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Research Report

The effect of various concentrations of HA-TCP derived from cockle shell synthesis on scaffold porosity

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ABSTRACT

Background: *Porosity is an important property that must be possessed by scaffold due to its role in new bone growth. Hydroxyapatite is a scaffold material with a composition resembling that of bone that can be synthesized from cockle shell (Anadara granosa).* ***Purpose:*** *This research aimed to determine the effects of various HA-TCP concentrations (wt%) derived from cockle shell synthesis on scaffold porosity.* ***Methods:*** *HA-TCP was synthesized from cockle shells using a hydrothermal method at 200^{o} C with a 12-hour sintering process period. An XRD test was subsequently carried out to determine the composition of hydroxyapatite (HA) and tricalcium phosphate (TCP) compounds. Eighteen scaffold samples (n=6) were then produced using a freeze dry method and divided into three groups, namely; Group 1 (K1) treated with 5% HA-TCP, Group 2 (K2) treated with 25% HA-TCP and Group 3 (K3) treated with 50% HA-TCP. Thereafter, a scaffold porosity test was conducted using liquid displacement method. Scaffold porosity was observed by means of an SEM image. A One-Way ANOVA test was subsequently performed, followed by an LSD Post-Hoc test ($p < 0.05$).* ***Results:*** *The results of the XRD test showed that the percentage of HA was 51.5%, while TCP was 16.8%. The porosity of the scaffolds was within the range of 67.24% - 80.17%. The highest porosity was found in Group 1, while the lowest occurred in Group 3. There were significant differences in all groups.* ***Conclusion:*** *The concentration of HA-TCP derived from the synthesis of cockle shells affects the porosity of scaffold. The lower the concentration of HA-TCP, the higher the scaffold porosity.*

Keywords: *HA-TCP concentration; gelatin; porosity; scaffold; cockle shells*

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INTRODUCTION

Repairing bone damage remains a problem for medical personnel since the bone healing process often proves ineffective, culminating in the need for a material to promote it. One method commonly used to restore the function of lost or damaged bone tissue is the application of bone graft[1], a biomaterial used to fill damaged bone cavities which disappears when new bone cell growth has occurred.[2] In general, there are three properties that a bone graft requires in order to form new bones, namely: osteoconductivity, osteoinductivity and osteogenetic.[3] However, bone graft has been widely used in dentistry to overcome the problem of bone resorption by regenerating lost or severely damaged bones.

Bone grafts can be grouped into four types: autographs, allographs, xenographs and alloplasts. The most commonly used are xenografts, bone grafts from different species that possess osteoconductive properties and demonstrate high levels of biocompatibility.[4] Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is the main inorganic component in bones and teeth generally used as a bone graft due to its excellent biocompatibility, osteoconductivity in relation to the chemical and biological affinity to bone tissue.[5,6] Synthetic hydroxyapatite currently constitutes an expensive imported product, costing approximately one million rupiah per gram. Although hydroxyapatite can be synthesized from readily available natural ingredients, these have yet to be utilized.[7] Moreover, hydroxyapatite unfortunately demonstrates certain weaknesses, including fragility and poor absorbency.[8]

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Tricalcium phosphate is a compound possessing physical and chemical properties similar to the mineral structure of human bones and teeth.[9] Tricalcium phosphate has bioactive, biocompatible and osteoconductive properties, while being readily absorbed. The combination of hydroxyapatite and tricalcium phosphate produced is assumed to possess higher quality and more stable compounds.[10] The manufacture of HA-TCP combination compounds with a 12-hour sintering period produces a high tricalcium phosphate compound in which the longer the sintering time, the higher the production of tricalcium phosphate compounds.[11]

One of the natural ingredients possessing a high calcium content is cockle shell with a calcium carbonate level of 98%.[12] Cockles constitute one of the marine food commodities with a high commercial value that are favored by consumers in Indonesia and throughout Asia. The high consumption of cockles will, consequently, produce large quantities of shells as waste. The use of accumulated cockle shell waste can enhance waste management in reducing pollution and improving environmental aesthetics.[13] This research, therefore, focused on converting cockle shell waste into HA-TCP bone graft.

