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PREPARATION, CHARACTERIZATION AND IN VITRO BEHAVIOUR OF HIGHLY POROUS CALCIUM PHOSPHATE SCAFFOLD FOR BONE TISSUE ENGINEERING

RINGKASAN: Dalam kejuruteraan tisu tulang, kalsium fosfat berliang mewakili bahan perancah menjanjikan penyelesaian dalam membaiki tisu tulang yang rosak atau berpenyakit. Walau bagaimanapun masalah utama struktur ini adalah sifat-sifat mekanikal yang rendah dan sangat rapuh. Fokus utama kerja-kerja ini adalah untuk membangunkan perancah menggunakan teknik buih replikasi untuk menghasilkan struktur yang sangat berliang, terbiodegradasi dan sifat mekanikal yang kompeten serta untuk menentukan kadar kemerosotan selepas rendaman di dalam cecair badan simulasi (SBF) dan cecair penampan fosfat (PBS). Struktur "polyurethane" dengan dimensi (20 x 10 x 20) mm dilarutkan di dalam buburan serbuk apatit dan poli vinil alkohol (PVA), yang kemudiannya meratakan struktur yang membawa kepada suatu lapisan homogen partikel kalsium fosfat di permukaan substrat polimer. Selepas pengeringan, badan hijau (polimer) perlahan-lahan di bakar pada suhu 1150 °C selama 3 jam untuk mendapatkan perancah porous, diikuti dengan salutan dengan larutan PDLLA (poly-D-L-Lactic acid) pada kadar 1, 3, dan 5 % bagi tujuan meningkatkan kestabilan mekanikal dan seterusnya dikeringkan pada suhu bilik selama 24 jam. Perancah ini akan dianalisis di bawah belauan sinar-x (XRD), mikroskop elektron imbasan (SEM) dan mesin tegangan universal (UTM). Spektrum belauan sinar-x (XRD) mendedahkan kewujudan puncak tajam fasa hidroksiapatit (HA) $[Ca_{10}(PO_4)_6(OH)_2]$ sebelum dan selepas proses pembakaran. SEM menunjukkan struktur perancah dengan keliangan tinggi dan saling berinteraksi. Hasil penurunan berat menunjukkan perancah kalsium fosfat masing-masing terlarut selama 136 hari di dalam SBF dan 142 hari di dalam larutan PBS.

ABSTRACT: In bone tissue engineering, porous calcium phosphate represents promising scaffolding materials to repair diseased or damaged bone tissue (Chen *et al.*, 2008). However the main problem of these structures is due to its slow mechanical properties and high brittleness. The main focus of this work is to develop a scaffold using foam replication technique to produce highly porous, biodegradable and mechanically competent structures. In order to determine degradation rate, the scaffolds were immersed in Simulated Body Fluid (SBF) and Phosphate Buffer Saline (PBS). A polyurethane (PU) foam with a dimension of (20

x 10 x 20) mm was immersed in a slurry mixture of apatite powder and poly vinyl alcohol (PVA), which subsequently infiltrates into the porous structure while forming a homogeneous coating of calcium phosphate particles on the surface of the PU form. After drying, the green body was slowly burned off at sintering temperature of 1150 °C for 3 hours to obtain porous scaffolds, followed by dipping in PDLLA solution at 1, 3 and 5 wt/v.% in order to improve mechanical stability, and dried at room temperature for 24 hours. The scaffolds were analysed under X-ray Diffraction (XRD), Scanning Electron Microscope (SEM) and Universal Testing Machine (UTM). The X-ray diffraction (XRD) spectrum revealed the presence of sharp peaks of hydroxyapatite (HA)[Ca₁₀(PO₄)₆(OH)₂] phases before and after sintered scaffolds. SEM micrograph showed the structure of scaffold with highly porous and interconnected pores. The weight loss results indicated that calcium phosphate scaffold was degraded after 136 days in SBF and 142 days in PBS, respectively.

Keywords: Hydroxyapatite, porous calcium phosphates, replication technique, simulated body fluid.

INTRODUCTION

In this era of the modern technology, tissue engineering is regarded as one of the designing and fabrication of new tissues for guiding body to regenerate, when organ and tissue lose or failure due to cancer, disease or trauma (Chien and Wang, 2008). As a temporary supporting structure of tissue engineered constructions, the scaffold needs to be selected carefully. The materials for the scaffold must be biocompatible and bioresorbable with control degradation rates to match tissue replacement (Goldstein and Moalli, 2001).

To produce scaffolds with interconnected pore networks, there are various of porous biodegradable synthetic materials, such as polyvinyl alcohol (PVA), poly-D-L-Lactic acid (PDLLA), poly-Lactic acid (PLA) and poly-glycolic acid (PGA) where these are currently tested as filler or additive for the regeneration of damaged and diseased tissues (Lu *et al.*, 2000). The available synthetic biodegradable calcium phosphate ceramics for bone tissue regeneration also include hydroxyapatite (HA), β -tricalcium phosphate (β -TCP), tetra-calcium phosphate (TTCP) and calcium polyphosphate. Among these bioactive materials, HA is one of the most promising materials for clinical use due to the fact that its chemical and crystallographic structure are similar to the bone mineral. However, the major problem of the HA is the brittleness and poor mechanical properties, which prevents it to be used in load-bearing applications (Weng *et al.*, 2002). To overcome this problem, biodegradable polymer was used as a binder or filler to enhance the mechanical strength.

