RESEARCH ARTICLE

Antibacterial Properties of Silver Nanoparticle (AgNPs) on Stainless Steel 316L

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ABSTRACT

Objective(s): In Indonesia, the incidence of bone injuries as a result of traffic accidents is quite high. This necessitates the use of bone implants, which are frequently made of Stainless Steel 316L (SS316L). The probability of contracting an infection when implanting an SS316L implant has been increasing. Infection due to implant placement is called osteomyelitis which is bone inflammation caused by biofilms formed by pyogenic bacteria. Biofilms can be prevented by giving antibacterial agents. This research aims to explore silver nanoparticles (AgNPs) as an antibacterial agent in SS316L implants.

Methods: AgNPs are synthesised using the Gallic acid reduction technique. AgNPs solution added with gelatin was misted on SS316L with five different precursor concentrations (0.1, 1, 10, and 100 mM) using the airbrush spray coating approach with a distance of 20 cm between the nozzle and the substrate and a pressure of 40 psi.

Results: AgNPs solutions produced from various concentrations of AgNO3 precursors have a broad spectrum of excitation maximums (λmax = 401.5 nm-424.5 nm) and crystallite size in the range of 0.97 - 4.88 nm. The AgNPs layer on SS316L was characterized for their crystalline phase, crystal size, and antibacterial activity. It has a cubic structure with a phase fraction of 6.5-19%. The inhibition zone radius for AgNPs coated samples is in the range of 12-16 mm. The combination coating of AgNPs (10 mM) and gelatin layer seemed to have the best antibacterial ability, with an inhibition zone diameter of 16.63 mm.

Conclusions: It is imperative to generate concentration variation of the 10 mM AgNPs precursor- Gelatin to be used to as coating layer on the SS316L restorative surface.

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INTRODUCTION

Fracture is a condition in which bone discontinuity occurs mostly caused by accidents at work, traffic, or other places. Other factors that may cause fractures include degenerative processes and pathology. With a population of 238 million people, the fracture cases in Indonesia have reached 1.3 million each year, which is the highest number in Southeast Asia [1]. Medical treatment for fractures is usually performed with surgical operations by installing bone implants commonly made of Stainless Steel 316L (SS316L). This type of stainless steel is used due to its low price, abundant availability, and relatively easy manufacture [2].

The increasing number of fractures has an impact on the high prevalence of metal implant use indicating the probability of acquiring infections associated with the increase of implant placement surgery. 2.6 million implants were installed each year in America, and approximately 112,000 (4.3%) of them contracted infections. It was due to osteomyelitis, an inflammation of the bones caused by biofilms formed by pyogenic bacteria.

Implant-related infections occur at the cellular level as a result of bacterial adhesion to the surface

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of the biomaterial implant. This occurs because, following implantation, there will be a competition between the implant material's ability to integrate with the surrounding tissue (osteointegration) and bacteria's ability to adhere to the biomaterial surface. If the materials and tissues are integrated first, bacteria have no chance of colonisation. On the other hand, if bacteria attach to the implant surface of the biomaterial, the tissue's immune system is frequently unable to prevent colonisation, which results in the formation of biofilms.Gristina in Evan M Hetrick [3] stated that 6 hours post-implantation is the period for preventing bacterial adhesion and a critical period for the long-term success of the implant. Bacteria that have colonized will form biofilms on the implant surface, and biofilms are highly resistant to immune response, and both systemic or local antibiotic therapy. The development of bacteria from adhesion, colonization, to biofilms is the main cause of infection in the bone implantation process. Pharmacy experts as well as other researchers realized that biofilms caused by bacterial colonization are resistant to antibiotics [4], therefore new antibacterial materials or agents are developed.

Nanomaterials are the next generation of antimicrobial agents because, in addition to having a high surface to volume ratio, they also possess exceptional chemical and physical properties [5]. Metals based nanomaterials such as copper, zinc, titanium, magnesium, gold, and silver are representative of nanomaterials utilized as antimicrobial agents [6-8]. Silver nanoparticles (AgNPs) are the most effective because they have good antimicrobial properties against bacteria, viruses, and other eukaryotic micro-organisms [8]. One of its applications in the health sector is as a material for implant surface modification with the aim for the implant to be more resistant to corrosion, to have osteointegration properties, and most importantly to be more resistant to bacteria causing infections.

Furthermore, research regarding AgNPs coating conducted by [4], using the electrophoretic deposition (EPD) method with various concentrations of AgNO3 and chitosan and gelatin on titanium alloy, showed that the addition of Ag as a coating material did not significantly change the mechanical properties between the coating and the substrate.

