

Biomimetic Apatite Deposition in Modified Dulbecco's Phosphate-Buffered Saline Solution on the Alkali-Heat Treated Ti6Al4V

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Abstract: Simulated Body Fluid (SBF) solution has been used in biomimetic apatite coating. In some cases, the apatite formation on the metallic surface may take up to 4 weeks in SBF solution. Increasing the concentration of SBF seems to work in shortening the period of apatite formation yet the high concentration of unstable SBF solution is not easy to handle. Another potential solution that mimics human blood plasma to hasten the apatite formation is Dulbecco's Phosphate Buffered Saline. The aim of this research was to investigate the formation of apatite in Dulbecco's Phosphate Buffered Saline (DPBS) solution modified with CaCl₂ and MgCl₂ on the surface of alkali treated Ti6Al4V. The bioactive surface on Ti6Al4V was prepared by alkali etching using 5M NaOH and 5M KOH and consolidated by heat treatment at 500 and 600°C. The morphology structure, phase changes, wettability and chemical composition of apatite coating were characterised. The results demonstrated that apatite coating in globular rosette-like shape was formed on the surface treated with NaOH and subsequently heat-treated at 500°C within 3 days of immersion time in DPBS solution. Significantly, the DPBS solution can be used as a substitute in biomimetic solution for apatite coating at low processing temperatures for bioactive coating of the medical implant.

Key words: Ti6Al4V, alkali treatment, heat treatment, biomimetic, Dulbecco's Phosphate Buffered Saline (DPBS), medical implant

INTRODUCTION

The bioactive surface has a special attention in orthopaedics and dental implants. The main advantage of the bioactive surface is its ability in promoting a good interfacial bonding between bone tissue and implants that are greater or equal to the host bone. There are various kinds of bioactive materials such as synthetic hydroxyapatite, bioglass and groups of Calcium Phosphate (CaP). However, the bioactive ceramic material alone could not withstand the load-bearing situations since it has a low mechanical strength as compared to human bones. Thus, a metallic implant such as Co-Cr alloys, stainless steel 316L and Titanium (Ti) and its alloy are a wise choice for the load-bearing application. In medical implant surgery, Ti and its alloy were used widely due to its good mechanical strength owing to their light weight quality, excellent biocompatibility and high

corrosion resistance. Ti and its alloy are also classified as a biotolerant material which formation of connective tissue does not adhere to the implant surface driving to implant rejection (Geetha *et al.*, 2009). Mismatch of elastic moduli of Ti and host bone may lead to inappropriate stress distribution at the bone-implant interface which induces stress-shielding effects consequentially leading to bone degeneration and loosening of the implant (Engh Jr. *et al.*, 2003). Rejection of implants could be avoided through various method of surface modification.

Coating metallic implants with bioactive materials such as Hydroxyapatite (HA) is an ideal approach in medical implant. Ti contribute to a good mechanical strength while HA enhances osseointegration and cellular interlocking which make the implant more suitable for load bearing application. Calcium phosphate-based coating that accelerates bone healing and bone apposition around the implant encourage to rapid biological fixation

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(Cosma *et al.*, 2015). A classic method of Calcium Phosphate (CaP) coating on metallic implant has been developed including anodizing, electrochemical deposition (Ducheyne *et al.*, 1990; Ban and Maruno, 1993), sol-gel precipitate (Kaneko *et al.*, 2009; Montenero *et al.*, 2000) and plasma spray coating (De Groot *et al.*, 1987; Klein *et al.*, 1991). However, high processing temperature method of plasma spray will render to decomposition of hydroxyapatite (Jaworski *et al.*, 2009). As reported by Huang *et al.* (2015), plasma spray coating produced a non-uniformly HA coating on the internal surface of porous Ti. With this limitation, an intense progress in surface implant modification has brought to the biomimetic methods, a promising technique for bioactive coating at low processing temperature.

A biomimetic solution of Simulated Body Fluid (SBF) was introduced by Kokubo *et al.* (1996). The ionic composition of SBF solution mimics human blood plasma with a pH of 7.2~7.4 at 37°C. The mechanism of bone-like apatite formation in biomimetic solution induced a uniformed coating on the porous and complex metal surface in room temperature conditions. Besides, the apatite deposition which acts as a bioactive interface layer between implant and cell can stimulate the growth of juxtaposed bone and the healing process. With this advantage, a various type of substrate has been tested including bio-glass and polymer-based implants in preparing bioactive composite implants for bone-like apatite forming.

CaP coatings on the metallic implant act as a barrier between the implant and bone tissue which provide a surface for bone easily growth, enhance mechanical interlocking and chemically bond at the bone-implant interface (Pasinli *et al.*, 2010). CaP apatite coating via SBF solution is a challenging process as the process could be considered as complex where high ion concentration in SBF is difficult to control due to the instability of the solution. SBF coating is an intricate process as many parameters need to be taken into account such as the pre-treatment steps (i.e., chemical, thermal) and control of SBF concentration in order to produce a calcium phosphate coating layer at the fastest rate. Moreover, the nucleation rate of apatite formation in a biomimetic solution of SBF takes more than 24 h. Therefore, another potential biomimetic solution has been explored by researchers in CaP coating. One of the promising solutions is Phosphate Buffered-Saline (PBS) which has a similar composition with human blood plasma and SBF. Forsgren *et al.* (2007) used Dulbecco's 1×PBS as a medium to deposit HA on the commercially pure Titanium (CpTi) surface. The result indicated that HA was

deposited on CpTi after exposure to PBS within seven days of soaking. According to Chen *et al.* (2010), DPBS has great potential as a bone-like apatite self-induction on HA coated CpTi disc in 15 min of exposure time without the additional fibronectin. Meanwhile, the feasibility of biomimetic HA coating on Cobalt-Chromium (CoCr) substrates using PBS was studied by Lindahl *et al.* (2015). A homogenous coating composed of small flower-like crystal aggregates on the porous CoCr surface was formed which show the ability of PBS in nucleation of apatite after 3 day's soaking time. The HA layer was examined as nanocrystalline and amorphous HA.

