures of penicillin, streptomycin, and penicillin G sodium salt.

1.4.2. Drug Loading Strategy and Methods

Of course, for these drugs to be useful, they must be incorporated into or onto currently-used titanium implants. Potential approaches, including a conventional soaking method and two other novel methods, electrodeposition and co-precipitation, are described below.

1.4.2.1. Drug Physical Adsorption and Mediating Surface Wettability

For this approach, the surface wettability of titanium implants is of great interest due to the fact that in biological systems, the nature of hydrophobic and hydration forces play a key role in the mediation of protein adsorption leading to cell adhesion [87]. Anodizing titanium in an HF electrolyte solution creates a very hydrophilic titanium surface [88] and this is possibly one of the reasons why hydrophilic proteins (like vitronectin) favor anodized titanium surfaces [89].

Physical adsorption of drugs is a well accepted method to load drugs onto and into anodized titania nanotubular structures. There are a few considerations here to improve the adsorption of drug molecules onto the titanium surface. First, a super-hydrophilic surface would be beneficial towards wetting the titania nanotubes and, thus, favor interactions with hydrophilic drug molecules. Second, since not all drug molecules are hydrophilic, a hydrophilic surface is not always a favorable approach. Moreover, interactions between a hydrophobic surface and hydrophobic drug molecules are stronger than those between a hydrophilic surface and hydrophilic molecules [90], which will certainly affect the release of drug molecules. As a result, for a specific drug molecule, there may be a range of optimal surface wettability properties (hydrophilicity/hydrophobicity) that achieve the best loading efficiency and release rates.

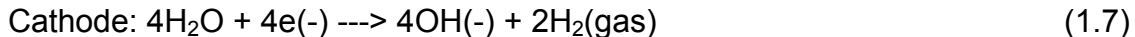
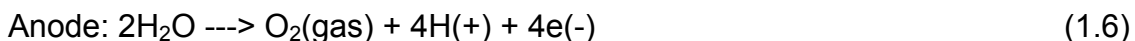
For this purpose, there are some existing methods to mediate titanium surface wettability. One of them is to coat the titanium surface with a layer of hydrophobic organic molecules (such as octadecylsilane or octadecylphosphonic acid) [87]. UV irradiation could then cause chain scission and removal of the unpolar portion of the molecule so the surface wettability gradually changes. The other method, adopted in the present study, involves silanization that covalently links various functional groups and ligands onto a titanium surface [91-94]. This method has been used for various metal oxides surfaces because they contain hydroxyl groups which attack and form a covalent -Si-O-Si- bond on silane. Different functional groups (such as amine, methacrylates or epoxides) introduced by

silanization onto titanium surfaces could result in different degrees of hydrophobicity. It is proposed here to introduce amine groups via silanization that will result in a certain controllable level of hydrophobicity and substitute such amine groups with methyl groups to result in a higher degree of hydrophobicity. Anodized titanium surfaces with minimal (non-silanization), medium, and maximum hydrophobicity can then be chosen based on the properties of a specific drug.

1.4.2.2. Drug Cathodic Electrodeposition

During physical adsorption, the loading of drug molecules into titania nanotube structures depends on the wetting process and the diffusion/sediment of drug molecules. It is reasonable to consider that the driving force for drug loading might not be significant enough if the nanotube structures are more difficult to penetrate (due to surface tension). In this light, another method, electrodeposition, has been adopted in the current study in an attempt to increase the loading efficiency of specific drug molecules and decrease the processing time.

An electrodeposition system is similar to anodization in many ways except that the cathode is the working electrode. In this cathodic process, positively charged molecules are directed by an applied electrical field and are deposited on the cathode. The primary electrochemical process which occurs during aqueous electrodeposition is the electrolysis of water.



There are several mechanisms by which drug molecules can be deposited on the electrode: charge destruction and decreased solubility, concentration coagulation, and salting out. For charge destruction, when the salt of the base as a charge bearing group is formed by protonation of the base, the protonated base will react with the hydroxyl ions formed by electrolysis of water to yield the neutral charged base (charge destruction). The uncharged polymer is less soluble than it was when charged and, thus, will precipitate onto the cathode. Concentration coagulation means aggregating colloidal and fine particles into a settleable material. The salting out is based on the principle that proteins are less soluble at high salt concentrations.

There are several factors that affect the quality of the deposited coatings in cathodic electrodeposition. First of all, voltage is the most important parameter to control the amount of coating. This is because the electrochemical resistance decreases proportionally to the thickness of the deposited film, and thus, at a given voltage, the electric current finally reaches a point where deposition has stopped occurring. Second, increase of the coating temperature increases the conductivity of both the bath and deposited film. Temperature also has an effect on the viscosity of the deposited film. Third, the coating time usually determines the film thickness and the quality of the deposited film. Depending on the type of

the object being coated, coating times of several seconds up to several minutes may be appropriate.

1.4.2.3. Drug Co-precipitation with Calcium Phosphate

As mentioned in section 1.3.5, apatite-like minerals can be formed through anodization followed by chemical precipitation treatments. For example, Yang et al. soaked titanium in SBF for 6 days after H_2SO_4 anodization and observed uniform apatite formation [52]. One advantage of this method is that the composition and surface morphology of the resulting apatite layer is very close to that in natural bone. Similarly, a two-step procedure was used to produce nanoscale HA on anodized titanium possessing nanotubular structures [74]. Specifically, the anodized titanium was treated with NaOH to form nanofibers of bioactive sodium titanate structures on the top edge of the nanotube wall, which was then immersed in a SBF solution to induce the formation of nanoscale calcium phosphate and then HA. This technique could be useful as well-adherent bioactive nano-HA layers on titanium-based implants are created which simulate the size and shape of natural HA in bone. The advantage of introducing nano-HA onto titanium anodized structures was supported by previous work revealing greater osteoblast adhesion, proliferation, and differentiation on nano-HA compared to conventional, or micron grain size, HA [95].

The dissolution of HA is much longer when it is relatively high in crystallinity. The hypothesis of the present study is that during the apatite formation process, pre-

mixed drug molecules into SBF can be integrated into those apatite structures probably via molecular interactions. After that, drug molecules could be released by a diffusion process or by the degradation of apatite and result in a longer release period from weeks to months.

1.4.3. Controlled Release

A successful drug delivery system in this study requires controlled or sustained drug release kinetics. For an ideal diffusion controlled system (membrane system), the drug release rate is independent of time, so that the release kinetics is called zero-order (see Equation 1.6).

$$Mt = A \cdot \frac{DK\Delta C}{\ln \frac{r}{R}} \cdot t \quad (1.6)$$

Where A is area, D is diffusion coefficient, K is partition coefficient, ΔC is concentration difference, and r/R is inner/outer radius ratio in a cylinder reservoir.

There are other equations to describe the release kinetics. A first-order equation describes the release from systems where dissolution rate is dependent on the concentration of the dissolving species ($Mt \sim [A]$). The Higuchi square root equation describes the percent dissolved as a function of the square root of time ($Mt \sim t^{0.5}$).

In the present study, when using adsorption or the electrodeposition method, this anodized titanium matrix loaded with drugs cannot be considered as a

membrane system. The drug molecules loaded into titania nanotubes are directly exposed to the body fluid and may release much more quickly than zero-order kinetics. On the contrary, the co-precipitated drug molecules are expected to undergo more sustained release kinetics. Their release kinetics will be studied and compared to existing models in the present study.

1.5. Objectives

As discussed above, the demands for designing novel orthopedic implants come from the fact that current titanium-based implants lack sufficient bioactivity and are subjected to long-term failure rates due to insufficient bone bonding with juxtaposed bone. In this manner, a new approach based on anodizing titanium is proposed here to modify the surface properties of current titanium-based implants. Moreover, nanotube arrays on anodized titanium implant surfaces can be used for novel drug delivery purposes further ensuring the efficacy of such implant materials. The specific aims of this study are, thus, to:

Part 1: Anodize metals for use as a novel bone implant surface.

- (i) Anodize titanium to possess nanotubular structures on its surface.
- (ii) Test the versatility of anodization on Ti6Al4V and CoCr alloys towards creating nanoscale features.
- (iii) Characterize the surface properties of anodized titanium, Ti6Al4V, and CoCr.

- (iv) Characterize the in vitro osteoblast and chondrocyte responses on anodized titanium, Ti6Al4V, and CoCr and underlying initial protein interactions that may explain such trends.

Part 2: Anodize titanium for use as a novel drug delivering system.

- (i) Chemically functionalize anodized nanotubular titanium surfaces to possess varied wettability suitable for loading a wide range of drugs.
- (ii) Load antibiotics into modified nanotube arrays using appropriate methods including soaking, electrodeposition, and co-precipitation with calcium phosphate.
- (iii) Characterize the drug release behavior in vitro.
- (iv) Characterize the ability of such antibiotics loaded anodized titanium to inhibit bacteria responses.
- (v) Characterize in vitro bone cell responses to different drug-loaded anodized nanotubular titanium.

It is important to point out that although in this study the first attempt will only include antibiotics, it is anticipated that several different kinds of drug molecules and motifs would be incorporated into anodized nanotubular titanium to be released in a controlled manner. The goal is to eventually control drug release corresponding to the respective requirements by the bone remodeling phenomena (Figure 1.5). For example, it is eventually envisioned that the first drugs released from titania nanotubes will attract bone marrow cells and the region of drugs released later will promote their differentiation into osteoblasts.

Further, it is anticipated that the nanotube arrays should be able to load drugs in a deliberate spatial arrangement so that multiple drugs can be loaded in specific areas of the anodized titania nanotube arrays to correlate with specific regions of the cell. That is, in the future, it is envisioned that drugs which act on specific internal cellular processes (such as the mitochondria, nucleus, etc.) can be spatially matched with underlying nanotubes and, thus, release drugs locally to the site of need. However, the first series of steps in this direction is to create such nanotubes, examine osteoblast functions on such nanotubes, load drugs into these nanotubes, and control drug release.

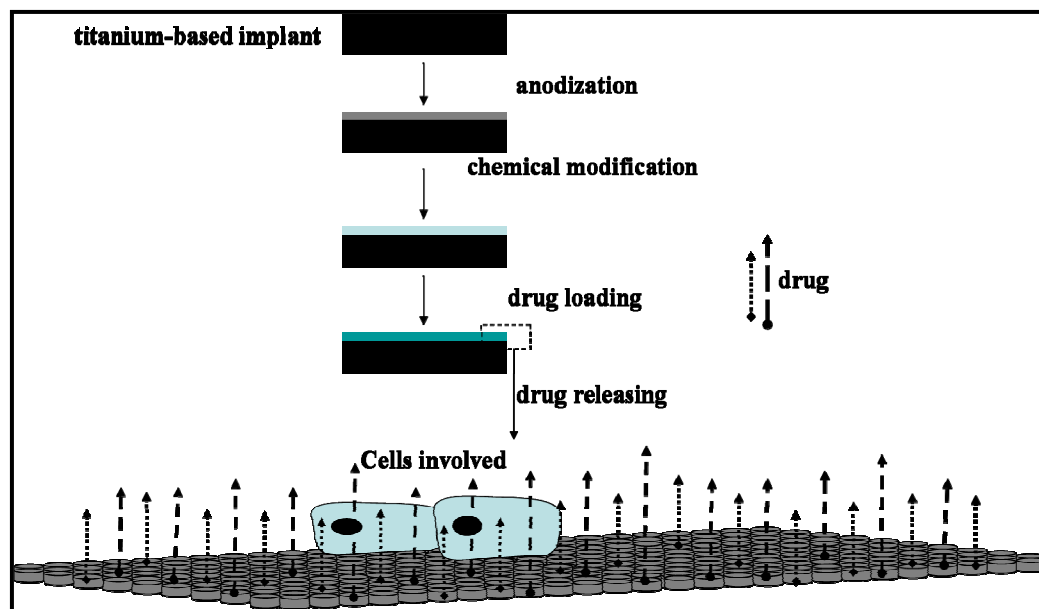


Figure 1.5 Flow chart of using anodized nanotubular titanium implants for local drug delivery.

CHAPTER 2. MATERIALS AND METHODS

2.1. Material Preparation

2.1.1. Titanium

Titanium foil (10 x 10 x 0.2 cm; 99.2 % pure) was purchased from Alfa Aesar, Inc. and was cut into 1 x 1 cm squares using a metal abrasive cutter (Buchler). All the substrates were then cleaned with liquid soap (VWR) and by soaking in 70 % ethanol (AAPER) for 10 min in an aqua sonicator (VWR). Substrates were then dried in an oven (VWR) at about 65 °C for 30 min, and were then immersed in an acidic mixture of 2 ml of 48 wt. % hydrofluoric acid (HF, Mallinckrodt) plus 3 ml of 70 wt. % nitric acid (HNO₃, Mallinckrodt) and 100 ml of deionized (DI) water for 5 min to remove the natural oxide layer that formed on the surface of the titanium. The acid-polished substrates were then immediately treated by anodization as described in section 2.2.1. After anodization, the titanium substrates were rinsed with DI H₂O and dried in an oven at about 65 °C for 30 min. The dried titanium samples were sterilized in an autoclave (VWR) at 120 °C for 30 min if used for in vitro studies.

Unanodized titanium substrates were used as control samples. Borosilicate glass (Fisher Scientific; 1.8 or 1.2 cm diameter) was used as a reference material in the present study. The glass coverslips were degreased by soaking in acetone

(Mallinckrodt) for 10 min, sonicating in acetone for 10 min, soaking in 70 % ethanol for 10 min, and sonicating in ethanol for 10 min. Lastly, the coverslips were etched in 1 N NaOH (Sigma) for 1 hour at room temperature. For use in cell experiments as described in section 2.7, glass coverslips were then rinsed thoroughly with DI H₂O, dried in an oven at about 65 °C for 30 min, and sterilized in an autoclave at 120 °C for 30 min.

2.1.2. Titanium Alloy

1 cm diameter circular discs of Ti6Al4V (alpha-beta Ti alloy) obtained from DePuy, Inc. were cleaned with liquid soap and by soaking in 70 % ethanol for 10 min in an aqua sonicator. Substrates were then dried in an oven at about 65 °C for 30 min, and were then immersed in an acidic mixture of 2 ml of 48 wt. % HF, plus 3 ml of 70 wt. % HNO₃ and 100 ml of DI water for 5 min. The substrates were then immediately treated by anodization as described in section 2.2.2. After anodization, the titanium substrates were rinsed with DI H₂O and dried in an oven at about 65 °C for 30 min. The dried titanium samples were sterilized in an autoclave (VWR) at 120 °C for 30 min if used for in vitro studies.

2.1.3. Cobalt-Chromium Alloy

CoCrMo alloy samples obtained from Arthrosurface, Inc. were cleaned with liquid soap and by soaking in 70 % ethanol for 10 min in an aqua sonicator. Substrates were then dried in an oven at about 65 °C for 30 min. The dried substrates were

directly treated by anodization as described in section 2.2.3. After anodization, the titanium substrates were rinsed with DI H₂O and dried in an oven at about 65 °C for 30 min. The dried titanium samples were sterilized in an autoclave at 120 °C for 30 min if used for in vitro studies.

2.2. Anodization for Titanium and Other Materials

2.2.1. Titanium Anodization

Anodization was carried out in a two electrode electrochemical system. The titanium-based substrates cleaned as described in section 2.1.1 served as an anode in the anodization treatment while a high purity platinum sheet (Alfa Aesar) was used as a cathode (Fig 2.1). The anode and cathode were connected by copper wires and were linked to a positive and negative port of a 36V / 3A power supply (Circuit Specialists Inc.), respectively. During processing, the anode and cathode were kept parallel with a separation distance of about 1 cm, and were submerged into an electrolyte solution in a Teflon beaker (VWR). Dilute HF (either 0.5 wt % or 1.5 wt. %) was used as an electrolyte.

To study the evolution of resulting titanium-based anodized structures, different time periods of anodization were utilized. Anodization was conducted in 0.5 wt% HF for 10 sec, 30 sec, 1 min, 2 min, 5 min, and 20 min at a constant voltage of 20 V.

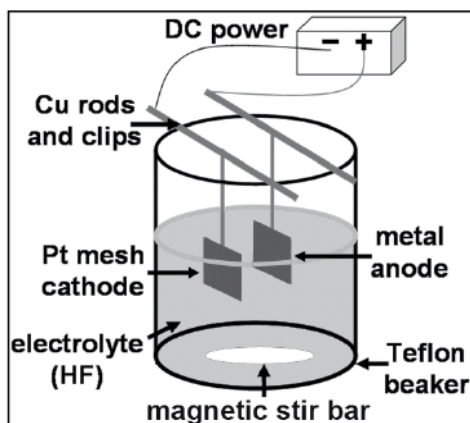


Figure 2.1 The anodization system.

To study the effect of voltage on the anodized titanium-based surfaces, a series of voltages ranging from 5 V to 25 V were used during the experiments. All anodization runs lasted for 20 min in this study.

To study the effect of electrolyte concentration on the resulting anodized structures, 0.5 wt% HF and 1.5 wt% HF were both used. The anodization processes were conducted for 20 min at room temperature for both concentrations.

Finally, two kinds of anodized titanium substrates were created for further characterization in this study. They were titanium substrates anodized in 0.5% HF under 20 V for 20 min (termed anodized titanium with nanotubular structures) and titanium substrates anodized in 0.5% HF under 10 V for 20 min (termed anodized titanium with nanoparticulate structures).

2.2.2. Titanium Alloy Anodization

The cleaned titanium alloy samples served as an anode in the anodization treatment as described above. The anodization conditions used included a 20 V applied voltage, 5 min duration, and 1.5% HF electrolyte according to the above study anodizing titanium.

2.2.3. CoCr Alloy Anodization

The cleaned CoCr alloy samples were anodized in the same system as described in section 2.2.1. To study the evolution of the resulting anodized CoCr structures, different time periods of anodization were utilized. Anodization was conducted in a 1.5 wt% HF solution for 5 s, 10 s, 30 s, and 2 min at a constant voltage of 10 V. To study the effects of voltage on the resulting anodized CoCr structures, an applied voltage ranging from 2 to 10 V was used. To study the effect of electrolyte composition on the resulting CoCr anodized structures, 0.5 wt. % HF, 1.5 wt. % HF, 1 M sulfuric acid (H_2SO_4), 1 M H_2SO_4 /0.25 wt. % HF, 1 M phosphoric acid (H_3PO_4), 1 M H_3PO_4 /0.25 wt.% HF and 0.1 M NaOH were used.

2.3. Post-Anodization Chemical Modifications

In an effort to explore the possible use of anodized titanium with nanotubular structures to deliver drugs, various surface modification techniques were completed in this study.

There were three steps of chemical reactions in this study. To introduce hydroxyl groups on anodized titanium, anodized titanium substrates were soaked in a mixture of sulfuric acid and hydrogen peroxide (1:1, Sigma), so called Piranha solution, for 10 minutes (step 1). After that, silanization was conducted by immersing samples in 100 ml of a non-aqueous solution of 10 % amino-functional organosilane (APTES, Sigma) in toluene (step 2, Figure 2.2). The reaction was heated by an oil bath at 110 °C for 4 h. This silanization reaction resulted in the formation of amine groups terminated on anodized titanium surfaces. Finally, some of the samples underwent further chemical reactions with acetic anhydride (Sigma) for 30 min with stirring to substitute amine groups with methyl groups (step 3).

After chemical modification, five different types of titanium substrates were used for the following experiments. They included: unanodized Ti (U), anodized titanium (A), anodized titanium terminated with hydroxyl groups (A-OH), anodized titanium terminated with amine groups (A-NH₂), and anodized titanium terminated with methyl groups (A-CH₃). These abbreviations will be used often in the following chapters for convenience.

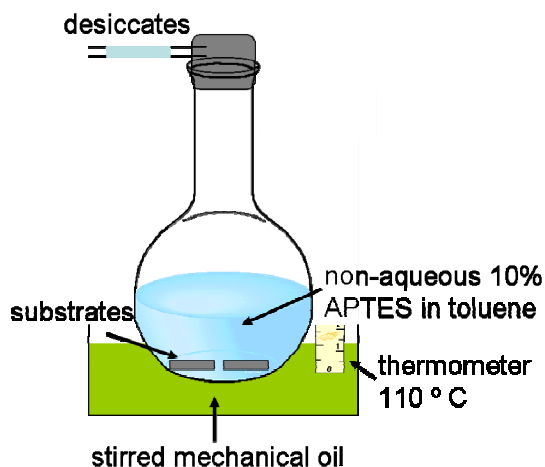


Figure 2.2 Schematic of the silanization process for anodized titanium.

2.4. Drug Loading

2.4.1. Drug Physical Adsorption

To assess drug loading, anodized titanium substrates of different surface chemistry were immersed into 1ml of either a P/S solution (containing 6.25 mg penicillin and 10 mg streptomycin per ml) or a P-G sodium salt (6.25 mg penicillin per ml) for a predetermined time (24 hours) under room temperature in a vacuum oven (-20 inch Hg, equaled to -0.67 atmospheric). Samples were then taken out of the oven, rinsed with enough DI water to remove the excessive drug solutions remaining on the surface. These samples were vacuum dried until use. Some of the samples were imaged by a scanning electron microscope to observe the morphology of the drugs adsorbed onto and into titania nanotube structures. The other samples were used for drug release experiments.

2.4.2. Drug Electrodeposition

Another method used to load drugs into the substrates of interest to the present study was cathodic electrodeposition. In this method, titanium substrates (or modified titanium substrates described in section 2.3) were used as a cathode in an electrochemical cell similar to that of anodization. A 5% penicillin solution in DI water (P/S or P-G) was used as an electrolyte. 0.9 wt. % NaCl was used as a control electrolyte.

The applied voltage was constant at 5 V or 8V according to experimental observations. The deposition time was 5 min.

2.4.3. Drug Co-precipitation with Calcium Phosphate

A third method used to load drug molecules into the substrates of interest was a co-precipitation method. This method was distinctive from the previous physical adsorption method and used different post-anodization treatments (Figure 2.3). Specifically, after cleaning, the anodized titanium samples were soaked in a 6.0 M sodium hydroxide for approximately 1 hour to form sodium titanate on the surface. The samples were then removed and placed in a furnace at 500 °C in the air for approximately 2 hours. After this, the samples were allowed to cool to room temperature in air. These samples are abbreviated as ASH samples for the rest of this thesis. Once the samples were prepared they were allowed to soak in 1.5X Simulated Body Fluid (SBF, containing 11.994 g NaCl, 0.525 g NaHCO₃, 0.336 g KCl, 0.342 g K₂HPO₄·3H₂O, 0.458 g MgCl₂·6H₂O, 0.417 g CaCl₂, 0.107 g

Na_2SO_4 , and 9.086 g $(\text{CH}_2\text{OH})_3\text{CNH}_2$ in 1000 ml dH_2O , pH 7.25) or a mixture of P/S (5 vol.%, 10 vol.%, 20 vol.%) in 1.5X SBF for 3 days. After soaking, they were dried at room temperature overnight and prepared for observation and analysis using a scanning electron microscope.

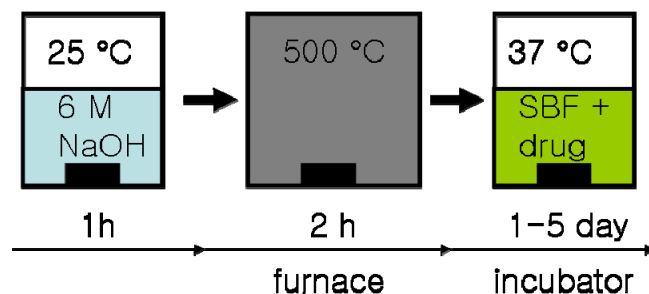


Figure 2.3 Steps to co-precipitate antibiotics with apatite crystals in a 1.5 X SBF solution.

2.5. Drug Release

Drug loaded samples were transferred to a new tissue culture plate, immersed into 0.5 ml of PBS and incubated under 37 °C. The buffer solution was collected and replaced with the same amount of fresh buffer solution after select time periods (1h, 2h, 24h, 48h and up to 3 weeks). The amount of drug released from the sample surface was analyzed using a commercially available assay kit (microBCA, Pierce). Specifically, 150 μl of each supernatant was added to same amount of BCA reagent and incubated for 2 hours. A purple-colored reaction product was formed afterwards. Finally, the optical absorbance was measured at 562 nm wavelength using a spectrophotometer.

2.6. Surface Characterization

2.6.1. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectrometry (EDS)

The surface morphology of the unanodized and anodized titanium-based substrates as well as CoCr alloys was characterized using a LEO Field Emission Scanning Electron Microscope (FE-SEM) for ultra-high magnifications. SEM was also used to characterize cell morphology on various titanium substrates and to characterize titanium morphology after further modifications. It was also used to characterize titanium substrates loaded with drugs. For all non-conductive samples (like those with adherent cells), samples were sputter-coated with AuPd before imaging using a sputter-coater for 2 min. An energy dispersive spectrometer attached to the SEM system was used to detect surface chemistry of various substrates.

2.6.2. Atomic Force Microscopy (AFM)

Surface roughness of the substrates was measured by AFM (Multimode SPM Digital Instruments Veeco). The typical tip (NSC15; Mikromasch) curvature radius of the AFM probe used in the present study was less than 10 nm. The measurements were conducted in ambient air under tapping mode with a scan rate of 2 Hz. The scan area was 1 x 1 μm for titanium/anodized titanium, 5 x 5 μm for unanodized/anodized Ti6Al4V, and 10 x 10 μm for unanodized/anodized

CoCr. The root mean square (rms) roughness and relative surface area were estimated with the aid of the Nanoscope imaging software (Veeco).

2.6.3. CBQCA Assay

ATTO-TAG CBQCA (3-(4carboxybenzoyl)quinoline- 2-carboxaldehyde) derivatization reagent (Invitrogen) reacts specifically with primary amines to form conjugates that can be analyzed by electrophoretic or chromatographic methods (Figure 2.4). This was used in the present study to qualitatively confirm the efficiency of the silanization process (i.e., the formation of A-NH₂ samples). Specifically, anodized titanium, anodized titanium terminated with hydroxyl groups, amine groups, and methyl groups were covered with 200 μ l of a reagent solution (including 1 volume of 10 mM CBQCA in DMSO solution and 2 volume of 10 mM potassium cyanide solution) for 1 hour at room temperature. After that, the samples were observed under a fluorescence microscope (Leica DM5500B) at 450 nm wavelength. The appearance of green fluorescence signified that primary amines were on the titanium surfaces.

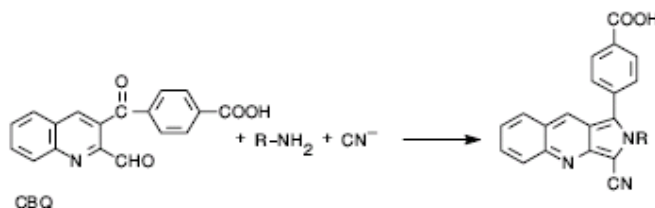


Figure 2.4 CBQ molecule reacts with primary amines to form CBQCA conjugates.

2.6.4. Contact Angle Measurements

Sessile contact angles on each substrate were measured 20 seconds after placing 3 μ l of DI water or polyethylene glycol (PEG, Sigma) onto the sample surface at room temperature. Images of drops were recorded by a contact angle analyzer (EasyDrop, Brüss) and contact angles were calculated by the Drop Shape Analysis software using a circle fitting method (the drop outline is adapted mathematically to a circular segment shape). The surface energy of various substrates was also calculated with the help of the Drop Shape Analysis software using the Owens-Wendt method. This method uses two pairs of contact angle values of two liquids with known disperse and polar fractions of the surface energy to calculate surface energy of a solid divided into dispersive and polar components. In this method, the surface tension of each phase can be split up into a polar and disperse fraction:

$$\sigma_l = \sigma_l^P + \sigma_l^D \quad (2.1)$$

$$\sigma_s = \sigma_s^P + \sigma_s^D \quad (2.2)$$

The surface energy of the solid is calculated using the following equations:

$$\gamma_{sl} = \sigma_s + \sigma_l - 2(\sqrt{\sigma_s^D \cdot \sigma_l^D} + \sqrt{\sigma_s^P \cdot \sigma_l^P}) \quad (2.3)$$

$$\sigma_s = \gamma_{sl} + \sigma_l \cos(\theta) \quad (2.4)$$

2.6.5. X-ray Photoelectron Spectroscopy (XPS) Analysis

All XPS spectra were taken on a Perkin Elmer 5500 Multitechnique Surface Analyzer. This instrument has a monochromatized Al K α X-ray (1486.6 eV). X-ray spot size for these acquisitions was on the order of 300 μ m. Pressure in the

analytical chamber during spectral acquisition was less than 5×10^{-9} Torr. The take-off angle (the angle between the sample normal and the input axis of the energy analyzer) was $\sim 55^\circ$ (55° take-off angle means about 50 Å sampling depth).

XPS was used to detect the surface chemistry changes after each step of the surface modification techniques. A sputter gun was used to blast a certain thickness of the outermost surface layers to analyze the chemistry changes along the depth of the sample.

2.7. In Vitro Cytocompatibility Tests

Since titanium, titanium-based alloys and CoCr alloys have been used for both hip and knee replacements, both bone-forming cells (osteoblasts) and cartilage-synthesizing cells (chondrocytes) were used for the present study.

2.7.1. Cells

Human osteoblasts (CRL-11372; American Type Culture Collection) were used at population numbers between 6~12. When necessary, frozen cells stored in cryogenic vials were quickly brought to 37°C and transferred to cell culture dishes. Then, the osteoblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% P/S (Hyclone) under standard cell culture conditions, i.e., a humidified, 5%

CO₂ / 95% air environment at 37 °C. After 6 hours, the media was replaced for the first time.

Human articular chondrocytes (Cell Applications Inc.) were cultured in chondrocyte growth medium (Cell Applications Inc.). Cells were incubated under standard cell culture conditions. Chondrocytes used for the following experiments were at population numbers below 10.

2.7.2. Osteoblast Responses

2.7.2.1. Osteoblast Adhesion on Different Anodized Materials

This study compared osteoblast adhesion after 4 hours of culture between:

(1) unanodized titanium (control) and two kinds of anodized titanium (one anodized in 0.5 % HF, 20 V, 20 min and the other in 0.5 % HF, 10 V, 20 min); (2) unanodized Ti6Al4V (control) and anodized Ti6Al4V (anodization condition: 1.5 % HF, 20V, 5 min); and (3) unanodized CoCrMo (control) and anodized CoCrMo (1.5% HF, 10 V, 2 min).

To determine osteoblast adhesion, substrates of interest to the present study were placed in 12-well cell culture plates (Corning) and were quickly rinsed with phosphate buffered saline (PBS; a solution containing 8 g NaCl, 0.2 g KCl, 1.5 g Na₂PO₄, and 0.2 g KH₂PO₄ in 1000 ml DI water adjusted to a pH of 7.4; all chemicals from Sigma). Osteoblasts at a density of 3500 cells/cm² were seeded

onto all samples in 2 ml of DMEM supplemented with 10% FBS and 1% P/S.

The samples were then incubated under standard cell culture conditions for 4 hours.

After that time period, the culture medium was removed from all wells and the substrates were gently rinsed with PBS three times to remove any non-adherent cells. The adherent cells were then fixed with formaldehyde and were stained with Hoescht 33258 dye (Sigma). The cell numbers in five random fields were counted under a fluorescence microscope (Leica; excitation wavelength 365 and emission wavelength 400 nm).

All experiments were run in triplicate and repeated at three separate times. Cell adhesion was evaluated based on the mean value of the number of adherent cells with standard error of the mean (SEM).

Osteoblast morphology on the unanodized and anodized titanium-based substrates was observed using SEM. For this purpose, after the 4 hour adhesion test, the osteoblasts on the substrates were fixed with 2 % glutaraldehyde (Electron Microscopy Sciences) in 0.1 M cacodylate (pH 7.4; Electron Microscopy Sciences) for 30 min at 4 °C. After washing with cacodylate buffer, the cells were secondarily fixed with 1 % osmium tetroxide (Electron Microscopy Sciences) in 0.1 M cacodylate (pH 7.4) for 30 min at 4 °C. The cells were dehydrated through a series of ethanol solutions (from 30, 50, 70, 90, to 100 %;

AAPER) and were finally dried by critical point drying (CPD; LADD Research Industries). Specifically, the specimens were immersed into liquid CO₂ until there was a complete exchange of liquid CO₂ for the ethanol in the specimens. The specimens were then heated above 34 °C under 7.6 MPa where all liquid CO₂ was converted to gaseous CO₂ and the specimens were dry. Before imaging, all the samples were sputter-coated with AuPd for 3 min using a HUMMER I sputter-coater.

2.7.2.2. Osteoblast Long Term Functions on Anodized Titanium

Although adhesion of anchorage dependent cells is important for bone deposition, it does not ensure bone deposition. Thus, osteoblast long term functions (such as total protein synthesis and calcium deposition) on the anodized titanium were determined in the present study.

Osteoblasts were seeded (100,000 cell/cm²) onto the substrates and were cultured in complete DMEM (that is, DMEM supplemented with 10% FBS, 1% P/S, 50 µg/ml ascorbate (Sigma) and 10 mM β-glycerophosphate (Sigma)) under standard cell culture conditions for 7, 14, and 21 days. During the long term osteoblast function experiments, the media was replaced every other day. At the end of the prescribed time periods, the substrates were first rinsed with Tris-buffered saline (TBS; a solution consisting of 42 mM Tris-HCl, 8 mM Tris Base and 0.15 M NaCl adjusted to a pH of 7.4; all chemicals from Sigma) three times

and then the osteoblasts were lysed using distilled water and three freeze-thaw cycles.

