Antibacterial Activity of Silver Nanoparticles Capped by *p*-Aminobenzoic Acid on *Escherichia coli* and *Staphylococcus aureus*

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Abstract: *This paper describes the antibacterial performance of silver nanoparticles (AgNPs) which have been synthesized by using* p-*aminobenzoic acid as a reducing and stabilizing agent simultaneously. The silver nitrate with various concentrations was reacted with pH 11-adjusted* p-*aminobenzoic acid with a concentration of* 5×10^{-3} *mol* L^{-1} *for 30 min in a boiling water bath. The synthesized AgNPs were characterized by UV-Vis spectrophotometry, Transmission Electron Microscope (TEM), and Particle Size Analyzer (PSA). The antibacterial performance of the synthesized AgNPs was evaluated by agar well diffusion method on* Escherichia coli *and* Staphylococcus aureus*. The higher silver nitrate concentration, the bigger the nanoparticle size, the wider particle size distribution, and the higher number of AgNPs formed. AgNPs synthesized from higher silver nitrate concentration had higher antibacterial activity. It is an indication that the antibacterial activity of AgNPs is mainly controlled by the silver ion concentration which influences the AgNPs particle size and the existence of silver ion in the AgNPs colloidal solution.*

Keywords: *antibacterial activity;* p-*aminobenzoic acid; silver nanoparticles*

■ INTRODUCTION

Silver has been known as an antibacterial agent for centuries. The advances in science and technology have been enabling the transformation of the bulk form of silver to become silver nanoparticles (AgNPs). Silver nanoparticles are preferable to be used as an antibacterial agent than silver in bulk form because they have a larger surface area to contact with bacteria [1]. Furthermore, AgNPs are also more desirable than using silver ions as antibacterial agents. The reason is that silver ions are relatively reactive and their binding with another ion may induce precipitation (for example, AgCl) and their interaction with protein (for example, albumin) can decrease their antibacterial efficacy and limit their applications [2-3].

In this research, *p*-aminobenzoic acid was used as a reducing and stabilizing agent in AgNPs synthesis. This synthesis has some advantages in the environmental perspective. First, it only uses one chemical reagent which can act simultaneously as reducing and stabilizing agents. Second, it provides a less toxic route of AgNPs synthesis because *p*-aminobenzoic acid is non-toxic and has been widely used in sunscreen and pharmaceutical products [4]. Third, this synthesis uses water as a solvent which is more environmental friendly than other solvents that have been used for AgNPs syntheses, such as ethylene glycol [5] and methanol [6]. Those three reasons make this synthesis fulfill the green chemistry principles which should be a concern in nanoparticle synthesis [7].

The study of AgNPs antibacterial activity on *Escherichia coli* (gram-negative bacteria) and *Staphylococcus aureus* (gram-positive bacteria) has been performed previously. AgNPs which were synthesized by $NaBH_4$ with size of about 13 nm had an excellent bactericidal performance on both bacteria [8]. However, this synthesis is less environmentally friendly because $NaBH_4$ is a hazardous material. AgNPs which were synthesized by chitosan had good antibacterial activity on *Escherichia coli* and *Staphylococcus aureus* [9]. Nevertheless, this synthesis took about 8 h. AgNPs which were biosynthesized using *Garciana mangostana* leaf extract as a reducing agent was found to be highly

effective against *Escherichia coli* and S*taphylococcus aureus* [10]. However, the effect of silver nitrate concentration and AgNPs particle size difference had not been explained yet.

The synthesis of AgNPs using *p*-aminobenzoic acid as a reducing and stabilizing agent was found to be environmentally friendly because of the use of a non-toxic chemical as a reducing and stabilizing agent and because the reaction happened in a short time. The synthesized AgNPs also had excellent stability for more than 16 weeks [11]. In this study, the antibacterial activity of AgNPs which were synthesized using *p*-aminobenzoic acid was evaluated. The concentration of silver nitrate was varied in order to study the effect of silver nitrate concentration on the particle size and antibacterial activity of the synthesized AgNPs. The synthesized AgNPs were characterized by UV-Vis spectrophotometry, Transmission Electron Microscope (TEM), and Particle Size Analyzer (PSA). The antibacterial performance was evaluated by in vitro agar well diffusion method against two bacteria, namely *Escherichia coli* (gram-negative bacteria) and *Staphylococcus aureus (*gram-positive bacteria*)*.

