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Research Report

Variations of gelatin percentages in HA-TCP scaffolds as the result of 6- and 12-hour sintering processes of blood cockle *(Anadara granosa)* shells against porosity

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ABSTRACT

Background: *Porous scaffold is one type of biomaterial primarily employed as a bone substitute material which demonstrates superior osteoconductive and osteointegrative properties than solid scaffold since it can stimulate and accelerate the growth of new tissue. For the purposes of this study, porous scaffold was produced using hydroxyapatite-tricalcium phosphate (HA-TCP) powder derived from a synthesis of blood cockle (Anadara granosa) shells and gelatin.* ***Purpose:*** *The aim of this study was to reveal the effects of the percentage of gelatin in HA-TCP scaffolds derived from 6- and 12-hours sintering processes involving blood cockle shells on porosity.* ***Methods:*** *HA-TCP powder was derived from a synthesis of Anadara granosa shells using a hydrothermal method at 200^{o}C with sintering periods of 6 and 12 hours. A XRD test was subsequently conducted to reveal the compositions of HA-TCP powder. The 24 scaffold samples (n=6) employed were manufactured using a freeze dry method before being divided into four groups, namely; Group 1 using 25% HA-TCP powder (a six-hour sintering process) combined with 20% gelatin, Group 2 using 25% HA-TCP powder (a six-hour sintering process) combined with 10% gelatin, Group 3 using 25% HA-TCP powder (a twelve-hour sintering process) combined with 20% gelatin; and Group 4 using 25% HA-TCP powder (a twelve-hour sintering process) combined with 10% gelatin. A scaffold porosity test was subsequently carried out using a liquid displacement method. A one-way ANOVA test was performed using SPSS, followed by a Post-Hoc LSD ($p<0.05$).* ***Results:*** *The statistical results for scaffold porosity were within the range of 67.21 -77.51%. The highest porosity was found in Group 3, while the lowest was in Group 4. Significant differences were also present in all groups.* ***Conclusion:*** *Variations in the percentage of gelatin can affect the porosity of HA-TCP scaffolds derived from 6-and 12 hours sintrering processes blood cockle shells. The smaller the percentage of gelatin, the higher the porosity.*

Keywords: *Anadara granosa shell; HA-TCP; percentage of gelatin; porosity; scaffold*

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INTRODUCTION

Bone substitute material serves to assist reconstruction, stabilize the structure and bonding of bone and stimulate osteogenesis and healing processes within bone defects.[1] Bone substitute material must, therefore, be biocompatible, non-toxic, non-cariogenic and non-allergenic, while possessing a biological mechanism that is osteoconductive, osteoinductive and osteogenic.[2] In general, there are four types of bone substitute material (bone graft), namely; autograft, allograft, xenograft and alloplast. Autograft is a substitute for bone material taken from the patient's body, while allograft is derived from that of another human being. Contrastingly, xenograft is extracted from the body of an animal or a different species, while alloplast is composed of synthetic material.[3] Hydroxyapatite (HA), with the chemical formula Ca_{10} $(PO_4)_6$ $(OH)_{2,}$ is an inorganic compound capable of binding to bone[4] which can be produced synthetically from blood cockle (*Anadara granosa*) shells.

Indonesia is one of the countries producting significant amounts of seafood, one example being blood cockle.

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According to data released by the Directorate General of Capture Fisheries in 2011, shellfish production in Indonesia during the previous year reached 34,929 tons of which the volume of blood cockle production amounted to 34,482 tons.[5] Until recently, blood cockles had been harvested only for their meat, the consumption of which results in high levels of unutilized shell waste. As a result, hydroxyapatite derived from the synthesis of blood cockle shell waste is used to produce scaffold.

Scaffold applied to tissue engineering requires open porosity construction and adequate pore size to support cell nutrient transport, cell proliferation and migration resulting from tissue vascularization.[6] A porous surface also serves to facilitate mechanical interlocking between the scaffolds and surrounding tissue to improve the mechanical stability of the implant. In addition, the network structure of the pores assists in guiding and promoting new tissue formation.[7] To promote ideal scaffold production the addition of supporting materials is required, one of which is gelatin.

