



Effect of Alkaline-Acid Treatment on the Biomimetic Formation of Calcium Phosphate Layers on Titanium Substrates: An *In Vitro* Study

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Abstract

The aim of this study was to investigate the effect of alkaline and heat treatment and acid etching of titanium substrates on the formation of calcium phosphate layer and bioactivity in simulated body fluid (SBF) solution. In this research, at first the titanium surfaces were treated with 5N NaOH, 15% HNO₃-10%HF-75% H₂O and 5N NaOH followed by heating at 600 °C for 1h. The samples were immersed in the SBF for 28 days to investigate the influence of surface treatments of titanium on *in vitro* bioactivity. The modified substrates were characterized using scanning electron microscopy, X-ray diffraction and profilometry of samples were investigated by light interferometry. The *in vitro* assessment was carried out through the immersion of samples in SBF. According to the results obtained in this work the deposit of calcium phosphate in alkali and alkali/heat treated samples were more stable than acid etched samples. Porous network structure containing the sodium titanate and the titanium oxide was characterized on the surface after the titanium was subjected to NaOH aqueous solution. Results have illustrated the fact that chemical surface treatment improves *in vitro* behavior of titanium.

Keywords: Alkaline-acid treatment; Bioactivity; Simulated body fluid.

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1. Introduction

Titanium has been widely used in orthopedic and dental materials because of their superior mechanical property and high corrosion resistance. However, bioactivity of metal surface is not high enough to induce

growth and fixation of the bone tissue [1]. The bioactivity of materials is the ability to induce the direct adherent and strong bonding between the materials and the bone tissue [3]. In order to evaluate this bioactivity of materials, it has been proposed that the materials, which form a bone-like apatite on their surfaces in the Simulated body fluid (SBF), can also form apatite in a living body and can bond to bone through the apatite layer.

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Table 1. Details of surface preparation procedures and designation of samples.

Designation of sample	Treatment
S ₀	No treatment
S ₁	5 N NaOH , 60 °C, 24h
S ₂	5 N NaOH, 60 °C, 24 h, 600 °C, 1h
S ₃	15 % HNO ₃ +10 %HF +75%H ₂ O, 25 °C, 2 min.

This means that the apatite-forming ability in the SBF is a measure of the *in vivo* bioactivity [2, 4, 5]. More recently, bioactive porous TiO₂ hydrogel layers have been prepared on titanium implant surfaces by simple chemical treatments like immersion in alkali or H₂O₂ solution [6]. It was foreseen that an ideal bioactive surface for bone-bonding biomaterials might be the surface with the required negative charge and porous structure which could induce the rapid formation of physiologically stable Hydroxyapatite (HA) layer after immersing in SBF or even in real body fluid [3].

The bone formation around an implant is a complex process and it is not fully understood. The physicochemical and topographical surface characteristics of materials are some of the most influential factors in the improvement of osseointegration [7]. The surface energy also seems to be crucial for the cell attachment, even though the relationship between this parameter and the osseointegration event has only recently started to be discussed [8]. A simple method to estimate the bone-bonding potential of the materials is the immersion in SBF. In other words, the *in vivo* behavior can be predicted by using *in vitro* tests such as immersion of samples into SBF solution [9, 10].

This SBF affect the precipitation reaction and play a decisive role in the chemical and biological events that take place on metal surfaces [11]. The aim of this study was to investigate the effect of alkaline and heat treatment and acid etching of titanium substrates on the formation of calcium phosphate layer and bioactivity in SBF.

2. Materials and methods

2.1. Sample preparation

The samples of cp-titanium were cut into sheets of (10×10×1 mm). The samples were washed with distilled water and sequentially polished with abrasive papers (400, 600, 800, 1000, 1200 and 1500 grit). They were cleaned in acetone for 15 min., in 70% alcohol solution for 20 min. and finally in distilled water for 20 min. Three samples were kept untreated and used as control. A total of three treated samples of each of the three types were used for a total of 9 treated samples. The detail of treated samples is summarized in Table 1.

