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Experimental

Antimicrobial effects of silver nanoparticles

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Abstract The antimicrobial effects of silver (Ag) ion or salts are well known, but the effects of Ag nanoparticles on microorganisms and antimicrobial mechanism have not been revealed clearly. Stable Ag nanoparticles were prepared and their shape and size distribution characterized by particle characterizer and transmission electron microscopic study. The antimicrobial activity of Ag nanoparticles was investigated against yeast, *Escherichia coli*, and *Staphylococcus aureus*. In these tests, Muller Hinton agar plates were used and Ag nanoparticles of various concentrations were supplemented in liquid systems. As results, yeast and *E. coli* were inhibited at the low concentration of Ag nanoparticles, whereas the growth-inhibitory effects on *S. aureus* were mild. The free-radical generation effect of Ag nanoparticles on microbial growth inhibition was investigated by electron spin resonance spectroscopy. These results suggest that Ag nanoparticles can be used as effective growth inhibitors in various microorganisms, making them applicable to diverse medical devices and antimicrobial control systems.

Key words: Ag nanoparticle; Antimicrobial effects

With the emergence and increase of microbial organisms resistant to multiple antibiotics, and the continuing emphasis on health-care costs, many researchers have tried to develop new, effective antimicrobial reagents free of resistance and cost. Such problems and needs have led to the resurgence in the use of Ag-based antiseptics that may be linked to broad-spectrum activity and far lower propensity to induce microbial resistance than antibiotics [1].

The antibacterial effects of Ag salts have been noticed since antiquity [2], and Ag is currently used to control bacterial growth in a variety of applications, including dental work, catheters, and burn wounds [3,4]. In fact, it is well known that Ag ions and Ag-based compounds are highly toxic to microorganisms, showing strong biocidal effects on as many as 12 species of bacteria including *E. coli* [5]. Recently, Mecking and co-workers showed that hybrids of Ag nanoparticles with amphiphilic hyperbranched macromolecules exhibited effective antimicrobial surface coating agents [6].

Reducing the particle size of materials is an efficient and reliable tool for improving their biocompatibility. In fact, nanotechnology helps in overcoming the limitations of size

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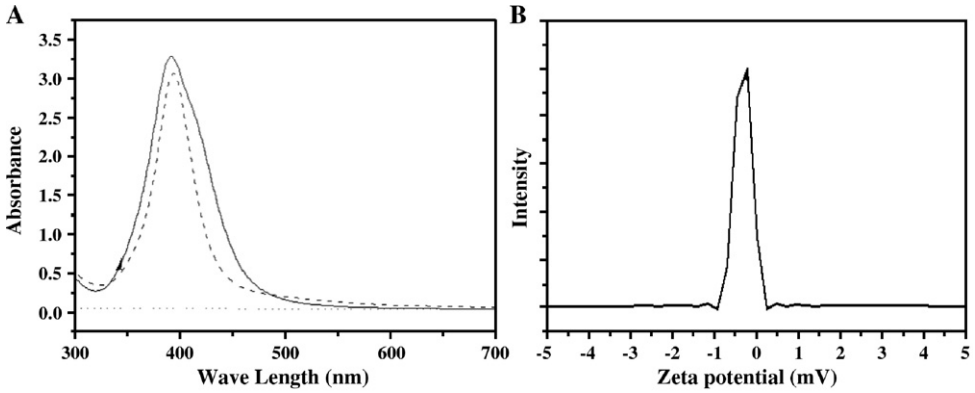

Fig 1. Absorption spectra of Ag nanoparticle solutions (**A**). The solid line is for Ag nanoparticle solution as prepared, the dashed one is for the ten-time concentrated one after diluted back to the original concentration, and the dotted one is for the solution left after the Ag nanoparticles are removed by sedimentation. The maximum potential peaks of Ag nanoparticles were measured at -0.33mV (**B**). The effective zeta-potential in aqueous solution were measured by particle characterizer "Delsa 440 SX" (Coulter Ltd., Miami, FL), and the mean values were averaged from 3 times assay data.

