

Synergistic effects between silver nanoparticles and antibiotics and the mechanisms involved

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Silver nanoparticles (nano-Ags), which have well-known antimicrobial properties, are used extensively in various medical and general applications. In this study, the combination effects between nano-Ags and the conventional antibiotics ampicillin, chloramphenicol and kanamycin against various pathogenic bacteria were investigated. The MIC and fractional inhibitory concentration index (FICI) were determined to confirm antibacterial susceptibility and synergistic effects. The results showed that nano-Ags possessed antibacterial effects and synergistic activities. The antibiofilm activities of nano-Ags alone or in combination with antibiotics were also investigated. Formation of biofilm is associated with resistance to antimicrobial agents and chronic bacterial infections. The results indicated that nano-Ags also had antibiofilm activities. To understand these effects of nano-Ags, an ATPase inhibitor assay, permeability assay and hydroxyl radical assay were conducted. The antibacterial activity of nano-Ags was influenced by ATP-associated metabolism rather than by the permeability of the outer membrane. Additionally, nano-Ags generated hydroxyl radicals, a highly reactive oxygen species induced by bactericidal agents. It was concluded that nano-Ags have potential as a combination therapeutic agent for the treatment of infectious diseases by bacteria.

Received 13 May 2012
Accepted 5 September 2012

INTRODUCTION

Silver and its compounds are known to have antimicrobial properties. Early in the 19th century, 0.5 % AgNO₃ was used for the treatment and prevention of microbial infections such as Ophthalmia neonatorum (by German obstetrician Carl Crede). When the era of antibiotics began with the discovery of penicillin, the use of silver slowly diminished (Klasen, 2000), but currently, due to the lack of effectiveness of conventional drugs, the use of silver for treating infections has regained importance. However, the use of ionic silver has one major flaw: it is easily inactivated by complexation and precipitation. As a result, the use of silver ions has been limited (Atiyeh *et al.*, 2007).

Silver nanoparticles (nano-Ags), which are zerovalent, can be a valuable alternative to ionic silver (Sintubin *et al.*, 2009). Nano-Ags are a non-toxic and safe antibacterial agent for the human body. In addition, nano-Ags are also reported to possess antifungal activity (Kim *et al.*, 2009), anti-inflammatory properties (Nadworny *et al.*, 2008), antiviral

activity (Rogers *et al.*, 2008) and anti-angiogenic activity (Gurunathan *et al.*, 2009). Nano-Ags can be applied safely in therapy when the effective concentrations against various types of organisms have been determined. Recently, we demonstrated that nano-Ags exhibit antifungal activities, including apoptotic cell death. In particular, we showed that hydroxyl radicals play a significant role in apoptosis (Hwang *et al.*, 2012). In addition, several studies regarding the synergistic activity of nano-Ags in combination with other compounds have been reported: a combination of amoxicillin and nano-Ags showed greater bactericidal efficiency towards *Escherichia coli* than when they were applied separately (Li *et al.*, 2005), and interactions between nano-Ags and polymyxin B showed synergistic effects for Gram-negative bacteria (Ruden *et al.*, 2009).

Some bacteria have become resistant to commonly used antibiotics. They are able to survive and even multiply in the presence of an antibiotic. The production of biofilm is also related to the antibiotic resistance problem (Lynch *et al.*, 2007). Bacteria frequently adhere to surfaces through a biofilm matrix, a 3D, gel-like, highly hydrated and locally charged environment. Adhesion of these bacteria to other organs may contribute to the pathogenesis of infection (Archer, 1998). Biofilms are associated with dental plaque

Abbreviations: CLSI, Clinical and Laboratory Standards; DCCD, *N,N'*-dicyclohexylcarbodiimide; FIC, fractional inhibitory concentration; FICI, FIC index; HPF, 3'-(*p*-hydroxyphenyl) fluorescein; nano-Ag, silver nanoparticle; TCP, tissue culture plate.