■ EXPERIMENTAL SECTION

Materials

The materials used in this research were silver nitrate (Merck) as precursor of AgNPs, *p*-aminobenzoic acid (Sigma Aldrich) as reducing and stabilizing agents, sodium hydroxide (Merck) as pH adjuster, Mueller-Hinton Agar (Merck), Brain Heart Infusion Broth (Merck), and gentamicin sulfate as positive control in antibacterial activity evaluation. All reagents were used as received without further purification.

Instrumentation

The characterization of AgNPs was performed by using UV-Vis Spectrophotometer (Shimadzu UV-Pharma Spec), TEM (JEOL JEM-1400), and PSA (Nanoparticle Analyzer Horiba SZ-100). The inhibition zone in antibacterial activity assay was observed using Darkfield Quebec Colony Counter.

Procedure

Synthesis of silver nanoparticles

The synthesis of AgNPs was performed according to the previous study [11] with some modification. Briefly, *p*-aminobenzoic acid (Mr = 137.14 g mol^{-1}) was weighed and dissolved in distilled water. After dissolved completely, the pH of the solution was adjusted to 11 by adding sodium hydroxide and an amount of distilled water was added to the solution in order to make a solution that has a concentration of 10×10^{-3} mol L^{-1}. The pH meter was calibrated with pH buffer of 4.01, 7.00, and 9.21 right before use. The pH-conditioned *p*-aminobenzoic acid solution was then poured to a reaction tube and added by silver nitrate solution (Mr = 170 g mol^{-1}) with various concentrations (0.2, 0.6, 1.0, 2.0, and 3.0×10^{-3} mol L^{-1}). The volume ratio of the reagent is 1:1 so the final concentration of reagents in the mixture are 5×10^{-3} mol L^{-1} for *p*-aminobenzoic acid and 0.1, 0.3. 0.5, 1.0, and 1.5×10^{-3} mol L^{-1} for silver nitrate. The solution was homogenized by shaking it several times and placed in a boiling water bath for 30 min. When the solution turned yellow, it meant that the AgNPs had been formed. After the synthesis was finished, the tube was cooled in tap water and the solution was moved to a storage bottle.

Characterization of silver nanoparticles

The synthesized AgNPs were analyzed by UV-Vis Spectrophotometry in order to monitor their surface plasmon resonance (SPR) absorbance. The resulted AgNPs were poured into a quartz cuvette with 1 cm optical path length and scanned at 200–800 nm wavelength. The scanning speed was fast with the wavelength interval of 1 nm. The SPR absorbance was shown by the absorbance peak in wavelength around 400 nm [12-13].

The shape of the synthesized AgNPs was analyzed by TEM. The AgNPs colloid was immersed by a copper grid and dried at room temperature. The image was taken by using 120 kV accelerating voltage. The size of the AgNPs was measured by using PSA based on dynamic light scattering principle.

Evaluation of the antibacterial performance of silver nanoparticles

The antibacterial performance of the synthesized AgNPs was evaluated by in vitro agar well diffusion method [14-15] with some modification against *Escherichia coli* (gram-negative bacteria) and *Staphylococcus aureus* (gram-positive bacteria). The culture media was made by dissolving Brain Heart Infusion Broth in water, heated, and boiled for 1 min in order to make sure it has been dissolved completely. The solution was then poured in a reaction tube and sterilized by autoclave with a pressure of 15 psi and a temperature of 121 °C for 15 min. The sterilized media was then inoculated by adding the bacteria and stored in the temperature of 37 °C. The Mueller-Hinton Agar was dissolved in water by heating and boiling it for one minute in order to dissolve the medium completely. It was sterilized using autoclave with the same condition as culture media sterilization and cooled in room temperature. The sterilized agar solution was then poured into sterile petri dishes with uniform depth and allowed to cool in room temperature. The plates were stored at 2–8 °C. The surface of the agar was inoculated entirely using a sterile swab which was stepped in the prepared bacteria suspension. A ring with a diameter of 6 mm was placed on the inoculated agar in order to form a well with a certain distance to the other wells. Each plate contained seven wells. Each well was injected by 50 µL solution of synthesized AgNPs as the sample, *p*-aminobenzoic acid as the negative control, and gentamicin 0.2 g L^{-1} as the positive control. The plates were then incubated at 37 °C for 24 h. After incubation, the inhibition zone diameter was measured by a ruler in mm unit. The obtained value was reduced by a correction factor of 6 mm as the well diameter. All tests were performed in duplicate.