2.2. Surface characterization

Surface morphology was evaluated via a scanning electron microscope (SEM-LEO-440I). The topography of samples with an area 350 μm×350 μm was measured using a White Light Interferometer (WLI-Taylor Hobson CCI 3000A). The technique is truly contact less, clean, rapid and reproducible. The results were obtained employing a Gaussian filter with cut-off of 0.8 mm, which discriminates waviness from roughness. R (two-dimensional) parameters describe the mean value of only one profile. Amplitude parameters that are measures of variations in profile height can be grouped into two subclasses: Averaging parameters and peak to valley parameters.

1- Averaging parameters: The most widely used parameter to quantify surface texture is R_a the arithmetical mean deviation of the absolute ordinate values, Z(x) of the profile from a midline Equation 1:

$$R_a = \frac{1}{l_r} \int_0^{l_r} |z(x)| dx \quad \text{Equation (1)}$$

Table 2. Ion concentration of SBF in comparison with those in human blood plasma

Ion	Blood plasma (mM)	SBF (mM)
Na ⁺	142.0	142.0
K ⁺	5.0	5.0
Mg ²⁺	1.5	1.5
Ca ²⁺	2.5	2.5
Cl ⁻	103.0	147.8
HCO ₃ ⁻	27.0	4.2
HPO ₄ ²⁻	1.0	1.0
SO ₄ ²⁻	0.5	0.5
pH	7.2-7.4	7.4

where L_r is the sampling length over which the surface profile has been measured. The parameter, R_a is typically used in engineering to describe the roughness of surfaces.

2- Peak and valley parameters: The distribution of peak heights and valley depths can provide valuable information about surface texture. The dimensionless skewness parameter, R_{sk} , is used to detect and quantify bias in the shape of this distribution where Equation 2:

$$R_{sk} = \frac{1}{Rq^3 L_r} \int_0^{L_r} z(x)^3 dx \quad \text{Equation (2)}$$

The skewness of a perfectly random surface with a wide range of peak heights and valley depths is zero [12, 13].

2.3. SBF preparation and samples immersion in SBF

The bioactivity of titanium surfaces was assessed through the immersion of samples in SBF solution for 28 days. The SBF solution had a pH value of 7.4 and an ion concentration nearly equal to those of human blood plasma. The SBF solution was prepared by dissolving the reagent grade chemicals of NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, CaCl₂ and Na₂SO₄ into distilled water and buffering at 7.4 with Tris-(hydroxymethyl)-amino methane (TRIS) and hydrochloric acid 1 M at 37 °C. Throughout the experiments, reagents grade chemicals were used (Merck). Solution was prepared by dissolution of the

salts into 1000 ml of demineralized water. The SBF solution was renewed every 2 days. The ion concentrations of SBF are summarized in Table 2.

The surface of the samples after soaking in the SBF was examined via a scanning electron microscope (SEM). The Ca/P ratio of the calcium phosphate layer on the surface of samples was estimated by EDAX analysis. Phase characterization of the treated samples and coatings was carried out using X-ray diffraction analysis (D4-Brocke).

3. Results and discussion

Figure 1 illustrates SEM images and 3D profiles of all treated titanium samples in different conditions before soaking in SBF. For the sample S₁, homogeneously distributed nanoporous (100-300 nm) can be observed (Figure 1b). The treatment in alkaline media and then heating at 600 °C resulted in that pores. It seems the heat treatment at 600 °C led to increase of both width and depth of pores (Figure 1c). Large amounts of submicrometer-sized pores were observed after acid treatment at 25 °C for 2 min. (Figure 1d).

The 2D roughness parameters obtained by white light interferometry (WLI) for all treated samples are summarized in Table 3. The roughness data values showed despite depth of profile (R_a), irregularity shape (R_{sk}) for S₁ and S₂ group is lower than S₃ groups. That means the surface roughness was more affected by acid etching than alkaline/heat or alkali treatments. These results can be