Total protein content in the cell lysates was determined spectrophotometrically using a BCA Protein Assay Reagent Kit (Pierce Chemical Co.) and following manufacturer's instructions. Specifically, 25 μ l of each sample lysate was incubated with 200 μ l of working reagent (containing cupric sulfate and bicinchoninic acid) at 37 °C for 30 min. Then, the light absorbance of these samples was measured by a spectrophotometer (SpectoMAX; Molecular Devices) at 562 nm. Total intracellular protein synthesized by osteoblasts cultured on the substrates was determined from a standard curve of absorbance versus known concentrations of albumin run in parallel with experimental samples. The total intracellular protein synthesis was normalized by substrate surface area.

Collagen is a well-known protein contained in the extracellular matrix of bone. To test this, the cell lysates were dispensed into a 96-well plate (Corning), 50 μ l per well. The collagen was allowed to dry on the plate through incubation at 37 °C for 16 hours and then was incubated at 37 °C for 24 hours in the presence of a desiccant (W.A. Hamond Drierite Company LTD.). Thereafter, the 96-well plate was rinsed three times with distilled water (1 min. per wash and 200 μ l per well). 100 μ l of 0.1% Sirius Red stain (Sirius Red powder in picric acid; Sigma) was dispensed into each well and was allowed to sit for one hour at room temperature. After that, each well was washed 5 times with 200 μ l of 0.01 M HCl for 10

seconds per wash. 200 μ l of 0.1 M NaOH was added into each well and was allowed to sit for 5 minutes. Finally, the solution in each well was mixed, transferred to a second plate, and absorbance was read at 540 nm in a spectrophotometer. The total intracellular collagen synthesized by osteoblasts cultured on the substrates was determined from a standard curve of absorbance versus known concentrations of collagen run in parallel with experimental samples.

Alkaline phosphatase is an enzyme whose synthesis indicates the differentiation of osteoblasts from non-calcium depositing to calcium depositing cells. To test this, a commercial Alkaline/Acid Phosphatase Assay Kit (Upstate) was used to determine the concentration of alkaline phosphatase in cell lysates following all manufacturer instructions. Aliquots of the distilled water supernatants were first mixed and incubated with 40 mM NiCl_2 , 5 mg/ml BSA, 1 mM phosphopeptide solution, and Pnpp Ser/Thr Assay Buffer at 37 °C for 10-15 min. Then, they were incubated with Malachite Green solution for 15-20 min at room temperature. The optical absorbance values were measured by a spectrophotometer (SpectoMAX; Molecular Devices) at 650 nm. Alkaline phosphatase synthesized by osteoblasts cultured on the substrates was determined from a standard curve of absorbance versus known concentrations of potassium phosphate monobasic run in parallel with experimental samples. The alkaline phosphatase activity was normalized by substrate surface area. All experiments were run in triplicate and were repeated three different times.

The ultimate indication of osteoblast differentiation is their ability to deposit calcium. To test this, the substrates (and remaining calcium deposited on them) were incubated with 0.6 N HCl (Sigma) at 37 °C overnight. The amount of calcium present in the acidic supernatant was quantified by a spectrophotometer using a Calcium Quantification Kit (Sigma) and following manufacturer's instructions; light absorbance of the samples was measured using a spectrophotometer at 575 nm. Total calcium (mg/dl) was calculated from standard curves of absorbance versus known concentrations of calcium measured in parallel with the experimental samples. Calcium concentration values (total) were normalized by substrate surface area. Same procedures were completed with titanium substrates to calculate calcium precipitation from medium ingredients when no osteoblasts were cultured in the medium. The final value of calcium deposited by osteoblasts was calculated by subtracting this value from the total calcium concentration.

All experiments were run in triplicate and were repeated three different times.

2.7.3. Chondrocyte Responses

2.7.3.1. Chondrocyte Adhesion

For adhesion experiments, chondrocytes were seeded at 3,500 cells/cm² per well and were allowed to attach for 4 hours. After the prescribed time point, non-adherent cells were removed by rinsing with PBS. The adherent cells were then

fixed with formaldehyde and were stained with DAPI (Sigma) and counted under a fluorescence microscope. Five random fields were counted per substrate and all experiments were run in triplicate, repeated three different times.

2.7.3.2. Chondrocyte Proliferation

For proliferation experiments, chondrocytes were seeded at 10,000 cells/cm² onto the substrates of interest to the present study and were allowed to proliferate for 1, 3, or 5 days. Cells were counted using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) based in vitro toxicology assay kit (Sigma-Aldrich). The MTT system is a simple, accurate, reproducible means of measuring the activity of living cells via mitochondrial dehydrogenase activity. Specifically, 100 µl of MTT solution was aseptically added into each cell culture well and incubated for 4 h. During this period, mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of MTT and yield purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals then were dissolved by adding 1 ml of acidified isopropanol. The resulting purple solution was spectrophotometrically measured at wavelength of 570 nm. An increase in cell number resulted in an increase in the amount of MTT formazan formed and, thus, an increase in absorbance. All experiments were run in triplicate, repeated three different times.

2.7.4. Mechanism of Cell Adhesion

Proteins are required for the adhesion of anchorage-dependent cells (such as osteoblasts and chondrocytes). To explore the effect of the titanium-based anodization treatment on protein adsorption, well-established ELISA (Enzyme-Linked Immunosorbent Assay) techniques were used in the present study. Two specific proteins, vitronectin and fibronectin, were chosen since they have been shown to play an important role in the adhesion of osteoblasts and chondrocytes onto material surfaces [96].

To test this, titanium substrates were separately exposed to either 500 μ L of DMEM (Hyclone) supplemented with 10% FBS and 1% P/S or serum free media overnight under standard cell culture conditions. The substrates were then rinsed with PBS twice and were blocked with 2% BSA (Sigma) for 1 hour. After that, the substrates were rinsed with PBS and incubated with an anti-bovine vitronectin antibody (6 μ g/mL in 1% BSA solution; Accurate Chemical) or an anti-bovine fibronectin antibody (6 μ g/mL in 1% BSA solution; Chemicon) for 1 hour under standard cell culture conditions. Thereafter, the substrates were rinsed with Tris buffered saline-0.1% Triton X-100 (Sigma) three times and were incubated with horse radish peroxidase conjugated with an anti-rabbit secondary antibody (1:100 BSA solution; Biorad) for 1 hour. The substrates were rinsed with Tris buffered saline-0.1% Triton X-100 three times, transferred to new cell culture plates and rinsed one more time with Tris buffered saline-0.1% Triton X-100. Finally, an ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6sulfonic acid) soluble

substrate kit (Vector Labs) was used to detect secondary antibodies using a spectrophotometer (SpectoMAX; Molecular Devices) at 405 nm. Light absorbance readings were used to highlight the difference between protein adsorption trends on the various substrates of interest to this study. All experiments were run in triplicate and were repeated three different times.

2.8. Bacteria Adhesion Test

2.8.1. Bacteria Culture

A bacteria cell line of biofilm-producing *S. epidermidis* was obtained in a freeze-dried form from the ATCC (35984). The pellet was rehydrated in 1 ml Luria broth (sterile and composed of 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter of distilled water, all chemicals from Sigma) and transferred to a test tube of 6 ml fresh Luria broth. The cells were propagated in an incubator at 37 °C until they reached a stationary phase (around 24 h). The cells solution were then passed to another test tube of 6 ml Luria broth at a 1:200 ratio (first passage cells solution to fresh Luria broth). Once the second passage of bacteria reached its stationary phase (around 24 h), the second passage was frozen in one part Luria bacteria broth and one part 50% glycerol (Sigma).

One day before bacteria seeding, a sterile 10 µl loop was used to withdraw bacteria from the thawed frozen stock and to inoculate a glass test tube with 3 ml of Luria broth. The test tube was agitated for approximately 16 h at 37 °C and

about 250 rpm in an incubator. Cell solution were then passed to a second 3 ml test tube of Luria broth and incubated for another 5 h under the same conditions as previously described. The bacteria concentration was assessed via optical density readings by a spectrophotometer (Molecular Device). According to previous studies, 30% transmittance was equated with a three on the McFarland scale and an estimation of 900,000,000 cells/ml. If necessary, the concentrated Luria bacteria broth was diluted with fresh Luria broth until achieving a 30% transmittance. This was done by reading a light absorbance of 0.52 at 562 nm wavelength using a spectrophotometer. Finally, the diluted bacteria was further diluted to a concentration of 10,000,000 with DMEM supplemented with 10% fetal bovine serum, 50 µg/ml ascorbate and 10 mM β-glycerophosphate (but without antibiotics).

2.8.2. Bacteria Adhesion

Substrates including (a) unanodized titanium, (b) anodized titanium with nanotube structures, (c) anodized titanium terminated with hydroxyl groups, (d) sample c loaded with P/S using an adsorption method as described in section 2.4.1, (e) sample c loaded with P-G as described in section 2.4.1. Prior to seeding, substrates of interest were placed into a 12-well cell culture plate and were rinsed with PBS twice. *S. epidermidis* were seeded at 20,000,000 per well and were allowed to adhere for 1 h in a standard bacteria culture condition (5% CO₂ / and 20% O₂ at 37 °C). At the end of the prescribed time period, the media was aspirated and the substrates were rinsed with TBS to remove unattached

cells. The bacteria were then fixed with formalin and stained with a Syto 9 (Molecular Probe) nuclei stain for 15 min under room temperature. Bacteria were counted using a fluorescence microscope with the help of Image Pro software. Ten fields were averaged for each substrate. Experiments were completed in triplicate and were repeated three different times.

2.9. Osteoblast Attachment on Antibiotic Loaded Surfaces

One main concern of the antibiotic drugs used in the present study is whether they will be harmful for osteoblast function. To determine this, substrates of interest to the present study were loaded with P/S as described in 2.4.1 or co-precipitated with P/S and HA as described in 2.4.3. Following that, all of the substrates were placed in 12-well cell culture plates (Corning) and were quickly rinsed with PBS. Osteoblasts at a density of 3500 cells/cm² were seeded onto all samples in 2 ml DMEM supplemented with 10% FBS and 1% P/S. The samples were then incubated under standard cell culture conditions for 4 hours.

After that time period, the culture medium was removed from all wells and the substrates were gently rinsed with PBS three times to remove any non-adherent cells. The adherent cells were then fixed with formaldehyde and were stained with DAPI (Sigma). The cell numbers in five random fields were counted under a fluorescence microscope.

All experiments were run in triplicate and repeated at three separate times. Cell adhesion was evaluated based on the mean value of the number of adherent cells with standard error of the mean (SEM).

2.10. Statistical Analysis

All numerical data were analyzed using standard student t-test; statistical significance was considered at $p < 0.1$.

CHAPTER 3. RESULTS

3.1. Anodized Orthopedic Implant Surfaces

3.1.1. Anodized Titanium Surfaces

Commercially pure titanium was a main interest of this research because of its availability and ease of processing. In this study, both bone and cartilage related cell functions were tested on anodized titanium to explore the potential applications of such novel implant surfaces. Ti6Al4V and CoCr were also tested due to their prominence in orthopedic applications.

3.1.1.1. Surface Characterization: Influence of Voltage, Time, and Electrolyte Solution

The unanodized titanium as purchased from the vendor possessed micron rough surface features as shown by the SEM image (Fig. 3.1 a). Upon closer examination, it can be seen that the titanium surface was rather smooth at the nanoscale (Fig. 3.1 b). After anodization in 0.5 % HF for 20 min (constant voltage at 20 V), the titanium surface was oxidized (Figs. 3.1 c and d). Upon closer examination, the titanium surface appeared to possess nanotubular structures

uniformly distributed over the whole anodized titanium surface. As estimated from these SEM images, the inner diameter of the tubes was from 70 to 80 nm.

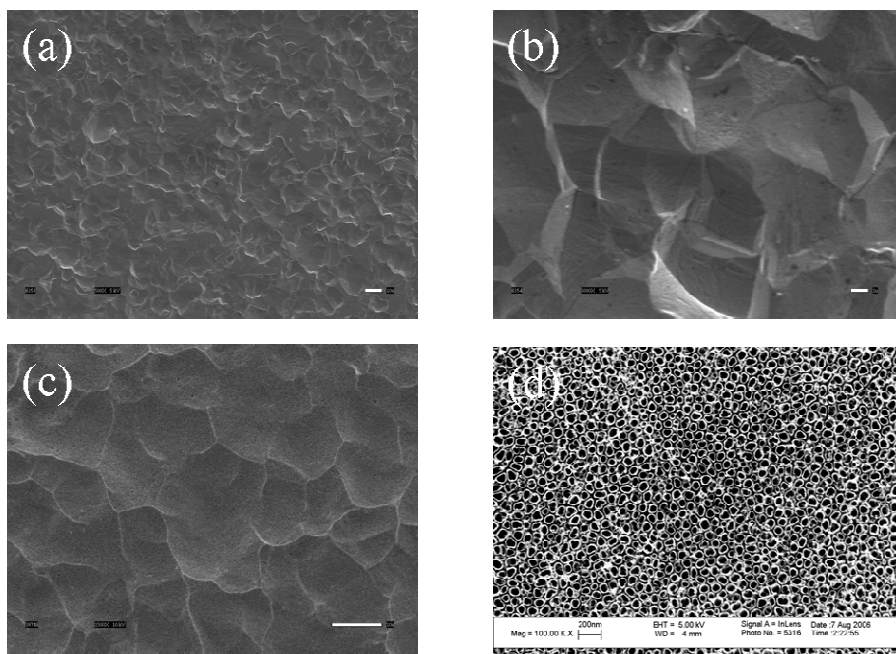


Figure 3.1 SEM micrographs of the titanium surface before and after anodization: (a) unanodized titanium surface under low magnification, scale bar = 10 μm ; (b) unanodized titanium surface under high magnification, scale bar = 1 μm ; (c) anodized titanium surface under low magnification, scale bar = 10 μm ; and (d) anodized titanium surface under high magnification, scale bar = 200 nm. The condition used for anodization was 20 V for 20 min in 0.5 % HF.

To study the mechanisms of nanotubular formation, the titanium substrates were subjected to several different time periods of anodization. Representative SEM fields for each time period are shown in Figure 3.2. Results demonstrated that when 0.5% HF was used as the electrolyte solution in the anodization process, after 10 seconds, a uniform oxide layer already covered the titanium surface. Holes and other surface defects seen in Figure 3.2 (a) to (c) were the result of

machining. The titanium surface was further etched and some particulate features were apparent as the anodization time increased (Figure 3.2 b and c). Importantly, the nanotubular structures appeared between 2 and 5 min of anodization (Figure 3.2 d and e). After that, the nanotubes further developed until they became uniformly distributed across the titanium surface after 20 min of anodization (Figure 3.2 f).

To study the effect of electrolyte concentration on the anodized titanium surface features, similar experiments were carried out in 1.5 % HF instead of the above mentioned 0.5 % HF. When 1.5 % HF was used as the electrolyte solution in the anodization process, similar to previous results, an oxide layer immediately formed on the titanium surface. Compared to anodization in 0.5% HF, the titanium surface was etched in 1.5 % HF after 10 seconds (Figure 3.3 a). The nanotubular structures appeared between 30 and 60 sec in 1.5 % HF (Figure 3.3 b and c); this was much earlier than that observed when 0.5 % HF was used. After that, the nanotubes further developed and seemed uniformly distributed over the titanium surface after 5 min of anodization (Figure 3.3 e). This is in contrast to anodization times of 20 min in 0.5 % HF needed for the uniform distribution of nanotubes across the titanium surface. The morphology of the anodized titanium after 20 min in 1.5 % HF was similar to that observed after 5 min of anodization (Figure 3.3 f).

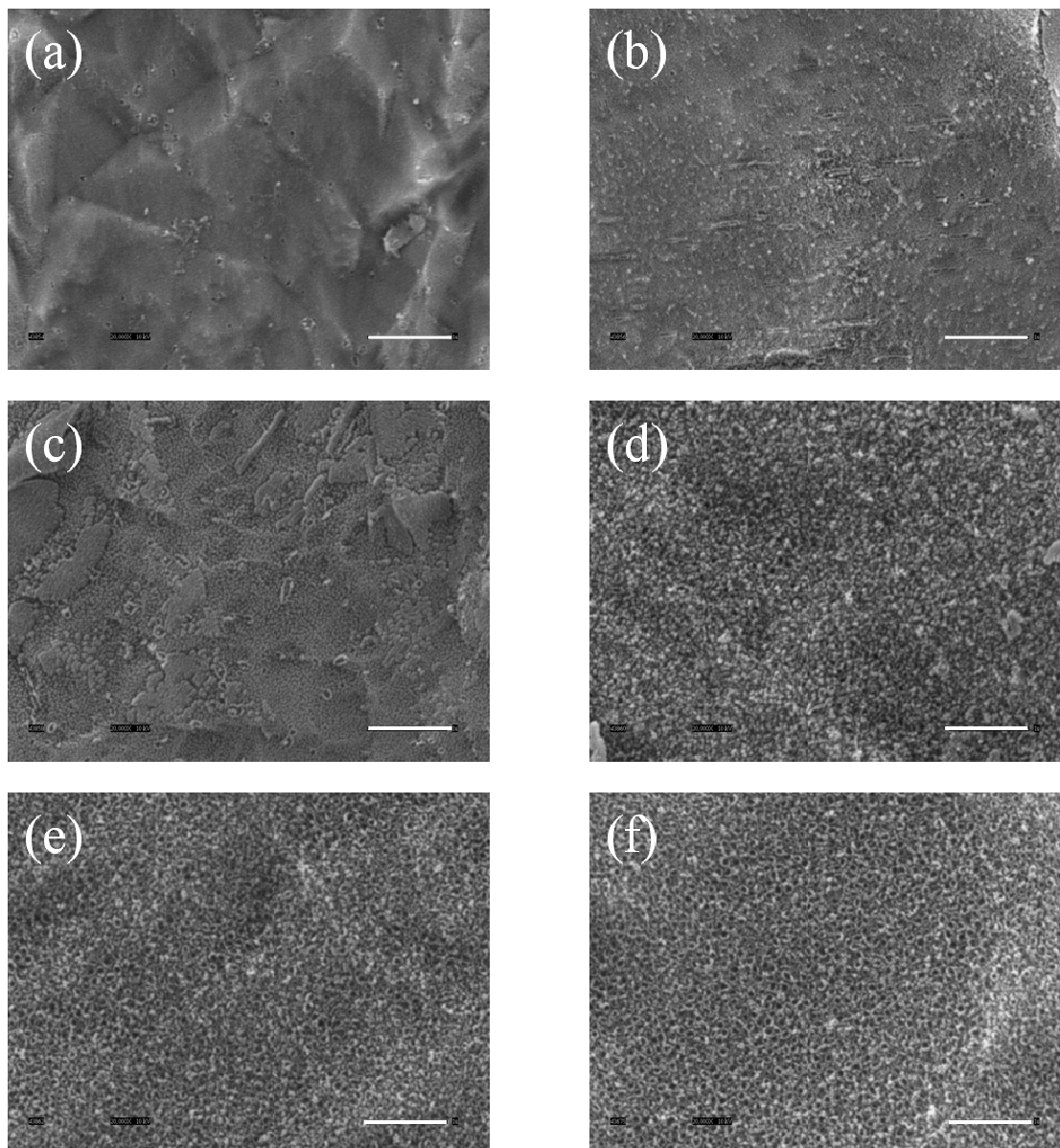


Figure 3.2 SEM micrographs of the titanium surface anodized in 0.5% HF under 20 V for (a) 10s; (b) 30s; (c) 1min; (d) 2 min; (e) 5 min; and (f) 20 min. Scale bars = 1 μm .

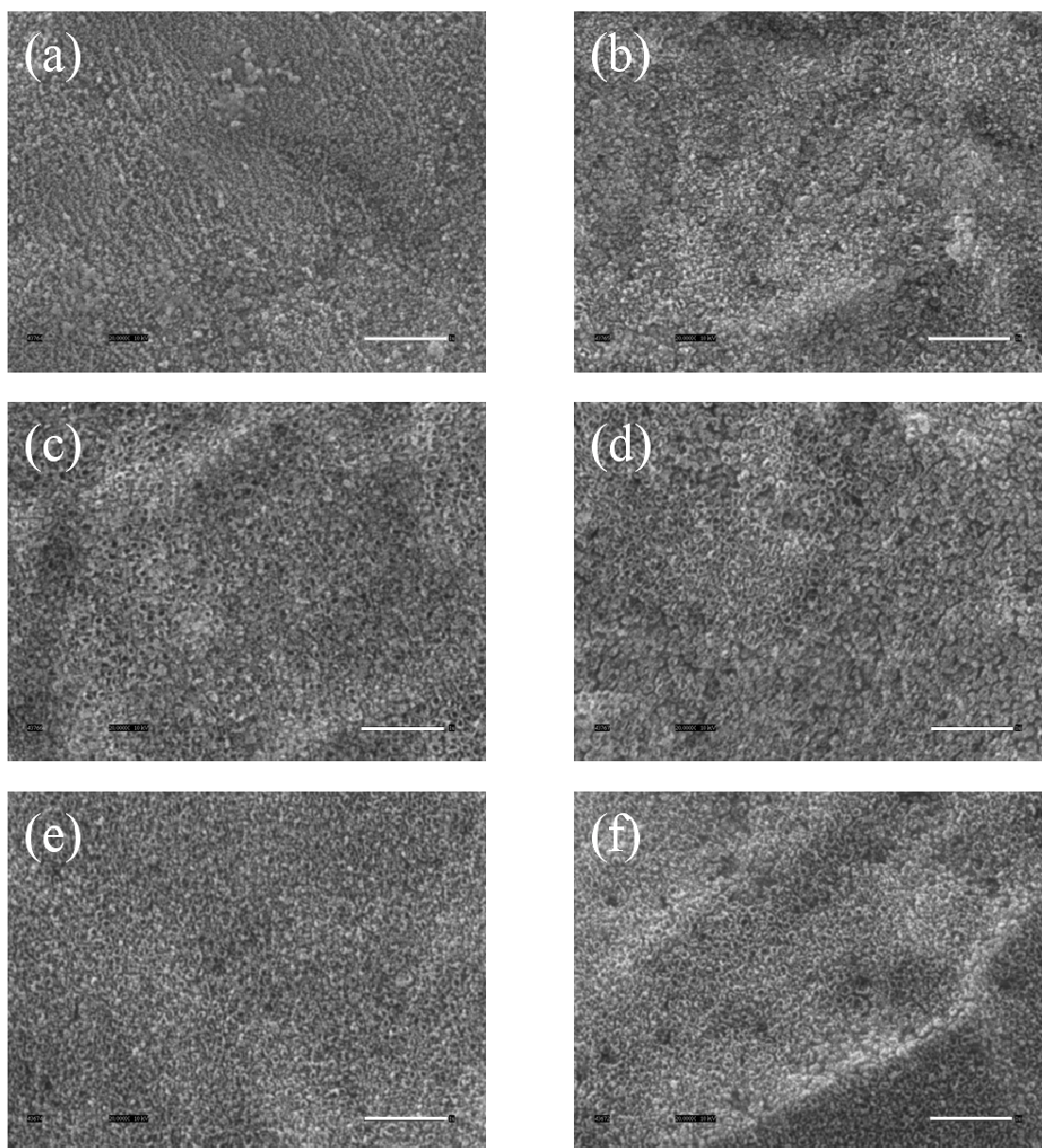


Figure 3.3 SEM micrographs of the titanium surface anodized in 1.5% HF under 20 V for (a) 10s; (b) 30s; (c) 1 min; (d) 2 min; (e) 5 min; and (f) 20 min. Scale bars = 1 μm .

The applied voltage was an important parameter that determined the resulting titanium surface morphology. In this study, the effect of voltage on the anodized

titanium surface features was studied by varying the applied voltages from 5 to 25 V. In 0.5 % HF for 20 min at 5 V, an oxide layer covered the titanium surface and it appeared particulate in nature (Figure 3.4 a). When the voltage increased to 10 V, the titanium surface roughness was clearly greater as the features were still present in a particulate morphology (Figure 3.4 b). From 15 to 20 V, the nanotubular structures formed and became more uniform at 20 compared to 15 V (Figure 3.4 c and d). Finally, when the voltage increased to 25 V, the nanotubular structures disappeared as the surface features were disordered (Figure 3.4 e).

Similar to what was done for 0.5 % HF, the anodized titanium surface features at different voltages were investigated under a higher electrolyte concentration (1.5 % HF). This part of study represented the highest voltage, electrolyte concentration, and time of anodization. The results of anodizing in 1.5% HF at 5 V (Figure 3.5 a) were similar to those in 0.5% HF at 5 V (Figure 3.4 a). A layer was uniformly created on the titanium surface and the surface features were particulate after anodization in 1.5 % HF for 20 min (Figure 3.5 a). However, when the voltage increased to 10 V, some pits were created on the titanium surface (Figure 3.5 b). As the voltage increased, the unique nanotubular structures were produced at both 15 and 20 V (Figure 3.5 c and d). At 25 V, the nanotubular structures were less ordered (Figure 3.5 e).

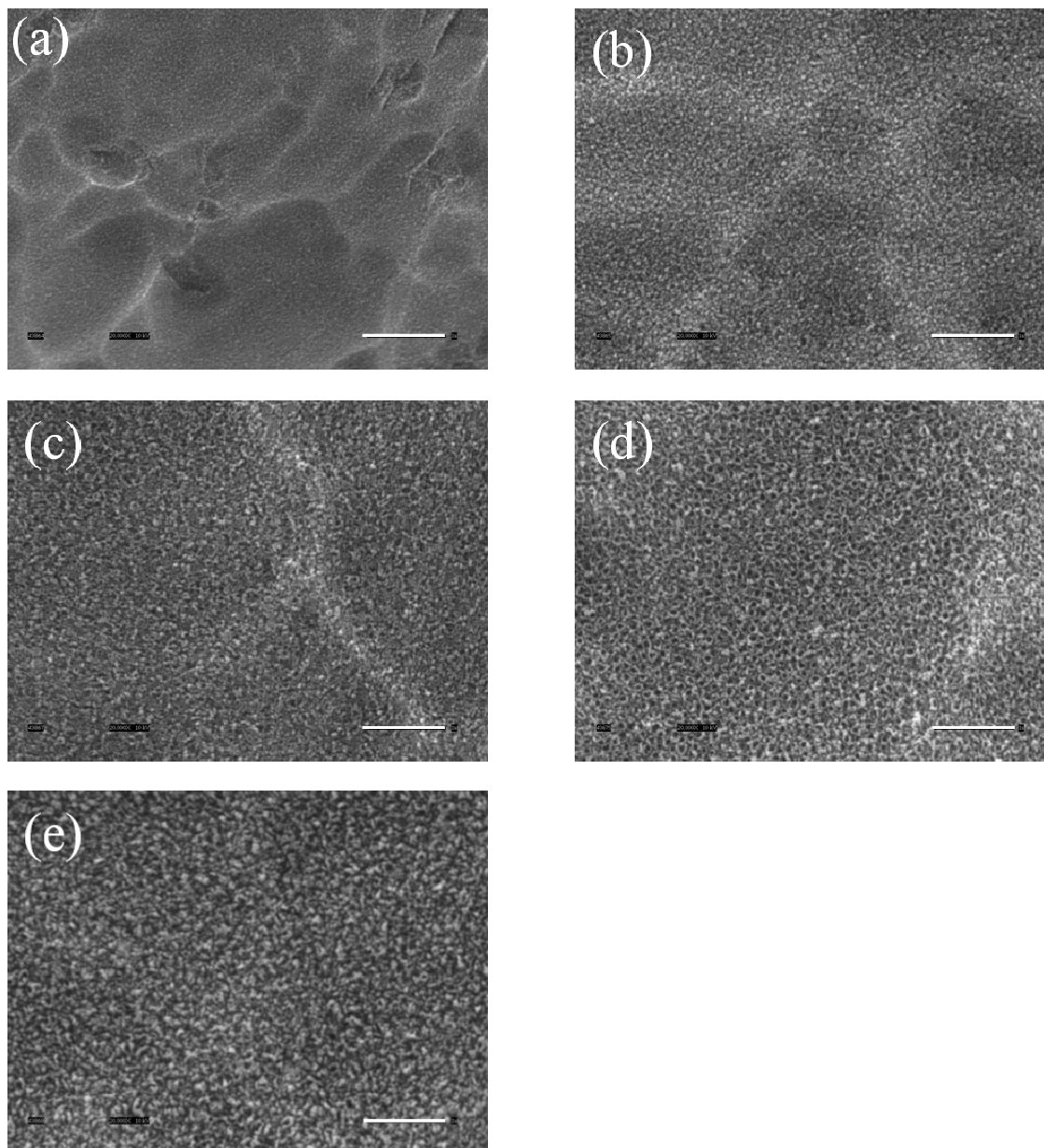


Figure 3.4 SEM micrographs of the titanium surface anodized in 0.5 % HF for 20 min under (a) 5 V; (b) 10 V; (c) 15 V; (d) 20 V; and (e) 25 V. Scale bars = 1 μm .

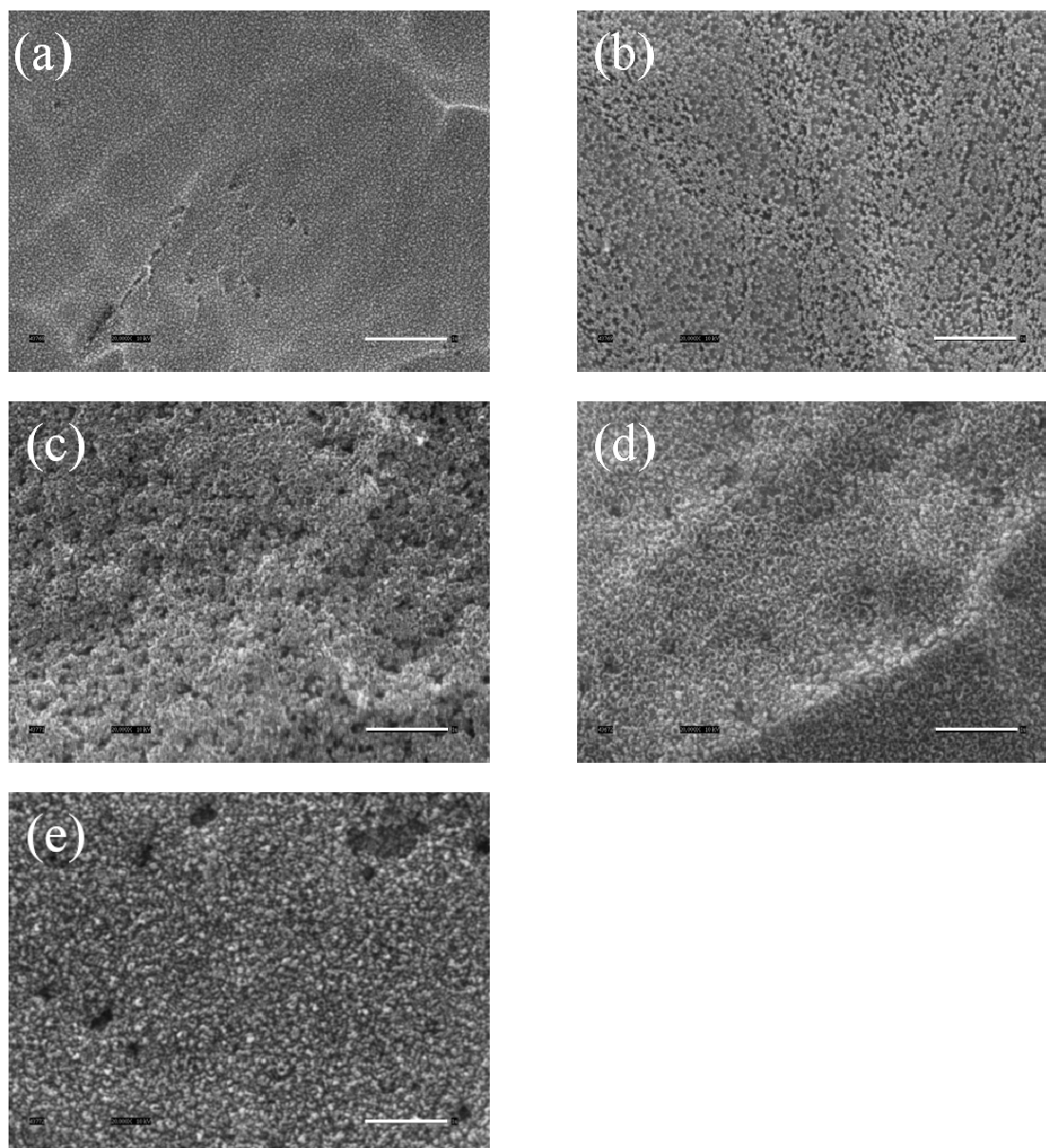


Figure 3.5 SEM micrographs of the titanium surface anodized in 1.5 % HF for 20 min under (a) 5V; (b) 10V; (c) 15 V; (d) 20V; and (e) 25 V. Scale bars = 1 μ m.

Within the potential window that titania nanotubular structures can be formed, the diameter of such nanotubes increased with voltage (Figure 3.6). When using 0.5 % HF, under 12 V, the average inner diameter was about 35 nm estimated from

the SEM images. This diameter increased to about 50 nm, 65 nm, and 80 nm when voltage was increased to 15 V, 18V, and 20 V, respectively.

