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Advances in calcium phosphate coatings – anodic spark deposition: a review

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1. ABSTRACT

High voltage anodization of titanium in the presence of an electrolytic medium containing calcium and phosphate ions has shown improved osteointegration and biocompatibility compared to untreated titanium. Processing parameters influence the unique porous microstructure developed during anodization. These parameters tailor the specific properties of the surface to achieve improved osseointegration of an implant. In addition, subsequent treatment following anodization further alters the microstructure. Numerous studies have examined the influence of these properties on the cellular response and the mechanical properties in terms of coating adhesion and pull-out strength in bone; however, there are conflicting reports on the cellular responses. This review examines those processing parameters and the related influence on cellular responses and mechanical properties. In addition, this review provides a summary of published reports regarding the work related to the advancement of calcium phosphate coatings achieved through high voltage anodization.

2. INTRODUCTION

Over the past sixty years, titanium has been investigated for biomedical applications based on its good biocompatibility (1, 2), stability (2-4), and mechanical properties (2, 3). In fact, titanium and titanium alloys osseointegrate with the surrounding bone without forming a fibrous intermediate layer between bone and the implant (5). Fibrous intermediates can delay healing and result in micromotion, ultimately leading to implant failure. However, research has also suggested that the cytocompatibility of titanium prevents long term implant functionality (6).

Several researchers have suggested that the high degree of biocompatibility of titanium is due to the presence of titanium oxide, TiO_2, at the bone-implant interface (3, 7-13). TiO_2 is naturally and spontaneously formed on the surface of titanium in ambient air, as well as in oxidizing media such as human body fluids (10). The natural oxide layer is approximately 5 nm thick, is self-healing and prevents direct contact of bone to titanium (14,

15). TiO_2 is responsible for the excellent corrosion resistance of titanium implants (6, 16), and is also believed to increase cellular attachment (16, 17). The oxide layer changes the surface characteristics of the titanium, and surface energy, structure, crystallinity, chemical composition and roughness, which all influence cellular responses (13, 15, 18-20). Additionally, the crystalline phase of the TiO_2 influences cellular response and will be discussed. Thicker titanium oxide layers have favorable cellular responses (11). However, the native TiO_2 layer alone does not significantly improve osseointegration. Therefore, increased roughness and incorporation of a thicker titanium oxide layer is ideal for titanium biomedical implants.

Calcium phosphates are often applied to titanium in an effort to improve its osteoconductive properties (5, 21-25) and improve the bone-implant interface. Rapid bone formation with calcium phosphates (Ca-P) enhances the strength between bone and coatings via chemical bonding. Calcium phosphates come in many different chemical formulas with the most well-known being hydroxyapatite. The difference between the types of calcium phosphates is the Ca/P stoichiometric ratio, as hydroxyapatite has a Ca/P ratio of 1.67 with ranges between 1.5 and 1.7. Hydroxyapatite has been reported to enhance bone formation due to the chemical similarity of bone mineral (5, 26). Commercial hydroxyapatite is often plasma sprayed on a grit-blasted surface generally to a thickness of 100 to 150 microns (27). This method relies on interlocking at the coating-substrate interface; however, the bond strength between the coating and substrate must be higher than the bond strength of the coating and substrate interface (22). Thus, the low adhesion strength (5, 21-24) from mechanical interlocking at the implant-coating interface via plasma spray may be an inadequate coating design in plasma spray. Additionally, the weak interface produces HA debris at the surface (28). Plasma-spray creates micron-grain sized HA, whereas nano-grain sized HA, as developed from anodization, improves bone growth and is more natural. Finally, a lower crystallinity results in a higher dissolution of the coating (29), lowering implant success.

Therefore, alternative methods to plasma spray are of particular interest for biomedical applications. Surface modification that integrates calcium phosphates with titanium oxide may provide an optimal coating layer for bone applications. One possible method to achieve this structure is through the anodization of titanium. This review focuses on the work that has been completed related to titanium surface modification by depositing calcium phosphates through anodization. Although there has been a renewed interest in this area, no recent review exists for this topic (15). This review is intended to report the current trends of anodization with calcium phosphates and their subsequent investigation for orthopedic applications.

2. ANODIZATION

Anodization is an electrochemical process, which modifies the surface of a metal. For titanium anodization, the applied voltage at the anode excites the metal, electrons enter into the aqueous medium, and leads to the formation of titanium oxide, TiO_2, as described by the following set of equations:

Anode: $Ti \rightarrow Ti^{4+} + 4e^-$
Cathode: $4H_2O + 4e^- \rightarrow 4OH^- + 2H_2$
$Ti^{4+} + 2H_2O \rightarrow TiO_2 + 4H^+ + 4e^-$

As the applied external field is passed through the titanium at the anode, the growth of the oxide occurs through redox reactions. A thicker oxide has a higher resistivity and requires more voltage to continue growing until the dielectric breakdown is reached (15). The dielectric breakdown depends on the electrolyte and varies between 100 and 160V for titanium (15). Continual increase of the voltage results in visible “micro-arcs” or “sparks” rapidly occurring at the surface (11, 15, 30). This breakdown is known as micro-arc oxidation (MAO) (11, 31) and anodic spark deposition or discharge (ASD) (15, 30, 32, 33) in the literature, and is responsible for forming a porous microstructure at the surface. Low voltage anodization often leads to the anatase form of the oxide, while high voltage leads to rutile (34). It has been reported that anatase possesses antibacterial properties (35). Additionally, the micropore formation at the dielectric breakdown allows for the integration with ions from the electrolyte solution. For example, calcium and phosphate ions can be integrated within the microporous structure of titanium oxide formed through ASD. Thus, the cellular response of titanium may be improved through high voltage anodization by the following: (1) forming thicker titanium oxide layers, (2) increasing the roughness, (3) improving the chemical function of the microporous surface, and (4) creating nanometer-scale surface features.