Gelatin is a collagen-rich polypeptide bond derived from the hydrolysis of bone and animal skin. It is non-toxic, biocompatible, biodegradable and nonimmunogenetic in character, rendering it useful as a coating for implants, wound dressings and scaffold in cell culture. Therefore, as a natural polymer, gelatin can be used as a scaffold for tissue engineering.[8,9] A mixture of 10% gelatin and 80% nanoparticles cockle shells bone graft is effective in increasing the number of osteoblast cells in the bone healing process. 10% gelatin produces a high viscosity liquid with significant potential for tissue engineering.[10,11] On the other hand, scaffold containing 20% gelatin shows a strong expansive character and induces biological responses such as cell attachment, cell proliferation and effective cell spread.[12] Gelatin plays an important role in making scaffold because of its ability to cross-link and modify other materials that can significantly change their mechanical properties to become stronger and porous.[13] The addition of gelatin in the manufacture of scaffold is also intended to increase adhesion, migration and mineralization of osteoblast cells that play an important role in bone formation process.[14] The pore structures in each sample of macroporus scaffold will differ.

Furthermore, variations in sintering period will also affect the composition and structure of the HA powder produced, while also causing differences in crystallinity. The longer the sintering period, the greater the crystallinity produced which affects the regularity of HA atom arrangement.[15] In this research, porous HA scaffold was derived from the synthesis of blood cockle shells and 25% HA and 10% or 20% gelatin after 6- and 12-hour periods of hydrothermal circulation. Hence, this study aimed to determine the effect of adding variations of gelatin percentage on the porosity of hydroxyapatite-tricalcium phosphate (HA-TCP) scaffold as a result of blood cockle shells synthesis with hydrothermal sintering of 6- and 12-hour duration.

MATERIALS AND METHODS

The sample manufacture stage includes producing scaffold from the synthesis of blood cockle shells. HA was obtained by processing blood cockle shells which involved boiling them for 30 minutes before cleaning and drying. The shells were subsequently pounded to form a powder which was filtered through a 100-mesh sieve. The powder was calcined at 100°C for three hours to produce calcium carbonate ($CaCO_3$) powder, 10 grams of which were then dissolved in distilled water to produce 1M $CaCO_3$. Thereafter, 1M of $CaCO_3$ was mixed with 0.6M of $NH_4H_2PO_4$ obtained from 6.9 grams of NH4H2PO4 which was dissolved in 100 ml of distilled water. By means of a hydrothermal method at 200°C for 6 hours for P1 and 12 hours for P2, $CaCO_3$ powder was further processed and rinsed using distilled water to obtain pH ± 7. Thereafter, final rinsing was performed using methanol PA after which it was warmed at 50°C for 4 hours before being subjected to the final sintering process at 900°C for 3 hours until HA powder was produced. Scaffold was then produced by mixing up to 25% (wt%) HA powder with 10% or 20% (wt%) gelatin and putting it into 360 µl (6–10 mm) well plates. The scaffold material was subsequently frozen at -80° C for 5 hours before being freeze dried for 30 hours.

A x-ray diffraction (XRD) test was carried out to determine the crystal system, lattice parameter, crystallinity degree and substance type contained in each sample. X-rays were directed at the sample inducing the XRD detector to rotate according to the range of diffraction angles employed. A diffractogram graph depicting the relationship between the intensity and diffraction angle was created, before being presented on the computer screen. The diffractogram graph was subsequently interpreted using Software Match which provided information about the crystal structure contained in the sample in the form of percentage results.

A porosity test was carried out to determine the percentage volume of void space contained in the samples. In this research, the samples were divided into 4 groups (n = 6) according to differences in the ratio between HA and gelatin. 96% absolute ethanol was subsequently used as a liquid to determine the wet mass value. Moreover, a digital balance was also used to quantify the mass of objects. A porosity test was carried out using the liquid displacement method which involved soaking the samples in 96% absolute ethanol for 48 hours before wet mass measurement was carried out.[16] The percentage porosity of each sample was then quanitified after the object mass measurement results of the wet mass of the samples had been obtained by means of the following equation (Figure 1).

