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IN-VITRO BIOCOMPATIBILITY STUDY OF HYDROXYAPATITE COATED ON CO-CR-MO WITH OXIDE INTERLAYER

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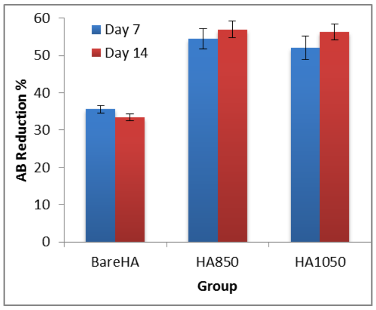
H. Mas Ayu[a*], S. Izman[b], R. Daud[a], A. Shah[c], Mahmood Anwar[d], G. Krishnamurithy[e], T. Kamarul[e]

[a]Faculty of Mechanical Engineering, Universiti Malaysia Pahang, 26600 Pekan, Pahang, Malaysia
[b]Faculty of Mechanical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia
[c]Faculty of Technical and Vocational, Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia
[d]Faculty of Engineering and Science, Curtin University, 98009 Miri, Sarawak, Malaysia
[e]Department of Orthopaedic Surgery, NOCERAL, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

*Corresponding author
masszee@ump.edu.my

Graphical abstract



Abstract

The effects of oxide interlayer on cobalt-chromium-molybdenum substrate were investigated in order to improve the quality of hydroxyapatite (HA) coating as well as enhance the cell responses. Substrates were oxidized at temperature of 850 °C and 1050 °C for 3 hours. Oxidized substrates were then coated with HA slurry using dip coating technique. Analysis of surface morphology, thickness and chemical composition of oxide interlayer prior to HA coating were performed using field-emission scanning electron microscopy, energy dispersive X-ray spectroscopy and grazing X-ray diffraction respectively. It seems that rough surface of oxide interlayer provides better mechanical interlocking of HA particles to the substrate surface with no visible micro-cracks. In addition, the HA coated substrates with oxide interlayer also demonstrate strong attachment and better proliferation of cells compared to HA coated substrates without oxide interlayer. The results also demonstrates that cells were spread out more actively as earlier as day 7 and have greater extensions of filopodium on HA coated substrates with oxide interlayer. It is concluded that the introduction of an intermediate oxide layer on Co-Cr-Mo substrate prior to HA coating has shown a positive effect in terms of improving the quality of HA coating as well as cell bioactivity performance.

Keywords: Co-Cr-Mo alloy, thermal oxidation, hydroxyapatite, cell attachment, dip coating

Abstrak

Kesan lapisan oxida ke atas substrat kobalt-kromium-molibdenum dikaji dengan tujuan mempertingkatkan kualiti salutan hidrosiapatit (HA) sekaligus mempergiatkan tindakbalas sel. Substrat dioksidakan pada suhu 850 °C dan 1050 °C selama 3 jam. Substrat yang telah teroksida kemudian disalut dengan HA menggunakan teknik salutan bercelup. Analisis ke atas permukaan morfologi, ketebalan dan komposisi kimia bagi lapisan oxida sebelum salutan HA dijalankan menggunakan mikroskop imbasan pelepasan elektron, serakan tenaga X- ray spektroskopi dan ragutan X- ray pembelauan. Ia seolah-olah permukaan kasar lapisan oksida telah menyediakan pautan mekanikal yang lebih baik kepada partikal HA ke atas permukaan substrat tanpa ada mikro-retak yang boleh dilihat. Tambahan lagi, salutan HA substrat dengan lapisan oksida juga menunjukkan lekatan yang kuat dan percambahan sel yang lebih baik berbanding salutan HA substrat tanpa lapisan oksida. Keputusan juga menunjukkan yang penyebaran sel yang lebih aktif iaitu seawal 7 hari dan mempunyai lanjutan filopidia yang lebih besar di atas salutan HA subtrat dengan lapisan oksida. Kesimpulannya, pembentukan pengantara lapisan oksida di atas Co-Cr-Mo substrat sebelum salutan HA menunjukkan kesan positif dari segi peningkatan kualiti salutan HA dan juga prestasi bioaktiviti sel.

