



# MORPHOLOGICAL AND MECHANICAL STUDY OF GELATIN/HYDROXYAPATITE COMPOSITE BASED SCAFFOLDS FOR BONE TISSUE REGENERATION

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## ABSTRACT

Gelatin/hydroxyapatite (GHA) composite has been synthesized as a scaffold in bone tissue engineering. The purpose of this study was to find the optimal composition of the GHA scaffold composite which has the best mechanical properties. The independent variable in this study was the composition of HAp. Hydroxyapatite was synthesized by precipitation method from  $\text{Ca}(\text{OH})_2$  and  $(\text{NH}_4)_2\text{HPO}_4$  as raw materials. Scaffold from GHA Composite was made by freeze-drying technique with freezing time for 8 hours at  $-80^\circ\text{C}$  and drying with lyophilizer. The results were characterized using XRD, SEM and tested for compressive strength. The results of the XRD showed that there was no change in a compound or the formation of new bonds on the GHA scaffold when it became a composite which was indicated by the absence of new peaks. It is also known that the peaks decrease in intensity as the amount of polymer in the composite increases. The highest degree of crystallinity was found in the 1:3 GHA sample because it had the highest concentration of HAp. The results of observations with a SEM showed that the most homogeneous pore surface morphology was GHA 1:2 with an average pore size of  $225 \pm 17 \mu\text{m}$ . From the results of the compressive strength test, the best value for the 1:2 GHA scaffold was  $18.1 \pm 0.6 \text{ MPa}$ . The pore size and compressive strength values obtained meet the minimum scaffold requirements needed so that it can be used as a scaffold candidate in bone tissue engineering.

Keywords: composite scaffolds; compressive strength; gelatin; hydroxyapatite

## INTRODUCTION

Natural bone is composed of inorganic salts and collagen. Since calcium and phosphate are the main components of inorganic salts, calcium phosphate-based ceramics seem to be the perfect material for scaffolding bone tissue<sup>[1]</sup>. Calcium phosphate ceramics are widely used in the construction and repair of hard tissues because of their biological and physical and chemical similarities with human bones and teeth<sup>[1,2]</sup>. Various phases of calcium phosphate including hydroxyapatite (HAp) and tricalcium phosphate (TCP) have good osteoconductivity and osteoinductivity properties<sup>[3]</sup>.

Due to its superior biological activity, biocompatibility, osteoconductivity, osseointegration, and non-toxicity, HAp is preferred as a biomaterial in bone repair, bone implants, and bone drug delivery systems<sup>[4,9]</sup>. Hydroxyapatite (HAp) is composed of calcium, phosphorous, oxygen, and hydrogen atoms with the chemical formula  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , having a hexagonal unit cell and  $\text{P6}_3/\text{m}$  space group and a stoichiometric molar ratio Ca/P of 1.67<sup>[5,6]</sup>. HAp naturally hardens bones and makes them resistant to stress, but pure HAp is limited by its

fragility and stiffness. To improve the biological activity and mechanical properties, several bioactive polymers and ceramic composite materials for bone tissue engineering have been developed. Among these composite materials, the HAp/polymer composite material has osteoconductivity due to the presence of HAp. Therefore, HAp/polymer scaffold composites are very attractive for biomedical applications [7,10].

The collagen/HAp scaffold has a microstructure similar to that of real bone and exhibits good osteoclast absorption and osteoconductivity, but the high cost of collagen may limit its clinical application in healing bone defects [8]. Therefore gelatin emerged as a candidate for polymer composites with HAp. Gelatin is a water-soluble and biocompatible material in the body [9]. Gelatin is a mixture of peptides and proteins produced by the hydrolysis of collagen through the extraction process from the skin, bone, connective tissue, and animal organs [10,11]. In particular, gelatin has been used as a composite matrix because its composition is very similar, but its cost is lower than that of collagen, and hydroxyapatite (HAp) was chosen as a favorable filler for reinforcing composite materials [12]. The report shows that increasing the content of HAp nanoparticles in gelatin/HAp composites will increase cell attachment, proliferation, and increase alkaline phosphatase levels, and osteogenic differentiation gene expression [13].

The good performance and reliability of biomedical implants are not only determined by the choice of material, but also by the synthesis technique used in biomedical implant applications. Several studies describe various methods for HAp syntheses, such as the hydrothermal method, sol-gel method, microemulsion method, precipitation method, solid-state method, and microwave method [14]. The precipitation process is a wet chemical method that involves a chemical reaction between two or more solutions to produce a metal hydroxide precipitate. This wet chemical process has several advantages, including (a) low energy consumption due to low process temperature; (b) high yield purity, and (b) flexibility to apply other post-sol-gel and precipitation processes; and (d) equipment that is relatively much cheaper than other techniques [15].