A scanning electron microscope (SEM) test was performed to identify the microstructure of the samples with the results being observed through a photo. A sample preparation had previously been produced for observation using the SEM imaging device. Coated scaffold samples were then cut on the side to be studied and observed through

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$$\text{Porosity (\%)} = \frac{m_b - m_k}{\rho_{liquid} \times V_b} \times 100$$

Figure 1. Porosity test equation.[16]

Note:

m_b = wet mass of sample (gram)

m_k = dry mass of sample (gram)

V_b = volume of test object s(cm^3)

ρ_{liquid} = density of liquid

an electron microscope and displayed on a computer screen. An interpretation was subsequently conducted by searching for a field of view, selecting pores randomly and measuring them with the SEM imaging device at 1000x magnification. A statistical test analysis was carried out using one-way ANOVA, followed by LSD Post-Hoc test ($p<0.05$).

RESULTS

XRD was performed to determine the lattice parameters, crystallinity degree and substances contained in a sample.

The results of an XRD test on HA-TCP powder derived from the synthesis of blood cockle shells with a 6-hour sintering period produced a diffractogram with sharp peaks, the highest being in the range of 30°-40°, as well as high intensity. This signifies the sample having undergone perfect crystallinity. Moreover, this diffraction pattern also indicated that the predominant content of the sample was HA.

The results of an XRD test on HA-TCP powder derived from the synthesis of blood cockle shells during a 12-hour sintering period produced a diffractogram similar to that of a 6-hour sintering period with sharp peaks, the highest being in the range of 30°-40° as well as elevated intensity. The similarity between the XRD results of the two samples indicated that HA was the main content of HA-TCP powder derived from the synthesis of blood cockle shells.

Table 1 shows that HA-TCP powder derived from the synthesis of blood cockle shells with a 6-hour sintering period had the most dominant HA level of 54.5% and a TCP level of 9.1%. The table above also indicates that HA level in the group with a 12-hour sintering period was less than in the group with a 6-hour sintering period. However, the TCP level in the group subject to a 12-hour sintering period was double that of the group subject to one of 6 hours.

Table 2 shows that K4-generated scaffold had the highest percentage of porosity compared to the other three sample groups. Furthermore, the porosity test results revealed that the group using 25% HA-TCP combined with 10% gelatin and a 6-hour sintering period demonstrated a higher average percentage porosity than the group using 25% HA-TCP combined with 20% gelatin and a 6-hour sintering period. Similarly, the group using 25% HA-TCP combined with 10% gelatin and a 12-hour sintering period had a higher average percentage of porosity than the group using 25% HA-TCP combined with 20% gelatin and a 12-hour sintering period.

Table 1. List of chemical compounds contained in scaffold based on XRD test on HA-TCP powder derived from the synthesis of blood cockle shells sintering times lasting 6 and 12 hours.

Compounds	Chemical Formula	Sintering Period (hours)	Percentage (%)
HA	$Ca_5(PO_4)_3(OH)$	6	54.5
TCP	$Ca_3(PO_4)_2$	6	9.1
HA	$Ca_5(PO_4)_3(OH)$	12	51.5
TCP	$Ca_3(PO_4)_2$	12	16.8

Table 2. The porosity of the HA-TCP scaffold with variations in the percentage of gelatin and the results of the comparative test between groups

Groups	Scaffold sample codes	Mean ± SD	p
K1	25% HA-TCP combined with 20% gelatin (with a 6-hours sintering period)	67.65 ± 0.872	0.000*
K2	25% HA-TCP with 10% gelatin (with a 6-hours sintering period)	72.98 ± 2.250	
K3	25% HA-TCP with 20% gelatin (with a 12-hours sintering period)	67.21 ± 1.977	
K4	25% HA-TCP with 10% gelatin (with a 12-hours sintering periiod)	77.51 ± 2.858	

Note: * $p<0.05$ (significantly different)

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Subsequently, a one-way ANOVA test with a significance level of 95% (0.05) was performed, followed by an LSD Post-Hoc test. The one-way ANOVA test showed significant differences between groups, where the value was one of $p<0.05$.

In Table 2, the results for significance between groups were 0 with a p value of less than 0.05 (0 <0.05). It indicates that there was a significant difference in the mean percentage of porosity between all groups. Therefore, an LSD Post Hoc test was then performed.