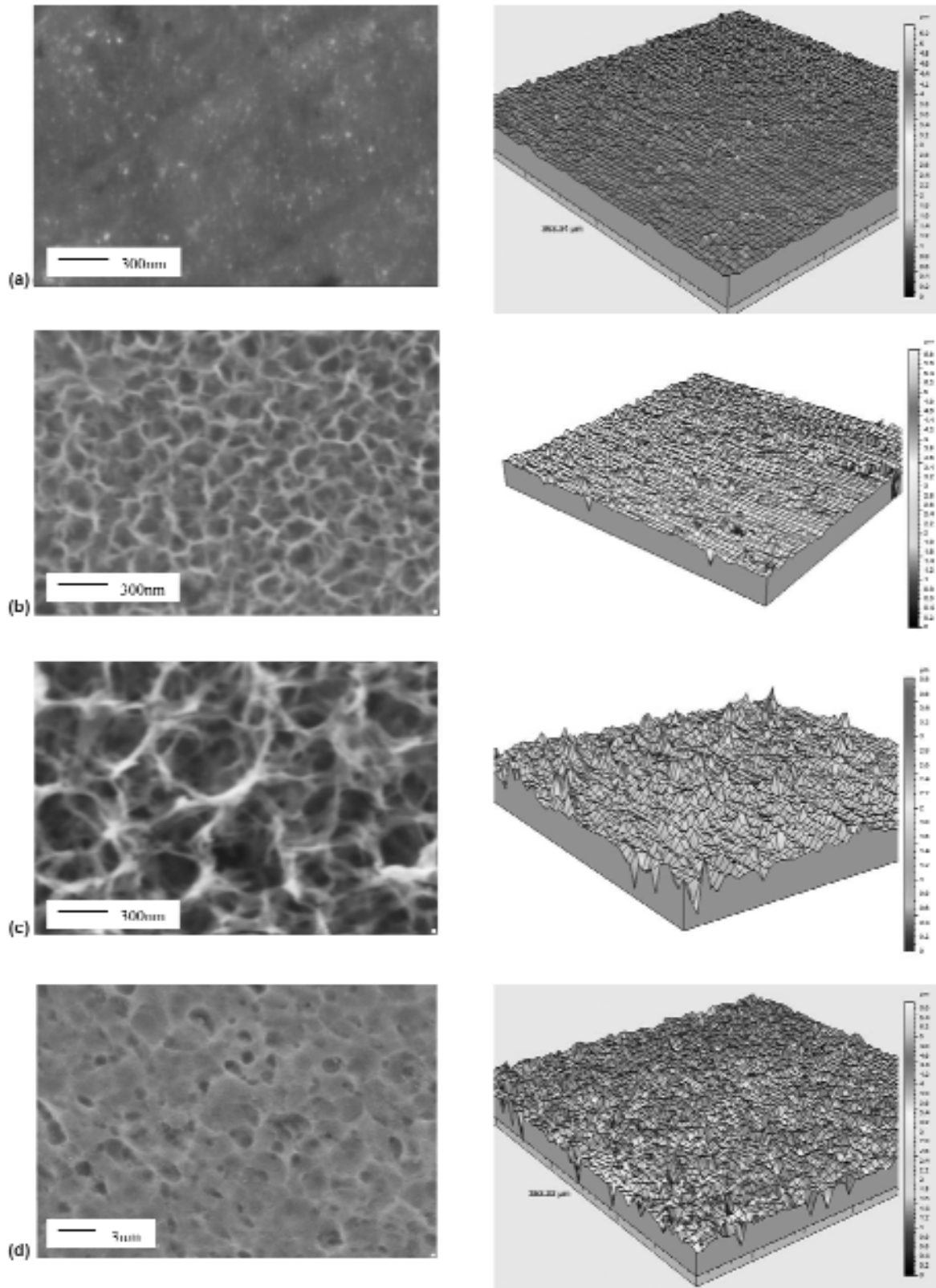


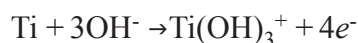
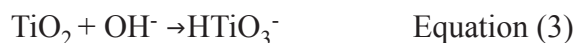
Figure 1. SEM micrographs and 3D profiles of titanium surfaces (a) untreated, (b) alkali-treated titanium, (c) alkali and heat treated titanium, (d) acid etched.

Table 3. 2D roughness parameters of all treated samples

Samples	R _a (μm)	R _z (μm)	R _{sk}
S ₀	0.015	0.060	363.4
S ₁	0.014	0.059	550.6
S ₂	0.018	0.073	360.6
S ₃	0.021	0.085	638.8

attributed to the formation of nano and micro-porosities as shown in Figure 1. For the comparison of the data related to the controlled and treated samples, it should be also taken into account the effect of polishing scratches on the profilometry parameters. Recent developments in measurement for an optical technique such as WLI have increased the vertical resolution of these instruments to give a capability of better than 0.01 nm but the ultimate horizontal resolution is limited to the wavelength of the source ($\sim 0.35\text{-}0.5\ \mu\text{m}$) [13, 14]. So, the absolute parameters related to spacing of irregularities along the surface obtained especially in the alkaline treated samples have not been acceptable. R_{sk} is a measure of the symmetry of a profile about a mean line. Negative skew R_{sk} indicates a predominance of valleys, while positive R_{sk} is seen on peaky surfaces.