Thus, in the present study, Dulbecco's Phosphate-Buffered Saline (DPBS) was used as a substitute for SBF solution in order to observe apatite deposition on the treated Ti surface. In order to initiate a bioactive surface, a simple combination step of alkali and thermal treatment were imposed on the metal substrate. Furthermore, the effect of potassium hydroxide (KOH) on the topography of metal substrate was not widely discussed by the researcher as much as sodium hydroxide (NaOH). Kim *et al.* (2013) demonstrated that KOH treatment has a great potential in creating a bioactive surface on the commercial pure Ti with numbers of osteoblast-like cell declining as temperature treatment increases. In this study, the apatite forming ability in DPBS solution was studied using two different types of alkaline treatment with the same concentration at temperatures of 500 and 600°C. The effect of the alkali-heat treatment on calcium phosphate deposited on substrate was evaluated based on surface morphology, phase composition, wettability and the functional group of the apatite formation on the substrate Ti.

MATERIALS AND METHODS

Material preparation: Titanium alloy discs Ø9.4×2 mm, Ti6Al4V ELI Grade 23 purchased from STC Company Taiwan were cleaned ultrasonically (NEYTech, 208 H, USA) in a clean glass beaker for 10 min with the presence of acetone, ethanol and distilled water consecutively. Then, surface modification of Ti alloy was conducted using alkali-heat treatment as reported by Kim *et al.* (1997) and Qu and Wei (2008). Alkali treatment was performed by soaking Ti6Al4V disc in 50 mL of 200 g/L NaOH (R&M Chemicals) and 50 mL of 280.53 g/L KOH (R&M Chemicals) solution for 24 h at 60°C was identified as S and P, respectively. This alkali treatment concentration was chosen based on the optimum alkali etching concentration of metallic implant resulting in porous network structure (Fatehii *et al.*, 2009). Then, Ti6Al4V discs were rinsed with distilled water and dried overnight

at room temperature. After the alkali treatment in NaOH and KOH, Ti6Al4V discs were heated at 500 and 600°C for 1 h with the heating rate of 3°C/min in a CARBOLITE furnace (CARBOLITE, ELF 11/14B, UK) and allowed to cool down in furnace after that. Samples were coded as S500 (heat treated at 500°C), S600 (heat treated at 600°C) for NaOH treatment while P500 (heat treated at 500°C) and P600 (heat treated at 600°C) for KOH treatment.

Apatite deposition: The apatite deposition was prepared by immersion of alkali treated Ti6Al4V in Dulbecco's Phosphate-Buffered Saline (DPBS) with additional calcium chloride and magnesium chloride (D8662, SIGMA). The ion concentration in the solution was: Na⁺ (145 mM), K⁺ (2.7 mM), Mg²⁺ (0.5 mM), Ca²⁺ (1.0 mM), H₂PO₄ (1.5 mM), Cl⁻ (141 mM). Each sample was immersed in 50 mL of DPBS solution at 37°C in the incubator for 72 h which was subsequently removed and washed thoroughly with deionized water. Samples were dried in a desiccator overnight at room temperature. Changes of pH DPBS solution were measured using a basic pH meter (Sartorius, PB-11, USA) every 12 h. Sodium and calcium ion concentration in DPBS solution were evaluated by atomic absorption spectroscopy (Hitachi, Z2000, Japan) every 12 h. About 2 mL of DPBS solution were extracted out at 12 h intervals and then diluted using distilled water. A standard solution was carried in order to get a linear standard curve before doing sample solution analysis. Hollow cathode lamp of Ca, Mg and Na was used for analysis.

Sample characterization: Morphology and trace element for all samples of Ti6Al4V were observed by using Field Emission Scanning Electron Microscope (FESEM, Carl Zeiss, SUPRA 40VP, Germany). The images of gold sputter coating samples were captured at the magnification of 1 and 10 k at accelerated voltage of 10 kV. Meanwhile, the elemental mapping is carried out by using EDX attached with FESEM. Surface structural changes on Ti6Al4V treated specimen were examined by X-ray diffraction (XRD, Rigaku, D/Max 2200 PC, Japan) using CuK α radiation at scan range of (2 θ) = 20-60° (scan speed: 1°/min and scan step: 0.02°). The functional group presence in the apatite deposition was determined using Fourier Transform Infra-Red spectroscopy (FTIR, Perkin-Elmer, Spectrum One, USA). FTIR absorption peak was recorded in the 4000-500 cm⁻¹ range with 4 cm⁻¹ resolution. Wettability of the surface treated was determined by contact angle goniometry (AST Products, Inc, VCA-3000S, Massachusetts USA) using distilled water drops at room temperature. Image of water droplet was captured for measurement after 10 sec by using static

sessile drop method. The angle was calculated manually from the baseline of surface-water contact and the reading was conducted in triplicate for the average value.

RESULTS AND DISCUSSION

Morphology structure: The morphology of the alkali treated surface of Ti6Al4V is illustrated in Fig. 1. A nested porous network structure was observed on the surface of Ti6Al4V that was treated with 5 M NaOH (Fig. 1a) and 5 M KOH (Fig. 1b) at 60°C for 24 h. Apparently, the porous network structure created on the surface treated with NaOH is finer than KOH treated. Reactivity of potassium earth metal was known to be slightly reactive than sodium. Thus, the chemical effect on the surface etching using KOH created a large macroporous network structure. A similar cellular-looking morphology on NaOH-treated Ti6Al4v was observed by Kim *et al.* (2013) on commercial Ti treated with 5M NaOH. According to Cai *et al.* (2010), regardless of the size, porous network structure will increase the surface area of treated Ti6Al4V which then could enhance cell anchoring to adhere on the surface of the titanium implant. Then, when a porous network structure of alkali-heated Ti6Al4V was immersed in DPBS solution, the deposition of the apatite was detected on the surface of alkali-heat treated Ti6Al4V.