and can change the outlook of the world regarding science [7]. Furthermore, nanomaterials can be modified for better efficiency to facilitate their applications in different fields such as bioscience and medicine. In this study our research team, consisting of experts in several disciplines, has investigated the antimicrobial effects of Ag nanoparticles against representative microorganisms of public concern. A possibility of free-radical involvement near the Ag nanoparticle surface in the antimicrobial activity of Ag nanoparticles was discussed based on electron spin resonance (ESR) measurements. Here, we report that Ag nanoparticles can be applied effectively in the control of microorganisms and the prevention of deleterious infections. Our results support the hypothesis that Ag nanoparticles can be prepared in a simple and cost-effective manner and are suitable for formulation of new types of bactericidal materials.

Materials and methods

Preparation of Ag nanoparticles

Ag nanoparticles were made according to the recipe described in the literature [8,9]. Briefly, a 100-mL aqueous solution of 1.0×10^{-3} M silver nitrate was mixed with a 300-mL aqueous solution of 2.0×10^{-3} M sodium borohydride. Triply distilled water was used for solutions, and both solutions were chilled to ice temperature before mixing. By mixing both solutions, Ag ions were reduced and clustered together to form monodispersed nanoparticles as a transparent sol in aqueous medium. The Ag solution was yellow because of the absorption at ~390 nm. The solution was stirred repeatedly whenever some dark color appeared for approximately an hour until it became stabilized. At this point this solution of Ag nanoparticles was so stable that it did not change color for as long as several months without any stabilizing agent. Because the particle concentration of the solution is only 3.3 nM, it was concentrated 10 times using a rotary vacuum evaporator. Then, by diluting this solution, each sample of different concentration was used to investigate the concentration dependence of the antifungal effect of Ag nanoparticles.

Assay for antimicrobial activity of Ag nanoparticles against microorganisms

The antimicrobial activity of Ag nanoparticles was evaluated against yeast (isolated from bovine mastitis), *E. coli* O157:H8 (ATCC 43886), and *S. aureus* (ATCC 19636) by modification of the agar disk diffusion method of the National Committee for Clinical Laboratory Standards (NCCLS; now renamed as Clinical and Laboratory Standards Institute, CLSI, 2000). Approximately 10^7 colony-forming units of each microorganism were inoculated on Muller Hinton agar (MHA) plates, and then 20 μL of Ag nanoparticles were spread in a concentration of 0.2 to 33 nM. Itraconazol (for yeast, 33 nmol) and gentamicin (for *E. coli* and *S. aureus*, 33 nmol) were used as positive controls. The plates were incubated for 24 hours at 37°C.

To evaluate the growth inhibition of Ag nanoparticles, we designed a new method. After a 24-hour incubation, each plate was analyzed by LAS3000 (FUJI, Tokyo, Japan); Briefly, we analyzed the microorganism density at the center of the plate with Ag nanoparticles (A) and at the outer edge of the plate without Ag nanoparticles (B). The differences of microorganism density between (A) and (B) were measured and divided by the number of areas analyzed. The results on the three plates corresponding to a particular sample were averaged and this value regarded as the minimal inhibitory concentration (MIC) of Ag nanoparticles against each microorganism.