■ RESULTS AND DISCUSSION

Synthesis and Characterization of Silver Nanoparticles

The AgNPs formation was indicated by the change of solution color from transparent to yellow and confirmed by their SPR absorbance. The SPR absorbance of AgNPs can be measured by UV-Vis Spectrophotometer based on many previous studies [16-21]. Synthesis of AgNPs was performed by reacting 5 × 10^{-3} mol L^{-1} *p*-aminobenzoic acid and silver nitrate at various concentrations, ranging from 0 to 1.5 × 10^{-3} mol L^{-1}. The synthesis was performed in the optimum condition based on the previous study [11]. The pH was 11 because the reaction can only take place in this condition. The reaction time was 30 min because longer reaction time did not give any significant increase on SPR absorbance.

The UV-Vis absorbance spectra of the synthesized AgNPs is shown in Fig. 1. The higher silver nitrate concentration will increase the SPR absorbance of the synthesized AgNPs. SPR absorbance values were 0.17, 181,

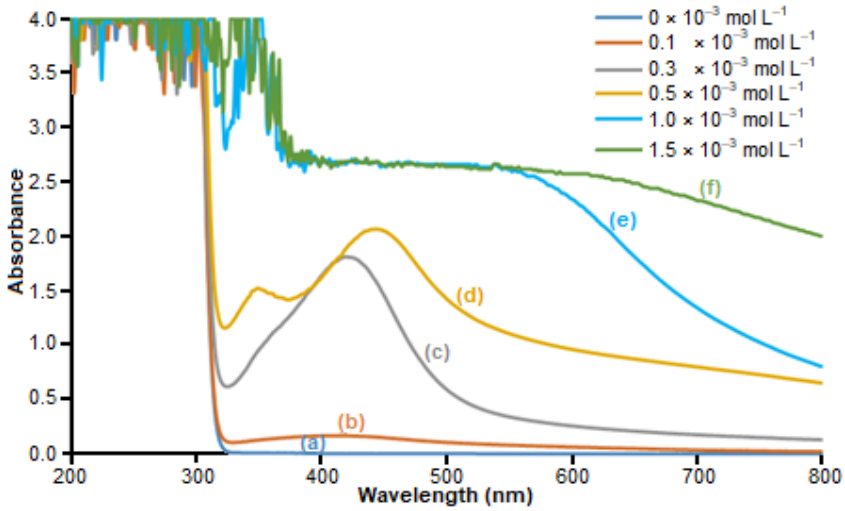

Fig 1. The UV-Vis absorbance spectra of silver nanoparticles synthesized from 5 × 10^{-3} mol L^{-1} *p*-aminobenzoic acid and silver nitrate 0 (a), 0.1 (b), 0.3 (c), 0.5 (d), 1.0 (e) and 1.5 (f) × 10^{-3} mol L^{-1}

and 2.066 for AgNPs synthesized from silver nitrate with a concentration of 0.1, 0.3, and 0.5 × 10^{-3} mol L^{-1}, respectively. The SPR absorbance for the AgNPs which were synthesized from silver nitrate with the concentration of 1.0 and 1.5 × 10^{-3} mol L^{-1} could not be determined because they were too high to be measured by the instrument. The higher SPR absorbance indicates the higher number of the synthesized AgNPs in the solution [22]. The concentration of the AgNPs was calculated based on the SPR absorbance referring to Paramelle et al. [21]. The molar concentrations of AgNPs were 21, 30, 40, 49, and 51 × 10^{-12} mol L^{-1} with yield percentage of 31, 49, 64, 44 and 33% for AgNPs synthesized from silver nitrate with concentration of 0.1, 0.3, 0.5, 1.0, and 1.5 × 10^{-3} mol L^{-1}, respectively. The AgNPs molar concentrations were increased with the silver nitrate concentration increment even though the yield percentage did not give the same pattern. It means that the higher silver nitrate concentration used in the synthesis will give a higher number of synthesized AgNPs. The same result was also published by previous studies where higher silver nitrate concentration would produce more AgNPs [19,23-25].