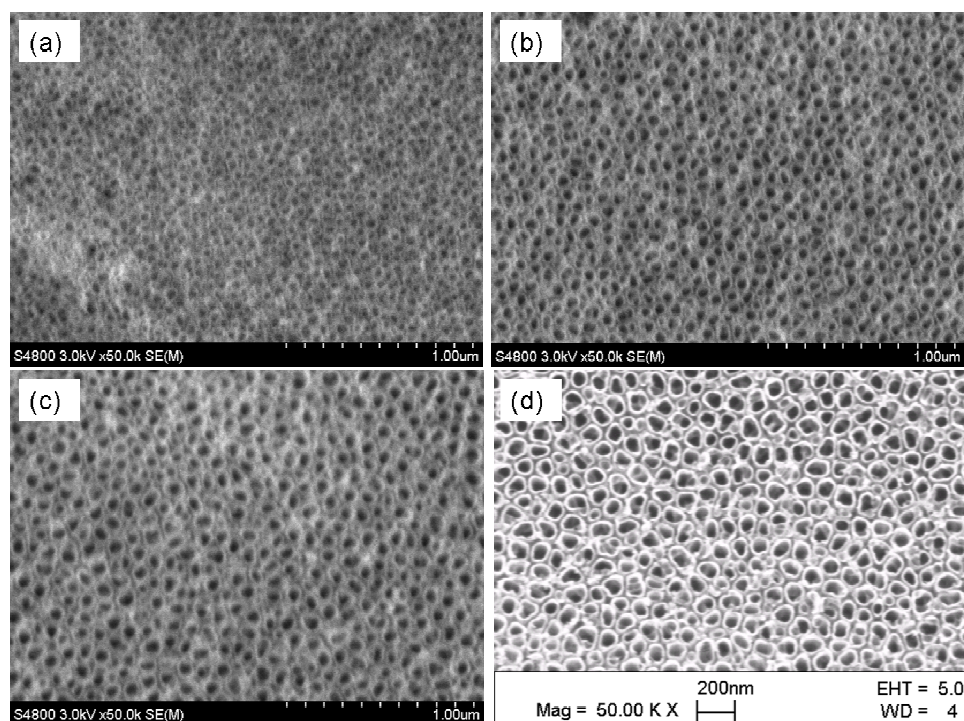


Figure 3.6 The diameter of nanotubes increased with voltage after anodization in 0.5% HF for 20 min. Voltages were 12 V, 15 V, 18 V, and 20 V in (a) to (d).

Representative AFM images of the unanodized titanium and anodized titanium possessing two distinctly different surface morphologies (nanotubular structures created under 20 V in 0.5 % HF for 20 min and nanoparticulate structures created under 10 V in 0.5 % HF for 20 min) were obtained (Figure 3.7). The resulting surface roughness was characterized by root mean square (rms) and relative surface area (Table 3.1). Results (rms / relative surface area) showed that the unanodized titanium surface was relatively smooth (4.74 nm / 1.018)

compared to any of the anodized titanium surfaces. Moreover, the roughness value was the largest for the nanotubular structures (25.54 nm / 1.811) compared to any other substrate; the roughness for the nanoparticulate structures was only 9.75 nm / 1.210.

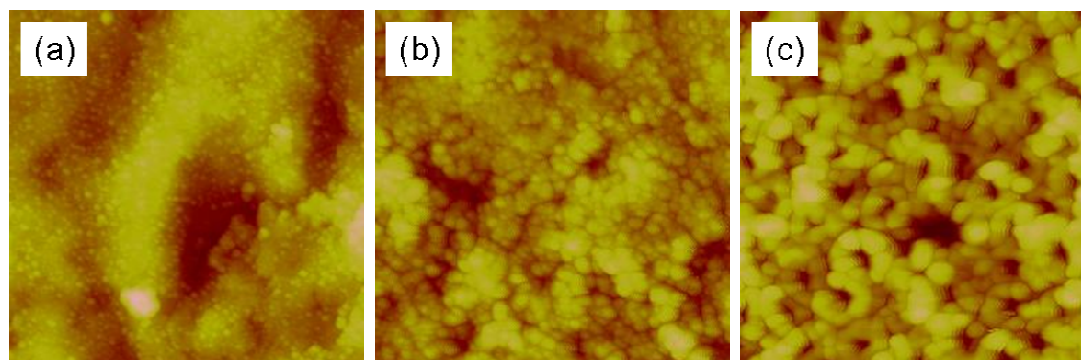


Figure 3.7 AFM images of unanodized and anodized titanium surfaces: (a) unanodized titanium; (b) anodized titanium with nanoparticulate structures (0.5 % HF, 10V, 20 min); and (c) anodized titanium with nanotubular structures (0.5 % HF, 20V, 20 min). The AFM scan area is 1 x 1 μ m.

The diameter of the nanotubes can be calculated from the AFM images. It was estimated that the nanotubes had an inner diameter approximately 70 to 80 nm when anodized under a voltage of 20 V for 20 min in 0.5 % HF. The diameter for the nanoparticulate structures was about 30 to 40 nm.

Table 3-1 Surface roughness (rms) of unanodized and anodized titanium surface. AFM scan size was 1 x 1 μ m.

Substrates	Relative surface area	rms (nm)
Unanodized titanium	1.018 $\pm$ 0.008	4.74 $\pm$ 1.87
Anodized titanium with nanoparticulate structures	1.210 $\pm$ 0.027	9.75 $\pm$ 2.92
Anodized titanium with nanotube-like structures	1.811 $\pm$ 0.133	25.54 $\pm$ 3.02

The surface chemistry of the anodized titanium substrates (as confirmed by XPS) was listed in Table 3.2. High resolution Ti 2p spectrum also indicated that the titanium oxide was TiO_2 . No other titanium species like TiO and Ti_2O_3 appeared to be present.

Table 3-2 Atomic percentages of elements in outermost layers of the unanodized and anodized titanium substrates. Data are mean $\pm$ sd. n/d = not detected.

Substrates	C	O	Ti	N	F	S	Cu
Unanodized titanium	43.2 $\pm$ 0.6	41.7 $\pm$ 1.2	8.3 $\pm$ 0.2	2.5 $\pm$ 0.2	2.2 $\pm$ 0.3	1.4 $\pm$ 0.1	1.0 $\pm$ 0.2
Anodized titanium (0.5%HF/10V/20min)	36.0 $\pm$ 0.8	45.7 $\pm$ 1.2	15.0 $\pm$ 0.5	1.0 $\pm$ 0.1	2.1 $\pm$ 0.2	n/d	n/d
Anodized titanium (0.5%HF/20V/20min)	30.8 $\pm$ 1.5	47.9 $\pm$ 1.4	16.7 $\pm$ 0.8	0.8 $\pm$ 0.1	2.8 $\pm$ 0.2	n/d	1.5 $\pm$ 0.4

The contact angle and surface energy of the unanodized titanium were 71.4° and 56.14 mN/m while those of the anodized titanium were 58.5° and 66.89 mN/m, respectively. These results will be compared to the other anodized titanium samples (A-OH, A-NH₂, and A-CH₃) in section 3.2.1.3.

3.1.1.2. Osteoblast Responses on Anodized Titanium Surfaces

As a critical initial step for cell and implant interactions, osteoblast adhesion was determined on the unanodized and anodized titanium substrates (as shown in Figure 3.8). All the anodized titanium samples increased osteoblast adhesion compared to the unanodized titanium. Specifically, osteoblast adhesion increased 33 % on the titanium substrates possessing nanotubular structures compared to the unanodized titanium while the anodized titanium substrates possessing nanoparticulate structures only increased osteoblast adhesion 17 %

compared to unanodized titanium. Osteoblast adhesion also significantly increased when comparing anodized titanium surfaces possessing nanotubular structures to anodized titanium surfaces possessing nanoparticulate structures.

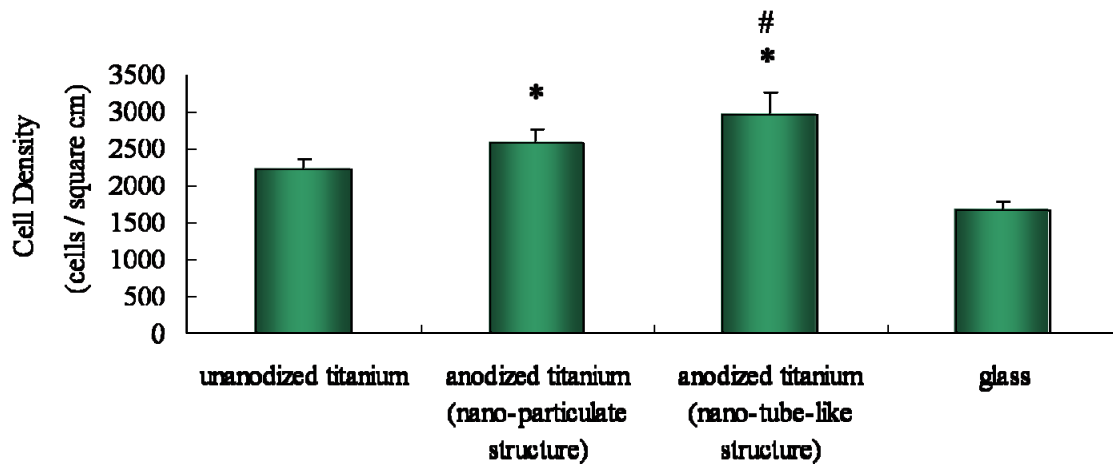


Figure 3.8 Osteoblast adhesion on unanodized titanium; anodized titanium with nanoparticulate structures (10 V, 0.5 % HF, 20 min); anodized titanium with nanotube structures (20 V, 0.5 % HF, 20 min); and glass (reference). Values are mean + SEM; n = 3; * p < 0.1 compared to unanodized titanium and # p < 0.1 compared to anodized titanium with nanoparticulate structures.

Osteoblast morphology after 4 hours of culture was characterized by SEM.

Figure 3.9 illustrates the typical morphology of adherent osteoblasts on the unanodized titanium (Figure 3.9 a) and the anodized titanium with nanotubular structures (Figure 3.9 b). Generally, it was observed that osteoblasts were rounded on the unanodized titanium while, in contrast, osteoblasts were more well spread on the anodized titanium. It was observed that the cell filopodia were extending along the surface in the case of anodized titanium possessing

nanotubular structures and some extended into the nanotubes (Figure 3.9 c and d).

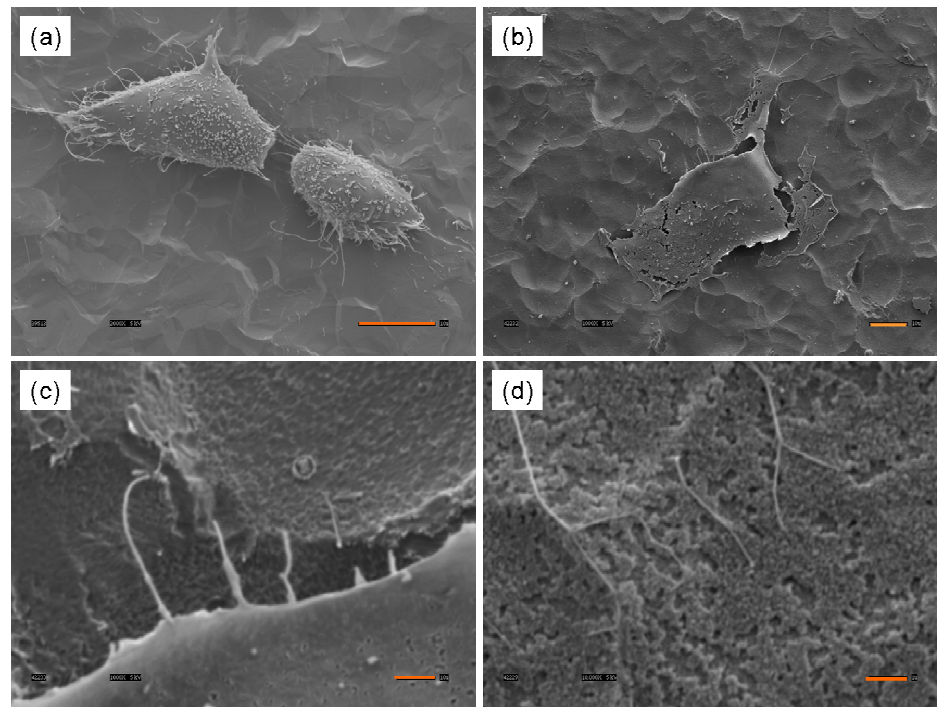


Figure 3.9 SEM images showing osteoblast morphology on (a) unanodized titanium, (b) anodized titanium with nanotubular structures, (c) and (d) filopodia of osteoblasts on anodized titanium with nanotubular structures.

The amount of intracellular proteins synthesized by osteoblasts increased with time for all of the samples tested in this study (Fig. 3.10). However, the results of this study did not show any statistically significant differences in total protein synthesis when comparing the anodized and unanodized titanium surfaces after 7, 14, and 21 days.

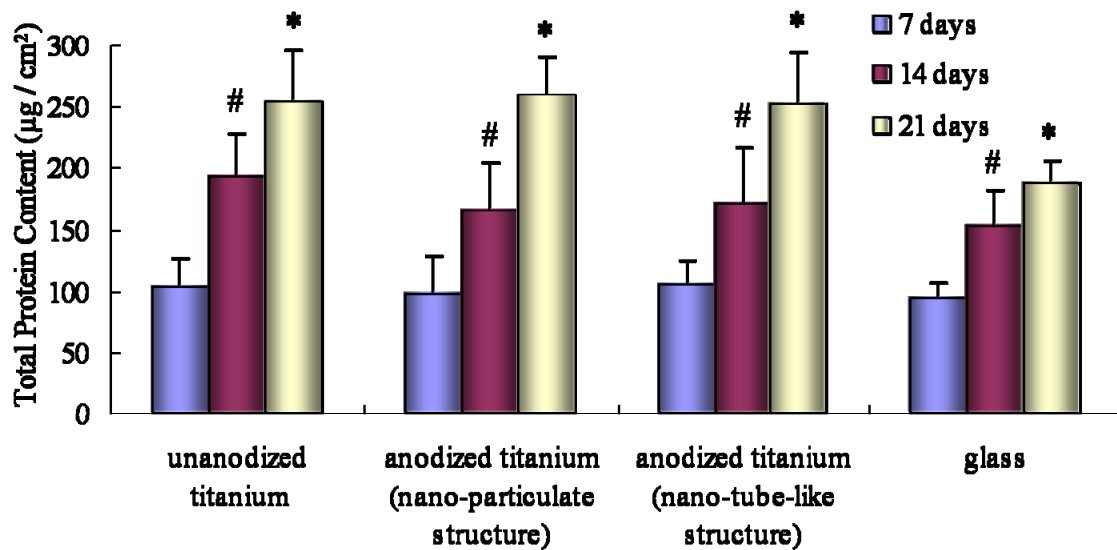


Figure 3.10 Total protein content after 7, 14, and 21 days of osteoblast culture on unanodized titanium; anodized titanium with nanoparticulate structures (10 V, 0.5 % HF, 20 min); anodized titanium with nanotubular structures (20 V, 0.5 % HF, 20 min); and glass (reference). Values are mean $\pm$ SEM; n = 3. * p < 0.1 compared to 14 days and # p < 0.1 compared to 7 days for each sample.

Similar to intracellular protein synthesis, the intracellular collagen secreted by osteoblasts increased with time on all substrates tested in this study (Fig. 3.11). No significant differences in collagen synthesis were measured between anodized and unanodized titanium substrates after 7, 14, and 21 days. In contrast to total protein and collagen synthesis, alkaline phosphatase activity by osteoblasts on all substrates except glass was observed to greatly increase after 14 days compared to 7 days (Fig. 3.12). This may indicate that at this time period osteoblasts began to differentiate into mature calcium depositing osteoblasts. Moreover, the glass substrates showed significantly low alkaline phosphatase activity at 14 and 21 days compared to any of the titanium substrates. The alkaline phosphatase activities of osteoblasts on anodized titanium possessing

nanotubular structures were higher than that of anodized titanium possessing nanoparticulate structures at 21 days. However, no statistically significant difference was observed between these three (specifically, unanodized titanium, anodized titanium with nanotubular structures, and anodized titanium with nanoparticulate structures) at any time point.

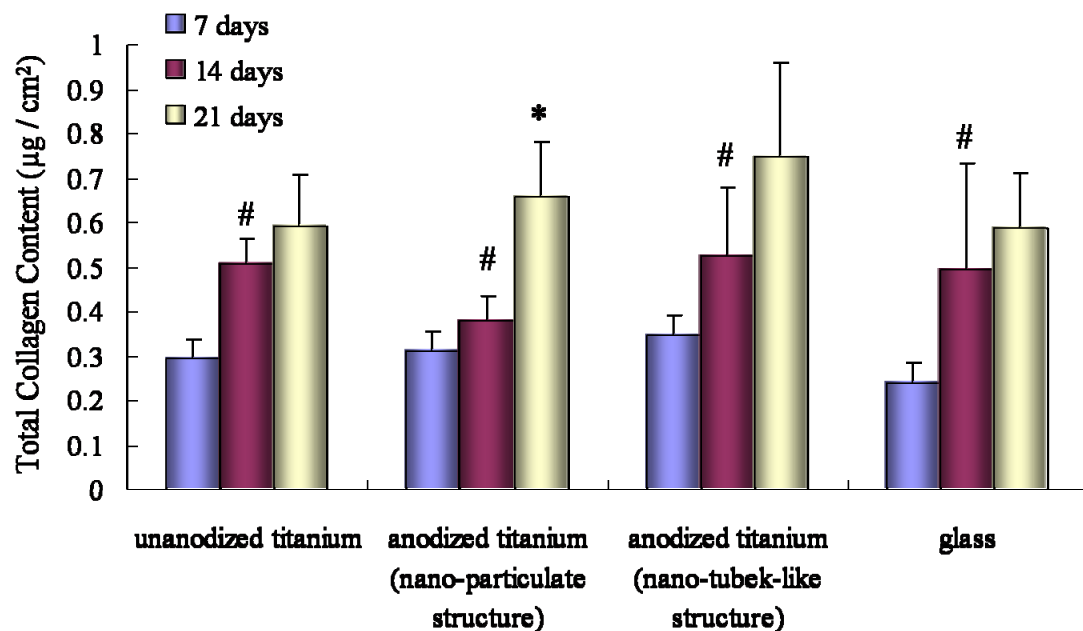


Figure 3.11 Intracellular collagen content after 7, 14, 21 days of osteoblast culture on unanodized titanium; anodized titanium with nanoparticulate structures (10 V, 0.5 % HF, 20 min); anodized titanium with nanotubular structures (20 V, 0.5 % HF, 20 min); and glass (reference). Values are mean $\pm$ SEM; n = 3. * p < 0.1 compared to 14 days and # p < 0.1 compared to 7 days for each sample.

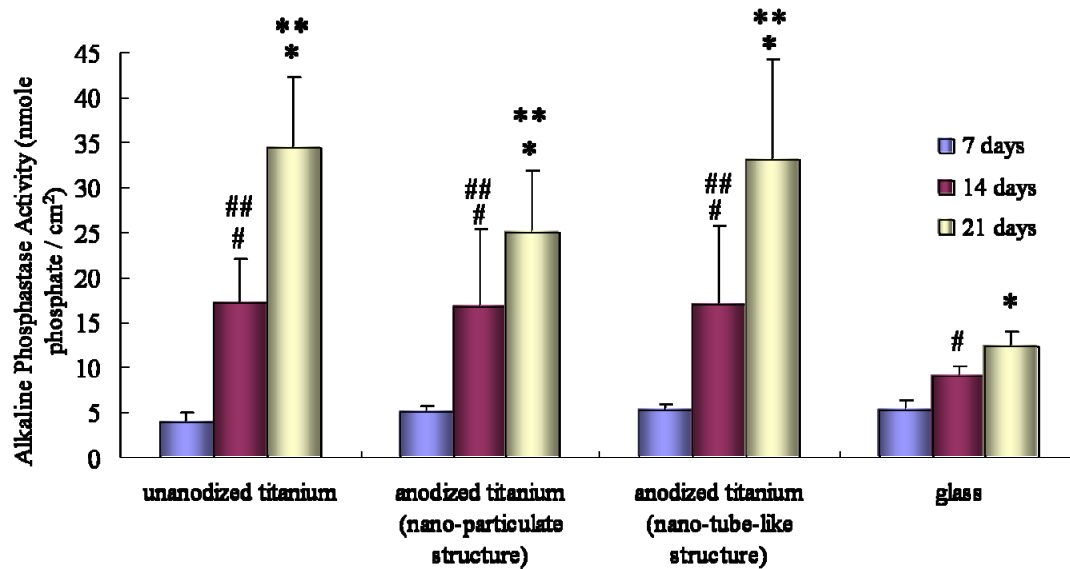


Figure 3.12 Alkaline phosphatase activity after 7, 14, and 21 days of osteoblast culture on unanodized titanium; anodized titanium with nanoparticulate structures (10 V, 0.5 % HF, 20 min); anodized titanium with nanotubular structures (20 V, 0.5 % HF, 20 min); and glass (reference). Values are mean $\pm$ SEM; n = 3. * p < 0.05 compared to 14 days and # p < 0.05 compared to 7 days for each sample; ** p < 0.1 compared to glass at 21 days and ## p < 0.1 compared to glass at 14 days.

Most importantly, it was observed that anodized titanium substrates possessing nanotubular structures increased calcium deposition by osteoblast after 21 days when compared to both unanodized titanium and anodized titanium possessing nanoparticulate structures (Figure 3.13). This corresponded to the results found during the osteoblast adhesion tests, where anodized titanium substrates possessing nanotubular structures demonstrated the greatest adhesion. In contrast to osteoblast adhesion results, through, the calcium deposition by osteoblasts on anodized titanium possessing nanoparticulate structures was unexpectedly lower than unanodized titanium substrates after 21 days. Lastly,

less calcium deposition was detected for each time period on glass compared to any titanium substrate tested in this study.

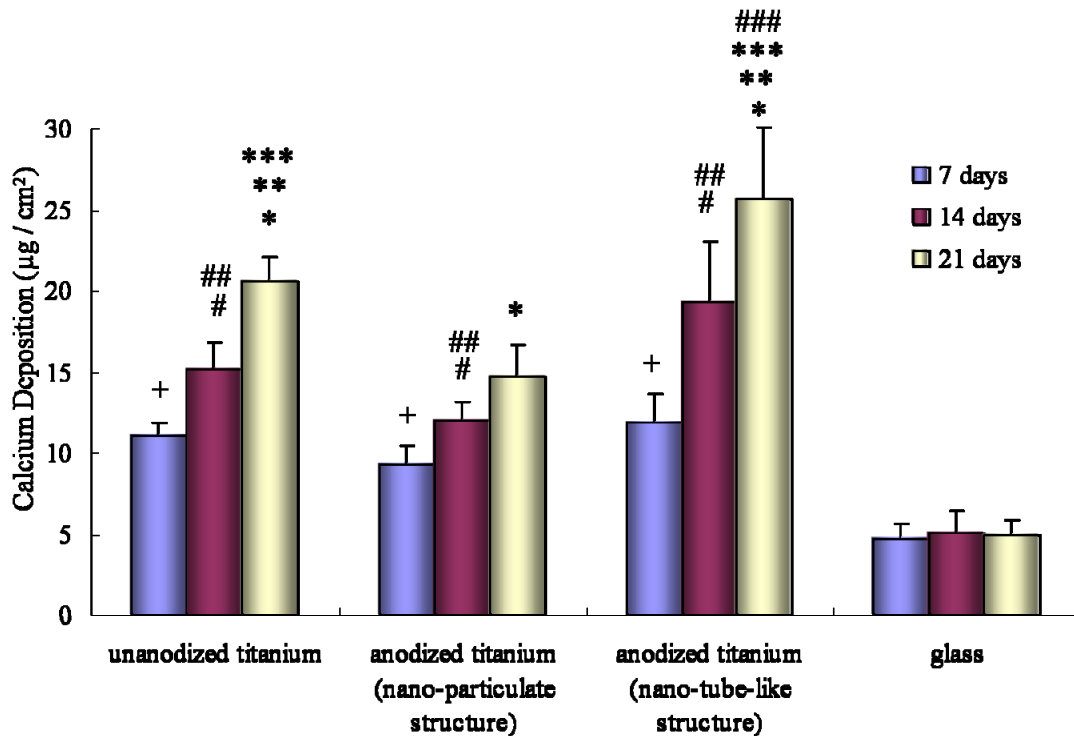


Figure 3.13 Calcium deposition after 7, 14, and 21 days of osteoblast culture on unanodized titanium; anodized titanium with nanoparticulate structures (10 V, 0.5 % HF, 20 min); anodized titanium with nanotubular structures (20 V, 0.5 % HF, 20 min); and glass (reference). Values are mean + SEM; n = 3; + p < 0.05, # p < 0.05, and * p < 0.05 compared to glass at 7, 14, and 21 days, respectively; ** p < 0.05 compared to 14 days and ## p < 0.05 compared to 7 days for each sample; *** p < 0.01 compared to anodized titanium possessing nanoparticulate structures; #### p < 0.05 compared to unanodized titanium.

3.1.1.3. Chondrocyte Responses on Anodized Titanium Surfaces

For the first time, the results of this study also demonstrated greater chondrocyte adhesion (31% more) on the nanotubular anodized titanium compared to unanodized titanium (Figure 3.14). Since chondrocytes are anchorage-

dependent cells, increased adhesion on nanotubular anodized titanium may lead to greater proliferation and synthesis of a cartilage extracellular matrix. Typical chondrocyte morphology on titanium and anodized titanium is shown in Figure 3.15.

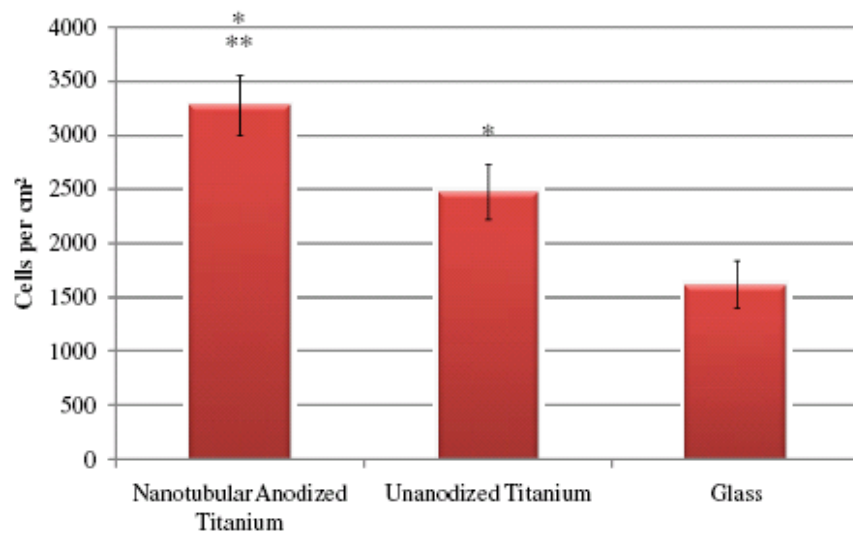


Figure 3.14 Increased chondrocyte adhesion on nanotubular anodized Ti. Values are mean + SEM; n = 3; * p < 0.1 compared to glass (reference); ** p < 0.1 compared to unanodized Ti.

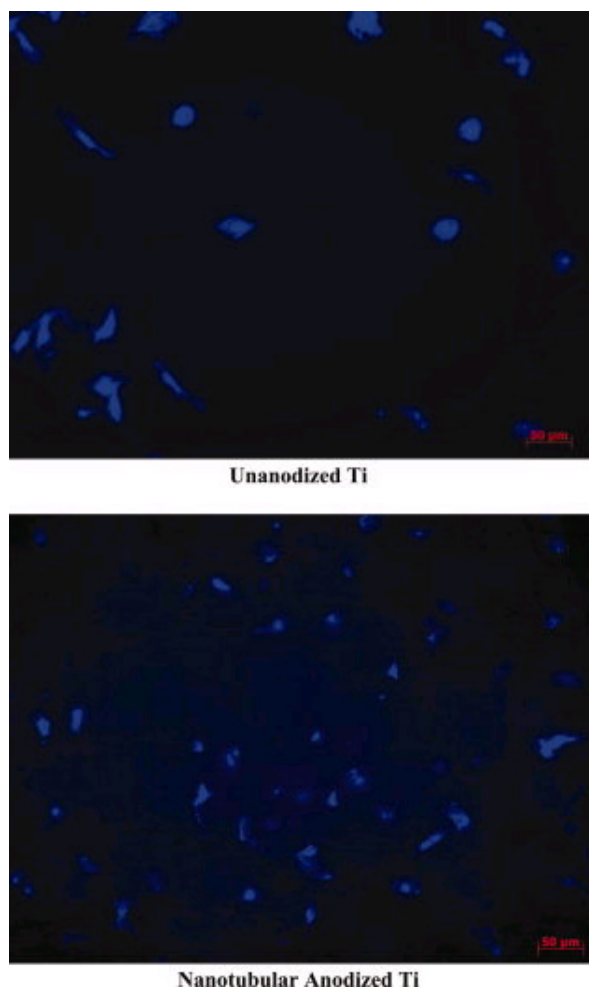


Figure 3.15 Fluorescent images of increased chondrocyte adhesion on nanotubular anodized titanium. Stain is rhodamine phalloidin. Bars = 50 μ m ([97]).

Chondrocyte proliferation results matched adhesion results. Specifically, after 1, 3 and 5 days, the number of cells on the anodized titanium was 20.02%, 32.86%, and 32.13% higher than on unanodized titanium, respectively (Figure 3.16).

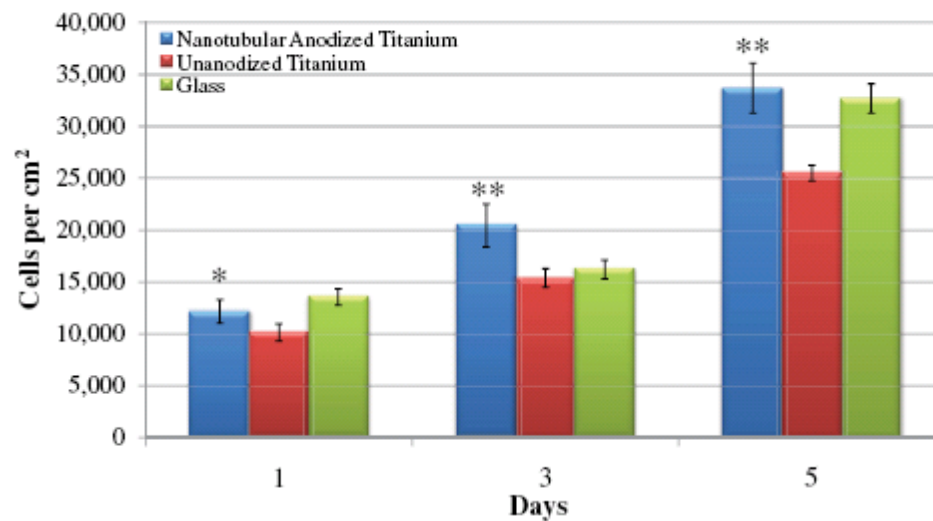


Figure 3.16 1, 3, and 5 day chondrocyte proliferation on nanotubular anodized titanium. Values are mean + SEM; n=4. * $p < 0.1$ for anodized compared to unanodized Ti; ** $p < 0.05$ for anodized compared to unanodized titanium.

3.1.1.4. Mechanisms of Enhanced Cell Adhesion: Protein Adsorption

Protein (specifically, fibronectin and vitronectin) adsorption on nanotubular samples was examined in the present study to explore the mechanisms of enhanced cell (osteoblast and chondrocyte) adhesion on anodized titanium. Fibronectin and vitronectin are two major proteins that are involved in cell adhesion. Results showed significantly increased fibronectin (30%) and vitronectin (18%) adsorption on anodized titania nanotubular structures compared to unanodized titanium samples (Figure 3.17). Also, 15 % more fibronectin absorbed on the anodized titanium possessing nanoparticulate structures compared to unanodized titanium. Because cells adhere to titanium surfaces via pre-adsorbed proteins, increased fibronectin and vitronectin

adsorption on anodized titanium substrates with nanotubular structures may explain the previously observed enhanced cell functions.

This increased protein adsorption on anodized titanium may be due to increased surface energy after anodization. This issue will be discussed in the next chapter.

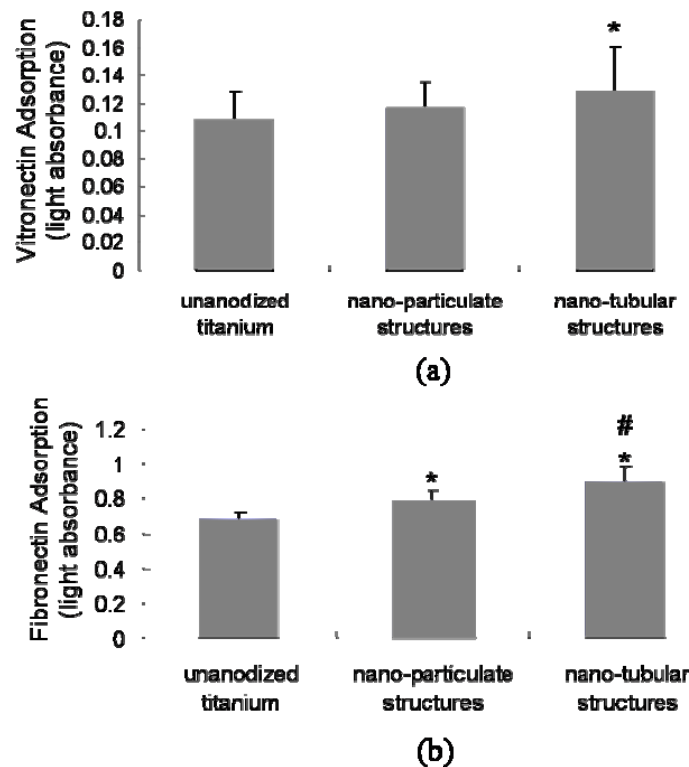


Figure 3.17 (a) Fibronectin and (b) vitronectin adsorption on unanodized titanium, anodized titanium possessing nanoparticulate structures (0.5 % HF, 10 V and 20 min), and anodized titanium possessing nanotubular structures (0.5 % HF, 20 V and 20 min). Values are mean $\pm$ SEM; n = 3; *p < 0.1 (compared to unanodized titanium) and #p < 0.1 (compared to nanoparticulate structures).