There are several techniques to produce scaffold with high porosity. The most popular technique is foam replication technique. For these purposes, an artificial

scaffold with certain criteria including mechanical and structural properties are crucial as it serves as templates for cell interaction and the formation of bone. The foam replication technique is a process developed for the manufacture of ceramic foams in 1963 (Schwartzalder and Somers, 1963). In this process, the scaffolds (green body) is prepared by coating a polyurethane foam with HA particles by slurry infiltration, before it is slowly burned off from the polymeric foam through sintering process. Additionally, the foam replication technique does not involve the use of toxic chemicals and is more rapid and cost effective compared to other standard processing techniques such as rapid prototyping and freeze drying.

Polymer-ceramic scaffold needs to meet some requirements before it can be used in bone tissue engineering such as ability to deliver cells, excellent osteoconductivity, biodegradable, sufficient mechanical properties, high porosity (> 90%), desired fabrication capability and commercialization potential (Rezwan *et al.*, 2006). In this study, we have developed a biodegradable ceramic calcium phosphate scaffold coated with PDLLA to enhance the mechanical properties using foam replication technique for bone tissue engineering application. The porosity and the mechanical properties of the scaffold were revealed and the *in vitro* of the scaffold was evaluated by immersing in simulated body fluid and phosphate buffer solutions.

MATERIALS AND METHODS

Synthesis of Apatite Slurry

Apatite slurry was obtained using synthesis of raw calcium hydroxide (Fluka, Germany) and phosphoric acid (Mallinckrodt, 85 % concentration) via wet precipitation technique at 1.60 Ca/P ratio. Phosphoric acid was slowly added into calcium hydroxide solution at a controlled additional rate. pH (maintained below 9) and temperature (below 80 °C) were controlled during reaction to obtain a stable suspension. Gelatinous apatite slurry was obtained at the end of the process and ready for spray drying. Polyurethane foam (PU) with size of 45 ppi (pores per inch) was purchased from Recticel, Belgium. Polyvinyl alcohol (PVA) (Sigma-Aldrich) with 99 % hydrolysed were added to the apatite slurry as a binder. PDLLA with inherent viscosity of 2.15 dL/g was purchased from Purac, Biochem, Netherland. Dimethyl carbonate (DMC) was purchased from Sigma-Aldrich, Malaysia.

Fabrication of Scaffolds

The starting materials were prepared by dissolving 3 g of PVA in 50 ml H₂O in a 250 ml beaker (beaker 1) and heated at 80 °C, while stirring for at least 30 minutes. 20 g of apatite powder and 2 g of alumina (Al₂O₃) were then mixed in beaker 2, before adding the mixture into the solution in beaker 1 until a homogeneous mixture was achieved. Solution in beaker 2 was added into solution in beaker 1 until homogeneous slurry was achieved for at least 5 minutes. The PU foam (dimension of 20 x 10 x 20 mm) was

then immediately immersed in this solution until the slurry covered PU foam and the remaining slurry was squeezed out by hand. The obtained green body was then oven dried at 60 °C overnight, before burning off the PU foam at 1150 °C for 3 hours using box furnace (CMTS). The heat treatment profile of the green body is shown in Figure 1. The burning off of PU foam was at 550 °C for 3 hours with heating rates at 2 °C/min and cooling rates at 5 °C/min (Chen, 2008).