Electron spin resonance spectroscopy

The Ag nanoparticles were aggregated by stirring the yellow colloid solution with a Zn bar. Stirring with a Zn bar induced aggregation of the Ag colloid particles by breaking the charge balance between Ag nanoparticles without adding anything else. Other methods of obtaining aggregated Ag nanoparticles, such as adding salt or chemicals, were excluded so as to permit an accurate evaluation of the effect of Ag nanoparticles alone. Upon stirring the solution of Ag nanoparticles with a Zn bar, the solution turned dark brown, after which the aggregated Ag nanoparticles slowly settled down. The precipitated Ag nanoparticles were collected as a powder and packed into a glass capillary tube. Free-radical

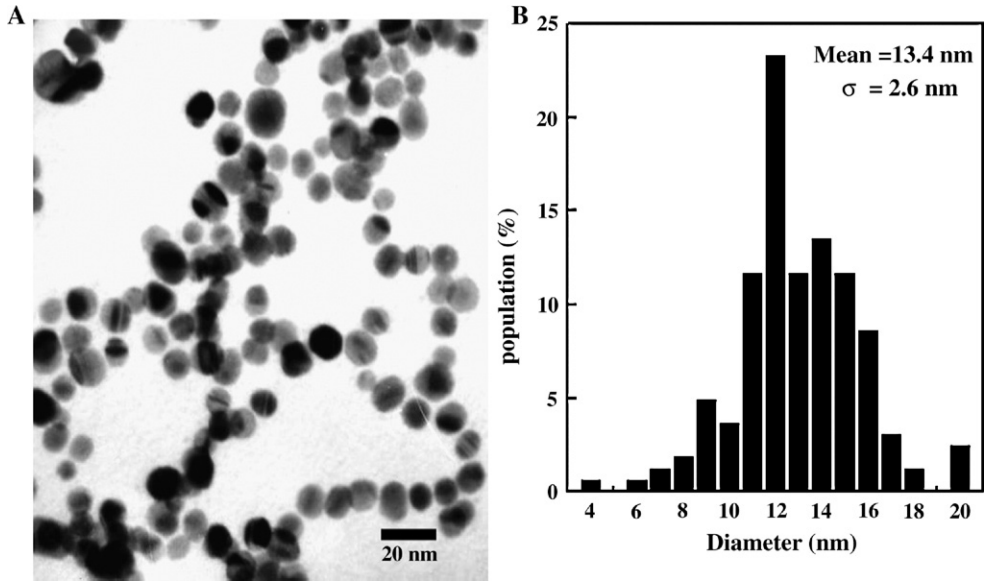

Fig 2. (**A**) A TEM image of Ag nanoparticles dispersed on a TEM copper grid (a, scale bar: 30 nm). (**B**) A histogram showing size distribution of Ag nanoparticles.

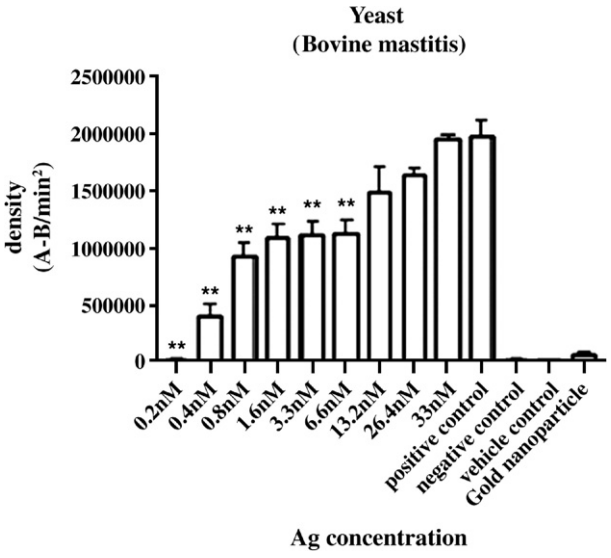

Fig 3. Growth inhibition of Ag nanoparticles against yeast. Itraconasol, distilled water, and solution devoid of Ag stand were used for the positive control, negative control, and vehicle control. The concentration of gold nanoparticles was 30 nM. Each point represents the mean ± SD. **: Significantly different from positive control ($P < .01$).

generation from Ag nanoparticles were recorded in an ESR spectrometer JES-TE 200 (JEOL, Tokyo, Japan). The ESR setting and experimental conditions are described in the figure legends.