Katakunci: Co-Cr-Mo aloi, pengoksidaan terma, hidroksiapatit, lekatan sel, salutan celup

1.0 INTRODUCTION

Co-Cr-Mo alloys have been extensively used as materials in orthopaedic and dental implants [1, 2]. They are preferred mainly due to their superior mechanical properties such as good wear resistance and high surface hardness [3]. When implanted in the body, these materials tend to undergo surface modification as the result of passive oxide film formation. This fine oxide film appears to be resistant to corrosion and host tissue interaction [2]. In fact, passive oxide film has also been shown to have greater tolerance to host immune response. Excessive immune response has implication in the increasing incidence of implant loosening [4]. In the absence of passive oxide film, the biocompatible characteristics of these materials can be altered. In effect, the exposed implant releases metallic ions into the host body as the result of direct metal-body fluid contact that causes ionic exchange event [5].

To overcome this issue, hydroxyapatite (HA) has been introduced as a potential coating material which is used as a protective layer, preventing any physio-chemical ionic reactions to occur. In addition, HA has osteoconductive properties which is preferred for bone reconstruction procedure [6, 7, 8]. Many studies have reported that HA coated on Co-Cr-Mo alloy enhances the osteointegration properties further when it is in contact with host bony tissue [1, 9, 10]. However, there have been reports of implant failure attributed to the dislodging of the HA coating from the implant surface [11]. To overcome this issue, surface modification techniques which includes sand blasting [12], grit blasting [13] and thermal oxidation [14, 15, 16] have been introduced to increase the surface roughness of metal with the aim to improve adhesion strength of HA coating. Other techniques such as wet chemical methods involving acid [17] and alkali treatment [1] have also been used to enhance the chemical interaction between metal and HA. Nevertheless, several studies have shown evidences that these physical and chemical methods still pose an unfavourable option that lacks improving the HA bonding on metal implant [17, 18, 19]. In spite of this, high temperature driven processes such as thermal spray, plasma spray, electron beam deposition and pulse laser deposition, which lead to melt the coating material during deposition process and solidifies during cooling of the substrate, have shown enhanced metal-HA bonding [19]. However, there were several issues such as variation in surface coverage, coating composition, crystallinity, homogeneity and surface morphology involved in applying these methods [19, 20].

Owing to this problem, thermal oxidation technique has been suggested and used in several studies [20-23]. Although this method has been adapted to study various core metal such titanium and stainless steel [16, 19], research on Co-Cr-Mo alloy is somehow still limited especially in evaluating the performances of the oxide interlayer on Co-Cr-Mo alloy in improving the quality of HA coating and their responses to the bioactivity cell. With regards to aforementioned evidences, therefore this study was presented to report the outcome of adopting thermal oxidation technique to create oxide interlayer on Co-Cr-Mo alloy prior to HA coating deposition. We anticipate that the formation of oxide interlayer would demonstrate better quality of HA coating with micro-crack free surface on Co-Cr-Mo alloy as well as enhance cell attachment and proliferation.

2.0 METHODOLOGY

2.1 Substrate Preparation

In the present study, a Cobalt-Chromium-Molybdenum alloy (Co-Cr-Mo) bar with ASTM standard of F1537 and chemical compositions of (in wt.%): C: 0.24; Cr: 29.6; Mo: 6.5; Si: 0.7; Ni: 0.1; Fe: 0.12; Mn: 0.7; N: 0.16 and Co: balance was used. The alloy bar was cut using precision cutter into disc shape with dimension of 14 mm diameter and 2 mm thickness (Buehler, Isomet 4000). The variation in surface roughness was reduced using #500 grit SiC paper in wet grinding method. The surface roughness of alloy discs was then measured using surface profilometer (Mitutoyo SJ-301) and 0.1 ± 0.02 µm surface roughness was achieved.