(Paju & Scannapieco, 2007; Hojo *et al.*, 2009), endocarditis (Palmer, 2006), lung infection (Lau *et al.*, 2005; Wagner & Iglewski, 2008) and infection through medical devices (Khardori & Yassien, 1995; Venkatesh *et al.*, 2009).

Moreover, recent articles have shown that lethal doses of bactericidal antibiotics create genetic and biochemical changes and promote the formation of highly detrimental oxidative radical species (Kim *et al.*, 2011). For instance, the various different classes of bactericidal antibiotics induce hydroxyl radical formation, whilst the other classes of bacteriostatic drugs do not (Kohanski *et al.*, 2007).

In the present study, we investigated the antimicrobial and antibiofilm activities of nano-Ags alone and in combination with conventional antibiotics against pathogenic bacteria. Additionally, we conducted experiments to measure hydroxyl radical formation and permeability in order to investigate the important causative factors for the synergistic effects.

METHODS

Agents, bacterial strains and culture conditions. Most agents used in this study were purchased from Sigma-Aldrich. The fluorescent reporter dye 3'-(*p*-hydroxyphenyl) fluorescein (HPF) was purchased from Invitrogen. *Enterococcus faecium* (ATCC 19434), *Staphylococcus aureus* (ATCC 25923), *E. coli* O157 (ATCC 43895), *E. coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from the American Type Culture Collection. *Streptococcus mutans* (KCTC 3065) was obtained from the Korean Collection for Type Cultures. Cells were grown in Luria–Bertani medium (Difco) at 37 °C and cell growth was monitored by measuring the optical density at 620 nm.

Preparation of nano-Ags. Solid silver (100 g) was dissolved in 100 ml 100% nitric acid at 90 °C and cooled, and 1 l distilled water was added. By adding sodium chloride to the silver solution, the silver ions were precipitated and then clustered together to form mono-dispersed nanoparticles in the aqueous medium. The size and morphology of the nano-Ags were examined using a transmission electron microscope (H-7600; Hitachi). The results showed that the nano-Ags were spherical in form and had a mean size of 3 nm (data not shown). The final concentration of colloidal silver was 60 000 p.p.m. (ICP Spectrophotometer, Optima 7300DV; PerkinElmer). This solution was diluted and then used to investigate antimicrobial effects.

Determination of MIC. Susceptibility tests with nano-Ags, ampicillin, chloramphenicol and kanamycin were carried out in 96-well microtitre plates using a standard twofold broth microdilution method of the antibacterial agents in Mueller–Hinton (MH) broth (Difco), following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2005). Briefly, bacterial cells were grown to mid-exponential phase in MH medium. Aliquots of 100 µl of the bacterial cells were then seeded in the wells of a 96-well microtitre plate at a density of 1×10^6 ml⁻¹; 10 µl each of the serially diluted solutions of the compounds was then added to the bacterial cells. The MIC was defined as the lowest concentration of drug inhibiting visible growth after overnight incubation at 37 °C.

Combination assay. The MICs of each antibiotic substance alone or in combination were determined by a broth microdilution method in accordance with CLSI standards using cation-adjusted MH broth modified for a broth microdilution chequerboard procedure (Eliopoulos & Moellering, 1991; CLSI, 2003). For the double

treatment, a 2D chequerboard with twofold dilutions of each drug was used to test the different combinations as follows. A chequerboard with twofold dilutions of nano-Ags and antibiotic (ampicillin, chloramphenicol or kanamycin) was set up as described above for the combined treatment. Growth control wells containing the medium were included in each plate. Each test was performed in triplicate. For the first clear well in each row of the microtitre plate containing all antimicrobial agents, the fractional inhibitory concentration (FIC) was calculated as follows: FIC of drug A = MIC_{drugA} in combination / MIC_{drugA} alone, and FIC of drug B = MIC_{drugB} in combination / MIC_{drugB} alone (Pankey & Ashcraft, 2005). The FIC index (FICI), calculated as the sum of each FIC, was interpreted as follows: FICI < 0.5, synergy; 0.5 ≤ FICI < 1, partial synergy; FICI = 1, additive; 2 ≤ FICI < 4, indifferent; 4 < FICI, antagonism (Odds, 2003).