3.1.2. Anodized Titanium Alloy Surfaces

3.1.2.1. Surface Characterization

The non-modified Ti-6Al-4V substrate possessed micron rough surface features due to the presence of some machined grooves under high magnification (Figure 3.18 a). This was most likely due to grinding and polishing. After etching in a mixture of HF / HNO₃ as previously described, the original surfaces were etched away and the β phase protruded from the α phase due to different etching rates of those phases (Figure 3.18 b). The anodized Ti6Al4V (0.5% HF, 20 V, 20 min) displayed nanotubular structures similar to the anodized titanium all over the surface except where the β phase was present (Figure 3.18 c and d).

AFM images of unanodized and anodized Ti6Al4V are shown in Figure 3.19. The anodized Ti6Al4V, similar to anodized titanium, exhibited higher surface roughness (45.0 nm, rms of a 5 x 5 μ m area) than unanodized Ti6Al4V (15.2 nm).

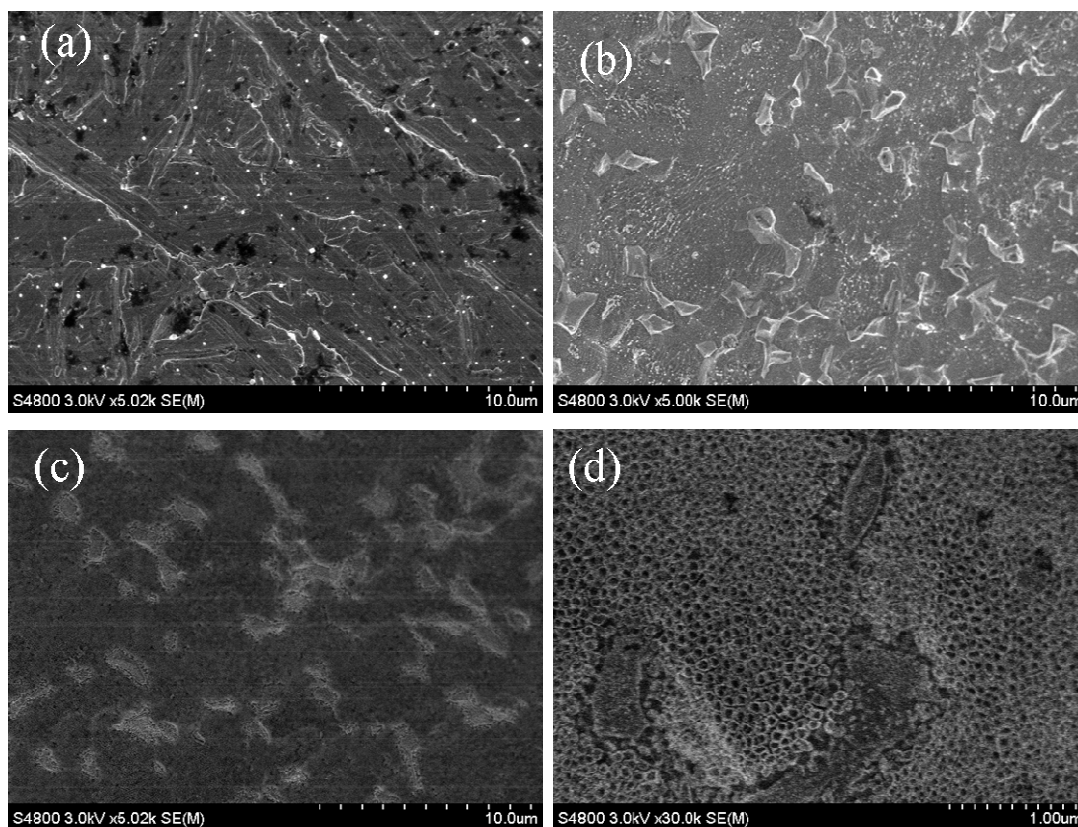


Figure 3.18 SEM micrographs of the Ti6Al4V surface (a) as received, (b) after etching in an acidic mixture (1% HF and 2% HNO₃ in DI water) for 5 min, (c) after anodizing in 0.5% HF for 20 min at 20 V, and (d) high magnification of (c).

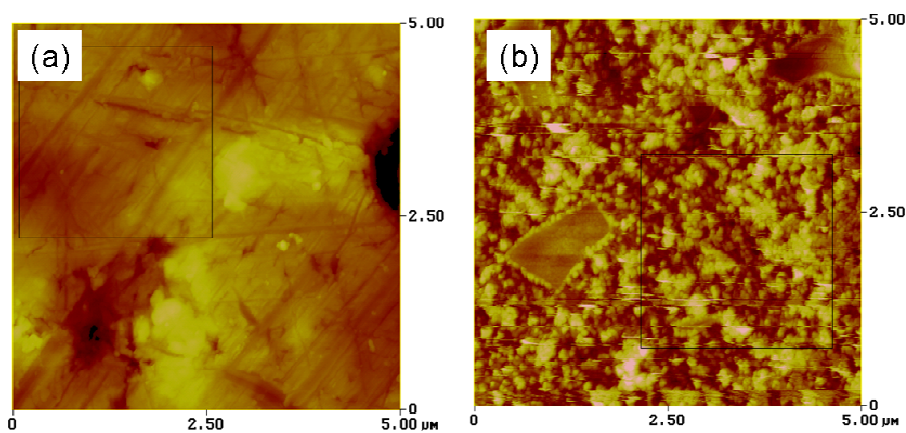


Figure 3.19 AFM images of (a) unanodized Ti6Al4V and (b) anodized Ti6Al4V in 0.5% HF at 20 V for 20 min. AFM scan area was 5 X 5 μm .

EDS results showed that the oxygen amount greatly increased after anodization of Ti6Al4V (Table 3-3).

Table 3-3 Element percentage on unanodized and anodized Ti6Al4V revealed by EDS.

Sample	Normal weight of element (%)			
	Ti	Al	V	O
Ti6Al4V	87.74	6.91	5.35	—
Anodized Ti6Al4V	54.62	2.30	3.09	39.99

The original Ti6Al4V surfaces had water contact angles around 60°. In comparison, Ti6Al4V anodized in dilute HF had water contact angles around 54° (Table 3-4). There was little surface energy change before and after anodization but it was noted that the polar component of the total surface energy increased by 40% after anodization.

Table 3-4 Contact angle and surface energy of Ti6Al4V and anodized Ti6Al4V (in 0.5 % HF under 20 V for 20 min). PEG stands for polyethylene glycol, E, Ed and Ep are the total surface energy, the dispersive and polar components of the total surface energy, respectively. Θ is the contact angle.

Sample	Θ (water)	Θ (PEG)	E (mN/m)	Ed (mN/m)	Ep (mN/m)
Ti6Al4V	59.9 $\pm$ 4.9	39.2 $\pm$ 1.8	57.27 $\pm$ 0.83	47.30 $\pm$ 0.44	9.96 $\pm$ 0.39
Anodized Ti6Al4V	54.2 $\pm$ 5.1	43.2 $\pm$ 4.8	58.24 $\pm$ 1.78	44.35 $\pm$ 1.16	13.89 $\pm$ 0.62

3.1.2.2. Osteoblast Responses on Anodized Titanium Alloy Surfaces

Similar to titanium, the anodized Ti6Al4V samples also significantly increased osteoblast adhesion (13 %) compared to the unanodized Ti6Al4V samples

(Figure 3.20). This provided evidence that anodization may be a useful technique to increase osteoblast adhesion on both pure titanium and α - β titanium alloys.

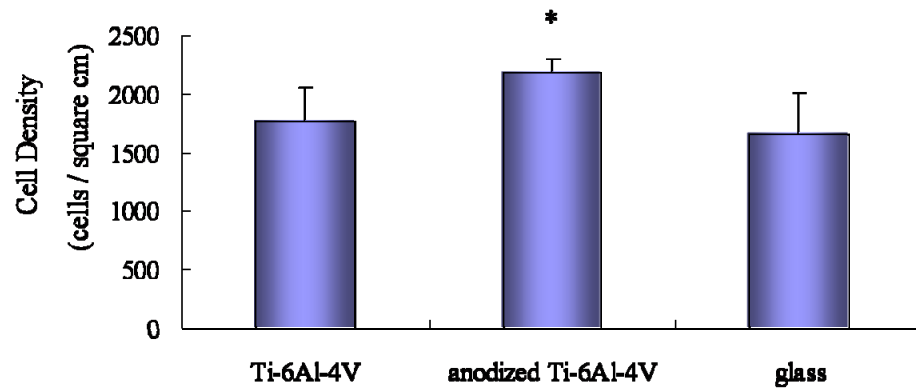


Figure 3.20 Osteoblast adhesion on unanodized Ti6Al4V, anodized Ti6Al4V (1.5 % HF, 20 V, 5 min), and glass (reference). Values are mean + SEM; n = 3; * p < 0.1 compared to unanodized Ti-6Al-4V.

3.1.2.3. Chondrocyte Responses on Anodized Titanium Alloy Surfaces

In addition, similar to titanium, chondrocyte attachment was 6% higher after 4 hours of culture on anodized Ti6Al4V than the unanodized surface (Figure 3.21).

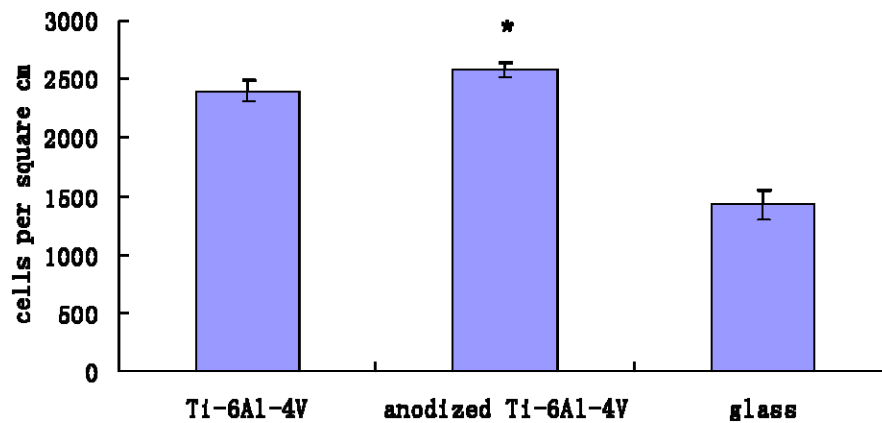


Figure 3.21 Chondrocyte attachment after 4 h of culture on the original unanodized Ti6Al4V, Ti6Al4V anodized in 0.5% HF under 20V for 20 min, and glass. Data = mean + SEM; n = 3; *p<0.1 compared to the original Ti6Al4V and glass.

Chondrocyte morphology after 4 hours of culture was characterized by SEM (Figure 3.22). Chondrocytes were usually elongated when they attached onto unanodized Ti6Al4V, probably due to the micro-grooves on the surface (Figure 3.22 a). The chondrocytes attached on etched Ti6Al4V and anodized Ti6Al4V were more spread and those on the anodized Ti6Al4V exhibited the highest cell density (Figure 3.22 b and c).

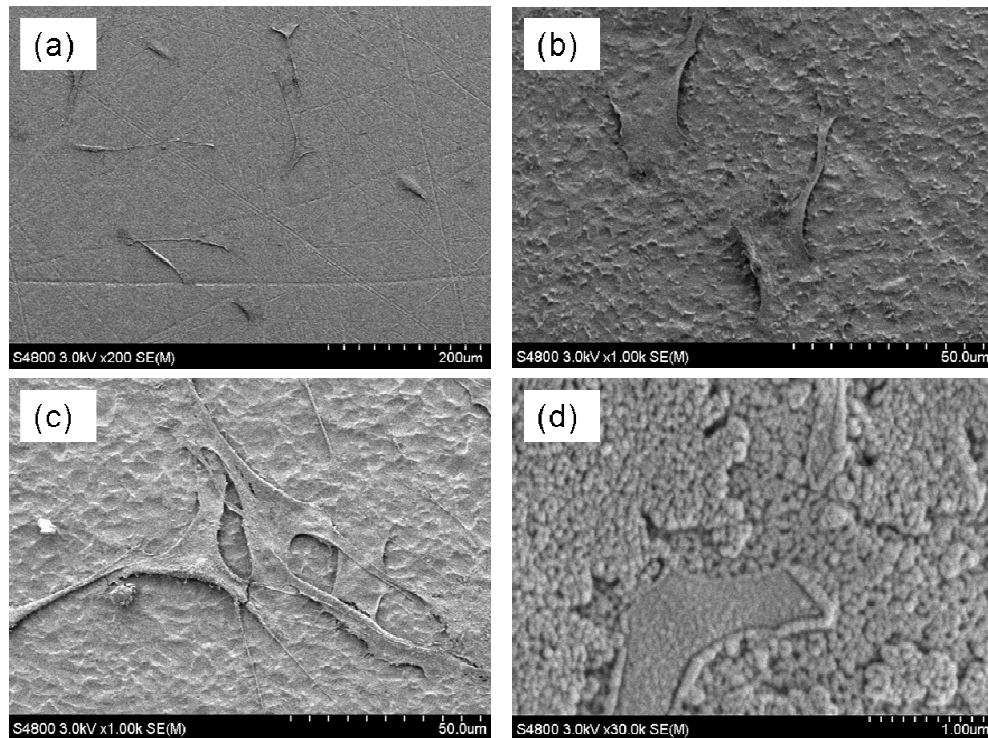


Figure 3.22 SEM micrographs of chondrocytes on (a) unanodized Ti6Al4V, (b) Ti6Al4V etched in an acidic mixture (1% HF and 2% HNO₃ in DI water) for 5 min, (c) anodized Ti6Al4V in 0.5% HF for 20 min at 20 V, and (d) a close look at the nanotubular structures after 4 hours of cell culture.

3.1.3. Anodized CoCr Alloy Surfaces

3.1.3.1. Surface Characterization

Anodizing CoCr required extensive investigation since the same anodization parameters used for titanium did not work for CoCr. For example, the voltage first used (10V) was too high and produced a fierce electrochemical reaction on the CoCr electrode (Figure 3.23).

The original CoCr shiny surface was very smooth with a minor amount of surface scratches. As anodization time increased, the surface possessed numerous micron features. This trend continued and after 3 min, as the anodized surface was very rough while it had nanoporous structures all over the surface. The size of such heterogeneous porous structures was about 20 to 30 nm estimated from SEM images. The smooth irregular shape particles embedded in the CoCr alloys were likely the breakdown points where the surface was no longer resistant to the applied voltage.

Under lower voltages (2 V), the topography was less rough in the micron-scale while the nano rough features looked similar to the surfaces created under 10 V (Figure 3.24).

Electrolytes other than HF were also used and the resulting surfaces were compared with those using the HF electrolyte. Figure 3.25 showed that the CoCr surfaces anodized in 1 M H_2SO_4 (Figure 3.25 a and b) were similar to those anodized in 1 M H_2SO_4 / 0.25 vol.% HF (Figure 3.25 c and d). CoCr surfaces anodized in 1 M H_3PO_4 (Figure 3.25 e and f) were also similar to those anodized in 1 M H_3PO_4 / 0.25 vol.% HF (Figure 3.25 g and h). Alkaline electrolyte solutions like 0.1 M NaOH lead to different CoCr surface morphologies compared to acidic electrolytes (Figure 3.26). These results indicated that not only HF but acidic electrolytes can all create such multi-rough surfaces on CoCr alloys. This may be important for industrial applications since HF may be viewed as too hazardous.

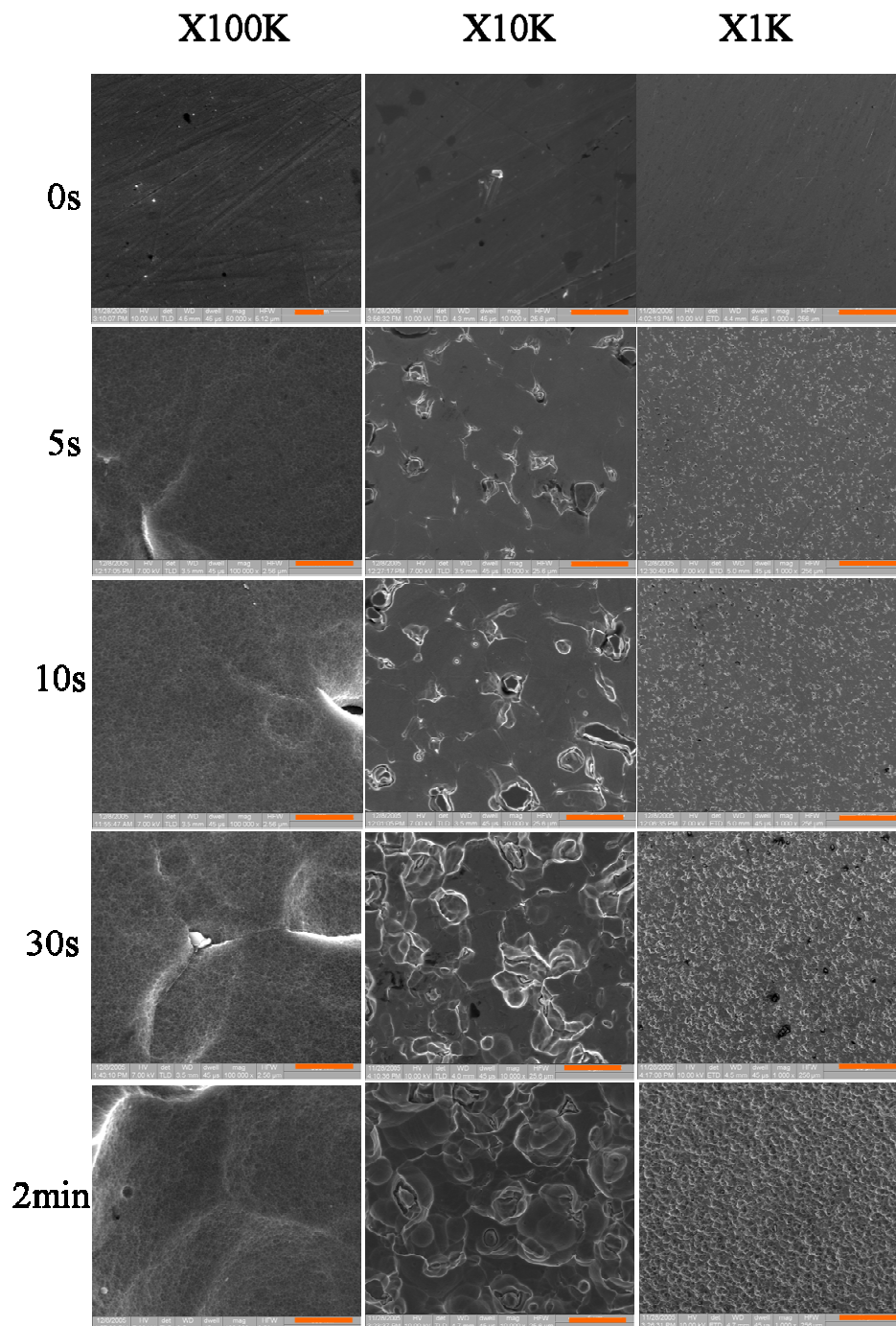


Figure 3.23 SEM images of CoCrMo anodized under 10 V for different time periods. Scale bars equal 500 nm for X100K, 5 μm for X10K, and 50 μm for X1K.

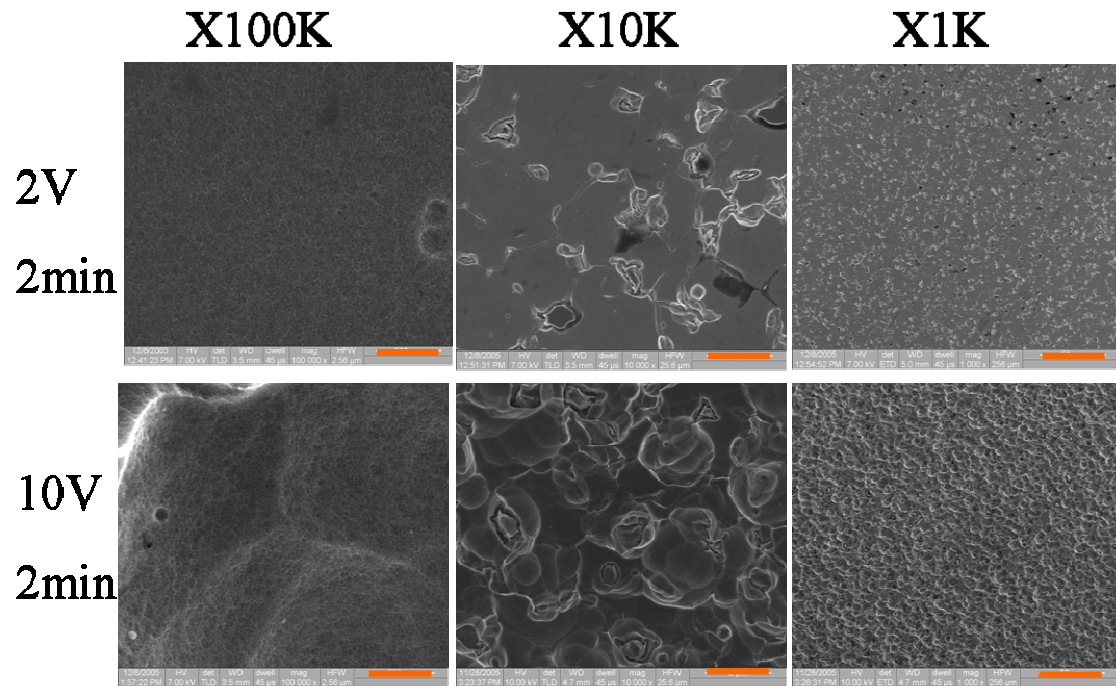


Figure 3.24 SEM images of anodized CoCrMo under different voltages.

When looking at the interface between unanodized and anodized CoCr, color differences could be seen (Figure 3.27 a). The anodized CoCr surface was no longer silver but grey. When the anodized CoCr surface layer was removed and the underlying substrate revealed, it can be estimated that the coating created by anodization in 0.5% HF at 10 V for 2 min was very thin, probably less than 100 nm (Figure 3.27 b).

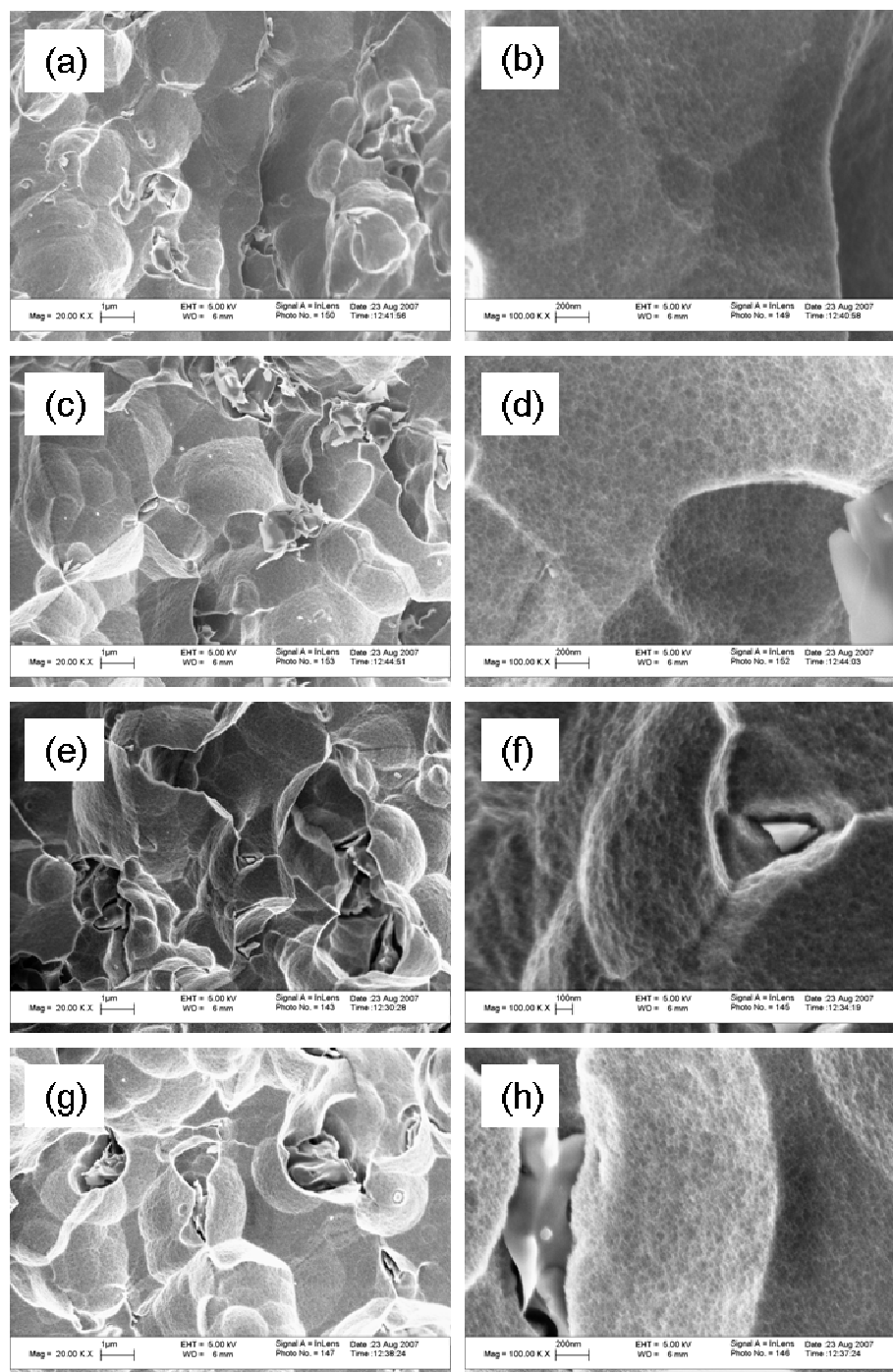


Figure 3.25 SEM images of CoCr anodized in 1 M H_2SO_4 (a & b), 1 M H_2SO_4 and 0.25% HF (c & d), 1 M H_3PO_4 (e & f), and 1M H_3PO_4 and 0.25% HF (g & h) at 2 V for 2 min. Scale bars = 1 μm in (a)(c)(e)(g), scale bars = 200 nm in (b)(d)(f)(h).

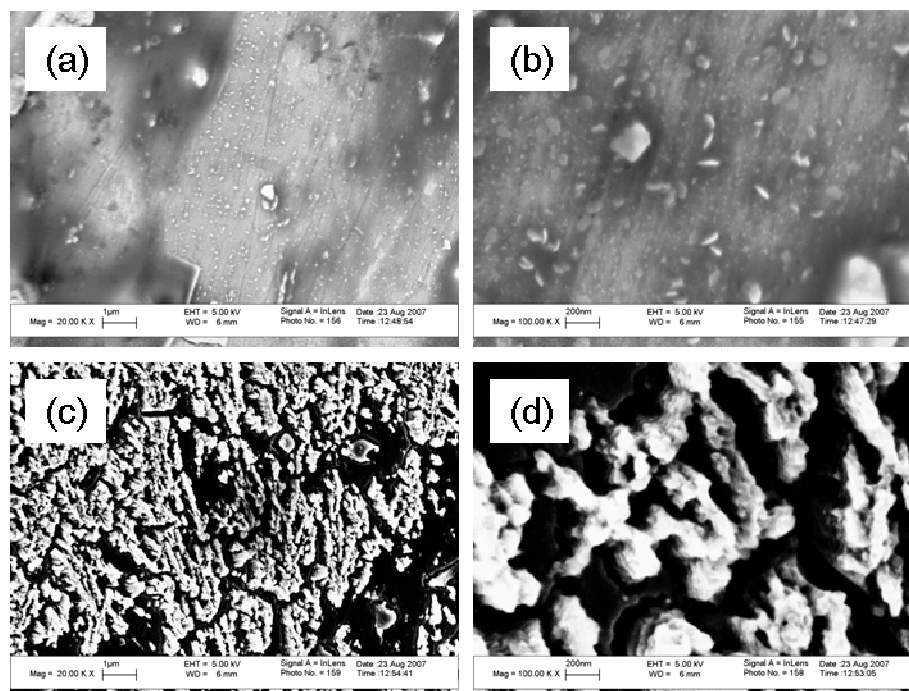


Figure 3.26 CoCr anodized in 0.1 M NaOH solution under 2 V (a & b) and 5 V (c & d) for 2 min. Scale bar equals 1 μm in (a)(c) and 200 nm in (b)(d).

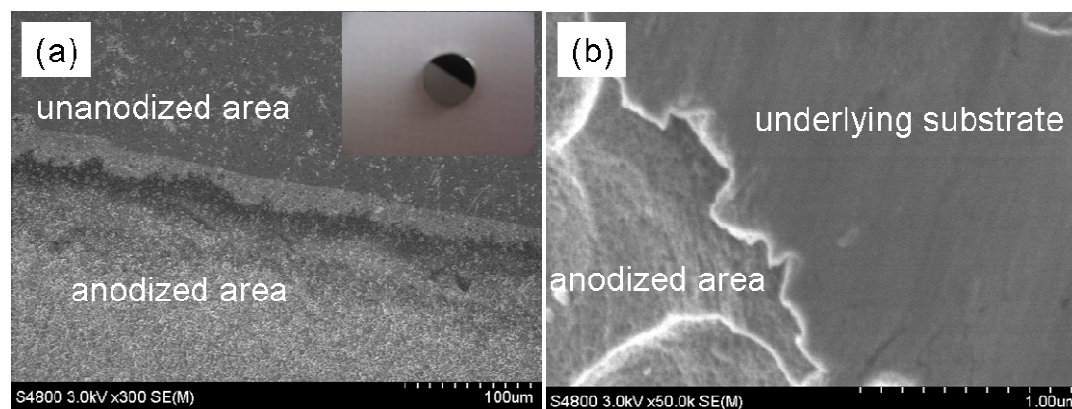


Figure 3.27 SEM images of (a) the interface between the anodized CoCr alloy and unanodized CoCr and (b) the anodized CoCr and the underlying substrates.

The rms roughness of unanodized CoCr (in a 10 x 10 μm area) was 41.6 nm while the rms value for anodized CoCr was 725.3 nm (see morphology in Figure 3.28).

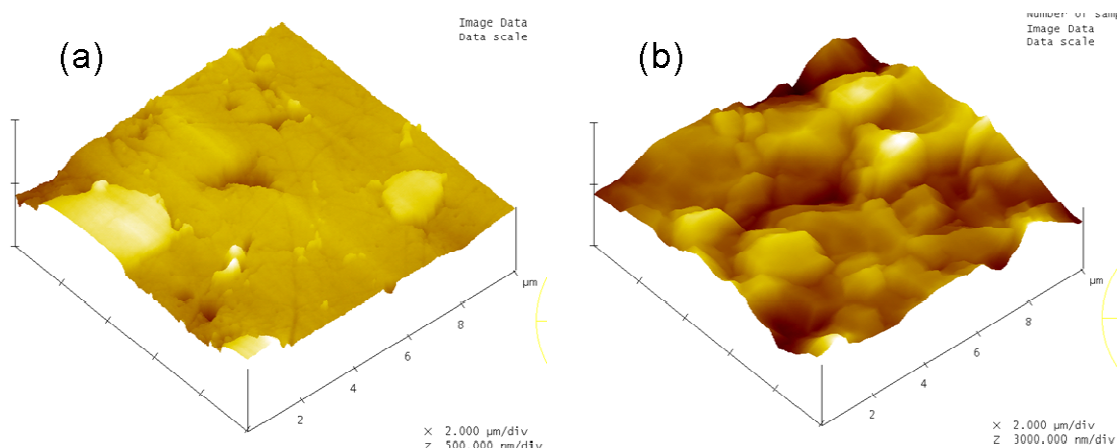


Figure 3.28 AFM 3D images of (a) unanodized CoCr and (b) anodized CoCr in 0.5% HF at 10 V for 2 min. AFM scan area was 10 x 10 μm .

EDS data showed that the chemistry of the CoCr surfaces were not greatly changed after anodization (Table 3-5). The main composition of this alloy was CoCrMo.

The original CoCr surfaces were hydrophobic, with water contact angles around 97° (Table 3-6). In comparison, CoCr anodized in dilute HF showed greatly decreased water contact angles around 67° , and, thus, were more hydrophilic than the unanodized CoCr surfaces. There was about a 9% increase in surface energy when comparing anodized and unanodized CoCr and it was noted that the polar component of the total surface energy greatly increased, from 0.27 to 3.89 mN/M after anodization.

Table 3-5 Atomic percentages of elements on unanodized and anodized CoCr as revealed by EDS.

