

Synthesis and Characterization of Composite Hydroxyapatite-Silver Nanoparticles

Charlena^{1*}, N A Nuzulia^{2*} and Handika¹

¹ Department of Chemistry, Bogor Agricultural University, INDONESIA.

² Department of Physics, Bogor Agricultural University, INDONESIA.

Email: charlena.ipb@gmail.com , aisyahnuzulia@apps.ipb.ac.id

Abstract. Hydroxyapatite (HAp) is commonly used as bone implant coating recently; however, the material has disadvantage such as lack of antibacterial properties, that can cause an bacterial infection. Addition of silver nanoparticles is expected to be able to provide antibacterial properties. Silver nanoparticles was obtained by reduction of AgNO₃ using glucose monohydrate with microwave heating at 100p for 4 minutes. The composite of hydroxyapatite-silver nanoparticles was synthesized using chemical methods by coprecipitation suspension of Ca(OH)₂ with (NH₄)HPO₄, followed by adding silver nanoparticles solution. The size of the synthesized silver nanoparticles was 30-50 nm and exhibited good antibacterial activity. Nevertheless, when it was composited with HAp to form HAp-AgNPs, there was no antibacterial activity due to very low concentration of silver nanoparticles. This was indicated by the absence of silver nanoparticles diffraction patterns. Infrared spectra indicated the presence of chemical shift and the results of scanning electron microscope showed size of the HAp-AgNPs composite was smaller than that of the HAp. This showed the interaction between HAp and the silver nanoparticles.

Keywords: antibacterial activity, composite, coprecipitation, nanoparticles

1. Introduction

Bone damage such as broken bones or fractures can be treated with the implants. Planting the implant will be able to replace the damaged bone or assist in the formation of new bone tissue. The implants can be made of metal, ceramic, polymer, or composites [1]. The field of medicine has been used as an implant metal as the metal has good mechanical properties, strong and malleable. Metals commonly used as an implant material of which is stainless steel, titanium alloys, and cobalt. But the form of metal implants when used in the long term would be harmful to the body. The release of ions from the implant-based metals such as iron ions, chromium, and nickel in the body led to allergy and demonstrated carcinogenic properties. Additionally, in the form of metal implants can cause swelling and pain around the bone. It is caused by a reaction of metal corrosion in body fluids and the low level of biocompatibility of these metals [2].

Prevention of corrosion and metal-based implants to increase of biocompatibility is necessary that successful to increases implantation of bone. This can be done by coating the implant with a metal-based material that is non-corrosive and have a good biocompatibility such as Hydroxyapatite [3]. biocompatibility of the hydroxyapatite due to the composition of hydroxyapatite has similarities with



the composition of biological hard tissue such as bone and teeth [4]. Hydroxyapatite not only has good biocompatibility, in addition to hydroxyapatite is non-toxic, osteoconductive and bioactive. Therefore, the formation chemical bond between the implant and the bone tissue will be increases and accelerates the healing process of bone [5].

Hydroxyapatite is one type of bioceramics are widely applied in the fields of medical because of the similarity of its properties with chemical structure of the bone [6]. However hydroxyapatite has some disadvantages, among which it is fragile and antibacterial. This can reduce of the success of implantation and increased risk of death due to the infection by bacteria. Prevention of infection after bone implantation can be done by creating a composite of hydroxyapatite and material that is antibacterial. Nanoparticles of metals such as Zn, Cu, and Ag showed good antibacterial properties [7]. One of the most studied nanoparticles are silver nanoparticles. Silver nanoparticles have stable properties and potential applications in various fields such as catalysts, optical sensors detectors and antibacterial agents [8].

Hydroxyapatite can be synthesized using waste blood shells, animal bones, stone gypsum, egg shells or *Bellamya javanica* shell [9]. *Bellamya javanica* shell of an abundant waste but has not been used commercially significant. This waste contains a variety of minerals one of which is calcium that can be used in the synthesis of hydroxyapatite [10]. In this study, hydroxyapatite synthesized from the utilization of waste *Bellamya javanica* shells through the wet method (precipitation). after obtained hydroxyapatite, hydroxyapatite-silver nanoparticle composite (HAp-AgNPs) synthesized by precipitation method using silver nitrate solution as the source of silver nanoparticles formed after reduced use glucose monohydrate. The research aims to synthesize silver nanoparticles as active substances that have antibacterial activity and synthesizing composite HAp-AgNPs potential as implant materials with antibacterial properties.

