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REVIEW ARTICLE

Anodization of titanium alloys for orthopedic applications

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Abstract In recent years, nanostructured oxide films on titanium alloy surfaces have gained significant interest due to their electrical, catalytic and biological properties. In literature, there is variety of different approaches to fabricate nanostructured oxide films. Among these methods, anodization technique, which allows fine-tuning of oxide film thickness, feature size, topography and chemistry, is one of the most popular approaches to fabricate nanostructured oxide films on titanium alloys, and it has been widely investigated for orthopedic applications. Briefly, anodization is the growth of a controlled oxide film on a metallic component attached to the anode of an electrochemical cell. This review provides an overview of the anodization technique to grow nanostructured oxide films on titanium and titanium alloys and summarizes the interactions between anodized titanium alloy surfaces with cells in terms of cellular adhesion, proliferation and differentiation. It will start with summarizing the mechanism of nanofeatured oxide fabrication on titanium alloys and then switch its focus on the latest findings for anodization of titanium alloys, including the use of fluoride free electrolytes and anodization of 3D titanium foams. The review will also highlight areas requiring further research to successfully translate anodized titanium alloys to clinics for orthopedic applications.

Keywords titanium alloys, anodization, biocompatibility, orthopedics

1 Introduction

The value of the global orthopedic implant market was USD 4.3 billion in 2015 and it is estimated to maintain a growth rate of 3% until 2020 [1]. Future projections indicate that in the US alone, the number of total hip replacements will grow by 174% (572000 procedures) and the number of total knee arthroplasties will grow by 673% (3.48 million procedures) by the end of 2030 [2]. The constantly rising geriatric population has primarily been driving the growth of orthopedic market since people aged above 65 years are at a high risk of developing orthopedic complications and about one out of every four 65-year-old person today is estimated to live past age 90 [3]. For the case of total hip arthroplasty, considering the lifetime of a hip implant to be 15–20 years, most people having a hip replacement surgery require at least one revision surgery; in fact multiple surgeries might be needed for younger and more active patients [4,5]. Clearly, implants with prolonged lifetime are required in orthopedic applications.

Currently, titanium and its alloys are widely used for fabrication of implants to replace hard tissue in orthopedics. Though orthopedic implants made of titanium alloys have been used in clinics for many years, limited lifetime of these implants has been a major issue, as stated in the previous paragraph. Integration of the implant with the juxtaposed bone tissue (osseointegration) has been one of the leading reasons for orthopedic implant failure. In fact, the data for hip replacement revision surgeries performed in the United States in November, 2017 showed that implant failure rate due to osseointegration reached more than 25% [6]. Better integration of orthopedic implants with the surrounding bone tissue would improve the implant stability and its lifetime; reducing the number of revision surgeries. To promote osseointegration of titanium based orthopedic implants, anodization method received specific attention due to its versatility in creating nanophase topographies by tailoring the electrochemical parameters in the process. For instance, Zwilling et al. [7] successfully fabricated self-organized array of nanotubes on titanium upon anodization. In the following years, scientists observed improved bone cell functions *in vitro* and *in vivo* on these nanofeatured titanium oxide film; making anodized titanium alloys one of the promising materials in the orthopedic research field. Before providing an in-depth description of the anodization process and chemical mechanism of titanium anodization, the next

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section will briefly discuss the use of titanium and its alloys in the orthopedics field.

2 Why titanium and its alloys are used for orthopedic applications?

Titanium exhibits hexagonal close packed crystal structure at room temperature (HCP, α-phase). As temperature of α-phase titanium (hexagonal close packed structure, HCP) increases, it transforms to β-phase (body centered cubic structure, BCC,) at beta transus (882.5°C) and β-phase titanium melts at 1678°C [8]. Titanium has low density, high biocompatibility and corrosion resistance due to the naturally forming oxide layer on its surface, making it the material of choice in orthopedic applications. However, bioinert nature of titanium, along with its suboptimal mechanical properties limit the lifetime of titanium based implants to 15–20 years [5]. To remedy these issues, titanium alloys have emerged as alternative implant materials. Titanium alloys can be categorized as α, near-α, $\alpha+\beta$, metastable β and stable β depending on their room temperature microstructures. Alloying elements incorporated into titanium are classified as three parts: (i) α-phase stabilizers (i.e., Al, O, N and C), (ii) β-phase stabilizers (i.e., Mo, V, Nb, Ta, Fe, W, Cr, Si, Ni, Co, Mn and H), and (iii) neutral alloying elements (i.e., Zr). α and near-α titanium alloys show superior corrosion resistance, yet the ability to control their mechanical properties is limited due to their non-heat treatable nature to preserve α phase microstructure. On the other hand, β titanium alloys can be shaped even at lower temperatures because of its BCC crystal structure, making them ideal for complex geometries. Coupling the benefits of having both phases, $\alpha+\beta$ titanium alloys exhibit higher tensile strength, fracture toughness, wear resistance and their heat treatable nature allows fabrication of complex geometries making them ideal for orthopedic implants [9,10].

Among titanium alloys, Ti6Al4V based orthopedic implants have extensively been utilized to replace bone tissue in hip and knee replacements and bone fixation devices. Ti6Al4V has high strength and ductility and low density. In fact, strength of Ti6Al4V alloy can be increased with an increase in β stabilizer content, which further improves its fatigue limit. However, the major concern of using Ti6Al4V alloy in clinics is the presence of vanadium in its chemistry, which can potentially increase the expressions of pro-inflammatory factors and cause osteolysis, exhibiting toxic effect in the body [11]. Studies on biological response of metallic elements demonstrate that chemical composition of alloys used in biomaterial applications, including orthopedic implants, must be optimized to limit adverse body reactions. Furthermore, there has been an extensive push by the regulatory bodies to direct manufacturers toward fabricating implants having minimum heavy metal content. Thus, alternative titanium alloys having different alloying elements and concentrations have been utilized toward improving the biocompatibility of the orthopedic implants. One such chemistry showing promise in patients is Ti6Al7Nb. Ti6Al7Nb has both α and β phases, where aluminum stabilizes α phase and niobium, replacing vanadium in Ti6Al4V alloy, stabilizes β phase. Ti6Al7Nb alloy is more ductile than Ti6Al4V, which permits easier fabrication into components having complex contours and has superior corrosion resistance compared to Ti6Al4V [12]. In fact, due to having superior corrosion resistance, mechanical strength and biocompatibility, Ti6Al7Nb alloy has gained significant popularity for femoral components of hip prostheses. Although incorporation of alloying elements into titanium's chemical composition as Al, V, Nb, etc. satisfied some of the physical and chemical requirements to improve the lifetime of orthopedic implants, bioinert nature and the inability of titanium based implants to integrate with the juxtaposed bone (osseointegration) is still a major concern and among the major reasons for orthopedic implant failure.

To enhance osseointegration of orthopedic implants, researchers modified the surfaces of implants which are in direct contact with bone tissue and demonstrated that when the implant surfaces provide a better environment for bone cell functions, integration of the implant with the juxtaposed bone tissue can be improved [13]. For this purpose, various surface modification techniques to alter physical and chemical properties of titanium alloy components have been investigated, i.e., hydrothermal treatment, sol-gel, sandblasting, physical and chemical vapor deposition, etc. Among these techniques, anodization has been capturing the attention of multiple research groups for over a decade due to its simplicity, low cost and ability to control surface features at the nanoscale. In the anodization process, surface of a metallic component connected to the anode of an electrochemical cell is oxidized inside a suitable electrolyte, while an inert material, i.e., graphite or platinum, is used as the cathode. An electrical potential is applied between the electrodes to induce oxidation at the anode surface. In this process, electrochemical parameters including anodization time, applied voltage, temperature, electrolyte composition and concentration, etc., can be controlled. By adjusting these electrochemical parameters, a nanostructured oxide film having an array of pits, wires, pores or tubes can be grown on the surface of the anode material and the dimensions of these surface features can be fine-tuned within the nanoregime.

Fabricating surface features having nanoscale dimensions is specifically important in tissue engineering where feature size of tissues is all in nanoregime. For instance, natural bone has inorganic constituent made up of 2–5 nm thickness and 20–25 nm wide hydroxyapatite crystals [14]. Another example is the vascular basement membrane which consists of a complex meshwork of pores and fibers

in the submicron (100–1000 nm) and nano (1–100 nm) range [15]. By modifying biomaterial surfaces to possess features having nanophase topography, implant surfaces mimic the feature size of natural tissues, providing a more realistic niche to promote cellular functions on implant surfaces. Since nanophase surface features similar to natural bone can be engineered upon anodizing titanium alloy surfaces, it provided researchers the rational to investigate them in orthopedic applications. In the next chapter, the mechanism of anodization and formation of aforementioned nanophase surface features will be discussed.

3 Mechanisms of anodization

Anodization has been used to fabricate stable, protective and dense oxide layers on valve metals. Pure metals that can be anodized include aluminum, titanium, zinc, magnesium, zirconium, tantalum, and hafnium. Basically, anodization is the oxidation of these metals in certain electrolytes by generating an electrical field at metal/electrolyte interface. As stated previously, it is possible to fabricate nanofeatures having different morphologies and sizes on metallic surfaces via anodization. Figure 1 shows schematic of the anodization process and different stages for nanotubular oxide formation on a Ti6Al7Nb alloy. Though this schematic indicates Ti6Al7Nb alloy as the anode, the mechanism of anodization is similar for all titanium alloys [16].

