Synthesis and characterization of hydroxyapatite from duck eggshell modified silver by gamma radiolysis method

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Abstract. Crystalline hydroxyapatite (HA) prepared from duck eggshell using the precipitation method. Duck eggshell used because it has higher calcium oxide (CaO) purity than another eggshell. The amount of duck eggshell calcinated to obtain calcium precursor then dropped with phosphoric acid wisely. The hydroxyapatite result modified with a silver (HA-Ag) using gamma radiolysis reduction. Hydroxyapatite powder added with an alcoholic silver ion precursor and followed by high energy gamma irradiation. This modification is to increase the antibacterial activity of hydroxyapatite for further medical applications. The results of the bacterial inhibitory test, showed the largest antibacterial clear zone in 25 kGy irradiation dose of HA-Ag. HA and HA-Ag characterized by FTIR, XRD, SEM and EDX. All characterization result support the forming of pure hydroxyapatite and the presence of a silver particle.

1. Introduction

The potential of eggshell waste in Indonesia is quite large, ie in a year, it can produce 178,566.33 tons [1]. In general, people consider eggshells as unused waste, so most of them are just thrown away. Even though eggshell contains high calcium, it reaches ± 94%. Therefore, techniques for processing and utilizing eggshell is necessary to increase economic value and reduce the burden on the environment [2].

One of the utilization of calcium eggshell waste is in the biomedical field, namely for the synthesis of hydroxyapatite as bioceramics material. Hydroxyapatite, Ca_{10}(PO_4)_6(OH)_2, is the most dominant mineral component in human teeth and bones with a 65% fraction [3]. This mineral is composed of calcium and phosphate with a Ca/P atomic ratio 1.67. Hydroxyapatite has the same biocompatibility, bioactivity, and osteoconductivity properties as well as structural and chemical components of bone and tooth minerals, so that they are widely used as biomaterials for implants [4] [5].

The abundant of eggshell waste can be used as a source of calcium, so that hydroxyapatite can be obtained with organic matter. Eggshell has the advantage as a raw material for hydroxyapatite synthesis because it has a better degree of dissolution, biomineralization, and osteointegration than some other sources. The results of calcination of duck eggshell have a higher calcium oxide (CaO) purity than the results of calcination of chicken eggshell and quail eggshell, so this study uses duck eggshell as the main ingredient in making hydroxyapatite [6] [7].

Hydroxyapatite synthesis can be done by mixing calcium and phosphate precursor compounds. There are some eggshell-based hydroxyapatite synthesis methods, such as wet precipitation method [8], hydrothermal method [9], mechanochemical method [10], and the microwave irradiation method [11] [12]. In this study, the hydroxyapatite synthesis method used is the wet chemical precipitation.
method, because the process is simple, low-cost, suitable for large-scale industrial production, and does not cause pollution to the environment [13] [14].

On the other hand, hydroxyapatite material has disadvantages especially in its antibacterial ability. Hydroxyapatite modification by using an antibacterial agent was carried out to improve the performance of its antibacterial properties. The selected antibacterial agent is silver (Ag). This is because silver is the most superior antibacterial with several advantages: having the highest efficiency at low concentrations, low levels of cytotoxicity, good biocompatibility, intrinsic stability, and has an oligodynamic effect on Gram-negative and Gram-positive bacteria [15] [16] [17].

The silver modification process for hydroxyapatite has been done by several methods, such as the microwave method, ultrasonic spray pyrolysis, sol-gel technique, ion exchange, and coprecipitation. In this study, silver doping modification used the gamma radiolysis method known as a simple, clean, and efficient method to produce silver nanoparticles with uniform particle size distribution [16] [15]. Radiation on the system will cause the formation of free radicals such as solvated electrons. Solvated electrons will reduce Ag+ ions to form Ag0 metal. The formation of Ag0 metal is expected to modify the surface of the hydroxyapatite sample to form HA-Ag. The formation of Ag atoms is followed by the combining of several ions and the dimerization process, which is terminated by the aggregation process in the sample to form a larger nucleus [18].

The objective of this study is to obtain hydroxyapatite synthesized from duck eggshell waste, to find the optimum irradiation dose for silver-modified hydroxyapatite, and to know the effect of this modification on its antibacterial ability.