3.1. History

The first work involving the integration of calcium phosphates with titanium by anodization was introduced by Ishizawa, *et al.* (22). In this research, the aqueous electrolyte medium used during anodization (150 – 400 V at 50 mA/cm^2) of commercially pure titanium contained sodium beta-glycerophosphate (beta-GP) and calcium acetate (CA). The resulting film was referred to as an anodic titanium oxide film containing calcium and phosphorous (AOFCP). Through this method, it was shown that there was a similar Ca/P ratio to hydroxyapatite in the AOFCP layer. Other work by the same group focused on determining the optimal molar concentration of CA to develop a film with a stoichiometric Ca/P ratio similar to HA with 0.04 M beta-GP anodization at 350V (21). In addition, other groups have characterized AOFCPs and explored biological responses (5, 11, 17, 19, 36-40), which will be reviewed later. ASD has also been investigated with other electrolytic media, such as phosphate anions and calcium cations followed by a subsequent anodization in calcium cations (41) and followed by alkali etching (32, 33, 41-43); ethylenediamine tetraacetic acid (EDTA) as a chelating agent to the Ca-P to increase the solubility of the calcium (44); $Ca(H_2PO_4)_2$ at a spark voltage ~165V (30); acetic acid, phosphoric acid, calcium hydroxide and sodium hydroxide up to 100V (45); as well as H_3PO_4 up to 350V (39) or up to 180V (46). Other anodization methods have

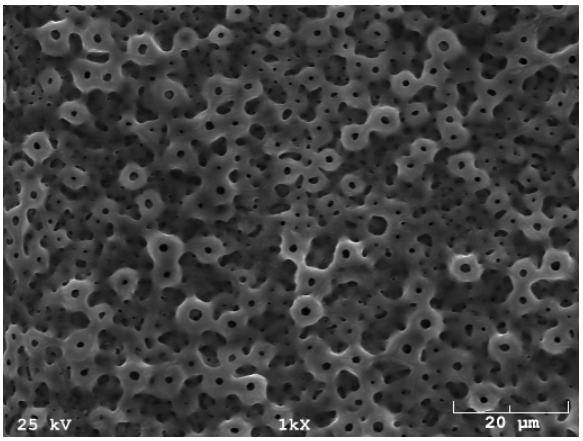

Figure 1. SEI-SEM plan view image of the anodized Ti surface containing Ca-P ions.

Table 1. Pore development observed on the anodized surface for various anodization voltages in 0.15M CA and 0.02 Ca-GP, as reported by (11).

Voltage, V	Microstructural Features
<190	No porosity
190<V<230	Pores first formed
230<V<270	Increased number of pores Non-uniform porosity
270<V<350	Uniform porosity Surface composed of craters
350<V<450	Pores and craters interconnected Microcracks observed
450<V<600	Linear growth of pores and craters

been developed at 20V in an electrolyte containing HF resulting in nanotubular titanium oxide with excellent cellular responses (6, 47-53), in an electrolyte containing HF and $(NH_4)_2SO_4$ (54), in various fluoride containing electrolytes (55), as well as between 10 and 25V in electrolytes with buffered fluorides KF and NaF (56), and at 60V with H_3PO_4 (57). However, due to the numerous approaches of electrolyte compositions used during ASD, this review will attempt to focus on those that incorporate calcium-phosphates in the anodic film.

3.2. Nanostructural, microstructural, chemical and mechanical properties

Material biocompatibility and bioresponses can be improved based on the structural, chemical and mechanical properties at the bone-implant interface. In anodic oxidation, these features are controlled based on processing parameters associated with the anodization such as the electrolyte, current density and anodization voltage. Specifically, the thickness, porosity, TiO_2 crystalline form and chemical composition, as well as the roughness can be designed for optimal biointegration based on the anodization processing parameters.

A typical microstructure after ASD (350V) with titanium in beta-GP and CA is represented in (Figure 1). As shown, homogeneous micropores with diameters in the range of 1 - 2 microns are formed at the surface. Spark anodization is a plasma-like electrochemical process (30) that is responsible for the increased thickness of the oxide layer due to localized melting during the sparking at dielectric breakdown. One group has reported micro-crack formation with an increased thickness layer of the coating (11, 26, 38). In the dielectric breakdown, Ti^{2+} ions from the implant and OH^- ions in the electrolyte move in opposite directions to reform TiO_2 (11). With Ca-P present in the electrolyte, the ions embed during the melting phase of dielectric breakdown. Thicker oxide coatings and incorporation of Ca-P ions enhances biocompatibility properties (45). In fact, the incorporation of Ca-P into the layer has been shown to increase the adhesion to titanium, compared to plasma sprayed HA coatings using ISO test 13779-2 (58). In addition to the bone promoting Ca-P ions, roughness is also increased on the anodized titanium, which is known to increase bone formation.

Typically, anodization is conducted in a constant current mode; however, the type (alternating or direct) of current reportedly has no influence on the ASD outcome (59). As the voltage linearly increases, the thickness of the oxide initially increases linearly, until the electric field is not strong enough to force electrons through the oxide. If the voltage increases, dielectric breakdown occurs (38), and rate of this growth is ~1.5 – 3 nm/V, depending on the electrolyte (10, 38). The trend of increased oxide thickness with increased anodization voltage has been reported elsewhere (15, 22, 38, 45), and thicker oxide films have been suggested to have improved biocompatibility (45).

Li *et al.* (11) examined the influence of applied DC fields (190 – 600V) for 3 minutes on titanium specimens anodized with 0.15 M CA and 0.02 Ca-GP on the film microstructure. A summary of the microstructural changes for increased anodization voltages are shown in (Table 1). Below 190V, a thin oxide layer was observed with no porosity, indicating the possible retention of the dielectric strength up to this applied field. However, above 190V, pores were formed in the oxide layer and above 230V, the entire surface was porous. Upon further increases to 270V the layer was not uniformly porous, at 350V microcracks were observed and the pores became interconnected (as in Figure 1), at 450V the holes were larger and appeared less integrated with the film, and the same trend was observed until the final voltage of 600V. In addition, it was observed that the thickness of the layer was ~1 micron at 230V, ~2 microns at 270V, ~7 microns at 350V followed by linear growth to ~14 microns at 600V. Anodic films on titanium formed at 350V in an electrolyte containing 0.1M CA and 0.02 – 0.05M beta-GP had interconnected pores of 0.5 – 2 microns in diameter (38). Increasing the voltage in that study resulted in an increased pore diameter, as previously described by (11).

The anodization voltage also influences the Ca/P ratio expressed in the film, such that the value is increased up to ~400V (depending on the particular electrolyte), but decreased beyond 450V (60). Furthermore, (39) showed that the Ca/P ratio for 0.15M CA and 0.03M Ca-GP continually increased from 0.33 at an anodization voltage of 140V to 1.00 at 300V, as measured by XPS. In a study of various combinations of CA and calcium-GP at anodization voltages above 300V and varying current densities, (19) showed that the combination of 0.02M Ca-GP and 0.15M CA resulted in near stoichiometric values of

Table 2. Summary of Ca/P ratios obtained from various anodization voltages and electrolyte concentrations of beta-GP and CA. Approximate values are estimated from graphs

	Electrolyte		Voltage	Approximate
Study	beta-GP (M)	CA (M)	(V)	Ca/P
(60)	0.04	0.40	250	1.3
			300	1.6
			350	1.7*
			400	1.8
			450	1.5
(21) *Anodization only	0.04	0.1	350	0.60
		0.2		1.55
		0.3		2.40
		0.4		3.25*
		0.5		3.75
(21) *Anodization + hydrothermal treatment	0.04	0.1	350	0.60
		0.2		1.40
		0.3		1.45
		0.4		1.50
		0.5		2.25

*A large variation is noted for identical anodization voltage and electrolyte concentration

Table 3. Summary of Ca/P ratios obtained from various anodization voltages and electrolyte concentrations of Ca-GP and CA. Influence of current density on Ca/P ratio is also described. Approximate values are estimated from graphs.