Figure 2 illustrates FESEM images on untreated Ti6Al4V after being immersed in DPBS solution at 37°C for 3 days. Figure 2a shows the polished and shiny untreated Ti6Al4V surface that was directly immersed in DPBS solution. The globular shape apatite deposition was observed after 3 days in DPBS solution as shown in Fig. 2b. The distribution of apatite globular was unevenly formed on the surface as the smooth surface of untreated Ti6Al4V could be seen at low magnification image. While, Fig. 3 shows a FESEM images of treated Ti6Al4V after 3 days incubation in DPBS at 37°C. Sample S, S500 and P600 surfaces were covered with a multilayer globular shape of calcium phosphate coating as shown in Fig. 3a, b and f, respectively. Furthermore, a rosette petal-like crystal structure was captured clearly, especially for sample S treated with 5 M NaOH that has a slightly homogenous apatite globular than S500 and P600. Tas and Bhaduri (2004) reported similar findings on the rosette-like structure by a biomimetic coating of Ti6Al4V strips pre-treated with 5 M KOH and heat-treated at 600°C. The results were also concurred by Chen *et al.* (2010) in which a homogenous apatite plate has successfully formed on the calcium phosphate coated surface after 15 min of incubation in DPBS solution in the presence of fibronectin. Meanwhile, for P, P500 and S600, the absence of globular shaped formation was observed on the surface

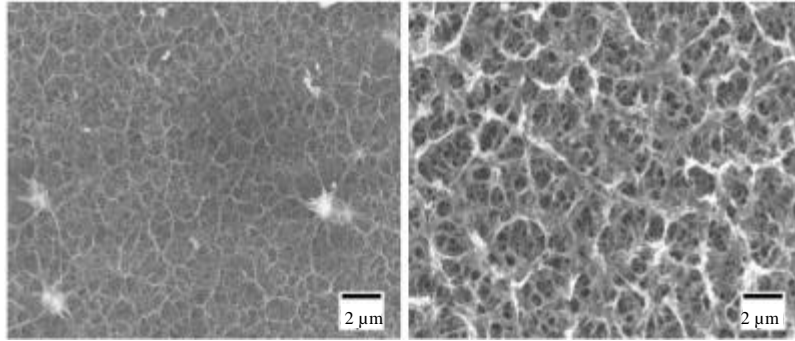


Fig. 1: FESEM image of Ti6Al4V soaked in 5 M NaOH; a) and 5 M KOH and b) at 60°C for 24 h

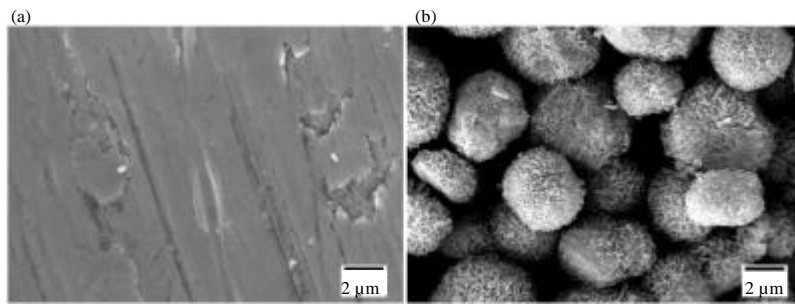


Fig. 2: a) FESEM photographs of untreated Ti6Al4V; b) A globular rosette-like structure of apatite forming on the surface of Ti6Al4V after 3 days being incubated in DPBS with CaCl₂ and MgCl₂ at 37°C

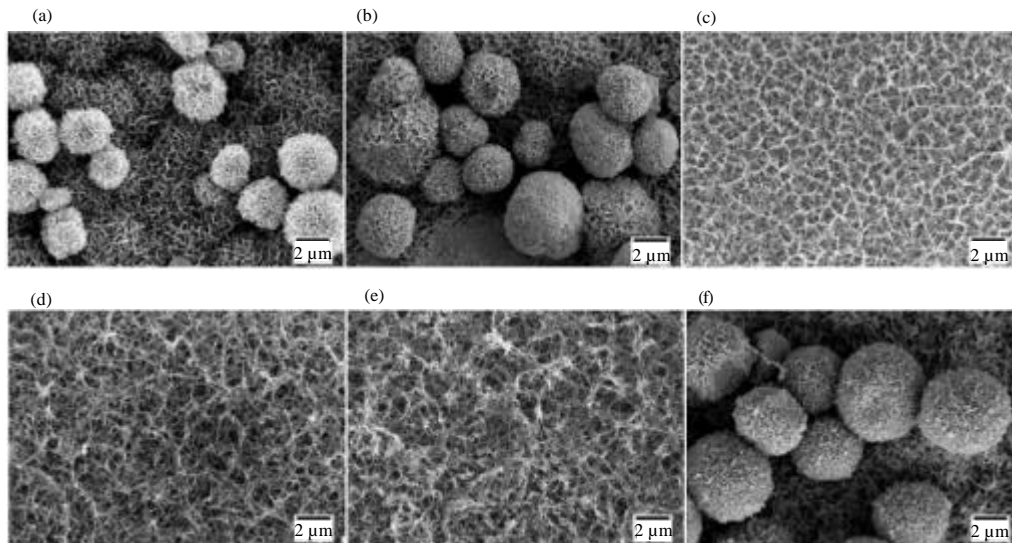


Fig. 3: a) FESEM photographs of sample S; b) S500; c) S600; d) P, P500; e) P600 and f) incubated in DPBS with CaCl₂ and MgCl₂ at 37°C for 3 days

of alkali-heat treated Ti6Al4V. On the other hand, the mesh network fibre structure on the sample S600, P and P500 as seen in Fig. 3c-e remain unchanged after immersion in DPBS solution.

