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Study on Antibacterial of Chitosan/Hydroxyapatite Doped Magnesium Composite as a Material for Bone Graft Applications

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Abstract

Hydroxyapatite (HAp) is one of the constituent minerals of bone and teeth, that has been widely used for synthesizing bone graft. Due to the limitation on properties of the hydroxyapatite, it is doping with Magnesium (HAp-Mg). The addition of Chitosan (Chi) was expected to improve the antibacterial properties of HAp-Mg. The present research aims to study the influence of Chitosan with 0, 5, 15, and 25 wt% addition on biocompatibility properties of Chi/HAp-Mg composite. HAp-Mg was synthesized using the sol-gel method; meanwhile, Chi/HAp-Mg composite was manufactured by mixing Chitosan in acetic acid, and HAp-Mg was added into the mixture. The synthesized samples were characterized using XRD and SEM. In vitro antibacterial activities of the Chi/HAp-Mg composite were evaluated against Escherichia coli bacteria. Biocompatibility analysis from antibacterial activity showed that composite with the optimal composition on the addition of 15 wt% Chitosan has the best ability in inhibiting the growth of E. coli bacteria.

Keywords: HAp-Mg, chitosan, composite, antibacterial, biocompatibility

1. Introduction

Nowadays, the using of bone graft continuously increased recently. This is due to increased accidents that cause fractures and congenital disorders and non-congenital disorders. Besides, bone fracture can also cause osteoporosis. According to the European Journal of Rheumatology, one in three women over the age of 50 years and one in five men over 50 years suffering osteoporosis fractures in their lifetime [1]. Disruption of the health and function of these organs can lead to a decline in the quality of human life. Appropriate treatment of bone fractures is essential because bone provides a body frame to support the body, so the proper use of the material is a factor of bone implantation and their success.
Hydroxyapatite (HAp, Ca_{10}(PO_4)_{6}OH_2) is a significant component of human bone and teeth. It is utilized in bone reconstruction and dental prosthesis applications due to bioactive and osteoconductive properties in the process of bone mineralization. Because of its superiority, HAp is considered a good substitute for bone graft application [2]. The most important application of bioactive ceramics such as hydroxyapatite has been the coating of orthopaedic metal implants, at locations where a strong interface with bone is required [3]. However, HAp synthetic has several disadvantages; it is not osteoinductive in the body and has brittle properties [4]. The development of HAp with various additives has been one of the solutions in the field of biomaterials to overcome its weakness. In this study, HAp is doped with magnesium.

Magnesium (Mg) is one minor constituent element in bone and teeth due to its ability to trigger cell proliferation in bone and to improve the mechanical properties of bone. Enamel, dentin, and bone contain 0.44, 1.23, and 0.72 wt.% of Mg, respectively [5]. However, considering its application in living bodies, the biocompatibility of HAp doped Mg must be attended in concerning the antimicrobial properties. This is for the prevention of infection caused by microorganisms that appear in the human body. The most common bacteria is E. coli. One of the treatments for improving biocompatibility properties of bone graft is the addition of chitosan.

Chitosan is one of the most abundant natural polymeric materials. The biocompatibility of chitosan has been studied and can enhance biological properties and can be degraded and absorbed in the body and has an excellent antibacterial activity for implantation in HAp-Mg [6]. However, there is a lack research about the effect of the variation of chitosan in wt.% to combine with HAp-Mg and become a composite for bone graft application.

This research aimed to study the effect of the presence of chitosan with consideration on the biological properties of HAp-Mg, presenting potentially interesting features for medical applications such as a bone graft. Each composite was determined by X-ray diffraction (XRD) and the morphology of composite through Scanning Electron Microscopy (SEM). Biocompatibility testing performed by an antibacterial test against E.coli bacteria with a diffusion disk method.

2. Methods

Calcium oxide (CaO; SAP Chemicals), diammonium hydrogen phosphate (NH_4)_2HPO_4; SAP Chemicals), magnesium chloride (MgCl_2; SAP Chemicals), ammonium hydroxide (NH_4OH; SAP Chemicals), nitric acid (HNO_3; SAP Chemicals), and acetic acid (CH_3COOH; SAP Chemicals) were supplied by PT. Sumber Ilmiah Persada Surabaya, East Java, Indonesia. Chitosan from shrimp shell with a degree of deacetylation 95.2% obtained from CV. Biochitosan Indramayu, West Java, Indonesia.