In the present study, the coating process of AgNPs on SS316L substrate was carried out via the airbrush spray method. Gelatin, a type of protein that is mostly obtained from natural collagen found in skin and bones, is needed to adhere AgNPs on the SS316L substrate. It can be used as a stabilizer, gelling agent, binder, thickener, emulsifier, adhesive, and wrapper [9]. Moreover, this protein can improve implant biocompatibility [4], The airbrush spray coating method is a coating technique where the coating liquid is applied through a spray gun with a certain pressure to the material surface. The method was selected based on the ease of operation of the airbrush, the results that were relatively smoother and easier to dry, and its ability to paint textured materials. This study will investigate the potential of the AgNPs-gelatin layer on the SS316L substrate as a result of coating via the airbrush spraying method as an antibacterial agent on bone implants.

MATERIAL AND METHODS

Materials

The materials used in the research are Stainless Steel (SS316L) with a diameter of 5 mm (ASTM A240/A240M), gelatin (SAP-G 003) Extra Pure for Analysis, 96% ethanol solution, distilled water, $AgNO_3$, 99%), 3,4,5-trihydroxy benzoic acid (Gallic acid, GA), Sodium Hydroxide (NaOH, 1%), and bacterial suspense of staphylococcus aureus.

AgNPs Synthesize

The synthesis of AgNPs was conducted using a chemical reduction method with gallic acid as the reducing agent (e.g. Fig. 1). 1.7 grams of $AgNO_3$ powder was dissolved in 100 ml of distilled water (100 mM) using a magnetic stirrer. Subsequently, gallic acid as much as 0.01 gram was dissolved in 10 ml of distilled water then mixed into the $AgNO_3$ solution to form AgNPs. Followingly, 1M NaOH solution was added gradually by using a dropper until the pH of the solution reached 11 [10]. Lastly, it was diluted 3 times to obtain AgNPs with different concentrations, namely 10 mM, 1mM, and 0.1 mM.

For this research, silver nanoparticles were synthesized by reducing them in the form of gallic acid.

The process of $AgNO_3$ reduction with gallic acid as the reducing agent, as shown in Fig. 2, begun when the dissolved gallic acid released 2 electrons and 2 H^+ ions forming the phenol functional group. Afterward, Ag^+ ions accepted electrons from gallic acid and left 1 H^+ ion which then reacted with

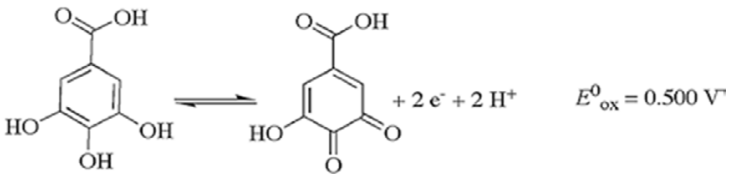

Fig. 1. The reduction process of $AgNO_3$ by Gallic Acid [11]

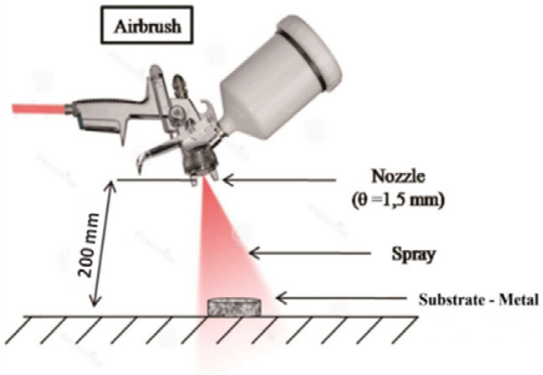

Fig. 2. The Set-Up of Coating Tool with Air Brush Spray Coating Method

NO_3^- ions to form nitric acid [11]. The addition of NaOH until reaching pH 11 aims to accelerate the reduction process because at that pH level the phenol functional group will be oxidized and release electrons [12]. Thereafter, the formed AgNPs were characterized by measuring their wavelength absorption using a UV-Vis spectrometer (Shimadzu UV-1800) as well as measuring the particle size of AgNPs using a Particle Size Analyzer (Malvern).