Table 1 shows the weight percentage of Ti, Calcium (Ca) and Phosphate (P) as detected by EDX mapping analysis. Calcium to phosphate ratio of untreated Ti6Al4V, S, S500 and P600 were in the range of 1.7-2.2

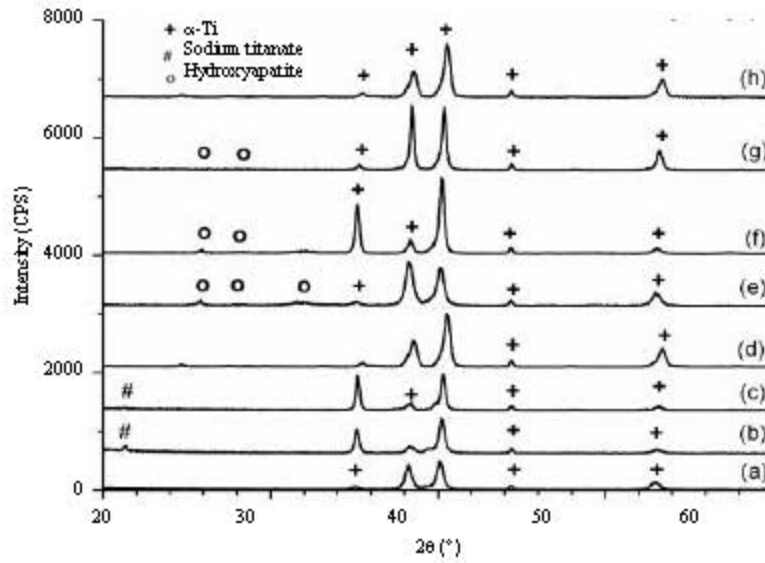


Fig. 4: a) X-ray diffraction data of untreated Ti6Al4V; b) S; c) S500; d) S600; e) After soaking in DPBS of untreated Ti6Al4V; f) S-DPBS; g) S500-DPBS and h) S600-DPBS samples

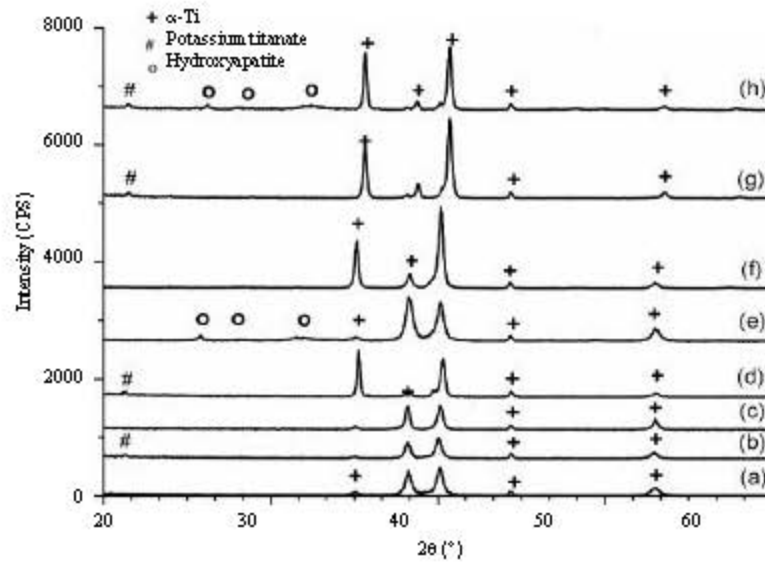


Fig. 5: a) X-ray diffraction data of untreated Ti6Al4V; b) P; c) P500; d) P600; e) After soaked in DPBS of untreated Ti6Al4V; f) P-DPBS; g) P500-DPBS and h) P600-DPBS samples

Table 1: Calcium and phosphate weight percentage (wt.%) on coated Ti6Al4V

Sample name	Ti	Ca	P	Ca:P
Ti	27.81	17.79	8.53	2.09
S	5.07	15.00	8.73	1.72
S500	6.26	10.87	5.89	1.85
S600	56.13	0.00	0.00	-
P	47.46	3.74	2.01	1.86
P500	47.36	0.68	0.00	-
P600	0.00	28.52	12.77	2.23

which indicates that the apatite deposition is Ca-rich amorphous calcium phosphate. Furthermore, the weight percentage of Ti has decreased as the globular shape

multilayer CaP was completely covered on the Ti6Al4V surface. Even though there is an absence of globular apatite structure on S600 and P500 surfaces, yet the EDX analysis detected the presence of small amount of Ca element but lacking the phosphate element. The phase composition of apatite layer deposited on the treated surface was analysed using XRD in order to validate the presence of apatite deposition.