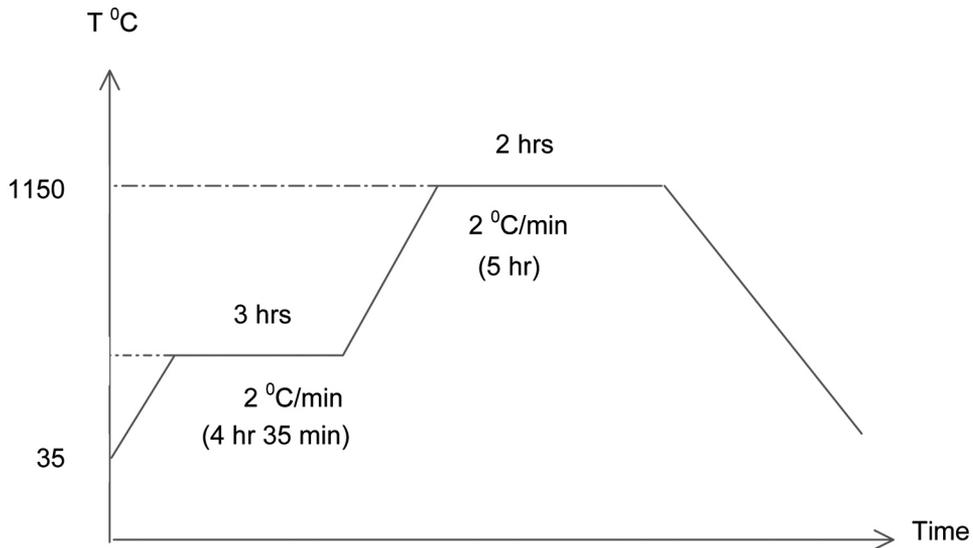


Figure 1. Sintering profile at 1150 °C for porous scaffold

Coating of Porous Apatite Scaffolds

The porous scaffolds were weighed using analytical weighing balance (Precisa, Germany) before coating with PDLLA to enhance their mechanical properties. The scaffolds were then slowly immersed in a solution of 1, 3 and 5 % wt/v of PDLLA-DMC (di-methyl carbonate) solution and dried at ambient temperature for 24 hours. The dried scaffolds were measured again using analytical balance and the dimension was measured using digital caliper (Mitutoyo, UK). Finally, the scaffolds were ready for characterizations and immersion process in order to determine the mass loss (Chen, 2008).

In-Vitro Chemical Evaluation

For *in-vitro* chemical evaluations, each scaffold was sterilized using autoclave HVE-50 (Hirayama) for 20 minutes at 121 °C. Samples were then immersed in vials containing 30 ml of Simulated Body Fluid (SBF) (pH 7.4 ± 0.1) and Phosphate Buffer Saline (PBS) (pH 7.3 ± 0.1) and maintained at 37.0±0.5 °C in oven throughout the tests for various durations (1, 5, 10, 15, 20, 25 and 30 days). SBF and PBS were prepared according to

the standard procedure as reported elsewhere (Takadama and Kokubo, 2006). The preparation of the samples was conducted in biohazard safety cabinet (TELSTAR BIO-II-A). After predetermined immersion duration, scaffolds were withdrawn from the vials of SBF and PBS, and subsequently dried with laboratory tissue for 24 hours at ambient temperature. Prior to sending the dried samples to SEM and XRD for characterisations, the dried samples were weighed using analytical balance to determine the rate of mass loss. After immersion, upon withdrawal of the scaffolds, the SBF and PBS solutions were stored in a chiller. Then, the solution were maintained at 37.0 ± 0.5 °C in a water bath for pH measurement. Mass loss was calculated according to Equation 1.

$$\text{Mass loss: } \frac{\text{Dry weight} - \text{Initial weight}}{\text{Initial weight}} \times 100 \quad (1)$$

Characterisation

Porosity

The dimension of the scaffolds were measured using a digital caliper (Mitutoyo, Japan) and analytical balance (Precisa, Germany). Five specimens of 1, 3 and 5 % wt/v of PDLLA were used to compare and determine the total porosity. The calculation for porosity is as given in the equation below:

$$\text{Bulk density } (\rho_B) = \text{weight of the sample} / \text{volume of the sample}; \quad (2)$$

where theoretical density of the calcium phosphate ($\rho_{\text{CaP}} = 3.14 \text{ g/cm}^3$); relative density ($\rho_{\text{relative}} = (\rho_B / \rho_{\text{CaP}}) \times 100 \%$); and total porosity = $100 \% - \rho_{\text{relative}}$.

Physical Characterization of Scaffolds

X-Ray Diffractometer (XRD) Bruker D8 Advanced was carried out to determine the crystallinity of the sintered samples and to evaluate the formation of hydroxyapatite (HA) after firing at 1150 °C, to reveal the differences between the sample in pre and post immersion process. Scanning Electron Microscope (SEM) LEO 1500 series was used to observe the microstructure and interconnected pores of the samples before and after immersion in SBF and PBS.

Mechanical Testing

The compression strength of the coated scaffolds with 1, 3 and 5 % wt./v of PDLLA at 1150 °C was carried out using Universal Testing Machine (UTM, Instron 3369, USA) with crosshead speed of 2 mm / min. At least five samples were tested throughout the process to get the average results.

RESULTS AND DISCUSSION

Fabrication of apatite porous scaffolds was successfully carried out using foam replication technique. This process has many advantages over other fabrication techniques. It is easy to fabricate scaffolds which does not involve toxic chemicals and able to produce scaffolds with highly porous structure. Figure 2 shows the structure of polyurethane (PU) after immersion with apatite slurry (green body) and scaffolds obtained after firing at 1150 °C. The PU foam that was burnt off has changed to blue indicating that PU was possibly replaced by hydroxyapatite phase after sintering process. This phase was confirmed in XRD results, (Figure 10 and Figure 11).