In this study, HAp-Mg prepared by a sol-gel method. The following stages of synthesis by the sol-gel method: (1) 4.2 grams of calcium oxide (CaO) was dissolved in 75 mL of 2M HNO_3 in a beaker glass. (2) The mixture of a solution was stirred using a magnetic stirrer for 15 minutes at a temperature of 60 °C. (3) The results of the mixture then were filtered by using Whatman filter paper. The filtered was produce as a filtrate (Ca(NO_3)_2). (4) Filtrate (Ca(NO_3)_2) was added dropwise (NH_4)2HPO_4 0.18 M as much as 250 mL using a separating funnel. The result of this mixing was formed a white precipitate, and the mixture was stirred using a magnetic stirrer. (5) About 1/3 of the 2HPO_4(NH4) solution had been added, as much as 0.753 grams of MgCl_2 was added to the mixture using a spatula. During mixing was observed the pH to remain 10 with the addition of NH_4OH. (6) After mixing was complete, the resulting mixture could stand for 24 hours. (7) The gel was formed, then filtered using Whatman filter paper, and the resulting sludge was condensed for 20 hours using a crucible at a temperature of 110 °C. (8) The obtained HAp was sintered at 600 °C for 2 hours to increase its crystallinity. (9) The magnesium doped HAp produced was identified for the element/compound using the XRD characterization.

The further stages, synthesis process of composite Chi/HAp-Mg, was performed using a wet mixing method with a weight percent ratio of chitosan 0, 5, 15, and 25 %wt. First, chitosan was dissolved in 100 mL of 3% acetic acid solution and stirred using a magnetic stirrer at a constant temperature of 70 °C for one hour. Then HAp-Mg powder was added to the chitosan mixture for one hour until the result was a slurry. The slurry was then sterilized for approximately 24 hours and after that placed in a 70 °C oven for more than overnight to dry. The synthesis sample was then crushed using mortar and pestle until Chi/HAp-Mg composite powder was obtained.

The chemical composition of Chi/HAp-Mg composite powders was characterized using X-ray diffraction (XRD; PANalytical X'Pert Pro) with a monochromatized CuKα as a radiation source. The surface morphology of the Chi/HAp-Mg composite powders was characterized using Scanning Electron Microscopy (SEM; FEI INSPECT 550). Evaluation of antibacterial activity in vitro measured by the amount of inhibition zone diameter on antibacterial testing with 12 samples (3 tests for each variation of chitosan with a continuous test for incubation time). First, made a 0.5 McFarland standard solution used as a comparison of bacterial
turbidity. This solution was prepared by mixing 1% 
BaCl₂ and 1% H₂SO₄ with a ratio of 0.5:0.95 (1.5 x 10⁸ 
CFU/mL). Then, E.coli bacteria dissolved in 2 mL of 
0.85% NaCl. One mL of bacterial suspension was 
dropped into Mueller-Hinton Agar (MHA) and spread 
using a sterile spatula. The characterization was 
performed using the paper disc diffusion method by 
dipping 0.6 cm diameter paper disc into Chi/HAp-Mg 
composite solution using pH 7.2 phosphate buffer 
solutions with a concentration of 800 mg/mL. Further, 
the paper disc placed on the top of that Mueller-Hinton 
Agar (MHA). Finally, the sample was incubated at room 
temperature for 24-48 hours in the incubator.

3. Results and Discussion

The synthesis process of Magnesium doped 
Hydroxyapatite (HAp-Mg) was carried out using the 
so-gel method. The principle of the Hydroxyapatite sol-
gel method is to form a solution of the desired 
compound elements (precursors) in an organic solvent 
so that mixing occurs at the molecular level. This 
mixing can increase the chemical homogeneity of the 
powder particles and polymerize the precursors to form 
a gel. The obtained gel was then dried and sintered to 
remove the organic components contained [7]. The 
synthesis process began with the dissolution of calcium 
oxide powder as a source of 4.2 grams of calcium into 
75 ml of nitric acid solution. The mixture was then 
stirred using a hot plate and magnetic stirrer for 15 
minutes, as shown in Figure 1. Stirring using a hot plate 
and the magnetic stirrer was done to heat the solution; 
thus, chemical reactions can occur, and the results 
obtained can be more homogeneous.