Antioxidant effect of Ag nanoparticles and silver nitrate in antimicrobial activity

To confirm the effects of Ag nanoparticles, a comparative study of Ag nanoparticles and silver nitrate on antimicrobial activity against *E. coli* O157:H8 was performed. Approximately 10^7 colony-forming units of *E. coli* were inoculated on MHA plates, and then 20 μL of Ag nanoparticles and silver nitrate were spread

Table 1
MIC results of Ag nanoparticles

	MIC of Ag nanoparticles
yeast (ATCC19636)	>6.6 nM
E.coli (ATCC43890)	>3.3 nM
St. aureus (Bovine mastitis)	>33 nM

in the same concentration of 33 nM. *N*-acetylcysteine (NAC) as antioxidant was added in the plates at 10 and 50 nM [10]. The plates were incubated for 24 hours at 37°C.

Results

Characterization of the synthesized Ag nanoparticles

The prepared aqueous solution of Ag nanoparticles showed an absorption band at 391 nm as shown in Figure 1, which is a typical absorption band of spherical Ag nanoparticles due to their surface plasmon [8]. The stability of the concentrated solution was checked by observing its absorption spectrum after rediluting 10 times. The absorption spectrum of the rediluted solution depicts almost identical spectral features to the spectrum of the original solution of Ag nanoparticles (Figure 1, *A*). This confirms that the Ag nanoparticles are not further dimerized or agglomerated with many particles together, in that a new absorption band, appearing to the red side of the band at ~390 nm because of a localized surface plasmon between two or more Ag nanoparticles in contact with one another when dimerization or aggregation of Ag nanoparticles occurs, is not observed. The absorption spectrum of the solution remaining after Ag nanoparticles had been completely sedimented and removed by stirring the solution with a Zn rod was obtained to confirm that there were no Ag nanoparticles in the solution. Then this solution was used as

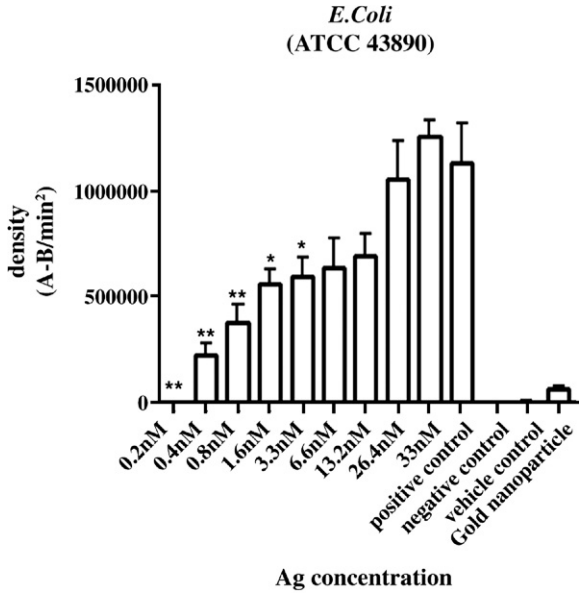

Fig 4. Growth inhibition of Ag nanoparticles in *E. coli*. Gentamycin, distilled water and solution devoid of Ag stand for a positive control, negative control and vehicle control. The concentration of gold nanoparticles is 30 nM. Each point represents the mean ± SD. *: Significantly different from positive control ($P < .05$). **: Significantly different from positive control ($P < .01$).

