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The Effect of Sintering Process on the Characteristics of Hydroxyapatite from Cuttlefish Bone (*Sepia Sp*.)

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ABSTRACT

This study aimed to determine the effect of the sintering process on the characteristics of hydroxyapatite which was produced from cuttlefish bone (*sepia sp*.). Hydroxyapatite was obtained by a hydrothermal reaction between 1M $CaCO_3$ of cuttlefish bone lamellae and 0.6M $NH_4H_2PO_4$ at 200^oC in 12 hours. Followed by a sintering process with variations in the temperatures : 600°C, 800°C, and 900°C for 1 hour. Furthermore, the best results were taken from variation in time of 2 and 3 hours. XRD, SEM-EDX, and compressive strength test as well as MTT assay were performed to determine the characteristics of HA. The result of this study showed that an increase in crystallinity, crystal size, morphology, and compressive strength occurs by increasing the sintering temperature. In addition, an increase in temperature does not cause toxic effects on the HA as shown by cell viability which is more than 60%. Meanwhile, the increase in sintering time showed no significant changes to HA crystallinity, yet the mechanical properties of HA could still be improved. Based on the analysis, the optimal HA sintering temperature and time which are 900^oC and 1 hour respectively, yielded the highest crystallinity with the high maximum diffraction peak of 1163.02 and compressive strength of (11.79900 ± 0.00057) MPa which is suitable for the application of cancellous bone.

Keywords : Hydroxyapatite, Cuttlefish bone, Hydrothermal, Sintering

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INTRODUCTION

Bone is a powerful tissue which forms the framework of the human body. Bone has four main functions namely mechanical, protective, metabolic and hematopoietic function. Protective function acts as a protector of vital organs in the body and the bone marrow. Metabolic function acts as a backup and site of metabolism of many essential minerals such as calcium and phosphate. Hematopoietic function acts as a venue for the process of formation and development of blood cells [1].

Damages and bone disorders can affect the activities and functions of other organs. To overcome various damages that occurs in the bone, some treatment with therapy or an implant to replace or support the function of the actual bone was performed. There are several sources in medical biomaterials for implantation such as autograft, allograft and xenograft. Sources of biomaterials for bone implantation has a weakness that synthetic hydroxyapatite as an alternative materials to implant from nature must be developed [2].

Hydroxyapatite (HA) is the largest component (60%) of the total mineral phase of bone. HA has osteoconductive and bioactive properties that can support the process of bone remineralization [3]. Research on HA synthesis of natural products have been made such as HA synthetic of coral [4] and from bovine bone [5]. HA sources that have been mentioned above have weaknesses namely the imbalance of requirements and donors supply as well as the emergence of problems in terms of infection control. Therefore, the researchers tried to find the source of HA which was expected to be more available with characteristics that were not inferior to other sources, and one of which is by using cuttlebone.

Study on Hydroxyapatite synthetic with $CaCO_3$ source of cuttlebone has been done by Istifarah [6] using a hydrothermal method. The study yielded HA with quite well characteristics. However, the crystallinity of the resulting HA still needs to be increased, as in bone repair applications, HA with very good crystallinity to produce good mechanical properties is necessary. Crystallinity of a material depends on several factors such as the temperature and time of sintering.

Several studies have been conducted to determine the effect of the sintering process on the characteristics of HA. Muralithran and Ramesh [7] proved that sintering temperature affects the phase stability, densification properties, microstructure and hardness (hardness) of hydroxyapatite. Prokopiev and Sevostianov [8] found that changes in the mechanical properties of the sintered HA correlated with grain size, the bonds between the grains, the density of the specimen and pore shape. Monmaturapoj and Yatongchai [9] proved that the microstructure and mechanical properties increased with increasing sintering temperature, whereas the effect of sintering time is still very small.

Based on the preliminary studies a synthesis of HA from cuttlebone with variations in temperature and sintering time was conducted to determine the effect of these parameters on the characteristics of hydroxyapatite which include crystallinity, morphology, compressive

strength and toxicity of the material. sintering temperature and time were then optimized to obtain HA with the appropriate characteristics for its application in bone repair.

MATERIAL AND METHODS

Materials and Equipments

Materials used in the manufacture of the sample in this study was cuttlefish bone (*Sepia sp.),* ethanol, ammonium dihydrogen phosphate ($NH_4H_2PO_4$) and methanol.

