Synthesis and Characterization of Porous Biphasic Calcium Phosphate Scaffold From Different Porogens For Possible Bone Tissue Engineering Applications

A. Amera¹, A. M. A. Abudalazez², A. Rashid Ismail¹, N. Hayati Abd Razak¹, S. Malik Masudi¹, S. Rizal Kasim², Z. Arifin Ahmad²*)
¹School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
²School of Materials & Mineral Resources Engineering, Engineering Campus, University Sains Malaysia, 14300 Nibong Tebal, Penang, Malaysia.

Abstract:
By using the wet precipitation method, Biphasic calcium phosphate granules were synthesized with Ca/P ratio 1.52 and controlled porosity, pore size distribution, and granule size. Microporosity was then obtained by adjusting sintering temperature while macroporosity was prepared by adding 1:3 wt% ratio of two normally used porogens (naphthalene and sugar) and 2 newly introduced porogens (sago and lentil). Samples from each ratio were pressed into pellets and were fired at 500°C for 2 hours with 0.5°C/minute heating rate (for removal of porogens) and further sintered at 850°C for 2 hours with 5°C/minute before cooling down to room temperature. The granules were prepared by crushing and sieving BCP sintered pellets to get granules of sizes ranging from 250-500μm. X-rays diffraction (XRD), field emission scanning electron microscope (FESEM), particle size and porosity analyses were employed in order to characterize the granules. A round to oval shape pores with 200-400 μm size were obtained and identical to the prepared porogens’ particle size. This approach gives the desirable properties near to normal bone leading to a perfect osteogenesis for the purpose tissue engineering.

Key words: Synthesize; Biphasic Calcium Phosphate; Macroporosity; Tissue engineering

1. Introduction

Biphasic calcium phosphate (BCP) a biomaterial consists of a mixture of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) which belong to calcium phosphate ceramics (CaPCs) [1-3]. It is biocompatible, biocontactable material and possesses reasonable biodegradable properties, depending on HA: β-TCP ratio. In addition to it osteoinduction properties in the micro-macroporous BCP forms [4-5], BCP is also used in bone grafting due to its similarity in chemical composition to that of bone’s [6], for drug delivery in association with therapeutics agents (bone morphogenetic proteins, human growth hormone, antibiotics, anti-osteoporotic, anticancer drugs, insulin, steroid hormones etc.) [7-8] and as a scaffold in tissue engineering to ensure stem cells remain in the recipient site [9]. The desired scaffolding by BCP is facilitated by osteogenic and/or growth factor to optimize tissue in which the process requires a biocompatible carrier to transfer the stem cells [5]. A number of

*) Corresponding author: zainal@eng.usm.my
researchers have turned their attention to the use of synthetic CaP ceramics in order to engineer trabecular bone-like scaffold [10]. Macro architecture can be achieved by changing the porosity, pore size, and pore interconnectivity, to match the characteristics of the native tissue whilst retaining scaffold integrity [11].

Preparation of BCP with the required phase compositions is not straightforward because several experimental factors can account for the final result, such as pH, temperature and initial Ca/P ratio [12-13]. Optimization of the macroporosity is one of its serious challenges in terms of the technology to optimize the performance of calcium phosphate and therefore, the frequency of use. The term porosity is defined as the percentage of void space in a solid [14]. Several processes are therefore developed to manufacture porous ceramics with various pore characteristics, such as polymeric sponge method by using different sponges [13], and compaction methods in which they include porogens such as naphthalene, polymethyl methacrylate acrylic and sucrose [15-16].

Porosity and pore size, both at macroscopic and microscopic levels, are important morphological properties of a biomaterial scaffold for bone regeneration. High porosity and large pores enhance bone ingrowths because they allow migration and proliferation of osteoblasts and mesenchymal cells, as well as vascularization [17]. Based on the early work of Hulbert et al. [18], the minimum recommended pore size for a scaffold is around 100 µm, but subsequent studies have shown better osteogenesis for implants with pores >300 µm [19-20]. Relatively, larger pores favor direct osteogenesis, since they allow vascularization and high oxygenation; while smaller pores result in osteochondral ossification, although the type of bone ingrowths depends on the biomaterial and the geometry of the pores. For fabricating biomaterial scaffolds that can meet the requirements set by the specific site of application, there has been an attempt to create a porosity gradient in BCP scaffolds both in the macroporous (>100 µm) as well as in the microporous (<1µm) scales [21].

Generally, there are two main methods to synthesize BCP ceramics: (1) sintering the calcium-deficient hydroxyapatite (CDHA) powder [22-23], and (2) directly mixing HA and TCP powders [24-25].