HA-TCP bone graft derived from cockle shells is formed into a scaffold enabling it to accelerate the process of biomineralization in the bone. An important property that must be possessed by scaffold is porosity. The porous characteristics of scaffold are very important to the process of new bone growth. Scaffold represents a site to which growth cells can attach and develop to form new bone tissue. Scaffold with high porosity, optimal pore size and pore interconnectivity plays a very important role in the growth of bone cells.[14] Hence, the hydroxyapatite present in porous scaffold is assumed to demonstrate more effective osteoconductivity and greater absorbability than dense scaffold. Effective pore size for bone cell growth is approximately 40-100μm.[15] The porosity of scaffold effectively employed as a bone graft is between 30% and 90%.[16] Consequently, there is a great demand for the development of porous scaffold synthesis.

Research conducted by Narbat (2006) using HA scaffold at concentrations of 30%, 40% and 50% showed that the highest porosity was found in scaffolds with an HA concentration of 30%. However, HA has difficulty in absorbing.[17] As a result, this research was conducted to synthesize HA-TCP from cockle shells (Anadara granosa). Theoretically, TCP will be more easily degraded and absorbed by the body, leading to the expectation of more rapid new bone formation.[18] However, it has yet to be confirmed whether variations in the concentrations of HA-TCP synthesis derived from cockle shells can affect scaffold porosity.

In this research, porous HA-TCP scaffold was produced with HA-TCP concentrations of 5%, 25% and 50% combined with gelatin, a biodegradable, biocompatible and soluble protein-based material..[19] Gelatin contains many protein bonds in aginine-glycine-apartic acid (RGD) which can increase cell attachment and cell growth.[20] The addition of gelatin to scaffold with hydroxyapatite combination was intended to increase bone-forming cell differentiation and improve the mechanical properties of the scaffold.[21] Thus, this research aimed to determine the effects of HA-TCP concentration variations (wt%) derived from cockle shells synthesis on scaffold porosity.

MATERIALS AND METHODS

This research constituted an experimental laboratory study with a posttest-only group design and was conducted in two stages, namely, a sampling process stage and a sample testing stage. The raw material used consisted of cockle shells (Anadara granosa) extracted from waste present on the coast at Probolinggo. The cockle shells were pulverized and converted to HA-TCP by a hydrothermal method at 200°C with a 12-hour sintering period, including calcination, sintering, PA methanol rinsing and drying processes.

HA-TCP compounds were obtained from cockle shells which had been cleaned and ground to form a smooth texture. The filtered powder was then calcined at 100° for three hours in an oven furnace (Naberterm, Germany). As part of the hydrothermal process, the hydroxyapatite powder was mixed with Ammonium Dihydrogen Phosphate (Daishin, Japan) and heated at 200°C in an oven furnace (Naberterm, Germany) with a sintering time of 12 hours. After completion of this process, the powder was rinsed with distilled water until the pH reached ±7, and was also rinsed with methanol PA (Emsure, Germany). The powder was then heated to a temperature of 50° for four hours and at 900°C for a further three hours. Before manufacture of the scaffold, HA-TCP powder had been filtered with a 200-mesh filter to produce a powder size of <74 μm. Scaffold was subsequently produced by mixing HA-TCP with 10% gelatin (Sigma, Germany) (wt%) (1:1). In this research, three varieties of HA-TCP concentration (wt%) were used, namely 5% HA-TCP (K1), 25% HA-TCP (K2) and 50% HA-TCP (K3). The results of the mixing process were then deposited in a 6mm-diameter mold 10mm in height, frozen at a temperature of -80° C for five hours and freeze dried for 30 hours.

An XRD test was conducted using an Xpert-Pro PANalytical at an angle of 2θ= 5°- 60° to identify the crystallization phase and content of the material using X-ray electromagnetic radiation. The XRD test results were then presented in the form of spectrum charts and tables. The spectrum diffraction pattern of the XRD test results provides information about the angle of diffraction in the atomic material (2θ) on the horizontal axis and the intensity result on the vertical axis. An identification phase was then performed by comparing the hydroxyapatite diffraction pattern with data from International Center for Diffraction Data (ICDD).