The tools used for the manufacture of the sample in this study were High Energy Milling HEM-E3D, electric oven, magnetic stirrer, autoclave, pH meter and furnace.

Research Procedure

The study was carried out experimentally with the following stages.

Extraction of $CaCO_3$ from cuttlebone

Aragonite ($CaCO_3$) was obtained from the lamellae of cuttlefish bone powder which was made using HEM-E3D or mortar, then heated in a furnace at 350 ° C for 3 hours. To determine the content of $CaCO_3$, the results were characterized by XRD.

Preparation Materials

$CaCO_3$ solution (Mr = 100) 1M was obtained by adding 100 grams of $CaCO_3$ to 1 liter of distilled water. The solution of $NH_4H_2PO_4$ (Mr = 115) 0.6 M was prepared by dissolving 69 grams to 1 liter of distilled water.

Synthesis of Hydroxyapatite by Hydrothermal Method

In this synthesis process, a reaction that was expected to occur is as follows [10].

$$10\ CaCO_3 + 6\ NH_4H_2PO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 3\ (NH_4)_2CO_3 + 7\ H_2CO_3$$

Hydrothermal synthesis method was carried out by mixing 1M $CaCO_3$ and 0.6 M $NH_4H_2PO_4$ solution with a magnetic stirrer for 30 minutes. Solution mixture was transferred to the reactor and put in an Electric Oven to be heated until the temperature of 200^{o}C with the duration of 12 hours. The results obtained, were cooled at room temperature and then washed with distilled water using a magnetic stirrer. Washing performed repeatedly until the results of the reaction were separated from distilled water, was indicated by the return of the neutral pH (pH = 7). This assisted to eliminate the acidic byproducts. The last washing was performed with methanol to limit the agglomeration of HA during drying. Next, the sample was filtered with a filter paper and dried in an electric oven at a temperature of 50°C until dry. Hydroxyapatite

samples that have been formed is named sample 1, which further was characterized by XRD to ensure the formation of HA.

Stages of Sintering

Hydroxyapatite resulting from previous stages was further sintered using a furnace. Products of sintering conducted with variations in the temperature of 600°C, 800°C, and 900°C for 1hour, are respectively named sample 2, 3, and 4. Next, the best sintering temperature based on the characterization chosen as the sintering temperature for the second sintering with time variation of 2 and 3 hours, are respectively named sample 5 and 6. Sintering results of time variation were then characterized.

Characterization

The entire sample was characterized by the crystal structure and crystalinity with XRD, morphology and the ratio Ca / P with SEM-EDX, compressive strength and cytotoxicity by MTT assay using fibroblast cell cultures.

RESULTS AND DISCUSSION

Test Results of XRD

The XRD test results of the cuttlebone lamellae powder that have been heat-treated at 350 ° C for 3 h showed 100% content of calcium carbonate (aragonite, $CaCO_3$) (Figure 1).XRD spectrum of the sample showed suitability with ICDD 01-71-4891. This is in line with the research results of Paljar et al.[11] which indicates that the heat treatment on the cuttlefish bone lamellae does not change the content of aragonite into calcite, not as part of dorsal.Aragonit is more easily transformed into HA than calcite. Furthermore, in this study aragonite from the cuttlefish bone lamellae to synthesize HA was used.

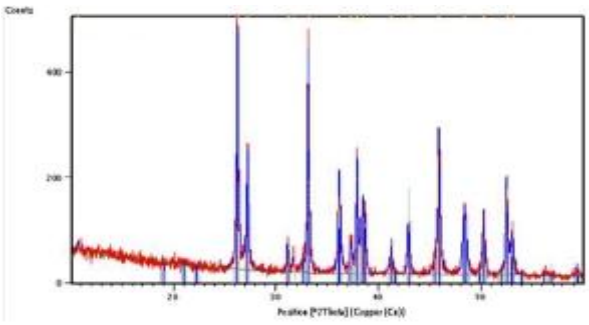

Figure 1: XRD spectrum of cuttlefish bone lamella powders

Hydroxyapatite was characterized and analyzed by XRD reference ICDD 01-074-0565. The analysis showed that the content of the tested samples was 100% hydroxyapatite (Ca_{10}

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$(PO_4)_6$ $(OH)_2$). The entire XRD spectrum formed on the HA samples correspond to ICDD reference.