Determination of biofilm formation by a tissue culture plate (TCP) method. To determine the efficacy of nano-Ags and their combination with antibiotics in the elimination of formed biofilm, a TCP method was carried out with suitable modifications (Christensen *et al.*, 1985). Individual wells of sterile, polystyrene, 96-well, flat-bottomed TCPs were filled with 190 µl of the single populations of the bacterial species (1×10^6 cells ml⁻¹). After 18 h of overnight culture, 10 µl nano-Ags alone or in combination with antibiotic was added so that the final concentration was the MIC or FIC, respectively. The TCPs were incubated for 6 h at 37 °C. After incubation, the wells were washed four times with 0.2 ml PBS to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in the plates were fixed with sodium acetate (2%) and stained with crystal violet (0.1%, w/v). Excess stain was rinsed off by thorough washing with deionized water and the plates were dried. After drying, 95% ethanol was added to the wells and the A₅₉₅ of stained adherent bacteria was measured with an Emax microtitre ELISA reader (Molecular Devices). The percentage of biofilm inhibition was calculated using the equation: $[1 - (A_{595} \text{ of cells treated with nano-Ags} / A_{595} \text{ of non-treated control})] \times 100$ (Wei *et al.*, 2006). Experiments were performed in triplicate, and means ± SD were determined.

Antibacterial activity with detergents and ATPase inhibitors. To understand whether the antibacterial activity of nano-Ags was associated with altered membrane permeability or the action of an energy-dependent protective function including multidrug-resistant pumps or repair systems, the antibacterial susceptibility of nano-Ags was examined in the presence of detergents or ATPase-inhibiting agents. To increase the permeability of the outer membrane, the concentration of nano-Ags, as the FIC determined in a combination assay with other therapeutic agents, was used to treat bacterial cells in the presence of 0.001% Triton X-100 or 30 mM Tris (Jung & Lee, 2008). NaN₃ and *N,N'*-dicyclohexylcarbodiimide (DCCD) were used as inhibitors of ATPase (Linnett & Beechey, 1979). Bacterial susceptibility using nano-Ags in the presence of 0.001% NaN₃ and 25 µM DCCD was also determined under the same conditions. Experiments were performed in triplicate in a plate and in three independent assays, and the results were expressed as means ± SD.

Hydroxyl radical formation assay. Bacterial cells (1×10^6 ml⁻¹) were treated with each substance or combination so that the final concentration was the MIC or FIC, respectively. Samples were incubated for 1 h at 37 °C. To detect hydroxyl radical formation, we used the fluorescent reporter dye HPF at a concentration of 5 µM, and used a spectrofluorometer (RF-5301PC; Shimadzu) to measure excitation at a wavelength of 490 nm and emission at 515 nm (Kohanski *et al.*, 2007). The percentage hydroxyl radical increase was calculated using the equation: $\{[(A_{490} \text{ of cells treated with coprisin} - A_{490} \text{ of non-treated control}) / A_{490} \text{ of non-treated control}] \times 100\}$. Experiments were performed in triplicate and means ± SD were calculated.

Table 1. Antibacterial activity of nano-Ags against human pathogenic bacteria

Species	MIC ($\mu\text{g ml}^{-1}$)			
	Nano-Ags	Ampicillin	Chloramphenicol	Kanamycin
Gram-positive species				
<i>Enterococcus faecium</i> ATCC 19434	0.25	2	4	2
<i>Staphylococcus aureus</i> ATCC 25923	0.5	4	2	4
<i>Streptococcus mutans</i> KCTC 3065	2	2	4	4
Gram-negative species				
<i>E. coli</i> O157 ATCC 43895	0.5	4	2	4
<i>E. coli</i> ATCC 25922	2	8	4	4
<i>P. aeruginosa</i> ATCC 27853	0.5	2	1	2