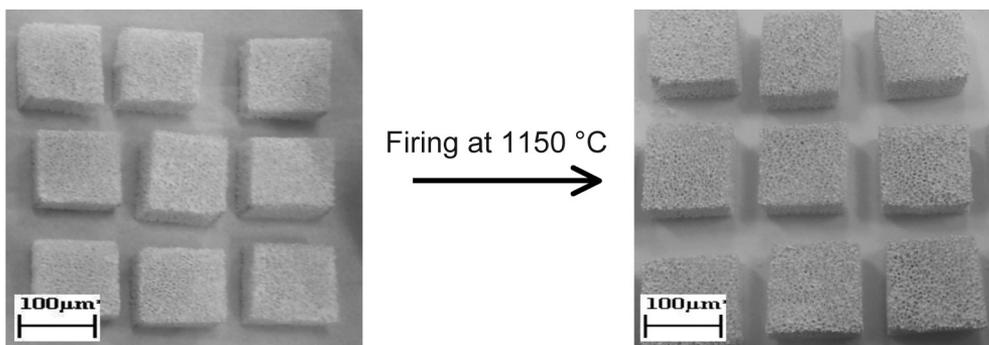


Figure 2. Porous scaffold [(20 x 20 x 10) mm] via foam replication technique using apatite slurry

After the sintering process was completed, the porous scaffolds were coated in a solution of 1, 3 and 5 % wt./v PDLLA-DMC and left overnight before measuring the porosity. Figure 3 (A-C) shows the porous scaffolds after coating with 1, 3 and 5 % wt./v PDLLA-DMC. It was found that the strength of the scaffolds after coating with PDLLA was significantly enhanced compared to the value without PDLLA coating. However, there were significant differences compared to physical properties for 1, 3 and 5 % wt./v PDLLA-DMC. The structure of scaffold coated with 1% wt./v (Fig. 3A) expanded after dipping with PDLLA-DMC, and it immediately showed unstable phase and easy to collapse. The physical properties of the scaffolds coated with 3 % wt./v (Figure 3B) was more stable and possessed a tough surface compared to Figure 3A. However, the structure was still fragile and easy to collapse. Figure 3C with 5% wt./v showed the desired properties such as hardened surface layer of the porous structures due to high concentration of PDLLA. The polymer was believed to cover and fill the microcracks on the struts surfaces and improved the mechanical stability of the scaffolds. It has also been found that HA crystals formed inside the polymer coating layer during immersion of PDLLA coated apatite foams in simulated body fluid and phosphate buffer saline. Finally, porous scaffold coated with 5% wt./v of PDLLA-DMC was chosen for immersion process due to appropriate physical properties and its ability to enhance the structure for bone tissue engineering application.

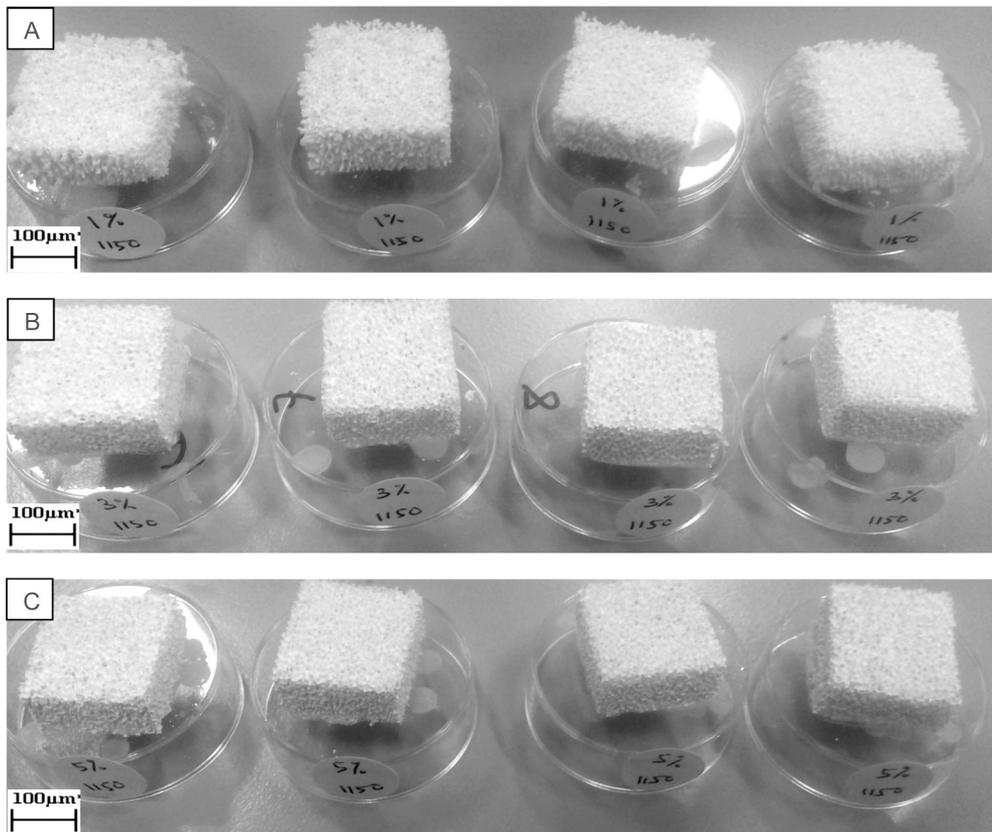


Figure 3. Porous scaffold (20 x 20 x 10) mm coated with various concentration of PDLLA-DMC: A) 1 % wt./v; B) 3 % wt./v and C) 5 % wt./v

Porosity

The porosity of the apatite porous scaffold is shown in Figure 4. The porosity of porous scaffold coated with 5 % wt./v is 0.935, although the porosity decreased, it is still more than 90 % compared to as sintered. The decreased in porosity was expected due to the polymer filled the porous scaffolds during coating process. The pore size is expected to decrease due to the shrinkage of the scaffolds after sintering at 1150 °C for two hours. Although porous scaffold with 1, 3 % wt./v and as sintered has a higher porosity as compared to 5 % wt./v, they are not suitable for bone engineering application as mentioned before. Paola (2010) stated that high porosity is valuable for bone engineering application because it allows for cell penetration, growing tissues and vascularisation and delivery of the nutrients to the bone (Paola *et al.*, 2010).