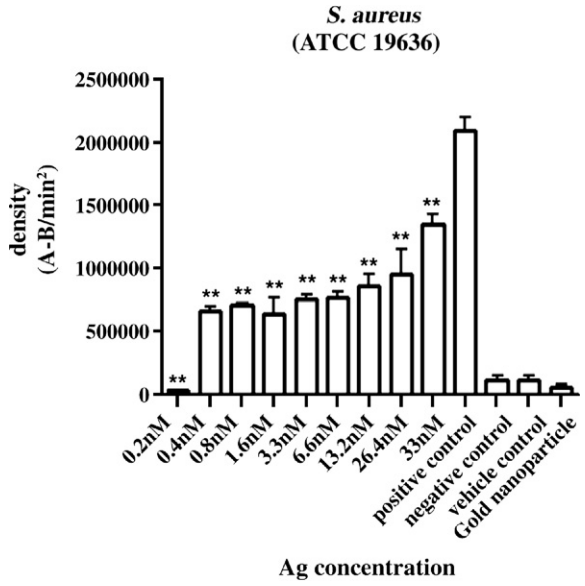

Fig 5. Growth inhibition of Ag nanoparticles in *S. aureus*. Gentamycin, distilled water and solution devoid of Ag stand for a positive control, negative control and vehicle control. The concentration of gold nanoparticles is 30 nM. Each point represents the mean ± SD. **: Significantly different from positive control ($P < .01$).

a vehicle control to confirm that the other salts such as nitrate, borate, and sodium ions included in the Ag nanoparticle solution during preparation of the nanoparticles did not affect the antimicrobial activity. Surface zeta potential of Ag nanoparticles was measured to be slightly negative (Figure 1, *B*). In the colloid solution, there exist nitrate and borate ions adsorbed on the surface of Ag nanoparticles with the result that the surface charge of the Ag nanoparticles will be slightly negative. Shape and size distribution of the synthesized Ag nanoparticles were characterized by transmission electron microscopic (TEM) study. A few drops of Ag nanoparticle solution were dropped onto a TEM grid, and the residue was removed by a filter paper beneath the TEM grid. The TEM image shown in Figure 2, *A* was obtained by high-resolution TEM (JEOL, JEM-2000E7). As can be seen by the shape and size distribution in Figure 2, *B*, the particles are highly monodispersed with an average diameter of 13.5 nm and a standard deviation of 2.6 nm.

Antimicrobial activity of Ag nanoparticles against microorganisms

Antimicrobial tests were performed against yeast, *E. coli*, and *S. aureus* on MHA plates treated with different concentrations of Ag nanoparticles (from 0.2 to 33 nM). Yeast was isolated from bovine mastitis. Comparing with the positive control, itraconazole, Ag nanoparticles of 33 nM showed a similar growth inhibition effect against yeast, and significant growth inhibition was observed from 13.2 nM (Figure 3). These results revealed that the MIC of Ag nanoparticles against yeast may be estimated between 6.6 nM and 13.2 nM in this condition (Table 1). For *E. coli*, we used *E. coli* O157:H7, which is known as a notorious pathogen causing hemorrhagic enteritis. In our results, Ag nanoparticles were most effective against *E. coli* (Figure 4 and Table 1). The MIC of Ag nanoparticles against *E. coli* may be estimated between 3.3 nM and 6.6 nM, and the growth inhibition effect was observed in a concentration-dependent manner. For *S. aureus*, however, Ag nanoparticles showed a mild growth-inhibitory effect even in high concentration, and there was no statistically significant inhibitory effect compared with the control (gentamicin) in this condition (Figure 5). MIC of Ag nanoparticles against *S. aureus* was estimated to be more than 33 nM (Table 1). Also, there is no antimicrobial activity in solution devoid of Ag nanoparticles used as a vehicle control, reflecting that antimicrobial activity was directly related to the Ag nanoparticles. To determine whether the growth-inhibitory effect of Ag nanoparticles is a specific event or not, we used gold (Au) nanoparticles (~30 nM) as another control of nanosized metals. Au nanoparticles showed no growth-inhibitory effect against various microorganisms in our experimental conditions (Figures 3-5).