The content of Hydroxyapatite with Sintering Temperature Variations

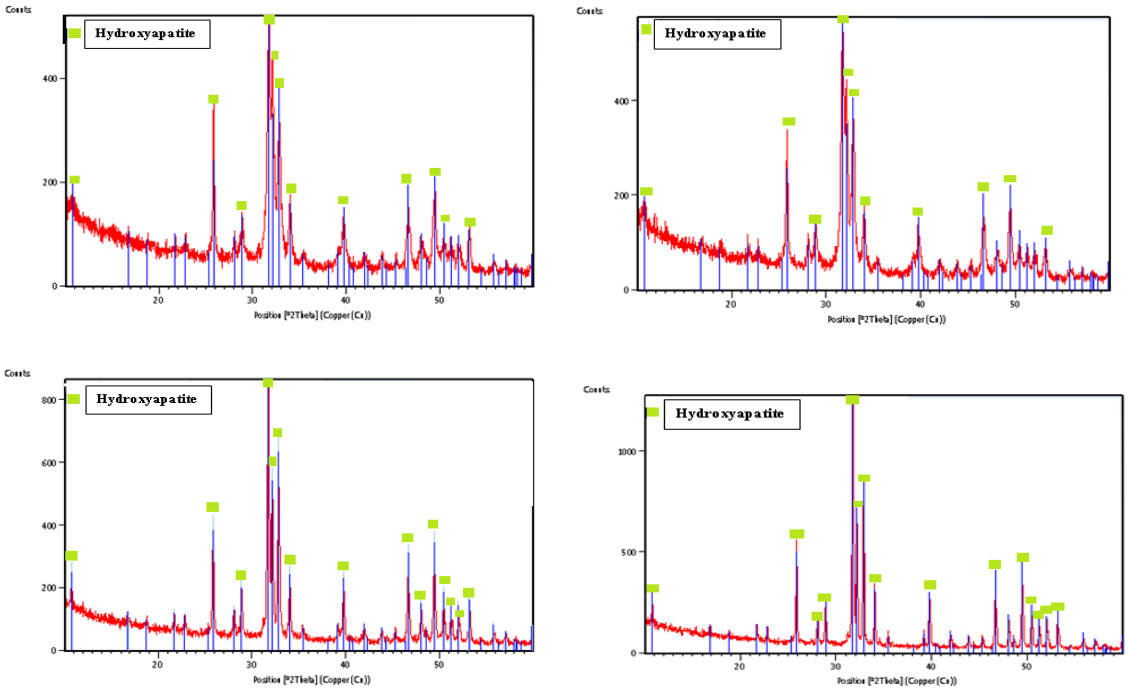

Figure 2.XRD spectrum of Hydroxyapatite with Sintering Temperature Variation
(a) Without sinter, (b) 600°C, (c) 800°C, (d) 900°C

Based on the test results of XRD (Fig. 2) shows that the HA samples sintered at a temperature of 900°C (Figure 2d) has the highest intensity of the diffraction peak, which is 1163.02. Therefore, the sample can be said to have a higher crystallinity compared to the HA samples before sintering and sintered at a temperature of 600 °C and 800 °C.Crystallinity of a material was figured out by looking at the parameters peak height and FWHM bandwidth, but bcause HA samples produced a single crystal phase and no other phases formed, the height of peaks can represent the HA crystallinity.

The content of Hydroxyapatite with Duration Sintering Variation

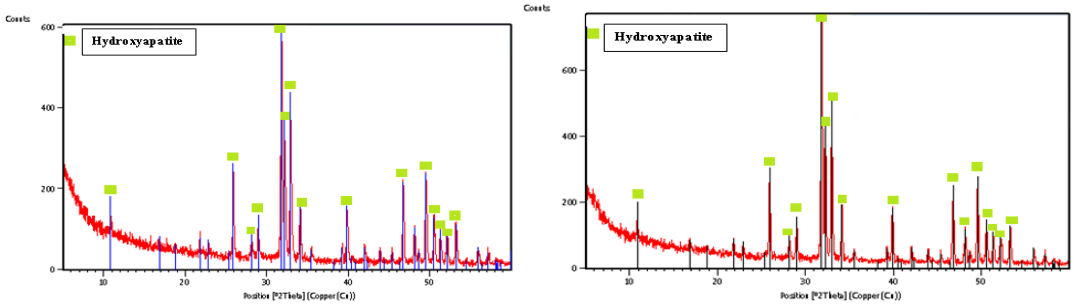

Figure 3. XRD spectrum of Hydroxyapatite at 900°C with durationVariation: (a) 2 hours, (b) 3 hours