2.2 Thermal Oxidation Process

The purpose of thermal oxidation process on the Co-Cr-Mo substrate was to create oxide interlayer (chromium oxide, Cr_2O_3) on the substrate surface, which acts as an intermediate layer between the substrate and hydroxyapatite (HA) coating. In the present study, the thermal oxidation temperature of

850 °C and 1050 °C were assigned and performed in a muffle furnace for 3 hours under atmospheric condition, followed by a cooling process inside the furnace. The heating and cooling processes were constantly maintained at the rate of approximately 10 °C/min. The debris or impurities interfering during thermal oxidation process on oxidized substrates were removed in acetone using ultrasonic cleaning for 30 minutes, followed by steam cleaning and finally drying using a stream of compressed air. The substrates were then left to dry overnight in oven at 50 °C and analyses such as surface morphology, thickness and chemical composition of oxide interlayer were performed using field-emission scanning electron microscopy (FESEM), energy dispersive X-ray (EDX) and X-ray diffraction (XRD) respectively. Co-Cr-Mo substrate without thermal oxidation process (untreated sample) was used as control for the purpose of comparison.

2.3 Characterization of Hydroxyapatite Preparation

In the present study, a commercial hydroxyapatite (HA) powder ($Ca_3(PO_4)_2$) (Sigma Aldrich, UK) was used as the coating material. The purity, chemical composition and morphology of HA were pre-determined using EDX (Figure 1a) and XRD (Figure 1b), respectively. The HA powder was sieved using RX-B6-1 Sieve Shaker to obtain particle size of ≤ 71 µm and in order to prevent agglomeration of HA powder during mixing with ethanol (CH_5OH). The sieved HA powder was then collected and kept in the desiccator for further use.

2.4 Sol-gel Dip Coating Deposition

The HA coating deposition process was carried out using HTWL-01 Desktop Dip Coater (MT1 Cooperation, USA) at room temperature and under controlled humidity. The substrates with and without oxide interlayer were assigned for dipping and withdrawal steps in the HA slurry with fixed speed of 200 mm/min. This step was repeated for five cycles with an interval of 3 minutes for drying step in every cycle. This step is mainly used to obtain a desired coating thickness of HA on the substrates. The substrates were then sintered in the muffle furnace at temperature of 750 °C for 1 hour to evaporate the residual solvent and densify the coating layer. This step is crucial to improve the bonding strength of HA coating onto surface of Co-Cr-Mo substrate. Substrates oxidized at 850 °C and 1050 °C and were coated with HA were recognized as HA850 and HA1050, respectively, whilst substrate coated with HA without oxide interlayer, which serves as control group, was recognized as BareHA.

2.5 Mesenchymal Stem Cell Culture and Alamar Blue Assay

Mesenchymal stem cells (MSCs) were harvested and cultured as described previously by other researchers [24]. Cell attachment analysis was performed on untreated sample (no. of samples, n = 12), BareHA (n = 12), HA850 (n = 12) and HA1050 (n = 12). The analysis was done at two time intervals, i.e. at day 7 and 14. Six samples were drawn from each group of samples to be analyzed on the cell activities. Observation on the cell growth and cell attachment was done using Field Emission Scanning Electron Microscope (FESEM), Zeiss Supra 35VP. Analyses of each sample groups were evaluated on 6 different samples under the same condition to minimize variance of cell morphologies. However, before viewing under FESEM, all samples need to be prepared in order to fix the cell on the sample through dehydration process [25]. In addition, the cell proliferation assay was analyzed based on percentage of alamar blue (AB) reduction.