ESR study of Ag nanoparticles

The mechanism of the growth-inhibitory effects of Ag nanoparticles on microorganisms has not been well understood. One possibility is that the growth inhibition may be related to the formation of free radicals from the surface of Ag. Uncontrolled generation of free radicals can attack membrane lipids and then lead to a breakdown of membrane function [11]. To obtain insight into this possibility, we measured the ESR spectra of Ag nanoparticles. Ag samples

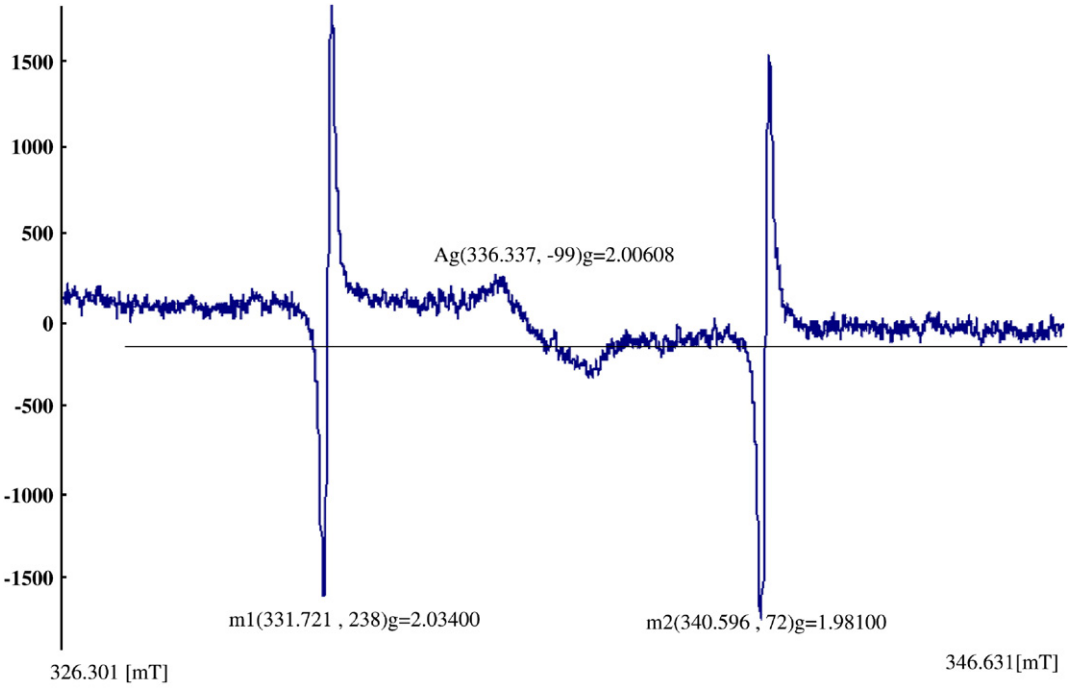

Fig 6. The ESR spectrum of Ag nanoparticles recorded at room temperature. m1 and m2 indicate the control peak of Mn and the peak (mT: 336.337) indicates the released free radical from Ag nanoparticles. The ESR spectral was obtained at room temperature. Instrumental setting of JEOL JES-TE 200 spectrometer: microwave power, 8.00 mW, MOD, 100 kHz, and time constant: 0.3 sec.

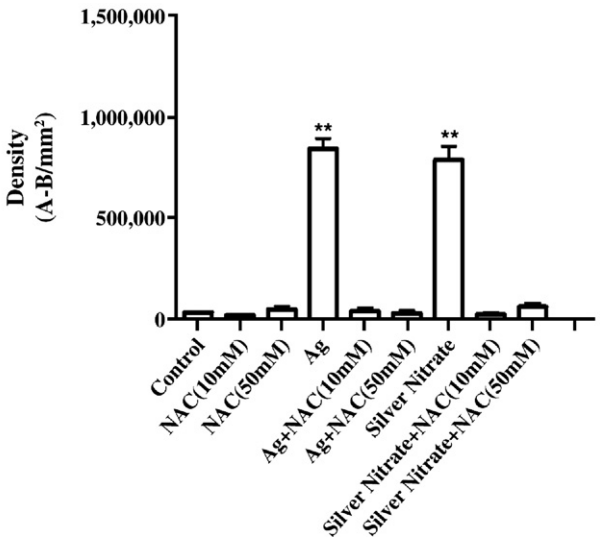

Fig 7. Comparative study of Ag nanoparticles and silver nitrate in growth inhibition with/without *N*-acetylcystein (NAC). Solution devoid of Ag stand for a control. The concentration of Ag nanoparticles and silver nitrate is 33 nM. Each point represents the mean ± SD. **: Significantly different from control ($P < .01$).