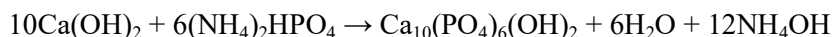
2. Methods

2.1. Synthesis of Silver Nanoparticles

AgNO₃ solution 10⁻² M is made by powder weighed as much as 0.1700 g AgNO₃, dissolved in 100 mL flask with distilled water and homogenized. A total of 16 mL of the solution was put in a beaker, then added 14 mL solution of glucose monohydrate 0.5 M. The solution was prepared by weighing 9.9000 g glucose monohydrate, dissolved in 100 mL flask with distilled water and then homogenized. After AgNO₃ 10⁻² M and 0.5 M glucose monohydrate were mixed, added 10 mL of distilled water, and then homogenized. The mixture was heated in the microwave heating for 2 minutes then stirred using a magnetic stirrer for 20 minutes. Reheat for 1 minute and stir again for 20 minutes, repeat 2 times [11].

2.2 Synthesis of Hydroxyapatite

The powdered of calcium hydroxide from calcination *Bellamya javanica* shell used in the synthesis of HA as a source of calcium which is reacted with a solution of (NH₄)₂HPO₄ through the following reaction:



The solution of (NH₄)₂HPO₄ 0.3 M dropped on the suspension of Ca(OH)₂ 0.5M using buret at 40±2 °C and stirred using a magnetic stirrer. In this synthesis, the pH is monitored and corrected to obtain a pH of 10. The solution was then sonicated for 3 hours, then decantation for 24 hours at room temperature. Furthermore, the solution was decanted and centrifuged at 4500 rpm for 15 minutes and then the precipitate is separated and rinsed with distilled water. Then, the precipitate is dried at 105 °C for 3 hours. After drying, the precipitated finely pulverized using a mortar and then sintered with a temperature of 500 °C for 3 hours. HA powder then cooled in the desiccator [12].

2.3 Synthesis of Composite HA-AgNPs

Powder $\text{Ca}(\text{OH})_2$ from calcination *Bellamya javanica* shell used in the synthesis of composite HA-AgNPs as a source of calcium. Composite HA-AgNPs synthesized through the reaction between the suspension of $\text{Ca}(\text{OH})_2$ solution $(\text{NH}_4)_2\text{HPO}_4$ and AgNPs solution. A total of 100 mL $(\text{NH}_4)_2\text{HPO}_4$ 0.3 M dropped to 100 mL suspension of $\text{Ca}(\text{OH})_2$ 0.5 M using a buret at a temperature of 40 ± 2 °C and stirred using a magnetic stirrer. Furthermore, as much as 50 mL AgNPs dripped on HA suspension using a buret. The solution was then sonicated for 3 hours and decantation for 24 hours at room temperature. The solution was decanted and centrifuged at 4500 rpm for 15 minutes and then the precipitate was separated and rinsed with deionized water. Furthermore, the precipitate is dried at 105 °C for 3 hours. After drying, the precipitated finely pulverized using a mortar and then sintered with a temperature of 500 °C for 3 hours. Powder HA-AgNPs then cooled in the desiccator [13].

2.2. Characterization

Silver nanoparticles has been synthesized and absorbance was measured with UV-Vis spectrophotometer at wavelength of 300-500 nm to estimate the size of AgNPs before being used in the synthesis of composite HA-AgNPs. Then HA and composite HA-AgNPs that has been synthesized are characterized using FTIR, XRD and SEM. FTIR characterization was performed to identify the functional groups on HA and the effect of adding AgNPs with the functional groups in the composite HA-AgNPs. XRD characterization was conducted to determine the phase contained in the composite HA and HA-AgNPs that has been synthesized. The results were compared with Joint Committee on Powder Diffraction Standards (JCPDS). Characterization using SEM was conducted to determine the morphology of HA and composite HA-AgNPs.