Besides SPR absorbance, SPR wavelength of the synthesized AgNPs was also observed. The higher SPR wavelength indicates the bigger particle size [5]. This phenomenon was also reported in AgNPs which were synthesized using sodium citrate [26], AgNPs-chitosan nanocomposites [19], AgNPs synthesized using guar gum [27] and citrate capped AgNPs [21]. In this study, AgNPs which were synthesized from higher silver nitrate concentrations had higher SPR wavelengths. They were 406, 420, and 444 nm for AgNPs which were synthesized from silver nitrate at the concentration of 0.1, 0.3, and 0.5 × 10^{-3} mol L^{-1}, respectively. This indicated that the AgNPs size increased along with the increase of silver nitrate concentration. The SPR absorbance band also became more extensive, which means that the particle size distribution also became broader along with the increase of silver nitrate concentration.

Further characterizations were performed in order to support the UV-Vis spectrophotometry results for AgNPs which were synthesized from lowest and highest silver nitrate concentrations. The AgNPs characterization was performed by TEM to observe the AgNPs morphology and PSA to measure the AgNPs diameter based on dynamic light scattering principle. The characterization result of the synthesized AgNPs can be seen in Fig. 2 and 3. TEM result showed that AgNPs which were originated from 0.1 × 10^{-3} mol L^{-1} silver nitrate had a uniform spherical shape with an average size of 35.9 nm and a polydispersity index of 0.277. While AgNPs which were originated from 1.5 × 10^{-3} mol L^{-1} silver nitrate had a spherical but not uniform shape with an average size of 113.0 nm and a polydispersity index of 0.283. These results were in agreement with the UV-Vis spectrophotometry result where higher silver nitrate concentration resulted in bigger size of AgNPs and broader particle size distribution.

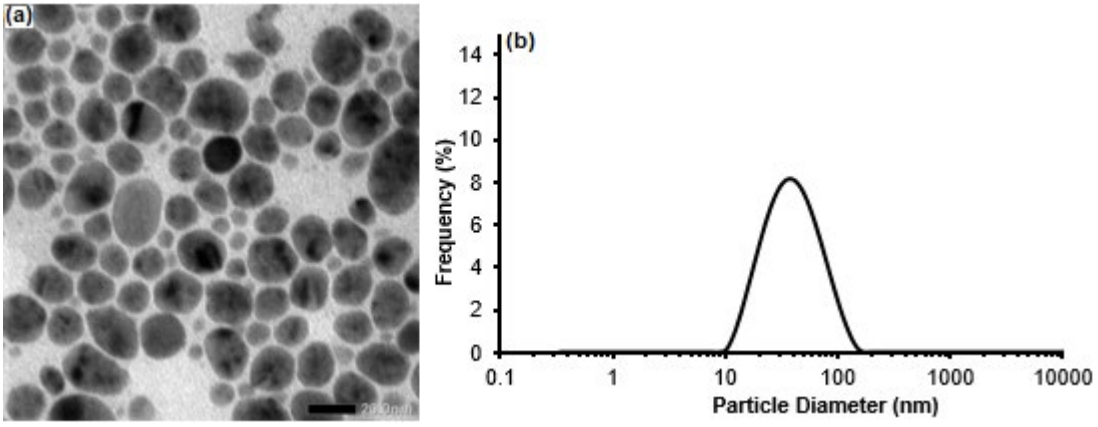

Fig 2. TEM image of silver nanoparticles synthesized from 0.1 × 10^{-3} mol L^{-1} $AgNO_3$ and 5 × 10^{-3} mol L^{-1} *p*-aminobenzoic acid with a magnitude of 150000× (a) and particle size distribution from PSA analysis (b)