Basically, titanium and titanium alloys have a naturally occurring 2–5 nm thick passive oxide layer [17]. Electrical potential applied during anodization process increases the thickness of this natural oxide layer on titanium surfaces. Once voltage is applied to titanium alloys connected to the anode of an electrochemical cell, an oxidation reaction will start at the metal/metal oxide interface (Eq. (1)) and generate Ti^{4+} ions. These Ti^{4+} ions migrate outwards under the applied voltage, while O^{2-} ions present in the electrolyte migrate toward the metal/metal oxide interface. During migration of negatively charged O^{2-} ions toward the metal/metal oxide interface, they encounter Ti^{4+} ions ejected from the surface to produce a compact titanium oxide film (Eq. (2)). Due to having a higher electrical resistivity than the underlying titanium substrate, the applied voltage will decrease across the oxide film forming on the surface. During anodization, the oxide film will continue to grow as long as the electric field is strong enough to drive the ionic conduction across the oxide [18].

$$Ti \rightarrow Ti^{4+} + 4e^{-} \quad (1)$$

$$Ti^{4+} + 2H_2O \rightarrow TiO_2 + 4H^{+} \quad (2)$$

The formation of self-assembled nanostructures is generally associated with the presence of fluoride ions (F^{-}) inside the electrolyte [19]. F^{-} ions are small and capable of chemically reacting with the oxide layer, producing water-soluble metal fluoride complexes. In addition, these F^{-} ions can migrate inside the oxide film similar to O^{2-} ions. During anodization process, when titanium alloy at the anode is inserted inside the electrolyte, there is an immediate growth of oxide film on the surface of titanium alloy. When there are fluoride ions inside the

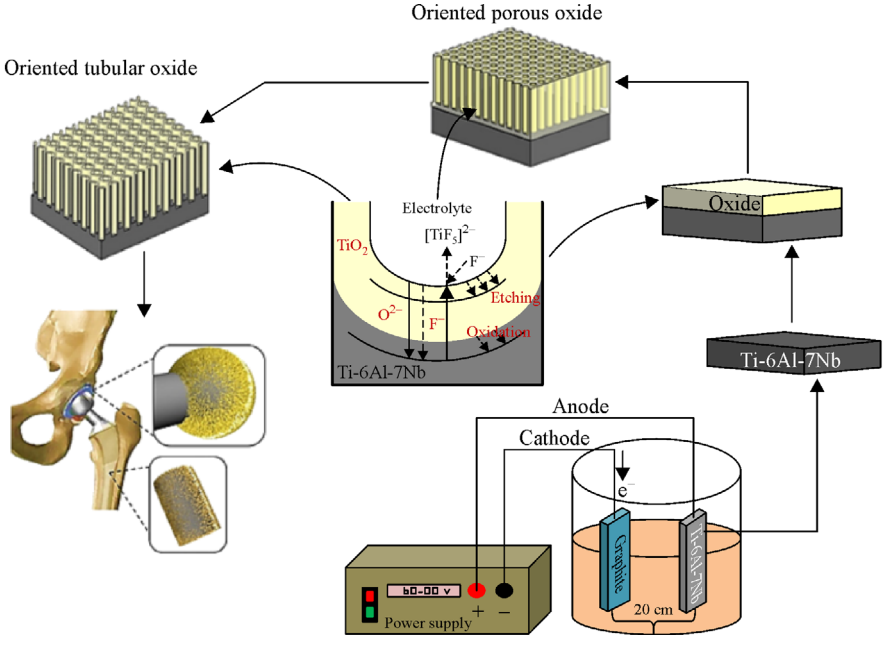

Fig. 1 Schematic depicting the growth of nanostructured oxide film on Ti6Al7Nb alloy. Image is reprinted with permission from reference [16]

electrolyte, pitting and decomposition of the compact oxide film occurs via Eq. (3), forming nanofeatures on the titanium alloy surfaces.

$$TiO_2 + 6HF \rightarrow [TiF_6]^{2-} + 2H_2O + 2H^+ \quad (3)$$

As nanostructures (specifically nanotubes) grow, once a critical film thickness is reached, current density in the electrochemical cell stabilizes and the rate of oxide film growth at the titanium/titanium oxide interface and the rate of oxide film dissolution at the titanium oxide/electrolyte interface reach equilibrium. At this point, the oxide film does not get thicker any more, reaching its maximum thickness. It is important to realize that nanotubular structures on the surface will still continue to growth with oxide film steadily moving further into the titanium alloy, yet formation and dissolution rates are equal and thus prevent increase in oxide film thickness. Higher anodization voltages increase the oxidation and field-assisted dissolution, and thus provide oxide layers with different maximum layer thicknesses.

Depending on fluoride concentration of the electrolyte, three distinct morphology regimes can be obtained for the oxide film forming on titanium alloys. If the fluoride content is very low, a compact oxide film forms above a certain electrical potential, U_p (passivation potential). If the fluoride concentration is at an intermediate level, nanoporous/nanotubular structures can be obtained; and if the fluoride concentration is high, all the Ti^{4+} cations react with fluoride to form soluble $[TiF_6]^{2-}$, forming self-organized nanotubular layers. Having this said, current density-time curves obtained during anodization of titanium alloys (Fig. 2(a)) demonstrate different regimes, each of them corresponding to distinct oxide film morphology (Fig. 2(b)). Specifically, in the first regime, a compact oxide film is produced on titanium alloys, leading to a significant drop in the current density. In the second regime, there is an increase in current density, which is attributed to the formation of nanoporous structures in the oxide film. In the third and last regime, current density decreases gradually, indicating growth of nanoporous/nanotubular structure [20].

By tailoring the anodization parameters, i.e., electrolyte type, electrolyte concentration, pH, temperature, applied voltage and time, it is possible to control physical and chemical properties of the oxide film forming on the titanium alloys [22–24]. In literature, nanostructures in the form of compact oxide films, disordered porous films and self-organized porous and nanotubular films were fabricated by anodization using various electrolytes, such as NH_4F, CH_3COOH, H_2SO_4, HF, Na_2HPO_4, NaF, NaOH, NH_4Cl [25]. In addition, it is well-established that different electrolytes have different ionic conductivities, and thus form different electrical fields inside an electrochemical cell. When the electrical field is high, it will be easy to induce breakdown fields, which are localized on rough surfaces during the initial step of anodization. Furthermore, pH of the electrolyte is an important parameter to control growth and self-organization of nanotubular structures. For example, Feng et al. [26] demonstrated using a NH_4F/water/glycerol electrolyte system that the basic environment (pH 8–9) is much more efficient for self-organization of longer nanotubes than the commonly used acidic condition (pH 3–5) for titanium. In another study, nanotubular formation rate was correlated with the pH of the electrolyte where increase in pH decreased the nanotubular length [27]. Another parameter that controls the microstructure of the oxide film on titanium and titanium alloys is the temperature of the electrolyte, which was found to effect dissolution rate of nanostructures [25]. It is interesting to note that while nanotubular diameters of oxide films do not depend on anodization temperature of aqueous electrolytes, they were found to increase with an increase in temperature once organic electrolytes are used. This phenomenon was explained with increasing electro-

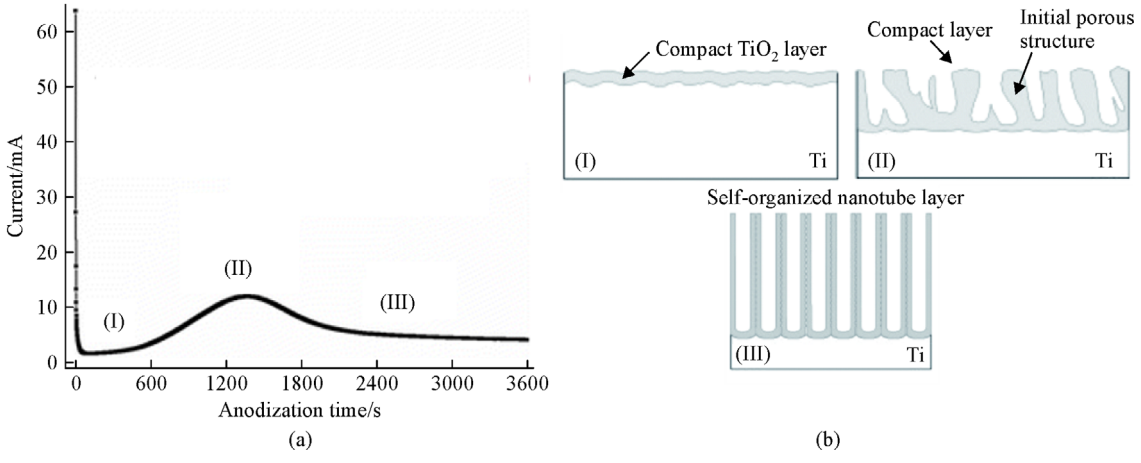

Fig. 2 (a) Current density-time graph (*j-t*) obtained during anodization of titanium using an electrolyte containing fluoride ions and (b) the representation of compact oxide layer (fluoride free electrolyte) and nanoporous/nanotubular oxide layers (fluoride containing electrolyte). Images are adapted with permission from references [20,21]

lyte viscosity and decreasing ionic migration at a lower temperature [28]. Applied voltage is another anodization parameter influencing surface microstructure, pore morphology, pore diameter, inter-pore distance and oxide film thickness forming on the titanium and titanium alloy substrates [29,30]. As anodization voltage was increased, the web-like oxide surface morphology forming at lower voltages was lost and the microstructure of the oxide film gradually transform to nanotube like structures. Additionally, duration of anodization alters nanotube morphology, increasing the oxide film thickness, while decreasing the nanotubular wall thickness at longer anodization times [20]. To sum all these findings, during anodization of titanium and titanium alloys, changes in electrochemical process parameters alter growth kinetics, microstructure and formation of nanofeatures on the substrate surfaces, influencing the physical and chemical properties of the oxide film, which will be covered in the next section.