Phase composition determination: Figure 4 and 5 show the XRD pattern analysis on coated samples after

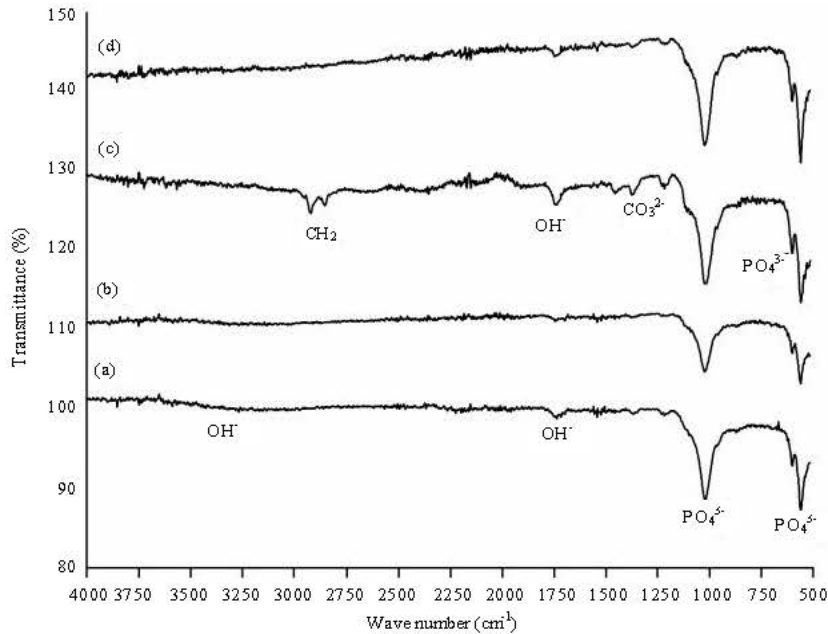


Fig. 6: a) FTIR wavelength data of DPBS-apatite deposition on untreated Ti6Al4V; b) For sample S; c) S500 and d) P600

immersion in DPBS which were being etched initially with 5 M NaOH and 5 M KOH and subsequently heat-treated at 500 and 600°C, respectively. The untreated Ti6Al4V (Fig. 4a) pattern is recognised as a hexagonal closed-packed α -Ti (ICDD 044-1294). Treatment with 5M NaOH initiates a reaction with the surface of Ti6Al4V leading to the formation of sodium titanate layer as pointed out at peak $2\theta = 21.3530^\circ$ shown by Samples S and S500 in Fig. 4b and c, respectively. Whereas, the samples treated with 5 M KOH initially revealed a monoclinic potassium hexatitanate ($K_2Ti_6O_{13}$) phase peak at $(2\theta) = 21.299^\circ$ of sample P and $(2\theta) = 21.3481^\circ$ of P600, respectively. The reflection peaks observed are similar to the ICDD 01-074-0275 file. The oxide layer of Ti6Al4V has been exposed to the concentrated KOH which led to the formation of potassium titanate layer. However, a corresponding peak of titanate layer was undetected in sample P500 (Fig. 5c) which was possibly due to a thin layer of titanate development.

After 3 days of DPBS incubation period, new peaks at low intensity were detected on XRD pattern for untreated Ti6Al4V, S, S500 and P600 samples. The presence of new peaks detected at $(2\theta) = 31.80, 25.84, 28.85^\circ$ with respective miller indices of (211), (002) and (210) could probably be recognized as a thin deposition of apatite that corresponds to a hexagonal HA phase matching the ICDD 09-0432 file data. Meanwhile, sharp peaks detected on the diffraction pattern of all samples after $(2\theta) = 35.18-60^\circ$ is similar to untreated Ti6Al4V

(Fig. 4a). A diffraction peak intensity of (α -Ti) phase slightly decreased which indicates that the surface of Ti6Al4V is concealed by the apatite deposition. A rosette petal-like structure on FESEM images of untreated Ti6Al4V, S, S500 and P600 sample was a calcium phosphate apatite group as confirmed by XRD pattern. Likewise, Ceng *et al.* also reported that the diffraction peak near $(2\theta) = 25^\circ$ was recognized as bone-like apatite after incubated commercial pure titanium coated with calcium phosphate film in DPBS (magnesium free) at 37°C for 1 and 12 h.

Functioning group analysis: The apatite deposited on the Ti6Al4V surface was exerted to Fourier-Transform Infrared Spectroscopy (FTIR) detector for functional group analysis. Figure 6 illustrates the bandwidth peak that is presented in the untreated Ti6Al4V, S, S500 and P600 samples. An amorphous calcium phosphate identified in XRD is known to display the $(PO_4)^{3-}$ modes. Three peaks of $(PO_4)^{3-}$ functional group in the IR spectrum of Fig. 6 attributed to (ν_3) stretching vibration modes occurring at 1021-1024 cm^{-1} and (ν_4) bending modes of $(PO_4)^{3-}$ at 559 and 600 cm^{-1} . There are small absorption peaks adjacent to 1024 cm^{-1} (around 960 and 1125 cm^{-1}) which is combined to form a broad peak of P-O stretching modes. Meanwhile, spectra of S500 (Fig. 6c) shows an extra absorption peak of 1369 and 1455 cm^{-1} indicating the incorporation of the CO_3^{2-} group into the calcium phosphate layer structure.

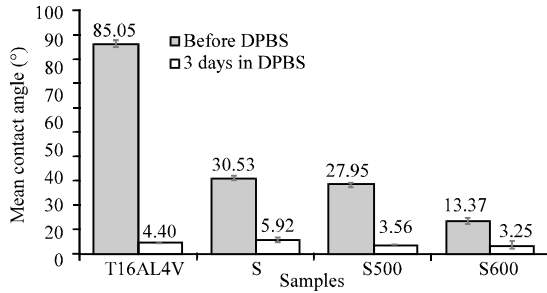


Fig. 7: Average contact angle measurements before and after DPBS immersion period of untreated, S, S500 and S600 samples. Vertical error bar indicates a standard deviation