The reactions that occur during the dissolution process 
can be seen in the equation below:

\[
\text{CaO(s) + 2HNO}_3\text{(aq) \rightarrow Ca(NO}_3\text{)_2 (aq) + H}_2\text{O} \quad (1)
\]

This mixing process will produce white Ca(NO₃)₂ 
filtrate obtained by filtering the mixture using a funnel 
and filter paper as in Figure 2.

Ca(NO₃)₂ filtrate produced, then added with 
diammonium hydrogen phosphate as a source of 
phosphate as much as 250 mL. Through this mixing 
white sediment will be produced which was stirred 
using a magnetic stirrer. Subsequently, 0.753 grams of 
magnesium chloride was added to the mixture after 1/3 
the solution of diammonium hydrogen phosphate was 
added. In this mixing process, the pH of the solution 
was kept 10 by adding ammonium hydroxide and 
measuring it using a pH indicator paper. The reason is 
HAp compounds that were not soluble will easily form 
under alkaline conditions. The reactions that took place 
during this mixing process can be seen in the following 
equation:

\[
10\text{Ca(NO}_3\text{)_2 \cdot 4H}_2\text{O + 6(NH}_4\text{)}_2\text{HPO}_4 + 2\text{NH}_4\text{OH \rightarrow Ca}_{10}\text{(PO}_4\text{)}_6\text{(OH)}_2 + 20\text{NH}_4\text{NO}_3 + 46\text{H}_2\text{O} \quad (2)
\]

Meanwhile, the reactions that take place between 
Magnesium and Hydroxyapatite are as follows: 
(10-x)Ca(NO₃)₂·4H₂O + xMgCl₂ · 6H₂O + 6(NH₄)₂ 
HPO₄ + 2NH₄OH \rightarrow \text{Ca}_{10-x}\text{Mgx(PO}_4\text{)₆(OH)}₂ + 
20\text{NH}_4\text{Cl}_2 + (6 + 4(10-x) + 6x)\text{H}_2\text{O} \quad (3)

The resulting mixture formed during this process was 
then allowed to stand for 24 hours to form HAp-Mg 
deposits. The comparison of HAp-Mg before and after 
settling for 24 hours can be seen in Figure 3. The 
resulting sludge was then filtered using Whatman filter 
paper; thus it will produce HAp-Mg gel as shown in 
Figure 4.

The HAp-Mg gel was then ground for 20 hours at a 
temperature of 110 °C to remove the water content 
contained. Also, it aims to save energy and keep HAp-
Mg samples from being degraded and damaged when 
roasted [8]. The obtained HAp-Mg was then sintered at 
600 °C for 2 hours in the furnace.
Sintering is the binding process between powder particles at high temperatures. The process of sintering can occur through the mechanism of atomic transport in solid conditions [9]. This process aims to produce bonds between HAp-Mg particles; thus, they are coherent and increase the crystallinity of HAp-Mg. The results of the HAp-Mg powder obtained after the sintering process will be white as shown in Figure 5.

The synthesized HAp-Mg powder will then be mixed with chitosan to form a Chi/HAp-Mg composite. Chitosan used was based on shrimp obtained commercially from CV. Bio Chitosan, Jakarta, Indonesia. This Chi/HAp-Mg composite synthesis was performed using a simple mixing method. The synthesis process begins by dissolving 0.25 grams (5% Chitosan) with 100 ml of 3% acetic acid into a beaker glass, then stirring using a magnetic stirrer at a constant speed at 70 °C as shown in Figure 6. The results were confirmed by the theory that chitosan dissolves well in organic acids such as 3% acetic acid solution [10].

The chitosan solution was then added to distilled water which was preheated to a temperature of 70 °C. Furthermore, the previously synthesized HAp-Mg powder was slowly added to the chitosan mixture with distilled water. The addition of chitosan slowly aims to make the mixture homogeneous. This mixture was stirred using a magnetic stirrer for 1 hour to obtain a slurry as shown in Figure 7.