Tissue engineering is a relatively new field that seeks to regenerate human tissues through the use of some combination of cells, bioactive molecules such as drugs or growth factors, and a biomaterial support system or scaffold [26]. An extensive study on porous BCP to be used as a scaffold has been done, however; very limited data is available on the necessary characteristics as a trabecular bone-like scaffold for tissue engineering applications. Moreover, each technique used to produce macroporosity has its own limitations, such as naphthalene method which suffers from several drawbacks including toxicity associated to PAHs, fire setting, air pollution and difficulties of naphthalene grinding and sieving [16]. Therefore, the current study focuses on synthesizing and modifying the microporous BCP granules with Ca/P ratio of 1.52, and adjusting the macroporosity parameter by adding new porogens (lentil and sago) to provide wider range of safety and biocompatibility. These two materials are cheaper and safer than naphthalene. In addition, maintaining the particle size during compaction with BCP under high pressure can be achieved even though when using isostatic compression to control the pore size.

2. Materials and Methods
2.1. BCP synthesis

BCP powder was synthesized by wet precipitation with titration process and followed by calcination method. In this study, different Ca/P ratios were prepared and a Ca/P ratio 1.52 was chosen. This ratio was confirmed with EDX analysis of the prepared BCP powder. Ten ml/minute of 1 M H₃PO₄ was added then drop wisely into 1.52 M CaCO₃ suspended under continuous stirring at 400 rpm for 2 hours, and where the temperature was maintained at
28 ± 3 °C (room temperature). This process was followed by heating at a temperature of 85-90°C for an hour with stirring before cooling it down to room temperature for an hour. Initially, the reaction was kept at pH 5 and reached at pH 7 towards the end of the reaction. A white precipitate was obtained at the end of the reaction. The precipitate was aged for 48 hours without stirring, then filtered with washing (3 times) using Buchner funnel. The filter cake was then oven dried at 100°C for 24 hours and finally crushed to granule form. About 5g of the as-dried powder was calcined at 850°C for 2 hours in order to confirm the formation of BCP with the correct Ca/P ratio (1.52). In its final process, the remaining as-dried powder was used to produce porous BCP.

2.2. Fabrication of porous BCP

The as-dried cake was crushed and sieved for the size between 200 – 400 µm. The sieved powder was then mixed with porogens of the same particle size at 1:3 wt% ratios, respectively, for 2 hours using alumina balls. The porogens used were naphthalene (M. K. Industries, India), sugar (Malayan Sugar Manufacturing, Malaysia), lentil (Econsave Cash & Carry, Malaysia), and sago (Nee Seng Ngeng & Sons Sago Industries, Sarawak, Malaysia). The mixture was pressed at 146 MPa in a 32 mm die to form 6 mm thickness pellets. The pellets were fired at 500°C for 2 hours with 0.5°C/minute heating rate (for removal of porogens) and further sintered at 850°C for 2 hours with 5°C/minute before cooling down to room temperature. The sintered pellets were crushed using pestle and mortar and sieved to prepare granules of 250 - 500µm. Granules from each sample were then analysed using both qualitative and quantitative XRD analysis (Bruker D8 Advance using CuKα radiation, Eva and XpertHighscore Plus softwares). The microstructure was studied using VPFESEM SUPRA 35 VP and particle size analysis using Sympatec GmbH.

2.3. Characterization of porosity

Both macro- and micro-porosity conditions were evaluated by using FESEM micrograph with 50X and 10kX magnifications, respectively. The laser magnification was used to observe pore structure and to measure pore size. A total porosity percentage was measured following the Archimedes principle. The sample was tied by nylon string and weighed in the air (Wa) by electronic balance Precisa XT 220A which has accuracy up to four decimal places. The sample was then placed in a beaker containing water in a vacuum glass container which was vacuumed to release the air from porosities of the sample for 1 hour. While it was sunk in the water (Wb), the sample then was weighed. The excessive water on the surface of the sample was wiped by filter paper before the sample was weighed in air (Wc). The percentage of porosity value can be calculated based on the following equation:

\[
\text{Porosity} \% (P) = \frac{W_c - W_a}{W_c - W_b} \cdot 100 \%
\]  

3. Results and Discussion

3.1. BCP synthesis

XRD pattern of the synthesized BCP calcined at 850°C for 2 hours is shown in Fig. 1. As for the presence of the two phases of β-TCP [ICDD 01-070-2065] and HA [ICDD 01-089-1943] they are clearly shown. For β-TCP it shows three high intensity peaks located at 27.9°, 31.2° and 34.4° while the highest peaks for HA are at 31.8° and 32.9°. From the Fig., high
crystallinity is indicated by the narrow diffraction peaks described as sharp peaks. This result is in good agreement with other researchers’ works who found that biphasic mixing on a crystallite level is feasible using this method [3, 27-28]. The quantities of HA and β-TCP, as measured by Xpert Highscore Plus software, was 16.3 and 83.7, respectively. This result is equivalent to Ca/P ratio of 1.52 as calculated by using the following equation [13]:

\[ \text{Expected Ca/P} = \text{wt. % HA (Rietveld)} \times 1.67 + \text{wt. % TCP (Rietveld)} \times 1.50 \]  