	Electrolyte		Voltage	Approximate
Study	Ca-GP (M)	CA (M)	(V)	Ca/P
(39)	0.03	0.15	140	0.33
			200	0.40
			260	0.67
			300	1.00
(19)	0.03	0.15	362	1.27
	0.02	0.15	340*	1.63
			348**	1.69
	0.04	0.10	362*	0.55
			365**	0.76

Current density *50 A/m^2 and **70 A/m^2.

HA at 1.63 and 1.69 with current densities of 50 mA/cm^2 and 70 A/m^2. However, this also suggests that a slight increase in the Ca/P ratio was observed with a higher current density, and in fact, was observed for all combinations with identical electrolytes at differing current densities. The Ca/P ratio was consistently four times higher for electrolytes containing EDTA compared to the standard Ca-P electrolyte (44).

In addition to the anodization voltage and current density, the particular electrolyte plays an important role in determining the properties of the oxide film. Acid and non-acidic mediums have been used during anodization of titanium (38); however, the focus in this review is on electrolytes such as beta-GP, CA, $Ca(H_2PO_4)_2$, H_3PO_4 and Ca-GP that are capable of depositing Ca-P into the oxide layer. In addition, anodization in the presence of HA nanoparticles has also been investigated (61) (37). During anodization, as described by Kim, calcium ions are repelled by the polarized titanium surface during anodization due their positive charge, while phosphate ions are attracted to the positively charged surface (38). This deficit leads to a low Ca/P ratio, but can be controlled by proper modification of solution concentrations. A summary of the Ca/P ratios obtained by anodization in electrolytes beta-GP and CA is shown in (Table 2), while (Table 3) shows the summary for Ca-GP and CA. Interestingly, work by two different groups using the same electrolyte concentrations (0.04M beta-GP and 0.4M CA) at 350 V obtained very different Ca/P ratios: 3.25 (21) and 1.7 (60). Both of these results were obtained by analyzing the Ca/P ratio with energy dispersive x-ray spectroscopy (EDS)

For example, in the preliminary work by Ishizawa, H_3PO_4 was used with various calcium containing compounds and the Ca/P ratio varied from 0.02 with lactate to 0.33 with GP (62). Ishizawa later showed that by a combinatorial approach to the selection of beta-GP and CA, the Ca/P ratio could be modified to that near HA. The amounts suggested by Ishizawa to achieve a film with complete conversion across the surface and a stoichiometric Ca/P ratio near that of HA was 0.03–0.05M beta-GP with 0.25 CA or 0.06 beta-GP with 0.3 CA (21). Additionally, through this work, it was also observed that the film thickness was influenced by the electrolyte, such that increased beta-GP resulted in increased anodic film thickness. For example, with 0.03M beta-GP, the thickness of the film formed after 350V with subsequent hydrothermal treatment was 9.0 microns but with 0.05M beta-GP the thickness was 10.5 μm. The thickness influences the adhesive strength, such that increased charge-transfer results in a decrease in the adhesive layer (44). Furthermore, anodization in 0.025M $Ca(H_2PO_4)_2$, 0.075M $Ca(OOCCH_3)_2$ and 0.12M $Na_2(EDTA)$ with currents between 80 and 290 mA resulted in films that all passed ISO 13779-2 for adhesive strength of hydroxyapatite coatings. Additionally, (21, 22) found that the adhesive strength at the interface increased with decreased electrolyte concentration. At lower concentrations, fewer Ca and P ions disrupted the normal bonding of the oxide layer. At the lowest electrolyte concentrations reported by (21), the adhesive strength reached ~40 MP, and they reported that the adhesive strength of plasma sprayed HA on titanium was 10 – 20 MPa (63). The increase in adhesive strength resulted from the chemical bond formed in the AOFCP, compared to the mechanical locking mechanism associated with the HA plasma-spray application. In fact, the adhesive strength was not altered over a 300 day soak time in SBF (62). However, Chiesa *et al.* (15), claimed that the Ishizawa method contained a number of unresolved inherent issues such as poor adhesion of the outer film layer.

In addition to the influence on the Ca/P ratio and film thickness, the electrolyte also affects the crystallinity of the film. For example, (64) reported that a higher beta-GP concentration of the titanium resulted in lower crystallinity characterized by X-ray diffraction (XRD). XRD is commonly used to assess the crystallinity of the film surface and to determine the titanium oxide phase at the surface. Anatase is reported to form at low voltages ~190V depending on the electrolyte, while rutile is detected by XRD above 270V (11). Continued increases in the voltage resulted in increased intensity of rutile peaks.

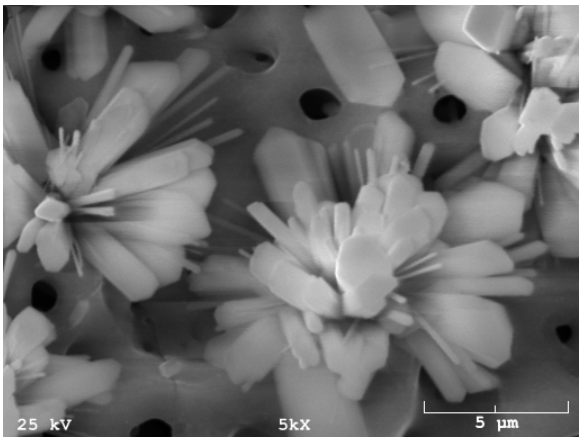

Figure 2. SEI-SEM plan view image of the anodized surface after 20 hours of hydrothermal treatment at 225ºC in high pressure steam.

The increased surface roughness of an implant encourages bone formation. ASD of titanium is an excellent processing method to develop a roughened surface compared to smooth titanium. The roughness of the anodic film formed by ASD was reported to be 0.3-1.5 microns (17, 38). The roughness was reported to increase rapidly above 300V (11).

4. ANODIZED FILM TREATMENT

After anodization, the implant can be subsequently treated to tailor a bioresponse. For example, hydrothermal treatment for the conversion of the AOFCP into a crystalline HA is one such method. Other groups have developed alternative subsequent processing methods for the conversion of HA to enhance the bone response at the interface. Alternative methods for subsequent treatment to anodization include submersion in alkali etching in potassium hydroxide (KOH) or sodium hydroxide (NaOH) and high pH levels. Furthermore, as an initial bioresponse evaluation, the anodized surface is often soaked in simulated body fluid (SBF). Interestingly, after SBF soaking, a negative charge is developed on the anatase form of the oxide and is suggested to facilitate the absorption of calcium ions then phosphate ions from subsequent solutions (34). Therefore, in addition to the preliminary evaluation in plasma-like solution, SBF soaking may also influence the *in vivo* response, as the surface chemistry is altered.