	Si (%)	Cr (%)	Mn (%)	Co (%)	Mo (%)
CoCr	1.4	33.37	0.91	60.91	3.35
Anodized CoCr	1.23	33.43	1.11	61.03	3.2

Table 3-6 Contact angle and surface energy of CoCr and anodized CoCr (in 1.5 % HF under 10 V for 2 min). PEG stands for polyethylene glycol. E, Ed and Ep are the total surface energy, the dispersive and polar components of the total surface energy, respectively. Θ is the contact angle.

Sample	Θ (water)	Θ (PEG)	E (mN/m)	Ed (mN/m)	Ep (mN/m)
CoCr	96.9 $\pm$ 2.6	57.7 $\pm$ 4.9	37.15 $\pm$ 1.14	36.88 $\pm$ 1.08	0.27 $\pm$ 0.07
Anodized CoCr	66.9 $\pm$ 6.2	69.5 $\pm$ 6.3	40.46 $\pm$ 1.95	26.58 $\pm$ 1.17	13.89 $\pm$ 0.78

3.1.3.2. Osteoblast Responses on Anodized CoCr Alloy

The anodized CoCr alloy surfaces promoted osteoblast attachment (23%) after 4 h of culture when compared to unanodized CoCr (Figure 3.29).

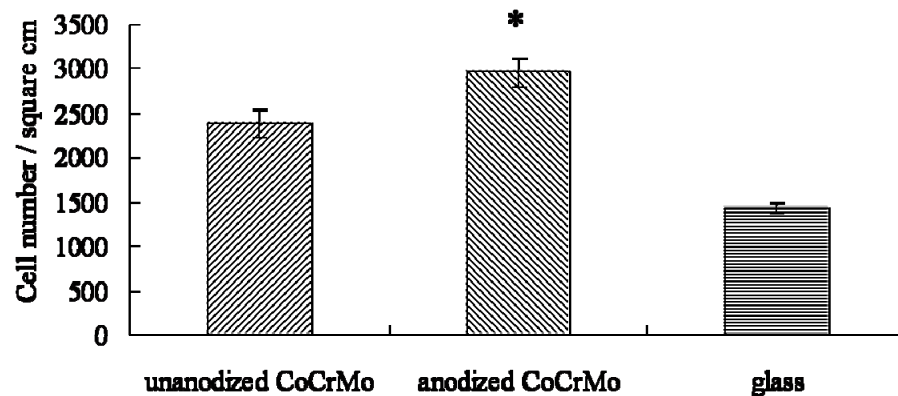


Figure 3.29 Osteoblast adhesion on unanodized CoCr, anodized CoCr (1.5 % HF, 10 V, 2 min), and glass (reference). Values are mean + SEM; n = 3; * p < 0.1 compared to unanodized CoCr.

3.1.3.3. Chondrocyte Responses on Anodized CoCr Alloy

The Anodized CoCr alloy surface promoted chondrocyte attachment after 4 hours of culture (Figure 3.30). Furthermore, CoCr surfaces anodized for shorter periods of time (10 s), which exhibited less rough surfaces, outperformed CoCr anodized for longer periods of time (2 min) in terms of cell attachment. The typical chondrocyte morphology on these substrates is shown in Figure 3.31.

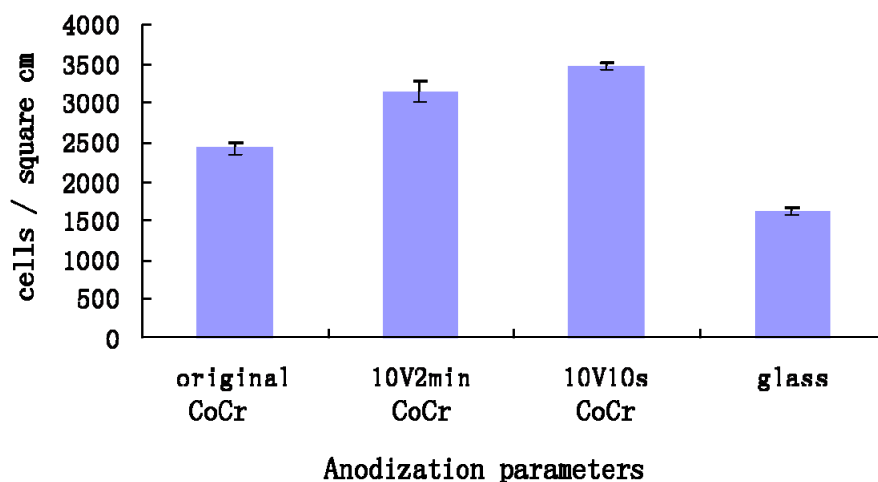


Figure 3.30 Chondrocyte attachment after 4 h of culture on the original CoCr, CoCr anodized under 10V for 2 min, CoCr anodized under 10 V for 10 s, and glass. Data = mean + SEM; $n = 3$; * $p < 0.01$ compared to original and glass, # $p < 0.05$ compared to 10V/2min.

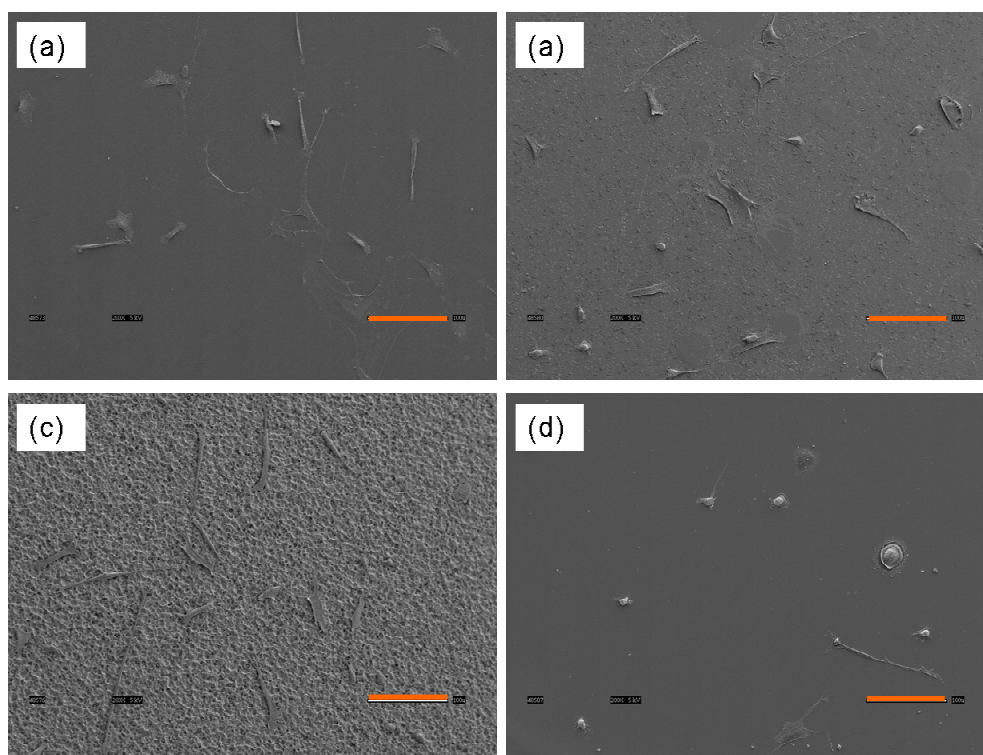


Figure 3.31 Chondrocyte morphology on (a) unanodized CoCr surfaces, (b) CoCr anodized in 1.5% HF at 10 V for 10 s, (c) CoCr anodized in 1.5% HF at 10 V for 2 min, and (d) glass. Scale bars = 100 μm.

3.2. Anodized Titanium as a Drug Delivering System

The survey of both osteoblast and chondrocyte responses on anodized titanium, a titanium alloy, and a CoCr alloy make it clear that anodization is a universal method possible to create a favorable surface on numerous metals used for orthopedic applications. However, the nanotubular surface structures resulting from anodization may have an additional potential to be utilized as a novel metallic drug delivering system, which could further ensure the efficacy of bone implants.

3.2.1. Characterization of Functionalized Anodized Titanium

3.2.1.1. Confirmation of Silanization

For drug delivery applications, it is important to maintain the nanotubular structures during any surface modifications necessary for loading drugs. Under SEM, it was clear that none of the reactions (introducing hydroxyl, amine, and methyl functional groups) significantly changed the nanotubular structures (Figure 3.32).

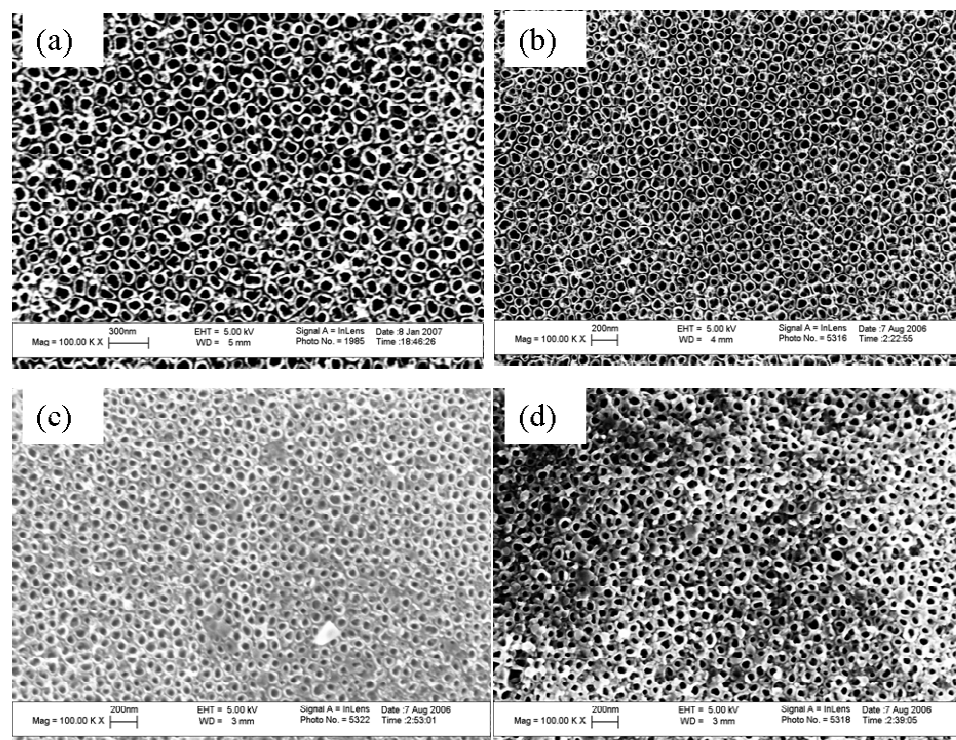


Figure 3.32 SEM micrographs revealed unchanged nanotubular structures after three steps of chemical modifications. (a) Original anodized titanium in 1.5% HF for 10 min, (b) anodized titanium underwent hydroxylation in a Piranha solution for 5 min, (c) sample in (b) underwent silanization, and (d) surface in (c) underwent replacement of amine groups with methyl groups. Scale bars = 200 nm.

The efficiency of silanization on anodized titanium with nanotubular structures was qualitatively confirmed by the CBQCA reagent kit. The following figure shows fluorescence signals uniformly over the anodized titanium with nanotubular structures where amine groups were introduced (Figure 3.33). This indicated a good efficiency of silanization on the anodized titanium with nanotubular structures. In contrast, none of the unanodized titanium, unmodified anodized titanium, and anodized titanium terminated with hydroxyl groups showed a fluorescent signal. During the CBQCA assay, the anodized titanium

terminated with methyl groups were shown to have good fluorescence intensity, indicating that the efficiency of the step 3 reaction described in section 2.3 was not high enough to replace all the primary amines with methyl groups.

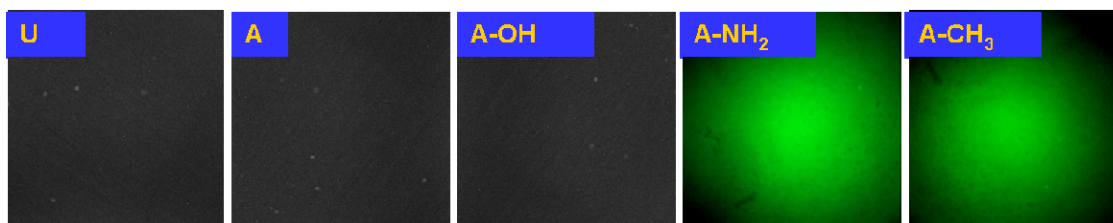


Figure 3.33 The CBQCA reagent confirmed the amine termination after silanization of the anodized titanium.

3.2.1.2. Surface Chemistry Changes

The anodized titanium was found to be embedded with a small amount of fluorine which may have come from the electrolyte solution (Figure 3.34 a). There was also around 20% carbon on the titanium surface most likely due to carbon contamination from the air. The surface chemistry of the anodized titanium with nanotubular structures terminated with hydroxyl groups was similar to that of the anodized titanium except two small peaks (Figure 3.34 b). First, a small amount of sulfur was found which may be the residue of sulfuric acid used during the respective step of chemical modification. Second, the fluorine peak disappeared after soaking in Piranha solution. After silanization, the appearance of silicone and nitrogen peaks were confirmed on the anodized titanium terminated with amine and methyl groups (Figure 3.34 c and d). Also, a much higher carbon percentage (about 31% for the anodized nanotubular titanium terminated with

amine groups and 36% for the anodized nanotubular titanium terminated with methyl groups) appeared due to the introduction of carbon chains on the surface.

A sputter gun can remove a thin layer from the surface and reveal the uncontaminated underlying layers. Approximately one minute of sputtering can remove a 20 nm thick layer. In this study, unanodized titanium with a naturally formed TiO_2 thin layer showed O/Ti atomic percentage ratios around 70/30 before sputtering. After sputtering for 30 and 60 seconds, the ratios decreased to around 15/85, which indicated the removal of the outmost oxide layer (less than 10 nm) reaching the underlying titanium substrates (Figure 3.35 a).

When sputtering anodized titanium surfaces, a decrease of the O/Ti ratio to its lowest level lasted much longer than 60 seconds, indicating a much thicker oxide layer formed during the anodization process, which was estimated to be larger than 100 nm (Figure 3.35 b).

When performing sputter removing on A- NH_2 and A- CH_3 samples, there were two differences when comparing their outmost surfaces. First, the peak of fluorine reoccurred after a short period of sputtering (12 s), meaning that the distribution of fluorine was not limited to the top surface. Second, it was found that after 1 min of sputtering, the signal of silicone and nitrogen disappeared, meaning that such surface modifications were on the top portion of the nanotubular titanium structures (around a depth of 20 nm).

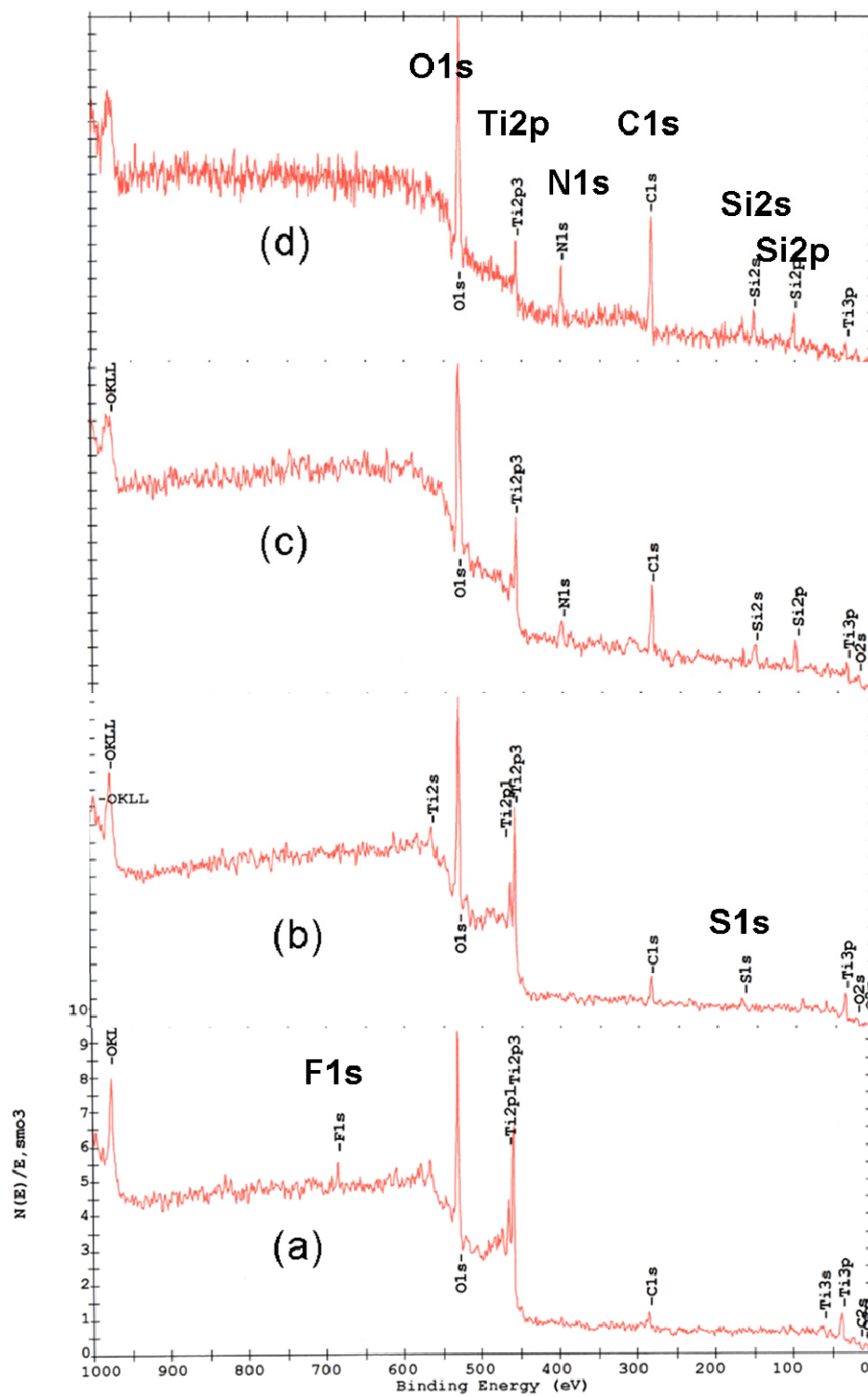


Figure 3.34 XPS spectrum of the outermost surfaces on the various anodized titanium including (a) A, (b) A-OH, (c) A-NH₂, and (d) A-CH₃.

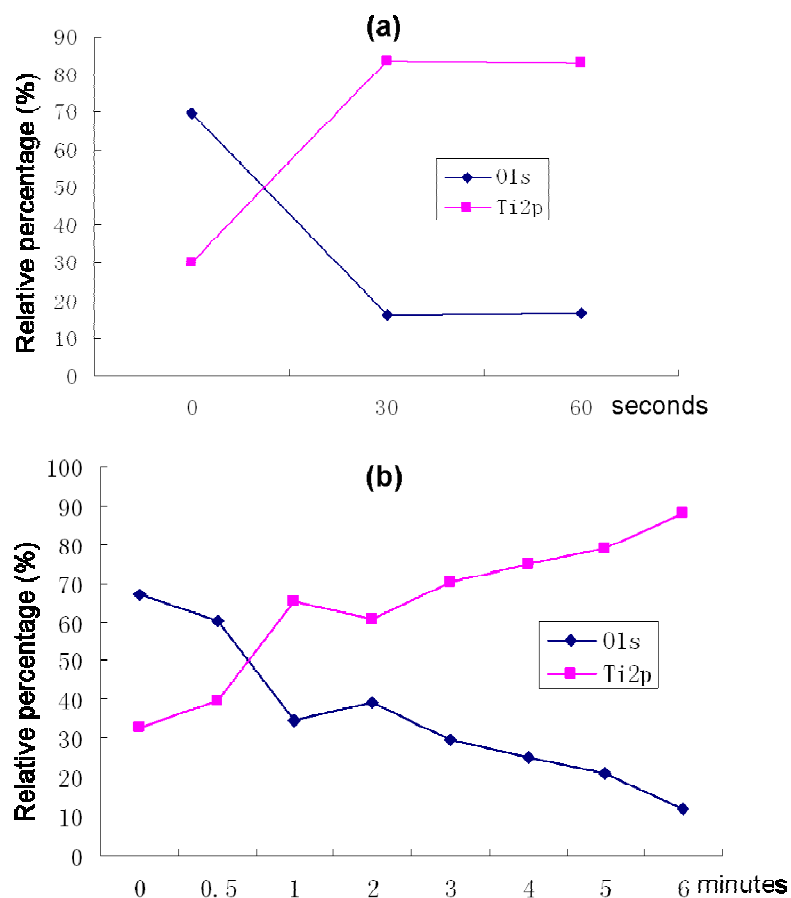


Figure 3.35 Relative percentages of oxygen and titanium on (a) unanodized titanium and (b) anodized titanium after different periods of sputter gun bombardment.

3.2.1.3. Contact Angles and Surface Energy

Contact angle measurements are indirect ways to confirm surface chemistry changes and evaluate the wettability of a surface, which is important for drug loading efficiency.

Results of this study showed that unanodized titanium surfaces had water contact angles around 70° (Table 3-7). The as-prepared anodized titanium had very small contact angles while after complete drying, contact angles were

around 15° lower than unanodized titanium. This maybe due to whether or not air bubbles were trapped in the nanotubular structures; this issue will be discussed in detail in the next chapter. Treating anodized titanium with the Piranha solution resulted in a much more hydrolyzed surface and, thus, a much lower surface contact angle (around 10°). When the silanization reactions occurred, the greater numbers of carbon chains and various termination groups changed the surface wettability of anodized titanium to various levels. Specifically, the A-NH₂ surface had contact angles of around 30° while the contact angles for A-CH₃ were around 40° . This difference implied that the amine groups were functionalized to methyl groups during the step 3 reaction although the efficiency was not as high as during the silanization reaction (step 2).

Surface energies were calculated from contact angles (using water and polyethylene glycol) and are listed in Table 3-7. The surface energy of anodized titanium increased 19% compared to unanodized titanium, in which the dispersive component increased 14% and the polar component increased 84%. A-OH samples had a much higher surface energy (47% more than U and 23% more than A). Importantly, this study provided evidence that this increment primarily came from the change in the polar component of the total surface energy. After silanization, the surface energy of A-NH₂ was 5% less than that of A-OH and the surface energy of A-CH₃ was 11% less than that of A-OH. It can also be noted that it was the polar component that was responsible for this decrease in total surface energy.

Table 3-7 Contact angles and surface energy of various titanium substrates. PEG stands for polyethylene glycol, Ed and Ep are dispersive and polar components of the total surface energy.

Sample	Θ (water)	Θ (PEG)	E (mN/m)	Ed (mN/m)	Ep (mN/m)
U	71.4 $\pm$ 4.2	32.4 $\pm$ 4.7	56.14 $\pm$ 1.56	52.13 $\pm$ 1.26	4.01 $\pm$ 0.30
A	58.5 $\pm$ 9.5	11.7 $\pm$ 2.4	66.89 $\pm$ 1.31	59.51 $\pm$ 0.68	7.38 $\pm$ 0.68
A -OH	12.9 $\pm$ 3.0	84.1 $\pm$ 10.5	82.52 $\pm$ 1.52	55.56 $\pm$ 0.82	26.96 $\pm$ 1.34
A -NH ₂	29.0 $\pm$ 5.4	16.1 $\pm$ 0.3	78.17 $\pm$ 0.68	56.72 $\pm$ 0.08	21.45 $\pm$ 0.59
A -CH ₃	41.7 $\pm$ 2.4	13.3 $\pm$ 4.6	73.46 $\pm$ 1.75	58.09 $\pm$ 1.27	15.36 $\pm$ 0.36

3.2.2. Drug Loading and Release Behavior

3.2.2.1. Drug Physical Adsorption

After soaking in the P/S or P-G solution overnight, titanium substrates with different surface chemistry (and, thus, different surface wettability) showed different drug adsorption morphologies under SEM (Figure 3.36). Generally, there was no uniform coverage over any of the substrates by P/S or P-G except for the A-OH titanium. Unfilled nanotubes can be seen in some areas of A, A-NH₂, and A-CH₃ titanium substrates.

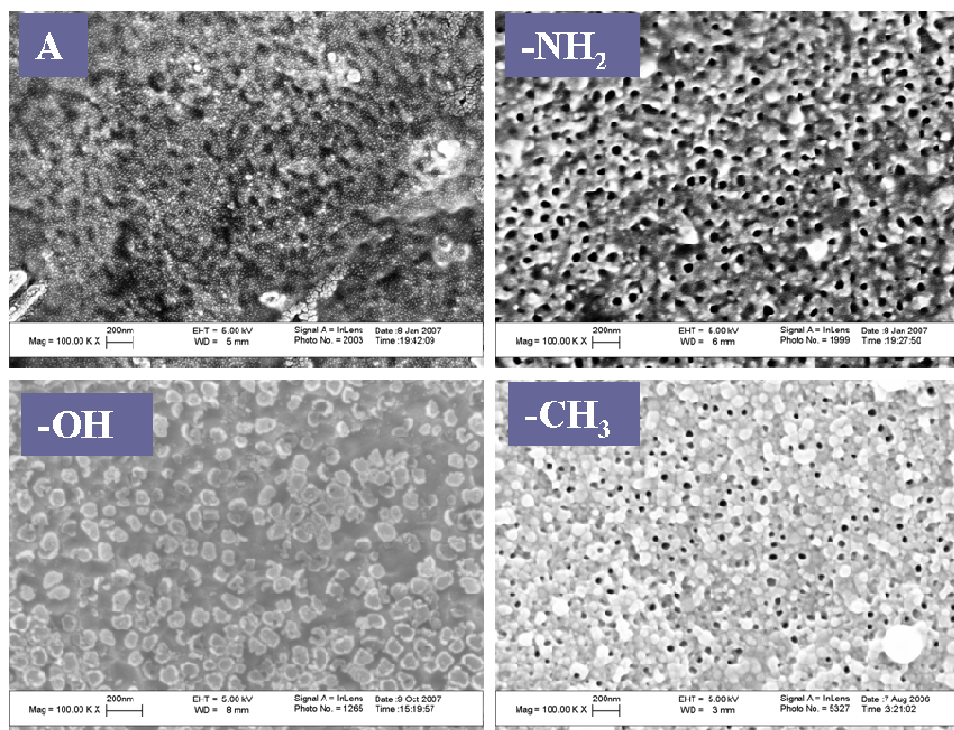


Figure 3.36 SEM micrographs showed filled/unfilled nanotubes after being loaded with penicillin drug molecules on A, A-OH, A-NH₂, and A-CH₃ substrates.

However, top-view images could not indicate whether or not the depth of the nanotubes was filled with drug molecules. For this reason, some top portions of the titania nanotubes were mechanically abraded to reveal the deeper portions of the nanotubes. As shown in Figure 3.37 (a), anodized titania nanotubular structures without loaded drugs were empty. The nanopores on the inclined surface (i.e., the edge area) could be seen since there was nothing loaded into the nanotubes. In other words, the nanopores on the edge area would not be seen if they were filled with drugs. For anodized titanium sample loaded with penicillin, some nanopores were seen, which is in agreement with SEM observation in Figure 3.36 that not all the nanotubes were filled with drug molecules (Figure 3.37 b). However, some of the nanopores were filled and

could not be seen. Importantly, for the A-OH titanium samples, no nanopores were seen on both the top and the middle of the nanotubes, indicating their filling with drugs (Figure 3.37 c). The A-NH₂ and A-CH₃ titanium samples were similar to the aforementioned anodized samples, with some empty nanopores seen (Figure 3.37 d and e).

To quantitatively determine how much drug was loaded onto/into such titanium substrates, drug release profiles were characterized. First of all, the release behaviors of two antibiotics were very similar to each other (Figure 3.38). Taking the A-OH titanium sample for example, most of the total amount was released within one hour (about 60 µg for P/S and 90 µg for P-G). Then, the released amount dropped quickly to around 10 µg for P/S and 15 µg for P-G during the second hour. Finally, the released drug amount decreased to only a few µg or close to zero after two days. The most significant result here is that anodized titanium terminated with hydroxyl groups outperformed unanodized and anodized titanium in terms of drug release amount after 1 h and 2 h.

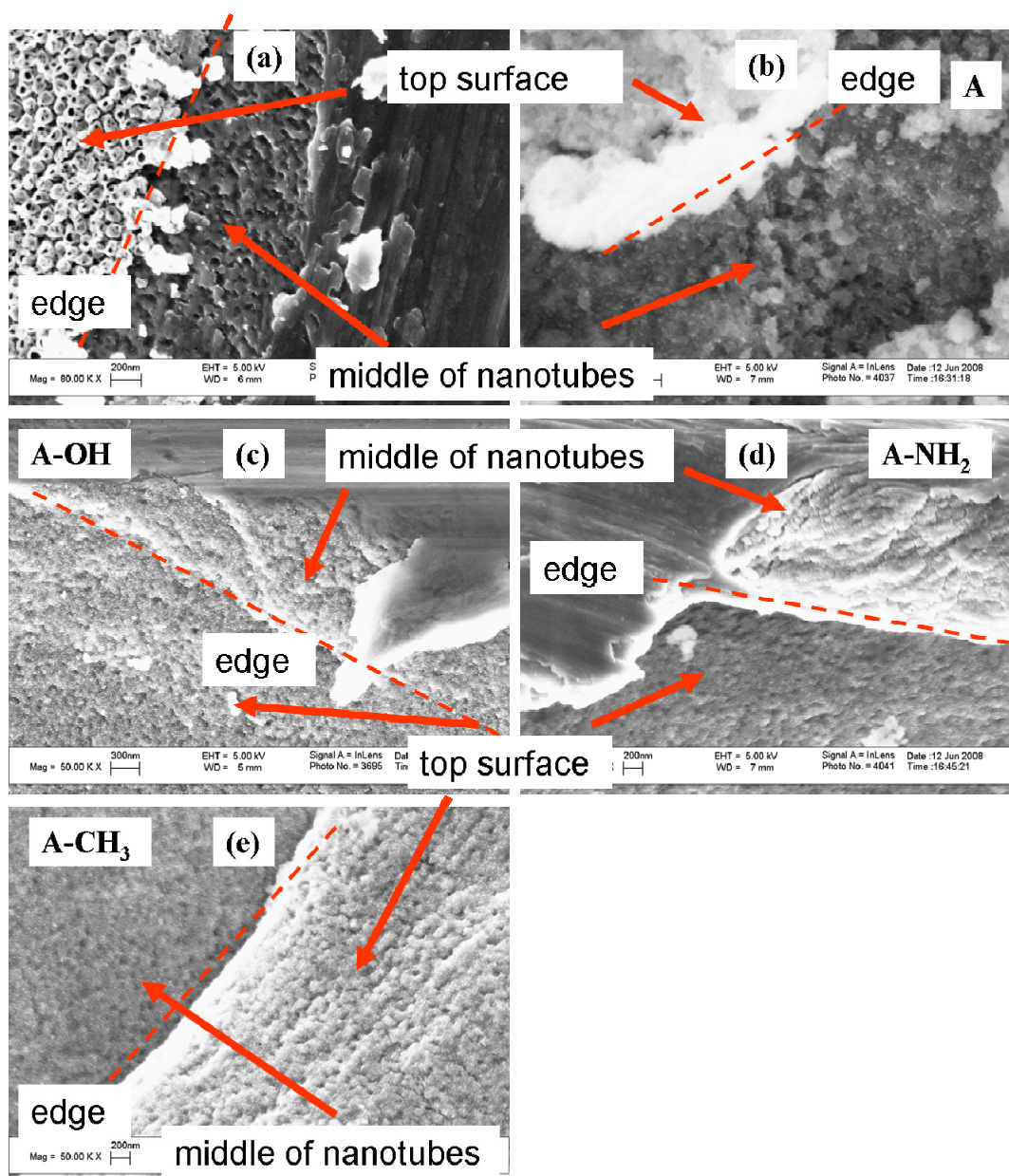


Figure 3.37 SEM images of the partially abraded titania nanotubular structures: (a) anodized titanium possessing nanotubular structures, (b) anodized titanium loaded with P/S showed some unfilled nanotubes in the middle portion, (c) A-OH loaded with P/S showed filled nanotubes, (d) A-NH₂ loaded with P/S showed some unfilled nanotubes on the top and in the middle portion, and (e) A-CH₃ loaded with P/S showed some unfilled nanotubes on the top and in the middle portion.