The creation of a negatively charged metallic surface is an effective way to initiate apatite nucleation from an SBF solution. The titanium oxide surface has been generally accepted to be negatively charged by OH⁻ in aqueous solution [3]. During the chemical treatment in alkaline solution, the surface passive TiO₂ layer partially dissolves into alkaline solution because of the corrosive attack of hydrogel group in accordance with the Equation (3):



When titanium is treated with NaOH aqueous solution, a titanate hydrogel layer can be formed on its surface in which negatively charged HTiO₃⁻nH₂O incorporating with sodium ions can be found [15, 16].

Figure 2a shows the XRD pattern of the sample treated in NaOH solution with a concentration 5N after heat treatment at 600 °C (S₂). The traces of characteristic peaks of sodium titanate compounds (Na₂Ti₅O₁₁) are identified. As reported elsewhere [1, 4] heat treatment at 600 °C led to the crystallization of sodium titanate layer.

The heat treatment led also to reduce the release rate of Na⁺ ions from the surface when it was soaking in SBF. In fact, with the immersion in the SBF solution, the sodium ions on the surface layer could replace the H₃O⁺ ions in the SBF, producing Ti-OH groups on the titanium surface to enhance the apatite nucleation. The Ti-OH groups would absorb Ca²⁺ by electrostatic force at first and, then, HPO₄²⁻ could reach the surfaces to form the apatite crystals. The Ca²⁺ and HPO₄²⁻ ions were consumed and diffused from the solution to the surface [4]. Figure 2b demonstrates the XRD patterns of the powders taken from the deposited layers on the surfaces of the alkaline and heat treated samples (S₂) revealed hydroxyapatite and calcium phosphate hydrate was formed during the soaking in SBF for 28 days .

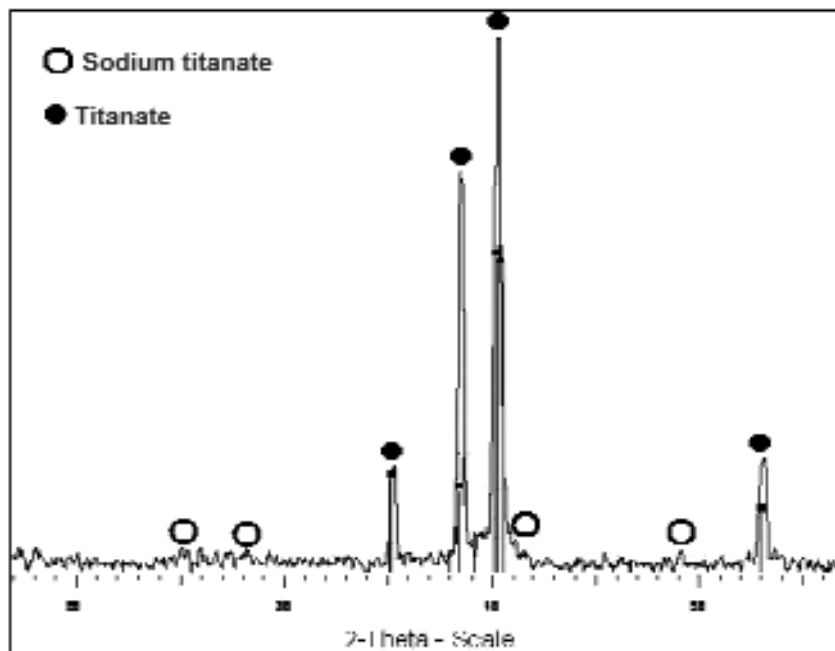
Table 4. EDAX analysis of samples soaked in SBF for 28 days.