4.1. Hydrothermal processing

Hydrothermal processing by Ishizawa *et al.* was performed in high pressure steam at 300ºC for 2 hours in an autoclave (22). After hydrothermal conversion, many HA crystallites precipitated on the surface; however, the crystallites were very inhomogeneous across the surface. For example, (Figure 2) shows the microstructure of a film after hydrothermal conversion in high pressure steam. To induce high pressure steam, a hydrothermal treatment was performed at 200ºC in a Parr® acid digestion bomb (#4748, Moline, Illinois) with 7 mL of deionized H_2O in a 125 mL polytetrafluoroethylene (PTFE) liner at 200ºC for 20 h. The AOFCP in (Figure 2) was developed at 350V with 0.04 M beta-GB and 0.25M CA (65), and as shown, the nucleated Ca-P rich crystals are inhomogeneous and very coarse. Coarsening of the crystallites is observed and faceted planes are developed with the longer processing time compared to the commonly reported 2 hours. Unlike other reports of the crystallites having hexagonal morphologies (23, 66), the nucleated Ca-P rich regions have a plated microstructure.

Ishizawa reported that the Ca/P ratio of both the AOFCP and hydrothermally treated film increased with increasing CA (0 to 0.5M) concentrations with a constant 0.04M beta-GP electrolyte solution (21). Concentrations between 0.15 and 0.35M CA resulted in HA crystal nucleation; however, a diminishing rate of return was observed between the Ca/P ratio of the AOFCP and the hydrothermally treated film in this range. In a later study by the same group, the effect of hydrothermal temperature (250ºC or 300ºC) was examined on the film with anodization up to 350V with electrolyte concentrations of 0.01M beta-GP and 0.15 CA, and it was found that at lower processing temperatures, the HA crystals were in an earlier phase of growth compared to higher processing temperatures (66). Furthermore, the amount of water:volume ratio altered the resulting precipitated HA crystallites. In their study, (66) determined that 15 vol% water always resulted in uniform HA nucleation, while the HA nucleation at 46 vol% was always non-uniform. However, the microstructure of the hydrothermal treatment with 15 vol% water contained many faceted crystals that did not appear to be homogenous. Alternatively, at 46 vol% the microstructure was both non-uniform and non-homogeneous. The smaller water vol%, according to the author, allowed for easier leaching of Ca^{2+} and $(PO_3)_4$ from the film to form the nucleated HA compared to the 46 vol%. The HA rich crystallites formed after hydrothermal treatment in high pressure steam were ~ 5 to 10 µm. Additionally, the authors never described the pressure during the hydrothermal treatment, only that it occurred in high pressure steam. The hydrothermal processing led to both an overall increase in film crystallinity and an increased concentration of calcium and phosphorus on the crystallites. Building on Ishizawa's hydrothermal processing (65) found that smaller Ca-P rich crystallites (~500 nm) were developed on the surface, as shown in (Figure 3), after hydrothermal treatment in solution with Ca and P ions. This hydrothermal process prevented the leaching of Ca-P from the AOFCP and prevented the nucleation of the large faceted Ca-P rich crystallites as observed in hydrothermal processing with water alone. This hydrothermal treatment was performed at 200ºC in the same acid digestion bomb with 44.5 mL of solution in the PTFE liner for 20 hours followed by a subsequent hydrothermal treatment at 225ºC with 7 mL of deionized H_2O in the same liner for 20 hours.

The porous microstructure developed during anodization was covered by Ca-P rich crystals. This treated layer is often described as hydroxyapatite; however, many authors do not obtain values in the range of stoichiometric

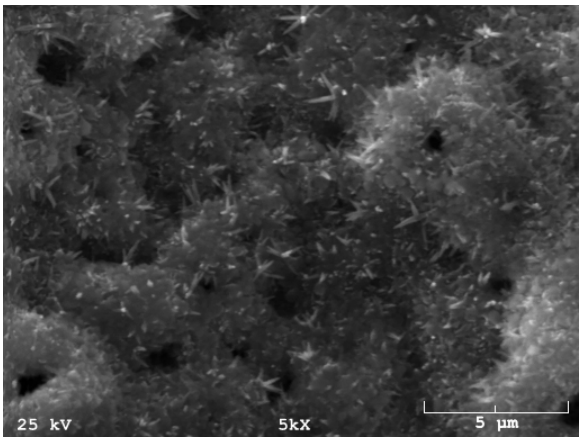

Figure 3. SEI-SEM plan view image of the anodized surface after 20 hours of hydrothermal treatment at 200 ºC in a Ca-P containing solution followed by a 20 hours hydrothermal treatment at 225ºC in high pressure steam.

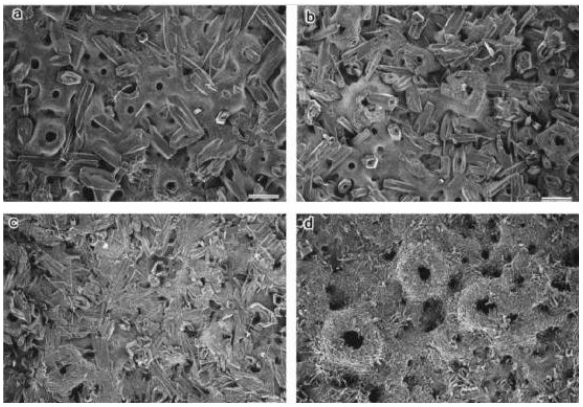

Figure 4. Surface microstructure after anodization at 350V in 0.04 beta-GP and a) 0.15M CA b)0.20M CA, c) 0.25M CA, and d)0.30M CA. HA rich crystallites are finer with increased CA concentration. All substrates were hydrothermally treated in high pressure steam at 300ºC. Reproduced with permission from (21).

HA (1.5 – 1.7). Near stoichiometric HA crystals have been reported to increase with decreasing beta-GP concentrations (21). As previously described, hydrothermal process parameters influence the surface covered, such that the temperature, time, vol% of water and anodization conditions all influence the final microstructure. In general, after hydrothermal treatment in high pressure steam at 300ºC, (22) found a HA rich layer on the AOFCP 1 to 2 microns thick. However, the resulting microstructure was not homogenous, as the Ca-P crystals nucleated out of the AOFCP. The nucleation may be attributed to an insufficient amount of Ca and P in the AOFCP prior to treatment; other work has suggested that hydrothermal treatment in medium containing Ca-P ions prevented this nucleation (65). The surface microstructure was examined under SEM, and it was found that HA crystals were finer with increasing Ca/P ratios (21). As shown in (Figure 4), anodization in 0.04M beta-GP with increasing amounts of CA concentration resulted in progressively finer HA crystallites; however the crystallites from the highest CA concentration (0.30M) were ~ 2-3 microns in length. The authors further suggested that the Ca and P ions moved to the surface during hydrothermal treatment for crystallization with hydroxyl groups in the high pressure steam. The pressure of the steam during hydrothermal treatment was not reported. The Ca/P ratio was 1.48 in the films, which although is near the stoichiometric value of HA, is lower than the generally accepted Ca/P ratio for HA. Although the adhesive strength of these anodized structures decreased with increased electrolyte concentrations, the adhesive strength after hydrothermal treatment was reportedly higher for higher electrolyte concentrations (21).