AgNPs-gelatin coating on SS316L substrate through the airbrush spray method

The SS316L with a diameter of 5 mm and 5 mm in thickness was firstly sanded and cleaned with ethanol then rinsed using distilled water. Before conducting the spray coating process, 2 grams of gelatin had been dissolved in 20mL distilled water at 50˚C for 30 minutes. The gelatin solution was mixed in the AgNPs solution as a binder with a mixture of AgNps and gelatin, 14 ml and 6 ml respectively. After the mixture had become homogeneous, spraying was carried out 3 times using airbrush spray with a distance between the nozzle and subtract of 200 mm at 40 psi (e.g. Fig. 2). Lastly, the AgNPs-coated SS316L samples were stored and dried at room temperature for 24 hours then the characterization process followed which was done through the XRD test and the antibacterial activity test.

Sample Characterization

Several tests were carried out to determine the characteristics of AgNPs and the AgNPs-gelatin layer. These tests include the UV-Vis spectrometer test (Shimadzu type UV-1800), Malvern Zetasizer (PSA) test, X-Ray Diffraction (XRD) test X-Pert Pro Analytical TYPE: PW3040/60 SN: DY 3574, and antibacterial test.

Uv- Vis Spectrophotometer Test

The purpose of the Uv-Vis spectrophotometric test is to determine the wavelength and the spectrum absorbance of $AgNO_3$ and AgNPs. The test was performed using the Shimadzu UV-1800 UV-Vis spectrophotometer. Initially, the sample of AgNPs solution was prepared then the absorbance level was determined at wavelength intervals of 355 - 550 nm for AgNPs and 200 - 300 nm for AgNO3 [13]. As stated before, the measurement data is in the form of absorbance level and wavelength, afterward, a comparison was made between the samples that had been made with the reference.

Particle Size Analysis (PSA)Test

The PSA test is used to ascertain the size and distribution of particles in a representative sample. The resulting image can be used to estimate the size distribution. For spherical-shaped particles, the size is expressed in radius. The characterization

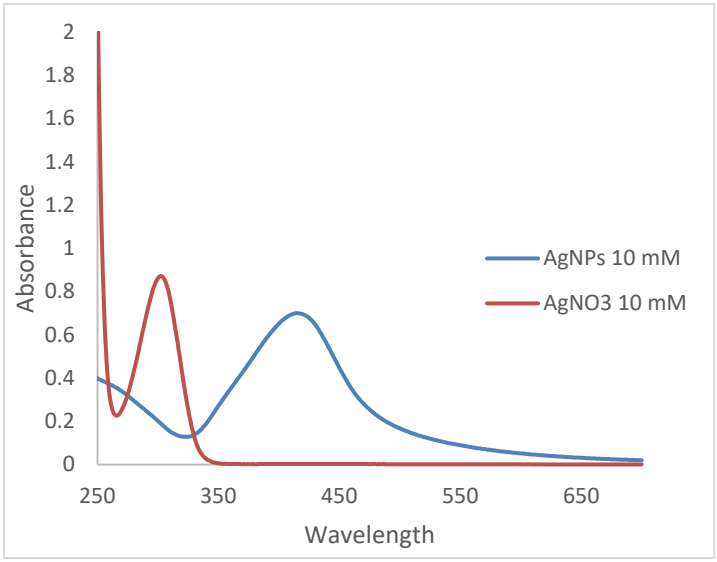

Fig. 3. The Spectrum Comparison of UV Vis Spectrophotometer Test Results between AgNPs and $AgNO_3$

in this test was carried out by using a Malvern Zetasizer tool. The sample tested is the $AgNO_3$ which has been reduced to be AgNPs by gallic acid. First, the AgNPs samples were dispersed with distilled water then they were put into a cuvet that later moved into the Malvern Zetasizer test kit. The measurement of the particle size and distribution via PSA test can also be done by ray scattering so that micro to nanometer particles can be measured [14].

Phase Analysis

X-ray diffraction (XRD, X-Pert Pro Analytical TYPE: PW3040 / 60 SN: DY 3574) was employed to verify the crystallographic properties of the AgNPs-gelatin layer. The XRD data were recorded this the range 2θ: 10-600 using Cu-Kα at 0.154 nm. The Crystal Size was determined using Equation (1):

$$D = \frac{K_B T}{6\pi\eta R_H} \quad (1)$$

Anti-bacterial Activity Test

This test was conducted using the diffusion method to determine the effect of increasing the precursor concentration on the samples' resistance levels in bacteria. A number of tested bacteria, namely *staphylococcus aureus*, were inoculated on agar media. *Staphylococcus aureus* bacteria were cultured on agar medium (TSA) for 24 hours at 37° C in an incubator, then dissolved in NaCl and the turbidity level was adjusted so that it gave a value comparable to 0.5 Mcfarland. Subsequently, the AgNPs-Gelatin-coated SS316L substrate was placed on the agar medium's surface that had solidified and then left in the incubator for 24 hours. After incubation, a clear zone would appear as an inhibition area that was not overgrown by bacteria around the substrate. The antibacterial potential was determined by the diameter of the inhibition area measured using a caliper. The larger the diameter of the inhibition area, the better the antibacterial activity of the nanosilver particles coated on the surface of the SS316L substrate. The five samples were subjected to this test which was done 3 times for each sample.