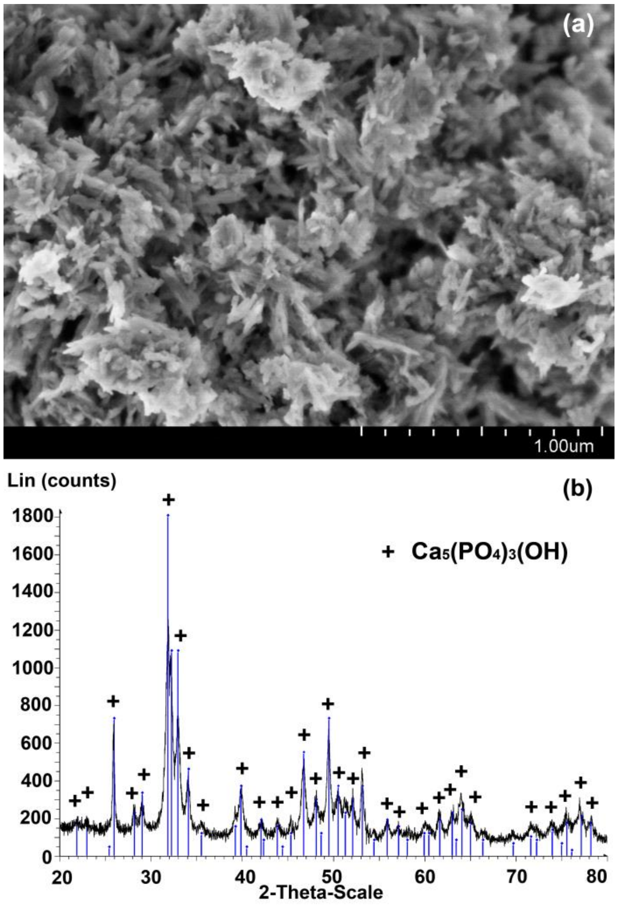


Figure 1 (a) FESEM and (b) XRD Bragg peaks of HA before sintering

3.0 RESULTS AND DISCUSSION

3.1 Characterization of Oxidized Substrate

Representation of the FESEM images of the oxide interlayer produced at 850 °C and 1050 °C is shown in Figure 2. The surface morphology and cross-section of oxide interlayer formed on Co-Cr-Mo alloy through thermal oxidation process is clearly shown that there were no micro-cracks, pores or spallation noticed in substrate oxidized at 850 °C (Figure 2a) as compared to substrate oxidized at 1050 °C (Figure 2c). The inset image in Figure 2a shows the oxide interlayer appears like spikes on the substrate oxidized at 850 °C, indicating the formation of compact composite material. This compact structure is proven to be highly essential to provide a suitable substrate surface for HA coating [6, 8, 23]. The cross-section of substrates oxidized at 850 °C and 1050 °C also showed that the thickness of oxide interlayer formed was 1.33 ± 0.2 µm and 3.49 ± 0.4µm, respectively.

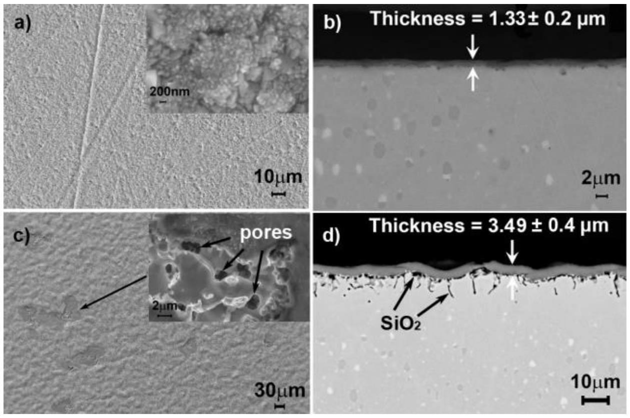
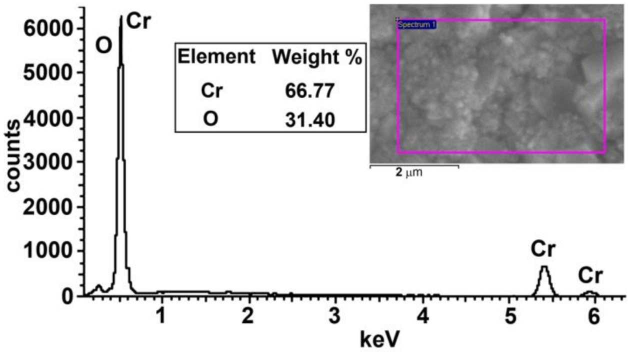


Figure 2 Surface morphologies and cross-section images of oxide interlayer prior to HA coating at temperature of (a, b) 850 °C, (c, d) 1050 °C

Chromium and oxygen were identified as predominant elements on substrate oxidized at 850 °C based on EDX results (Figure 3). This notion was further compared by a simple calculation of weight percentage of element using the following equation:

Cr has 2 atoms; 2 x 51.996 (atomic weight) = 103.992
O has 3 atoms; 3 x 15.996 (atomic weight) = 47.997
Cr % = 103.992/(47.997+103.992) = 0.684 ≈ 68%
O % = 100 – 68 = 32%