From the explanation above, it is known that HAp is one of the biomaterials that have osteoconductive characteristics, excellent biocompatibility, and is also bioactive. On the other hand, the polymer used to be composited with HAp must also have good properties. Researchers want to try to composite gelatin polymers to be used as scaffold-forming matrices which will later be composited with hydroxyapatite. This scaffold will later be made using the freeze-drying technique, which is a freeze-drying ethic that can form an interconnected porous structure [16]. It is hoped that this study will provide better results in terms of its mechanical properties and potential in bone tissue engineering.

## METHOD

### Synthesis of HAp

HAp was synthesized from  $(\text{NH}_4)_2\text{HPO}_4$  (Dwilab Mandiri, Bandung) and  $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$  by precipitation method.  $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$  was obtained by mixing  $\text{Ca}(\text{OH})_2$  and  $\text{HNO}_3$  (Duta Jaya, Malang).  $\text{Ca}(\text{OH})_2$  is made by mixing  $\text{CaO}$  (which is obtained from  $\text{CaCO}_3$  (Merck, Germany) which is calcined at a temperature of  $900^\circ\text{C}$ , and distilled water. Briefly, a 0.6 M  $(\text{NH}_4)_2\text{HPO}_4$  solution was prepared by dissolving  $(\text{NH}_4)_2\text{HPO}_4$  in 83 mL of distilled water, while a 1 M  $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$  solution was prepared by dissolving  $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$  in 83 mL aquadest. For the

synthesis of HAp, a solution of  $(\text{NH}_4)_2\text{HPO}_4$  was added dropwise to a solution of  $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$  and continuously stirred with a magnetic stirrer at a speed of 700 rpm for 2 hours and at a temperature of  $40^\circ\text{C}$ , then the pH was adjusted to 11 using  $\text{NH}_4\text{OH}$  (Duta Jaya, Malang) then the solution was allowed to stand at room temperature for 24 hours to settle. The resulting precipitate was then washed with distilled water 3 times and annealed for 3 hours at a temperature of  $100^\circ\text{C}$ .

### Synthesis of scaffold gelatin/HAp composite

To prepare the composite Gelatin/HAp scaffold, 300 mg of powdered gelatin (Merck, Germany) was dissolved in 10 mL at  $50^\circ\text{C}$  for 30 minutes to obtain a gelatin solution. 250 mg of gelatin was added to the gelatin solution and continuously stirred with a magnetic stirrer for 30 minutes to obtain a Gelatin/HAp mixture. The resulting mixture is then put into a molded tube and placed in the freezer (Sharp SJ-N182) overnight. Furthermore, this sample was subjected to a freeze-drying process (freeze-drying machine type MN12A) for 8 hours at a temperature of  $-80^\circ\text{C}$ . The sample resulting from this process was then annealed for 15 minutes at a temperature of  $100^\circ\text{C}$  until the sample was completely dry to prevent spoilage.

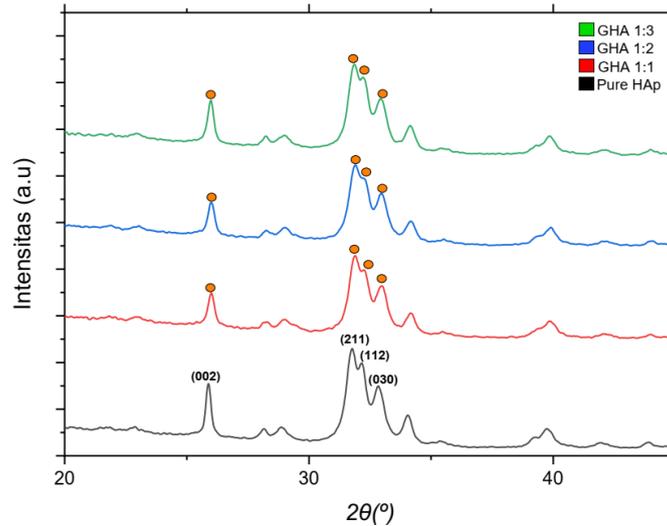
### Characterizations

The morphology, size, porosity, and chemical constituents of the samples were observed with an SEM (Quanta FEG 650). Pore size analysis using the ImageJ 1.53k software. The phase and crystal structures of the samples were identified by XRD (Panalytical Expert 3 Powder) with  $\text{Cu K}\alpha$  radiation ( $1.5406\text{ \AA}$ ) in the angular region of  $2\theta$  to be  $10 - 90^\circ$  at the voltage of 40 kV and current of 30 mA. Analysis of the phase and crystal structure formed using HighScore Plus software version 3.0.5. Phase identification was achieved by the PDF-2 database. The compressive strength of the porous scaffold was measured using a tensile strength machine (Imada Z2 Digital Force Gauge 50N).