In addition to liquid phase anodization, oxidation process can also be performed via plasma technique where anodic oxidation is performed by accelerating anions and electrons from an oxygen plasma onto the sample connected to an anodic potential [31]. The microstructure formed via plasma anodization is affected by the temperature of the sample; pressure, flow rate and composition of the gas; radio frequency (RF) power (if RF plasma is used), bias voltage and polarity; as well as field or sputter driven nature of the process. It is important to note that the main advantage of plasma oxidation is the lack of any acidic residue (i.e., fluoride) and hydrogen on the anodized surfaces as well as the enhanced oxide film growth rate on the substrate [31]. Göttlicher et al. [32] reported successful fabrication of oxide films having nanoparticles on the surface of Ti40Nb alloy using inductively coupled radio frequency oxygen plasma. Though nanotubular features or repeating arrays on titanium surfaces were not observed using plasma anodization, its applicability on titanium alloys holds promise for orthopedic applications.

4 Anodization of titanium and titanium alloys

Early works on anodization of titanium were conducted in hydrofluoric and chromic acid containing electrolyte mixtures to enhance corrosion resistance of the underlying titanium without any consideration to fabricate a nanofeatured oxide film [33]. In 2003, Schumiki et al. [34] reported the fabrication of nanotubular structures using a mixture of sulfuric acid and small amounts of HF (0.15 wt-%). In this study, they reported possibility of using alternative acid mixtures, i.e., phosphoric and acetic acid, in combination with HF or NH_4F as potential electrolytes for anodization to fabricate nanoporous surface oxides. In another study, self-assembled nanotubular oxide films as thick as 500 nm was fabricated for the first time using a chromic acid containing electrolyte with and without incorporation of hydrofluoric acid [34]. Later on, Grimes et al. [35] reported formation of nanotubular oxide having 4.4 μm length by anodizing titanium in a fluorinated solution having pH value of 4.5. Macak et al. [36] confirmed previous findings and showed that pH of the electrolyte influence nanotubular length and oxide film thickness, where lower pH values restrict the length of nanotubes by enhancing the tube dissolution rate. They also showed that while smooth nanotubes without any surface ripples could be grown using non-aqueous electrolytes, fabrication of hexagonally arranged nanotubular layers could be grown using an organic electrolyte, i.e., ethylene glycol.

Anodization of titanium alloys used in orthopedic applications, i.e., Ti6Al4V and Ti6Al7Nb, was has also been investigated. Chen et al. [28] anodized both Ti6Al4V and Ti6Al7Nb in 0.5 wt-% $(NH_4)_2SO_4$ + NH_4F solution and successfully obtained self-assembled nanotubular oxide films on these substrates. Since Ti6Al4V and Ti6Al7Nb are dual phase alloys, nanotube formation kinetics were found to be different for α and β phases, where Mazare et al. [37] earlier formation of nanotubular structures on β phase compared to α phase for Ti6Al7Nb alloy using and aqueous electrolyte containing CH_3COOH and 0.5 wt-% HF under 10 V potential. Kulkarni et al. [30] observed changes in the morphology of nanotubular oxide films on α and β phases depending on the fluoride concentrations used for the electrolyte. In an extreme case, only α phase exhibited nanotubular structures upon anodizing in 1 $mol \cdot L^{-1}$ H_3PO_4 and 0.2 wt-% HF electrolyte, while β phase did not form any nanostructures, indicative of different reactivities of each phase for fluoride containing electrolytes [38].

Park et al. [39] examined the growth behavior of nanotubular oxide on Ti6Al4V substrates in glycerol containing electrolytes. Anodized surfaces showed a wide distribution of nanotubular diameters, where nanotubes with smaller diameters formed across spaces present between nanotubes with larger diameters. Interestingly, diameters of the nanotubular features were increasing toward the base of the nanotubes as opposed to the commonly observed tapered nanotubular morphology. This study also highlighted the importance of electrolyte concentration used in anodization of titanium alloys, where diameters of the nanotubular features could be controlled by merely altering electrolyte concentration. Nanotubular features having 88.5 and 122.9 nm diameter were formed in glycerol electrolytes containing 1 wt-% NH_4F + 20 wt-% H_2O and 1 wt-% NH_4F + 30 wt-% H_2O at 20 V, respectively [39]. Another method to control tubular diameters was altering anodization parameters. For instance, Mohan et al. [40] formed self-assembled

nanotubular oxide films having 35, 100 and 125 nm average tubular diameters on the Ti6Al7Nb alloy by adjusting the applied voltage to 10, 20 and 30 V, respectively.

Though Ti6Al4V and Ti6Al7Nb are the main focus of this review, there are other titanium alloys in literature investigated for orthopedic applications, including binary TiAl, TiZr, TiNb, TiZr, TiTa, Ti-Ta-Nb alloys, ternary alloys (i.e., TiTaZr and Ti13Zr13Nb), quaternary alloys (i.e., different Ti-Nb-Ta-Zr alloy compositions), etc., where self-assembled nanotubular structures have been reported. Jha et al. examined nanotubular oxide growth on TiZr, TiNb and TiTa using chloride and perchlorate based electrolytes [41]. For these alloys, authors observed growth of nanotubular oxides reaching up to 10 μm in length, while nanotubular diameter varied between 10 and 15 nm for TiZr alloy, 20–100 nm for TiNb and 30–90 nm for TiTa. When Ti-7.5Mo alloys was anodized using glycerol containing 0.25% NH_4F [42], an amorphous oxide layer formed on alloy surface, where nanotubular size reached 80 nm and 110 nm for 20 and 30 V potentials, respectively. When the molybdenum content of the alloy increased to 10% (Ti-10Mo alloy) [43], anodization using 0.5% NH_4F and 2% H_2O in ethylene glycol at 20 V for 2 h successfully gave nanotubular structures, yet alternate oxide layer morphologies, i.e., continuous compact layer, continuous partial porous layer, porous layer, etc., were also forming on Ti-10Mo alloy surface. When ternary Ti-35Ta-xZr alloys were anodized in 1 $mol \cdot L^{-1}$ H_3PO_4 containing NaF solution, it was observed that average thickness of the nanotubes increased from 4.5 to 9.0 μm with an increase in Zr content from 3% to 15%. Increase in anodization voltage from 3 to 10 V had a similar effect which increased nanotube layer thickness from 0.5 to 9.5 μm [44]. When Ti-13Zr-13Nb alloy was anodized using 1 $mol \cdot L^{-1}$ H_3PO_4 with addition of HF at 20 V for 30 min to 1 h, nanotubular oxide layer formation was also achieved [45]. Having this said, one class of titanium alloys researched for orthopedic applications is gum metal. Gum metal (or TNTZO) is the name given to a set of β-titanium alloys with high elasticity, yield strength and ductility at room temperature. Though a range of chemical compositions are currently available, gum metal typically has a composition of Ti-23Nb-0.7Ta-2.0Zr-1.2O [46] where oxygen in its composition plays an important role giving rise to the unusual mechanical properties of this alloy. Though anodization of gum metal has not been investigated, Ti-Nb-Ta-Zr alloy (without oxygen in its composition) have been studied. For instance, Chiu et al. [47] anodized Ti29Nb13Ta4.6Zr using NH_4F-containing (0.3 wt-%) ethylene glycol solution under 20 V and systematically altered water content of the electrolyte from 20% to 0.9%. Results showed decrease in wall thickness of the nanotubular structures with a decrease in electrolyte water content. Literature results obtained from anodization of titanium alloys shows us that alloying titanium with different elements alter anodization process, influencing physical and chemical properties of the nanotubular oxide film forming on the alloy surfaces (Fig. 3), which in turn affects the biological properties of the material.

During anodization, fluorinated electrolytes were often used for the fabrication of nanotubular structures on titanium and titanium alloys. However, few researchers reported chloride ions (another element found in the 7A group of the periodic table) to play a similar role during anodization [48]. Since chloride ions provide a less hazardous alternative to the highly toxic fluoride based electrolytes, various issues in industrial applications can be avoided, i.e., corrosion, toxicity, environmental influence etc. Researchers observed that chloride containing electrolytes in combination with perchloric, oxalic or formic acid were successful in fabricating nanotubular surfaces on titanium, as seen in Fig. 4. Although the mechanism of anodization using chlorinated electrolytes was not clear, pits appear on titanium surfaces, as shown in Figs. 4(a–c) followed by rapid formation of nanotubular arrays inside the pits.