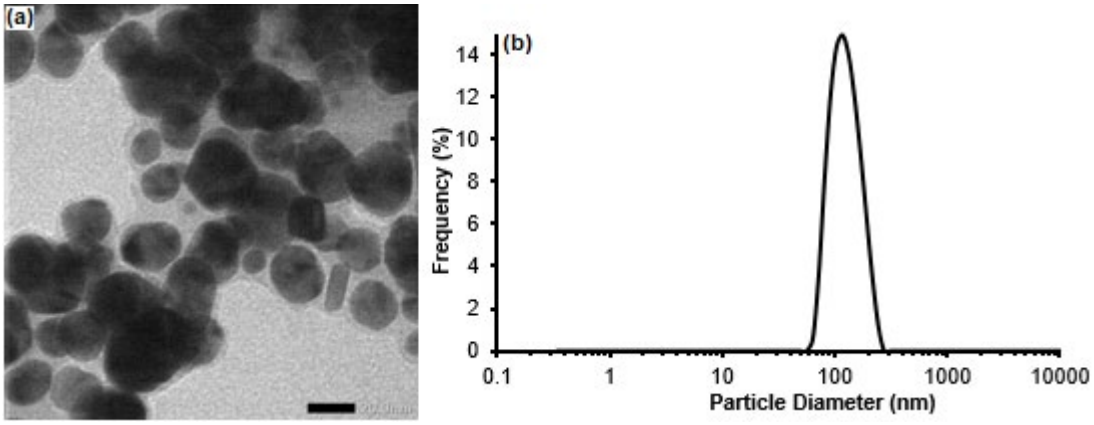

Fig 3. TEM image of silver nanoparticles synthesized from 1.5 × 10^{-3} mol L^{-1} $AgNO_3$ and 5 × 10^{-3} mol L^{-1} *p*-aminobenzoic acid with a magnitude of 150000× (a) and particle size distribution from PSA analysis (b)

The nanoparticle formation consists of three different processes; they are the reducing process, the particle growth, and the stabilizing process [28]. In this research, the reducing agent also acts as a stabilizing agent. In the appropriate reactant mole ratio, the reducing agent will reduce silver ion to become silver nanoparticle and stabilize it before it grows to become a bigger particle. The higher silver nitrate concentration will decrease the mole ratio of reactant. It means that the same amount of reducing agent molecules should react with more silver ions. In this condition, the reducing agent will be difficult to stabilize the formed silver nanoparticles. Thus, nanoparticles can grow to bigger particles. This phenomenon also has been reported in the synthesis of AgNPs by chitosan [29].

Antibacterial Activity of Silver Nanoparticles

Antibacterial activity of the synthesized AgNPs was evaluated by agar well diffusion method against two bacteria, namely *Escherichia coli* (gram-negative bacteria) and *Staphylococcus aureus* (gram-positive bacteria). The result is shown in Table 1. Gentamicin was used as a positive control corresponding to the previous study [8] because it has been widely used as a commercial antibiotic for *Escherichia coli and Staphylococcus aureus* [15]. Inhibition zones which were formed by AgNPs synthesized using higher silver nitrate concentration were bigger than inhibition zones which were formed by AgNPs synthesized using smaller silver nitrate concentration. Although the particles size was greater,

Table 1. The antimicrobial activity test result of silver nanoparticles (mean ± standard deviation)

Sample	*Escherichia coli*		*Staphylococcus aureus*	
	mm	%	mm	%
Gentamicin 0.2 g L^{-1} (positive control)	14.0 ± 0	100 ± 0	18.8 ± 0	100 ± 0
p-aminobenzoic acid 5 × 10^{-3} mol L^{-1} (negative control)	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.0 ± 0
AgNPs synthesized from $AgNO_3$ 0.1 × 10^{-3} mol L^{-1} and *p*-aminobenzoic acid 5 × 10^{-3} mol L^{-1}	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.0 ± 0
AgNPs synthesized from $AgNO_3$ 0.3 × 10^{-3} mol L^{-1} and *p*-aminobenzoic acid 5 × 10^{-3} mol L^{-1}	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.0 ± 0
AgNPs synthesized from $AgNO_3$ 0.5 × 10^{-3} mol L^{-1} and *p*-aminobenzoic acid 5 × 10^{-3} mol L^{-1}	1.0 ± 0	7.1 ± 0	0.0 ± 0	0.0 ± 0
AgNPs synthesized from $AgNO_3$ 1.0 × 10^{-3} mol L^{-1} and *p*-aminobenzoic acid 5 × 10^{-3} mol L^{-1}	2.0 ± 0	14.3 ± 0	3.8 ± 1.1	19.7 ± 5.6
AgNPs synthesized from $AgNO_3$ 1.5 × 10^{-3} mol L^{-1} and *p*-aminobenzoic acid 5 × 10^{-3} mol·L^{-1}	4.5 ± 0	32.1 ± 0	6.3 ± 0.4	32.9 ± 1.9