2.3. Antibacterial Activity Test

Antibacterial activity test performed on the active substance AgNPs, HA, and composite HA-AgNPs, the bacteria used were *Staphylococcus aureus*. Petri dishes are prepared and sterilized beforehand. Antibacterial activity test on AgNPs do with the disc method is to add 20 mL media Nutrient Agar (NA) into a sterile petri dish. Then, the bacteria is prepared at a concentration of 10^{-6} grown in media that have been made. Discs (diameter 6 mm) was added to the media. Furthermore, as many as 1 mL colloid AgNPs dropped into discs using a pipette. Then, incubated at 37 °C for 24 hours. Antibacterial activity test on HA and composite HA-AgNPs conducted using wells that the NA added 20 mL media into a sterile petri dish. Then, the bacteria is prepared at a concentration of 10^{-6} grown in media that has been made. A total of 0.2 grams of sample was mixed with 1 mL of distilled water, then put in the media. Furthermore, incubated at 37 °C for 24 hours. The antibacterial activity was calculated by the inhibitory zone diameter in mm. The test was done in duplicate. Value index of inhibition of bacteria can be calculated in the following equation:

$$\text{Index Inhibitory Control} = \frac{\text{diameter of inhibition zone} - \text{diameter discs}}{\text{diameter discs}}$$

3. Results and Discussion

3.1. Silver Nanoparticles Size Analysis

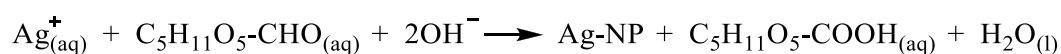
Silver nanoparticles sythesis in this research was done using chemical reaction method. AgNO_3 was reduced by monohydrate glucose. Synthesis of silver nanoparticle was done by adding monohydrate glucose into AgNO_3 solution, which is then heated gradually using microwave 100 W and after each heating phase finished, solution was stirred using magnetic stirrer. Gradual heating was done to prevent AgNPs agglomeration. Based on research by Sulistiawaty *et al.* (2015)[11], monohydrate glucose concentration was 0.5M and AgNO_3 concentration was 1×10^{-2} M. Formation of AgNPs was indicated by color changing from colorless to pale yellow. Pale yellow color showed that AgNPs started to form and when the heating phase ended, the solution will have yellow color (Figure1).



AgNPs

Figure 1. Synthesized silver nanoparticles.

Yellow color was caused by plasmon surface vibration excitation on AgNPs surface [14]. According to Solomon *et al.* (2007)[15], formation of nanoparticles was indicated by absorption peak at 400-500 nm, that peak involved electron cloud resonance on nanoparticles surface. Absorption appeared as result of inter bands transition in the system (4d-5sp) at higher energy level after exposed to light at UV-Vis wavelength [16]. Absorption peak at 420 nm indicated the formation of AgNPs. That absorption peak formed after AgNO₃ salt reacted with monohydrate glucose. Ag⁺ ion from AgNO₃ solution could be reduced to Ag with aldehyde group in monohydrate glucose. During reduction, addition of electron transformed Ag⁺ ion to become uncharged (Ag⁰) [17]. According to Dankovich (2014)[18], reaction of AgNPs formation with monohydrate glucose reductor could be concluded as:



According to Solomon *et al.* (2007) [15], AgNPs size could be estimated based on wavelength of the absorption peak (Tabel 1). Synthesized silver nanoparticle from AgNO₃ precursor with monohydrate glucose reductor had maximum wavelength of 420 nm (Figure 2). Based on this, synthesized silver nanoparticles size was estimated ranging from 35-50 nm. Synthesized AgNPs using ascorbic acid as the reductor for AgNO₃. Characterization result using UV-Vis spectrophotometer and TEM showed that synthesized AgNPs had absorption peak at 414.5 nm and average size of 28 nm.

Table1. Silver nanoparticles size range based on spectrum pattern [15].

Particle size (nm)	Maximum wavelength (nm)	Spectrum band width (nm)
10-14	395-405	50-70
35-50	420	100-110
60-80	438	140-150

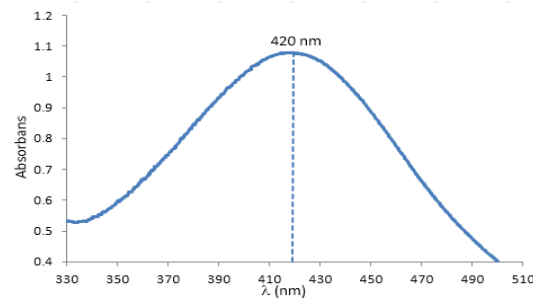


Figure 2. Silver nanoparticles UV-Vis Spectrum.