Through examination of SEM images, the topography changes through the hydrothermal processing of the AOFCP; however, no significant difference was observed in the roughness between anodized and hydrothermally treated surfaces (40). This work utilized a mechanical profilometer (Surftest-SV 402), and may not capture the small peak and valley changes after hydrothermal treatment; in addition, a significant limitation of mechanical profilomerty is that it is only a two-dimensional measuring tool. Due to the three-dimensional features created through the anodization and hydrothermal treatment, three-dimensional optical profilometry or AFM are more suitable to capture the nano-scale roughness. Similarly, after a two-step anodization process followed by an alkali treatment, the surface roughness actually decreased (R_a=0.29 µm) compared to the untreated surface (R_a=0.48 µm) (33). This anomaly is likely attributed to measuring the roughness with laser profilometry with a lateral resolution of 1 µm, which did not capture the sensitivity of the nano-roughness induced during hydrothermal treatment. Therefore, the characterization technique necessary to capture the sensitivity of roughness must be re-examined to include the nano-roughness scale.

In a manner similar to Ishizawa, (40) anodized titanium in 0.02M CA and 0.15M Ca-GP followed by several hydrothermal treatments at 300ºC in high pressure steam and found that anatase and rutile phases were present, and after hydrothermal treatment only trace amounts of rutile were observed with the anatase. As shown in (Table 4), higher Ca/P values were detected on the crystallite regions after hydrothermal processing compared to anodic oxide regions. In addition, the amount of titanium drastically decreases in the crystallite regions (~12 mol%) compared to the anodic oxide region (~40 mol%), as measured by EMPA. Furthermore, no significant difference was observed in chemical composition or roughness between hydrothermal treatments at 2 hours versus 4 hours. The roughness of the untreated titanium was 0.24 ± 0.12 µm, and was significantly different than those of the anodized and hydrothermally treated roughness, shown in (Table 4). There was no significant difference observed in the roughness between the anodized and the hydrothermally treated surfaces, which is surprising based on microstructural appearance. Due to increase in calcium at the surface, and increase in roughness compared to the untreated titanium, the bioactivity of the hydrothermally treated surface is expected to increase.

Table 4. Chemical composition (measured by EMPA) and roughness values (measured by mechanical profilometry) for titanium anodized in an electrolytic solution of 0.02M Ca-GP and 0.15M CA with a current density of 70 mA/cm^2 (40).

Hydrothermal treatment time (h)	Region of interest	Ca/P ratio mol%/mol%	Amount Ti mol%	Roughness-R_a (microns)
0	Anodic oxide	1.70	44.8 ± 0.6	0.73 ± 0.02
2	Crystallite	1.41	10.9 ± 6.7	0.79 ± 0.01
2	Anodic oxide	0.48	47.9 ± 7.0	
4	Crystallite	1.22	14.8 ± 12.7	0.77 ± 0.01
4	Anodic oxide	0.77	38.8 ± 10.9	

4.2. Alkali etching

Alkali treatment of titanium with 0.5M NaOH was described by (67) and was found to increase apatite formation after soaking in SBF. However, the authors state that the coating formed by alkali treated titanium is not mechanically stable, and thus unsuitable for clinical use. They investigated heat treatment of alkali treated titanium above 600ºC and found improved mechanical stability of the coating, but delayed SBF apatite formation after heat treatment at elevated temperatures. The coating stability was assessed in a rudimentary test by applying and removing Scotch® tape to the coating and qualitatively determining the amount of coating peeled away from the surface. Through this interesting assessment, the authors ascertained that since the alkali treatment group without further treatment peeled away and the alkali treatment group with annealed at 600ºC left remnants of the glue from the tape rather than pulling away, the heat treatment resulted in mechanically stable coatings. Other groups have subsequently examined the influence of alkali treatment on anodized titanium containing calcium phosphate (32, 33, 41-43). Alkali etching of anodized titanium has been conducted after a two-step anodization process, as described earlier, by etching in concentrated KOH at 60ºC (32, 33). The mechanical stability of these coatings is not reported. The microstructure of anodized surface after alkali etching is quite different than the as-anodized structure. In the etched structure, the "rings" associated with the anodization are disrupted to create a rougher surface. This has been described as an increased in the sub-micron roughness (42); however, actual values were not reported. In addition to the change in surface roughness, the oxide form of titanium may also be modified, leading to a slight increase in the hardness of the oxide layer after alkali treatment (210 compared to 193 HV for treated and untreated, respectively) (42). These microstructures are expected to have a strong effect on both *in vivo* and *in vitro* osteoblast response. As an attempt to assess this, these etched microstructures have been evaluated using simulated body fluid (SBF), as described next.

4.3. Simulated body fluid soaking

SBF soaking is commonly utilized to assess the ability of a biomaterial to form a mineralized apatite structure, determine the coating stability after a long period of time (i.e., 300 days) or to determine ion concentrations or pH change in the SBF, which would affect a biological response. According to (13), one requirement for *in vivo* success of bone growth on a biomaterial is the formation of a carbonated hydroxyapatite layer on the surface. SBF has a pH of 7.4, similar to that of human blood plasma and concentrations of ions similar to that found in plasma. Many different types of SBF solutions have been developed to assess the mineralization capability of a biomimplant For example, Kokubo's SBF (68) contains the following chemicals added such that the ion concentration is similar to human plasma with a pH of 7.4: $((HOCH_2)_3CNH_2)$, HCl, NaCl, $NaHCO_3$, KCl, K_2HPO_4, Na_2SO_4, $MgCl_2$ and $CaCl_2$.

SBF incubation has been extended to anodized titanium by many researchers. (42) claimed that anodization using Ishizawa and Zuhs' methods did not result in mineralized surfaces after SBF soaking for 14 days. Recently, (69) found that apatite formation on anodized titanium formed at 350 V in 0.01M beta-GP and 0.15M CA after 28 day soaking in SBF. However, (42) two-step method followed by soaking in SBF resulted in a new mineral phase after incubation of only 6 days in Vogel's SBF. This work suggests that the final alkali etching of the anodized microstructure influences the response to ion concentrations similar to that experienced *in vivo*. In fact, this follows work by (67) in that after NaOH alkali etching, apatite was formed on the surface during SBF incubation as a result of OH^- treated Ti releasing Na^+ and forming hydrated titania leading to apatite nucleation (70, 71).

