Biofunctionalization of Modified Surfaces of Titanium

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ABSTRACT

The aim of this study was to assess the viability of coating collagen to Hydroxyapatite (HA) coated commercially pure Titanium (cpTi) samples. Five samples of cpTi were immersed in a simulated body fluid (Tas-SBF) after stages of gritting, sandblasting and acid etching. One sample was taken out after 3, 5 and 7 days of immersion in Tas-SBF respectively. The 7 days Tas-SBF coated sample was coated with collagen by physical adsorption method. Analysis was performed to study the surface roughness, morphology and elemental analysis of the coated surfaces. The equipment used were Alicona, Scanning Electron Microscope (SEM), Energy-dispersive X-ray Spectroscopy (EDS) and Fourier Transform Infrared Spectroscopy (FTIR). Process of sand blasting and acid etching were able to produce desired morphology and surface roughness. The average surface roughness, Sa of sand blasted cpTi surface and acid etched cpTi were 2.17 ± 0.75 μm and 2.12 ± 0.15 μm respectively. Immersion in Tas-SBF produced a crystalline coating and morphology that were similar to bone-like apatite. Roughness value of cpTi after being immersed in SBF for 7 days was 2.23 ± 0.31 μm and the Ca/P ratio was 7.076. Collagen coated samples had the highest S_a value which was 3.91 ± 0.31 μm and the Ca/P ratio was 1.190. This study showed that it is possible to achieve apatite and collagen coating on a modified cpTi surface using physical adsorption method.

Key words: Titanium; Osseointegration; Acid-etching; SBF coating; Collagen; Hydroxyapatite.

INTRODUCTION

In this current world with the rapid growth of older population, dental implant has become increasingly popular due to its long record of success rate and predictable outcomes (1-2). Many materials have been used as dental implants but titanium has emerged as the most popular material due to its superior biocompatibility, resistance against corrosion, satisfactory strength and elasticity (3).

Osseointergration is the basis of dental implantology. It is described as direct structural and functional union between bone and implant interface where there is no interposed soft tissue layer (4-5). A successful osseointergration from the clinical point of view could be assessed though the implant stability assessment over time, as time is necessary to accomplish this process. Mobility of the implant 4-6 months after the implant was placed could give the indication of a failed osseointergration because the

chances of obtaining spontaneous stability after this period is extremely slim (6). The evidence of bone formation surrounding the implant surface shows a successful osseointergration, while the coating of fibrous tissue instead of bone formation indicates failure (7).

Surface characteristics such as surface morphology and chemical constituents of implant surface greatly affect the biocompatibility, and thus, the success rate of implants apart from the surgical technique, which is already standardized (3, 8). The surface between bone and implant can be influenced by the choice of materials used and methods of surface alteration, which includes morphological, physiochemical and biochemical methods (9-10).

Blasting which is one of the popular techniques to alter the morphology of the surface forms a layer of porosity by colliding the surface with particles that are minute in size. The desired thickness of

the porous layer created is altered according to the size of particles and force used to collide with the surface (5). However, the process of blasting also bring some disadvantages such as impurities and evoke surrounding inflammation reactions that are localised due to the abrasive particles that had been dissipated into the bone of the host (11). Research shows that the average surface roughness (S_a) of about 1-1.5 micrometer (μm) is known to produce a better osseointergration in comparison to smoother or rougher surfaces (10). The material that is commonly used in the process of blasting is alumina powder (12).

Besides that, physiochemical methods that are used in the alteration of implant surfaces will further influence the surface constituents, energy and charge. Blasting and acid etching, fluoride surface treatment, anodised surface implants, laser etching and micro arc oxidations are among physiochemical techniques that have been studied (10). Treating implant surfaces with acids after sandblasting could aid in the removal of surface oxide such as TiO_2 and contaminants, producing a clean, even and rough surface prior to other surface treatment that can lead to a better bone fixation (9, 10, 13). Commonly used acids include the combinations of Hydrochloric acid (HCl) and Sulphuric acid (H_2SO_4) or 2% Hydrofluoric acid (HF) and 10% Nitric acid (HNO_3) (10, 14).