3.2. Hydroxyapatite-Silver nanoparticles Composite Synthesis

Hydroxyapatite (HA) is a type of bioceramic that have wide application in medical field, one of the is as metal based implant coating material because it have similar properties with bone chemical structure [6]. This bioceramic has good biocompatibility, non-toxic, osteoconductive, and also bioactive. But HA doesn't have antibacterial properties, which can lead to infection by bacteria during implantation process. Infection can cause implantation failure or even death. Addition of antibacterial material can provide ideal condition for bone regeneration process because it can prevent infection and increase patient tolerance [7]. One of the material with antibacterial properties is silver nanoparticles [8].

HA-AgNPs composite can be synthesized using presipitation method. This method is easy and simple, has high rendement, and relatively low cost, which is by reacting calcium and phosphate source with concentration ratio of 1.67. Then, AgNPs solution is added. Composite formation mechanism is Ag ion combines with HA then AgNPs particle growth occurs inside and on the surface of HA (Figure 3). AgNPs particle growth on the surface of HA happen when AgNPs interacts with OH⁻ ion of HA [13].

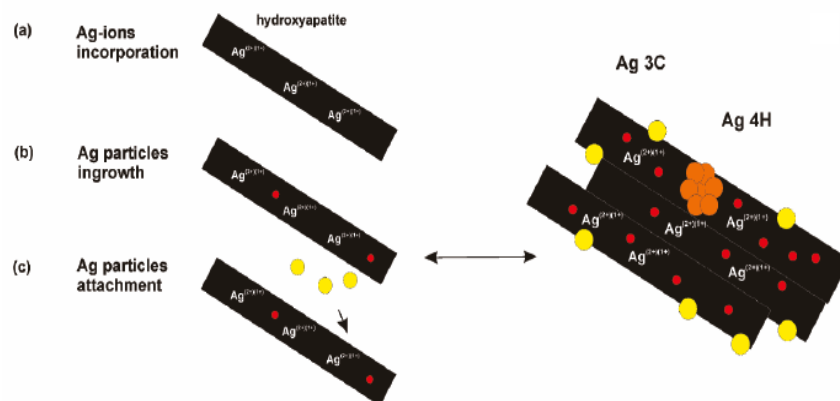


Figure 3. HA-AgNPs composite formation mechanism.

(NH₄)₂HPO₄ solution (Phosphate source) was added slowly into Ca(OH)₂ (calcium source) suspension under presipitation temperature of 40±2 °C and stirring for homogen mixing, then pH of the solution was measured until the pH was 10. Then AgNPs solution that has been synthesized before was added slowly into the suspension while stirred so that the mixture was homogen. That suspension was then sonicated. Sonication process was done to produce HA-AgNPs composite with high homogeneity. Precipitate was separated by sentrifugation. Precipitate was then filtered and washed with aquadest to get rid of unwanted solvent. Drying and sintering process could get rid of the water and other organic compound. Synthesized HA-AgNPs was in form of powder with yellow color. It had a

different color from HA that was synthesized as control using presipitation method (Figure 4). HA and HA-AgNPs composite was characterized using FTIR, XRD, dan SEM.

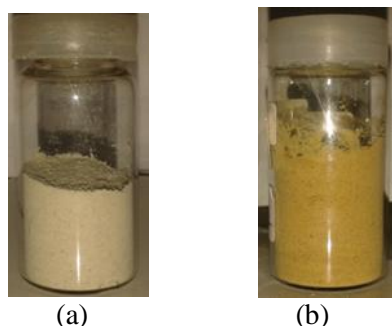


Figure 4. Synthesized HA (a) and HA-AgNPs composite (b).

3.3. Analysis of Hydroxyapatite and Hydroxyapatite-Silver Nanoparticle Composite

Characterization using XRD, FTIR, and SEM was done to HA and HAp-AgNPs composite. Characterization using XRD supported in the determination of HAp-AgNPs composite structure, especially in determining crystallinity of hydroxyapatite and silver nanoparticles. XRD characterization on HAp and HAp-AgNPs composite showed specific diffraction pattern of hydroxyapatite (Figure 5). That was because the concentration of silver nanoparticles that was added, which was 0.4% b/b, was too low. Yan *et al.* (2015) [7] reported that Hydroxyapatite composite, chitosan, and silver nanoparticle that coated TiO₂ with addition of 0.3% b/b silver nanoparticle did not show specific diffraction pattern of silver nanoparticles. But the antibacterial activity increased by 20% and the test on cell showed that cell durability increased by 10%.