MAO (250 – 500 V) of titanium in 0.04M beta-GP and 0.4M CA found a slight increase in calcium and phosphorous ions after soaking in SBF for up to 14 days, which was followed by a rapid decrease in longer soaking periods (60). Additionally, the pH value increased to 7.6 after soaking in SBF. The authors suggested that calcium and phosphorus ions were released into the SBF solution during the first 14 days then formed precipitates containing these ions. No apatite formation was observed for anodization below 450 V; however, at 450 V, apatite was induced in the oxide layer after SBF soaking. After anodization at 450 V and incubation for 28 days in SBF, carbonated hydroxyapatite was formed on the surface. Other work has found apatite formation after 3 days of SBF soaking with anodization at 155 and 180 V in H_2SO_4 (34). Furthermore, this work found that heat treatment at 600ºC following alkali etching strongly influenced the surface ability to form apatite. Even at 90 V, anodized and heat treated surfaces formed apatite after soaking in SBF for 3 days. This work also noted that for increased electrolyte concentrations (0.5 – 3M H_2SO_4) during anodization, more apatite was deposited on the oxide layer. Interestingly, one idea for tailoring the bioresponse could include SFB soaking prior to implantation, as mineralized apatite layer is nucleated on the surface.

Obviously, the factors involved in cellular studies both *in vitro* and *in vivo* are more complicated than SBF soaking; however, it is a useful tool for the initial assessment of the implant. To assess the true bioresponse of

the anodized material, cellular studies, followed by *in vivo* studies, are critical, which will be described next.

5. CELLULAR RESPONSES

5.1. Introduction

To understand the biologic response of anodic films containing calcium and phosphate ions, numerous *in vitro* and *in vivo* studies have been reported. *In vitro* studies are designed to monitor protein adsorption and subsequent cellular attachment, as well as assess toxicity and immunogenicity. For example, alkaline phosphatase (ALP) production indicates osteoblast differentiation, while differentiation of human mesenchymal stem cells (hMSC) can predict the osteogenic capabilities of the biomaterial. *In vivo* studies are performed to determine the true bioresponse of a material. In addition, torque and pull-out strengths can be measured after *in vivo* implantation into bone.

Finally, an interesting approach to determine the bacterial response of anodized titanium at 190 V in 0.025M $Ca(H_2PO_4)_2$, 0.075M CA, and 0.12 M $Na_2(EDTA)$ with pH = 14 was developed by (72) in which subjects volunteered to wear a buccal appliance containing 5 mm titanium disks for 24 hours, removed only for meals. In this work, no statistical difference in biofilm formation was observed between non-treated and treated titanium, suggesting bacterial response was not increased on the anodized surface. Recent work on nanotubular titanium developed at 20 V in HF found a significant increase in the *in vitro* bacterial responses (*S. aureus, S. epidermidis, P. aeruginosa*), compared to conventional titanium (48).

This section of the review will divide the cellular studies based on processing method and will be described next: i) anodization with hydrothermal treatment, ii) anodization without hydrothermal treatment, and iii) anodization with alkali-etching.

5.2. Anodization + hydrothermal treatment

The initial development of AOFCP by Ishizawa (22) included hydrothermal treatment; therefore, the cellular response will initially be examined for those films with subsequent hydrothermal treatment. (22) showed that after 8 weeks in rabbit femurs, tapered anodized and treated titanium rods exhibited higher push-out strengths compared to untreated titanium and was equivalent to conventional plasma-sprayed HA, as measured by the strength required to push the rod out of the adjacent bone. Failure occurred at the bone-implant interface at the new bone, indicating that new bone had directly bonded to the Ca-P containing layer. The amount of bone apposition was correlated with the amount of precipitated HA crystals, such that lower bone apposition was observed with lower electrolyte composition. Furthermore, the anodized titanium without hydrothermal treatment showed less bone apposition than the hydrothermally treated surface with precipitated HA crystals. According to these authors, hydrothermal treatment at temperatures lower than 300ºC or anodization in low electrolyte concentrations (less than 0.01M beta-GP and 0.15 CA), decreased bone apposition due to decreased amounts of HA crystals (22). However, recent work (65) showed that hydrothermal treatment at 225ºC in a calcium and phosphate ion containing solutions, resulted in a high amount of precipitated HA crystals.

Further cellular work with hydrothermal treatment in steam at 300ºC for 2 hours, as in Ishizawa's method, was carried out by Takebe and others (5, 23, 24, 36, 69, 73, 74) using 0.01M beta-GP and 0.15M CA as the electrolyte during anodization. Using murine spleen cells and human peripheral blood mononuclear cells, no influence was observed in terms of LPS-induced proliferation of lymphocytes or LPS-induced IL-1α production between the treated AOFCP, AOFCP plus hydrothermal treatment and the untreated titanium cultured for up to 66 hours (23). The aim was to further investigate the *in vitro* cellular response of rat bone marrow (RBM) cells on the anodized surface with hydrothermal treatment (24). After 30 min there was no significant difference in cellular attachment between the two groups; however, at 60 and 120 min there were significant decreases in the cellular attachment in the non-treated titanium. Surface morphology and chemistry significantly influenced the attachment behavior of RBM cells. The results of this *in vitro* work may indicate that treated titanium was more osteoconductive than non-treated titanium. Additionally, the treated surfaces were more hydrophilic than non-treated titanium, which increased the affinity for cellular spreading across the surface. Another group, (40), evaluated the *in vitro* response of an osteoblast-like cell line (rat osteosarcoma cell line) with incubation times of 6 hours, 24 hours and 4 days on anodized samples in 0.02M Ca-GP and 0.15M CA with hydrothermal treatment. The only difference observed at 6 hours was that osteoblasts partially or fully spread on the hydrothermally treated surface compared to the rounded morphology on the non-treated surface, which indicated that cells were not attached to the surface. Continuing the *in vitro* work with rat bone marrow cells, after 5, 7, 10 and 14 days, treated surfaces were shown to be stable as the chemical composition, binding energy of Ca and P in HA, and crystalline features remain unchanged (5). These results suggest that the HA interface is stable, biocompatible and osteoconductive. Morphology, spreading behavior and cell number were also analyzed on treated and non-treated discs *in vitro* with mouse cell line J774A.1 for 2, 4, 8, 24 or 72 hours (36). As shown in (Figure 5), cell spreading was enhanced on the hydrothermally treated surfaces. Specifically, a significant increase in cell adhesion was observed between the treated and non-treated surface at both 2 and 4 hours. Additionally, bone morphogenetic-protein 2 (BMP-2, known to induce osteoblast differentiation) secretions were significantly higher on the treated surface compared to the non-treated surface. After *in vitro* work with rat and mouse cells, the *in vivo* response to the anodized and hydrothermally treated titanium was examined in the rat maxilla *in vivo* for 14, 21 and 28 days (73). This work found that the hydrothermally treated HA crystals were stable *in vivo* during bone formation as suggested by the initial *in vitro* work (5). Recently, (69) compared the *in vitro* response of titanium anodized and hydrothermally treated with anodized titanium after soaking for 14 or 28 days in Hanks' balanced