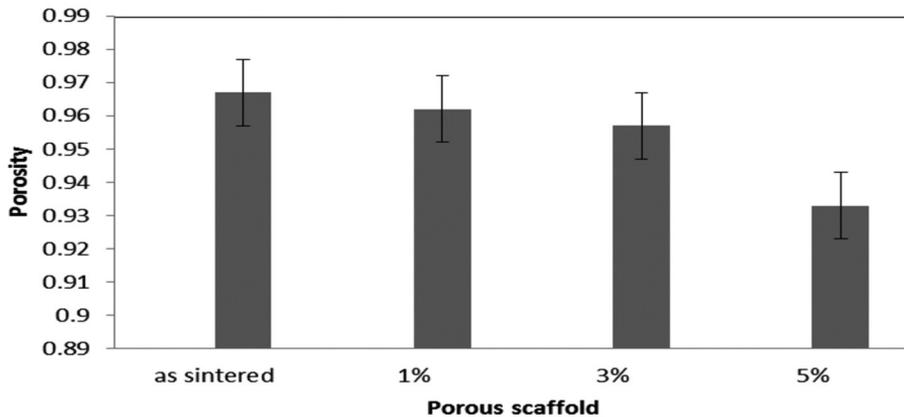


Figure 4. The porosity of the apatite porous scaffold before and after coating with 1,3 and 5 % wt./v of PDLLA-DMC

Mass Loss

Table 1 and Figure 5 show the mass loss / degradation rate of the porous scaffolds after immersion in SBF and PBS. It was observed that weight loss of 5 % wt./v of PDLLA-DMC in SBF and PBS is comparable. Initial weight loss for porous scaffold in SBF is 41.80 % and increase up to 55.88 % at day 30, while weight loss in PBS starting from 46.72 % until 56.32 % at day 30, respectively. However the results of R^2 based on gradient showed that the porous scaffold after immersion in PBS was more stable with $R^2 = 0.8745$ compared to SBF's $R^2 = 0.8344$, respectively. These results revealed that an increase of immersion time increased the weight loss. The porous scaffold will degrade in ~136 days in PBS and ~142 days in SBF based on formulation calculated and also gradient of the graph. The overall results showed that the porous scaffold samples will degrade faster in PBS compared to immersion in SBF, respectively.

Table 1. Weight loss of porous scaffold (5% wt./v of PDLLA-DMC) after immersion in SBF and PBS

Duration	SBF		PBS	
	Weight loss (g)	Loss (%)	Weight loss (g)	Loss (%)
Day 0	0.8540 ± 0.010	-	0.8540 ± 0.010	-
Day 1	0.3570 ± 0.070	41.80	0.3992 ± 0.036	46.72
Day 5	0.3616 ± 0.059	42.34	0.4050 ± 0.011	47.42
Day 10	0.4390 ± 0.016	51.41	0.4090 ± 0.052	47.89
Day 15	0.4021 ± 0.036	47.08	0.4151 ± 0.081	48.60
Day 20	0.4533 ± 0.044	53.08	0.4713 ± 0.009	55.15
Day 25	0.4651 ± 0.018	54.44	0.4751 ± 0.010	55.62
Day 30	0.4772 ± 0.011	55.88	0.4831 ± 0.003	56.32

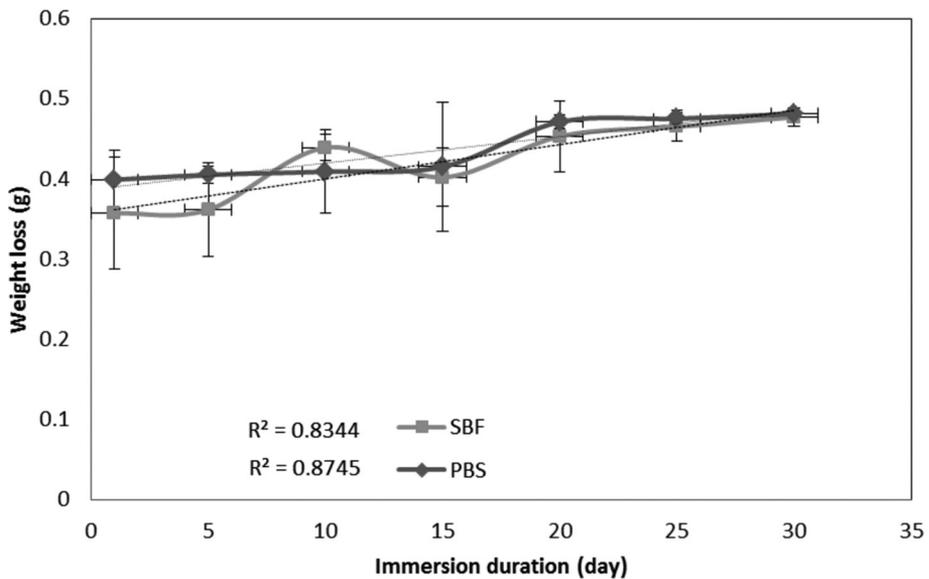


Figure 5. Mass loss / degradation trends of porous scaffold coated in 5% wt./v of PDLLA-DMC after immersion in SBF and PBS