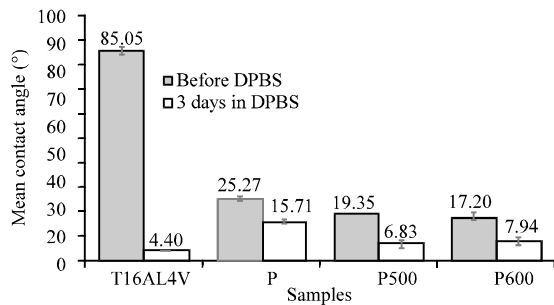


Fig. 8: Average contact angle measurements before and after DPBS immersion period of untreated, P, P500 and P600 samples. Vertical error bar indicates a standard deviation

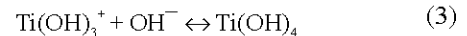
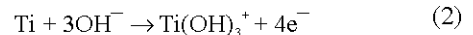
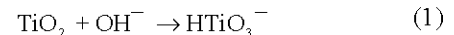
Surface wettability: The wettability of the surfaces of untreated and alkali-heat treated samples before and after incubation in DPBS solution was compared in the contact angle measurement as shown in Fig. 7 and 8, respectively. A contact angle value indicates the affinity of the Ti6Al4V surface towards water molecules. As the contact angle value increases, the surface properties become hydrophobic. Surface modification on Ti6Al4V with NaOH subsequently thermally treated at 500 and 600 has changed the surface hydrophilic as values of the contact angle were drastically reduced from 85.05±1.53° of untreated Ti6Al4V to the 13.37±1.23°. The decreasing data collected after alkali treatment was consistent with Lu *et al.* (2012). The contact angles of untreated Ti6Al4V before and after DPBS treatment were 85.05±1.53° and 4.40±0.14°, respectively.

On the other hand, S, S500 and S600 samples showed a lower contact angle after 3 days being immersed into DPBS with reduced percentage of 80, 87 and 76%, respectively. Zhou *et al.* (2013) has reported that a contact angle of an etched-Ti with 5 M NaOH declined as much as 73% after being soaked in SBF assisted with

microwave for CaP deposition. A low contact angle was influenced by the presence of hydroxyl group which attracts water molecules. Meanwhile, in the case of KOH etching as illustrated in Fig. 8, a similar downward trend of contact angle was observed but the value was slightly higher than NaOH etching (Fig. 7). Comprehensively, the wettability of Ti6Al4V surface properties decreased to <10° because calcium phosphate coating has a higher affinity towards water molecule-based components after DPBS treatment.

Ion composition study: In principal, deposition of apatite on the Ti6Al4V surfaces shows the presence of a chemical reaction. Hence, ion reduction or intensification of DPBS solution was examined using Atomic Absorption Spectroscopy (AAS) and the results are presented in Fig. 9. A drastically decreased calcium ion concentration in DPBS solution was recorded as shown in Fig. 9a. This is because ion Ca was consumed for apatite nucleation. While Mg²⁺ ion seems to remain constant with 15% ion reduction. In contrast, Na⁺ ion concentration increased and maintained after 36 h (Fig. 9b). Figure 9c demonstrated pH values of DPBS solution contain S samples that lie between 7.4 and 7.05 as a function of time. The rapid increase of Na⁺ ion with decreased pH solution indicated the released of ion Na from the alkali treated surface (5 M NaOH) due to ion exchange with hydronium ion as reported by Kim *et al.* (1999).

A conventional Ti6Al4V surface covered in passive titanium oxide film prevents negative ions from invading the matrix of the alloy. This oxide layer creates a great corrosion resistance on the Ti6Al4V surface yet, it is easily destroyed and reacted with an alkaline solution (e.g., NaOH and KOH aqueous) to form an alkaline hydrogel layer. The dissolution equation of the reaction is illustrated as follows (Lee and Yoo, 2015):



The further chemical interaction between the negatively charged species of HTiO₃⁻·nH₂O and the alkali ions (Na⁺/K⁺) in aqueous solution created a mechanically weak and unstable alkali titanate hydrogel layer on the treated surfaces. Heat treatment was applied to this layer in order to create a stable amorphous titanate layer as the