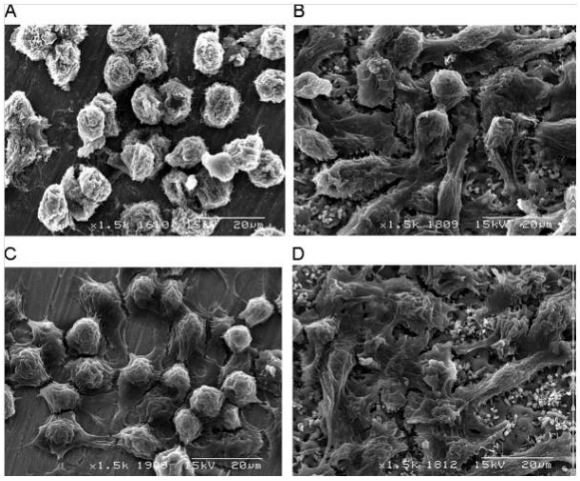

Figure 5. J774A.1 cell behavior and morphology on a) non-treated commercially pure titanium after 24 hours, b) hydrothermally treated anodized titanium after 24 hours, c) non-treated commercially pure titanium after 72 hours, and d) hydrothermally treated anodized titanium after 72 hours. Increased cell spreading is apparent on treated surfaces B and D. Reproduced with permission from (36).

salt solution (HBSS). The results of this work showed that HA deposits completely covered the hydrothermally treated surface, while the anodized layer did not show HA deposition. Deposition of HA may indicate that hydrothermally treated titanium improves osteoconduction.

Anodization plus hydrothermal treatment has been extensively examined in both *in vitro* and *in vivo* studies. These studies indicate that the process improves osteointegration with the incorporation of Ca-P into the oxidized titanium layer. In addition, equivalent pull out strengths have been shown compared to traditional plasma-spray hydroxyapatite through *in vivo* examination, indicating good bone integration. However, some concern may be the structure of the surface such that the HA crystals may be easily removed during implantation. Scratch tests and wear debris *in vivo* should be considered when evaluating these implants.

In 2003, the group associated with the two-step anodization method published a review of anodic spark anodization (15). Their review suggested that many of the ASD techniques were not followed with *in vitro* and *in vivo* investigations; however, the science was relatively new in 2003 and still under investigation to develop the optimal processing parameters. In fact, several reports had been published by 2003 specifically on the *in vitro* and *in vivo* response of Ca-P containing oxide layers developed by anodization (22-24).

As an important note to the following studies, the group associated with the two-step anodization method has incorporated the anodization parameters of (75) and Ishizawa's hydrothermal treatment to compare the cellular responses and mechanical properties to alternative surface modification treatments. However, a striking flaw in the methodology for comparison is the lowered anodization voltage (275 V) (25, 42) compared to Ishizawa's method (350 V). Additionally, the electrolyte concentrations during anodization do not reflect the refined values attained by Ishizawa and associates. In both (25) and (42) studies, the electrolyte was 0.03M beta-GP and 0.06M CA as in (75), and although initial work by Ishizawa (21) recommended this concentration, Takebe and others consistently describe using 0.01M beta-GP and 0.15M CA as the electrolyte medium (5, 23, 24, 36, 69, 73, 74). Clearly, altering the anodization voltage or concentration (and type) of the electrolytes will influence cellular responses as well as the mechanical properties. Therefore, it is not possible to interpret the comparison statements that are made for the "ISH" method in (42), as no comparison of mechanical or cellular differences between the two-step anodization and the Ishizawa method can be made. There must be a direct study that compares the optimal processing parameters developed by Ishizawa and associates to make a direct comparison.

(25) compared the *in vivo* response of three different types of modified titanium screws in ovine femurs after 8 and 12 weeks. These modified screws included anodization at 275V followed by hydrothermal processing, plasma spraying hydroxyapatite as well as acid-etching in HF followed by passivation in a 25 vol% HNO_3. The concentration of HF was not reported for the etching of the surface. After retrieval, histomorphometry analysis and push-out testing was conducted. Bone growth was observed in the threads of the screws for both the plasma and anodized surfaces. In addition, anodized surfaces exhibited accelerated bone-implant mechanical strength, measured by maximum push-out force and ultimate shear strength, at 8 weeks compared to HF and non-treated titanium; however, was comparable to the plasma sprayed surfaces. There was no significant difference reported for the osseointegration between the modified screws (25), and shorter time periods such as 2 and 4 weeks were suggested to show the specific influences of anodized surface modification on the osseointegration rate and process.

5.3. Anodization

Since the Ishizawa method included subsequent hydrothermal treatment, many researchers have focused on preparing AOFCP with subsequent hydrothermal treatment; therefore, only recently have cellular responses per alternative anodization methods without hydrothermal treatment been investigated as described below.

In vitro work by (11) with MAO up to 600 V in 0.15M CA and 0.02M Ca-GP found different behaviors for MG63 cells cultured *in vitro* for 7 days compared to those on untreated titanium. In particular, high anodization voltages (>300 V) resulted in a decrease in the number of proliferated cells, while 190 V reportedly resulted in the highest proliferation rate. However, the ALP activity increased with increasing anodization voltage associated with surface roughness and increased Ca-P in the oxide layer. In addition, preliminary *in vivo* work performed in rabbit tibias for 4 weeks found that the removal torques increased more than three times with the MAO surface

modification at 270 V compared to the as-machined titanium. The increase is due to the increased surface roughness, modified chemical composition and thickness of the oxide layer, as previously described. Further *in vitro* work by another group examined the proliferation and differentiation of human osteoblast like SaSO-2 cells after anodization at 200 – 350 V in 0.2M H_3PO_4 or 140 – 300 V in 0.03M Ca-GP and 0.15M CA (39). At early time points (1 and 2 h) cell attachment was enhanced by anodization. Interestingly, anodization in H_3PO_4 showed that an increased roughness resulted in decreased cellular attachment, while anodization in Ca-GP and CA showed the expected trend of increased SaSO-2 cellular attachment with increased surface roughness. The roughness values for anodization treatment was 0.1 – 0.5 μm, as measured by a surface profilometry. As previously described, the roughness should be characterized on the nano-scale. However, the pore size after anodization in H_3PO_4 was up to 0.5 μm, while pore size after anodization in Ca-GP and CA was up to 2 μm. In contrast to other work, they found surfaces formed under higher anodization voltages enhanced SaSO-2 cell attachment. The authors suggested that anodization of titanium increases cytocompatibility, specifically improving bone attachment and proliferation. Surface topography, roughness and chemistry may all play a role in this increase in cellular response. Additionally, the nano-structuring of titania has been shown to improve the immunogenicity (76).