AgNPs which were synthesized from higher silver nitrate concentration gave higher antibacterial activity. This may indicate that the silver ion is the main actor in the bactericidal mechanism of AgNPs rather than the AgNPs themselves. Silver nanoparticles may act as a reservoir of silver ions which would interact with the bacteria by attacking their membrane, attacking the respiratory chain in their mitochondria, creating free radicals and induce oxidative stress and leading to cell death [1,3,30].

In the previous study, smaller AgNPs would give higher antibacterial activity because smaller AgNPs size has a higher surface area which can increase the contact site with the bacteria [31]. However, the result in this study was different. The higher antibacterial activity came from larger AgNPs which were synthesized from higher silver nitrate concentration. This indicates that the silver nitrate concentrations which were used in the synthesis process had significant roles in the antibacterial activity of AgNPs because it influences the AgNPs particle size and the existence of silver ions in the AgNPs colloidal solution.

The synthesized AgNPs had antimicrobial activity against both gram-negative and gram-positive bacteria. The inhibition zone diameter induced by the synthesized AgNPs against *Escherichia coli* bacteria was relatively smaller than the inhibition zone diameter against *Staphylococcus aureus* bacteria. This may be caused by the different membrane composition of those bacteria. *Staphylococcus aureus* as a gram-positive bacteria have a cell wall which is entirely composed by peptidoglycan layer. This layer is composed of networks of plenty of pores. These pores enable foreign molecules to come into the cell. On the other hand, *Escherichia coli* as a negative-gram bacteria have a thin membrane of peptidoglycan and an outer membrane, which consists of lipopolysaccharide, lipoprotein, and phospholipids. This outer membrane is a potential barrier against foreign molecules [32-33].

Compared to the previous study, a similar result was obtained from AgNPs which were synthesized using *Garciana mangostana* leaf extract as a reducing agent. The inhibition zones were 20 mm for *Staphylococcus aureus* and 15 mm for *Escherichia coli.* Furthermore, the antibacterial activity of AgNPs in this study was not as good as AgNPs-chitosan, AgNPs-cellulose, AgNPs-microcrystalline cellulose, or AgNPs-carboxymethyl cellulose which have inhibition zones ranged from 15–22 mm on *Staphylococcus aureus* and 13–20 mm on *Escherichia coli* [33]. The lower antibacterial activity of AgNPs can happen because this research used a threefold lower concentration of silver nitrate in AgNPs synthesis. Besides that, the high antibacterial activity may come from the initial antibacterial property of carboxymethyl cellulose and chitosan. However, the AgNPs should be synthesized first using glucose as a reducing agent and then impregnated to chitosan, cellulose, microcrystalline cellulose and carboxymethyl cellulose. This process took more than 6 h, a very long time compared to the AgNPs synthesis in the present study.

■ CONCLUSION

The antibacterial activity of AgNPs which were synthesized from silver nitrate and *p*-aminobenzoic acid has been evaluated against *Escherichia coli* and *Staphylococcus aureus* bacteria. Higher silver nitrate concentration gave bigger AgNPs size, wider particle size distribution, and a higher number of the formed AgNPs. Conversely, smaller silver nitrate concentration gave smaller AgNPs size, narrower particle size distribution, and a smaller number of the formed AgNPs. Nevertheless, AgNPs which were synthesized from more silver nitrate concentration had higher antibacterial activity. It is an indication that the antibacterial activity of AgNPs is mainly controlled by the silver ion concentration which influences the AgNPs particle size and the existence of silver ions in the AgNPs colloidal solution.

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