RESULT

AgNPs Characterization with UV-Vis Spectrophotometer

The synthesis results of AgNPs were compared with $AgNO_3$ at the same concentration to ensure that Ag^+ from $AgNO_3$ was reduced evenly and formed AgNPs at the peak absorbance test. The data results are depicted in the line graph below (Fig. 3).

A significant difference can be seen between $AgNO_3$, as the precursor, and AgNPs, as the reduction reaction result of gallic acid. The maximum wavelength (λmaks) of AgNPs obtained is 415.5 nm with an absorbance peak value of

Table 1. Visible Light Spectrophotometer Analysis Results for Each $AgNO_3$ Concentration

No	$AgNO_3$ Concentration (mM)	λmax (nm)	Peak Absorption	Sample Color
1	0,1	424.5 nm	0.080	Clear Yellow
2	1	401.5 nm	0.445	Bright Yellow
3	10	415.5 nm	0.700	Brown – Red
4	100	401.5 nm	3.116	Blackish Brown

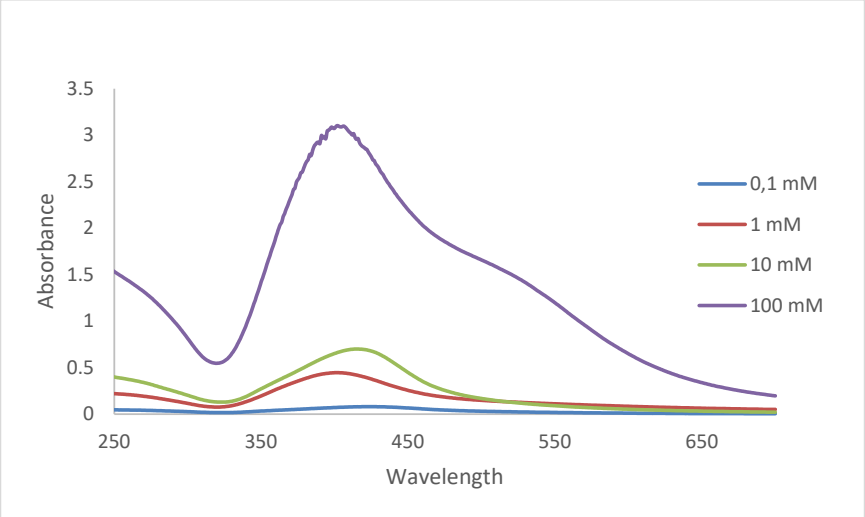

Fig. 4. The Spectrum Comparison of UV Vis Spectrophotometer Test Results of AgNPs at various concentrations

0.700. On the other hand, $AgNO_3$ has a maximum wavelength (λmaks) of 302.5 with an absorbance peak of 0.872. The results of the spectrophotometer analysis at 400 nm- 450 nm is the nanosilver of Ag^0, while Ag^+ was formed at 370 nm - 400 nm. This Ag^+ formation means that the chemical reduction process has not yet been completed. At 300 nm, the Ag particles in $AgNO_3$ did not undergo any reduction reactions.

From Table 1 and Fig. 4, it is known that the maximum wavelength of each sample ranges from 400-450 nm which is in accordance with the characteristics of the wavelength of AgNPs, namely 350 to 550 nm with a plasmon peak of approximately 450 nm on visible light spectrophotometer analysis [13]. Subsequently, the chemical reduction process by using $AgNO_3$ with various concentrations and gallic acid as the reducer has been successfully carried out and silver nanoparticles (AgNPs) have been obtained.

PSA Characterization

All concentration variations were subjected to PSA testing to determine its particle size distribution. The PSA test results are in the form of particle size and its distribution as presented in Table 2 and Fig. 4.