In addition, an *in vivo* study with anodic plasma chemical (APC) calcium-phosphate treated titanium Schanz screws in ovine bone showed that the infection rate was lower at 6 weeks compared to anodized titanium (58). In addition, the resorption was slower for titanium pins compared to the treated surface. The torque strength of the treated pins increased to nearly two times than that of the anodized titanium pins, suggesting that osseointegration was achieved faster in the APC treated surface. However, one concern is the particle detachment of the surface treated coating, but the authors suggested that the particles were likely debris from pin extraction since the particles were not found on the histological specimens (58). Unfortunately, the authors do not describe the fabrication method of either the APC-Ca-P or the anodized titanium used in their work. In contrast to this work, (77) published a report comparing the *in vivo* response of surface modified titanium in ovine bone. Essentially, they found that after 8 weeks, those specimen prepared by the anodic plasma-chemical surface modification method had significantly lower torque removal strength values than those prepared by alkaline or acid etching. However, it should be noted that the electrolytes and final anodization voltage was not mentioned, which has a direct impact on the film properties. In fact, the SEM images of the microstructure do not suggest that high voltage anodization was used in this study, which may have reduced the properties of the film and the torque strength.

In an attempt to find a commercial use for anodized titanium, (37) investigated the *in vivo* response of self-tapping screws and rectangular titanium plates modified by MAO through a voltage boost procedure (100 V in 1 min, 200 V in 4 min, 300 V in 5 min, 400 V in 7 min with a final voltage of 450 – 550 V in 9 min) in 0.02M Ca-GP, 0.15M CA, 0.01M NaOH and nano-scale hydroxyapatite powder deposition. This type of anodization allowed for the melting of the nano-hydroxyapatite powder during anodization and resulted in a rougher surface (R_a ~ 1.55 μm) than the typical anodization methods. The plates were implanted in rabbits for determining a subcutaneous bioresponse compared to non-modified titanium plates, while the screws were implanted into canine femurs. Osteoid deposition, necessary for osseointegration, was observed at 4 and 8 weeks in the MAO prepared surfaces. Interestingly, compared to other work by this group, they found the *in vivo* study to be much slower than the *in vitro* process due to the extensive additional biological molecular interactions *in vivo*. The work also indicated that a similar response was observed in the MAO and untreated plates implanted subcutaneously, such that essentially no inflammatory cells were observed. Additionally, the number of osteoblasts and Haversian formation was better in MAO implants than untreated titanium. The authors suggested that their MAO fabrication process provides good biocompatibility and excellent osteoid deposition *in vivo* (37).

As already mentioned, several papers report on the cellular response of the two-step anodization method (20, 33, 41, 43). In particular, *in vitro* protein adsorption demonstrated a preferential adsorption of fibronectin (an extracellular matrix protein) on the two-step anodization compared to an aspecific adsorption of both fibronectin and albumin (a surface passivating protein) occurred on the Ishizawa anodization (41). Furthermore, the proliferation rate of osteoblast-like cells was significantly increased by the two-step method followed by alkali-etching compared to commercially pure titanium (43) as described next.

5.4. Anodization + alkali etching

The two-step process with alkali-etching was investigated *in vivo* by implanting treated and untreated cylinders in cortical bone of the dog humeri (43). After 8 weeks, fibrous connective tissue with focal areas of mineralized matrix was found for all treated surfaces. However, both the control and the treated groups exhibited low bone-to-implant contact (less than 3%), without a significant difference observed between groups. Under a different name, but the same basic two-step process with alkali-etching, (33) developed surfaces for implantation in sheep tibiae. In this work, the bone-to-implant contact increased by 84% compared to untreated titanium after 4 weeks. The authors suggested that the two-step anodization method followed by the alkali etch enhanced osseointegration. Furthermore, the treated surface also exhibited the highest microhardness at the interface, suggesting that the treated surface resulted in mature bone formation.

Subsequent work by (32) compared the *in vitro* cellular response of the two-step ASD followed by alkali-etching to both untreated titanium as well as alkali- and acid-etched titanium at 1, 2, 4 and 7 days. This work actually showed a lower number of attached cells on ASD

treated surfaces after 24 hours compared to the untreated and the etched surfaces. Human SaOS-2 cell proliferation and growth was also higher in the untreated and the etched substrates. However, the ASD processed surface did not induce cytotoxicity.

6. PERSPECTIVE

TiO_2 formation on a titanium surface promotes the biocompatibility of titanium *in vivo*. In addition, the presence of Ca-P in a stoichiometric ratio near hydroxyapatite increases the osseointegration of implants due to a similarity to bone composition. Increased roughness adds to improved bone formation. Therefore, anodization in the presence of calcium and phosphate ions is an ideal method to achieve all three goals in developing a bioactive surface (increased TiO_2 thickness, incorporation of Ca-P and increased roughness). In 1995, Ishizawa proposed anodization with a subsequent hydrothermal process to nucleate hydroxyapatite crystals. From this research, many others have examined alternative processing parameters and subsequent treatment for optimal bone integration at the implant surface. Ultimately, advances in surface modification of titanium through anodic spark anodization in the presence of calcium and phosphate ions have evolved into a commercially developed process for dental applications. However, the fundamental mechanisms influencing the cellular response remain largely uncertain. Therefore, many conflicting reports are expressed through a lack of proper processing or anodization methods or understanding in direct comparison studies.

This review provided a summary of work in the development of anodized titanium containing calcium-phosphate ions, as well as the subsequent mechanical properties and cellular responses. Although *in vitro* cellular studies have shown promising results for osteointegration, the response *in vivo* is the critical determinant of implant success. Results of several studies showed equivalent or better responses of anodized and treated surfaces compared to non-treated titanium.

There is insufficient characterization of debris wear analysis for the anodic films, which could be analyzed by a scratch test or at the *in vivo* stage. Many studies have examined the stability of the hydroxyapatite layer *in vivo*, but this does not give an indication of the structural stability while in use. Additionally, surface profilometers generally used to evaluate surface roughness are not sensitive enough to capture the nano-scale roughness attained by anodization with subsequent treatments. Without proper characterization, the structural influences cannot be evaluated in terms of the cellular response. In terms of processing, hydrothermal treatment may be improved if conducted using an electrolyte medium containing Ca-P ions. This has been shown to prevent nucleation of individual crystallites, while inducing a homogeneous microstructure. Additionally, SBF soaking may improve the bioresponse of the implant with the mineralized apatite on the surface. Further work must address the deficiencies in the characterization of the anodized and treated films, continue to investigate the optimal processing parameters and expand on the current *in vivo* work.

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Abbreviations: ASD: anodic spark deposition; MAO: micro-arc oxidation; AOFCP: anodic titanium oxide film containing calcium and phosphorous; GP: glycerophosphate; CA: calcium acetate; HA: hydroxyapatite; Ca-P: calcium phosphates; SBF: simulated body fluid

Surface modification of titanium via high voltage anodization

Key Words: Anodization, Titanium, Calcium-Phosphate, Hydroxyapatite, High Voltage, Anodic Spark Deposition, Review

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