Element	Weight %
Cr	66.77
O	31.40

Figure 3 EDX spectra for oxidized substrate at temperature of 850 °C

The calculated percentage of chromium, Cr is 68% and oxygen, O is 32% which is not much different from EDX results obtained in Figure 3. Therefore, the presented calculation confirms that the compound observed on the oxidized sample was Cr_2O_3. This finding is also in line with XRD results as shown in Figure 4. The XRD spectrum obtained in the current study correlates with the Bragg peaks of standard Cr_2O_3 X-ray diffractogram (ICDD 38-1479). Besides that, the XRD spectrum also indicates the presence of other compound such as $Mn_{1.5}Cr_{1.5}O_4$ and $Co_{0.8}Cr_{0.2}$ on the oxide surface, which correlates with the standard $Mn_{1.5}Cr_{1.5}O_4$ X-ray diffractogram obtained from (ICDD 33-0892) and $Co_{0.8}Cr_{0.2}$ (ICDD 01-071-7109), respectively.

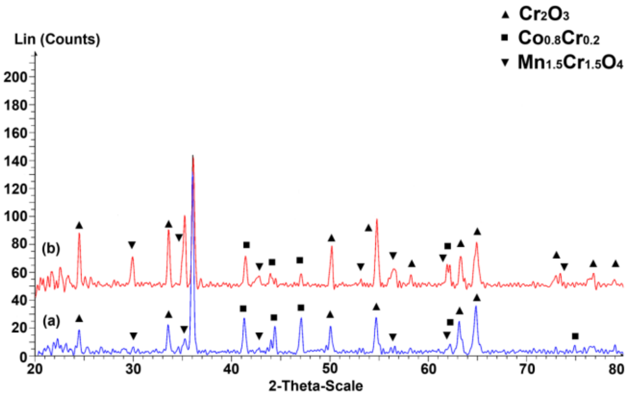


Figure 4 XRD peaks of oxidized substrate at temperature of (a) 850 °C and (b) 1050 °C

In the case of applying higher oxidation temperature of 1050°C on the Co-Cr-Mo alloy, pores were observed to scatter across the substrate surface. Moreover, it seemed that some of oxide interlayer formed at 1050°C was spalled off as referred in Figure 2c although most of them still remained adhered on the substrate. The XRD outcome provides further evidence that the little parts that spalls off from the substrate surface was $Mn_{1.5}Cr_{1.5}O_4$. It has been reported in many studies that this phenomenon might have occurred due to cooling process somewhere after oxidation process [23, 27-29]. Further, the observation on the cross-section images of oxidized substrate at 1050 °C (Figure 2d) has shown undulation surface morphology when compared with oxidized substrate at 850 °C (Figure 2b). Moreover, in cross-section of the substrate oxidized at 1050 °C there were myriad of black spots and some look like micro-cracks. This phenomenon happened may be due the effect of internal oxidation that occurred beneath the oxide interlayer [29]. By using EDX, the black spots and cracks-like patterns which distributed beneath the oxide interlayer were corresponding to non-continuous SiO_2 precipitates as shown in Figure 5 and the same calculation of weight percentage was done to confirm this hypothesis.

It is worth to note that the formation of the internal oxidation appeared to be the main reason in increasing the thickness of the oxide interlayers on the substrates [26, 27]. In addition, as the internal oxidation grows deeper to the bulk material, thicker oxide interlayer is produced. Similar outcome was also reported in previous studies where thicker oxide layer formed due to sintering of cobalt based alloy in thermal cyclic experiment using higher temperature (1100 °C) with extended holding duration [27].

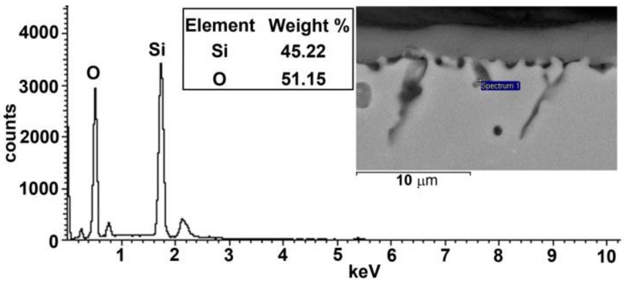


Figure 5 EDX spectra for oxidized substrate at 1050 °C

Si has 1 atom; 1 x 28.086 (atomic weight) = 28.086
O has 2 atoms; 2 x 15.999 (atomic weight) = 31.998
Si % = 28.086/(28.086 +31.998) = 0.467 ≈ 47%
O % = 100 – 47 = 53%