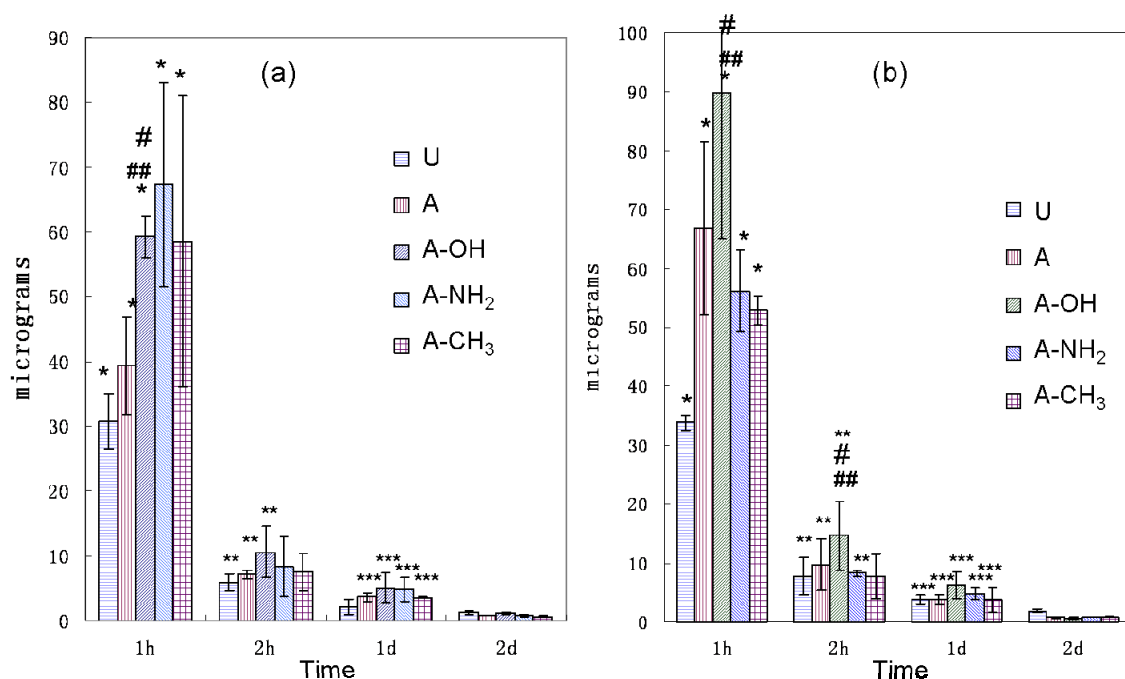


Figure 3.38 Release of (a) P/S or (b) P-G from various titanium substrates after 1h, 2h, 1d, and 2d using the physical adsorption method. #p < 0.1 compared to unanodized titanium, ##p < 0.1 compared to anodized titanium with nanotubular structures, *p < 0.1 compared to respective release amount after 2 h, **p < 0.1 compared to respective release amount after 1 d, ***p < 0.1 compared to respective release amount after 2 d. Data = Mean + SEM, N = 3.

3.2.2.2. Drug Electrodeposition

As previously mentioned, the anodized titanium with nanotubular structures was used as a cathode in an electrodeposition system in another attempt to promote drug loading and prolonged drug release. Without an applied voltage, it was shown that close to no drugs were deposited onto the anodized titanium substrates (Figure 3.39 a). Because the P/S solution contained 0.9% NaCl, an electrolyte containing only NaCl was used to determine the role of sodium salt in this deposition process. It was found that some salt crystals can be deposited onto the nanotubes along the edges but the nanotubes were not capped by such

crystals (Figure 3.39 b). In comparison, when the electrolyte was P/S and a voltage of 8 V was applied, SEM images showed a well covered surface in which the nanotube structures were barely seen (Figure 3.39 c). Other samples like A-OH, A-NH₂, and A-CH₃ had similar results to anodized samples (Figure 3.39 d to f).

The release of drugs from the electrodeposited substrates was much different from that of the physical adsorption method. The total amount of released drugs was less than 15 µg but the drug released after the first hour was much closer to that released in the first hour than in the adsorption method. Taking the A-OH titanium sample for example, most of the total amount was released within one hour (about 7 µg for P/S and 9 µg for P-G). Then, the released amount dropped quickly to around 2 µg for P/S and P-G during the second hour. Finally, the released drug amount decreased to less than 1 µg or close to zero after two days. There was no significant difference between A-OH titanium samples and the other anodized titanium samples (Figure 3.40).

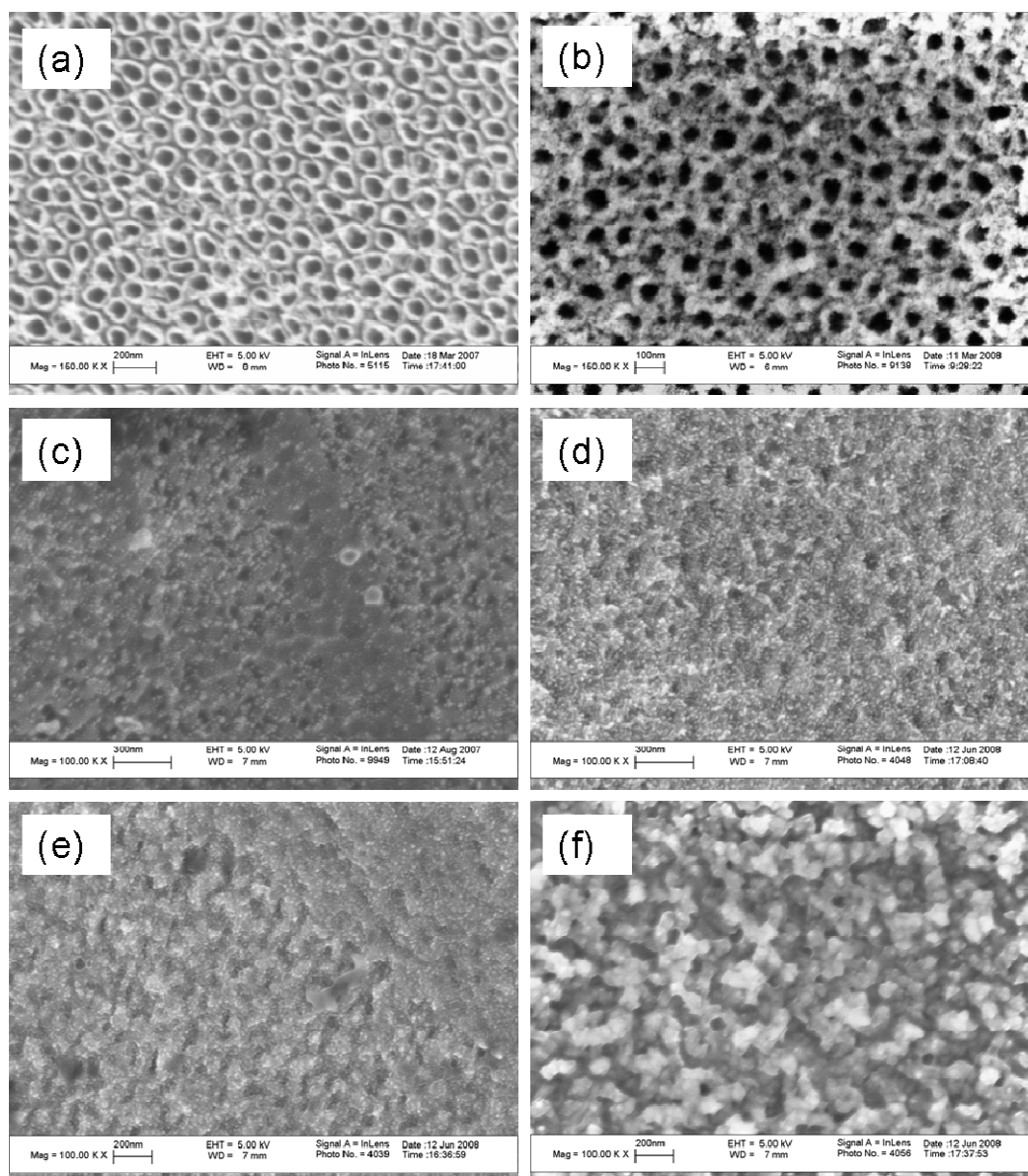


Figure 3.39 SEM images of (a) anodized titanium substrates soaked in a 5% P/S solution for 30 min, (b) anodized titanium electrodeposited in a 0.9% NaCl solution for 5 min under 8V, (c) anodized titanium electrodeposited in a 5% P/S solution for 5 min under 8 V, (d) anodized titanium terminated with $-OH$ electrodeposited in a 5% P/S solution for 5 min under 8 V, (e) anodized titanium terminated with $-NH_2$ electrodeposited in a 5% P/S solution for 5 min under 8 V, and (f) anodized titanium terminated with $-CH_3$ electrodeposited in a 5% P/S solution for 5 min under 8 V.

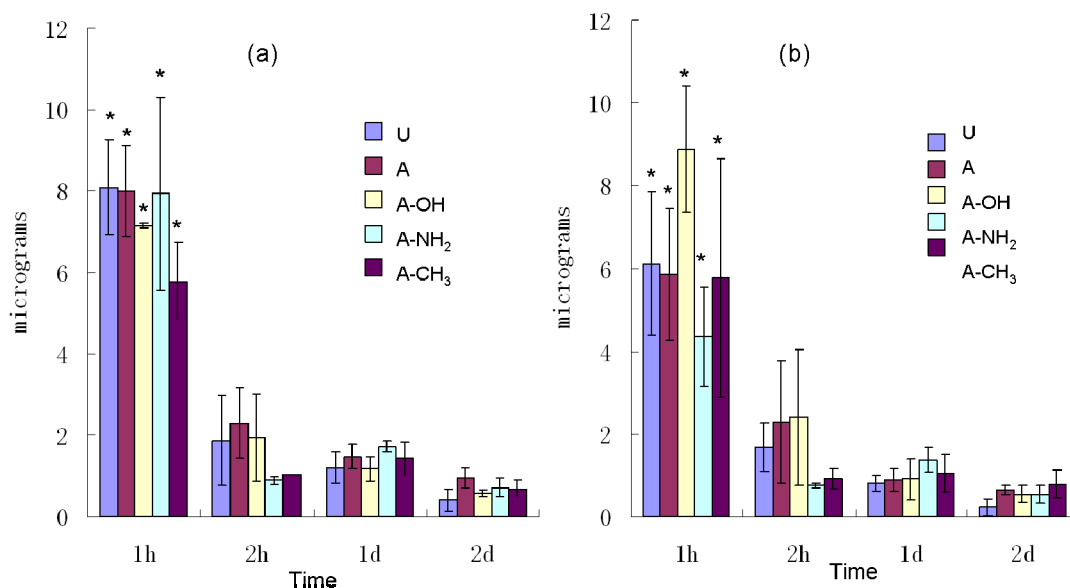


Figure 3.40 Release of (a) P/S or (b) P-G from various titanium substrates after 1h, 2h, 1d, and 2d using the electrodeposition method. Data = Mean + SEM, N = 3. *p < 0.1 compared to respective release amount after 2 h.

3.2.2.3. Drug Co-precipitation

A co-precipitation method was also used in the present study to promote drug loading and prolonged release. SEM images demonstrated the appearance of the anodized titanium substrates after they were soaked in sodium hydroxide for 1 hour (Figure 3.41 b). It can be seen that fiber-like crystals formed along the edges of the nanotubes. EDS results confirmed that these crystals were composed of appearance of sodium and titanium; thus, the crystals were considered sodium titanate. After being soaked in 1.5 X SBF for 3 days, needle-like minerals were observed on most of the anodized titanium surfaces (Figure 3.41 c). These areas were analyzed by EDS and were found to have calcium and phosphorous (Figure 3.42). Thus, these minerals were considered calcium phosphates. In some areas of the surface, there were coatings with a different

morphology, specifically, a more dense particulate structure (Figure 3.41 d).

When the SBF solution contained 20% P/S, the anodized titanium substrates soaked for 3 days had similar surface morphologies to those without P/S (Figure 3.41 e and f). Higher P/S concentrations in the SBF solution led to very dense coatings on the surface and, thus, were thought to interrupt the precipitation process of calcium phosphates.

The same abrasion method was used here to study the filling of titania nanotubes during co-precipitation. SEM images demonstrated that the co-precipitation of P/S and HA mainly formed on the top of the anodized titania nanotubes; unfilled nanopores were seen in the middle of these titania nanotubes structures (Figure 3.43 a).

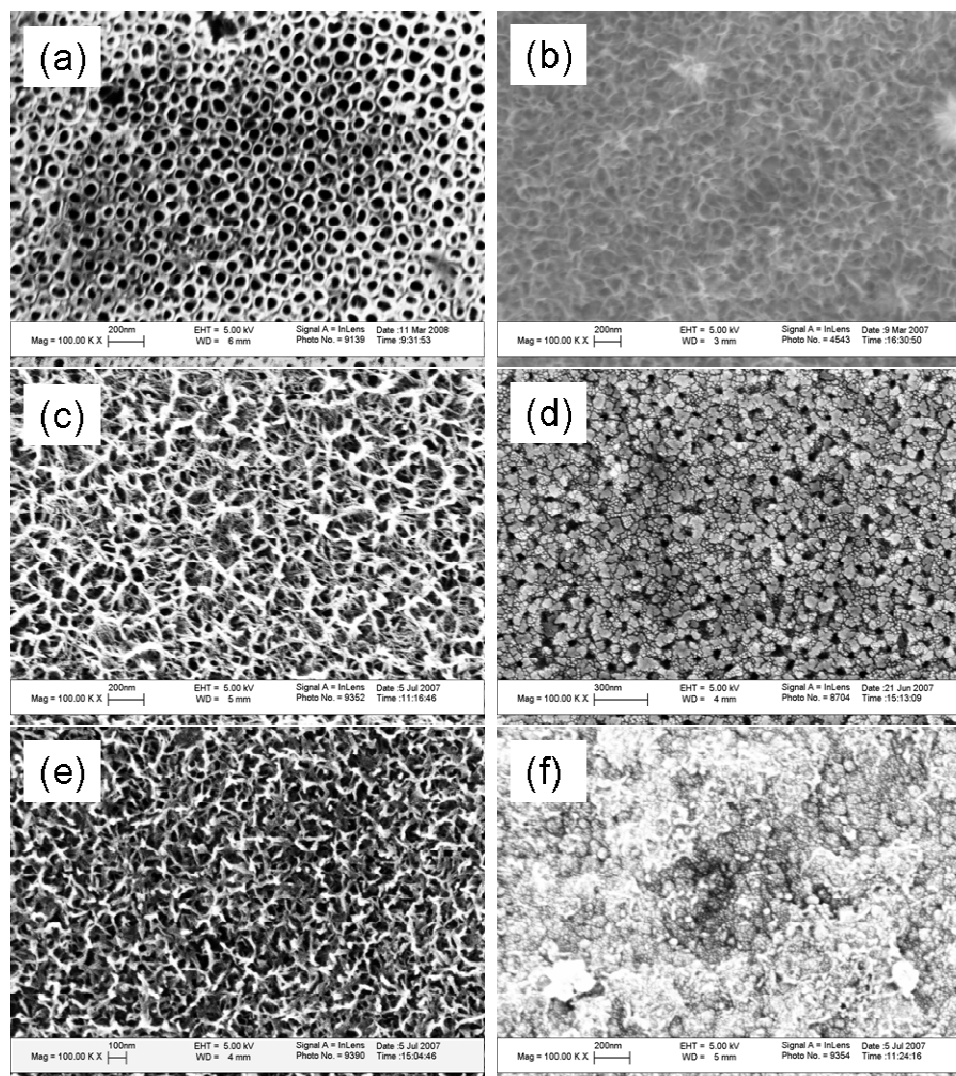


Figure 3.41 SEM images of (a) anodized titanium, (b) anodized titanium soaked in 6M NaOH for 1 h, (c) and (d) ASH samples soaked in 1.5X SBF for 3 days without P/S, (e) and (f) ASH sample soaked in 1.5X SBF for 3 days with 20% P/S. ASH = anodized, soaked in NaOH and heat treated titanium.

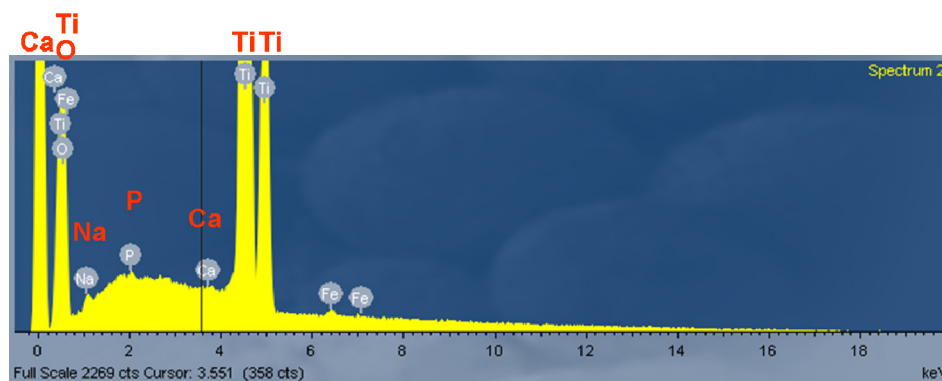


Figure 3.42 EDS spectrum of ASH samples revealed the existence of Ca and P in the coatings deposited onto anodized titanium surfaces during a co-precipitation method. ASH = anodized, soaked in NaOH and heat treated titanium.

During the drug release study, anodized titanium substrates were soaked in three concentrations of P/S in SBF solutions at 5 %, 10 %, and 20 vol. %. Then, these substrates were used to test the drug release behavior (Figure 3.44). Since the drug concentration was as low as 5%, the total release amount was comparable to the electrodeposition method, around 10 to 20 μg . The most obvious difference was that the release of drugs lasted much longer with the co-precipitation method than those in previous adsorption or electrodeposition methods. There was significant release within one hour (e.g., 4 μg for the 20% P/S solution), but nearly nothing during the second hour. A major release peak was found in the next week (about 10 μg for the 20% P/S solution) and was not completed until 21 days. It is clear that the calcium phosphate minerals remained on the substrates after the soaking (Figure 3.43 b to d).

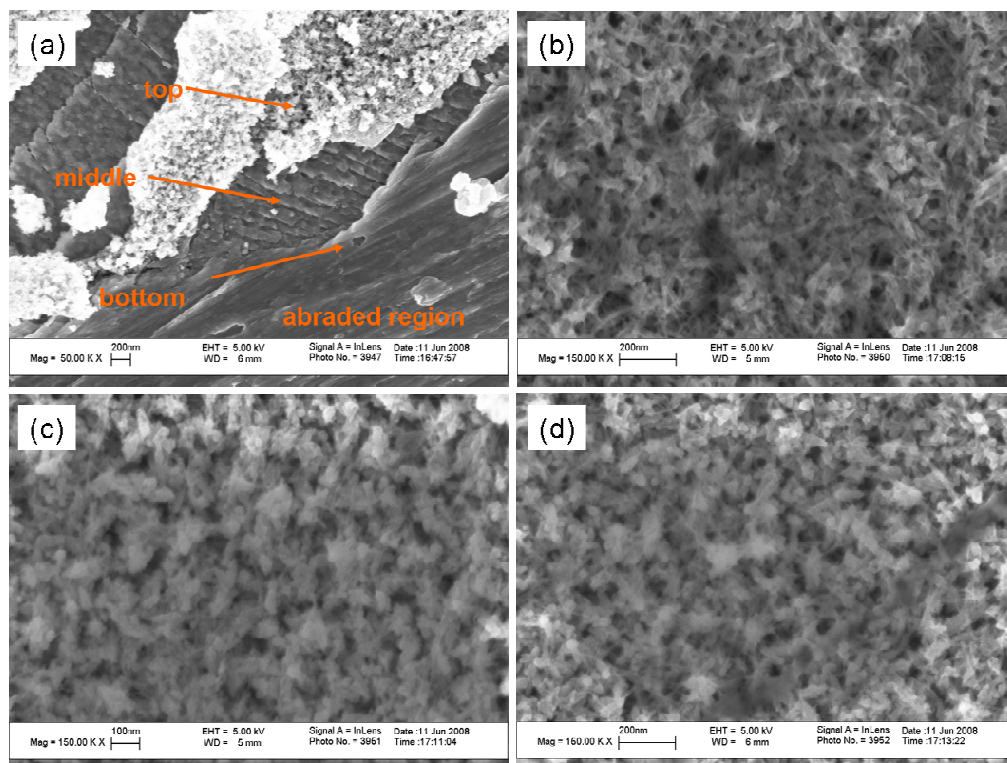


Figure 3.43 SEM image of anodized titanium surfaces co-precipitated with P/S and minerals: (a) the nanotube structures were abraded to reveal the cross-section and the middle portion of the titania nanotubes were not filled with drugs or minerals after the co-precipitation process; (b) to (d) are top views of anodized titanium co-precipitated with 5%, 10%, and 20% P/S in the SBF solution after 21 days of release.

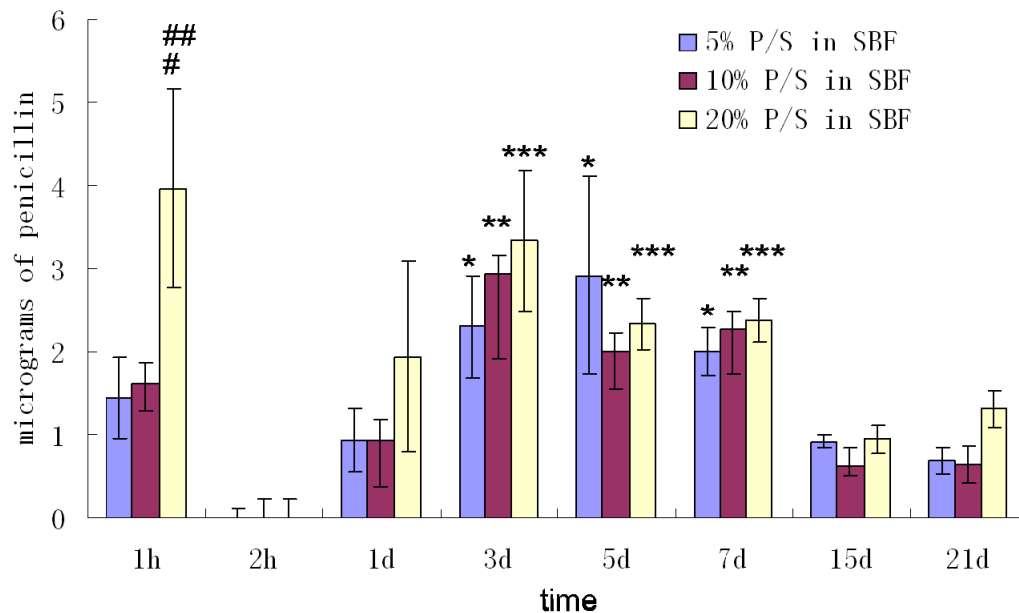


Figure 3.44 Released penicillin amounts after different time periods from anodized titanium co-precipitated with 5%, 10%, and 20% penicillin/SBF solution. # $p < 0.1$ compared to 5 and 10 % data after 1h, ## $p < 0.1$ compared to 2 h, 1 d, 5 d, 7 d, 15 d, and 21 d of 20 % data series, * $p < 0.1$ compared to 2 h, 1 d, 15 d, and 21 d of 5 % data series, ** $p < 0.1$ compared to 2 h, 1 d, 15 d, and 21 d of 10 % data series, *** $p < 0.1$ compared to 2 h, 15 d, and 21 d of 10 % data series. Data = Mean + SEM, N = 3.

3.2.3. Anti-bacteria Properties

Of course, loading drugs into/onto anodized titanium with nanotubular structures is meaningless unless such drugs perform their function (and, thus, retain bioactivity). This was investigated in this study. Unanodized and anodized titanium showed similar bacteria (*S. epidermidis*) attachment after 4 hours (Figure 3.45). When the A-OH titanium samples were loaded with antibiotics (either P/S or P-G) using the physical adsorption method, they inhibited bacteria attachment greater (53% and 57%, respectively) than the anodized substrates as well as other non-drug loaded substrates.

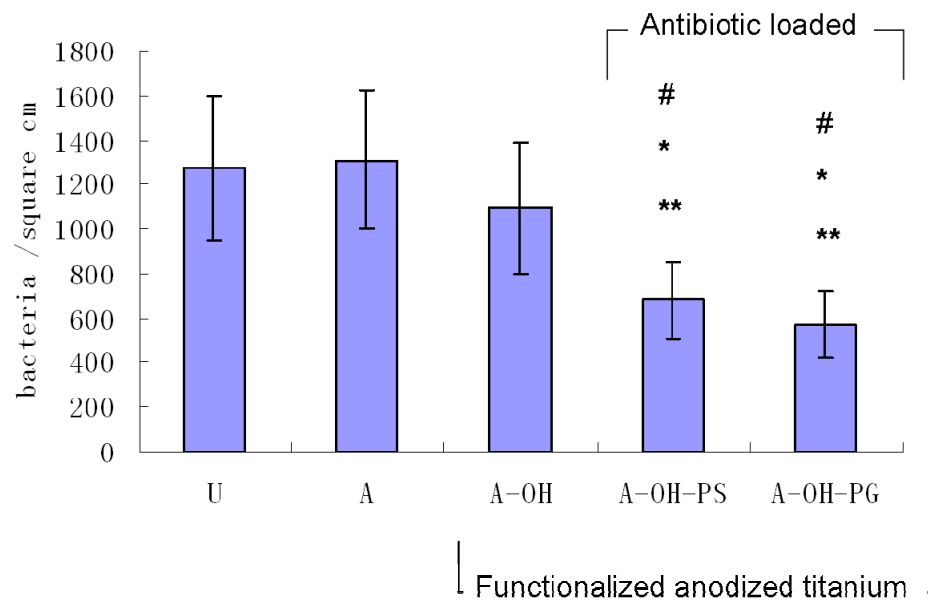


Figure 3.45 *S. epidermidis* attachment on antibiotic loaded into/onto titanium substrates. Abbreviations: OH-PS stands for A-OH samples loaded with P/S, OH-PG stands for A-OH samples loaded with P-G. * $p < 0.1$ compared to unanodized titanium and # $p < 0.1$ compared to anodized titanium. ** $p < 0.1$ compared to A-OH samples. Data = Mean + SEM, N = 3.

3.2.4. Osteoblast Attachment on Antibiotic Loaded Titanium Samples

For drug-unloaded samples, the anodized titanium, anodized titanium terminated with hydroxyl and amine groups all showed increased osteoblast attachment compared to unanodized titanium (Figure 3.46). When all substrates were loaded with penicillin using the physical adsorption method, they all outperformed their respective drug-unloaded samples in terms of osteoblast adhesion. Also, there was no significant difference in osteoblast attachment among the various substrates after drug loading.

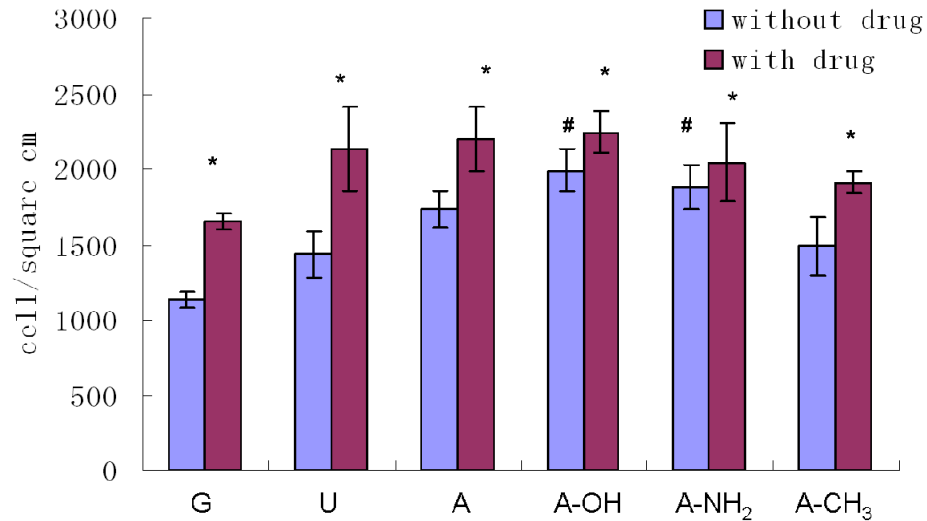


Figure 3.46 Osteoblast attachment after 4 h of culture on drug-unloaded and drug-loaded titanium substrates. * $p < 0.1$ compared to their respective drug-unloaded substrates and # $p < 0.1$ compared to unanodized titanium. G stands for glass. Data = Mean + SEM, $N = 3$. G = glass.

Anodized titanium substrates loaded with penicillin using a co-precipitation method were also tested to determine if osteoblast adhesion was influenced by the loaded antibiotics. Results showed that both anodized titanium precipitated with calcium phosphate minerals and anodized titanium co-precipitated with calcium phosphate and P/S had increased osteoblast attachment compared to unanodized titanium and anodized titanium (Figure 3.47). However, there was no significant difference between these two, indicating that the co-precipitated P/S did not impair osteoblast attachment.

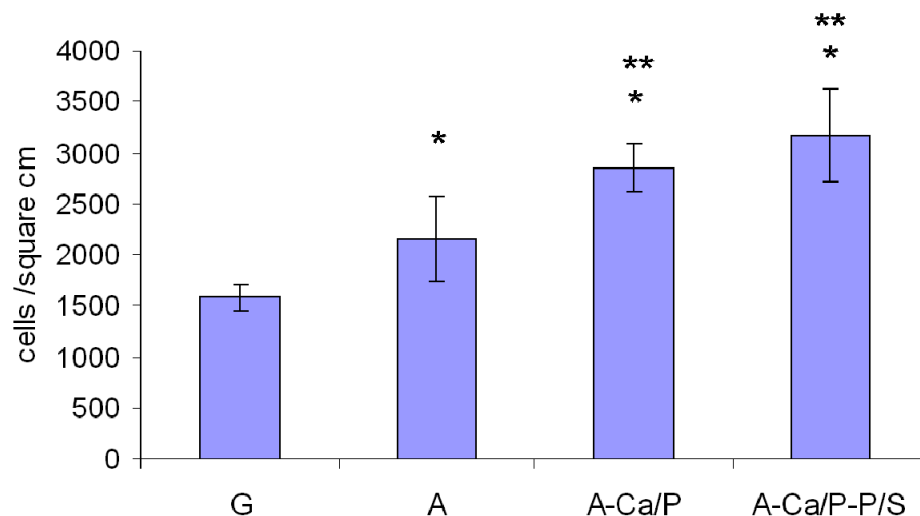


Figure 3.47 Osteoblast attachment after 4 h of culture on anodized titanium and anodized titanium precipitated with calcium phosphate minerals, and anodized titanium co-precipitated with calcium phosphate and P/S. * $p < 0.1$ compared to glass and ** $p < 0.1$ compared to anodized titanium. Data = Mean + SEM, N = 3. G = glass, A-Ca/P = anodized titanium precipitated with calcium phosphates, and A-Ca/P-P/S = anodized titanium co-precipitated with calcium phosphates and P/S.

CHAPTER 4. DISCUSSION

4.1. Versatility of Anodization to Modify Different Metallic Bone Implants

As shown in the last chapter, anodization of titanium or Ti6Al4V resulted in the formation of ordered titania nanotubular structures on their surfaces; anodization of CoCr alloys resulted in heterogeneous nanoporous structures. In addition, studies by others have shown that anodization of aluminum resulted in ordered aluminum oxide nanopore arrays [98]. These results suggest a wide applicability for anodization to improve properties of a wide range of current metal orthopedic implants. In this chapter, the mechanisms of these modifications will be discussed.

4.1.1. Anodizing Titanium Based Materials

4.1.1.1. Mechanism of Titania Nanotube Formation

Of particular importance to the present study is how the unique nanometer features on titanium were created. When a constant voltage is applied to the anode and cathode, ions are driven by the electric field so that a current flows in the circuit (as shown earlier in Figure 1.3). Specifically, Ti^{4+} in a metal substrate moves through the metal/oxide interface and is then ejected towards the

oxide/electrolyte interface as it dissolves into the electrolyte. Meanwhile, O^{2-} and OH^- migrate from the solution to form an oxide on the surface. As a result, during anodization, an oxide layer grows down to the substrate (called field-enhanced oxidation) and at the same time the oxide layer dissolves in the electrolyte solution (HF) due to polarized and weakened Ti-O bonds (called field-enhanced dissolution). It is important to note that the chemical dissolution of titanium oxide by HF plays a critical role in the anodization of titanium. According to the observations and other models proposed by previous researchers [62, 64], a modified mechanism of titania nanotube formation during anodization is presented here. As a voltage is applied, a thin barrier layer of oxide forms on the substrate surface. In the model proposed by Mor et al., the titanium surface was smooth and pores formed by localized dissolution due to presence of stress, impurities, and dislocations. However, for the titanium substrates used in the present study, the initial oxide layer somewhat retained the original surface morphology, i.e. there were nano-scale surface features of a particulate morphology between micron ridges on the surface (Figure 4.1 a). At the concave regions with a small radius of curvature, the charges tended to accumulate and this resulted in a higher surface charge density than the gently curved region, which in turn increased the electric field around these points. Although this charged field is small compared to the external electric field, it may also have contributed to the localized dissolution around these points thus leading to the formation of pores (Figure 4.1 b). Since the pore had a curved bottom surface, the field-enhanced dissolution made the pores simultaneously wider and deeper.

On the other hand, the inter-pore region etched more slowly than at the bottom of the pore (Figure 4.1 c). Voids were also formed in the inter-pore regions which further dissolved due to field enhanced dissolution; this produced thin walls between pores and finally transforms the pores into tubes (Figure 4.1 c and d). Recently, Raja et al. also suggested that the instability of the oxide layer and the self-ordered structures could be explained by the perturbation theory; thus, the separation of individual nanotubes of titanium oxide from the inter-connected nanopores could be attributed to the repulsion forces of the cation vacancies [71].