Samples	Na	Mg	P	Ca	Ti	Total (%)
S1	0.53	1.28	34.73	61.29	0.08	100
S2	0.71	1.58	35.82	60.06	0.03	100
S3	0.51	1.29	35.16	62.75	0.08	100

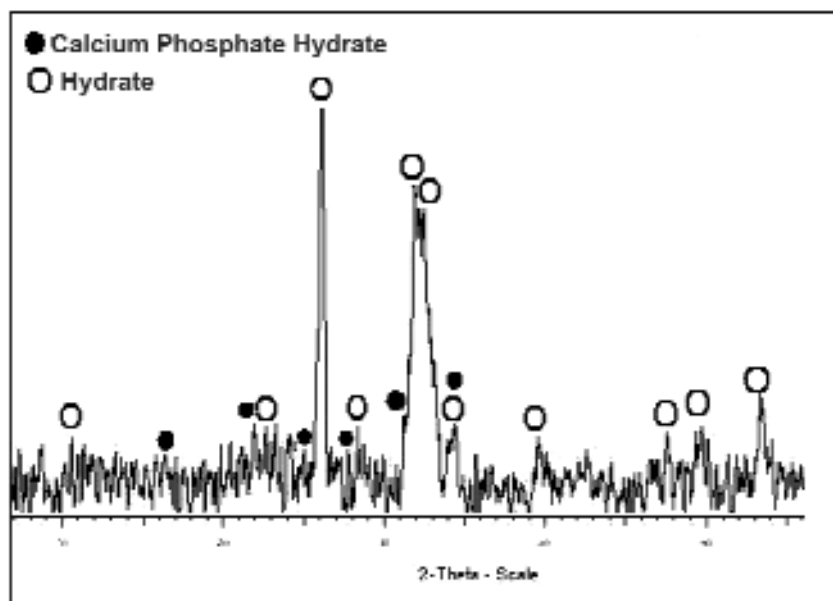
The first step of the bioactive layer deposition seems to begin due to heterogeneous nucleation. Heterogeneous nucleation is of great importance in many processes. In bio-mineralization, potential nucleates such as proteins, cells, other tissue components and porous surface of implant

materials are in contact with mineralizing solutions and promote the growth of bio-minerals on their surface [17].

The SEM images of the samples, soaked in SBF for 28 days, revealed the formation of a calcium phosphate coating (Figure 3). For the non-treated titanium samples, deposition



(a)



(b)

Figure 2. XRD pattern of titanium treated with NaOH 5N at 60 °C and heated to 600°C (a) before soaking in SBF, (b) After soaking in SBF for 28 days.

layer was not visible. The spherical grain morphology was formed on the surfaces of all treated samples. The reduction of apatite grains after heat treatment has been related to decreasing of the number of TiOH groups due to less sodium ions released from the substrate [4] (Figure 3a and 3b). Large grains in the treated samples HF/HNO₃ solution (S₃) could be due to the low active nucleation sites on the surface (Figure 3c).

The results obtained this work reveal that the surface treatment has a key role in the improvement of *in vitro* behavior of titanium. Surface topography is an important factor in creation of calcium phosphate layer and adhesion of cells. This will influence the behavior and function of the cells. Table 4 summarized the data emanated from the EDAX analysis of samples soaked in SBF for 28 days. The difference between the results of Ca or P values between the samples S₁ to S₃ can be due to the precision limit of this method for estimation of chemical composition.

4. Conclusion

In this study different chemical and thermal treatments of titanium surface were performed. Then, the *in vitro* behavior of treated samples was examined via soaking in SBF solution. The treatment in alkaline solution led to the formation of sodium titanate layers. Treatment in the alkaline solution with a concentration 5N led to the formation of homogeneous nanoporous (100-300nm). The treatment in HNO₃/HF led to the formation of large amounts of micrometer-sized pores. The amplitude parameters of surface roughness such as R_a, R_z and R_{sk} have been discussed and the correlation of SEM images and WLI data were established. The thickness of apatite layer increased with soaking time.

According to the results obtained in this work the apatite grain in alkali and alkali/heat treated samples were smaller than acid etched samples. The formation of nanoporous structure after alkaline treatment of titanium

provides more favorable sites for calcium phosphate nucleation. The calcium phosphate microstructure was found to be affected by both the chemical compounds and topography of the surface. The SEM images and the XRD results have illustrated the fact that the bone-like apatite has successfully been deposited on the titanium surface and surface treatment seems to play a key role in the improvement of *in vitro* behavior of titanium.

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