The main inorganic composition of normal bone is calcium phosphates. The molar ratio of Ca/P in bone is around 1.71 (15). SBF is a solution that is similar to the constituent of the human plasma without the presence of proteins and cells which was first made popular by Prof. Tadashi Kokubo (16). SBF mimics human body plasma in terms of pH, temperature and inorganic composition (17). Supersaturated calcium phosphate solution of the SBF with a pre-treated titanium implant surface allows bone deposition on the surface of titanium implants forming a layer of hydroxyapatite. Due to its bioactivity, it could be made as a carrier for biologically active molecules such as peptides and collagens (13). Besides that, osteoblast adhesion and proliferation are also highly encouraged with the presence of calcium phosphate that are major part of this biomimetic hydroxyapatite layer (18). However, the major drawback could be the poor mechanical properties exhibited by this layer (19).

Obtaining an optimum osseointergration has become central to the clinical success of implants. However, the importance of having a biological seal which involve a layer of soft tissue seal surrounding the implant is also known as the contributing factor to a successful implant procedure for a good seal is important to avoid the colonisation of bacteria that leads to peri-implantitis that fails an implant (20). One of the approaches that could improve biological seal is through biomimetic modification of implant surface with collagen (20).

Collagen is one of the most abundant proteins in the human body that acts as the major structural protein of our connective tissue and aids in mechanical stability, migration of osteoblasts and the control of cell adhesion. Methods tested in immobilising collagen to the surface of implants are via covalent bonding and physical adsorption. Covalent bonding is shown to have higher stability and effective in immobilizing a greater amount of collagen onto the pre-treated implant surface (20). The amount of collagen being immobilised and its stability onto the surface is also greatly influenced by the physical chemical properties and roughness of the surface.

The aim of this study was to assess the viability of coating collagen to hydroxyapatite (HA) coated commercially pure Titanium (cpTi) samples. The objective of this study was to evaluate how changes in the surface morphology of titanium would affect the coating of collagen via physical adsorption method. The coating duration was also investigated to ascertain the details of how collagen adhered onto the pre-treated titanium surface that is layered by hydroxyapatite and ultimately how they affect osseointergration in dental implants.

MATERIALS AND METHOD
Materials

5 samples of 10 mm x 10 mm pre-cut commercially pure grade 2 titanium (cpTi) discs from E Steel Sdn. Bhd., Malaysia were used. Different chemicals reagents and materials were used for various procedures (Table 1).

Table 1. Chemicals reagents and materials used for cpTi samples surface modifications.

Method	Materials/ Chemicals	Purity	Supplier
Gritting	Waterproof silica carbide paper – 400 grit	NA	NA
Sand blasting	Brown alumina powder	Grade A	NA
Acid etching	Hydrochloric acid	37%	Fisher Scientific UK
	Sulphuric acid	>95%	Fisher Scientific UK
Alkaline treatment	Sodium hydroxide pellet	NA	Fisher Scientific UK
SBF apatite coating	See Table 2		

Table 2 shows the chemicals that were used for the coating of samples with hydroxyapatite using SBF solution.

Table 2. Order and quantity of chemicals reagents supplied by Sigma Aldrich Sdn. Bhd., Malaysia and then mixed into SBF solution.

Order	Chemical reagents	Weight (g/L)	Purity/ concentration
1	NaCl	9.8184	99.5%
2	$NaHCO_3$	3.4023	99.5-100.5%
3	KCL	0.5591	99%
4	Na_2HPO_4	0.2129	99%
5	$MgCl_2.6H_2O$	0.4574	99%
6	1M HCl	15mL	10mol
7	$CaCl_2.2H_2O$	0.5513	99%
8	Na_2SO_4	0.1065	99%
9	TRIS	9.0855	99.8%
10	1M HCl	50mL	10mol

Methods

Five samples of 1 cm x 1 cm pre-cut commercially pure titanium (cpTi) were selected for this study. Samples were labeled using a high speed hand piece. Samples were then gritted with waterproof silicon carbide sandpaper 400 grit to creat an initial surface roughness using the Buehler (Illinois, Unites States of America) twin variable speed grinding and polishing machine. Samples were then washed with distilled water and ethanol for 30 seconds respectively and then air-dried for 30 seconds using compressor (Model no: DR-115, Swan Oilless Compressors, Tong Cheng Iron works Co.Ltd, Malaysia). Using the Alicona Infinite Focus Optical 3D Measurement Device G4 (Alicona Imaging GmbH, Austria) in 20X magnification, the surface roughness values were measured at seven points. The average value and the standard deviation of the surface roughness (S_a) were obtained and tabulated.