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Figure 3 above shows that crystallinity decreased when viewed from maximum diffraction peak height. The HA samples sintered at 900°C for 3 hours (Figure 3b) has a higher crystallinity compared with the HA samples sintered at 900°C for 2 hour (Figure 3a). Based on the XRD data obtained, it can also note the sample size of HA crystals. To determine the size of the crystal, Scherrer equation was used.

$$t = \frac{k\lambda}{BCos\theta} = \frac{0.9\lambda}{BCos\theta} \qquad (1)$$

Where t is the crystal size (Å), λ is the wavelength of x-ray diffraction (equivalent to 1,54 Å), B is the width of the diffraction peak FWHM (radians), and (θ) is the diffraction angle. According to the results of this study, the crystal size in the sample is as follows.

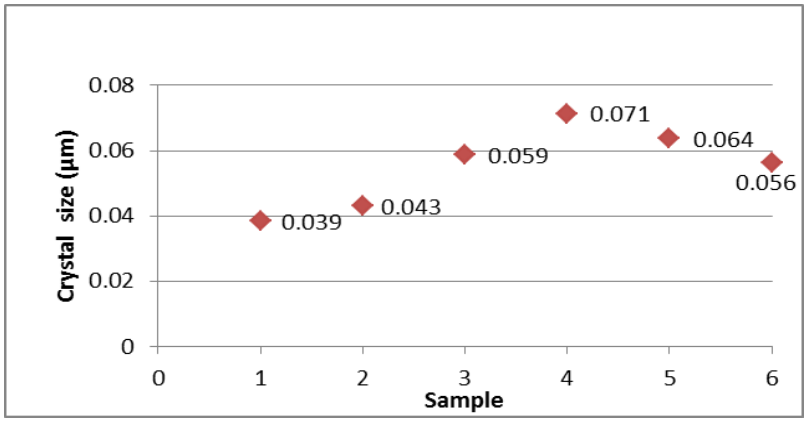

Figure 4. Comparison of crystal size with Sinter Temperature and duration variation

Figure 4 shows an increase in crystal size due to an increase in sintering temperature. This is in contrast with the measured crystal size of the sample with sintering time variations. On the graph, the crystal size decreased while the sintering was increased.

In this study, a refinement using the program Powder Cell of Window (PCW) was also carried out. One of the information obtained from this refinement is the crystal lattice parameters, as shown in table 1.

Table 1: Lattice parameters of HA Refinement Results

Parameter	ICSD	Sample					
		1	2	3	4	5	6
Rp	-	10,12	13,75	13,66	14,58	15,65	15,89
Rwp	-	15,25	15,67	19,46	18,76	20,83	21,32
Rexp	-	2,170	2,13	2,08	2.04	2,02	1.83
a = b	9,424	9,4167	9,4170	9,4185	9,4195	9,4193	9.4187
C	6,879	6,897	6.877	6,8807	6,8801	6,8819	6.8809

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The data presented in the table shows that there is a slight increase and decrease in the lattice parameters a and c. However, the overall sample 4 (HA sintered at a temperature of 900^{o}C for 1 hour) has a lattice parameter value closer to the value of the lattice parameters of HA reference (a = 9.424 Å and c = 6.879 Å).

SEM EDX Test

SEM characterization on samples with variations in temperature and length of time of each sinter using 500x magnification, 10000x, and 20.000x is shown in Figure 5.