After that, the resulting slurry was allowed to stand for 24 hours to remove air bubbles trapped in the solution. Then, the slurry was dried in a 70 °C oven for more than overnight; thus, the water content in the powder is lost. The resulting composite was then mashed by grinding using mortars and pestles. Synthesis results show that the difference in the color of chocolate that gets older with increasing chitosan content in the sample. The higher the concentration of chitosan, the color produced in the composite will be increasingly brown as shown in Figure 8.
Figure 8. Color Comparison of Sample Powder (a) HAp-Mg, (b) 5% Chi/HAp-Mg, (c) 15% Chi/HAp-Mg, (d) 25% Chi/HAp-Mg

Figure 9. XRD Pattern of HAp-Mg

All samples in the form of Chi/HAp-Mg composite powder with chitosan weight percent variation of 0, 5, 15, and 25 wt% were under XRD tests to see the compounds formed in these samples. This XRD pattern is depicted in the form of a diffractogram graph (curve with peaks), which provides information about the angle of diffraction occurring at the atomic material (2θ) on the horizontal axis and the magnitude of the intensity produced on the vertical axis [11]. The diffraction pattern of the entire sample is then matched with a pure hydroxyapatite (Ca5(PO4)3(OH)) according to ICDD 00-001-1008. From the XRD test results for the HAp-Mg composite can be seen in Figure 9. From the XRD results, there is a peak of 2θ at 32.2722° with the highest intensity, which is a hexagonal peak of hydroxyapatite (Ca5(PO4)3(OH)) according to ICDD 00-001-1008. It can be seen that the diffraction pattern resulting from the HAp-Mg synthesis process is shown at a value of 2θ = 25.9942°; 28.9937°; 32.2722°; 34.0539°; 46.4730°; 49.6072°; 53.3653°. It is known that the samples resulting from the sintering process all exhibit the characteristics of the peaks possessed by hydroxyapatite compounds (HAp) which correspond to the standard diffraction patterns of the ICDD 00-001-1008. From the diffraction pattern, it can be seen that hydroxyapatite is crystalline because it produces peaks that look sharp and clear. The absence of the peak of the individual Mg in the XRD pattern indicates that the Mg particles have joined microstructure with Hydroxyapatite [12].

The XRD pattern HAp-Mg and Chi/HAp-Mg composite shown in Figure 10. The XRD pattern of the sample was compared with calculated models in a Powder Diffraction File (PDF) database from the ICDD. The XRD pattern of prepared samples is similar to the peaks collected from pure calcium-hydroxyapatite (Ca10(PO4)6OH2) with PDF No. 00-001-1008. There is no individual peak of crystalline Mg detected in the XRD pattern indicates that magnesia particles have been micro joined with hydroxyapatite.

Furthermore, the composite XRD results in 5% wt. Chi/HAp-Mg composition found a peak at about 2θ = 11.6490° and 20.9607°, the composition of 15% wt. Chi / HAp-Mg at the position of 2θ = 11.6268° and 20.9982°, and composition 25% wt. Chi / HAp-Mg at position 2θ = 11.6321° and 20.9418°. These emerging peaks are not the peaks belong to Chitosan but are the peak of the brushite compound [CaHPO4(H2O)2]. The formation of CaHPO4 in this composite, probably due to the instability of the hydroxyapatite compound when chitosan added. The apatite crystals of hydroxyapatite exposed to the acidic solution of chitosan, in a certain period, indicate the formation of calcium hydrogen phosphate [CaHPO4(H2O)2] otherwise known as this brushite. The formation of such crystals may be possible given the apatite properties which are very tolerant of substitution [13].

Figure 10. XRD Pattern of HAp-Mg and Chi/HAp-Mg Composite
The result is under SEM micrographs on the surface of the HAp-Mg and Chi/HAp-Mg composite shown in Figure 11. It can demonstrate that the HAp-Mg sample consists of many agglomerations of spherical particles such as surface morphology with a relatively smooth surface showing non-uniform particle distribution as in Figure 11. The morphology of the HAp-Mg is a spherical shape. The addition of chitosan influences the composite morphology of Chi/HAp-Mg. In the micrographs of Chi/HAp-Mg composite showed that the composites with the addition of chitosan formed boulders or granules with a rough surface. Similar results were found in agreement with previous research by Groza et al. [14].