Gritted samples were then sandblasted using a sandblasting machine (Model no SB-8060-KP Techno Finishing Sdn Bhd, Malaysia). Grade A brown alumina of mesh 120 and an average abrasive grain size of 125 μm were blasted perpendicular to the surfaces of samples through an air gun at a distance of 5 cm for approximately 3 seconds, at pressure of 50 psi. Samples were then rinsed with ethanol and distilled water and then air-dried to remove sandblasted residua and contaminants from the samples surfaces. Then, the surface roughness values were measured at seven points again using the Alicona machine. The S_a values were obtained and tabulated.

The Memmert (Germany) water bath was preheated to 80°C. 37%HCL and >95% H_2SO_4 were used in the ratio of 1:3. 5 ml of HCL and 15 ml of H_2SO_4 was added into 80ml of distilled water. Samples were put into a beaker containing the acid solution. The beaker was sealed with parafilm and placed into the water bath of 80°C for 30 minutes. The solution was swirled for every 5 minutes. Samples were then taken out, rinsed with ethanol for 5 seconds and distilled water for 10 seconds and then air-dried. Surface roughness of samples was measured using Alicona machine again at seven points. The S_a values were obtained and tabulated.

50 ml of 5M NaOH was prepared by adding 10 g of NaOH pellet into 50 ml of distilled water. NaOH was stirred using magnetic stirrer (IKA@ RCT Basic, Malaysia) at 700 rpm at room temperature until all NaOH pellets dissolved. Samples were then placed in beaker containing NaOH solution, sealed with parafilm and placed in the precision thermal convection oven (Medcenter Einrichtungen GmbH, Germany) under 60°C for 24 hours.

SBF solution was prepared by adding the chemical reagents according to order and quantity (Table 2) into a beaker. The beaker was then sealed with parafilm and stirred using magnetic stirrer under 700 rpm, room temperature for 2 hours. Samples that were immersed in NaOH were taken out after 24 hours. Samples were then rinsed with distilled water for 10 seconds. Samples were put into a beaker and placed into oven at 40°C for 30 minutes to 1 hour until the samples were completely dry. Samples were then immersed in SBF solution. SBF solution was changed every 24 hours for the first two days, and then it was changed every 48 hours twice. One sample was taken out after 3 days, 5 days and 7 days for surface analysis.

The 7 days collagen coated sample was coated with collagen by physical adsorption. 0.1% (1 mg/ml) solution of calf skin collagen type I in 0.1 M acetic acid was obtained from Sigma Aldrich, Germany (C8919) and was kept at 2 - 8°C for storage purposes. The collagen solution (1 mg/ml) was diluted 10-fold with sterile water to obtain a working concentration of 0.01%. The diluted solution was stirred for 2 hours at room temperature and the protein was allowed to bind to the cpTi for 18 hours at 2-8 °C.

Afterwards, the cpTi substrate was carefully removed and rinsed with distilled water to remove weakly bonded COL off the surface. To perform the surface analysis, the sample was freeze dried using a freeze dryer (FreeZone, Labconco, USA). The temperature of the freeze dryer was set to -50°C and the vacuum pressure was around 0.043 mBar. The sample was freeze dried for 6 hours. Alicona,

SEM, EDS and FTIR were used to examine collagen coated sample.

RESULTS
Surface Roughness Measurements

Surface roughness measurements of all the samples at the various stages of processing are shown in Figure 1.
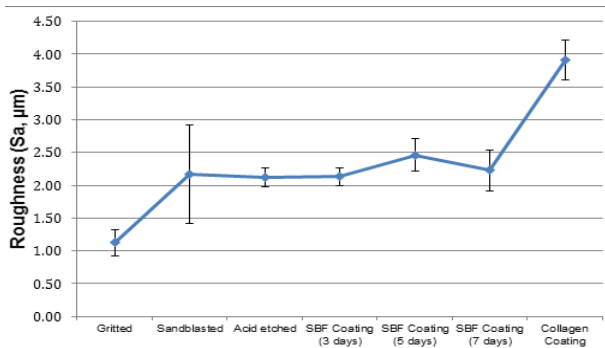

Figure 1. Chart of average surface roughness of cpTi samples after stages of gritting, sand blasting, acid etching and immersing in SBF for 3 days, 5 days and 7 days, as well as collagen coating.