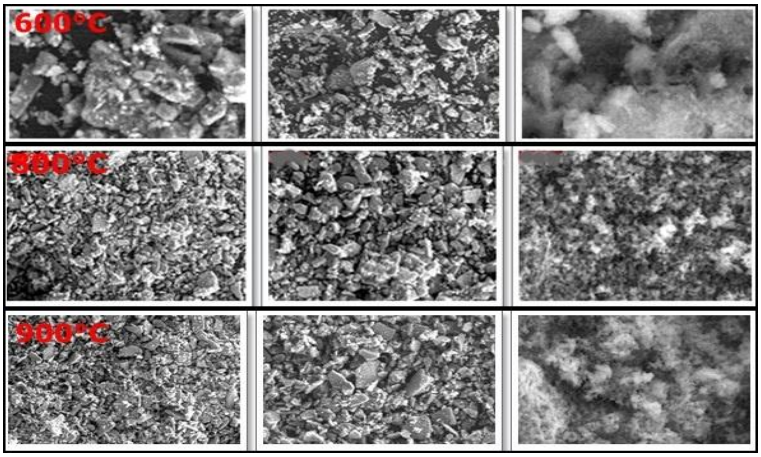

Figure 5.Morphology of hydroxyapatite with sintering temperature variations.

The SEM picture above showed no differences in morphological structure. However, there has been little changes in the form of granules.

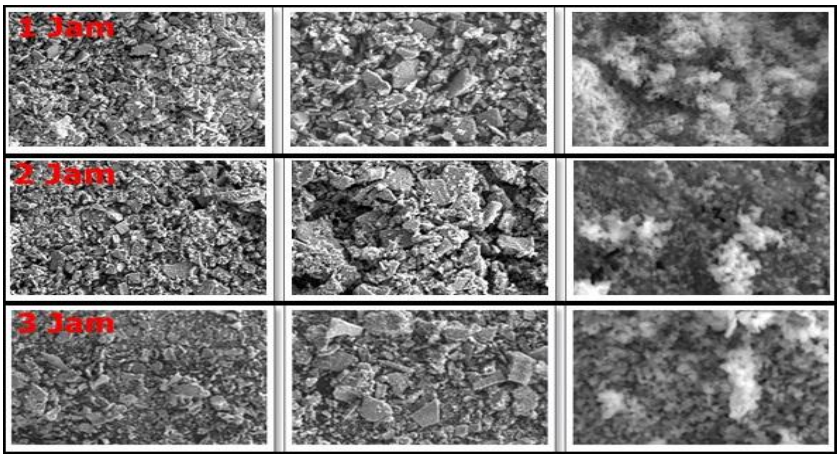

Figure 6. Morphology of hydroxyapatite with sintering duration variation.

Pore diameter size of the sample was measured using a line scale in the SEM images. The pore diameter is presented in Table 2.

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Table 2: The pore Diameter Size

Sample	Pore Diameter Size (nm)
2	152,1-418,7
3	102,0-123,6
4	70,46-102,0
5	65,16-88,63
6	32,58-52,30

Sintering temperature increases led to a more solid material and pore shrinkage. The longer time given in the sintering process, the smaller the size of the pores became.

Pore size of a material is closely related to the material strength. The larger pore size showed reduced density of the material, meaning that the material is not able to withstand the load given to it. In other words, a material which has a large pore size tends to be brittle.

Pore size of HA samples (Table 2) shows a decrease along with the increase sintering temperature and time. This occured because during the sintering process, bonding between grains occurred and caused the pore size to become smaller.

Compressive Strength Test

Compressive strength test was carried out using autograph. The magnitudes obtained were then calculated using the Equation:

$$\sigma = \frac{F}{A} \qquad (2)$$

F is the maximum force that can be accepted by the samples (kN), A is the sample surface area (mm^2) and σ is compressive strength (kN/mm^2 or MPa).

Compressive strength of HA sample with variations in temperature and time sintering is presented in the graph in Figure 7.