Table 2. AgNPs Sizes of Each $AgNO_3$ Concentration

No	$AgNO_3$ Concentrations (mM)	Particle Sizes (nm)	Particle Distributions (%)
1.	0,1	1,74	100
2.	1	1,92	100
3.	10	0,97	100
4.	100	4,88	100

All variations in the concentration of $AgNO_3$ formed AgNPs with sizes ranging from 0.9 to 4 nm, as presented in Table 2 and Fig. 5. Moreover, these synthesized AgNPs obtained from PSA have met the target. Hence, it can be said that these results are in line with the previous study results conducted by [15] with the same reduction method using gallic acid, namely AgNPs of 7 to 15 nm. The working principle of the particle size analyzer (PSA) is to

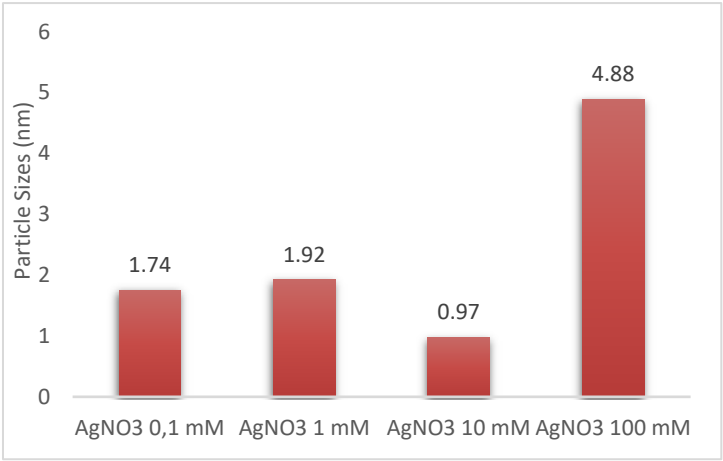

Fig. 5. Diagram of Precursor Concentrations and Particle Sizes of AgNPs

scatter laser light on the colloid-shaped sample so that the particle size distribution in the colloid can be revealed.

In orthopedic surgical procedure, the highly prevalent and serious complications is infection of the implant. Adhesion and bacterial colonisation are commonly associated with artificial implants and tissues nearby [16]. Ag treatment is a valid strategy for inhibiting biofilm formation and infection.In the form of ions or nanoparticles (AgNPs), Ag serves as a bactericidal agent that does not have resistance problems unlike some antibiotics [17]. Silver nanoparticles (AgNPs) are a developmental replacement for silver ions which have limited clinical applications due to their high cytotoxicity. They also have better physical and chemical stability than silver ions, as well as having excellent bactericidal properties, and low biological toxicity [18].

The synthesis process of AgNPs via the chemical reduction methods with variations in the concentration of the precursor and a reducing agent in the form of Gallic Acid produced particle sizes ranging from 0.97 nm to 4.88 nm (see Fig. 5). This is an advantage for the candidate material to be used as an antibacterial agent since the bacterial cell wall sizes ranging from 20 to 30 nm [19]. The synthesis of AgNPs using various concentrations as well as reducing agents conducted by [13] showed a linear trend. A higher concentration will be followed by an increase in particle size. However, in Figure 5, it can be seen that there was a decrease in particle size for the $AgNO_3$ precursor with a concentration of 10mM. Presumably, it was due to the on-going reduction reaction because of the absence of pH control [20].

X-Ray Diffractometer (XRD) Test of AgNPs

The XRD test on the solution was carried out to determine the phase difference between $AgNO_3$ and AgNPs. Initially, the synthesized AgNPs sample was dried to form powder using an oven at 60˚C for 36 hours.

As can be seen in the figure above, AgNPs have a cubical structure and show two intensity peaks, namely, at ($2\boldsymbol{\theta}$) 38.04° and 44.18°, respectively located in the Miller index hkl of (111) and (200). Based on Equation (1), the crystal size in (111) is 2.3659 Å and the crystal size in (200) is 2.0498 Å. These results are in accordance with the theory which states that the Miller index of the main peak owned by

Ag lies on d_{hkl} (111) and (200) which are respectively located at ($2\boldsymbol{\theta}$) 38° and 44° with a cubical structure [21]. Meanwhile, in the $AgNO_3$ spectrum, no peaks are formed at ($2\boldsymbol{\theta}$) 38° and 44° as a representation of the AgNPs peaks.

a. X-Ray Diffractometer (XRD) Test Results of AgNPs-gelatin layer

The X-Ray Diffraction (XRD) test was carried out to determine the formation of AgNPs in samples that have been successfully coated on the surface of the SS316L substrate by knowing their crystalline fields. This test was carried out on each sample as displayed in Fig. 7.