The corresponding current-time graph upon applying 10 V potential using 0.5 $mol \cdot L^{-1}$ HCl aqueous electrolyte is consistent with the graph shown in Fig. 2 [50]. However, nanotubular arrays formed via fluoride free anodization were inhomogeneous and disordered, not fully covering the titanium surfaces. Additionally, using fluoride free anodization, nanotubular features having diameters as small as 20 nm could be fabricated, while carbon contamination over 20% was found over the oxide

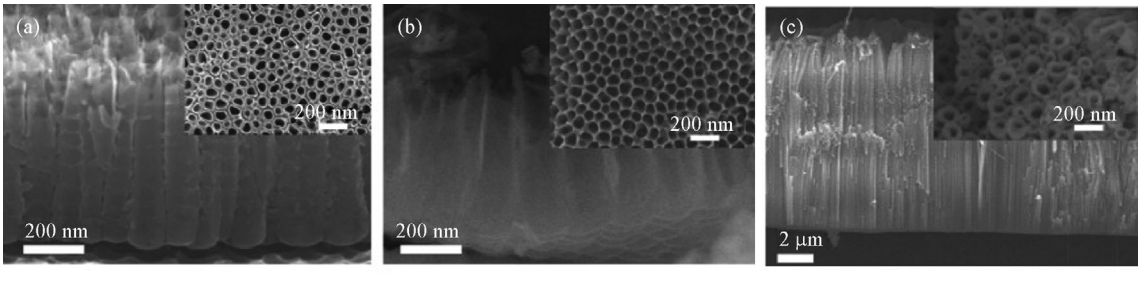

Fig. 3 SEM images showing nanotubular layers grown on (a) Ti6Al7Nb, (b) TiAl, and (c) TiZr using $(NH_4)_2SO_4$ electrolyte. Larger images show cross-sectional views and insets show top views of nanotubular layers. Images are adapted with permission from reference [21]

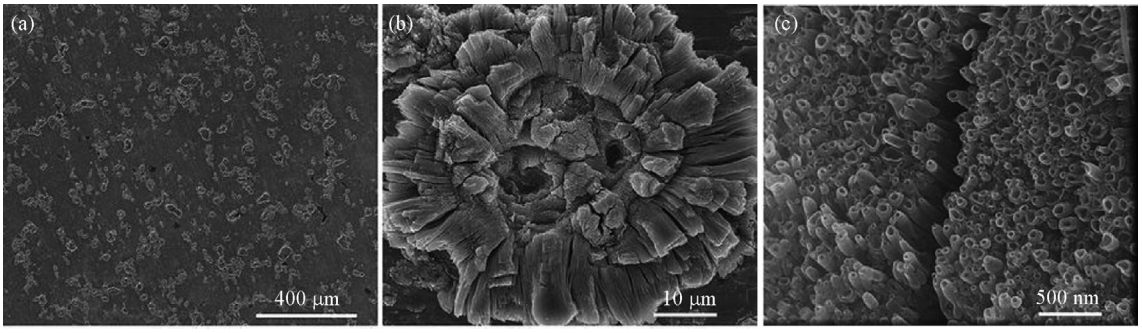

Fig. 4 (a), (b) and (c) SEM images of flower-like nanotubular structures on titanium at different magnifications. Titanium samples were anodized using an electrolyte containing 0.3 $mol \cdot L^{-1}$ sodium chloride and 6:4 distilled water to ethylene glycol ratio. Images are adapted with permission from reference [49]

films in several chlorine based electrolyte systems, i.e., 0.4 $mol \cdot L^{-1}$ NH_4Cl + 0.5 $mol \cdot L^{-1}$ formic acid, 0.4 $mol \cdot L^{-1}$ NH_4Cl + 0.05 $mol \cdot L^{-1}$ sulfuric acid and 0.4 $mol \cdot L^{-1}$ NH_4Cl + 0.5 $mol \cdot L^{-1}$ formic acid electrolytes [25,48]. Having this said, the role of other chemicals typically used in fluorinated electrolytes, i.e., ethylene glycol, for anodization of titanium and titanium based alloys in chloride based electrolytes is not clear and could be a key component to obtain uniformly distributed nanotubular features for chlorine based electrolyte systems. For the case of titanium anodization using halogen-free electrolytes, Kim et al. [51] suggested rapid breakdown mechanism, which is basically the local breakdown of oxide film by ions, for growth of nanotubular bundles. Perhaps altering the anodization conditions, which influence rapid breakdown anodization mechanism, i.e., applied potential, water content, chloride and other ion concentrations, may lead to the formation of self-organized nanotubular structures in both fluoride-free and halogen-free electrolytes [25]. Further research is required on fluoride-free and halogen-free anodization to identify formation mechanism of nanotubes and to obtain homogenous surface distribution.

Discussions up to this point focused on the formation of nanofeatured structures on titanium and its alloys on flat 2D substrates. However, implants used in orthopedics are rarely flat and have contoured surfaces to match the geometry of the bone. Furthermore, the portions of the implants that are in contact with bone tissue is fabricated to have porousand microstructure to permit infiltration of bone cells for enhanced osseointegration. Thus, anodized nanofeatured morphologies obtained on flat surfaces need to be translated on contoured porous surfaces for orthopedic applications. Since porous surfaces, i.e., foams, have higher surface area compared to their 2D counterparts, materials fabricated to have porous geometries provide a higher efficiency for the current collection during anodization. It requires adjusting electrolyte concentration, voltage and time, along with having an intricate control on anodization temperature to fabricate similar nanofeatures obtained for anodization of flat surfaces on porous material surfaces.

SEM images of titanium foams anodized under a constant potential of 40 V for 1.5 h are shown in Figs. 5(a–c). In this study, the nanotubular length was adjusted to be nearly 1.5 μm and the diameter of nanotubular structures were controlled to be between 80 to 150 nm [52]. In another study, anodization was completed under a constant potential of 80 V using ethylene glycol containing 0.25 wt-% NH_4F and 2 vol-% H_2O electrolyte for 30 min, where a nanotubular surface morphology was again fabricated [53]. After anodization,

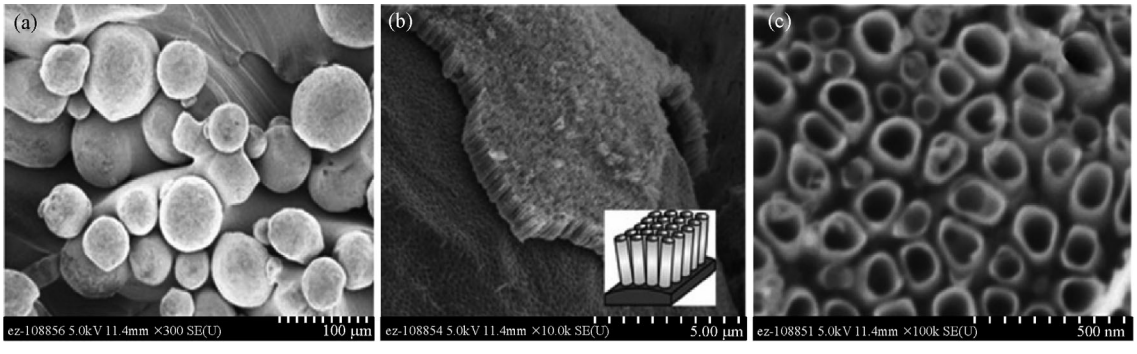

Fig. 5 (a), (b), and (c) SEM images of an anodized titanium foam. Images are reprinted with permission from reference [52]

foams were heat treated at 450°C for 4 h to transform amorphous oxide film to a polycrystalline anatase phase.

Though anodization of titanium foams have been completed for photocatalytic and energy storage applications, there are no studies investigating the anodized foams for orthopedic applications. Considering the increase in demand for implants with longer lifetime and enhanced osseointegration, porous titanium and titanium alloy scaffolds having anodized nanotubular surface morphologies can potentially offer various advantages where currently used implants fail to satisfy. This topic should further be investigated.

5 *In vitro* studies investigating anodized titanium alloys

In the orthopedics field, preventing implant failure is the ultimate challenge. To accomplish this aim, enhancing adhesion, proliferation and cellular functions of bone cells on currently used titanium based implants, and thus improving osseointegration of the implant is an important necessity. Since cellular functions are mediated by physical and chemical properties of the surface they interact with, bone cell-implant interface has been the focus of many studies [54].

When an orthopedic implant is inserted into the body, proteins in the plasma and the surrounding tissues adsorb onto the implant surfaces within the first couple of seconds. Depending on the surface properties of the implant, concentration and confirmation of the adsorbed proteins can significantly differ. Specifically, implant surface chemistry, energy, topography, crystallinity, texture, etc. influence adsorption of proteins onto its surfaces. Since adsorbed proteins play a key role mediating cellular functions for anchorage dependent cells, aforementioned surface properties of orthopedic implants have a major influence on how implant interacts with juxtaposed bone tissue, osseointegration and long-term success of the implant. The main mechanism of bone cell adhesion onto implant surfaces is the interaction of cell membrane receptors, called integrins, with RGD (R: arginine, G: glycine, D: aspartic acid) peptide sequence of the adsorbed proteins. The proteins in the blood plasma that have RGD peptide sequence are fibronectin, vitronectin, laminin and collagen, and their adsorption onto orthopedic implant surfaces is the dominant mechanism controlling osteoblast (bone cell) adhesion onto implant surfaces.