2. Materials and methods

2.1. Materials
Duck eggshell waste obtains from Condong Catur traditional market; H3PO4, HClO4, AgNO3, NH4OH, and absolute ethanol form Merck, distilled water, and Whatman filter paper number 41.

2.2. CaO precursor preparation
Duck eggshells are cleaned from dirt and the membranes. Furthermore, dried in the sun and calcined with a furnace at 1000 °C temperature for 3 hours. The calcined powder (CaO) was pulverized using a mortar and sieved with 120 mesh size.

2.3. Synthesis of hydroxyapatite
Hydroxyapatite was synthesized through a wet precipitation method. The Principle of this method is the reaction between calcium hydroxide and phosphoric acid with a molar ratio of Ca/P=1.67. Calcined CaO powder was mixed with distilled water to form a 1.67 M Ca(OH)2 solution. H3PO4 solution with concentration 1 M was added to 1.67 M Ca(OH)2 solution by dropwise through a burette and stirred. The pH of the mixed solution is maintained higher than 10 by periodically adding NH4OH solution. During the process of hydroxyapatite synthesis, temperature conditions, stirring velocity and time were maintained at 60 °C, 300rpm, and 3 hours. The solution to precipitation results is leaving for 24 hours. The aging solution is filtered and the precipitate is washed with distilled water. The precipitate, that is hydroxyapatite, was dried in an oven at 80 °C for 24 hours, mashed and sieved with 120 mesh size, then sintered at 900 ºC for 3 hours.

2.4. Modification hydroxyapatite with silver
Modification hydroxyapatite with silver is done by adding 0.02 M AgClO4 alcoholic solution into hydroxyapatite sample as much as 2% (w/w). Furthermore, the suspension was sonicated for 5 minutes and irradiated using irradiator type I Ob-Servo Ignis with 12 kCi Co-60 radioactive sources located in STTN BATAN. The irradiation process was done by varying the dose at 5 kGy, 10 kGy, 15 kGy, 20 kGy, 25 kGy, and 30 kGy.
2.5. Bacterial inhibition test

The bacterial inhibitory ability test was carried out by diffusion method using one of the gram-negative bacteria, *Escherichia coli*. Hydroxyapatite sample (HA) and hydroxyapatite with silver modification (HA-Ag) were respectively dissolved using 1 M HCl solution in the ratio of 0.1 grams of sample in a 5 ml solution. Culture media is prepared by mixing sterile nutrient agar media with *Escherichia coli* bacterial suspension. Culture media is poured into a petri dish and awaited until the media freezes and then perforated as a sample hole zone. Each suspension of the sample is dropped into the hole and leaves so that the sample can diffuse. The sample incubated at 37 °C for 24 hours. Then the area of the clear zone is measured as the zone of inhibition of bacteria. Clear zone as an indicator of the ability to inhibit bacterial growth can be determined by equation (1).

\[
\text{Inhibition zone} = D_{\text{clear zone}} - D_{\text{hole}} \quad (1)
\]

2.6. Characterization

Functional groups of HA and HA-Ag samples were analyzed by using Shimadzu Prestige 21 spectrophotometer in the range 400-4000 cm\(^{-1}\). The XRD pattern was recorded by using the D2 Phaser Bruker X-ray diffractometer in the 2\(\theta\) range 5-100°. The SEM-EDX was performed on a Hitachi SU 3500 to capture sample morphology.

3. Results

3.1. Hydroxyapatite synthesis

The first step in the hydroxyapatite synthesis is the calcination of cleaned duck eggshell. The purpose of this calcination process is to eliminate organic compounds in the eggshell and convert calcium carbonate (CaCO\(_3\)) to calcium oxide (CaO) as calcium precursor. In addition, calcination also plays a role in eliminating pathogens in the eggshell that has the opportunity to transmit the disease to people \[20\] \[19\]. The decomposition reaction is in accordance with equation (2).

\[
\text{CaCO}_3(s) + \text{heat} \rightarrow \text{CaO}(s) + \text{CO}_2(g) \quad (2)
\]

In this experiment, from 100.45 grams of duck eggshell was obtained 48.93 grams of CaO, that is mean the calcination yield value was 48.71%. These results indicate a reduction in mass caused by the release of organic compounds contained in the eggshell. In this process, there is a color change of the duck eggshell from greenish to milky white. The discoloration shows that there has been an organic decomposition \[19\]. Figure 1 shows the difference in color between raw and calcined duck eggshell.