The analysis results, from Fig. 6, show that the phase of AgNPs has been formed on each layer on the SS316L substrate. The phase peaks formed from each concentration can be seen in Table 3.

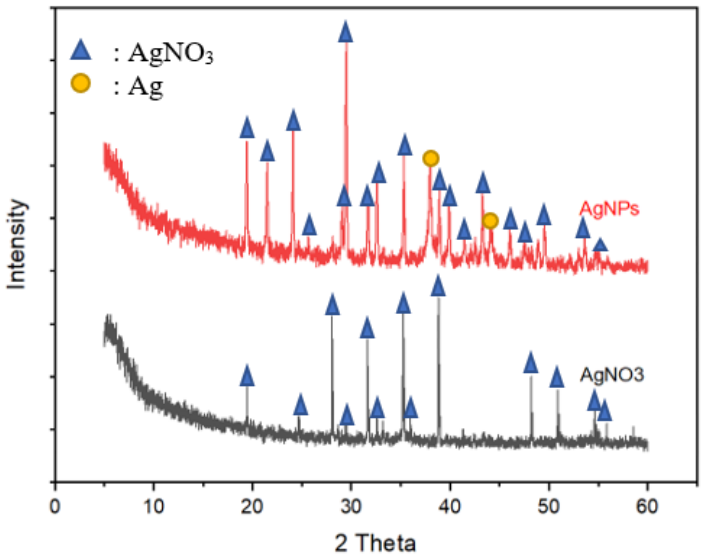

Fig. 6. The XRD Graph Plots between $AgNO_3$ Precursors and Synthesized AgNPs

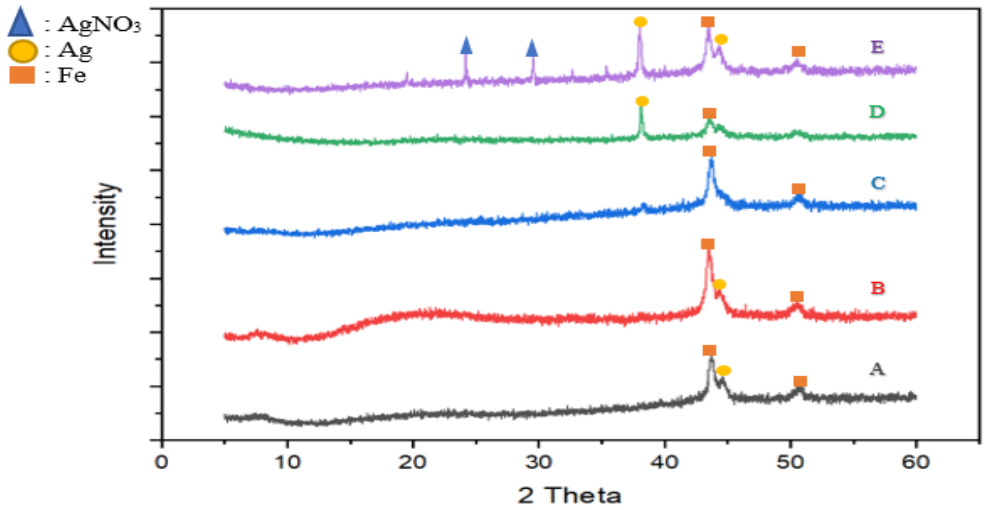

Fig. 7. The XRD Graph Plots of each sample with concentration variations, namely A (0,1 mM $AgNO_3$ + Gel), B (1 mM $AgNO_3$ + Gel), C (10 mM $AgNO_3$ + Gel), D (100 mM $AgNO_3$ + Gel), E (100 mM $AgNO_3$)

Table 3. Phase Identification of Silver Nanoparticle and SS316L

Sample	Phase (%)		
	AgNPs	*SS316L* (Fe)	$AgNO_3$
A	6,5	64,2	29,3
B	6,5	52,7	40,8
C	9,5	81,3	9,2
D	21,7	55,5	22,8
E	21,4	48,5	30,1