Fig.1. X-ray diffraction pattern of BCP calcined at 850°C for 2 h

Therefore, the experimental value used for precursor’s solution has shown a good coincidence with the calculated Ca/P ratio determined from phase content. This ratio was chosen believing that it is the most proper ratio for tissue engineering (TE) purposes. Daculsi [28] found that the rate of degradation or resorption of HA/TCP ceramics in vivo can be accelerated by increasing the amount of the more soluble phase, TCP [29].

This result that was obtained is in the same range found by Farina et al. [30] who claimed that the BCP with HA:β-TCP ratio of 15:85 had induced an earlier bone formation and more bone with higher degradation rate compared to BCP 85:15. This was explained as calcium phosphate materials can add values to the higher osteoinductivity potential of BCP with 15:85 ratios. Previously, Legeros et al. [31] found that the lower the HA/β-TCP ratio, the higher the extent of dissolution in which calcium and phosphate can promote mineralization of bone cells at specific concentrations. In addition, in Livingston’s [32] study, it was demonstrated that mesenchymal stem cells (MSCs) bone induction had occurred at the fastest rate in vivo when loaded on a HA/TCP ceramic having the ratio of 20/80, which is degrading faster than the 60/40 formulation. However, Bruder et al. [33] stated that for bone tissue engineering using MSCs, HA/TCP ceramics have been the material of choice, and when MSCs were combined with 60/40 bone formation was induced in large and long bone defect.

As depicted in Fig. 2, it shows the macrograph results of BCP powder. The scanning was done with 20KX magnifications. The particle shows irregular fibrous morphology with porous nature when sintered at 850°C for 2 hours.
Fig. 2. SEM picture of BCP sintered at 850°C for 2 h showing the microporosity.

Fig. 3 shows the effect of various porogens addition on the formation of macroporosity (Fig. 3(a), (c), (e) and (g)) and microporosity (Fig. 3(b), (d), (f) and (h)) of BCP when sintered at 850°C for 2 hours.

Therefore, the calcination temperature was fixed at 850°C for 2 hours to keep same quality of micropores (Fig. 3). Since the microporosity will be reduced at higher temperatures. LeGeros et al. [31], Daculsi and LeGeros [34] found that microporosity depends on sintering temperature or sintering program. Therefore, CaP sintered at 1200°C shows significantly less microporosity than that sintered at 1000 °C and a dramatic change in crystal size as well. Petrov et al. [27] mentioned in their study that controlling the sinterability of the powders can give some room to obtain dense/porous materials. Generally this microporosity is necessary to achieve excellent osteoinductivity for the Ca-P materials [35-36] also promoted the material resorption and bone substitute in vivo [37]. It result in a larger surface area that is believed to contribute to higher bone induction, protein adsorption as well as to ion exchange and bone-like apatite formation by dissolution and reprecipitation [33] and it was found that the influence of micropores on materials strength is lower than that of macropores [38].

The assessment of the interconnected macroporous structure with random pore size distribution indicated to the pore size range of 200–400 µm, and oval to round shape resembling the original porogens.

Since scaffolds for bone tissue engineering must have a porous structure [15], therefore, the most suitable sample fabrication method is compaction technique [37]. In this study, prior to compaction process, BCP powder was mixed with the commonly used porogens (naphthalene and sugar, respectively), and their effect was compared with the newly studied porogens (lentil and sago, respectively). In this method, pores were created during sintering, porogen burned out leaving pores identical to its size and shape [39]. These pores were found to be in the range of trabecular bone pore size of 200–400 µm [40] which either allow for restoration of vascularity or complete penetration of osseous tissue through the repaired site, or for other applications such as scaffolds for tissue engineering and systems for controlled delivery of drugs [13].

Porosity is one of the major factors influencing the osteoconductivity, in which, pore size and total porous volume are very important aspects [41]; but there are different perspectives among researchers regarding the macropores size for optimum bone growth. Some studies observed notable bone ingrowth in the macropores smaller than 100 µm [20]; while Hulbert et al. [18] recommended minimum pore size of 100 µm for a scaffold.
Subsequent studies showed better osteogenesis for implants when using pores $> 300 \, \mu m$ [42-43].