Once surface of a biomaterial is fabricated to possess nanostructures, it potentially exhibits altered physical and chemical properties compared to its conventional counterpart, and thus interact differently with serum proteins. In fact, this altered interaction with proteins results in enhanced biological properties for orthopedic applications. For instance, when surface of titanium samples was modified to possess nanophase surface roughness, enhanced fibronectin adsorption was observed compared to conventional titanium surfaces, altering adhesion of cells onto titanium surfaces [55]. Wang et al. [56] reported 15% increase in both fibronectin adsorption and 18% increase in vitronectin adsorption on nanophase titanium structures compared to conventional titanium. One of the pioneering works on interactions of osteoblasts with nanofeatured surfaces was reported by Webster et al. [57] which showed increased osteoblast density, alkaline phosphatase activity and calcium deposition on nanophase alumina and titania compared to their conventional counterparts. The same research group also reported that osteoblast adhesion, proliferation and long-term cellular functions were significantly improved on nanophase titanium, Ti6Al4V and CoCrMo alloys compared to their conventional counterparts having micron grain size [58]. In another study, nanophase titanium surface fabricated via electron beam evaporation showed enhanced osteoblast adhesion and cellular functions than conventional titanium surfaces [59]. Furthermore, nanostructured Ti6Al4V alloy prepared by severe plastic deformation supported increased osteoblast adhesion and expression of adhesion related integrin $\beta 1$ gene on nano alloy compared with conventional samples. These results suggest that enhanced osteoblast cellular function to nanophase surface topography is independent of the material type and controlled by the feature size of bone cell-material interphase [60].

Biological gains obtained by decreasing the surface feature size to nanoregime were also observed for anodized titanium and titanium alloys. Oh et al. [61] showed enhanced interaction of osteoblasts with anodized titanium surfaces where filopodia of propagating osteoblasts were growing into nanostructured titanium oxide film and proposed it as an alternate mechanism for enhanced osteoblast functions on nanotubular titanium oxide surfaces. Anodized Ti6Al4V surfaces promoted enhanced osteoblast densities, alkaline phosphatase activity and calcium deposition, which was correlated with greater vitronectin adsorption on anodized Ti6Al4V surfaces compared to its conventional counterpart [62]. Enhanced vitronectin adsorption was explained with increased hydrophilicity of Ti6Al4V alloy upon anodization, where sessile water drop contact angle measurements showed 60° for anodized samples, whereas it was 83° for the polished Ti6Al4V alloy [62]. These results were consistent with previous findings which demonstrated enhanced osteoblast functions on anodized nanotubular oxide samples compared to conventional titanium [63].

Recently, several research groups reported nanotubular feature size dependent changes in cellular functions for mesenchymal stem cells, hematopoietic stem cells, endothelial cells, osteoblast and osteoclast functions on anodized nanotubular titanium surfaces [64,65]. The influence of nanotubular size was initially studied by

Park et al. [66] where self-assembled layers of vertically oriented nanotubular oxide films having 15 to 100 nm diameters were fabricated on titanium (Fig. 6). While the average surface roughness (R_a) on conventional titanium substrates is nearly 6.54 nm, the surface roughness of anodized nanotubular samples was found to increase with increasing nanotubular diameter, reaching 12.62 nm for 40–60 nm diameter samples [67].

In vitro biocompatibility results showed mesenchymal stem cell adhesion, spreading, proliferation and osteogenic differentiation were all influenced by the diameter of nanotubular titanium oxide grown on titanium substrates. Specifically, mesenchymal stem cells density reached its highest value on nanotubular oxide films having 15 nm diameter arrays [66]. Brammer et al. [69] observed similar trends with osteoblasts, where nanotubular titanium samples having 30 nm diameter (the smallest diameter investigated in these studies) supported the highest cellular density compared to all other diameters after 2 days of culture (Fig. 7(A)). This study also confirmed elongation of osteoblasts with an increase in nanotubular diameter at 2 and 24 h of culture (Fig. 7(B)). Yu et al. [70] reported MC3T3-E1 preosteoblast behavior on anodized titanium oxide films with nanotubular diameters ranging between 20 to 120 nm. Results showed well-spread cellular morphology on nanotubular samples having 20–70 nm diameters, while cellular adhesion and spreading was decreasing on samples having diameters larger than 70 nm. Similar results were obtained by Oh et al. [71] who investigated the relationship between diameter of anodized nanotubular titanium and human mesenchymal stem cell (hMSC) morphology. Their findings indicated that hMSCs on flat titanium appeared well-spread, while the ones on anodized samples having 100 nm nanotubular diameter expressed unidirectional lamellipodia extensions and filopodia after 2 h of culture. After 24 h, hMSCs began to demonstrate an elongated cellular morphology where aspect ratios of cells increased with an increase in nanotubular diameter. Quantitative polymerase chain reaction (qPCR) analysis of osteocalcin (OCN), osteopontin (OPN) and alkaline phosphatase (ALP) gene expressions of hMSCs cultured on anodized samples for 14 days demonstrated upregulation of these osteogenic genes on titanium samples having 70 and 100 nm diameter nanotubular features compared to cells cultured on other nanotubular diameters and conventional titanium. Importantly, osteogenic induction media was not used in this study, highlighting the influence of surface nanofeature

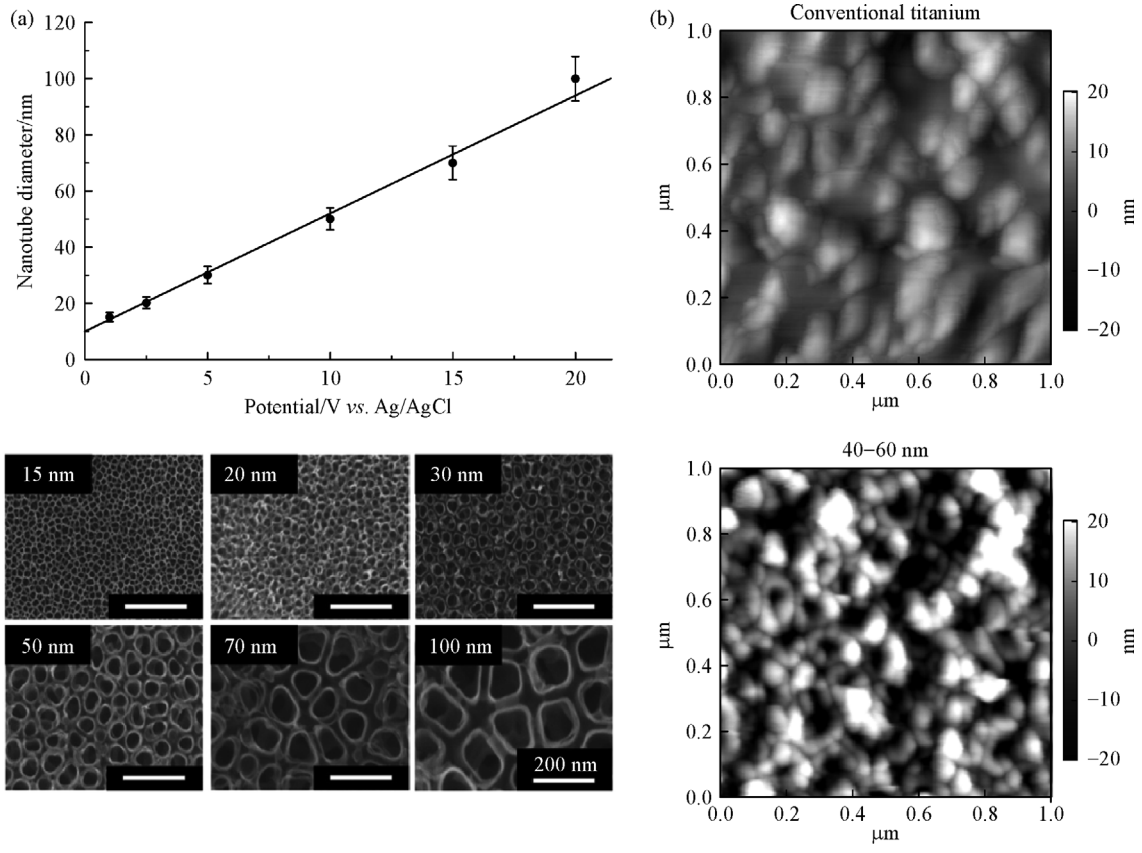

Fig. 6 (a) Change of nanotubular diameters on titanium surfaces with the applied voltage and the corresponding SEM images of the anodized surfaces, (b) AFM scans showing the topography of conventional titanium and anodized nanotubular titanium having 40–60 nm diameters. Images are adapted with permission from references [66,67]

size on osteogenic differentiation of hMSCs [71]. Park et al. [66] studied the underlying mechanism for altered cellular response on nanotubular surfaces with different diameters and observed that nanotubes having 15 and 30 nm diameters elicited MSC response through extensive formation of paxillin-positive focal contacts which were anchored to actin stress fibers, while MSCs cultured on 100 nm diameter nanotubular structures did not express distinct focal contacts (Fig. 7(C)).

Aside from cell density and morphology, nanotubular diameter of anodized titanium surfaces can also alter cellular functions. Malec et al. [72] observed higher calcium deposition from human adipose tissue derived MSCs cultured on 108 nm diameter anodized nanotubular surfaces after 21 days of culture compared to untreated titanium (S), electropolished titanium (W) and 80 nm diameter anodized nanotubular titanium surfaces.