RESULTS AND DISCUSSION

Determination of MIC

According to previous studies, nano-Ags exhibit potent antifungal effects (Kim *et al.*, 2009; Hwang *et al.*, 2012). We thought it might be possible that nano-Ags also have antibacterial activity and synergistic effects on diverse pathogenic bacteria. To examine the antimicrobial activity of nano-Ags, alone and in conjunction with ampicillin, chloramphenicol and kanamycin, antibacterial susceptibility testing was conducted against several human pathogens including *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus mutans*, two strains of *E. coli* and *P. aeruginosa* using the CLSI method. In the present study, ampicillin, chloramphenicol and kanamycin were used as positive controls against the bacteria: ampicillin is a β -lactam antibiotic that inhibits bacterial cell-wall synthesis, whilst chloramphenicol and kanamycin inhibit protein synthesis (Weisblum & Davies, 1968; Kohanski *et al.*, 2007).

As shown in Table 1, nano-Ags showed antibacterial activity against Gram-positive and Gram-negative bacteria with MIC values of 0.25–2 $\mu\text{g ml}^{-1}$. Generally, nano-Ags

were more potent against Gram-negative than against Gram-positive bacteria (Shrivastava *et al.*, 2007), although it has been reported that some strains of *E. coli* are more resistant to the bactericidal effects of nano-Ags than strains of *Staphylococcus aureus*, contradicting earlier studies (Ruparelia *et al.*, 2008). In this study, nano-Ags showed similar levels of MIC towards Gram-positive and Gram-negative bacterial strains. The bacterial strains showed MIC values of 2–8 $\mu\text{g ml}^{-1}$ for ampicillin, 1–4 $\mu\text{g ml}^{-1}$ for chloramphenicol and 2–4 $\mu\text{g ml}^{-1}$ for kanamycin. The nano-Ags and antibiotics showed different degrees of antibacterial activity in relation to the bacterial species used in this study. The Gram-positive bacteria tended to show more sensitivity than the Gram-negative bacteria to ampicillin. In particular, the Gram-negative *E. coli* strains were less sensitive to ampicillin than the other bacteria strains.

Combination assay

The synergistic effects of nano-Ags were also investigated with three conventional antibiotics against bacteria using a chequerboard method and the effects evaluated by

Table 2. Activities of combinations of nano-Ags and antibiotics

S, Synergistic; PS, partially synergistic.

Species	FICI ($\mu\text{g ml}^{-1}$)*		
	Nano-Ag + ampicillin	Nano-Ag + chloramphenicol	Nano-Ag + kanamycin
Gram-positive species			
<i>Enterococcus faecium</i> ATCC 19434	0.375 (S)	0.375 (S)	0.75 (PS)
<i>Staphylococcus aureus</i> ATCC 25923	0.5 (PS)	0.75 (PS)	0.375 (S)
<i>Streptococcus mutans</i> KCTC 3065	0.375 (S)	0.5 (PS)	0.375 (S)
Gram-negative species			
<i>E. coli</i> O157 ATCC 43895	0.5 (PS)	0.5 (PS)	0.5 (PS)
<i>E. coli</i> ATCC 25922	0.375 (S)	0.5 (PS)	0.375 (S)
<i>P. aeruginosa</i> ATCC 27853	0.5 (PS)	0.375 (S)	0.375 (S)

*FICI = (MIC_{drug A} in combination/MIC_{drug A} alone) + (MIC_{drug B} in combination/MIC_{drug B} alone).

determination of the FICI. The results of the combination assay are presented in Table 2. All of the combinations showed effectiveness against the bacteria tested. Synergistic interactions of nano-Ags and ampicillin were observed against *Enterococcus faecium*, *Streptococcus mutans* and *E. coli* ATCC 25922. Synergistic interactions of nano-Ags and chloramphenicol were found only against *Enterococcus faecium* and *P. aeruginosa*. Finally, synergistic interactions of nano-Ags and kanamycin were seen against *Staphylococcus aureus*, *Streptococcus mutans*, *E. coli* ATCC 25922 and *P. aeruginosa*. Other combinational activities of nano-Ags and antibiotics were seen as partially synergistic interactions. These synergistic activities of nano-Ags in the presence of conventional antibiotics suggest that it might be possible to reduce the viability of bacterial strains at lower antibiotic concentrations.