Figure 1 and Table 3 show the S_a, of the gritted, sand blasted, acid etched and SBF coated samples of cpTi samples. The gritted samples had a roughness value of 1.13 ± 0.20 µm. The relatively small standard deviation value was an indication of the importance of this process to obtain an even surface prior to sandblasting. The chart shows a drastic increment of roughness values from gritting stage to sand blasting stage. There was a small decrease of S_a values after the acid etching stage. The S_a value of the SBF coated samples fluctuated between 2.13 µm and 2.46 µm. The S_a value then increased to the highest value, which is 3.91 µm after the collagen coating stage.

Table 3. The Sa values (±SD) of the modified surfaces measured using Alicona.

Stages	Average surface roughness (µm)
Gritting	1.13 ± 0.20
Sand blasting	2.17 ± 0.75
Acid etching	2.12 ± 0.15
SBF coating (3 days)	2.13 ± 0.14
SBF coating (5 days)	2.46 ± 0.25
SBF coating (7 days)	2.23 ± 0.31
Collagen coating	3.91 ± 0.31

SEM Analysis

SEM images of the various surfaces coated with SBF as well as at different draf (3, 5, 7) were shown in Figure 2.

The lower magnification images (at 1000x) do not show any clear difference in the morphology of the 3 days. All of them show a rough surface with pores. Higher magnifications show the crystalline nature of the surface with fine nanostructural crystals forming a flower like structure. The structures are connected to form long and thick walls that have a network of pores.

The thickness of HA formed cpTi samples after immersion in SBF for 3, 5 and 7 days are shown in Figure 3. The thickness of HA increases from 3 days coated samples (10.79 ± 0.14 µm) to 5 days coated sample (12.77 ± 0.11µm). The HA layer for the 7 days coated samples is 10.43 ± 0.23 µm.

Figure 4 shows the morphology of the collagen-coated samples at different magnifications. The network of structures is evenly distributed on the surface and small fibrils are visible. The distribution of the collagen coating was dense and even throughout the coated samples indicating a good coating.

EDS Analysis

EDS analysis of the various samples coated with SBF and collagen are given in Table 4. It give the elements detected in the sample surface.

Table 4 shows the elements analysis of cpTi samples after immersing in SBF for 3 days, 5 days, 7 days and collagen coated samples after being immersed in SBF for 7 days. The analysis was in term of atomic %. Calcium content decreased from 3 days coatings to 5 days coatings, and then it increased again to the highest, which is 1.06 ± 0.50 for the 7 days coatings. Collagen coated samples have the lowest calcium content which is 0.25 ± 0.04. Phosphorus present on all the samples as well. The phosphorous content is 0.15 ± 0.02 on the 3 days coatings, the content then increases to 0.17 ± 0.06 on the 5 days coatings. The 7 days coated samples have the same phosphorous content as the 3 days coated samples. The phosphorous content is the highest in the collagen coated samples, which is 0.21 ± 0.05.