There were barely any differences noted in the chemical composition of oxide interlayer produced on substrates oxidized at 850 °C and 1050 °C. However, in Figure 4b it appears that the relatively higher intensity of $Mn_{1.5}Cr_{1.5}O_4$ peak produced by substrate oxidized at 1050 °C than the one oxidized at 850 °C. It is believed that the presence of relatively higher intensity of $Mn_{1.5}Cr_{1.5}O_4$ in oxidized substrate at 1050 °C may be due to the location of this compound which mostly was detected at the external interface of the substrate. Furthermore, the formation of thicker oxide interlayer tends to increase the intensity of $Mn_{1.5}Cr_{1.5}O_4$ diffraction peak of alloy.

Based on the observation made on the surface morphology, thickness and chemical composition analyses, substrates oxidized at 850 °C were deemed to be superior and warrants further investigation. However, 1050 °C oxidation temperature was also included for comparison purposes.

3.2 HA Coatings Deposition

FESEM and EDX analyses were carried out on BareHA, HA850 and HA1050 samples. Using FESEM, there were no differences noticed at surface morphology of all samples (Figure 6a, b and c) since they were prepared using the same HA slurry concentration and sintered at the same sintering temperature. It is expected no significant difference in those surface morphologies observed.

Generally, the irregular shape of crystallites HA particles and agglomerations in HA coating were observed on all samples. There are also many formations of pores in between the agglomerations and it is known that nano-sized HA crystallites are beneficial in the use of implant biomaterial.

However, EDX result analysis of BareHA (Figure 6d) revealed the peak for chromium element, with none was detected on HA850 and HA1050 samples. This phenomenon happened due to HA coating on bare material have experienced severe visible micro-cracks as shown in Figure 7a. The exposure of chromium element from the bulk material may be due to fairly thin coating of HA on BareHA which is about 1.28 µm (Figure 7b).

Nevertheless, there were only the predominant elements of HA such as calcium (Ca), phosphorous (P) and oxygen (O) shown in EDX peaks of HA850 and HA1050 (Figure 6e and 6f). This outcome provides preliminary evidence that the oxide interlayer between Co-Cr-Mo alloy substrate and HA that created, providing significant bonding between the surface alloy and HA coating. It is worth noting that this may also be due to the dense and thick coating of HA ranges from 20 to 25 µm on HA850 and HA1050 itself, thereby avoiding the formation micro-cracks of HA (Figure 7c to 7f).

Further observation on the FESEM images showed that the oxidized sample at much higher temperature (1050°C) exhibits few cracks on the HA coating surface (Figure 7e). Probably 20 µm thickness of HA coating is a threshold in this study therefore, above that thickness value HA1050 sample surface become more intention and caused cracks in HA coating. However, the tendency for micro-cracks decrease in HA1050 compared to BareHA sample can be considered due to enhancement of mechanical interlocking between the coating-sample interfaces produced by oxide interlayer.

3.3 In-vitro Cell Responses

3.3.1 Cell Attachment

The MSCs responses to the untreated sample of Co-Cr-Mo alloy and HA coated sample with and without oxide interlayer was assessed by FESEM and the resulting images are shown in Figure 7a-h. It is observed that MSCs were attached on untreated Co-Cr-Mo alloy at day 7 but the MSCs cultured still remained in their original round shape even up to 14 days of incubation as shown in Figure 7a and 7b, respectively. This phenomenon indicates slower speed of cell growth compared to HA coating samples. FESEM analysis demonstrated that MSCs were attached on HA coated Co-Cr-Mo alloy substrate in all group samples.