In spite of the interesting *in vitro* biological response obtained from cells cultured on anodized nanotubular titanium surfaces with varying diameters, there are only a few studies testing the effect of nanotubular diameters for different titanium alloy formulations for orthopedic applications. Li et al. [73] evaluated *in vitro* osteoblast response cultured on anodized Ti6Al7Nb alloy surfaces, where anodized nanotubular oxide films within 18 to 30 nm diameter range supported adhesion and proliferation of the osteoblasts up to 3 days of culture and maintained their integrity during *in vitro* cell experiments. For the case of Ti6Al4V, anodized samples had higher SAOS-2 densities compared to glass controls. Nanotubular surfaces having larger average diameters had the highest concentrations of both vinculin and talin at 3 days of culture. Unlike anodized titanium samples, the highest concentrations of alkaline phosphatase, type I collagen and osteopontin were found on cells cultured on Ti6Al4V samples having smaller average nanotubular diameter [66]. Using a different cell line in this study could also contribute to the observed differences in literature.

It is important to stress that when titanium and titanium alloys are anodized to obtain nanotubular features with

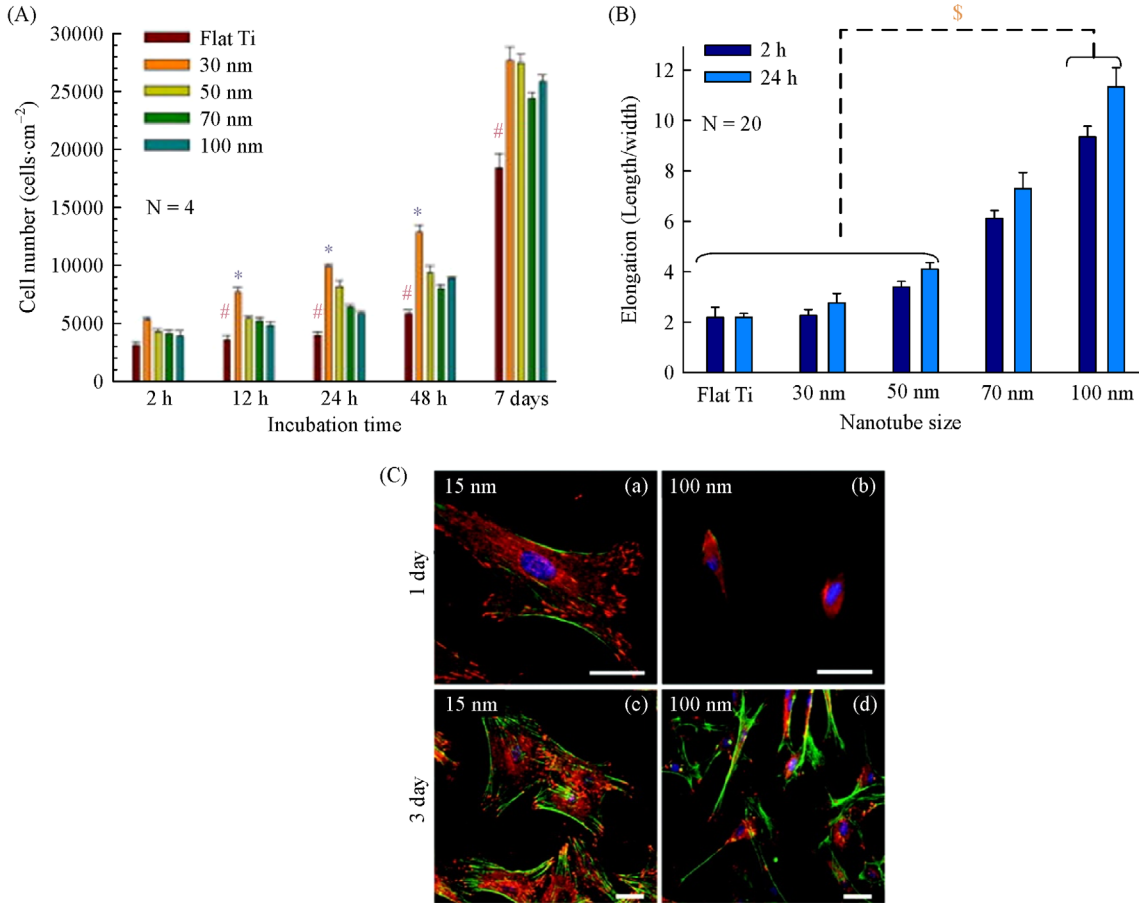

Fig. 7 (A) Osteoblast densities on anodized surfaces up to 7 days of culture. # $p < 0.05$ indicates significant difference between the flat titanium and anodized nanotubular surfaces; * $p < 0.05$ indicates significant difference between 30 nm diameter nanotubular features with larger size (50–100 nm) ones; (B) Influence of nanotubular diameter on osteoblast elongation, $ $p < 0.05$ compared to flat titanium and 30 and 50 nm diameter nanotubular titanium; (C) focal contact formation of MSCs on 15 and 100 nm diameter nanotubular surfaces at 1 and 3 days of culture. Cells were stained for paxillin (red), actin (green) and nucleus (blue). Scale bars are (a) and (b) 50 μm, (c) and (d) 100 μm. Images are reprinted with permission from references [66,68]

varying sizes, physical and chemical properties of the surfaces also change according to the electrochemical parameters used during anodization. For instance, root mean square roughness of the surface increases with an increase in nanotubular diameter, altering the hydrophobicity of the anodized surfaces, and as a consequence, the interaction of the anodized surfaces with serum proteins, which controls adherent bone cell functions, is altered. Furthermore, chemical compositions of the surfaces also change depending on the anodization parameters since voltage driven migration of ions into the titanium lattice is altered. It is the combining all of these factors which influences interaction of cells with anodized surfaces. Controlling only one surface property without changing the rest via anodization is not possible. Thus, altered *in vitro* cellular response should not be only attributed to changes in feature size of nanotubes, instead a combination of multiple physical and chemical property changes come to play different roles. In fact, newly formed anodized surfaces can have very different surface properties compared to conventional titanium and titanium alloys that it is impossible to predict biological response merely from *in vitro* assays. More realistic *in vivo* studies are required and the next chapter will detail various animal studies testing the effects of anodized titanium and titanium alloys for orthopedic applications.

6 *In vivo* studies of nanostructured titanium and titanium alloys

To translate nanotubular titanium alloys for clinical applications, in-depth animal studies are critical. Though there is plenty of published research on the use of anodized nanotubular titanium *in vitro*, little is known about how nanotubular titanium surfaces behave in more realistic *in vivo* scenarios. In one study, screw shaped implants were anodized using 1 $mol \cdot L^{-1}$ H_3PO_4 and 0.4 wt-% HF electrolyte at 20 V, followed by insertion into the femur condyle close to the knee joint in a rabbit model [74]. After six weeks of implantation, anodized nanotubular implants in rabbit femurs demonstrated significantly increased osseointegration strengths in removal torque tests and superior new bone formation in quantitative analysis of histological slides compared to their grit blasted counterparts, revealing more frequent direct bone contact at the bone–implant interface. Histological examinations revealed well developed trabecular architecture surrounding the implant, with some variations in the newly formed bone structures in periosteal and endosteal regions. In the periosteal region, active formations of woven bone were observed and in the endosteal regions, newly formed bone was clearly identified [74]. In another study, Sul [75] manufactured coin-shaped anodized nanotubular titanium implant disks (5 mm in diameter and 2.5 mm thick) for *in vivo* studies. After 4 weeks of implantation into the tibia of earloop rabbits, anodized nanotubular oxide films on titanium surfaces were found to enhance *in vivo* bone bonding strength up to 9-folds compared to grit blasted titanium surfaces. Histological analysis also confirmed greater bone-implant contact area, new bone formation and calcium and phosphorus levels on nanotubular oxide films. Bjursten et al. [76] compared of the osseointegration of machined, grit blasted and anodized nanotubular Ti6Al4V implants in rabbit models. After 4 weeks of implantation into the femoral condyles of New Zealand white rabbits, pull-out tests presented higher results for anodized nanotubular Ti6Al4V compared to other groups. Histology examinations supported direct apposition of bone tissue onto the nanotubular oxide film on Ti6Al4V alloy surfaces. Besides, nanotubular surfaces exhibited higher bone to implant contact and bone growth compared to machined and grit blasted surfaces. This study demonstrates that nanotubular surfaces on Ti6Al4V alloy can offer advantages over the grit blasted alloys, which have been widely used in clinics [77]. Furthermore, Puckett [78] investigated anodized Ti6Al4V pins using unilateral through the knee rat amputation model where *in vivo* results indicated that animals implanted with anodized nanotubular Ti6Al4V pins walked much earlier and had increased bone-to-implant contact showing enhanced bone growth on anodized surfaces compared to conventional Ti6Al4V pins.

Wang et al. [79] studied *in vivo* osseointegration of anodized surfaces at the molecular level, where performance of 30, 70 and 100 nm diameter anodized nanotubular titanium implants were compared with machined implants using a minipig model (Figs. 8(a,b)). Results showed upregulation of alkaline phosphatase (ALP), osterix (Osx), collagen-I (Col-I) and tartrate-resistant acid phosphatase gene expressions up to 5 weeks of implantation for anodized titanium implants compared to their conventional titanium counterparts. Moreover, histological analysis showed up to 3 fold increase in bone-implant contact on anodized samples. The highest increase in osteoblast differentiation and mineralization was observed for anodized titanium samples having 70 nm diameter, followed by anodized 100 nm diameter and 30 nm diameter titanium samples. In addition, sequential fluorochrome labeling inferred dynamic bone deposition, where fluorescent labels in the bone surrounding anodized titanium implants expressed higher signal intensity and continuity than the conventional titanium implants, especially those around 70 nm diameter nanotubes, as seen in Figs. 8(c–f). Results of this study identified 70 nm diameter nanotubular features to be the most favorable size for osteoconduction and osseointegration.