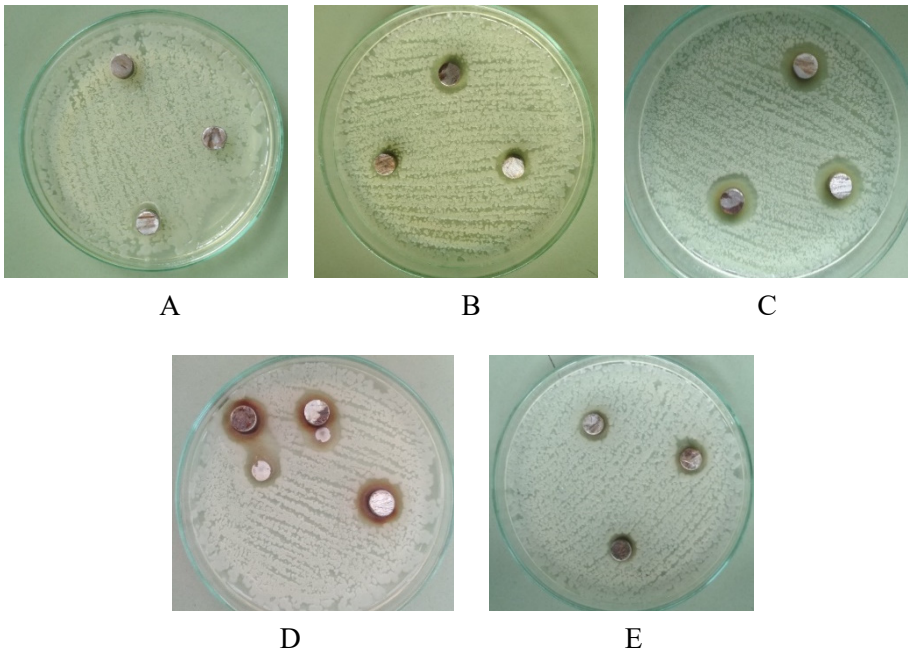

Fig. 8. Antibacterial Test Results for AgNPs - Gelatin Layer on SS316L Substrate (Personal Documentation, 2019)

Table 4. Inhibition Zone Diameter of AgNPs Layer on SS316L Substrate

Samples	Inhibition Zone Diameter (mm)
A	-
B	(12,41 ± 3,70)
C	(16,63 ± 0,90)
D	(16,26 ± 1,40)
E	(12,64 ± 3,50)

The characterization results of the two samples indicate that the layer covering the substrate was not completely Ag, but there were several peaks belong to $AgNO_3$. It was because there are particles experiencing agglomeration due to the ongoing reduction reaction, then in result subsiding its stability. The continuous reduction reaction results in smaller particle size. Furthermore, smaller particle sizes usually have worse stability [20]. The presence of the $AgNO_3$ phase is not a problem because $AgNO_3$ also has antibacterial abilities, although it is not as good as AgNPs [22]. In addition, $AgNO_3$ in certain concentrations is nontoxic to human body tissue, thus, it is safe to be applied as a coating for orthopedic implants [23].

b. Results of Antibacterial Characterization using the AgNPs-Gelatin Layer Diffusion Method

The antibacterial test was conducted to determine the samples' resistance level to bacteria. The gram-positive bacteria were used in this test, namely *staphylococcus aureus*. After the incubation process had been performed, the clear zone was observed and the diameter was measured as shown in Fig. 8 and Table 4.

AgNPs act as the antibacterial agent which was proven by the appearance of clear zones in the sample area (e.g. Fig. 8).

In Table 4, it is known that the analysis results on the clear zone diameter of each sample are quite good, marked by an inhibition zone of 10 - 20 mm

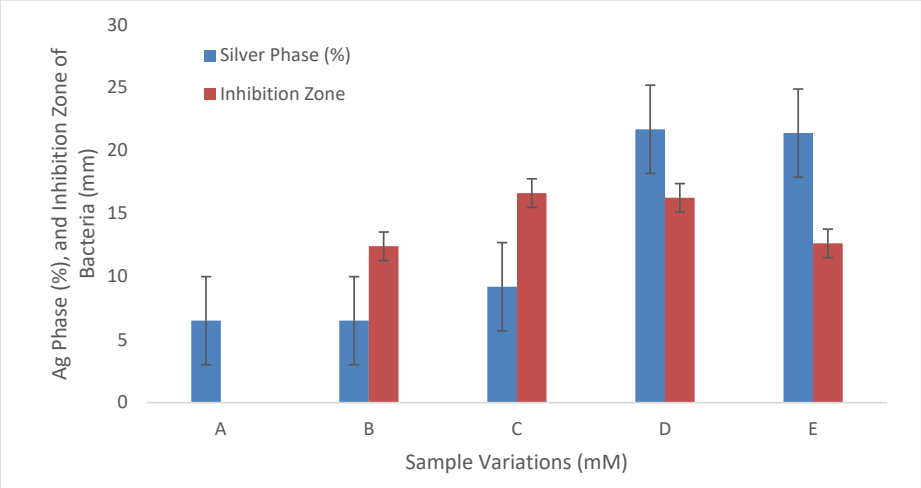

Fig. 9. Graph of Relationship between $AgNO_3$ Precursor's Concentrations, Ag Phase and Inhibition Zone Diameter