![Figure 1](image1.png)

**Figure 1.** (a) Raw eggshell, (b) calcined eggshell.

The calcium oxide dissolved with distilled water to form calcium hydroxide (Ca(OH)$_2$) according to equation (3).

\[
\text{CaO}(s) + \text{H}_2\text{O}(l) \rightarrow \text{Ca(OH)}_2(aq) \quad (3)
\]
Phosphor precursor used was phosphoric acid (H$_3$PO$_4$) that is relatively cheap and the by-product obtained in hydroxyapatite synthesis is water only. Alkaline (NH$_4$OH) also used to maintain the stability of the pH value during the reaction process because hydroxyapatite is stable under alkaline conditions. The reaction of hydroxyapatite formation according to equation (4).

\[
10\text{Ca(OH)}_2(\text{aq}) + 6\text{H}_3\text{PO}_4(\text{l}) \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2(\text{s}) + 18\text{H}_2\text{O}(\text{l}) \tag{4}
\]

In the synthesis process, the mixing time is carried out for 3 hours after phosphate addition was complete. The 3 hours reaction time is the best time to produce the optimum product [21]. If the stirring time is too short, it can lead to inhomogeneous phases so that reaction does not fully occur and can reduce the yield of the product.

3.2. Modification of hydroxyapatite with silver
The modification process is carried out by adding an alcoholic silver solution to the hydroxyapatite sample. Furthermore, the suspension is sonicated to mix and reduce the size of hydroxyapatite particles. In the silver radiolysis process, the solution produces active species such as solvated electrons (e$^{-}_{\text{aq}}$). These electrons directly reduce metal ion precursors (Ag$^+$) to lower valence (Ag$^0$) and eventually become metal atoms. The reduction reaction is shown in Equation (5). The Ag$^0$ metal which plays a role in doping the surface of the hydroxyapatite sample to form HA-Ag.

\[
\text{Ag}^+ + e^{-}_{\text{aq}} \rightarrow \text{Ag}^0 \tag{5}
\]

The gamma irradiation process results in a different color in the sample. The HA-Ag suspension color changes to a brownish color from pure white HA. This brown color shows the presence of silver in hydroxyapatite samples. Color changes that occur in silver modified materials are due to the color of Ag nanoparticles. Ag nanoparticles are reported to have a yellowish color [22]. The difference in color changes that occur in each sample shows the amount of silver doped in hydroxyapatite. This is influenced by the amount of irradiation dose to the sample, that is the amount of silver that modifies hydroxyapatite will increase according to the amount of irradiation dose received [16][23][24]. The change in color of a hydroxyapatite sample modified by silver using gamma irradiation can be shown in figure 2.

![Figure 2. Hydroxyapatite color change (a) before irradiation (HA), (b) After irradiation (HA-Ag).](image)

3.3. Characterization
FTIR spectrum results in raw duck eggshell showed that CaO appeared with a dominant intensity compared to other functional groups. There is also exist CO$_3^{2-}$ groups and H$_2$O groups with low-intensity values measured by FTIR. The presence of the CO$_3^{2-}$ group indicates that calcined eggshell still contains carbonate from raw duck eggshell. Meanwhile, the existence of hydroxyl ions indicates the sample is not completely dry. The combined FTIR spectrum of the characterization results on the CaO, HA, and HA-Ag samples can be shown in figure 3.
Figure 3 presented data from the results of FTIR analysis for hydroxyapatite (HA) and Ag-doped hydroxyapatite (HA-Ag) with an irradiation dose of 25 kGy. The spectrums of the two samples are then compared with the reference hydroxyapatite spectrum results obtained by several other researchers, such as Hardiyanti (2016); Meldha (2016); and Suryadi (2011). After comparing them, several suitable spectra were obtained and showed the presence of functional groups CO\(_3^{2-}\), PO\(_4^{3-}\) (\(v_1\)), PO\(_4^{3-}\) (\(v_2\)), PO\(_4^{3-}\) (\(v_3\)), PO\(_4^{3-}\) (\(v_4\)), OH\(^-\) and HOH. The presence of hydroxyl (OH\(^-\)) and phosphate (PO\(_4^{3-}\)) functional groups prove that hydroxyapatite has formed.