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Porosity is the volume of empty space contained in a sample tested using a liquid displacement method determined by the percentage of liquid absorbed by scaffold after being immersed in ethanol. Materials used in this test comprised HA-TCP scaffold derived from cockle shell synthesis and 96% absolute ethanol (Merck, Germany). The tools employed consisted of analytical scales, tweezers and vernier calipers (Krisbow, Indonesia). This research also used 18 cylindrical 6x10mm-sized (n=6) samples. A porosity test was subsequently carried out to determine the volume of empty space in each sample. A porosity test measured the volume of the samples, weighing their dry weight before soaking them in 96% absolute ethanol for 48 hours. The samples were weighed to determine the wet mass of the specimens. The porosity of the samples was then calculated using the following equation:[22]

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

Note:
m_b = wet mass of specimen (gram)
m_k = dry mass of specimen (gram)
V_b = volume of specimen (cm^3)
ρ_{liquid} = density of water (1 gr/cm^3)

Table 1. Chemical compounds contained in HA-TCP powder derived from cockle shell synthesis.

Name of compounds	Percentage (%)	Chemical formulas
Tri-Calcium Phosphate (TCP)	16.8	$Ca_3(PO_4)_2$
Hydroxyapatite (HA)	51.5	$Ca_5(PO_4)_3(OH)$
Aragonite (CaCO3)	20.8	$CaCO_3$
Calcium Hydroxide	3.0	$Ca(OH)_2$
Calcium Oxide	7.9	CaO
Calcite	-	$CaCO_3$

SEM was conducted with an electron microscope at 1000x magnification in order to identify pores in the samples. Pore size observed through an electron microscope lens was displayed on a computer screen using the SEM imaging device. A photo was taken of the selected part of each sample at the desired magnification and the pore size was measured. The scaffold pore was represented by black and its size measured using a scale line found in the SEM image.

Statistical analysis was performed on the porosity data using SPSS one-way ANOVA with a significance level of 95% (0.05), followed by an LSD Post-Hoc test.

RESULTS

The XRD results of HA-TCP powder in Figure 1 illustrated a diffractogram with high peaks indicating changes in the crystallization phase of the samples at each calcination temperature. The diffraction pattern of hydroxyapatite formation in the XRD results was shown

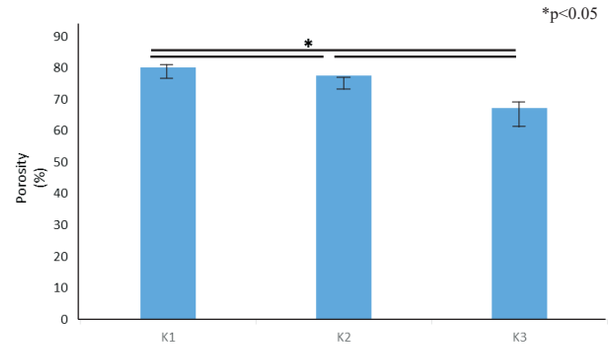

Figure 2. Percentages of porosity in 5% HA-TCP scaffold (K1), 25% HA-TCP scaffold (K2), and 50% HA-TCP scaffold (K3).