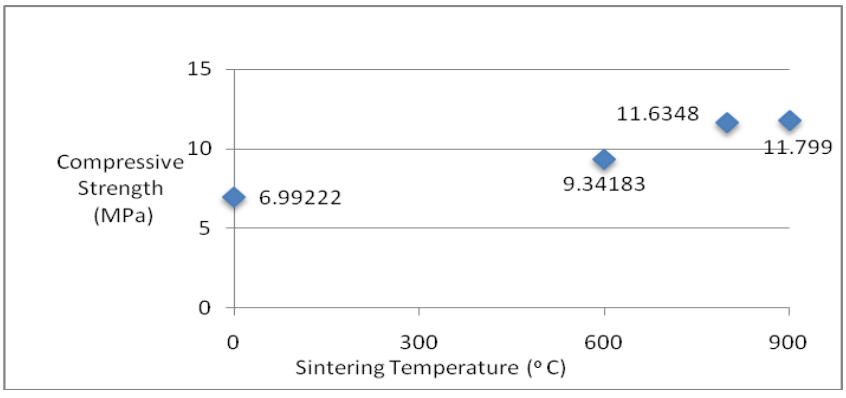

Figure 7. Graph of Hydroxyapatite with Sintering Temperature Variation

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Compressive strength of HA increased as the sintering temperature also increased as a result of the events on the grain growth and densification in Intermediate Stage. At this stage, grain growth occured and pore structure became smoother. Grain boundaries and pore geometry that occured at this stage depended on the rate of sintering process. Meanwhile, compaction (densification) that occured at this stage was followed by volume diffusion and grain boundary diffusion.The higher the sintering temperature was,the higher the density of the sample became. It made the sample able to withstand the load and pressure given [12]

The highest compressive strength is owned by the sample that was heated for 3 hours, which equals to (11.92357 ± 0.00057) MPa. An increase in the compressive strength was caused by a strong bond between the grains during the sintering process. This event could possibly occur due to a material movement mechanism between grain (diffusion process) and the source of energy to activate the movement. Longer time given in the sintering process, caused more particles to bond so that the material became stronger. Meanwhile, long sintering time affects the mechanical properties of the sample where the value of compressive strength samples increased by increasing the sintering time.

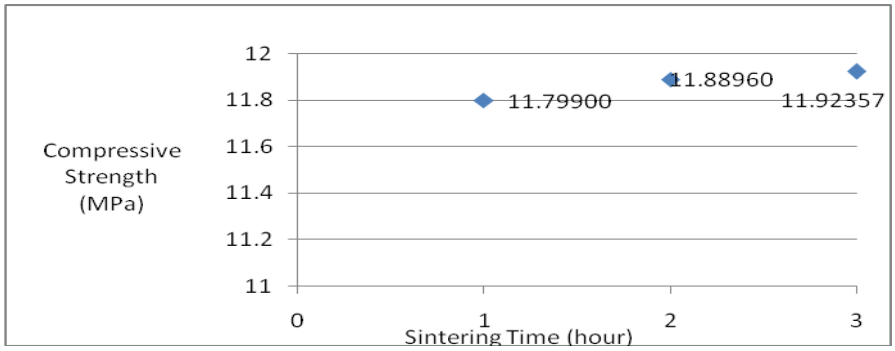

Figure 8: Graph of Hydroxyapatite with Sintering Time Variation

Cytotoxicity test by MTT Assay

MTT assay test results were read by Elisa reader absorbance value (OD). Cell viability was calculated with the equation of in vitro technologies. The cell viability of each sample obtained is presented in Figure 9.

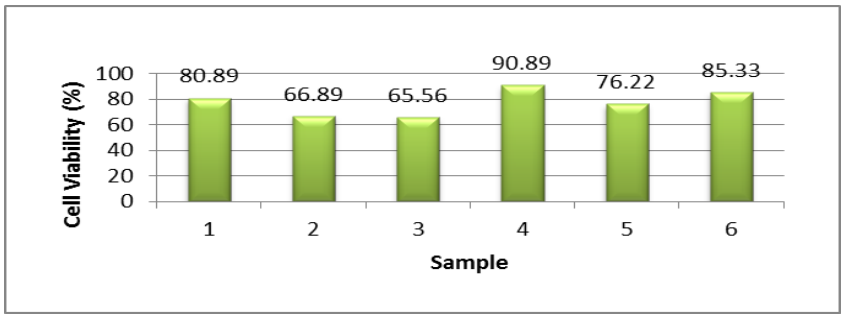

Figure 9. Cell Viability of Sample with Sintering Temperature and Time Variation

Hydroxyapatite that was synthesized from cuttlefish bones was not toxic.This is indicated by the value of live cell viability of which was above 60%.