[24]. The antibacterial ability of AgNPs is directly proportional to the particle size of the compounds formed. The smaller the particle sizes, the better the antibacterial ability, while larger particle sizes in a decrease in the antibacterial ability of AgNPs. Small-sized particles have a large surface area which allows excellent contact with microorganisms [13]. It can also be seen that sample C has the best antibacterial ability with a clear zone inhibition value of 16.63 ± 0.9. This is because the sample has the best size, namely 0.9 nm. Meanwhile, sample B layer has the lowest antibacterial ability indicated by several points; first, it does not have an inhibition diameter value because of the small concentration; and second, a very thin layer was formed so that the ability to repel bacteria is not visible. This result is in accordance with the theory stating that smaller particle sizes have a larger surface area, in other words, it has the good ability as an antibacterial agent. On the other hand, sample D has a large diameter due to a shift in the sample in agar, resulting in uncertainty in the measurement process. As the precursor concentration increases, the phase of silver nanoparticle coating also rises. The relationship between the $AgNO_3$ concentration, the Ag phase (%), and the clear zone diameter (mm) is presented in Fig. 9.

The antibacterial activity test showed that the inhibition zone diameters were rather large, ranging from 12 to 16 mm. Fig. 8 showed that the ability of AgNPs as the antibacterial agent can be classified as good. It is also found that Sample C holds the best antibacterial ability with an inhibition zone diameter of 16.63 mm. This is because the 10mM solution has the smallest particle diameter which supports the previous theory stating that smaller particle sizes will have a larger surface area, indicating a better antibacterial ability.

AgNPs have been shown to have good antibacterial abilities, where the inhibitory activity of AgNPs against bacteria occurs through 4 stages of mechanism. First, AgNPs stick to the surface of the bacterial cell wall, then they enter the cell and interfere with the intracellular structures of bacteria such as mitochondria, vacuoles, ribosomes, as well as damage proteins, DNA in the cell nucleus so that the ability of bacteria to replicate is disrupted. Afterward, AgNPs induce cellular toxicity and free radical production of Reactive Oxygen Species (ROS) until the cells are damaged then AgNPs modulate cell signaling resulting in cell deaths [25].

The discharged AgNPs bind to and build up on the cell membrane, oxidising surface proteins and causing architectural changes in the cell membrane. Meanwhile, the Ag+ released by AgNPs damages the cell membrane's integrity, resulting in morphological changes and a significant increase in membrane permeability. Additionally, AgNPs and Ag+ produce reactive oxygen species (ROS) that have the potential to damage cell membranes. Membrane leaks can prevent energy-consuming reactions such as ATP synthesis, ion transport, and metabolite sequestration from occurring. Gentamicin enters bacterial cells more easily due to the increased permeability of the cell membrane. Gentamicin inhibits protein synthesis

via biochemical binding to two sites on ribosomes (h44 and h 69 in the 30S subunit and 50S subunit, respectively), thereby eliciting an antibacterial effect.In addition, AgNP and Ag^+ that enter cells can interact with intracellular enzymes causing damages to DNA and cellular metabolic disorders by inducing intracellular ROS. The AgNPs antibiotics interact more strongly with bacteria generating greater release of Ag^+, thereby producing high concentrations of Ag^+ around the bacterial cell walls that inhibit bacterial growth. The synergistic antibacterial mechanism between silver and antibiotic combination increases ROS, resulting in membrane damage after protein release, as well as leading to K^+ leakage, and biofilm inhibition [26].

CONCLUSION

AgNPs produced from the reduction reaction of $AgNO_3$ with Gallic acid have λmax ranging from 401.5 to 424.5 nm and a particle size of 0.97 - 4.88 nm. The AgNPs layer on SS316L formed cubic crystals with angles of $2\boldsymbol{\theta}$ = 38° and 44° and a phase fraction of 6.5% -19.0%. In addition, the antibacterial activity test showed a good inhibition zone diameter of 12-16 mm. To conclude, the optimal concentration as an antibacterial agent belongs to the 10 mM AgNPs + Gelatin sample that produced an inhibitory zone diameter of 16.63 ± 0.9 mm.

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CONFLICTS OF INTEREST

There are no undisclosed conflicts of interest for the authors.

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