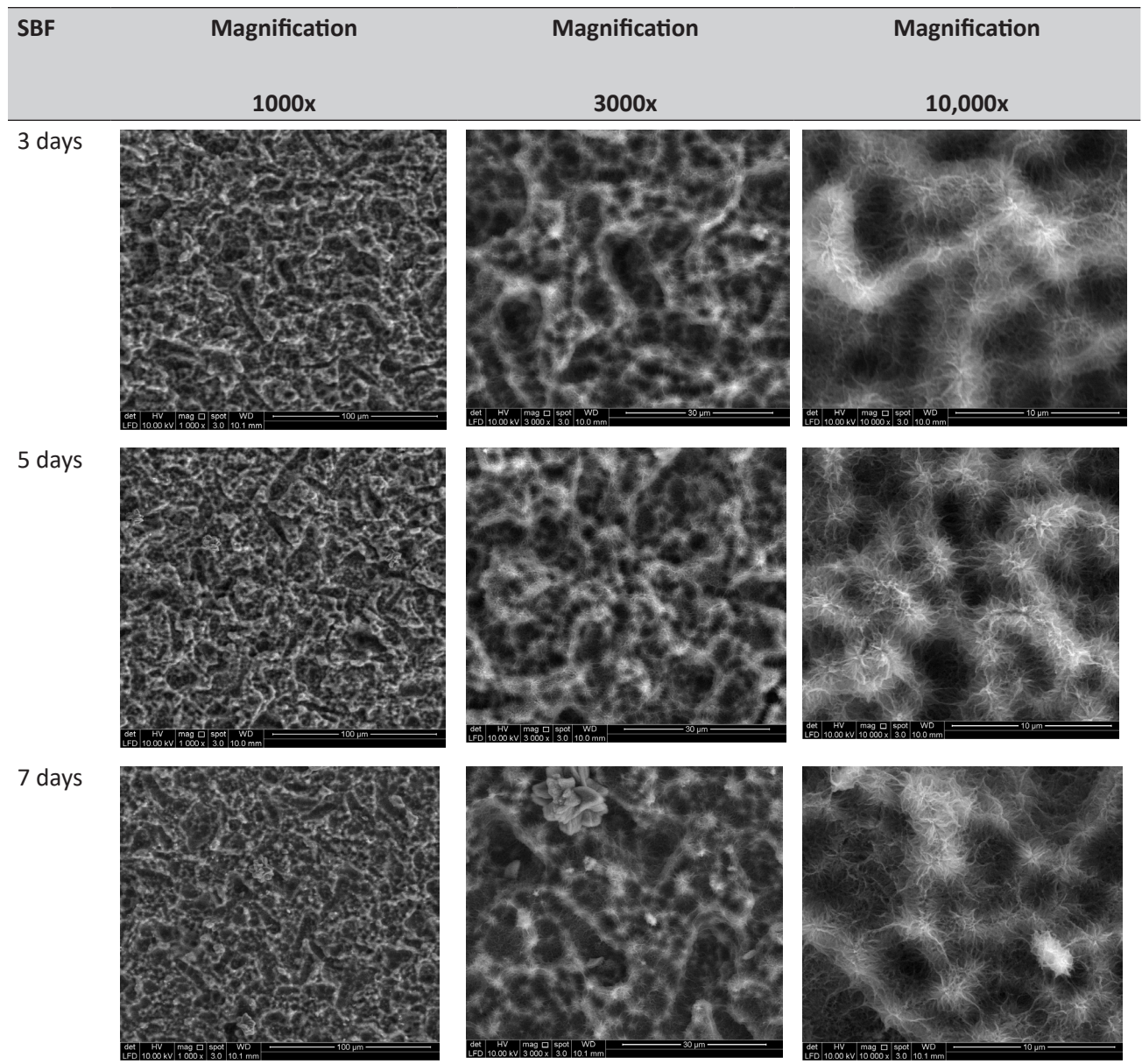| SBF	Magnification 1000x	Magnification 3000x	Magnification 10,000x
3 days			
5 days			
7 days			

Figure 2. SEM micrographs of the SBF coated commercially pure Ti under three different magnifications.
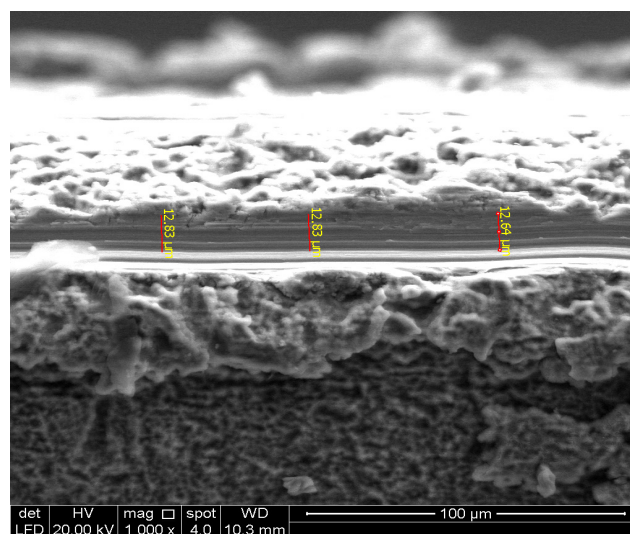