From Table 3 it can be seen that the results of the Post Hoc LSD test showed significant differences between each study group, except between K1 and K3 both of which showed a significance level of more than 0.05.

A SEM test at 1000x magnification was then conducted on pores derived from the randomly selected sample to be measured digitally through the computer. In all the samples tested, the scaffold pore size varied within the range of 41.02 µm to 73.63 µm.

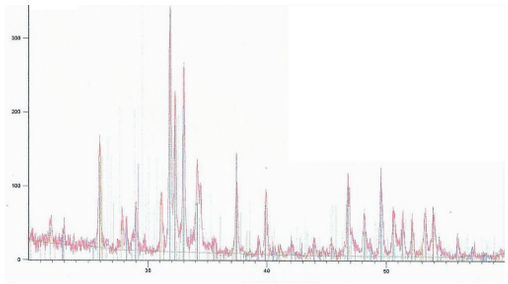

Figure 2. XRD graph of HA-TCP powder derived from the synthesis of blood cockle shells with a sintering period lasting 6 hours.

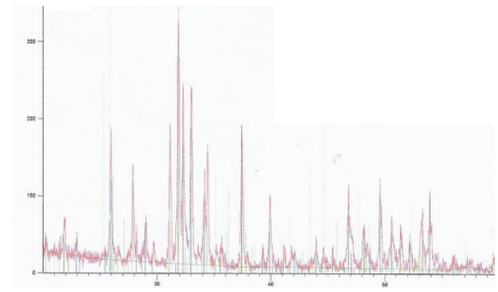

Figure 3. XRD graph of HA-TCP powder derived from the synthesis of blood cockle shells with a sintering period lasting 12 hours.

Table 3. Results of the Post Hoc LSD test

Groups	K1	K2	K3	K4
K1		0.000*	0.722	0.000*
K2			0.000*	0.001*
K3				0.000*

*p < 0.05 (significantly different)

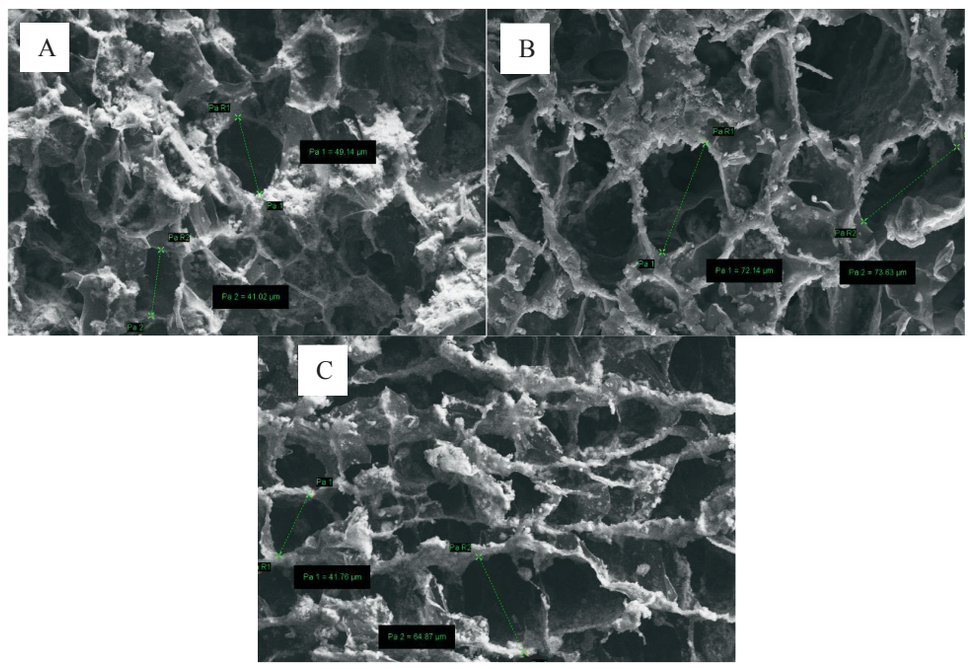

Figure 4. SEM results in scaffold porosity at 1000x magnification. (A) 25% HA combined with 10% gelatin after a 12-hours sintering period; (B) 25% HA combined with 20% gelatin after a 12-hours sintering period; (C) 25% HA combined with 10% gelatin after a 6-hour sintering period.