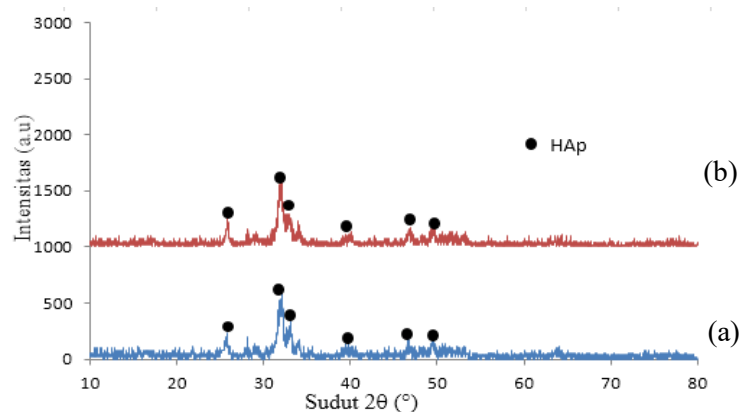


Figure 5. X-Ray diffraction pattern of HA (a) and HA-AgNPs composite (b).

Characterization using FTIR was done to determine functional group and interaction in HA-AgNPs composite. The result showed that the absorption bands of HA and HA-AgNPs composite was almost similar, that absorption band was dominated by apatite compound specific absorption but there was shift in wavenumber or the decrease of %transmittant of HA-AgNPs composite. Besides, there was absorption that showed the existence of aldehyde in HA-AgNPs composite (Figure 6). The shifting in wavenumber or decreasing of % transmittant could indicated the interaction between HA and AgNPs in the composite [7]. According to Vukamonovic *et al.* (2011)[13], shifting of OH⁻ ion strain that was in HA proved that there was interaction between HA and AgNPs. Besides, OH⁻ ion had an important role to control the size and stability of AgNPs in the composite. Decreasing of vibration from PO₄³⁻ indicated the decreasing of crystallinity that was caused by the addition of AgNPs.

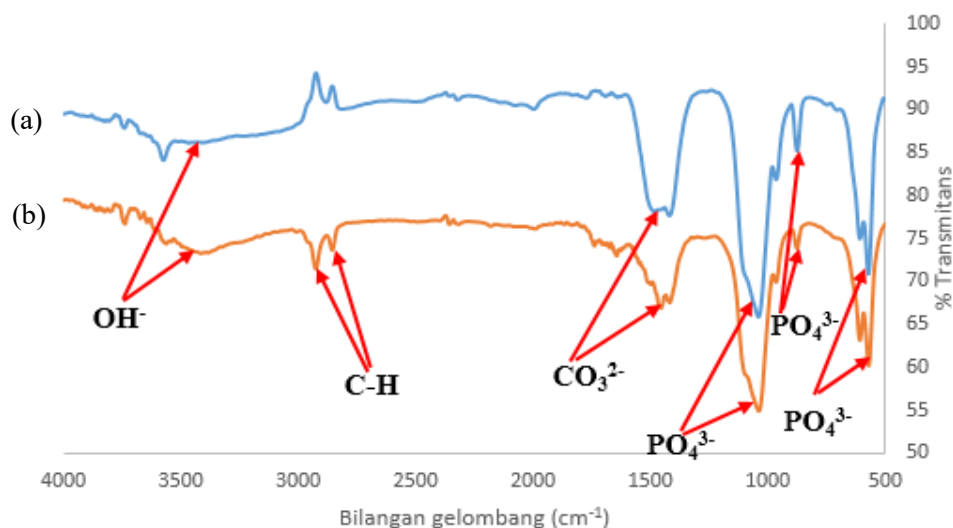


Figure 6. FTIR spectrum of HA (a) and HA-AgNPs Composite (b).

According to Meejo *et al.* (2006) [19], C-O stretching vibration for carbonate group usually appeared at wavenumber $1400\text{--}1600\text{ cm}^{-1}$. Characterization using FTIR on HA and HA-AgNPs composite showed the strain at 1423 cm^{-1} which was caused by CO_3^{2-} ion. CO_3^{2-} ion could cause the formation of carbonate apatite type A if CO_3^{2-} replaced OH^- position or carbonate apatite type B if CO_3^{2-} replaced PO_4^{3-} position in hydroxyapatite. Besides, HA-AgNPs composite showed C-H strain at 2850 and 2924 cm^{-1} . That strain resulted from monohydrate glucose that was used as AgNO_3 reductor. This caused HA-AgNPs composite had yellow color as the result of caramelization reaction of monohydrate glucose.