pH Study

pH is the measurement of the acidity or alkalinity of a solution. Table 2 and Figure 6 show the pH of the porous scaffolds after immersion in SBF and PBS solution, respectively. pH value of porous scaffold in SBF still maintain the neutral condition (7-7.5), from 7.27 at day 1 to 7.39 at day 30. The pH value of the porous scaffold in PBS started in acidic value starting from 6.73 at day 1 until 6.82 at day 20, before the pH value turned to 7.03 and 7.08 at day 25 and day 30, respectively. These results show an increase in duration increased the pH value. However, it was observed that an increase in immersion time to day 30, did not affect the pH value that much, where it was still maintained at neutral condition.

Table 2. pH value of porous scaffold coated with 5 % wt./v PDLLA-DMC after immersion in SBF and PBS

Duration	pH Value in SBF	pH Value in PBS
	Autoclave sterile	Autoclave sterile
Day 1	7.27 ± 0.023	6.73 ± 0.071
Day 5	6.96 ± 0.017	6.75 ± 0.163
Day 10	6.96 ± 0.035	6.78 ± 0.010
Day 15	7.13 ± 0.006	6.79 ± 0.087
Day 20	7.21 ± 0.001	6.82 ± 0.083
Day 25	7.45 ± 0.089	7.03 ± 0.030
Day 30	7.39 ± 0.125	7.08 ± 0.026

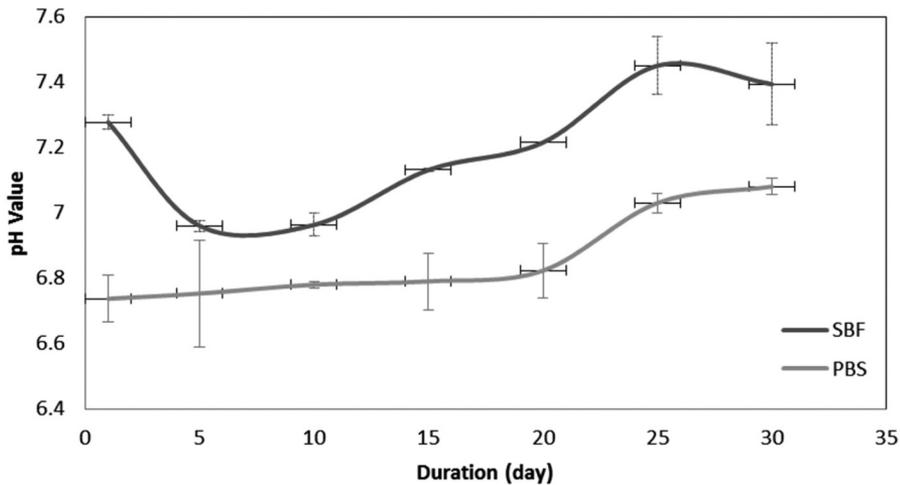


Figure 6. pH trend of porous scaffold coated in 5 % wt./v of PDLLA-DMC after immersion in SBF and PBS

Compressive Strength

The compressive strength of the porous scaffold after sintering at 1150 °C for 2 hours and coated with various %wt/v of PDLLA-DMC is revealed in Figure 7. The compressive strength increased almost ten times from as sintered (0.051 Mpa) to coated scaffold with 5 % wt./v of PDLLA (0.502 Mpa). The increase was due to the polymer infiltration into the porous structure of the sintered scaffolds (Chen *et al.*, 2006). It is shown that scaffolds coated with higher PDLLA-DMC will contribute to the thicker layer and desired mechanical stability and increased toughness of the porous scaffold. The compressive strength which used 1 % and 3 % of PDLLA did not show significant differences compared to as sintered, the scaffold was collapsed due to low concentration of PDLLA-DMC as shown in Figure 3.