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Figure 4 shows that pores od various sizes were interconnected in all groups. The porosity of HA-TCP scaffold combined with 10% gelatin and a 12-hour sintering period formed pores with an average diameter of between 41.02-49.14 µm. Meanwhile, the porosity of HA-TCP scaffold combined with 20% gelatin and a 12-hour sintering time formed pores with an average diameter of between 72.14 µm -73.63 µm. In addition, the porosity of HA-TCP scaffold combined with 10% gelatin and a 6-hour sintering period formed pores with an average diameter of between 41.76 µm - 64.87 µm.

DISCUSSION

In XRD characterization, the main property of a material observed using x-ray waves is crystallinity. The longer the processing time, the greater the semicrystalline nature of the calcium phosphate phase. This is because the more protracted the duration of calcium phosphate formation, the more the particles will combine with others to form agglomerates. This situation causes changes in the crystalline properties of calcium phosphate and also widens the XRD diffractogram. In other words, the longer the duration of calcium phosphate formation, the greater the crystallite size because particles will combine with others (agglomeration).[17] The HA-TCP samples with a 6-hours sintering period had the highest peak at 2θ = in the range 31˚-32˚ (Figure 2). Meanwhile, the HA-TCP samples with a 12-hours sintering period had the highest peak at 2θ = in the range 31˚-32˚ (Figure 3).

The majority of peaks identified from HA-TCP samples subject to varying sintering duration (6 and 12 hours) were the same, thereby signifying the presence of HA. This proves that, during the synthesis process, the HA composition is dominant. Although the HA level at the end of the 6-hours sintering period was higher than the HA level at the end of the 12-hours sintering period, the TCP level at the end of the 6-hours sintering period was less than the TCP level at the end of the 12-hours sintering period. This is due to the hydrothermal method. The highest peak of HA level was at the end of the 6-hour sintering period. At the end of the 12-hours sintering period, the HA level decreased while the TCP level increased. The HA level at the end of the 12-hours sintering period decreased since HA has a peak point after which HA will decrease and be converted to TCP with the result that the TCP percentage increases, while the HA percentage decreases.[18]

Porosity, the ratio of void space volume to the mass volume of a solid material, can be measured using the ratio between dry mass, wet mass and sample volume. There was a significant difference between porosity in HA-TCP scaffold combined with 25% gelatin (6-hours sintering) and that in HA-TCP scaffold combined with 10% gelatin (6-hours sintering). Moreover, there was also a significant difference between the porosity of HA-TCP scaffold combined with 25% gelatin (12-hours sintering) and that in HA-TCP scaffold combined with 10% gelatin (12-hours sintering).

The results of this research indicate that the composition of HA-TCP scaffold compounds is influenced by variations in the percentage of gelatin which is a natural polymer employed as a scaffold in tissue engineering. The combination of HA-TCP and gelatin will form covalent bonds between Ca^{2+} ions and $R\text{-}COO^{-}$ ions derived from gelatin molecules. This crosslinking will then cause a reduction in the distance between HA-TCP-gelatin fibers.[19] The greater the percentage of gelatin formed the more numerous the bonds resulting in a shorter distance between the fibers and reduced porosity.

The freeze dry method applied during the manufacture of scaffolds removed liquid from the scaffolds[20] with the result that rough structure patterns formed. This is because the HA-TCP particle powder used in this research was less than 74 µm in size, while the largest pore diameter was derived from HA-TCP scaffold combined with 20% gelatin. The largest pore diameter of ± 70 µm was found in the group using HA-TCP scaffold combined with 20% gelatin. Meanwhile, the smallest pore diameter of ± 40 µm was in the group using HA-TCP scaffold combined with 10% gelatin. Therefore, it is assumed that the addition of 20% gelatin can generate ± 70 µm pore diameter that is open and interconnected.

Finally, it can be concluded that the addition of various percentages of gelatin can affect the porosity of HA-TCP scaffolds as a result of the synthesis of blood cockle shells after a 6- or 12-hours sintering period. The lower the percentage of gelatin, the higher the porosity of the scaffold. The highest porosity is found in HA-TCP scaffold with the addition of 10% gelatin.

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