According to Santos *et al.* (2004) [12], synthesis of hydroxyapatite using coprecipitation method produced hydroxyapatite in the form of granule which size was not uniform. Characterization with SEM on HA and HA-AgNPs composite showed granules which was hydroxyapatite. But its size was still not homogen, granule size of HA was about $8\text{ }\mu\text{m}$, meanwhile HA-AgNPs composite size was $6\text{ }\mu\text{m}$. Surface morphology of HA-AgNPs composite was different from HA which was expected because the existence of silver nanoparticle on the surface of HA-AgNPs composite (Figure 7). Research by Vukamonovic *et al.* (2015) [20] showed that silver nanoparticle which was composited with hydroxyapatite caused hydroxyapatite size to become smaller and the silver nanoparticle was attached on the surface of hydroxyapatite.

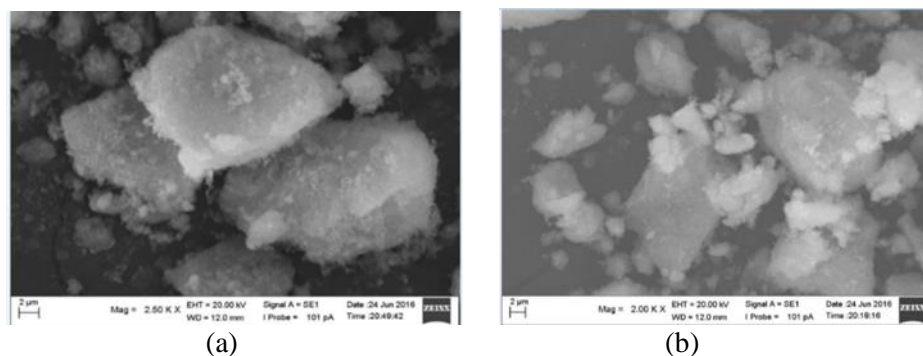


Figure 7. Surface morphology of HA (a) and HA-AgNPs composite (b).

3.4. Result of Antibacterial Activity Test.

Antibacterial activity test on silver nanoparticle colloid was done using disk method, meanwhile test on HA and HA-AgNPs composite was done using well method. The difference on the method was because silver nanoparticle colloid had liquid form meanwhile HA-AgNPs composite had powder form. But those two method had same principle which was antibacterial activity was showed by transparent zone diameter around the disk or well that was made. Transparent zone would appeared after incubation for 24 hours at 37°C. Bacteria that was used in this test was *Staphylococcus aureus*. Measurement was done twice with ruler.

Staphylococcus aureus was chosen because this pathogenic bacteria was the cause of most infection on bone implant. *Staphylococcus aureus* was positive gram bacteria and coccus- shaped. Important substances in bacteria cell wall structure was polysaccharide and protein. That substances, with peptidoglycan and teichoic acid formed a rigid cell wall. The main chain of teichoic acid had a lot of phosphate group, so it had negative charges and could interact electrostaticly with cationic antibacterial compound. Silver nanoparticle as antibacterial agent had been researched for quite a long time but the mechanism of AgNPs was only partly known. Some estimated mechanism of AgNPs was that AgNPs could prevent ATP formation and DNA replication in bacteria, and it could pass through the cell wall, make hole, and enter the cytoplasm. Then, AgNPs accumulation occurred on cell membrane and increased permeability which could cause the death of bacteria [21]. Other mechanism was that Ag ion penetrated cell membrane and reacted with -SH group of cell protein. This would cause uncoupling of ATP synthesis during cell respiration, proton loss, and phosphate efflux system disruption [15].

Antibacterial test result observation showed that there was transparent zone when AgNPs was tested, but test on HA and HA-AgNPs composite there was no transparent zone (Figure 8). Calculation of bacterial inhibition index on AgNPs was 0.33, meanwhile HA did not show bacterial inhibition index. When HA was composited with AgNPs, there was potential of forming antibacterial implant coating material. But the result showed that HA-AgNPs composite showed that there was no bacterial inhibition index. This was because the concentration of AgNPs was too low. Low AgNPs concentration caused released Ag ion could not damage bacteria cell wall so the growth of bacteria was not inhibited. According to Vukamonović *et al.* (2015) [20], composite of hydroxyapatite and silver nanoparticles with 10% b/b addition of silver nanoparticle had antibacterial properties because silver nanoparticle was released and could damaged bacteria cell wall. This was proved by SEM characterization result on bacteria that showed the damaged cell wall.