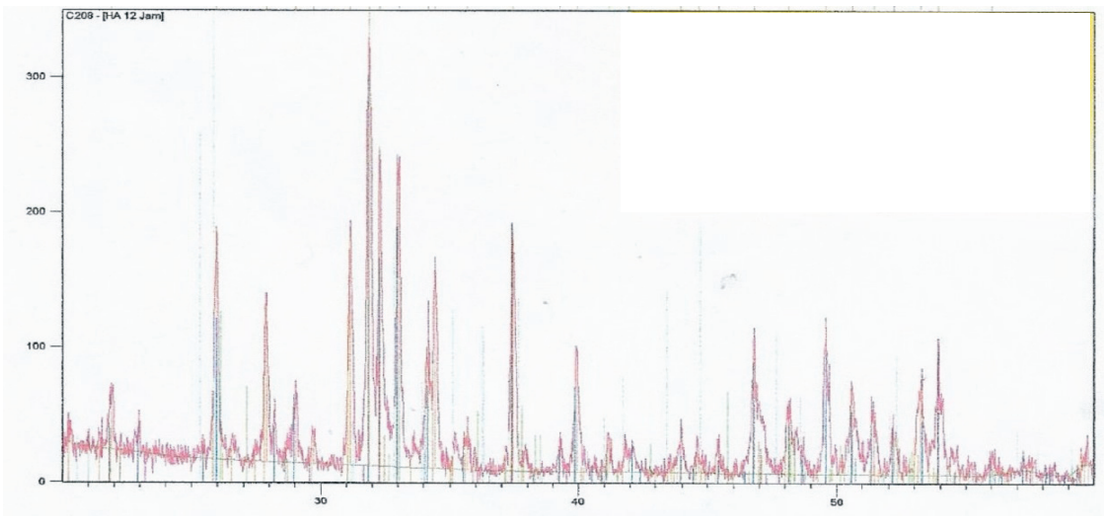

Figure 1. XRD spectrum graph of HA-TCP derived from cockle shell synthesis.

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at 2θ : 32°, and the formation of tricalcium phosphate at 2θ : 28°.

The XRD results in Table 1 showed that the levels of hydroxyapatite (HA) and tricalcium phosphate (TCP) produced were relatively high. Other compounds produced from blood clams (*Anadara granosa*) included: aragonite, calcium oxide and calcium hydroxide.

As shown in Figure 2, the percentages of porosity were high in all groups. The statistical test results showed significant differences between Group 1 and Group 2 ($p = 0.037$), Group 2 and Group 3 ($p = 0.000$), and Group 3 and Group 1 ($p = 0.000$). The percentage of porosity in the 5% HA-TCP group was 80.17%, 77.51% in the 25% HA-TCP group, and 67.24% in the 50% HA-TCP group.

Based on the SEM image in Figure 3, there were interconnected scaffold pores. In Figure 4, the pore size was small, while in Figure 5, the pore size varied. In general, the pore size in all groups varied and was unequally distributed. The average diameter of the 5% HA-TCP scaffold pore size was 75.99 µm, 25% HA-TCP scaffold was 45.08 µm and 50% HA-TCP scaffold was 90.31 µm.

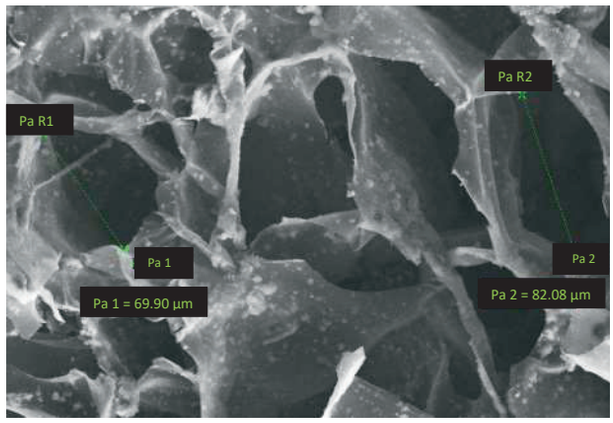

Figure 3. SEM image indicating porosity of 5% HA-TCP scaffold at 1000x magnification.

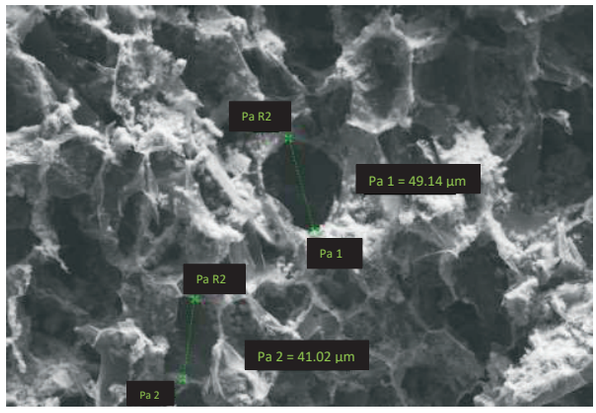

Figure 4. SEM image indicating porosity of 25% HA-TCP scaffold at 1000x magnification.