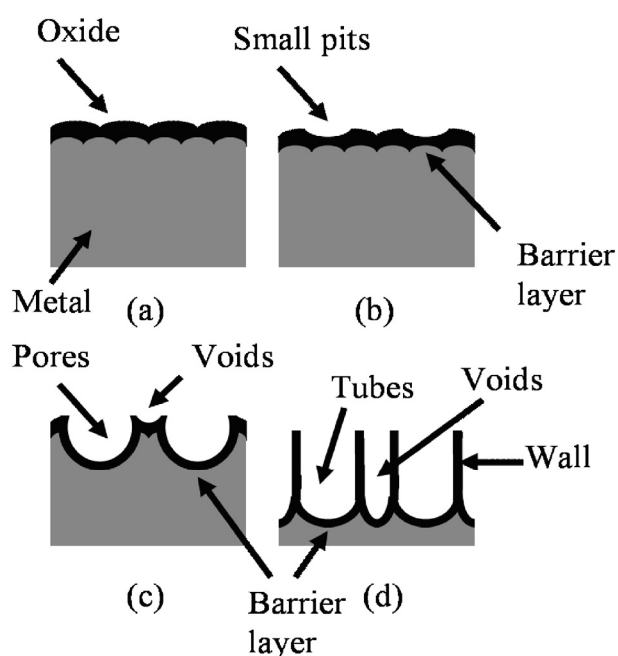


Figure 4.1 Schematic diagram of the evolution of nanotubular structures on the titanium surface during anodization in aqueous HF under constant voltage: (a) oxide formation; (b) pit formation in some concave sites; (c) pore formation and growth under field-enhanced dissolution leading to void formation; and (d) fully developed nanotubes. (Based on the model from [82] and modified according to current experimental observations).

Deepening of the tube ceased when the rate of dissolution of the oxide in the mouth of the tube equaled the rate of oxidation at the bottom-oxide/metal interface. It has been reported that anodizing titanium in HF had the key difference from anodizing aluminum (which has been well studied) in that the final pore depth will not increase with time after reaching a maximum [62-64]. This may be explained by the role of chemical dissolution of titanium in HF. When the oxide layer became thick enough, the strength of the electric field decreased and the field-enhanced dissolution became comparable to the chemical dissolution. Then, the oxide etching rate in the wall and at the pore bottom was balanced and resulted in a constant pore depth.

The ternary titanium alloy Ti6Al4V studied in this work consisted of two metallurgical phases, alpha and beta titanium. As mentioned before, aluminum is an alpha-stabilizing element while vanadium is a beta-stabilizing element. The different crystal structure chemical enrichment of the alpha phase (Al-rich, hcp) and the beta phase (V-rich, bcc) is responsible for the observation that the acid etching attack is visibly different for these two phases as shown in Figure 3.18. In other words, these two phases possess a different etch reactivity in fluoride-containing electrolytes. Macak et al. reported higher dissolution of V-rich titanium oxide in a fluoride-containing electrolyte [99]. As a result, the alpha regions formed homogeneous nanotubular structures after anodization, which had similar inner diameters to pure titanium under the same conditions (only determined by the applied voltage). In comparison, the beta phase suffered from complete

dissolution during anodization. Because it is known that the formation of nanotubes is controlled by a balance of dissolution and oxidation, there is no such nanotube formation occurring in the beta phase region.

4.1.1.2. Influence of Working Parameters

It was clearly shown that there was a range of voltages that produced nanotubular structures on titanium. When the voltage was under some threshold, the resulting structure on titanium was particulate. This was also confirmed in the work of Gong et al. and Mor. et al. [63, 64], where they both obtained nanotube arrays beginning at 10 V for 0.5 % HF but had nanoporous or nanoparticulate structures below that voltage. This is probably because the field-enhanced dissolution was not strong enough to promote pore formation. In other words, the chemical dissolution of the oxide was dominant in this situation and the dissolution rate for all the oxide surfaces (bottom and top) was almost the same. It is also important to note that the nanotubular structures disappeared and the surface contained disordered porous structures when the voltage exceeded 25 V in the present study. This was previously reported by other researchers [64] and they explained that thicker pore walls associated with higher voltages reduced the possibility of having a metallic interpore region and, thus, no voids formed during anodization.

The increase of nanotube diameters with increased voltage was comparable to the study of Park et al. [100]. In their studies, a voltage of 10 V, 15 V, and 20 V

corresponded to inner diameters of 45 nm, 70 nm, and 90 nm for anodized titania nanotubes, respectively. Also, there was a nearly linear relationship between the inner diameter of titania nanotubes and the applied voltage (Figure 4.2). Such capability of controlling titania nanotube diameter in anodization is advantageous when anodized titanium possessing nanotubular structures is used as a novel drug delivery system since drug molecules of different sizes can be loaded into the nanotubes.

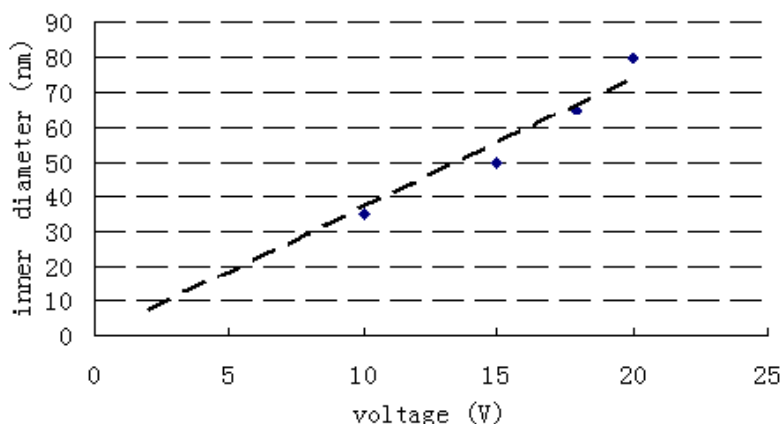


Figure 4.2 Linear relationship between the inner diameter of anodized titania nanotubes and the applied voltage.

As expected, compared with dilute HF concentrations (0.5%), the rate of nanotube formation under 20 V in higher HF concentrations (1.5 %) was much faster. The nanotubular structures did not develop until after 2 min for 0.5 % HF while they developed after just 30 s for 1.5 % HF. Clearly, this is because at higher concentrations (i.e., more ions in the electrolyte) higher currents are present and subsequently a higher rate of ion diffusion results; thus, the anodization process

was accelerated. When using 1.5 % HF as the electrolyte, the voltage threshold decreased compared to when using 0.5 % HF. As described in section 4.1.1.1, nanotube formation was related with the field-enhanced dissolution and the electric field should be large enough to initiate pit and void formation. At higher electrolyte concentrations the chemical dissolution of the oxide is stronger, hence the thickness of the initial barrier layer may be thinner than that when using a lower electrolyte concentration. As a result, the electric field across the thinner oxide layer, over which most of the applied voltage drop occurs, will be stronger. So even if the voltage decreases to 10 V for 1.5 % HF, the field-enhanced dissolution for 1.5 % HF can still form nanotubes on the titanium surfaces.

4.1.1.3. Physical Properties of Anodized Titanium/Ti6Al4V

Not surprisingly, AFM results showed that the anodization treatment significantly increased the rms roughness of the surface (as measured by AFM) for both titanium and Ti6Al4V. Comparing SEM pictures before and after anodization in Fig. 3.1 (b) and (c), one can see that the original surface morphology somewhat remained after anodization, which meant that the surface was still micro-rough. However, at a nanoscale magnification, the surface was covered with nanotubular structures with increased vertical dimensions (meaning that surface area also increased after anodization). Thus, it should be noted that the major contribution of increased roughness came from those nanotubular structures. These nanofeatures actually mimicked the properties of natural bone. For

example, Palin et al. revealed that the rms surface roughness of porcine femur (uncut side) was about 80 nm with numerous nanostructured features [101].

Besides rms and relative surface area, other parameters such as R_a (arithmetic average of the absolute height of all points), R_t (maximum peak-to-valley height) to describe amplitude, and S_m (arithmetic average spacing) to describe feature spacing are usually used by researchers [102]. If one comprehensively considers a combination of different parameters describing the feature size, height or depth, order and symmetry, the differences between unanodized and anodized titanium surfaces possessing nanotubular structures would be even larger than what the roughness values were reported.

Results of XPS spectra showed that the surface oxide of titanium after anodization was TiO_2 , the predominant oxide on the titanium surfaces. As reported by Zhu. et al. [54], the contamination of carbon on different substrates was similar in each experiment and, thus, the described difference in carbon content between the anodized and unanodized titanium substrates was due to the different oxides rather than to surface contaminations. Carbon content on the surfaces of most titanium implants are about 40 to 80 atomic % [54]. The relatively low carbon content on anodized titanium compared to unanodized titanium in this experiment may be ascribed to the acid-treating in the experiment. Fluorine was likely to be incorporated into the oxide layer during the anodization process.

The nanotubular structures formed during titanium anodization had interesting surface properties in terms of wettability and energy. The as-anodized titanium with nanotubular structures exhibited very low contact angles after short periods of drying (for example, 1 h) in an oven. As shown in Figure 4.3, the contact angle dynamically decreased from around 12° to around 2° in 400 milliseconds. In comparison, it was previously reported that anodized titanium with nanotubular structures had a static contact angle of 58.3° after complete drying. Other studies also reported different contact angles of anodized titanium with nanotubular structures compared to unanodized titanium, for example, $\sim 70^\circ$ (about 15° lower than the original titanium surface) [103], $\sim 58^\circ$ (about 12° lower than the original surface) [13], $\sim 27^\circ$ (about 38° lower than the original titanium surface) [104], and $\sim 7.5^\circ$ (about 62° lower than the original titanium surface) [105]. This discrepancy might be due to the formation of air bubbles in those titania nanotubes (Figure 4.4 a).

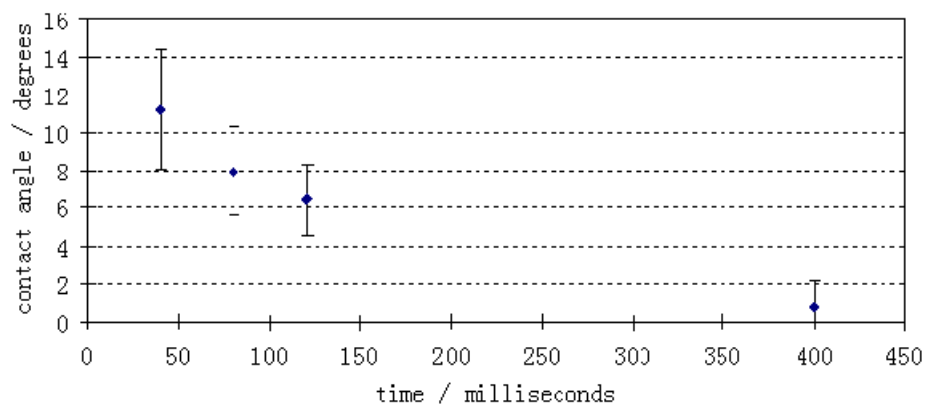


Figure 4.3 Dynamic contact angle measurements of anodized titanium with nanotubular structures after a short period of drying.

A similar structure to the titania nanotubes created in this study is the biomimetic micropatterned surfaces composed of microscopic pillars. They are known to be extremely water repellent (i.e., high contact angles). Researchers ascribed the hydrophobicity to the formation of air pockets within those patterned pillars and found the transition between the Cassie state (composite interface with air pockets trapped under the droplet) and the Wenzel state (complete wetting of the surface) by decreasing the D/P (pillar diameter/spacing) value between 0.083 and 0.111 (see Figure 4.4 b and c) [106, 107]. Compared to this patterned Si system with micron dimensions, the anodized titanium prepared in this study possessed nanometer tube structures which can trap air inside more easily. In this light, when the anodized titanium surface has water remaining inside the nanotubular structures, it can have less air bubbles and is, thus, more wettable; when the remaining water inside anodized titania nanotubular structures is heated, most nanotubes would be filled with air bubbles to prevent wetting of the surface.

The manipulation of titanium surface energy is important in the present study because surface energy of a titanium surface influences both its biocompatibility properties and the drug loading efficiency into anodized titania nanotubes. These issues will be discussed in detail later.

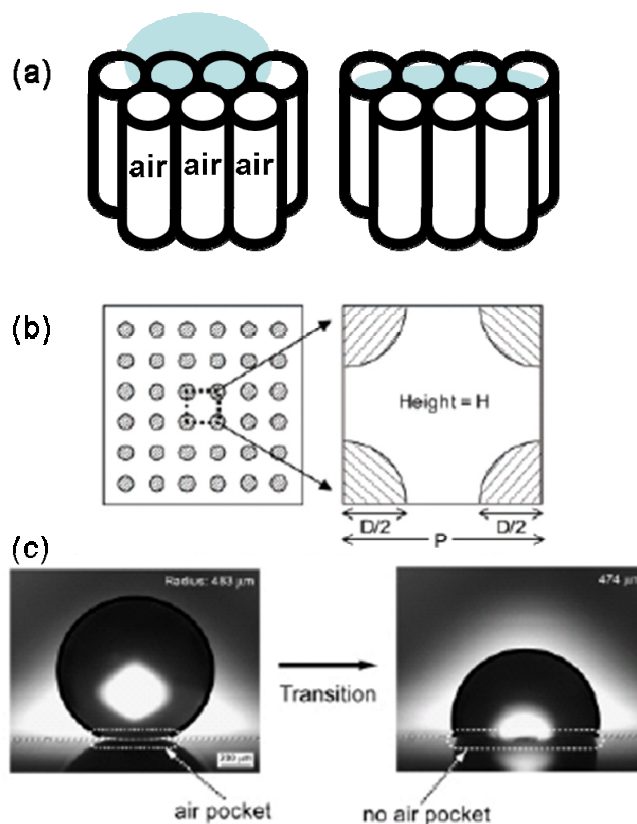


Figure 4.4 Schematic relationship of (a) air pocket formation in anodized nanotubular titania and increased contact angles, (b) a micro-patterned Si system with variable D (pillar diameter) and P (spacing between pillars), and (c) the transition from non-wettable to wettable surfaces by varying the D/P value to around 0.1. (b) and (c) are adapted from [106].

4.1.2. Anodizing CoCr Materials

This study also showed promise for anodizing CoCr for orthopedic applications.

The composition of the CoCr alloy used in the current study was Co-33.4Cr-3.3Mo according to chemical analysis. References reported that Co alloys containing 65% to 67% Co had a mixture of hcp (which is prevalent) and bcc Co phases [108].

The similarity in the resulting CoCr surfaces between fluoride-containing and fluoride-free electrolyte solutions indicated that CoCr alloys are not as sensitive to fluoride-induced chemical dissolution as titanium is. It is not surprising that the titania nanotube structures were not present in the CoCr anodization. On the other hand, in industrial applications, it is safer to use acidic electrolyte solutions like H_2SO_4 instead of HF during CoCr anodization to obtain the same resulting surfaces. The extreme rough structures (more than 15 fold increase in roughness compared to the original CoCr) formed in the present study, were the result of a relatively high applied voltage (10 V). It is clear in Figure 3.24 that the applied voltage is a key parameter for defining surface roughness and irregularity. Under a much more mild voltage (0.15 V) in sodium bicarbonate electrolyte solution used by Gallent et al., a much more smooth surface was obtained compared to that created in the present study [109]. They also reported an oxide thickness of 31 nm after 5 min of anodization. This result is comparable to Figure 3.27 b, in which the newly formed oxide layer was only at the level of tens of nanometers under a higher applied voltage (10 V) but shorter time (2 min). In the present study, the macroscopic color of anodized CoCr was semi-transparent only after a few seconds of anodization (with thinner oxide layers) and then became opaque until 2 min.

The thinness of the oxide layer after CoCr anodization may be explained by the EDS analysis which did not show significant difference in chemistry before and after CoCr anodization. It is likely that EDS did not detect the changes in oxygen

composition because of its detecting limit. According to the mechanism of anodization, it is considered that various forms of oxides (Co- and Cr- containing) are formed on the surface after anodization. CoO was reported as the main Co oxide after anodization, with much less Co_3O_4 in the outside layer [109]. The network of –Cr-O-Cr- bridges in the oxide of CoCr alloys are considered to be the main reason for their passivity [110]. The main form of Cr oxide on CoCr alloys was reported to be Cr_2O_3 [111]. In addition, the formation of CoCr_2O_4 oxide is also possible.

The water contact angle ($\sim 66.9^\circ$) on anodized CoCr is in agreement with other studies showing a value around 66° [112]. It was nearly 30° lower than the unanodized CoCr, and, thus, indicated a better wettability of the CoCr surfaces for proteins and cells attachment.

In summary, anodization is able to create nanotubular oxide structures on Ti / Ti6Al4V and micro/nano rough oxide structures on CoCr alloys. Although not investigated in the present study, another very promising metal in biomedical applications, tantalum, was successfully anodized to possess protective oxide layers [113]. These findings make anodization a very versatile platform for modifying current orthopedic implants to increase osteoblast/chondrocyte functions.

4.2. Increased Osteoblast/Chondrocyte Functions on Anodized

Titanium/Ti6Al4V/CoCr

In the first chapter, in-vitro and in-vivo studies on anodized titanium (ASD) were reviewed. However, since the anodized titanium surfaces (in fluoride-containing electrolytes) possess nanotubular structures instead of microporous structures (in Anodic Spark Deposition), it is interesting to investigate how cells respond on such novel surfaces. The present study answered this question.

As mentioned, more osteoblasts were counted on the anodized titanium substrates, especially those with nanotubular structures, than on the unanodized titanium substrates. Also, this was the first time osteoblast adhesion has been shown to increase on nanotubular compared to nanoparticulate anodized titanium substrates and unanodized titanium substrates. A similar trend was observed for chondrocytes, which indicated a much more favorable surface after anodization. Noting that the nanoscale surface roughness increased from a few nanometers to about 10 to 30 nanometers, nanometer roughness is considered to have a significant positive effect on this greater osteoblast/chondrocyte adhesion since similar crystallinity and chemistry were observed for the titanium substrates. This hypothesis was also suggested by Ejiofor et al. [114], who reported significant cell sensitivity to metal surface roughness values from 5 to 15 nm rms. However, it is hard to directly correlate roughness with the observed osteoblast or chondrocyte adhesion increase in this study. As mentioned before, not only did the overall roughness change but other parameters (like oxide

thickness, feature size, feature spacing and density) may have influenced osteoblast and chondrocyte adhesion [54, 115].

Cell adhesion mainly includes two events: attachment and spreading. Cell spreading is an essential step before the exponential cell growth phase. In the present study, a mixture of rounded and flattened osteoblasts/chondrocytes were found after 4 h of culture on both unanodized and anodized titanium substrates. But generally more well spread cells were observed on anodized titanium possessing nanotubular structures compared with unanodized titanium substrates. This difference in shape was definitely influenced by the morphology and roughness of the anodic oxide layer. In Zhu et al.'s work, porous titanium substrates resulting from anodization increased roughness (R_a) by 0.1 to 0.5 μm [54]. They found more irregular and polygonal osteoblasts on anodized titanium with most round cells on the control [53]. In Huang et al.'s work, they found extended lamellopodia and flattened osteoblast membranes on the anodized titanium with a nano- to submicron- scaled oxide network [56]. Das et al. also observed filopodia extensions coming out from cells to attach to the nanoporous surface of the nanotube for anchorage [105]. This is the same observation in the present study. Hence, some researchers have suggested that cells may be able to recognize nano-features on the surface and their adhesion is to some extent controlled by the size, regularity, and order of the nano-features [49]. Furthermore, studies showed that cells with a rounded shape divide at a lower rate than those well-spread and flat cells [116]. According to what was observed

in the present study, anodization could be helpful to achieve a desirable well-spread cell shape and hence promote the cell proliferation. For osteoblast, this proliferation stage (reproducing the living cells and synthesizing extracellular matrix) usually lasts about 12 days and then transits to differentiation stage [117]. Obviously, the proliferation rate of cells will influence the duration of the proliferation stage and, thus, influence the osteoblast differentiation from non-calcium depositing cells to calcium-depositing cells.

The enhancement of cell adhesion on anodized CoCr may also be partially attributed to the greatly enhanced surface roughness and the creation of many nano-features on the surfaces. The elongated cell morphology revealed in Figure 3.31 is in agreement with previous studies showing that CoCr alloys were less biocompatible than Ti/Ti6Al4V and, thus, supported less filopodia extensions [118].

Not only short term, but promoted long term responses of osteoblasts on anodized titanium were revealed in the present study. Importantly, it was observed that calcium deposition was enhanced on anodized titanium surfaces possessing nanotubular structures when compared to unanodized titanium substrates and anodized titanium substrates possessing nanoparticulate structures. This means that titanium surfaces possessing nanotubular structures were favored by osteoblasts and they may provide for better osteointegration than unanodized titanium surfaces in the long term. This finding was supported

by Popat et al. They did in vitro experiments similar to the present study and observed that the calcium deposition content on titania nanotubular surfaces by osteoblasts after 3 weeks was 50% more than on titanium surfaces [119].

To understand the role of nanofeatures and nanoroughness in mediating cell responses, an independent study conducting on anodized aluminum is worth discussing [47, 120]. Aluminum oxide is a kind of ceramic material usually used in femoral heads and sliding surfaces in contact with polyethylene in orthopedic applications [121]. It is less popular than titanium-based alloys or CoCr alloys due to its low flexion resistance. In this study, a two-step method involving aluminum anodization [98] created a well-ordered nanopore array on an aluminum surface. That study reported that the filopodia of osteoblasts attached to the pores of anodized aluminum and, thus, provided spatial evidence of cell adhesion [47]. They also observed increased osteoblasts proliferation, increased matrix production (higher Ca/Al, P/Al ratio) after 4 weeks of osteoblasts culture on anodized nano-porous alumina (pore diameter about 100 nm) compared to amorphous alumina and aluminum in vitro [120]. Thus, this study demonstrated the effect of nanoarchitectures on osteoblast functionality regardless of chemistry.

4.3. Increased Protein Adsorption on Anodized Titanium

Although there may be many reasons why osteoblasts/chondrocytes prefer anodized over unanodized titanium and Ti6Al4V, this study concentrated on elucidating initial protein adsorption events. Fibronectin and vitronectin are two

major proteins that are involved in osteoblast adhesion [96]. As mentioned before, cells adhere to adsorbed proteins via integrins. Hence, increased fibronectin and vitronectin adsorption on anodized titanium substrates may explain the observed enhanced osteoblast and chondrocyte adhesion on the anodized titanium substrates.

To further explain the mechanism of enhanced protein adsorption, several aspects can be considered. First of all, the changed topography after anodization resulted in increased surface roughness and provided more surface area for the proteins to adsorb. The unique nanotubular structures (inner diameter 70 to 80 nm), which is close to the size of proteins and protein bundles (vitronectin is 15 nm in length [122] and fibronectin is about 130 nm long [123]), may have promoted interactions (like physical interlocking effects) to enhance protein adsorption. As a result, as stated by previous studies, surface topography and roughness may upregulate the adsorption of certain proteins, and, thus, modulate the expression of fibronectin and vitronectin integrin receptors in osteoblasts, their aggregation, and finally determine their cell shape and spreading [124].

Another factor that may play an important role in enhancing cell functions is the wettability or surface energy of the anodized samples. It has been reported that cells prefer to attach on surfaces with higher surface energy [125, 126]. The decreased contact angles on anodized titanium/Ti6Al4V/CoCr in the present

study correlated to increased surface wettability and increased surface energy. Previous studies showed increased fibronectin and laminin adsorption on hydrophilic anodized titania nanotubular surfaces compared to chemically modified hydrophobic anodized titania nanotubular surfaces [127]. Similarly, the anodized titanium substrates possessing nanotubular structures in the present study were 19% higher in total surface energy than the unanodized substrates and they had 18% more vitronectin and 30% more fibronectin adsorption than unanodized titanium.

It is also noted that the polar component of the surface energy increased 84% after anodization. This indicated that anodization could allow more charged titanium surfaces. To determine surface charges, one of the important physico-chemical characteristics of a surface, zeta potential, should be studied. The Zeta (ξ) potential is the electric potential at the interface between a solid surface and a liquid. In the case of the titanium substrates in the present study, the Zeta potential of anodized titanium in culture depends on the thickness of the electrochemical double layer formed at the interface of the titanium oxide layer and the medium. Roessler et al. reported that a natural titanium oxide layer (about 5 nm) and a thicker, anodized amorphous titanium oxide layer (about 150 nm) had a Zeta potential around of -40 mV and -55 mV, respectively [128]. So it is very likely that the anodized titanium surface with nanotubular structures in the present study also had a more negative Zeta potential compared to the unanodized titanium with a thinner natural oxide layer. Such an increased

charged titanium surface is considered to be favored by hydrophilic proteins like vitronectin and fibronectin and, thus, can enhance their adsorption on anodized titanium surfaces.

4.4. Drug Loading into Anodized Titania Nanotubes

In the present study, three methods were used to load antibiotics onto/into anodized titania nanotubular structures. They all have advantages and disadvantages, which will be discussed in this and the following sections.

4.4.1. Drug Physical Adsorption

The first factor when considering physical adsorption is the wettability of the substrates, which can be quantitatively characterized by contact angles. This contact angle value can be calculated using surface tension values according to the Young's equation:

$$\gamma_{SG} = \gamma_{SL} + \gamma_{LG} \times \cos(\theta) \quad (4.1)$$

where γ_{ij} is surface tension between two different phases (s-solid, g-gas, l-liquid) and θ is the contact angle. This equilibrium at the interface of a solid, liquid, and gas is illustrated in Figure 4.5 (a).

Since the anodized titanium with nanotubular structures are not ideal flat surfaces, a modified model is illustrated in Figure 4.5 (b) and (c). In this illustration, the surface tension of the inner nanotube walls plays an important role in the Young's equation. When it is not large enough, to maintain the force

equilibrium, the contact angle will be larger than 90° and lead to non-wetting of the nanotubular structures (Figure 4.5 b). In contrast, when it is large enough, the contact angle will be less than 90° and lead to wetting of the nanotubular structures (Figure 4.5 c). Also considering the air bubble formation in the nanotubular structures, a very hydrophilic surface (higher surface tension) is probably required to wet the titania nanotubular structures.

To alter the surface tension of anodized titania nanotubes, more exactly, the surface tension of the upper nanotube inner wall surfaces, surface chemical modification was carried out in the present study. First, a Piranha solution, (a mixture of sulfuric acid and hydrogen peroxide), was used to hydroxylate the surface (add $-OH$ groups). The proton in the Piranha solution can break and attack the Ti-O bond in the natural oxide layer and eventually form Ti-OH bonds. This created a very hydrophilic surface according to contact angle and surface energy measurements.

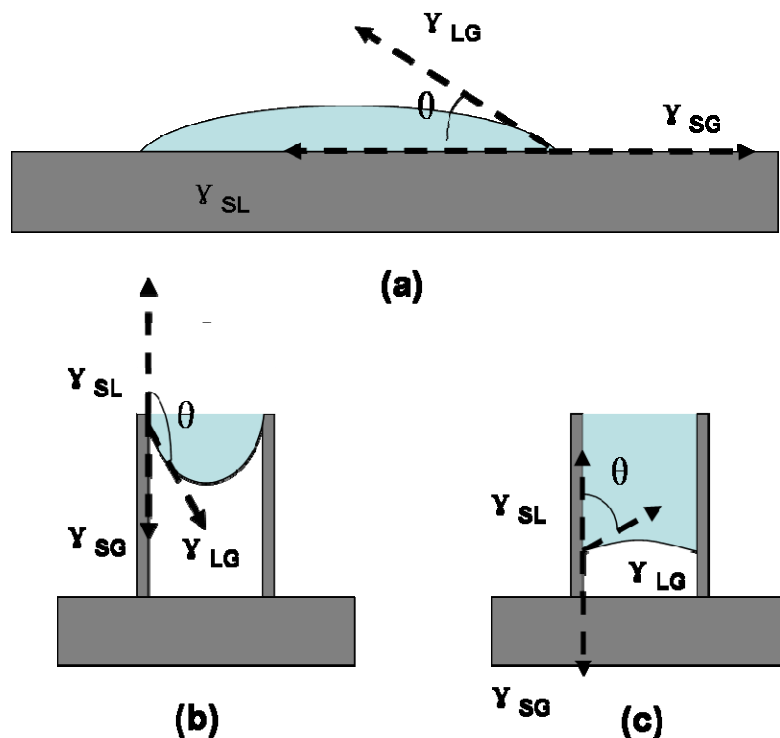


Figure 4.5 Illustrations of (a) a water contact angle on a flat surface, (b) non-wetting situation of anodized titanium with nanotubular structures, and (c) wetting situation of anodized titanium with nanotubular structures. S, L, G stand for solid, liquid, and gas, respectively. γ_{ij} is surface tension between two different phases and θ is the contact angle.

To study the role of surface wettability for drug loading, a gradient of surface wettability was created. This was completed by terminating the titanium surface with different functional groups other than $-\text{OH}$. The silanization reaction was used to introduce amine groups onto anodized titanium (Figure 4.6). CBQCA assay confirmed the efficiency of this reaction and the resulting surface had a contact angle around 30° , which is comparable to other studies [92]. A further reaction introduced methyl groups onto anodized titanium and led to a surface contact angle around 40° . The CBQCA assay showed an incomplete transformation of amine groups to methyl groups, indicating that more

hydrophobic surfaces can be created for methyl terminated surfaces [92]. However, this was acceptable for the current study because a gradient of wettability was still created to be compared with each other. Among the two antibiotics used in the present study, P-G is more hydrophobic due to the aromatic side chain in the structure while P/S is more hydrophilic. Their interactions with those functionalized titania nanotubes should be different and are expected to influence their release behaviors.

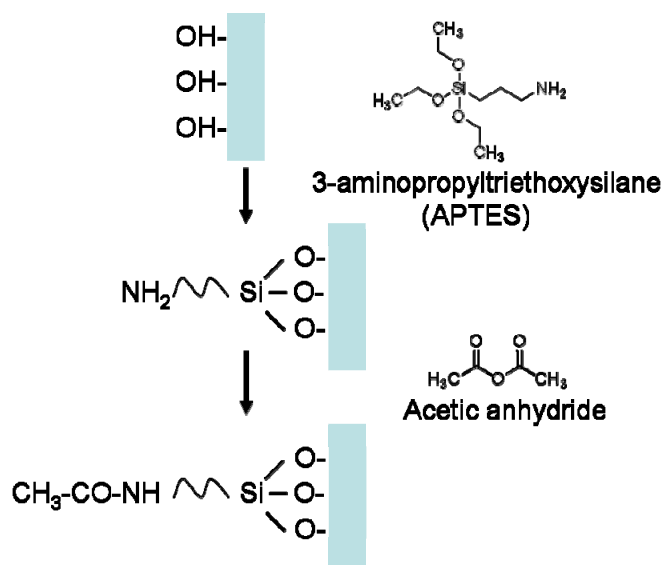


Figure 4.6 Chemical modification of anodized titanium.

For all the chemical modifications, XPS analysis found traces of the appropriate chemicals 20 nm down from the outermost surface, indicating that the upper portion of the nanotubes had been modified. To minimize the influence of bubble formation, the loading process was completed under vacuum conditions, which

may allow the air bubbles to escape from the nanotubes. SEM characterization confirmed the expected that the –OH terminated anodized titanium had the best filling of drugs into the nanotubular structures whereas more hydrophobic surfaces had unfilled nanotubes. Because the nanotubes were thin (less than 200 nm), drugs usually filled the nanotubes once the drug solution entered the nanotubes.

4.4.2. Drug Electrodeposition

The second method used to load drugs into the anodized titania nanotubular structures was electrodeposition. One of the major advantages of this process is quickness. It may take minutes to coat one sample using electrodeposition compared to hours for the adsorption method.

The P/S and P-G solutions in 0.045% NaCl were weakly acidic (pH 4.58 and 5.75, respectively), indicating they are not fully ionized in the water solution. During electrodeposition, the following reactions occur at titanium electrode:



The OH^- product at the interface of the electrode/solution plays a vital role in the penicillin precipitation on the anodized titanium cathode. It is proposed that a rise of the local pH decreased the solubility of penicillin and led to the precipitation. In

a previous study including collagen in the electrolyte, a bluish layer (collagen coatings) was observed on the electrode after 30 seconds [129].

Characterization of the antibiotics loaded into anodized titania nanotubes using an electrodeposition method showed similarity in surface morphology among the substrates with different functional groups. This observation indicated that the applied voltage and the electrochemical reactions occurring on the electrode were the major factors for controlling drug deposition whereas the influence of surface chemistry decreased compared to the adsorption method. In other words, the electrodeposition method promoted drug loading efficiency when the surface properties were not optimized (i.e., lower surface wettability) when compared to the adsorption method.

4.4.3. Drug Co-precipitation

Anodized titanium is known for its apatite formation ability in SBF [30, 52, 74, 130]. To induce calcium phosphate mineral precipitation in SBF, two different pretreatments were completed with anodized titanium. The first treatment was soaking anodized titanium with nanotubes in NaOH to form sodium titanate and was reported to help accelerating HA formation in SBF as well as in animals [131, 132]. It was observed here that very fine-scale fiber-like structures formed on the top of anodized titania nanotubes during this process (Figure 3.41); this was reported to be around 8 nm in a previous study using similar conditions [74]. These sodium titanate nanofibers nucleated and grew on the edges (walls)

of titania nanotubes, thus, the size and shape of such sodium titanate crystals were physically constrained by the underlying nanostructures, most likely less than or in the same level of the nanotube wall thickness (around 15 nm). The surface-tension-related difficulty and the existence of air bubbles inside the nanotubes may explain why sodium titanate nanofibers mainly formed on the top of nanotubes (Figure 4.7 a). The second pretreatment, that is heating the anodized titanium at 500°C for 2 hours to obtain anatase titania, was also reported to be helpful for HA nucleation and growth in SBF [74]. This occurred due to a better lattice structure matching between anatase titania and HA than amorphous or rutile titania [133]. Numerous studies have pointed out that annealing anodized titanium between 450°C to 500°C can transform the amorphous titania to anatase titania while temperatures above 600°C will start to form rutile titania and lead to collapse of some nanotubes [74, 134, 135]. The transformation from amorphous titania to anatase titania is also important in terms of cell attachment and growth. Some studies have examined bone cell functions on different crystalline forms of titania and found that anatase titania promoted bone cell attachment, proliferation, and differentiation [12, 13].