Figure 8. Antibacterial test result on AgNPs, HA, and HA-AgNPs.

4. Conclusion

Silver nanoparticles were successfully synthesized using reduction method with monohydrate glucose as reductor for AgNO_3 . Synthesized AgNPs size was ranging from 35-50 nm and had antibacterial activity which was stated in inhibition index of 0.33. But when composited to form HA-AgNPs, antibacterial activity was not detected. This was caused by low concentration of AgNPs and proved by XRD analysis result that did not show the diffraction pattern of AgNPs. FTIR analysis showed the interaction between HA and AgNPs. Characterization using SEM showed that HA-AgNPs nanoparticles were granules and had smaller size compared to HA, with size range between 1-6 μm .

References

- [1] Iwasaki T, Nakatsuka R, Murase K, Takata H, Nakamura H, Watano S 2013 *Journal Molecular Sciences* **14**(5) 9365-9378
- [2] Gopi D, Sathishkumar S, Karthika A, Kavitha L 2014 *Industrial & Engineering Chemistry Research* **53**(52) 20145–20153
- [3] Monmaturapoj N 2008 *Journal of Metals, Material and Minerals* **18**(1) 15-20
- [4] Ramli RA, Adnan R, Bakar MA, Masudi SM 2011 *Journal of Physical Science* **22**(1) 25-37
- [5] Sobczak A, Kowalski Z, Wzorek Z 2009 *Acta of Bioengineering and Biomechanics* **11**(4) 23-28
- [6] Agrawal K, Singh G, Puri D, Prakash S 2011 *Journal of Minerals & Materials Characterization & Engineering* **10**(8) 727-734
- [7] Yan Y, Zhang X, Li C, Huang Y, Ding Q, Pang X 2015 *Applied Surface Science* **332** 62-69
- [8] Haryono A, Dewi S, Harmami SB, Randy M 2008 *Jurnal Riset Industri* **2**(3) 156-163
- [9] Salomoni R, Leo P, Ro drigues MFA 2015 *The Battle Against Microbial Pathogens: Basic Sciences, Technological Advances, and Educational Programs* 851-857
- [10] Baby RL, Hasan I, Kabir KA, Naser MN 2010 *Journal of Scientific Research* **2**(2) 390–396
- [11] Sulistiawaty L, Sugiarti S, Darmawan N 2015 *Indonesian Journal of Chemistry* **15**(1) 1-8
- [12] Santos MH, de Oliveira M, SouzaL PF, Mansur HS, Vasconcelos WL 2004 *Materials Research*. **7**(4) 625-630
- [13] Vukomanović M, Bračko I, Poljanšek I, Uskoković D, Škapin SD, Suvorov D 2011 *Crystal Growth & Design* **11** (9) 3802-3812
- [14] Shankar SS, Rai A, Ahmad A, Sastry M 2004 *Journal of Colloid and Interface Science*. **275**(4) 496-502
- [15] Solomon SD, Bahadory M, Jeyarajasingam AV, Rutkowsky SA, Boritz C, Mulfinger L 2007 *Journal of Chemical Education* **84** (2) 322-325
- [16] Feldheim DL dan Foss CA 2002 *Metal Nanoparticles: Synthesis, Characterization, and Applications*. New York (US) : Marcel Dekker.
- [17] Shi Q, Vitchuli N, Nowak J, Noar J, Caldwell JM, Breidt F, Bourham M, McCord M, Zhang X 2011 *Journal of Materials Chemistry* **21** 10330-10335
- [18] Dankovich TA 2014 *Environmental Science Nano* **1** 367-378
- [19] Meejoo S, Maneeprakorn W, Winotai P 2006 *Thermocimica Acta* **447**(1) 115-120
- [20] Vukamonović M, Repnik U, Bergant TZ, Kostanjsek R, Skapin SD, Suvorov D 2015 *Biomaterials Science & Engineering* **1**(10) 935-946
- [21] Mocanu A, Furtos G, Rapuntean S, Horovitsz O, Flore C, Garbo C, Danisteanu A, Rapuntean G, Prejmerean C, Cotisel MT 2014 *Applied Surface Science* **298** 225-235