## RESULTS AND DISCUSSION

Characterization using XRD on samples of hydroxyapatite and composite Gelatin/HAp scaffold is presented in Figure 1. From the diffractogram it can be seen that all samples formed strong characteristic peaks of HAp at  $2\theta$  of  $25.88^\circ, 31.72^\circ, 32.19^\circ,$  and  $32.84^\circ$  with hkl (002) (121), (030), and (112) respectively without any foreign peaks (reference code: ICSD-98-018-1173) with  $\alpha=\beta=90^\circ; \gamma=120^\circ$  and the value of  $a=b= 9,441$  ;  $c = 6.875$  , and the value of the Ca/P ratio is 1.69. From the results of the analysis using the HighScore Plus software, it was also identified that all samples had the same crystal system, namely hexagonal. The addition of gelatin concentration did not shift the angle of  $2\theta$  too much. This indicates that the concentration of gelatin does not affect the HAp angle. However, the addition of gelatin to the sample caused a change in the peak intensity of the HAp sample. This is evidenced by the value of the degree of crystallinity of each sample, as can be seen in table 1. The value of the degree of crystallinity of the XRD of gelatin/HAp composite samples was identified with the help of Origin software and using the equation:

$$\text{Crystallinity} = \frac{\text{area of crystalline peak}}{\text{Area of all peak (crystalline+amorphous)}} \times 100\% \quad (1)$$



**Figure 1.** XRD measurement results of the Gelatin/HAp scaffold composite with variations in the composition of HAp

From table 1, it can be observed that pure HAp has the highest degree of crystallinity with 80.82%, followed by GHA 1:3 with 76.27%, GHA 1:2 with 74.85%, and GHA 1:1 with the lowest value with 68.38%. It can be seen from Table 1 that the crystallinity value of HAp obtained from formula (1) decreased with the addition of gelatin. As the concentration of HAp decreased, the crystallinity value also continued to decrease. This happens because the formation of HAp is not carried out in the gelatin matrix, and gelatin is an amorphous polymer so that by decreasing the concentration of HAp and keeping the concentration of gelatin fixed will cause the gelatin to diffuse more and cause the amorphous nature of the sample.

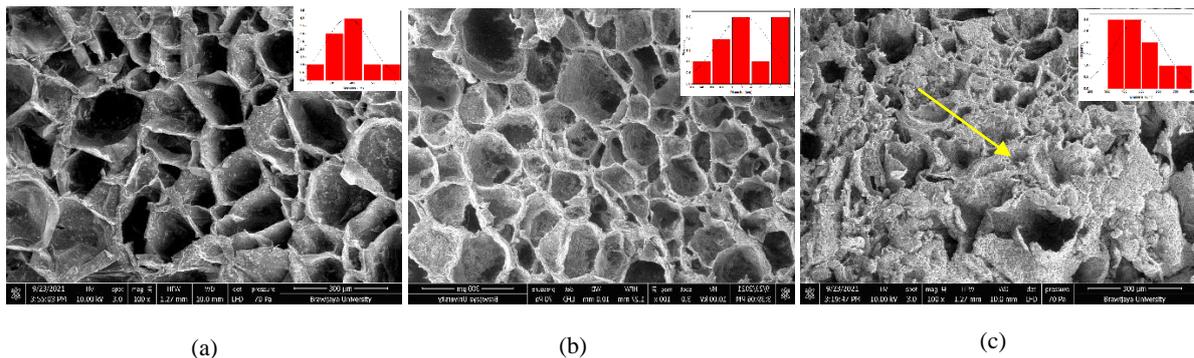
**Table 1.** Degree of Crystallinity

Sample	Degree of Crystallinity (%)
Pure HAp	80,82
GHA 1:3	76,27
GHA 1: 2	74,85
GHA 1:1	68,38

The results of the compressive strength test and pore size distribution on the gelatin/HAp scaffolds composite can be seen in Figures 2 and 3. The highest compressive strength value is  $18.1 \pm 0.6$  MPa in the 1:2 GHA sample. The pores of 1:1 GHA have a pore size range of 169.6 – 296.9  $\mu\text{m}$ . Meanwhile, the pore size in the range of 261.5 – 458.8  $\mu\text{m}$  is owned by GHA 1:2 and the pore size in the range of 305.2 – 747.9  $\mu\text{m}$  is owned by GHA 1:3. From this data, it can be seen that GHA 1:2 has the smallest pore size and GHA 1:3 is the largest. Pore size was measured based on the pore diameter of the scanning electron microscope (SEM) with the help of ImageJ software. Pore size measurement is done by selecting the largest porosity to represent the pores of the scaffold.