DISCUSSION

This research has been conducted on the synthesis of cuttlefish bone (*Sepia sp*.) Hydrothermally.Then, sintered HA was obtained by variation of sintering temperature and time.Variations in temperature and time of sintering aimed to produce HA with optimal characteristics in medical applications.

The characterizations, including XRD, SEM-EDX, compressive strength, and MTT Assay test, obtained good results. XRD test showed an increase in crystallinity with increasing temperature. Sample 4, HA sintered at temperature of 900^{o}C has a crystallinity with the highest diffraction peak, i.e the intensity of 1163.02. This diffraction peak height increase led to an increase in the size of the crystal. Crystal size was calculated using Scherre requation according to the XRD analysis data. The obtained results prove that when the sintering temperature increases, the grains in a material will bond together and form a larger grain structure. Meanwhile, the arising effect from the increase in sintering time could not be as certained due to fluctuating changes in the results.

Morphological structure of the sample showed a less significant change in both the sintering temperature and time variations. The HA samples synthesized was not homogeneous and has irregular grain and pore size. In addition, a decrease in pore size occurred after increasing the temperature and the sintering time. Overall, the pore size of the six samples is less than 10 microns. This proves the occurrence of densification, where the grains bind strongly and form a very solid structure, thereby reduce the pore size of the material.

The test results were then analyzed with SEM EDX to determine the composition of HA. Results were taken from one point so it could not be used as a benchmark of the ratio of Ca / P. Overall, there is an exception when it is a homogeneous sample. However, the EDX analysis showed that all samples have a ratio of Ca / P that were not exactly 1.67, but still close to that value. The composition of Ca / P is less appropriate because the starting material used as a source of CaO was cuttlebone that still contained $CaCO_3$, so as after the reaction between CaO and (NH_4) H_2PO_4, there were $CaCO_3$ which did not participate in reaction and affect the amount of Ca in the sample.

Compressive strength test results showed that increasing sintering temperature and time plays a role in increasing sintering temperature and that times play a role in increasing the compressive strength of material. The compressive strength of samples obtained in the range among the compressive strength forcancellous bone applications, and the highest is samples 6 (HA sintered at a temperature of 900^{o}C for 3 hours) equals to (11.92357±0.00057) MPa. Compressive strength is associated with a decrease in pore size due to densification events. This is supported by the SEM test results showing a decrease in pore size with increases in

temperature and sintering time. The higher the density of a material, the higher the material's ability to withstand a given load will be. Thus, the compressive strength value became higher.

Based on the results of MTT assay test, the highest cell viability was obtained on sample 4 with the percentage of 90.89%. Meanwhile, the lowest cell viability was seen in sample 3 with the percentage of 65.56%. The test results do not directly specify that a substance is toxic. To detect the toxic effects of the test material (e.g. the rate of growth, proliferation and differentiation of cells in the material) can depend on the contact and the spread of the cells on the surface of the material. If the cells do not interact with the material perfectly then there will be no cell growth, as well as for HA. Hydroxyapatite is effective to stimulate the growth of bone cells in case of interaction with cells.

CONCLUSION

1. Sintering temperature variation affects the crystallinity, crystal size, lattice parameter and morphology of hydroxyapatite. Crystallinity and crystal size increases with an increase of the sintering temperature. However, in this study the influence of sintering temperature on morphological changes in the structure of the HA of cuttlebone can only be seen from the size of the pore, where the pore size decreased with an increase of the sintering temperature. Meanwhile, long sintering time has an influence that is not linear to the lattice parameters, the crystallinity and crystal size of HA.
2. Sintering temperature and time affect the compressive strength of hydroxyapatite from cuttlebone. Higher temperature and longer sintering time, will decrease the pore size of the sample, so that compressive strength of the sample increases.
3. This study did not find the effect of sintering temperature and time on cytotoxicity. However, it is known that HA from the cuttlebone is not toxic because the cell viability is more than 60%.
4. Sintering temperature and time of HA are optimal at temperatures of 900^{o}C for 1 hour. This is evidenced by the high crystallinity with the height of maximum diffraction peak of 1163.02 and compressive strength which is suitable for the application on cancellous bone equals to (11.79900±0.00057) MPa.

Suggestion

To determine the effect of sintering time to the characteristics of hydroxyapatite more significantly, it is necessary to vary the time with a longer interval.

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