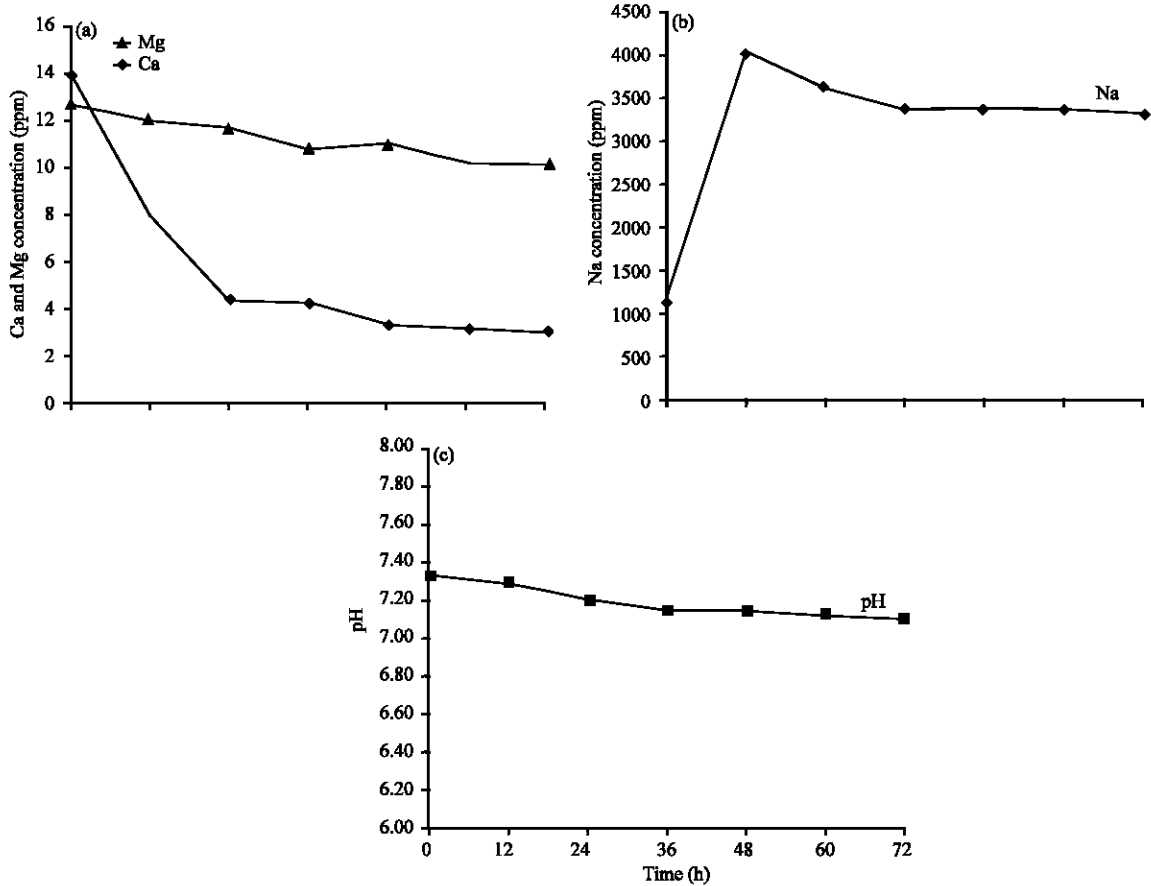


Fig. 9: a) Concentration of Ca and Mg ion; b) Na ion and c) pH monitoring values of DPBS solution at 37°C for 72 h on S sample

hydrogel layer is densified and dehydrated by heat. The presence of alkali titanate layer was confirmed by an XRD analysis. Nevertheless, subsequent heat treatment for samples S600 and P500 showed non-detected titanate peak. This might be due to the formation of a thin titanate layer. Simultaneously, sodium hydroxide treatment at high concentration produced a fine cellular-looking network structure rather than potassium hydroxide which renders to be a unique micrometre scale of nested porous morphology (Fig. 1). In basic chemistry, it is known that potassium is an alkali earth metal with reactivity slightly higher than sodium. Thus, the etching metallic metal implant in KOH solution creates a larger porous structure due to the reactive etching process that engraves or destroys the protective oxide layer on the metal implant surface.

During DPBS treatment, the spontaneous nanometre titanium oxide film forming on the untreated Ti surface became concentrated with Ti-OH groups as oxide surface is easily attacked by hydronium ion. The Ti-OH groups become deprotonated to form a negatively charged

surface at pH 7.4 once immersed in DPBS solution at 37°C. Meanwhile, the alkali treated surface grander with the Na⁺/K⁺ ions in the form of alkali titanate hydrogel layer is exchanged with hydronium ions, H₃O⁺ from the solution resulting in the Ti-OH layer formation (Fatehi *et al.*, 2009). It is noticeable that the amount of Na⁺ ion in DPBS increased after 12 h immersion (Fig. 9b). Simultaneously, Ca²⁺ ions in the solution were reduced (Fig. 9a) due to the incorporation of the Ca²⁺ ion with hydrated Ti-OH layer. Accumulation of Ca²⁺ ion led to the positive charge titanate surface. Positively charged is preferentially attracted to negatively charged species of PO₄³⁻ and CO₃²⁻ present in the DPBS to form a surface layer which is rich in an amorphous calcium phosphate (Jonasova *et al.*, 2004). Theoretically, the release of Na⁺ ions increased the surrounding pH (Kokubo and Yamaguchi, 2010). Indeed, in this case, the pH did not increase (Fig. 9c) at the beginning which indicates the formation of apatite nucleation or consumption of H₃O⁺ ions from DPBS was slowed down. Thus, the rate of apatite formation is considered to be slow. In the meantime, a small amount of

Mg ion reduction was plotted in Fig. 9a as magnesium potentially adhered on the Ti surface. However, the Mg element was washed out after the sample was immersed in DPBS rinsed thoroughly in ultra pure water.

The apatite coverage on the untreated surface seems to be less concentrated than S, S500 and P600 samples. The smooth surface of Ti can be observed under FESEM images while the major diffraction peak of apatite was detected in XRD (Fig. 2a). Even though the apatite was deposited on the untreated surface because of the Ti-OH group presence, the bonding of apatite on Ti surface is questionable due to its shiny surface of the metallic implant. Nested porous structure on the alkali treated Ti prepares a better apatite anchoring on the Ti surface.

In addition, releases of Na ion and increasing pH accelerates the apatite nucleation by increasing the supersaturation of the solution with respect to apatite (Fatehi *et al.*, 2009). Increasing of the pH due to the apatite growth rate on the sample surface has been reported by Azhar *et al.* (2015).

Relatively less amount of apatite formation on sample S500 and P600 by means of heat treatment stabilise the surface structure and less sodium ion was released from the treated surface, thus, less Ti-OH layer was formed (Fatehi *et al.*, 2009). This effect could be seen on sodium hydroxide treated samples of S, S500 and S600. As heating temperature increased, the number of apatite spherical shape formation decreased. XRD also illustrated peaks intensity of apatite detection becoming smaller and disappear as heating temperature rose. Apparently, a denser and uniform amorphous apatite was covered on the surface of the NaOH-treated sample (S) after 3 days of immersion in DPBS. High Na ion released as a thick layer of Ti-OH group on the alkaline-treated Ti promoted apatite nucleation at the fastest rate.