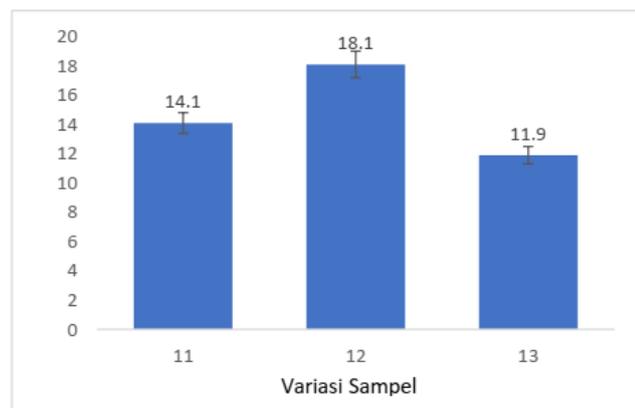
The scaffold with the highest concentration of HAp formed a few pores of a very large size and was less homogeneous, even forming a precipitate of HAp particles which can be seen in Figure 2 (c) (shown by arrows). This large pore is more appropriate if it is called a gap than a pore. This may be because the addition of too many HAp particles can cause defects in the material

causing particle deposition in the matrix, and the addition of excess particles when it reaches the saturation point of the matrix will cause matrix discontinuity. The same thing happened to the scaffold with the lowest concentration of HAp as shown in Figure 2(a). The gelatin matrix on this scaffold is still clearly visible and the pore size is less homogeneous. This happened because the gelatin matrix did not completely bind to the HAp due to the lack of HAp. It is seen that the gelatin matrix looks a little ruffled, uneven and irregular. Another reason for the lack of homogeneity of the pore size at 1:1 GHA is the effect of its viscosity. The scaffold made from a solution with low viscosity (GHA 1:1) contains more solvent, so that when the freeze-drying process is carried out, sublimation occurs, leaving large pores. So it is necessary to have an optimal composition of the gelatin matrix and HAp to form a good and homogeneous pore.



**Figure 2.** Morphology and pore distribution of an SEM with a magnification of 100x (a) GHA 1:1, (b) GHA 1:2 and (c) GHA 1:3.

The size of the appropriate porosity is an important point in the manufacture of scaffolds. The size of the porosity is related to cell adhesion and migration as well as the diffusion of nutrients and the removal of metabolic wastes. In this study, all samples had a pore size of more than 100  $\mu\text{m}$ , and the best average porosity was obtained by the GHA 1:2 scaffolds composite with size in the range of 305.2 – 747.9  $\mu\text{m}$ . This corresponds to the effective pore size for bone growth, which is 100-600  $\mu\text{m}$ . The size of the porosity that is too small will cause limited cell migration and will interfere with the diffusion of nutrients and metabolic waste. When this happens, it will cause scaffold necrosis. While the size of the porosity of the scaffolds is too large, it will cause the cells to be easily separated from the scaffolds.



**Figure 3.** Bar chart of compressive strength

In the results of the compressive strength value shown in the form of a graphic diagram above, it can be seen that the GHA 1:3 scaffold has the lowest compressive strength value. This is because the concentration of HAp is too high and HAp is a fragile ceramic material, so the results of the compressive strength measurement show that the scaffold with the highest HAp concentration has the lowest compressive strength value. This also happened to the GHA 1:1 scaffold. Even though there was an improvement in the compressive strength value, there was too much gelatin polymer in the scaffold causing the scaffold to be plastic because of the brittle nature of gelatin (when dry) and in the form of a gel when wet. So it can be concluded that too much concentration of HAp or gelatin is not good for making scaffolds, it takes a certain concentration of HAp and gelatin to obtain a scaffold with good mechanical properties. This is as shown by the GHA 1:2 scaffold. This scaffold has the highest compressive strength value of 18.1 MPa, this value has met the candidate as a cancellous bone scaffold which is required to have a mechanical strength of 17.7-27 MPa.

## CONCLUSION

The scaffold is a major component in tissue engineering that serves as a template for cell interactions and the formation of the extracellular matrix of bone that provides structural support for new tissue formation. Material can be said to be a candidate as a cancellous bone scaffold if it has an effective pore size for bone growth of 100-600  $\mu\text{m}$  and has a mechanical strength of 17.7-27 MPa so that it can be temporary support during the process of new tissue growth. In this study, a Gelatin/hydroxyapatite (GHA) composite scaffold has been obtained which has been perfectly formed without any foreign material following the XRD data results. The scaffold with the best properties is GHA 1:2 with a pore size of  $225.2 \pm 1 \mu\text{m}$  and a compressive strength value of  $18.1 \pm 0.61 \text{ MPa}$ , where these values are sufficient to make it a candidate scaffold for bone tissue engineering. An optimal composition of gelatin and hydroxyapatite is required to form a good and homogeneous pore.

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