Figure 3. SEM micrographs showing the thickness of HA on commercially pure Ti sample after immersion in SBF for 5 days under magnification of 1000x (Layer A: Coating, Layer B: Titanium)
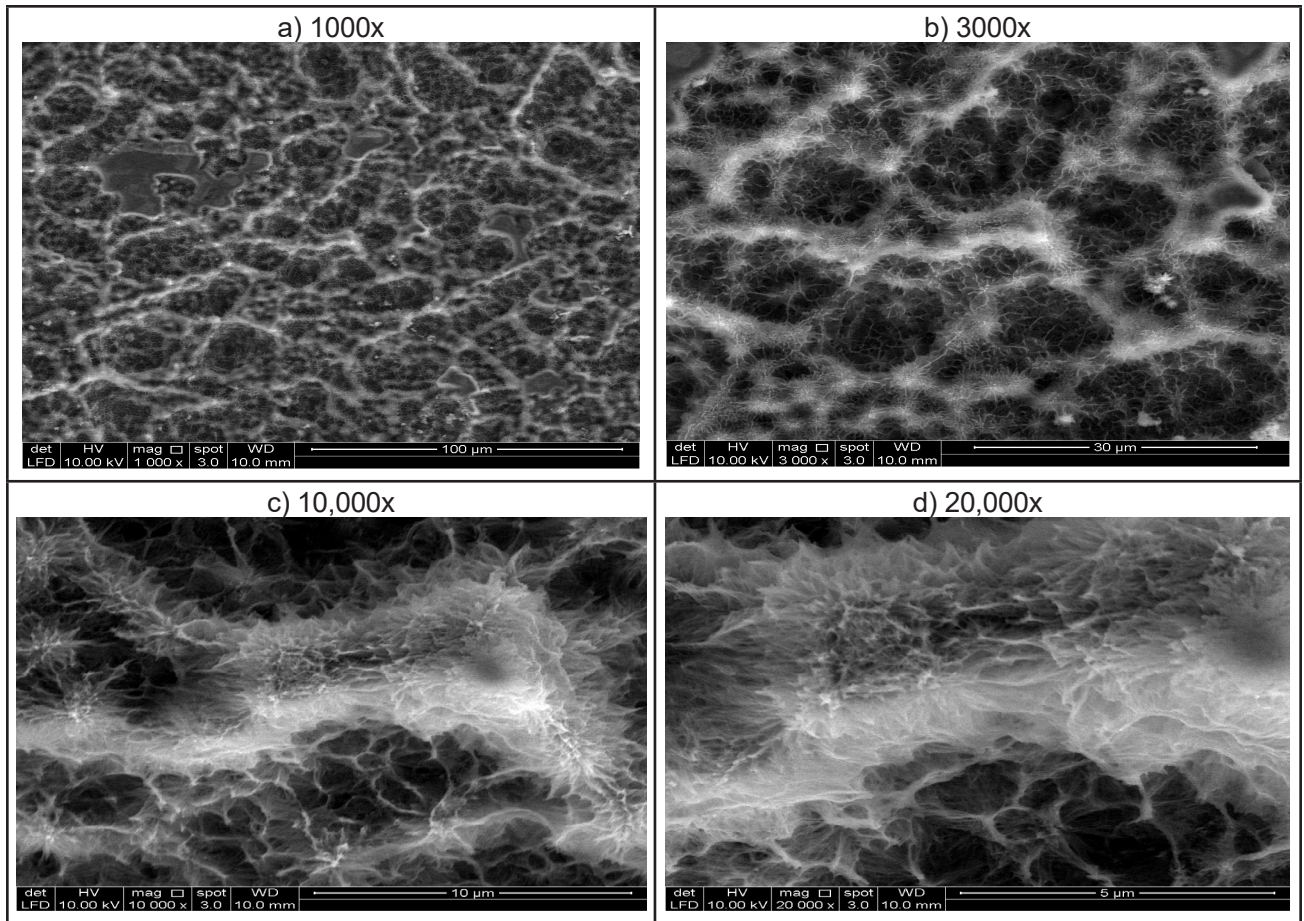
Figure 4. SEM micrographs of collagen coated surfaces of commercially pure Ti samples after being immersed in SBF for 7 days under magnification of a) 1000x, b) 3000x, c) 10,000x and d) 20,000x.

Table 4. The elemental analysis of cpTi samples in atomic % after immersing in SBF for 3 days, 5 days, 7 days and collagen coated samples after being immersed in SBF for 7 days.