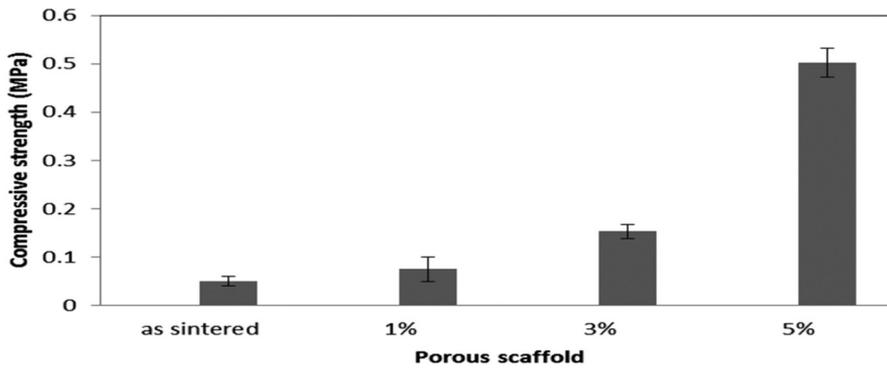


Figure 7. Compressive strength of porous scaffold sintered at 1150 °C after coated with PDLLA-DMC for 1, 3, and 5 % wt./v

Scanning Electron Microscope (SEM)

Figure 8 (A) shows the morphology of original PU foam with pore size in the range of 500 - 800 μm . Figure 8 (B) shows the morphology of as sintered porous scaffolds with pore size in the range of 350–600 μm after sintering at 1150 °C and Figure 8 (C) reveals a porous scaffold after coating with 5 % wt./v of PDLLA-DMC, respectively. The pore size of the porous scaffolds decreased due to the shrinkage of scaffolds after sintered at 1150 °C for 2 hours which is expected during the fabrication of foam replication technique (Goldstein and Moalli, 2001). However, the decrease in pore size is in the desired range for application in bone tissue engineering. Figure 8 (C) also reveals the strut's morphology of the porous scaffolds coating with 5 % wt./v of PDLLA-DMC. The polymer coating showed thicker surface on the micrograph compared to Figure 8 (B).

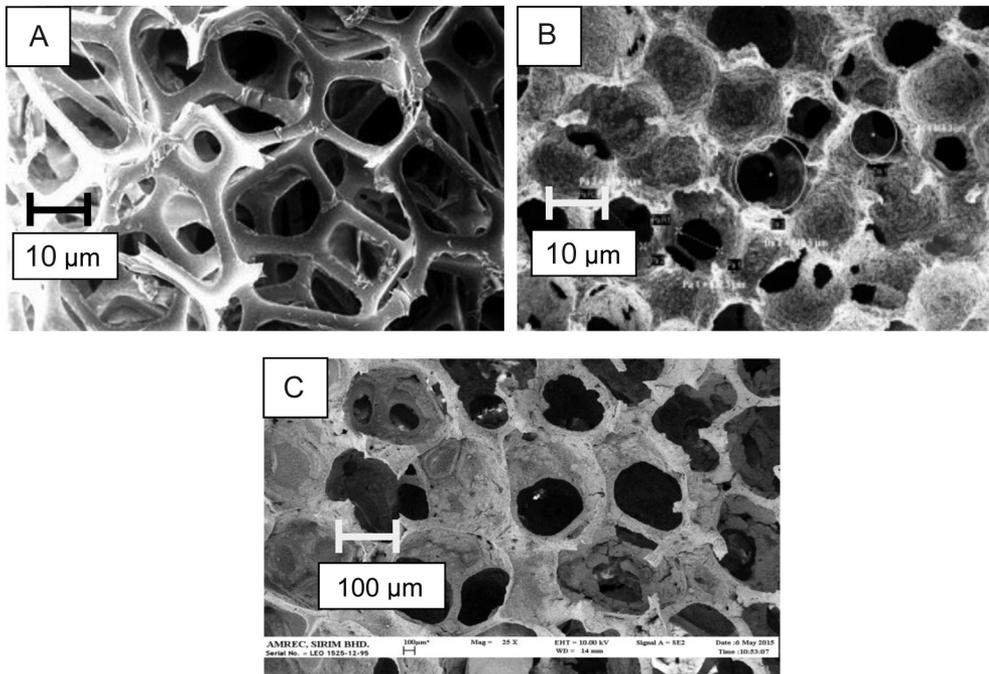


Figure 8. SEM micrograph of: A) Original PU foam; B) Sintered porous scaffold at 1150 °C and C) Porous scaffold after coated with 5 % wt./v PDLLA-DMC (25x)

Bioactivity Assessment of the Porous Scaffolds

As mentioned earlier, porous scaffolds sintered at 1150 °C followed by coated with 5 % wt./v of PDLLA, was immersed in 30 ml of SBF for 1, 5, 10, 15, 20, 25 and 30 days in an oven at 37 °C. The bioactivity assessment of the porous scaffolds was then confirmed by SEM and XRD. Figure 9 (A-D) shows the SEM micrograph of porous scaffolds coated with 5 % wt./v of PDLLA-DMC after immersion in SBF for 1-30 days, respectively. Hydroxyapatite was clearly formed on the struts even though at 1 day immersion. After 15 days of immersion, large amount of HA was covered at struts surface, showing the signs of bioactivity in SBF solution. After 30 days of immersion, the overall of the porous scaffolds was covered by apatite layer and HA crystals were formed clearly on the struts surface. The formation of HA phases were confirmed using XRD as shown in Figure 10.