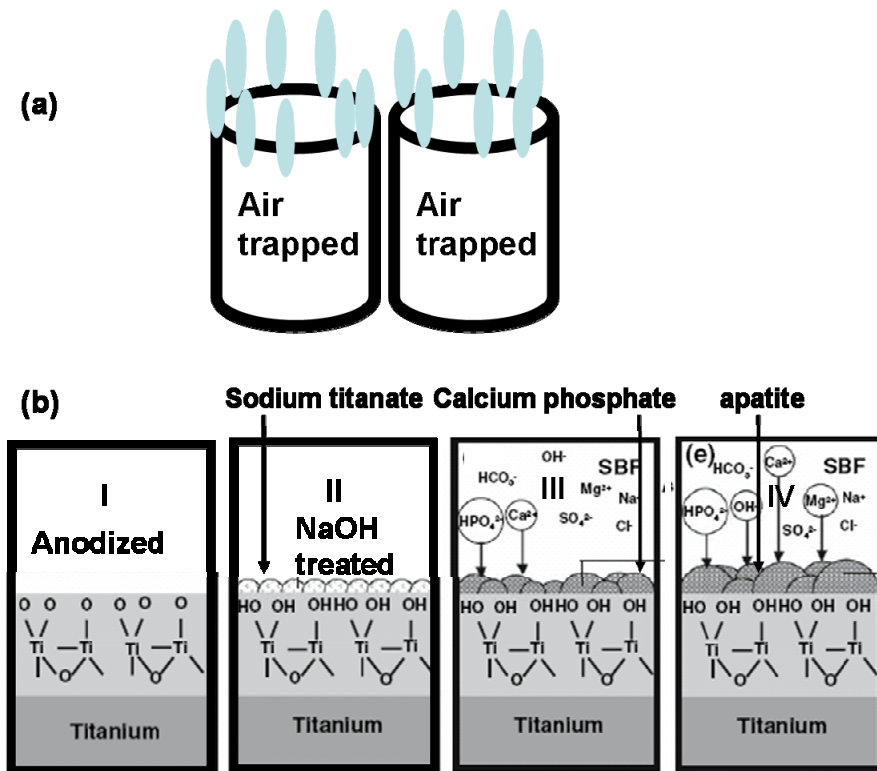


Figure 4.7 Illustration of (a) nanofiber like sodium titanate formation on the top of anodized titania nanotubes, and (b) the process of apatite formation in SBF (adapted and redrawn from [136]).

A 1.5 X SBF solution was used in the present study to accelerate the calcium phosphate mineral precipitation process. It was found that after 3 days, calcium phosphate minerals were deposited all over the anodized titanium surface (confirmed by chemical analysis). This phenomenon may be explained by the following model. As the ion exchanges between the Na^+ in the sodium titanate and Ca^{2+} in the SBF solution occurs, positive charges accumulate on the surface [137]. Then, negatively charged phosphate ions in the SBF solution are attracted to form amorphous calcium phosphate. Finally, the metastable amorphous

calcium phosphate transforms to crystalline apatite (Figure 4.7 b) [138]. Because the calcium phosphate minerals are nucleated and grown from the nanofiber-like sodium titanate, most of the resulting minerals had similar shapes to their precursors as shown in Figure 3.41. Of course, when penicillin was mixed in the SBF solution, these drug molecules may be incorporated into the mineral coating via molecular interactions. Thus, the co-precipitation method used in the present study would have two advantages compared to the adsorption method. First, the drug release from the co-precipitated coatings may be prolonged due to indirect contact with the buffer solution. Second, calcium phosphate or HA is well known for its better compatibility compared to titanium because it is the major inorganic component of natural bone. Li et al. reported that calcium phosphate deposited onto micro-arc anodized titanium by electron beam evaporation could transfer to apatite in a saline buffer solution and such coatings enhanced the osseointegration of titanium implants in vivo [139]. Bose et al. reported that bioactive tricalcium phosphate deposited on anodized titania nanotubes by laser techniques enhanced osteoconductivity [140]. Oh et al. reported increased osteoblast adhesion on HA coated anodized titania nanotubes (~400% after 48 h of culture) compared to the titanium control. It is considered that the co-precipitation method can provide a coating that releases antibiotic drugs and promotes bone cell functions simultaneously.

4.5. Drug Release Behavior from Anodized Titania Nanotubes

The drug release data obtained were plotted as the percent of release versus time to demonstrate release kinetics. When the antibiotics were loaded onto various anodized titanium and then exposed to PBS, an immediate drug release system was observed since there was no membrane or coating that could delay or prevent the dissolution of drugs into the buffer solution. In other words, it was a quick dissolution-controlled system. As shown in Figure 4.8 when the physical adsorption method was used, about 75 to 85% of the release occurred during the first hour, about 90 to 95% of the release occurred after two hours, and the release saturated after two days. The release kinetics of the two drugs, P/S and P-G, were similar. As shown in Figure 4.9 when the electrodeposition method was used, about 65 to 70% of the release occurred during the first hour, about 75 to 85% after two hours, about 95% after 1 day, and finally the release saturated after two days. Again, the P/S and P-G had similar release behavior to each other. These results are comparable to another study which showed that the antibiotic gentamicin physically adsorbed onto the anodized titanium (in the amount of 100 to 300 μg) usually had 100% of release after 1 to 3 h [119].

It is considered that the drug molecules may have different ionic or physical interactions with the titania nanotube inner surfaces with different functional groups. In a study of drug loaded magnetic nanotubes (MNT), the inner surfaces of the MNT were functionalized with APTS, i.e., terminated with amine groups [141]. Three different drug molecules, 5-fluorouracil, 4-nitrophenol, and ibuprofen,

loaded into the MNT had different release kinetics. Specifically, 10% of the ibuprofen was released in 1 h and 80% was released after 24 h, whereas more than 90% of 5-fluorouracil and 4-nitrophenol were released after 1 h. This observation was explained due to more ionization of ibuprofen in PBS (with negative charges) and their stronger Coulomb interactions with the positively-charged amine groups on the MNT inner surfaces compared to the other two drugs [141]. However, there was no significant difference in release kinetics among the four anodized titanium substrates of different surface chemistries in the present study. This could possibly be due to several reasons. First, the titania nanotubular structures formed in the present study had small diameters and depths, and, thus, their capacity of storing drugs was limited. So the release from different substrates may be too quick to be differentiated within one hour. Second, because the chemical modification was conducted mainly on the upper portion of the nanotubular structures (around 20 nm), the interactions between drug molecules and the nanotube inner surface may not be significant enough to influence the release kinetics in the current experiment. Nevertheless, this issue needs to be further investigated in a future study to better control drug release.

Although only antibiotics were used in the present study, such nanotube functionalization could also work for other important drug candidates such as dexamethasone (anti-inflammation) and knuckle region of BMP-2 protein (growth factor). Dexamethasone is hydrophobic in nature. A relatively hydrophobic nanotube wall surface would lead to increased interaction between

dexamethasone molecules and the nanotubes. For BMP-2 short peptide (KIPKASSVPTELSAISTLYL), because of the existence of acidic amino acids (K: lysine, E: glutamic acid) and polar amino acids (S: syrine, T: threonine), hydrophilic nanotube walls would have greater interactions with this peptide sequence.

For the release of P/S from the co-precipitation method, a prolonged release up to 3 weeks was observed (Figure 4.10). This was the longest release observed in this study. There was an initial burst after 1 h, probably due to the release of drugs attached on the outer surfaces of minerals (phase I). Then, there was nearly no drug released in the second hour. This could be a period when drug molecules diffused out of the mineral matrix. In the following week, a steady release of drugs was detected (phase II). The release of P/S slowed down after one week and saturated after 3 weeks (phase III). Regardless of the initial and ending regions in Figure 4.10, the steady release in phase II is quite interesting. In order to describe the release kinetics of the P/S co-precipitated with calcium phosphate mineral, various equations were used [142]. For example, the zero-order rate equation describes systems where the release rate is independent of the concentration of the dissolved species. The first-order equation describes the release from systems where dissolution rate is dependent on the concentration of the dissolving species. The Higuchi square root equation describes the release from systems where the solid drug is dispersed in an insoluble matrix.

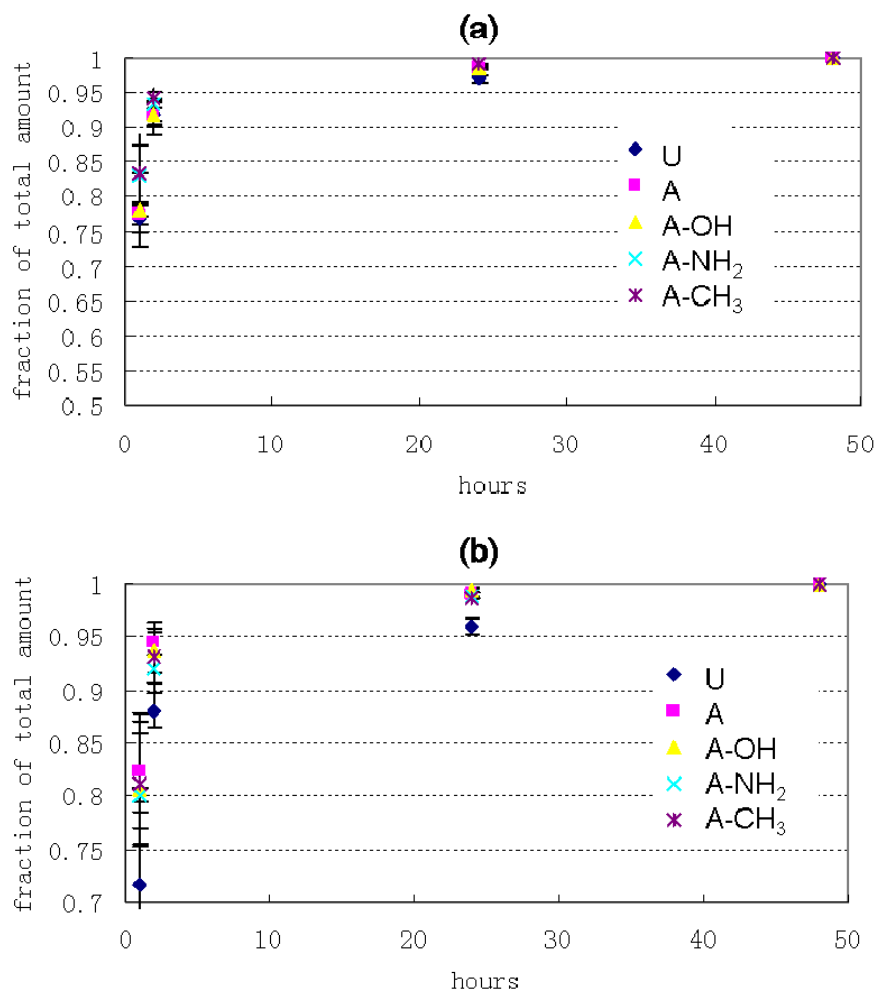


Figure 4.8 Release kinetics of (a) P/S and (b) P-G that physically adsorbed onto anodized titanium with nanotubular structures.

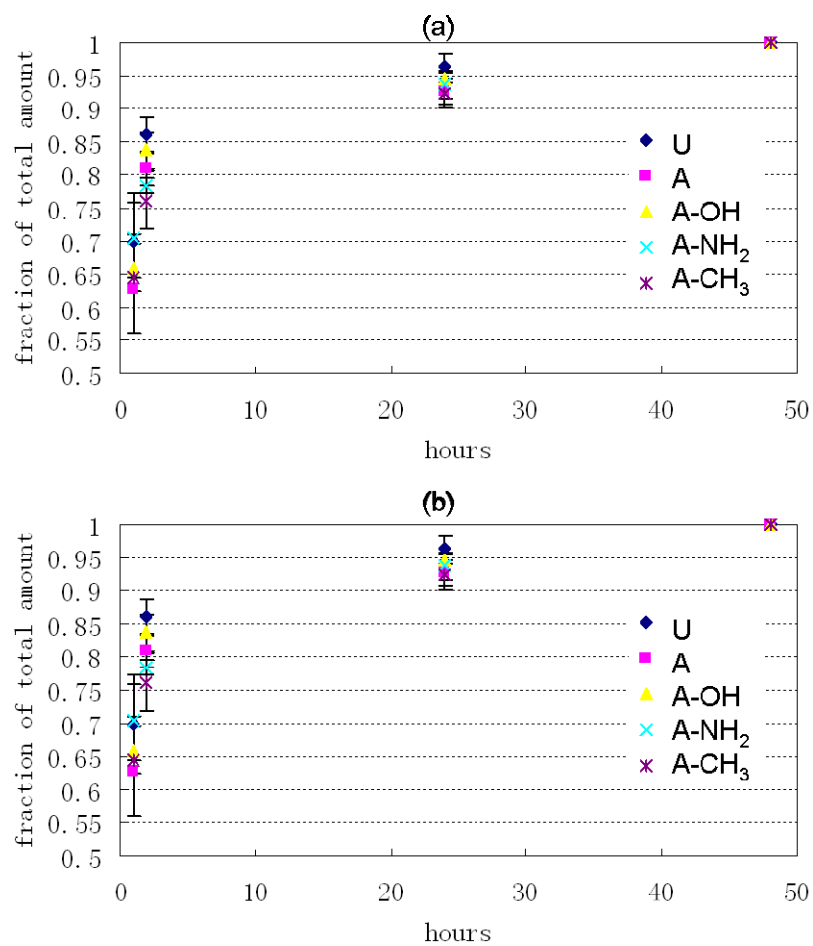


Figure 4.9 Release kinetics of (a) P/S and (b) P-G that electrodeposited onto anodized titanium with nanotubular structures.

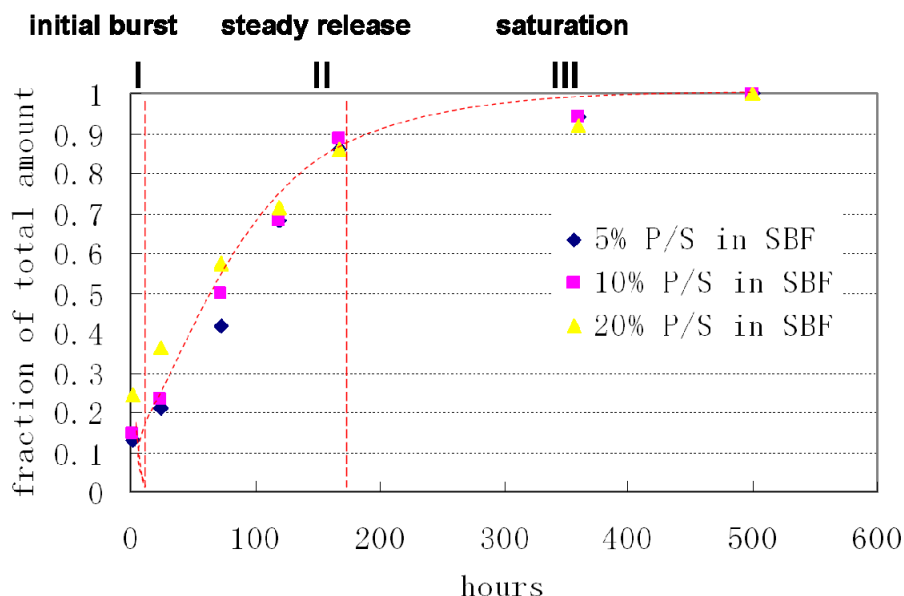


Figure 4.10 Three phase release kinetics of co-precipitated P/S in PBS for up to 3 weeks.

The dissolution data were plotted and compared with the theoretical zero-order equation, i.e., percent released as a function of time (Figure 4.11 a). It is evident that the plots are curvilinear and the release kinetics not zero-order in nature.

When the dissolution data were plotted and compared with the theoretical Higuchi's approach, i.e, percent released as a function of the square root of time, it is evident that the plots (between 2 h to 168 h) were close to the theoretical curve but deviated afterwards (Figure 4.11 b). This indicates that the Higuchi's model may not satisfactorily explain the release kinetics in the present study.

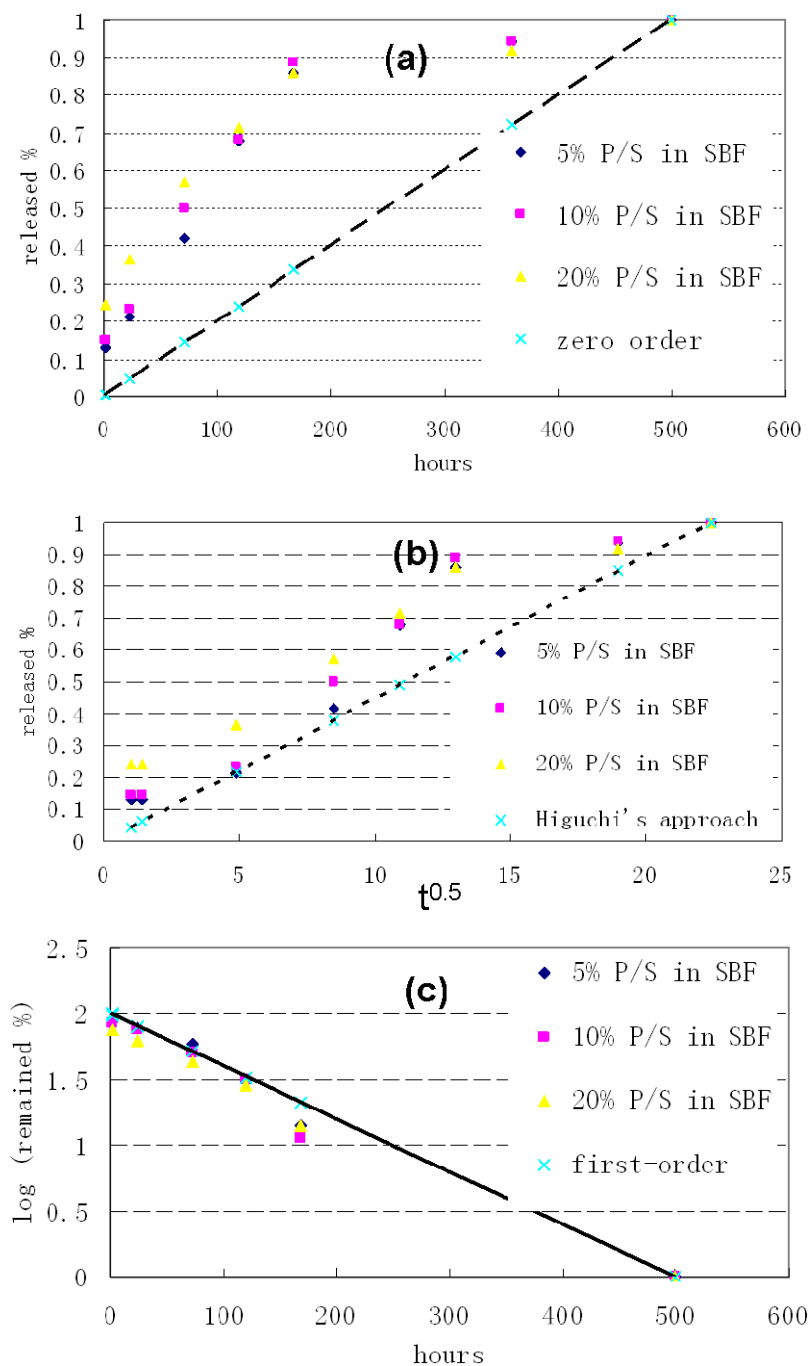


Figure 4.11 Comparison of the co-precipitated P/S release kinetics with (a) zero order kinetics, (b) Higuchi's approach kinetics, and (c) first order kinetics.

Finally, when the dissolution data were plotted and compared with the theoretical first-order equation (i.e., the logarithm of the percent of drug that remained as a function of time), it is evident that the experimental data fits the theoretical curve the best (Figure 4.11 c). This suggests that the amount of drug released is dependent on the matrix drug loaded.

Conventional antibiotic prescription usually lasts for 4 to 8 weeks and involves high dose. However, since local drug delivery is more efficient than other methods (such as oral), such antibiotic drug release model up to 3 weeks will be proper to fight bone infection. Of course, the loaded drug amount needs to be increased. This will be one of the future directions of the present study.

4.6. Biological Properties of Drug-loaded Anodized Titanium

The major purpose of loading antibiotics onto anodized titanium implant is to fight infection. The present study showed inhibited *S. epidermidis* attachment on P/S and P-G loaded anodized titanium surfaces using the physical adsorption method. Furthermore, such antibiotics loaded onto anodized titanium did not inhibit bone cell adhesion.

4.6.1. Anti-bacteria Properties of Drug-loaded Anodized Titanium

S. epidermidis is a member of Gram-positive bacteria, which means they are stained dark blue or violet by Gram staining. Gram-positive organisms are able to retain the violet stain because of the high amount of peptidoglycan in their cell

wall. Gram-positive cell walls typically lack the outer membrane found in Gram-negative bacteria. Penicillin and penicillin G are usually used to treat Gram-positive organism. The mechanism by which penicillin works to kill bacteria involves first binding of penicillin to the inner surface of the bacteria cell membrane and then interference with production of cell wall peptidoglycans and subsequent lysis of the cell. In the present study, these two antibiotics loaded on anodized titanium using a physical adsorption method inhibited *S. epidermidis* attachment 50% more than the drug-unloaded anodized titanium.

4.6.2. Osteoblast Attachment on Drug-loaded Anodized Titanium

The same substrates of interest tested in bacteria experiment were also tested for osteoblast adhesion. The main conclusion from the present study was that antibiotics loaded onto anodized titanium at a level of a few hundred micrograms did not impair osteoblast adhesion. In fact, osteoblast adhesion on all P/S loaded surfaces seems a little higher than those on non-P/S-loaded surfaces. The reason is not clear yet but there could be some possible explanations. The molecular structure of penicillin includes residue amino groups (-COOH, CO-NH). This similarity in chemical structures between penicillin and serum proteins composed of various amino acids may allow penicillin to have some degree of affinity with specific proteins. Thus, the pre-loaded P/S onto anodized titanium may be helpful for protein adsorption and subsequent cell attachment. However, as the release of P/S ends, no significant influence from the loaded P/S is expected on osteoblast responses.

Antibiotic loaded substrates using the co-precipitation method were also tested for osteoblast attachment in the present study. In the present study, the calcium phosphate coated anodized titanium showed significantly increased osteoblast attachment than anodized titanium and unanodized titanium. When P/S is co-precipitated with the mineral, there was no significant influence on osteoblast attachment, probably due to its prolonged and relatively low release compared to that during the physical adsorption method.

4.7. Future Directions

The study of anodized titanium, Ti6Al4V, and CoCr is still in its infancy and a lot more investigation is needed to optimize the use of anodization for orthopedic applications (as shown in Figure 4.12). Here, some of these future studies are listed:

1. It is important to optimize the properties of anodized titania nanotubular structures, including inner diameter, depth, etc. This is meaningful in terms of biocompatibility of the anodized materials, because the size and patterning of nanofeatures may direct cell fate [49, 100]. In vitro studies on roles of these parameters are definitely needed for future studies. Specifically, influence of the nanotube diameters (from about 30 to 100 nm that can be created in the present study) and depth (from a few hundred nanometers in the present study to a few micrometers [69] and a few hundred micrometers [143]) on osteoblast and chondrocyte functions will be studied. This is also critical in the

- design of drug delivery systems. As mentioned in the results, the release of drugs adsorbed onto/into anodized titania nanotubes was relatively short. It will be beneficial to increase the diameter and depth of the nanotubes for more drug loading capacity and longer drug release given these parameters are still appropriate for cells.
2. Carbon nanotubes can be grown from such anodized titania nanotube templates and used for sensor applications [144]. These carbon nanotubes combining with a conductive polymer layer (polypyrrole and poly-lactic-co-glycolic acid) can be used to apply and measure conductivity changes as new bone grows next to the implant [145].
 3. As stated earlier in this thesis, the inflammatory response is critical for bone and cartilage implants. Previously studies demonstrated that anodized titanium (microporous, no titania nanotubular structures) with or without Mg ions had a inflammatory response similar to tuned and blasted titanium [146]. However, there is a lack of information on inflammatory responses on anodized metal implants with nanotubular or nanoporous structures. Thus, it is necessary to carry our in vitro studies to see if such bone and cartilage cell favored anodized titanium surfaces lead to increased inflammatory cell (such as macrophages) adherence and, thus, excessive inflammatory responses.

4. Animal studies to evaluate the osseointegration of anodized titanium, Ti6Al4V implants with nanotubular structures or CoCr implants with nano-rough structures is a priority in the list since they are able to show direct evidence of new bone growth in vivo. To do this, a rabbit model can be used [81].
- Generally, defects will be carefully created in the femur and tibia of each rabbit by surgical drilling. Screw-shaped implants made of the above metals (both unanodized and anodized) will be inserted in the holes created in the femur and tibia. After 4 and 8 weeks, the rabbits will be sacrificed. Removal torque tests can be completed to investigate shear strength between the implants and bone; histological stains will be used to quantify the bone to metal contact area. The chondrogenesis of anodized metal implants could be studied in a similar way.

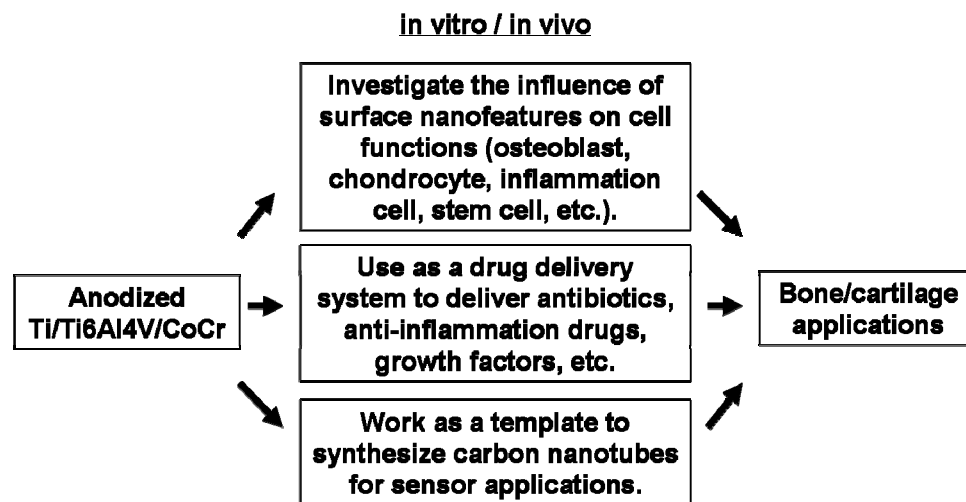


Figure 4.12 Investigations of anodized titanium, titanium alloys, and CoCr alloys for bone and cartilage applications.

5. The drug loading methods used in the present study can be improved to achieve better controlled (i.e., more sustained) release (Figure 4.13 curve a and b). For example, pretreatment can include hot water boiling or sonication to remove the air bubbles trapped in the anodized titania nanotubes. In this way, it would allow complete modification of the nanotube inner surfaces. In addition, a new drug loading method that combines electrodeposition and co-precipitation can be used in the future. In this method, an electrolyte containing both drug molecules, calcium and phosphate ions will be used in an electrochemical cell and simultaneous precipitation of drug / calcium phosphate can be achieved (Figure 4.14). The resulting coating would be similar to that using the co-precipitation method in the present study but it requires much less time and can have better control on the coating thickness and other properties.

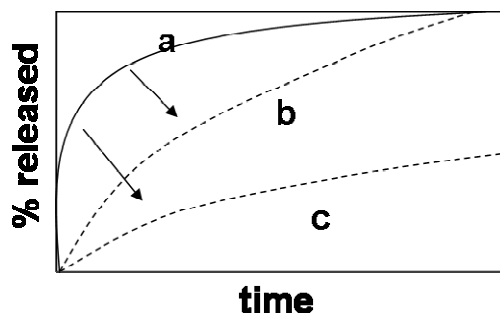


Figure 4.13 Desired drug release profile: (a) a release profile in the present study, (b) a slowed release profile that is suitable for short-term drug delivering (such as antibiotics), and (c) a much prolonged release profile that is suitable for long-term drug delivery (such as anti-inflammation drugs and bone growth factors).

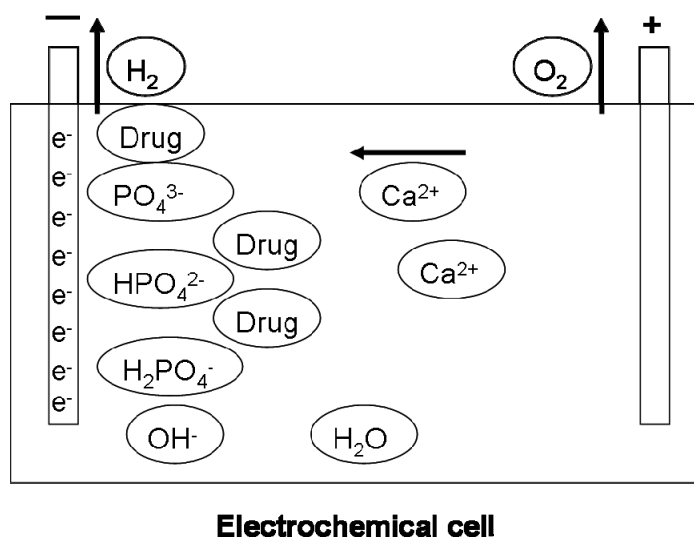


Figure 4.14 Schematic of an electrochemical cell that could co-precipitate calcium phosphate and drug molecules onto anodized titanium used as a cathode. The acid-base reactions near the cathode transform H_2PO_4^- ions to HPO_4^{2-} and then PO_4^{3-} . These products finally react with Ca^{2+} and forms CaP precipitations. Drug molecules could be co-precipitated via molecular interactions with the CaP mineral.

6. Drugs other than antibiotics can be used to increase the efficacy of implants.

Penicillin-based antibiotics used as model drugs in the present study have shown the capability of this anodized titanium drug delivery system. As the next step, drugs like dexamethasone (anti-inflammation drug) or peptide sequence of BMP-2 (growth factor) can be loaded into such anodized titanium substrates. For these applications, a much prolonged release behavior up to several weeks and months is needed (shown in Figure 4.13 curve c).

According to the result of the present study, co-precipitation with minerals would be a better way to achieve this goal. To increase the release period, the composition of the precipitation solution (i.e., the ratio of the drug solution to the Ca/P containing solution), the precipitation time, and the thickness and

crystallinity of the precipitated coating can be optimized. Their corresponding functions on either immune cells or bone cells should be studied in vitro.

Furthermore, multiple drug-loading implants should be tested (e.g., anti-infection and anti-inflammatory) to better support bone tissue growth.

7. Last but not least, it is anticipated in the long run that the nanotubes loaded with drugs which act on specific internal cellular processes can be deliberately managed to release drugs locally to the site of need.

CHAPTER 5. CONCLUSIONS

The present study made the following contributions to the design of better orthopedic implants:

- I. An electrochemical surface modification method, anodization, was developed to create unique biologically-inspired nano-surface-features on currently-existing orthopedic implant materials. Specifically, titania nanotubular structures were formed on anodized titanium and Ti6Al4V and micro- and nano- rough oxide structures were formed on CoCr alloys.
- II. Characterization of anodized titanium, Ti6Al4V, and CoCr revealed increased surface roughness, increased surface wettability, and increased surface energy after anodization, which were shown to be critical factors to mediate initial protein adsorption and subsequent cell (osteoblast and chondrocyte) responses.
- III. A complete in vitro study with bone-forming cells (osteoblasts) and cartilage-forming cell (chondrocytes) was carried out and results demonstrated enhanced osteoblast and chondrocyte responses on anodized titanium, Ti6Al4V, and CoCr compared to their unanodized counterparts for the first time.

- IV. Enhanced cell responses were determined to be due to increased vitronectin and fibronectin (cell-adhesion-mediating proteins) adsorption on anodized material surfaces compared to unanodized surfaces. This observation was further attributed to factors such as increased nano-roughness and increased surface energy after anodization.
- V. The anodized titanium with nanotubular structures were successfully used for prolonged drug delivery applications. Different loading methods including physical adsorption, electrodeposition, and co-precipitation were used in the present study to load penicillin-based antibiotics into anodized titania nanotubes. The present study showed the ability to control the release amount as well as the release kinetics using these different methods.
- VI. Functionalization of anodized titanium with nanotubular structures provided a way for drug immobilization. Different functional groups (such as hydroxyl, amine, and methyl groups) on the nanotube inner walls could have different ionic or molecular interactions with drug molecules which have specific properties such as charge (positive or negative) and hydrophobicity (hydrophobic or hydrophilic). Such interactions would influence the drug release behaviors.
- VII. Antibiotic-loaded anodized titanium surfaces inhibited *S. epidermidis* (one of the main bacteria causing orthopedic implant infection) attachment but did not influence bone cell attachment. Furthermore, the anodized titanium

co-precipitated with antibiotics and calcium phosphate showed increased osteoblast adhesion.

- VIII. Some of the present study was done starting with currently-used implants (Depuy & Arthrosurface). In this light, this in vitro work acted as a part of the invention process for improved orthopedic implants, which is beneficial for the patients who are in need of them.

In summary, anodization was found to be a quick and inexpensive technique to modify currently-used orthopedic implants to improve their cytocompatibility.

Anodized titanium possessing nanotubular structures also provides an innovative metallic-implant-based local drug delivery system to further ensure the efficacy of orthopedic implants.

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