*E. coli* is one of the two gram-negative pathogenic bacteria that is often found in cases of orthopaedic implant infection after *Pseudomonas aeruginosa*. *E. coli* contains special virulence (FV) factors such as bacterial ligands (adhesin), proteases, poisons, protein-immune-serum, and others. This virulence factor helps organisms to form colonies, attack host tissues, disrupt host immune mechanisms, take essential nutrients, and cause inflammatory reactions. In addition, *E. coli* is also known to form a biofilm that supports the attachment of organisms to the surface of the implant [15]. Therefore, it is important to know the antibacterial activity and the inhibitory mechanism that occurs.

According to Table 1, the observation on *E. coli* during the first 24 h showed that the addition of chitosan to HAP-Mg would affect the antibacterial activity of the Chi/HAp-Mg composite. However, along with the addition of chitosan, the inhibitory zone gets smaller. In the HAp-Mg sample, no inhibition zone was found, while for composite with 5%Chi/HAp-Mg, 15%Chi/HAp-Mg, and 25%Chi/HAp-Mg has 0.6 mm, 1 mm, and 1 mm inhibition zone. The results of antibacterial activity testing could be seen that the effect of adding chitosan on the antibacterial activity of the HAP-Mg sample will increase, as seen from the inhibition zone formed.

The antibacterial activity of *E. coli* microorganisms was observed from the formation of clear zones around the sample, as shown in Figure 12. The clear zone is an area formed due to inhibited microbial growth due to samples that diffuse into bacterial living media; thus, it can also be referred to as inhibitory zones. Each sample has a different ability to inhibit the growth of various species of bacteria. The ability of the sample was measured based on the diameter of the formed inhibition zone [16].

<table>
<thead>
<tr>
<th>Composition</th>
<th>Inhibition zone (mm)</th>
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<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>HAp-Mg</td>
<td>0</td>
</tr>
<tr>
<td>5%Chi/HAp-Mg</td>
<td>0.6</td>
</tr>
<tr>
<td>15%Chi/HAp-Mg</td>
<td>1</td>
</tr>
<tr>
<td>25%Chi/HAp-Mg</td>
<td>1</td>
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</tbody>
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*Figure 11. SEM Micrographs of Sample: (a) HAp-Mg; (b) 5%Chi/HAp-Mg; (c) 15%Chi/HAp-Mg; (d) 25% Chi/HAp-Mg*

*Figure 12. Visual Observation of Antibacterial Activity Testing of E. coli (a) 12 Samples with Different Variations (A1-A3 → HAp-Mg, B1-B3 → 5%Chi/HAp-Mg, C1-C3 → 15%Chi/HAp-Mg, D1-D3 → 25% Chi/HAp-Mg (b) Magnification of Visual Samples*
At the next 48 hours of observation, the antibacterial activity of composite against *E. coli* decreased. As time increases, the HAp-Mg and 5%Chi/HAp-Mg composite has no inhibition zone, while composite with composition 15%Chi/HAp-Mg and 25%Chi/HAp-Mg has 0.8 mm and 0.6 mm. These results confirm that chitosan on Chi/HAp-Mg composite has a bacteriostatic effect which is a condition caused by the antibacterial compound, so growth and development of bacteria are permanent (static), wherein this case composite is inhibiting for a specified time interval, in this case, the first 24 hours post-incubation. This may be due to the emergence of a subpopulation of resistant bacteria as a result of the physiological adaptation of the cell to chitosan exposures [12].

4. Conclusion

Magnesium doped HAp and Chi/HAp-Mg composite based on the different weight of chitosan namely 0, 5, 15, and 25% wt. prepared through the sol-gel method and wet mixing method. These samples were evaluated whether the addition of chitosan may affect the antibacterial activity and in vitro bioactivity properties of HAp-Mg. The addition of chitosan changes the increase of biocompatibility properties of HAp-Mg seen from the antibacterial activity test result. The bacterial inhibition activity of *E. coli* bacteria found to be greater in composite with composition 15%Chi/HAp-Mg with inhibition zone diameter of 1 mm. So, composite with this composition can be submitted to the material for bone graft applications.

References