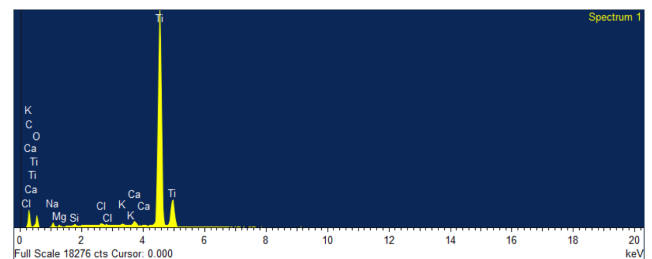| Element	Atomic % 3 days	Atomic % 5 days	Atomic % 7 days	Atomic % Collagen
C K	6.51 ± 6.11	19.87 ± 8.27	5.70 ± 0.60	20.05 ± 2.76
O K	63.21 ± 4.03	53.58 ± 5.68	65.48 ± 0.68	54.96 ± 2.23
Na K	2.28 ± 1.18	2.85 ± 0.84	0.73 ± 0.19	-
Mg K	0.52 ± 0.11	0.39 ± 0.08	0.63 ± 0.06	-
Al K	0.11 ± 0.15	0.11 ± 0.17	0.07 ± 0.10	-
P K	0.15 ± 0.02	0.17 ± 0.06	0.15 ± 0.02	0.21 ± 0.05
K K	0.06 ± 0.09	0.15 ± 0.11	-	-
Ca K	0.75 ± 0.08	0.47 ± 0.04	1.06 ± 0.50	0.25 ± 0.04
Ti K	26.41 ± 2.34	22.17 ± 1.87	26.07 ± 1.18	24.54 ± 0.61
Cl K	-	0.24 ± 0.08	-	-
Si K	-	-	0.07 ± 0.09	-

Figure 5 shows the EDS result of commercially pure Titanium (cpTi) after immersing in SBF for 3 days (a), 5 days (b) and 7 days (c). EDS results show that both 3 days coated samples and 5 days coated samples give a typical Ti peak with presence of K, C, O, Ca, Cl, Na, Mg, Si and K elements with extra of Al element for the 5 days coated sample. The 7 days coated sample shows a Ti peak as well, with the elements of C, O, Ca, Na and Mg. Collagen coated samples also shows a Ti peak with another small peak of C, O and Ca. The FTIR in Figure 6 shows the presence of amide functional groups indication the presence of collagen. The OH group shows the presence of HA.
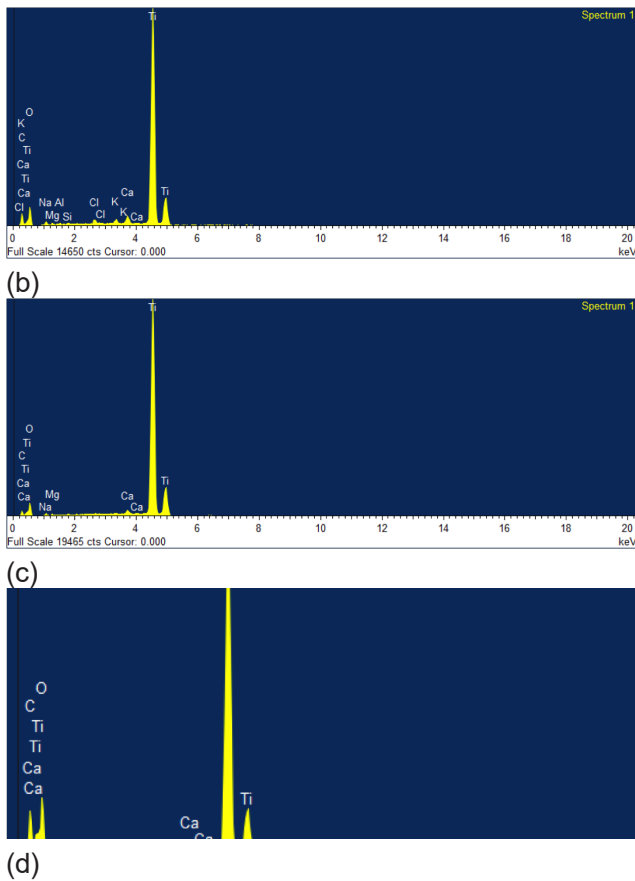

(a)

Figure 5. EDS result of cpTi after immersing in SBF for 3 days (a), 5 days (b), 7 days (c) and collagen coated samples after being immersed in SBF for 7 days (d).
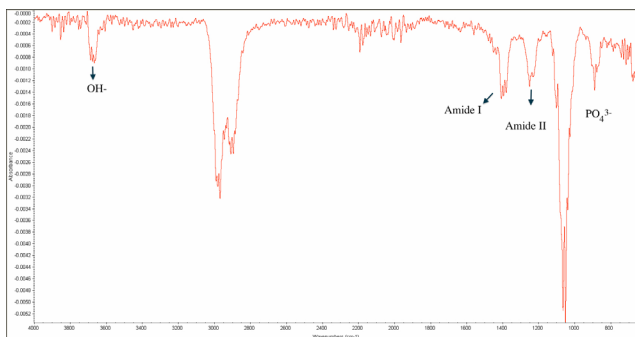

Figure 6. FTIR image of collagen coated samples after being immersed in SBF for 7 days.