Determination of biofilm formation by TCP assay

Extended cultivation of bacterial cells results in adherence to animal tissues and abiotic surfaces (Costerton *et al.*, 1981). This, in turn, allows the formation of a biofilm, a multilayered community of sessile bacterial cells. Biofilms provide a survival advantage over planktonic bacteria by enhancing nutrient trapping and colonization (Costerton, 1999). Currently, biofilms are a widespread problem in hospitals and healthcare facilities. Indeed, the National Institutes of Health found that 80 % of chronic infections are related to biofilms (Monroe, 2007).

To investigate the antibiofilm effects of nano-Ags and their combination with antibiotics, bacterial cells were grown to form biofilms and then treated with nano-Ags alone or in combination with the antibiotics (Fig. 1). The results

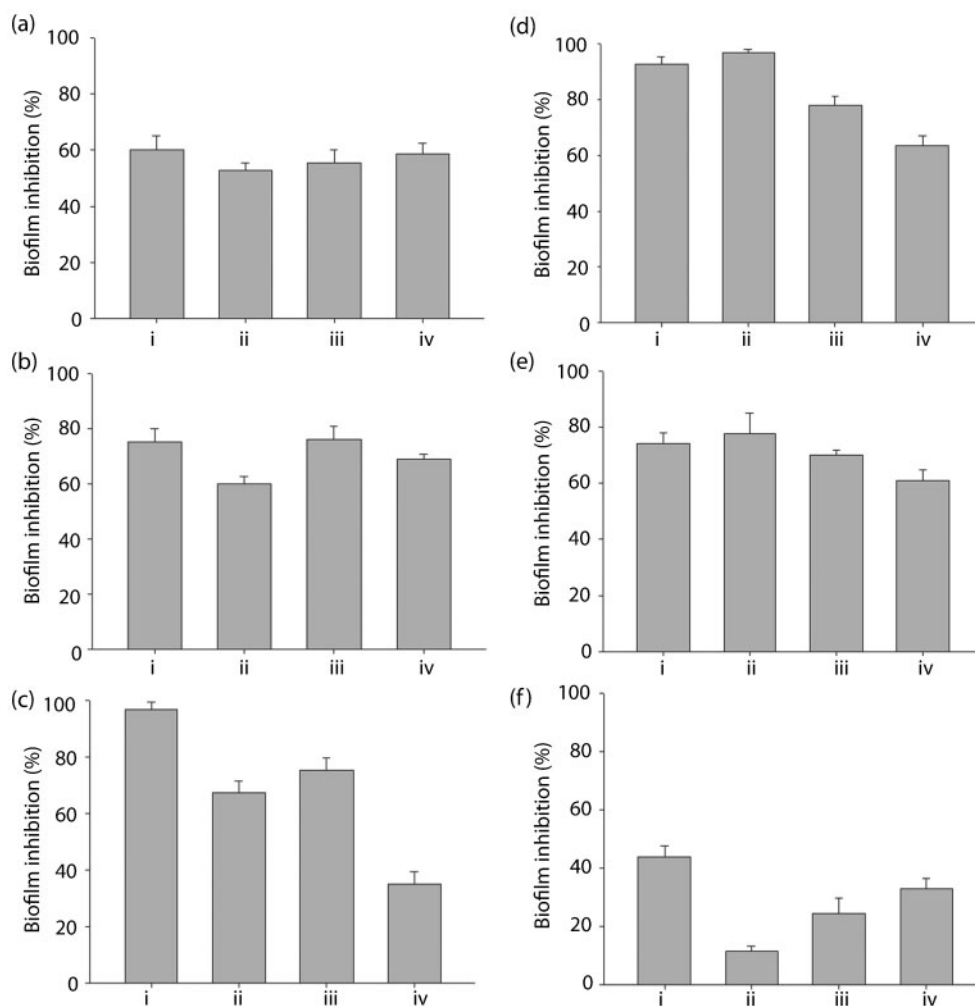


Fig. 1. Inhibition of biofilm formation determined using the TCP method, showing the effect of addition of nano-Ags alone or in combination with various conventional antibiotics. (a) *Enterococcus faecium*, (b) *Staphylococcus aureus*, (c) *Streptococcus mutans*, (d) *E. coli* O157, (e) *E. coli* ATCC 25922, (f) *P. aeruginosa*. Treatments were: (i) nano-Ags alone, (ii) nano-Ags + ampicillin, (iii) nano-Ags + chloramphenicol, (iv) nano-Ags + kanamycin.