In all the animal experiments, the potential of using anodized titanium and titanium alloys in orthopedics was confirmed. Results demonstrated enhanced bone synthesis and osseointegration, which could improve potential

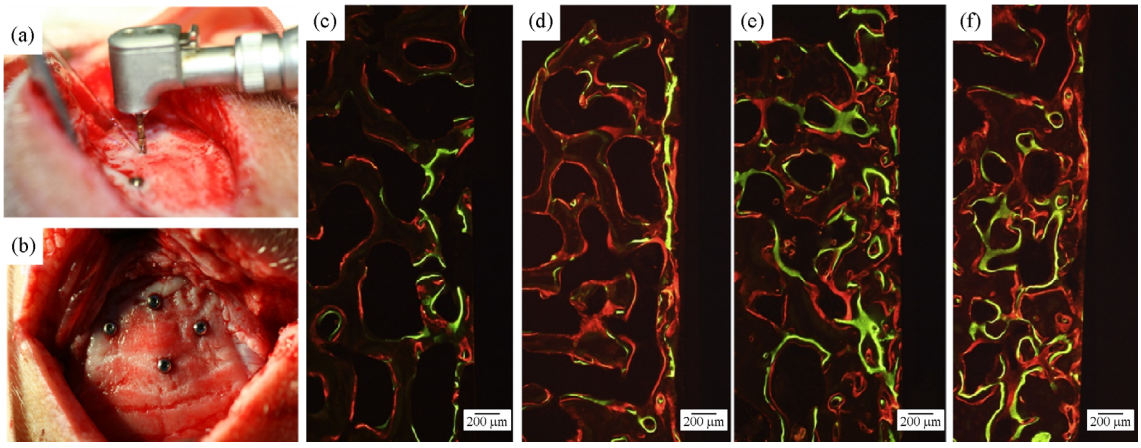

Fig. 8 (a) and (b) placement of four different implants in the frontal skull of minipig and fluorescence microphotographs of (c) machined implant, (d) 30 nm, (e) 70 nm, (f) 100 nm diameter anodized nanotubular titanium implants at 8 weeks of implantation. Markers used in this study were xylenol (orange), calcein (green) and alizarin (red). Alizarin-labeled lines were most visible due to the latest injection of this dye and the xylenol orange-label was hardly detected as a result of bone metabolism. Scale bars are 200 µm. Images are adapted with permission from reference [79]

failure issues observed for currently-used implants. Of course, these animal experiments only address biological response of bone tissue to anodized titanium implants up to a couple of months. Longer term and more detailed studies using larger animal models are required to assess the benefits, as well as unexpected failure mechanisms of using anodizing titanium and titanium alloys in orthopedic applications.

7 Antibacterial properties of anodized nanostructured titanium alloys

Infection is one of the leading failure mechanisms of orthopedic implants, accounting for 1.5%–2.5% of all hip and knee arthroplasties with an associated mortality rate as high as 2.5% [80]. Bacteria typically enter from the open wound during the implantation procedure. Once bacteria attach onto the surface of an orthopedic implant, they begin to secrete a polysaccharide based extracellular matrix, called biofilm. Though the most effective treatment method for bacterial infection is use of antibiotics, biofilm forming bacteria are 500–5000 times more resistant to commonly used antibiotics than planktonic bacteria, making it extremely challenging to treat biofilm. Besides, the efficacy of antibiotics to treat bacterial infections is decreasing due to the rise of antibiotic resistant bacteria strains, i.e., methicillin resistant *Staphylococcus Aureus* [81]. Thus, development of new antibacterial agents toward which bacteria cannot develop resistance is a pressing issue. For the case of orthopedic implants, development of new implant materials which prevent attachment and growth of bacteria would certainly decrease the number of failed orthopedic implants and improve the quality of life of patients.

One of the potential methods to limit bacterial attachment and biofilm formation is altering the nanophase topography of the implant surfaces. One of the first studies concerning the antibacterial properties of nanophase materials was completed by Colon et al. [82] where TiO_2 and ZnO surfaces were fabricated to possess nanostructures that decreased *Staphylococcus epidermidis* adhesion compared to their micro structured counterparts. Several studies investigated nanotubular titanium for its antibacterial properties. Heat treated nanotubular titanium having 20 nm diameter feature size (Anod_20_HT) presented a significant decrease in *S. epidermidis* and *S. Aureus* growth compared to heat treated 80 nm diameter nanotubular titanium (Anod_80_HT) and conventional titanium after 24 h of culture (Fig. 9(a)) [82]. The obtained difference in colonies counts between Anod_20_HT and conventional titanium were shown in the Fig. 9(b). In fact, when the samples were sterilized with UV radiation rather than ethanol immersion or steam autoclave, the lowest *S. epidermidis* and *S. Aureus* colony counts were observed (Fig. 9(a)). Interestingly, the highest *S. epidermidis* and *S. Aureus* colony counts were observed on Anod_80_HT compared to other samples. This data was in accord with Wang et al. [79] where nanorough titanium surfaces fabricated with electron beam evaporation express lower bacterial attachment compared to anodized 80 nm diameter titanium samples. This relation pointed toward a feature size dependent attachment of *S. epidermidis* and *S. Aureus* onto anodized nanotubular titanium surfaces, yet the underlying mechanism was not clear.

Ercan et al. [67] also investigated *S. aureus* biofilm formation on anodized 80 nm diameter nanotubular surfaces. The results showed higher biofilm formation on anodized nanotubular samples at 1, 4 and 8 h of culture. However, the observed trend up to 8 h began to change

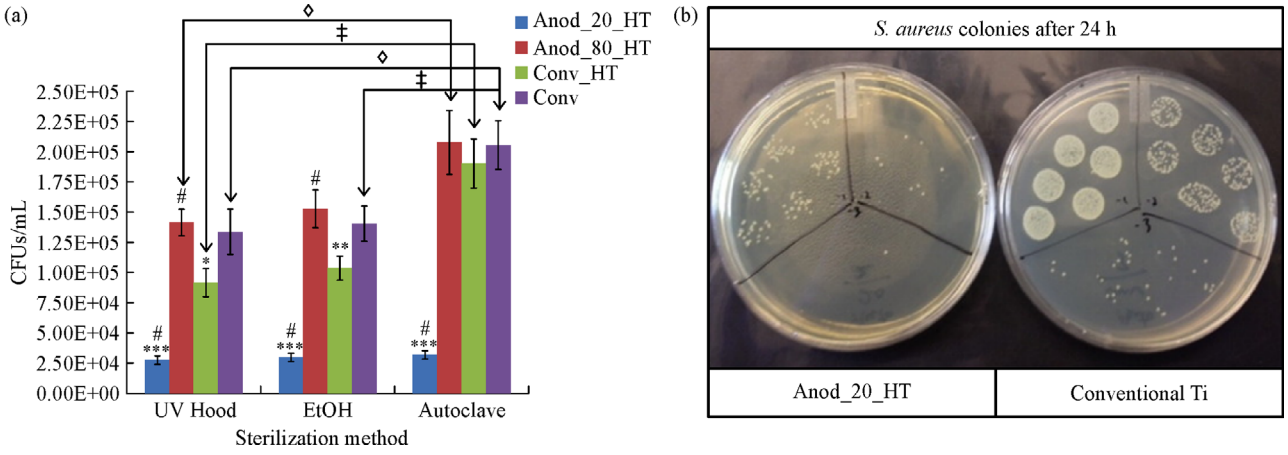

Fig. 9 (a) *S. aureus* growth after 24 h of culture on Anod_20_HT, Anod_80_HT, Conv_HT, and Conv Ti samples sterilized using ultraviolet light, ethanol immersion or steam autoclave. Values are mean±SEM, $N = 3$, * $p < 0.10$ compared to conventional Ti, ** $p < 0.05$ compared to conventional Ti, *** $p < 0.01$ compared to conventional Ti and # $p < 0.05$ compared to Conv_HT within each respective group. ◊ $p < 0.05$ compared to respective autoclaved samples; and ‡ $p < 0.05$ compared to respective autoclaved samples. Images are adapted with permission from reference. (b) *S. aureus* colonies grown on Anod_20_HT and conventional titanium (Conventional Ti) sterilized with EtOH immersion after 24 h of culture [82]

after 1 day where similar amounts of biofilm were observed on both anodized and conventional titanium samples. At 2 days of culture, a reverse trend was observed where there is more biofilm on conventional titanium samples compared to anodized 80 nm diameter nanotubular surfaces (Fig. 10). The reason for reduction in biofilm formation on 80 nm diameter nanotubular titanium surfaces was associated with the use of fluoride as an electrode during anodization process, which forms a titanium fluoride based water soluble layer on the anodized surfaces and gradually released to the culture media with time.

Peremarch et al. [83] studied the adhesion of *S. epidermidis* and *S. Aureus* onto anodized Ti6Al4V surfaces. Results showed greater adhesion of *S. epidermidis* than *S. Aureus*, independent of the surface treatment. However, depending on the strain of bacteria decrease in surface coverage for both *S. epidermidis* and *S. Aureus* was apparent on 20 and 100 nm diameter anodized nanotubular Ti6Al4V compared to conventional Ti6Al4V surfaces. Importantly, this study was in agreement with previously discussed ones with titanium where presence of fluoride decreased bacterial colonization on the surfaces and 20 nm diameter anodized nanotubes had the best antibacterial properties against clinical and non-clinical bacteria strains tested in this study [82].