DISCUSSION

Surface roughness of implants plays a crucial role in the success of osseointegration. It has been proven by various studies that there is a positive bone response with rough implant surfaces as compared to the earlier machined or polished surfaces (10). The S_a of the samples in the present study displayed surface roughness values that coincided with the reported ranges in other studies (9-10). After achieving a standardized gritted surface, the S_a value increased from 1.13 ± 0.20 µm to 2.17 ± 0.75 µm. Acid etching

ensured the removal of surface contaminants and introduced a uniform micro-roughened surface with a S_a of 2.12 ± 0.15 µm. The lower standard deviation seen in the acid etched surface is an indication that a more uniform surface roughness was achieved. This is important to this study as a uniform surface roughness favors a homogenous apatite deposition as reported in previous studies (14).

Biomimetic coatings have been proven to be beneficial for osseointergration and hence implant therapy success (21-22). This study employed a biomimetic HA surface coating using the Tas-SBF method because of its proven osseoconductive properties (23-24). An HA layer on titanium surfaces using Tas-SBF is capable of achieving crystallinity and morphology similar to that of bone-like apatite (25). Increased crystallinity of HA surface coatings appear to slow resorption of HA at the implantation site (26). This study follows a similar trend because it was successful in achieving uniform crystalline HA/ tricalcium phosphate layer on top of the cpTi. This is important because consequentially this layer could improve the surface area for cell attachment and proliferation. The FTIR spectrum analysis of the coatings detected –OH bonds which was an indication that the surface contained HA. Based on these results, it is evident that we were successful in obtaining a bioactive thin and relatively uniform coating of HA on cpTi disc.

Generally, biomimetic coating processes are performed for about 7 to 14 days (27). However, there are some treatment methods that temper with the concentration and constituents of Tas-SBF solutions and can considerably reduce the Tas-SBF immersion time to create a favourable HA/calcium posphate deposition (28-29). This study showed that it is possible to reduce the time of Tas-SBF immersion while maintaining the standard Tas-SBF solution concentration. Furthermore, this research was able to prove that a more uniform and thinner layer of HA/ tricalcium phosphate that was immersed in Tas-SBF for 7 days or less may be sufficient for implant surface coating. The biomimetic coating obtained for all the Tas-SBF immersion periods portrayed compositional and structural features that closely resembled human bone.

The EDS analysis showed a Ca/P ratio of 2.76 on the 5th day and a ratio of 7.067 on the 7th day of Tas-SBF immersion. This could indicate the presence of some CaO phase in the coating. Furthermore, in terms of the composition according to the EDS results that we obtained from this study, we also found the presence of Na+, K+ and Mg+ which is consistent with a previous study that used Tas-SBF to coat cpTi discs with HA (22).

In the final stage of this study, type I collagen was physically adsorbed onto a cpTi disc that was immersed in Tas-SBF for 7 days. Collagen has been proven to be beneficial for osseointergration (20). The authors are unaware of any other study that employs Tas-SBF cpTi samples in conjunction with collagen. The SEM micrographs showed beginnings of a collagen scaffolds on the surface of the sample. The EDS showed a high atomic percentage of C, that is 20.05 ± 2.76 % indicating the presence of collagen on the surface. Also, the FTIR showed presence of bands corresponding to groups PO_4^{3-} at 600 and 1100 cm^{-1}, group OH of P-OH bond at 870 cm^{-1}, groups OH of the structural and adsorbed H_2O molecules at 3570 cm^{-1} respectively. The FTIR also showed peaks at 1535 cm^{-1} for amide II and 1244 cm^{-1} for amide III. This proves that physical adsorption maybe a viable method to coat HA cpTi samples with collagen. Furthermore, the incorporation of both biomimetic HA and collagen on the surface of a titanium implant can provide the optimal basis for cell proliferation and addition.

CONCLUSION
This study was able to show the importance of different surface morphology in determining the viability of coating a biomolecule. This study was successful in showing that the coating of collagen on to a titanium sample with HA as the underlying coating can be effected by the surface features such as roughness and morphology. Further research needs to be conducted in investigating that benefits of collagen coated implant surface in the process of osseointergration.

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DECLARATION OF INTEREST
We have no conflict of interest to declare.

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