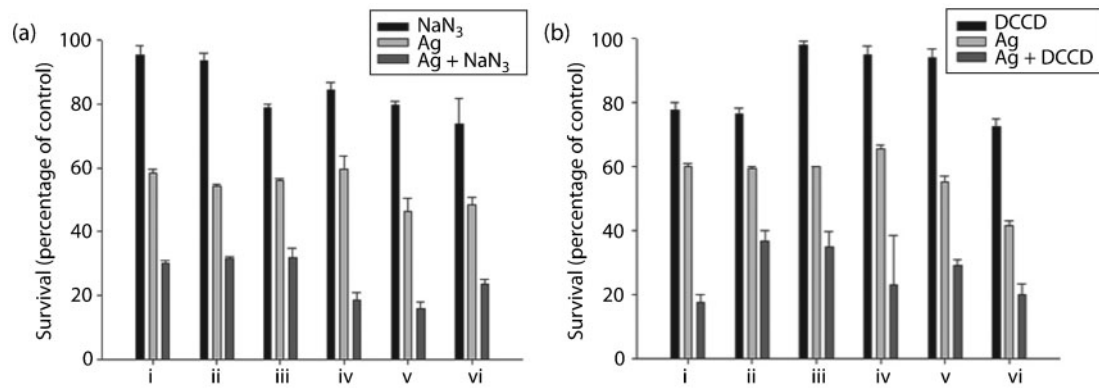


Fig. 2. Effect of NaN₃ (a) and DCCD (b) on the susceptibility of bacterial isolates. The viability of the bacteria was determined by counting c.f.u. on MH agar plates after incubation for 18 h with nano-Ags at the MIC and in the presence or absence of each agent. Results are shown as means \pm SD for three independent experiments. The bacteria were: (i) *Enterococcus faecium*, (ii) *Staphylococcus aureus*, (iii) *Streptococcus mutans*, (iv) *E. coli* O157, (v) *E. coli* ATCC 25922, (vi) *P. aeruginosa*.

indicated that nano-Ags mostly had an inhibitory activity on biofilm formation of $>60\%$. Combinations of nano-Ags and the three conventional antibiotics also appeared to actively inhibit biofilm formation to varying degrees, but generally showed a greater inhibitory activity than nano-Ags alone. This is an encouraging result, as it may be possible to achieve an effective antibiofilm effect at lower antibiotic concentrations. Thus, nano-Ags and their combination with antibiotics showed potential as an antibiofilm agent against mature bacterial biofilms.