DISCUSSION

An X-ray diffractometer (XRD) machine is essential not only to evaluate crystalline structures, crystallization phase and crystallinity degree, but also to determine types of elements or compounds contained in a material. The output of an XRD machine is a diffractogram with a high peak indicating the crystallization phase of a sample.[23] In this research, the XRD machine results relating to cockle shell synthesis confirmed the presence of HA compounds (51.5%), TCP (16.8%), and others such as calcium carbonate in the form of aragonite. This is due to the decomposition of hydroxyapatite during combustion as a result of imperfections in the reactants. Since natural material was used in this study, it proved difficult to achieve a high level of purity in the material.[24]

The results of this research indicated that the porosity of the samples varied. The highest porosity was demonstrated by Group 1 that had been treated with 5% HA-TCP scaffold, while the lowest occurred in Group 3 which had been treated with 50% HA-TCP scaffold. This signified that the porosity of HA-TCP scaffold samples decreased as the concentration of HA-TCP scaffold powder increased. The statistical test results also revealed significant differences between groups. In other words, the concentration of HA-TCP powder in the manufacture of scaffold affects the level of scaffold porosity. The higher the concentration of HA-TCP powder administered, the lower the level of porosity.[5] This occurs because the formation of a salt bridge and cross-linking due to the hydroxyapatite reaction absorbed on the material matrix fills the gap between the particles in the material.[25]

The porosity of the scaffold is also known to be affected by porogen (porous-forming material/gelatin) concentration and the sintering process. The porogen concentration is directly proportional to the porosity of a scaffold. The higher the concentration of porogen used in making scaffold, the higher the porosity produced.[26] In this research, the same concentration of porogen, 10% gelatin, was used in all groups. As a result, the effect of

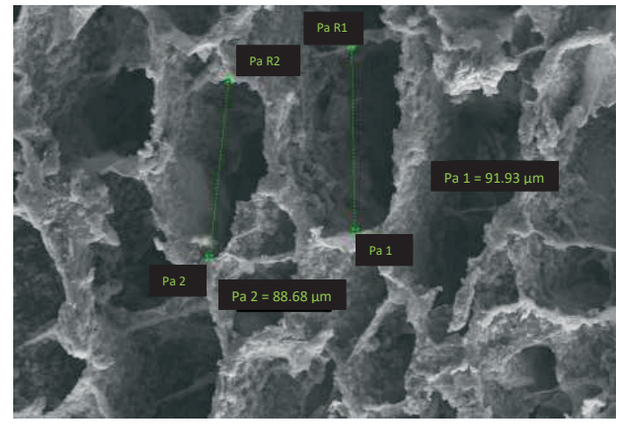

Figure 5. SEM image indicating porosity of 50% HA-TCP scaffold at 1000x magnification.

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the porogen factor on scaffold porosity was not detected. However, the porosity produced in this research was quite high. An effective level of porosity for bone cell growth is one of approximately 70%.[27] Thus, the manufacture of scaffold with 5% and 25% HA-TCP can produce porosity supportive of bone growth.

In addition, SEM images (Figures 3, 4 and 5) showed that pores of varying sizes in HA-TCP scaffolds were connected to one another and spread unevenly in all groups. The research findings reported here indicate that pore size is not directly proportional to porosity. Rather, a large number of small pores can increase porosity. The pore size of scaffold pores can be influenced by temperature and the freezing time required during the freeze-drying method. The lower the freezing temperature, the faster the ice crystal dendrite formed resulting in pores on the scaffold. The faster the freezing time, the larger the size of the pores formed.[28] Since variations in the freeze-drying method were not employed during this research, they did not affect scaffold porosity which has greater influence on bone formation compared to pore size. The number of endothelial cells, osteoblasts and bone mass is proportional to an increase in scaffold porosity.

In conclusion, this research indicated that the smaller the concentration of HA-TCP powder (wt%), the higher the porosity of the scaffold. The highest porosity occurs in scaffold with 5% HA-TCP (80.17%), proving that the concentration of HA-TCP powder derived from cockle shell synthesis affects the porosity of the HA-TCP scaffold.

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