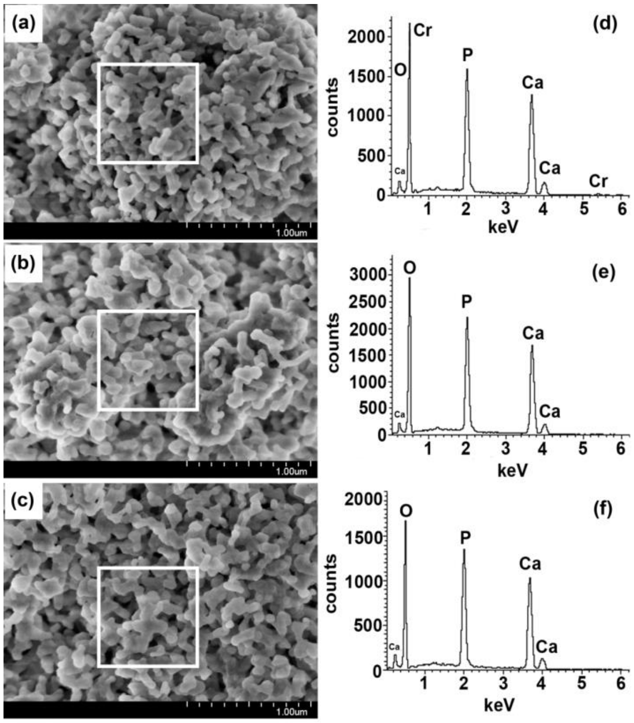


Figure 6 FESEM images of HA treated substrates (a) BareHA, (b) HA850 and (c) HA1050 (x50,000 magnification, scale =1 µm) and EDX spectra of (d) BareHA, (e) HA850 and (f) HA1050

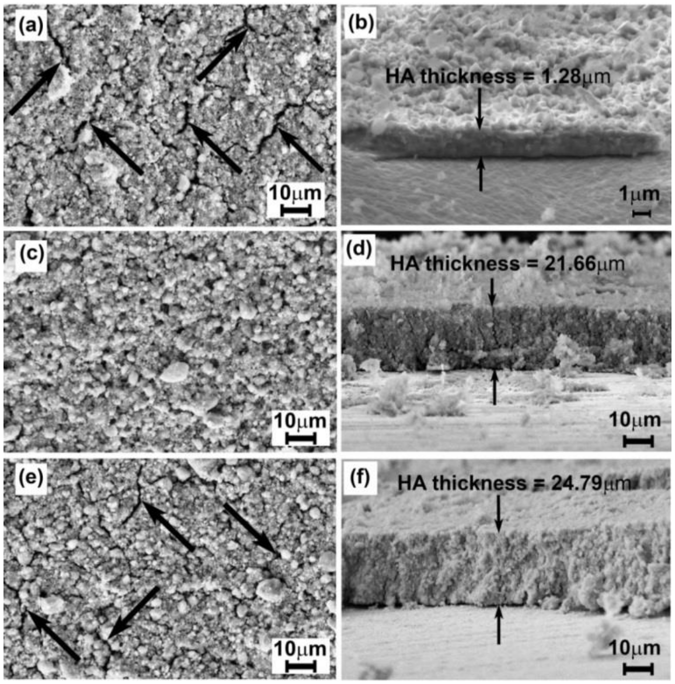


Figure 7 FESEM images of HA coating and cross-section of the HA coating thickness at three different sample conditions; (a) and (b) BareHA; (c) and (d) HA850; (e) and (f) HA1050

The MSCs morphologies on BareHA (Figures 7c and 7d) indicate that the cells appeared less extended of filopodium even after 14 days of incubation. However, there was different behavior of cells for HA850 (Figure 7f) and HA1050 (Figure 7h) as they demonstrated greater extension of filopodium and formed bridges across the substrate coating surface, appearing as canalicular-like cells. The formation of bridges among the cells indicates that the surface may be favorable for the cells to prolong the active metabolism for viability and proliferation [24]. Moreover, the architecture around the cells mimics extracellular matrix (ECM) and forms a continuous layer of fusiform over the surface of the HA850 (Figure 7e-f) and HA1050 (Figure 7g-h) samples.