were prepared in powder form by stirring the Ag nanoparticles solution with a Zn bar, causing the Ag nanoparticles to aggregate. In Figure 6, peaks of m1 and m2 indicate the control peaks of standard manganese, and the central peak (mT: 336.337) indicates the existence of free radicals from Ag nanoparticles, thus supporting that free-radical generation of Ag nanoparticles may be responsible for the antimicrobial effects.

Antioxidant effect of Ag nanoparticles and silver nitrate in antimicrobial activity

To determine the relationship between free-radical and antimicrobial activity, we used the antioxidant NAC to test whether the antioxidant could influence antimicrobial activity induced by Ag nanoparticles. In Figure 7, Ag nanoparticles and silver nitrate showed similar growth-inhibitory effect against *E. coli*. However, such inhibitory effect was abolished by the addition of NAC. NAC alone did not affect the antimicrobial activity.

Discussion

It is well known that Ag ions and Ag-based compounds have strong antimicrobial effects [12], and many investigators are interested in using other inorganic nanoparticles as antibacterial agents [4,12-14]. These inorganic nanoparticles have a distinct advantage over conventional chemical antimicrobial agents. The most important problem caused by the chemical antimicrobial agents is multidrug resistance. Generally, the antimicrobial mechanism of chemical agents depends on the specific binding with surface and metabolism of agents into the microorganism. Various microorganisms have evolved drug resistance over many generations. Thus far, these antimicrobial agents based on chemicals have been

effective for therapy; however, they have been limited to use for medical devices and in prophylaxis in antimicrobial facilities. Therefore, an alternative way to overcome the drug resistance of various microorganisms is needed desperately, especially in medical devices, etc. Ag ions and Ag salts have been used for decades as antimicrobial agents in various fields because of their growth-inhibitory capacity against microorganisms. Also, many other researchers have tried to measure the activity of metal ions against microorganisms. Studies of Russel and Hugo on the antimicrobial properties of Ag and Cu [15], and of Marsh on Zn were reported [16]. However, Ag ions or salts has only limited usefulness as an antimicrobial agent for several reasons, including the interfering effects of salts and the antimicrobial mechanism of [the continuous release of enough concentration of Ag ion from the metal form.] In contrast, these kinds of limitations can be overcome by the use of Ag nanoparticles. However, to use Ag in various fields against microorganisms, it is essential to prepare the Ag with cost-effective methods and to know the mechanism of the antimicrobial effect. Besides, it is important to enhance the antimicrobial effect. In this study we report that Ag nanoparticles can be prepared cost effectively and that these Ag nanoparticles are homogeneous and stable (Figures 1 and 2). The nanosize allowed expansion of the contact surface of Ag with the microorganisms, and this nanoscale has applicability for medical devices by surface coating agents.