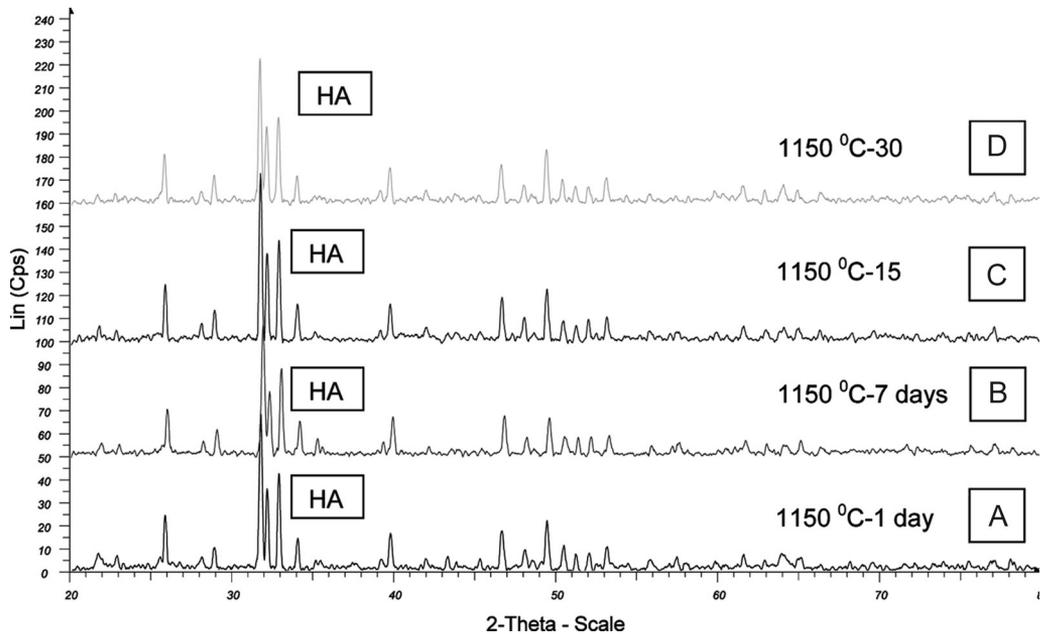


Figure 11. XRD results of porous scaffold sintered at 1150 °C and coated with 5% wt./v of PDLLA-DMC after immersion in SBF for A) 1 day; B) 7 days; C) 15 days and D) 30 days

CONCLUSION

Porous scaffold with high porosity was successfully developed using foam replication technique. The experimental results showed that porous scaffolds coated with 5% wt./v PDLLA-DMC gave desired properties and have the ability to enhance the structure and is suitable for bone engineering especially in osteoporosis treatment. The sharp peaks of crystalline hydroxyapatite was revealed by XRD spectra, while interconnected and highly porous characteristic was highlighted in SEM micrograph. Finally the weight loss of scaffolds showed degradation after 136 days in simulated body fluid and 142 days in phosphate buffer saline.

REFERENCES

- Chen, Q.Z., Boccaccini, A.R. (2006). Poly (D,L-Lactic acid) coated 45S5 Bioglass-based scaffolds: Processing and characterization. *Journal of Biomedical Materials Research*, 77A(3): pp 445-457.
- Chen, Q., J.A. Roether., and A.R. Boccaccini. (2008). Tissue Engineering Scaffolds from Bioactive Glass and Composite Materials. *Topics in Tissue Engineering, Chapter 6, Volume 4*.
- Goldstein, S.A., and Moalli, M.R. (2001). Current concepts in tissue engineering: cell, matrices and genes. *Current Opin. Orthopaedics*, 12: pp 424-427.

Kim, H.W., Knowles, J.C., Kim, H.E. (2005). Hydroxyapatite porous scaffolds engineered with biological polymer hybrid coating for antibiotic vancomycin release. *Journal of Materials Science- Materials in Medicine*, 16(3): pp 189-195.

Lu, L., B.L. Currier and M.J. Yaszemsk. (2000). Synthetic bone substitutes. *Current Opinion Orthopaedic*, 11: pp 383-390.

Paola, F., Valeria, C., Antonella, S., Andrea, D., Federica, C. (2010). Highly porous polycaprolactone-45S4 bioglass scaffolds for bone tissue engineering. *Composites Science and Technology*, 70: pp 1869-1878.

Rezwan, K., Chen, QZ., Blaker, JJ., Boccaccini, A.R. (2006). Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials*, 27: pp 3413-3431.

Schwartzalder, K., Somers, A.V. (1963). Method of making a porous shape of sintered refractory ceramics articles. *United States patent 3090094*.

Takadama, H. and Kokubo, T. (2006). How useful is SBF in predicting in vivo bone bioactivity. *Biomaterials*, 27: pp 2907-2915.

Wang Chien-Wen, Min, Yan. (2008). Degradation Behaviour of Porous Calcium Phosphate. *Journal of Medical and Biological Engineering*, 23(3):1 pp 59-164.

Weng, J., Wang, M., Chen, J.Y. (2002). Plasma-sprayed calcium phosphate particles with high bioactivity and their use in bioactive scaffolds. *Biomaterials*, 23: pp 2623-2629.