However, these results also show the presence of carbonate functional groups (CO\(_2^{2-}\)). Meldha (2016) and Suryadi (2011) convey in their report, that the presence of carbonate groups in hydroxyapatite can be influenced by several factors. The first factor, caused by the presence of CO\(_2\) during the synthesis process carried out in non-inert atmosphere conditions [25]. The presence of carbonate compounds can also be caused by the slow rate of acid addition, which causes the joining of carbonate with apatite. Carbonate ions entering the HA crystal lattice will replace hydroxyl (OH\(^-\)) or phosphate (PO\(_4^{3-}\)) ions and produce carbonated-HA (CHA) [25].

Carbonate compounds in hydroxyapatite need to be removed because it can reduce the thermal stability of hydroxyapatite. The method that can be done is to create an inert condition during the reaction of mixing calcium and phosphate precursors. Inert environmental conditions can be carried out by flowing inert gases such as nitrogen into the mixing reactor [25] [19].

There is observed wavenumber of the H\(_2\)O band. This can occur because the HAP sample absorbs water on its surface. The sample storage factor which does not include silica gel at the time of storage can be a contributing factor.

XRD pattern of each sample is compared to the Joint Committee on Powder Diffraction Standards (JCPDS) data standard. The combined diffractogram of XRD characterization results on CaO, HA, and HA-Ag samples shows in figure 4.

Figure 4. XRD diffractogram results from CaO, HA, and HA-Ag samples.
The results of CaO powder characterization from eggshell calcination were matched with JCPDS data number 00-37-1497 for CaO. Overall, the angle of 2θ calcined results has approached the angle value of 2θ standard CaO material. Based on the data from the analysis it is proved that the calcination process has succeeded in converting CaCO₃ into CaO. On the other hand, there is a Ca(OH)₂ phase which is measured in the XRD pattern with low intensity at 2θ angle of 34.1448°. The emergence of the Ca(OH)₂ phase is due to the interaction of CaO with the atmospheric air environment and the nature of the powder which is easy to absorb water vapor. Meanwhile, samples of silver hydroxyapatite (HA) and modified hydroxyapatite (HA-Ag) were matched with standard data.

These results confirm the results of the FTIR analysis which showed the presence of phosphate and hydroxyl functional groups as a characteristic of hydroxyapatite functional groups. Qualitatively, the width of the peaks in both samples can be seen that they are narrow and pointed. This shows that the sample is crystalline.

On the other hand, the HA-Ag diffractogram shows the spectrum of Ag peak inline with hydroxyapatite. This Ag peak shows that silver has been successfully formed in hydroxyapatite and also has a crystalline phase in the sample. When compared with JCPDS data, XRD diffractogram of HA-Ag samples, the silver phase detected at 64.192°.

The surface morphology of the HA crystal was investigated by SEM. The result of characterization SEM showed oval-shaped and homogeneous as shown in Figure 5. Modification of silver on HA does not have a significant effect on surface morphology with the average grain size of 1 micrometer.

![Figure 5. SEM images of hydroxyapatite: (a) HA (15000x), (b) HA (30000x), (c) HA-Ag (15000x), (d) HA-Ag (30000x)](image)

EDX characterization of HA and HA-Ag samples was carried out to determine the distribution of the elements contained in the sample as shown in Figure 6. While the quantitative results of EDX measurements are presented in Table 1. Based on quantitative data, the Ca/P ratio value in the HA sample is 1.77. This value approaches the theoretical value of the Ca/P ratio of pure hydroxyapatite which is 1.67. According to Suryadi (2011), the molar ratio of Ca/P affects the strength of hydroxyapatite. That is mean, greater Ca/P ratio, increases the strength of hydroxyapatite. This strength will reach a maximum value around the Ca/P ratio ~1.67 and its strength will decrease if the Ca/P ratio is larger than 1.67 [25].
Figure 6. EDX line spectrum of HA Sample.

Table 1. Quantitative EDX Results of HA Sample.

| Element | Weight % | Atomic % |
|---------|----------|----------|
| O       | 50,21    | 69,87    |
| P       | 15,09    | 10,85    |
| Ca      | 34,70    | 19,28    |

Silver concentration in HA-Ag samples was further confirmed by EDX analysis by measuring at two different area points shown in figure 7.

Figure 7. EDX image and spectrum in different region: (a) region 1, (b) region 2.
From the line spectrum image, it can be seen qualitatively that the intensity of silver concentration in the second analysis area is higher than the first analysis area. That is means the silver modification is not really homogeneous at all. This is supported by quantitative data from the EDX analysis results presented in Table 2.