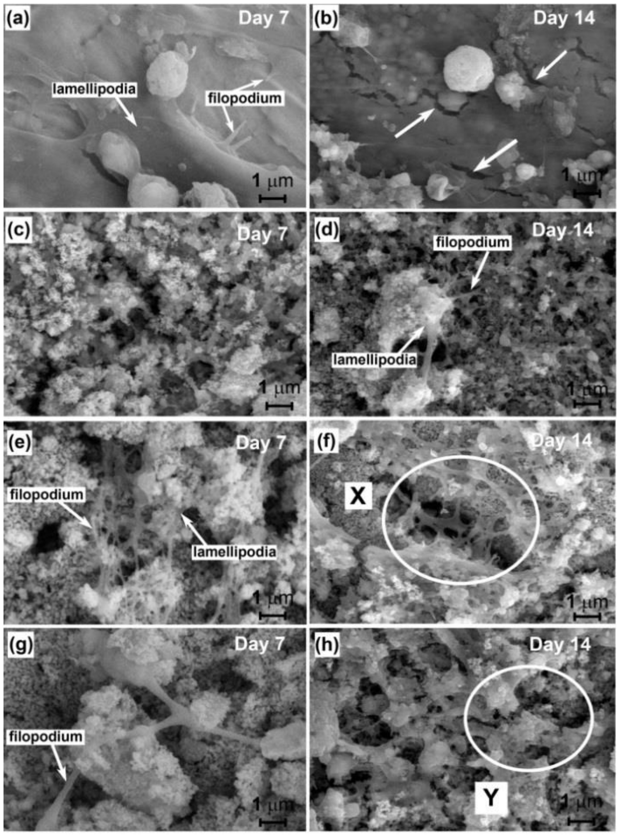


Figure 8 FESEM images of MSCs seeded on (a, b) untreated Co-Cr-Mo alloy, (c, d) BareHA, (e, f) HA850 and (g, h) HA1050

Even though the cells were spread out actively on both samples, further observation showed that the multiple microvilli of MSCs cultured on HA850 exhibited better attachment and extensions until the deepest layers of HA coating as shown in circle X (Figure 7f). However, after day 14, HA1050 showed breakage of filopodium in MSCs as shown in circle Y. This demonstrated that the attachment of filopodium is not as good as HA850 sample. Comparing all sample conditions, it is found that the best results were obtained for HA coating sample with oxide interlayer produced at 850 °C.

3.3.2 Cell Proliferation Assay

The percentage reduction of alamar blue was monitored on day 7 and day 14 (Figure 8). The data obtained showed a significant difference in cell proliferation between BareHA compared to HA850 and HA1050 samples. The proliferation rate was also found significantly higher in HA850 and HA1050 samples as compared to BareHA ($p < 0.01$). It is also noted that as early as day 7, cell proliferation performed much better in HA850 ($p < 0.01$) compared to HA1050. However, further observation until day 14 there were not much difference of cell proliferation rate in both samples occurred. Overall, there was no significant difference in cell proliferation rate between day 7 and 14 in all groups ($p > 0.05$).

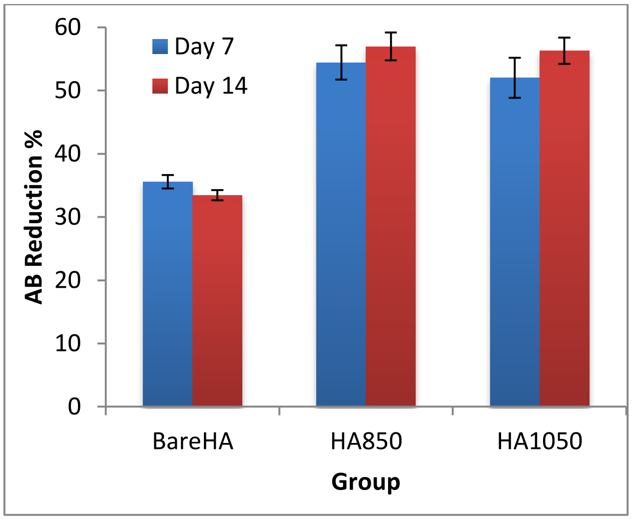


Figure 8 MSCs proliferation rate in BareHA, HA850 and HA1050 – Alamar blue assay

4.0 CONCLUSION

In conclusion, HA coating with oxide interlayer on Co-Cr-Mo alloy that oxidized at temperature of 850 °C provides the best quality of HA with no visible micro-cracks and thicker coating layer as well as supporting cells attachment and proliferation among other sample groups. The excellent growth of MSCs proven that HA850 sample has improved the biocompatibility with incorporated of oxide interlayer. However, the adhesion strength of HA coating on Co-Cr-Mo alloy has to be further investigated quantitatively in order to strengthen the findings of the present study.

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