The globular rosette petal-like structure of apatite observed are consistent with results from studies conducted by Lindahl *et al.* (2013) where globular rosette petal-like structure was formed after 7 days immersion of untreated Ti grade 2 in a 1×PBS solution. In this present study, DPBS solution used supplementary with calcium has an affinity for the apatite formation as early as 3 days of immersion. In contrast, the apatite globular shape was unable to be observed on the surface of sample P and P500. This might be due to the incompatibility of size, porous network distribution or lack of potassium titanate hydrogel layer on the treated surface. Fatehi *et al.* (2009) claim that the heat treatment caused changes of surface structure which can cause lacking apatite formation. Though an amorphous apatite peaks identified by XRD analysis of P500 (Fig. 5), sample g describes the presence of small amount apatite formation, yet it is unable to be observed under FESEM analysis.

Theoretically, there are 3 main functional group bandwidth peaks that made hydroxyapatite; a hydroxyl group, a phosphate group and a carbonate group. All apatite formation on the Ti surface shows a dominant band of phosphate at the almost similar intensity. An expected hydroxyl stretch band between 3570-3500 cm^{-1} in the spectra has a very weak vibration mode not even a spike of intensity was detected. The presence of weak carbonate vibration mode in biomimetic apatite formation may be attributing to the disappearance of hydroxyl ions in the spectrum (Rehman and Bonfield, 1997). Yet, the carbonate ion also did not show a strong spectrum which might be due to the lack of carbonate development in the biomimetic apatite. So, the substitution of CO_3^{2-} with OH^- as characteristic absorption band is ambiguous. It is also known that the liberation bands of stoichiometric apatite are strong compared to non-stoichiometry apatite (Muller *et al.*, 2007). Thus, biomimetic apatites deposited on the Ti surface probably are non-stoichiometry and non-carbonated hydroxyapatite.

Another aspect of concern which contributes towards a better bioactive implant is surface wettability. Alkali heat treatment, either sodium or potassium, both created an interconnected porous structure contributing to geometrical air trapping effects caused by wetting of hydrophilic pores (Whyman and Bormashenko, 2011). Immersion of Ti into DPBS solution for 3 days in the incubator has changed the wettability properties from slightly hydrophobic to hydrophilic. The formation of titanate layer in cooperation with hydroxyl group on the treated surface has the affinity to attract water molecule droplets from contact angle goniometer syringe. As detected by FTIR spectrum, the presence of a weak hydroxyl band on apatite deposited on the surface of Ti is superior hydrophilic. The hydroxyl group detected is from the apatite chemical composition itself and the remaining amorphous calcium titanate layer which has not been converted into amorphous calcium phosphate completely. Even though S600, P and P500 did not show apatite forming images, the amount of hydroxyl group up surged after DPBS treatment results in surface hydrophilicity. The interconnected porous structure that was not covered by apatite coating also contributed to the attraction of water molecules. Furthermore, the amount of hydroxyl group is insufficient in changing the negative charge surface which is inadequate for the attraction of the positive charge (Ca^{2+}) for further apatite formation. Besides, the porosity of size and morphology structure might disrupt the apatite nucleation rate. Meanwhile, Kim *et al.* (2013) reported a contradictory finding in which the calcium phosphate coating on the surface treated with concentrated potassium hydroxide have a slightly

hydrophobic characteristic in the range of 55-73° after 15 mins liquid-solid contact. There are no concrete studies describing the degree of hydrophilicity of biomimetic calcium phosphate in detail. At a glance, Zhou *et al.* (2013) reported that a microwave-assisted biomimetic apatite coating produced a superior hydrophilic surface which supports the presents study.

The human body consists of body fluid which contains 90% of water that carries proteins and ions. These components are hydrophilic and are directly in contact with the metal implant. Hence, the surface of the metallic implant is important to be in hydrophilic condition for a better adsorption of proteins which serve as substrate for bone cell growth. It is commonly reported that moderate hydrophilic characteristic of biomaterial surfaces (i.e., surfaces with high wettability) improved cell growth and implant-cell tissue interaction (Rosales-Leal *et al.*, 2010). So, taking this into consideration, the alkali etching process was performed to activate the surface with hydroxyl group thus increase the coated metal implant wettability.

Generally, the biomimetic apatite coatings are performed for approximately seven to 14 days in SBF solution (Ting *et al.*, 2015). There are several pre-treatment methods of manipulating the concentration and constituent of SBF solution in order to reduce the deposition time of calcium phosphate. SBF is a metastable solution especially in high concentration and it is not easy to handle its consistency and stability. Thus, in this present study, DPBS solution was used as an option for creating an environment that mimics human blood plasma to create a favourable calcium phosphate deposition. The biomimetic coating obtained for DPBS immersion represented structural features and ion composition that closely to human bone properties. In addition, surface modification in creating an active surface of Ti6Al4V was taken into consideration for further calcium phosphate deposition.

CONCLUSION

The deposition of CaP was detected on the alkali treated surface of Ti6Al4V after soaking in the DPBS solution for 3 days. The results showed a numerous apatite deposition after alkali treatment with 5M NaOH (S Sample) as well as on untreated Ti6Al4V, S500 and P600 samples. The surface wettability after alkali treatment and alkali-heat treatment decreased due to the presence of the interconnected porous structure. Apparently, lower contact angle after surface modification does not show a great influence on apatite deposition. DPBS solution has demonstrated apatite deposition on untreated Ti and treated Ti substrate within 24 h based on Ca ion reduction in the solution. In addition, Mg⁺ ion concentration hastens apatite forming within 24 h. The results of this

study confirmed that the simple NaOH-heat treatment on Ti substrate could change the topography of the metallic surface to bioactive surfaces which enhance bioactivity in DPBS solution. Coating HA via biomimetic techniques could be fast and easy handling with the appropriate approach.

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