Table 2. Quantitative EDX results of the HA-Ag sample.

| Element | Area 1 |  | Area 2 |  |
|---------|--------|--------|--------|--------|
|         | Weight % | Atomic % | Weight % | Atomic % |
| O       | 50.99   | 70.67   | 51.48   | 73.42   |
| P       | 14.73   | 10.54   | 12.75   | 9.39    |
| Ag      | 0.50    | 0.10    | 8.87    | 1.88    |
| Ca      | 33.78   | 18.68   | 26.90   | 15.31   |

The data can be used to determine the value of the Ca/P ratio. In region 1 Ag concentration is 0.5% (wt) with a Ca/P ratio 1.77. Whereas in region 2 Ag concentration is 8.87% (wt) with Ca/P ratio 1.63. The difference in value indicates that the HA-Ag sample is not homogeneous due to the occurrence of silver agglomeration. This agglomeration is influenced by the nature of silver nanoparticles (AgNP) that easily agglomerated and oxidized. For prevention agglomeration, the addition of other compounds requires as stabilizers. The compound that can be used as a silver stabilizer is trisodium citrate (C$_6$H$_5$O$_7$Na$_3$) [26]. Aside from being a stabilizer, trisodium citrate also acts as a reducing agent.

3.4. Bacterial inhibition test

Bacterial inhibition test was carried out using the diffusion method with gram-negative bacteria, *Escherichia coli*, which is a pathogen that could initiate bone infections. The test is done on HA-Ag samples which are obtained from gamma irradiation at doses of 0, 5, 10, 15, 20, 25, and 30 kGy in order to determine the optimum dose that produces the largest inhibitory zone.

The results showed that the presence of silver was able to increase the antibacterial ability of HA-Ag sample, so that silver was proven as an antibacterial agent. The quantitative test of antibacterial activity is presented in table 3.

Table 3. Bacterial inhibition test results of HA and HA-Ag.

| No | Sample name | Irradiation dose (kGy) | Clear zone area (cm$^2$) | Clear zone diameter (mm) |
|----|-------------|------------------------|--------------------------|--------------------------|
| 1  | HA          | 0                      | 54.62                    | 24.03                    |
| 2  | HA-Ag       | 5                      | 57.03                    | 24.77                    |
| 3  | HA-Ag       | 10                     | 67.03                    | 27.7                     |
| 4  | HA-Ag       | 15                     | 72.03                    | 29.08                    |
| 5  | HA-Ag       | 20                     | 64.03                    | 26.85                    |
| 6  | HA-Ag       | 25                     | 75.03                    | 29.89                    |
| 7  | HA-Ag       | 30                     | 56.03                    | 24.47                    |

The radiation dose plays an important role in determining the presence of the amount of silver in HA-Ag which is further evidenced by the antibacterial ability of the HA-Ag sample. In this study, the optimum dose for silver doping in hydroxyapatite is 25 kGy with a clear zone diameter of 29.89 mm.
The optimum dosage results were supported by Akhavan et al (2014). They reported that silver doping against hydroxyapatite produced the best products in the dose range between 20-40 kGy. The effect of the radiation dose on the zone of inhibition of bacteria formed tends to be directly proportional. However, at doses of 20 kGy and 30 kGy there was a decrease in the value of the clear zone area. Saion, Gharibshahi, & Naghavi, (2013) reported that in the high-dose radiolysis process, competition between the nucleation process is higher than the number of irreducible ions, so the system will become unstable. In this situation, the interaction of HA modification with Ag becomes disturbed. A large amount of radicals also increases the likelihood of release and binding (bound and unbound) in Ag dopant material against HA samples.

4. Conclusion
Hydroxyapatite with calcium precursor from duck eggshell and phosphate from HPO, was successfully synthesis by precipitation method. The characteristics of HA synthesis are in accordance with the criteria for hydroxyapatite material as evidenced by the results of the FTIR spectral wave numbers, angles of 2θ on XRD, and elemental content on EDX. The modification of Ag support for HA was successfully carried out by the gamma radiolysis method. However, Ag agglomeration occurs which is supported by EDX results. The optimum dosage for HA-Ag modification is 25 kGy with a bacterial inhibition zone diameter of 29.89 mm.

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