The optimal pore size varying from 50 $\mu m$ and 565 $\mu m$ is still a continuous debate among researchers [39], but in general many authors asserted that only the macropores larger than 100–150 $\mu m$ could facilitate ingrowth of mineralized bone [44], although Gauthier et al
[22] pointed out to the need for more suitable diameter of macropores of 250–600µm. However, Schopper et al. [45] recommended pore sizes higher than 300 µm due to the formation of capillaries. This macroporosity is important for bone formation. In the study by Kuboki et al. [17] it was found that no new bone had formed on the solid particles; while in the porous scaffolds, direct osteogenesis had occurred. According to Hing [46] the macroporous structure is necessary for cell growth and vascularization. While Kasten et al. [47] found that porosity and pore size of distinct TCP scaffolds influence not only protein production in vitro and in vivo but also specific alkaline phosphates ALP activity, as an important marker for osteogenesis.

3.2. The total porosity percentage

The total porosity, as measured by Archimedes method for all samples was around 79%. This high porosity and large pores can enhance bone ingrowths because they allow migration and proliferation of osteoblasts and mesenchymal cells, as well as vascularization [17]. It was shown that in vivo tests, the higher porosities of 65% and 75% yielded higher ALP activity than the 25% porosity. On the other hand, scaffolds fabricated from biomaterials with a high degradation rate should not have too high porosities (>90%), as rapid depletion of the biomaterial will compromise the mechanical and structural integrity before substitution by newly formed bone [48].

Generally, all the four additive materials used in the study had shown pore formation of different form and size, but no attempt has been made to determine their exact difference. Therefore, further investigation is needed in order to identify the best one in terms of biocompatibility and scaffolding aspects.

3.3. Granules

In this study, BCP is in granular form (granules) was chosen with the average size of 450 µm after sieving. Granules can be more easily combined with cultured osteogenic cells or loaded growth factors, which are known to promote bone repairing [49]. The granules are in the same range found by Higashi and Okamoto [50] and Kuroda [51], who stated that in transplantation of HA/TCP particles alone into hard tissue defects in rabbits and dogs was associated with superior bone formation around particles of 300–600 µm, while larger and smaller particles were associated with less bone formation.

Adjusting the particle size was recommended. This is in agreement with Mahesh et al. [52] who demonstrated that HA/TCP particle size plays a crucial role in determining the extent of bone formation by transplanted human bone mesenchymal stem cells (BMSCs) which form less extensive and poorer quality of bone when incorporated into blocks than when incorporated into particles. Apart from that, he also found that the two most important characteristics were particle size and shape [53-54]. Furthermore, granules were found to be a more preferable option in medical applications. When HA/TCP blocks are compared with HA/TCP particles which derived from the same blocks, human BMSCs form less extensive and poorer quality bone when incorporated into blocks than when incorporated into particles [53-55].

4. Conclusion

In this study, a new approach was developed to assure the desirable properties of BCP in terms of biocompatibility and scaffolding from different porogens to meet the osteogenesis
requirements of normal bone for tissue engineering purpose. Apart from that, the effect of adopting the Ca/P ratio of 1.52 recovered by wet precipitation in the laboratory have proved potential for biphasic calcium phosphate with higher degradation rate and inducing earlier bone formation as well as mixing on a crystallite level achieved with this method play an important role in protein adsorption, cell attachment, and dissolution of the biomaterials. Parameters of porosity, pore size distribution, and granule size, and the ultimate impact of such effect on bone formation process was assessed. The resulted pore size distribution was between 200 to 400 µm with the oval to round shape; both have an effect on increasing the similarity to trabicular bone form. Calcination at 850°C resulted in high microporosity which makes the methodology feasible for purposes of vascular and ion transport. The granular shape and HA/TCP particles size of 300–600 µm was also considered the best option for promoting bone formations.

5. References

величином гранула. Подешавањем температуре синтеровања постигнута је желена микропорозност, док је микропорозност регулисана додатком два порогена која се иначе користе у односу 1:3 тежинска % и то (нафтален и шећер) и два нова порогена (саго и лентил). Сви узорци испресовани су у таблете и жарени на 500ºC, током 2 сата са брзином загревања 0,5ºC/мин (да би се уклониле порогени) и даље су синтеровани на 850ºC, током 2 сата са брзином загревања 5ºC/мин, да би се након тога охладили до собне температуре. Да би се припремиле грануле величине 250-500 µm, синтероване таблете су ломљене и просејане. Методе рентгенске дифракције, ФЕСЕМ, расподеле величине зrna и пора су рађене ради карактеризације добијених гранула. Добијене су поре овалног и округлог облика величине 200-400 µm, које су идентичне са величином честица које су добијене употребом порогена. Овакав прилаз даје желена својства која су блиска својствима природне људске кости и води бољој остеогенези у инжењерингу човечијег ткива.

Кључне речи: Синтеза; двофазни калцијум фосфат; микропорозност; инжењеринг ткива.