In this study, to evaluate the antimicrobial effects against various microorganisms, we used three representative microorganisms, yeast, *E. coli* and *S. aureus*. There were distinct differences among them. When Ag nanoparticles were tested in yeast and *E. coli*, they effectively inhibited bacterial growth. In our results, Ag nanoparticles showed antimicrobial activity against yeast and *E. coli* (Figures 3 and 4) that was similar to that found by Sondi and Salopek-Sondi [17]. In contrast, the inhibitory effect of Ag nanoparticles was mild in *S. aureus* as compared with other microorganisms; these results suggest that the antimicrobial effects of Ag nanoparticles may be associated with characteristics of certain bacterial species. Gram-positive and gram-negative bacteria have differences in their membrane structure, the most distinctive of which is the thickness of the peptidoglycan layer. We think that the lower efficacy of the Ag nanoparticles against *S. aureus* may derive from the difference as a point of membrane structure. To confirm this hypothesis, further comparative study between various gram-negative and gram-positive bacterial species is needed. The peptidoglycan layer is a specific membrane feature of bacterial species and not mammalian cells. Therefore, if the antibacterial effect of Ag nanoparticles is associated with the peptidoglycan layer, it will be easier and more specific to use Ag nanoparticles as an antibacterial agent.

To identify the difference of antimicrobial activity between Ag nanoparticles and other metallic nanoparticles, we carried out the growth inhibition test with 30 nM Au nanoparticle. We also wanted to estimate the potential side effects of other components which might be included in the Ag nanoparticles solution. Thus, we prepared vehicle control, in which the Ag nanoparticles were removed by sedimentation. Our results indicate no crucial antimicrobial activity in either Au nanoparticles or vehicle control, suggesting that the antimicrobial activity of Ag nanoparticles is an Ag-specific event in our experimental conditions (Figures 3-5).

The mechanism of the inhibitory effects of Ag ions on microorganisms is partially known. Some studies have reported that the positive charge on the Ag ion is crucial for its antimicrobial activity through the electrostatic attraction between negative charged cell membrane of microorganism and positive charged nanoparticles [14,18,19]. In contrast, Sondi and Salopek-Sondi [17] reported that the antimicrobial activity of silver nanoparticles on Gram-negative bacteria was dependent on the concentration of Ag nanoparticle, and was closely associated with the formation of 'pits' in the cell wall of bacteria. Then, Ag nanoparticles accumulated in the bacterial membrane caused the permeability, resulting in cell death. However, because those studies included both positively charged Ag ions and negatively charged Ag nanoparticles, it is insufficient to explain the antimicrobial mechanism of positively charged Ag nanoparticles. Therefore, we expect that there is another possible mechanism. Amro et al. suggested that metal depletion may cause the formation of irregularly shaped pits in the outer membrane and change membrane permeability, which is caused by progressive release of lipopolysaccharide molecules and membrane proteins [20]. Also, Sondi and Salopek-Sondi speculate that a similar mechanism may cause the degradation of the membrane structure of *E. coli* during treatment with Ag nanoparticles [17]. Although their inference involved some sort of binding mechanism, still unclear is the mechanism of the interaction between Ag nanoparticles and component(s) of the outer membrane. Recently, Danilczuk and co-workers [21] reported Ag-generated free radicals through the ESR study of Ag nanoparticles. We suspect that the antimicrobial mechanism of Ag nanoparticles is related to the formation of free radicals and subsequent free radical–induced membrane damage. To confirm the production of free radical, we analyzed the Ag nanoparticles by ESR. In Figure 6, we observed an Ag-specific ESR spectrum. The obtained peak of Ag nanoparticles in an ESR assay corresponded with that obtained by Danilczuk et al [21]. To determine the relationship between free-radical and antimicrobial activity, we used the antioxidant NAC to test whether the antioxidant could influence Ag nanoparticles–induced antimicrobial activity. Figure 7 shows that similar antimicrobial activity was observed between Ag nanoparticles and silver nitrate against *E. coli*. However, the antimicrobial activity of Ag nanoparticles and silver nitrate was influenced by NAC. The results of ESR and antioxidant study suggest that free radicals may be derived from the surface of Ag nano-

particles and be responsible for the antimicrobial activity in our experimental conditions.

In conclusion, Ag nanoparticles prepared by the cost-effective reduction method described here have great promise as antimicrobial agents. Applications of Ag nanoparticles based on these findings may lead to valuable discoveries in various fields such as medical devices and antimicrobial systems.

Acknowledgments

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