To enhance the antibacterial properties of anodized nanotubular titanium, the fabricated nanotubular features were used as reservoirs for drug release. Zhao et al. [84] anodized titanium to form 50, 70 and 100 nm diameter nanotubular features and loaded silver onto the nanotubular features using a photo-reduction method for silver ion release from the nanotubular features. Silver loaded nanotubes were tested for antibacterial properties against *Escherichia coli* and *S. Aureus* in dark and under UV light irradiation. The results indicated that silver coated nanotube exhibited better antibacterial efficacy both against *E. coli* and *S. Aureus* independent of the nanotubular diameter. Furthermore, UV light irradiated samples exhibited the highest antibacterial efficacy. Contrary to the previously discussed studies, these experiments showed titanium samples having 100 and 50 nm diameter nanotubular features to have the highest and lowest antibacterial efficacy. Although the reasons behind these findings are not clear, changes in nanophase topography upon silver deposition onto the surfaces could be an influential parameter. To control drug release profile from the nanotubular features, Wang et al. [85] spin coated the surfaces with poly lactic co-glycolic acid (PLGA) after incorporation of model drug ibuprofen. By altering the size of nanotubular titanium from 80 to 140 nm and altering the concentration and thickness of the PLGA cap on the nanotubes, authors successfully expand the duration of drug release more than 5 folds, making it a potential approach to release antibacterial agents from the nanotubular reservoirs.

Multiple studies from different research groups all indicate antibacterial properties of nanotubular structures grown on titanium and titanium alloys. Though there are differences between the antibacterial efficacies of nanotubular features, these differences can be attributed to using different strains of bacteria as well as different anodization parameters, which alter the physical and chemical properties of the surface. Although the antibacterial efficacy of anodized titanium implants have not been investigated using *in vivo* animal models, preliminary *in vitro* studies highlight its potential to fight infection in orthopedic applications.

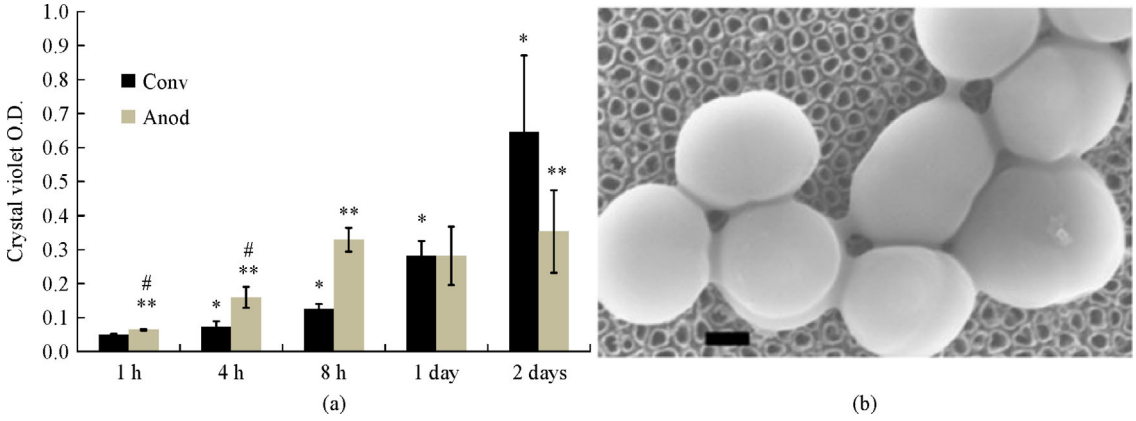

Fig. 10 (a) *S. aureus* growth on conventional (Conv) and nanotubular (Anod) titanium surfaces up to 2 days of culture. * $p < 0.01$ compared to conventional titanium; # $p < 0.01$ compared to nanotubular titanium samples; ** $p < 0.01$ compared to conventional titanium and (b) SEM images of *S. aureus* on nanotubular titanium surfaces. Scale bar is 2 μm. Images are adapted with permission from reference [67]

8 Potential drawbacks of using anodized nanostructured titanium alloys in orthopedic applications

Though there are biological advantages to use anodized nanostructured titanium alloys for orthopedic applications, several material based issues have to be addressed before they can be offered for clinical trials. For instance, anodically grown oxide on titanium alloys suffer from weak delamination strength between the metallic substrate and the surface oxide. Since implantation of orthopedic implants is an aggressive procedure involving exertion of mechanical loads onto the nanostructured oxide, i.e., press fitting to secure the implant into the bone, anodized surface films grown on titanium alloys should be able to withstand the implantation procedure without premature delamination. The delaminated oxide films in the body might potentially act like a wear debris and stimulate immune system and osteoclasts, leading to bone resorption and aseptic failure of the implant. Besides, once nanofeatured oxide film delaminates, the underlying titanium alloy substrate cannot provide the advantages of nanofeatured surfaces have to offer. Thus, findings regarding enhanced biocompatibility and bioactivity of the anodized nanotubular surfaces may only be limited to the *in vitro* experiments and fail to translate into successful clinical results. To enhance the delamination strength of the oxide layer, Crawford et al. [86] altered the thickness of the nanotubular oxide layer. The results showed that thin oxide films (nanotubular length up to 250 nm) were susceptible to delamination, while increase in thickness up to 600–650 nm improved adhesion strength. The authors suggested that increased thickness of the nanotubes retarded stress-build-up during the loading cycle of the nanoindentation test. Ossowska et al. [45] confirmed these results where delamination of nanotubular features on Ti-13Zr-13Nb alloy did not occur for 600 nm long nanotubes and elasticity of longer nanotubes was proposed as a potential mechanism. Another approach to enhance the adhesion strength of the nanotubular surface film was treating the anodized surfaces with non-protonated polar substances, i.e., petroleum ether and cyclohexane [87]. Zhao et al. [87] proposed hydrogen-assisted cracking mechanism as the main mechanism for oxide layer detachment from the substrate in which protons at the metal-metal oxide interface control the cohesion of the oxide layer and the underlying metal and suggested that solvents with high H_2 solubility could eliminate hydrogen-assisted cracking.

Having this said, depending on the anodization conditions (electrolyte type, concentration, voltage, temperature, etc.) and post-heat treatment temperature/duration nanofeatures with different physical and chemical properties can be fabricated. Currently, there is not enough information eliciting the effect of different anodized surface morphologies, topographies and chemistries on bone cell response. For instance, it is still not clear if tapered nanotubes or smooth nanotubes provide best osseointegration. Additionally, researchers used cell sources having different origins (i.e., species, extraction protocols, differentiation levels, etc.) to assess biological properties of the anodized surfaces, contradicting results are common [58,63]. Considering that minute changes in anodization parameters result in different physical and chemical properties for the oxide films, there is no consensus on the optimal physical and chemical properties of nanofeatures to enhance bone cell functions. Besides, studies regarding vascular growth, osteoclast functions and immune cell response on these surfaces are very limited. To successfully translate anodized titanium and titanium alloys for clinical use and benefit from the advantages that nanofeatures titanium films have to offer, a better understanding of the cellular response on anodized titanium

alloys, followed by detailed *in vivo* large animal studies are required. Only then scientists can have a better understanding of the advantages and potential hazards of using anodized titanium and titanium alloys for orthopedic applications.

9 Conclusions

Titanium and its alloys exhibit optimal mechanical properties, corrosion resistance and biocompatibility, yet they fail to provide osseointegration with the juxtaposed bone tissue due to their bioinert nature. Surface modification of these materials is required to improve their osseointegration with the juxtaposed bone tissue. Anodization is an electrochemical surface modification technique which provides control on surface topography, feature morphology and chemistry. By applying a constant voltage between the titanium alloy connected to the anode and an appropriately chosen cathode material, nanofeatures on the titanium alloys can be fabricated. In fact, it is possible to tailor surface properties, i.e., nanotubular morphology, diameter, feature size, etc., that alter the interaction of the surface with bone cells by controlling the electrochemical anodization parameters. *In vitro* tests, investigating nanostructured oxide films observed enhanced osteoblast and MSCs adhesion, proliferation and functions on titanium and its alloys compared to their conventional counterparts. As nanotubular feature size on titanium and titanium alloys decreased, osteoblast and MSC adhesion and proliferation increased, whereas cellular functions and osteogenic functions increased on nanotubular features having larger diameters. Animal experiments confirmed improved bone-implant contact and osseointegration with the use of anodized titanium implants compared to conventional titanium implants. *In vivo* experiments further indicated that anodized nanotubular titanium having 70 nm diameter to exhibit optimal bone formation and osteogenic gene expressions, making it a promising material for future studies. Though more detailed studies are required to translate anodized titanium and titanium alloys for clinical applications, *in vitro* and *in vivo* studies point to enhanced bone cell functions and osseointegration of anodized titanium surfaces, making them an ideal candidate to reduce orthopedic implant failure rate, and thus improve lifetime of orthopedic implants. Moreover, in an attempt to prevent bacterial infections on titanium implants, surface roughness can be altered from micronscale to nanoscale and results in the reduction of bacterial infections on nanoscale roughness with titanium samples. In addition to this, nanotubular diameters with 20 nm and loading Ag to nanotubular titanium structures and coating with PLGA on nanotubular titanium surfaces in several diameters can indicate the most promising features for antibacterial properties on titanium implants.

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