Antibacterial activity with detergents and ATPase inhibitors

We next used NaN₃ and DCCD to elucidate whether the antibacterial activity of nano-Ags was associated with an

ATP-dependent function such as the multidrug resistance pumps located in the periplasmic membrane. In several drug-resistant bacteria, multidrug resistance pumps, which can extrude toxic substances, including antibiotics, into the extracellular environment, have been reported as a major cause of antimicrobial resistance (Levy, 1992). NaN₃ is well known as a metabolic inhibitor that can decrease ATP levels by disrupting electrochemical proton gradients in the bacterial environment, whilst DCCD is an inhibitor of the F-F₀ ATPase (Swallow *et al.*, 1990; Gonçalves *et al.*, 1999).

Although bacteria treated with nano-Ags showed a decreasing percentage of survival, their viability decreased dramatically in the presence of NaN₃ and DCCD (Fig. 2). In contrast, there were no significant differences between the cells treated with nano-Ags in the presence or absence

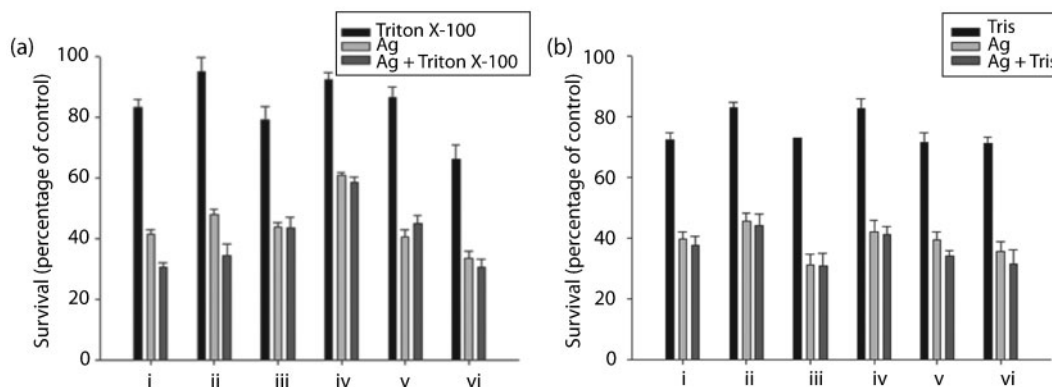


Fig. 3. Effects of membrane-permeabilizing agents on the susceptibility of bacterial isolates. The viability of bacteria was determined by counting c.f.u. on MH agar plates after incubation for 18 h with nano-Ags at the MIC and in the presence or absence of Triton X-100 (a) or Tris (b). Results are shown as means \pm SD for three independent experiments. The bacteria were: (i) *Enterococcus faecium*, (ii) *Staphylococcus aureus*, (iii) *Streptococcus mutans*, (iv) *E. coli* O157, (v) *E. coli* ATCC 25922, (vi) *P. aeruginosa*.

of the membrane-permeabilizing agents Triton X-100 and Tris (Fig. 3), which increase the permeability of the outer membrane (Levy, 1992). These results showed that increasing the permeability of the outer membrane is not required for the action of nano-Ags, but that the action of nano-Ags is potentiated by ATP-associated metabolism or protective actions.

Hydroxyl radical formation assay

Kohanski *et al.* (2007) suggested an intriguing mechanism by which antibiotics kill bacteria. They showed that three major bactericidal antibiotics (norfloxacin, ampicillin and kanamycin) stimulated hydroxyl radical formation in bacteria, and that this toxic chemical contributed to the killing efficiency of lethal antibiotics. This process was accompanied by hyperactivation of NADH dehydrogenases

and depletion of NADH. Based on the results, they concluded that generation of hydroxyl radicals is a common mechanism of bacterial cell death caused by antibiotics (Kohanski *et al.*, 2007; Kim *et al.*, 2011).

Consistent with this, we tested whether or not cells treated with nano-Ags generated hydroxyl radicals and how this affected the synergistic effects. As shown in Fig. 4, nano-Ags had the ability to generate hydroxyl radicals. Additionally, two different classes of bactericidal antibiotics, ampicillin and kanamycin, induced hydroxyl radical formation. In contrast, the bacteriostatic drug that we tested, chloramphenicol (a ribosome inhibitor), did not stimulate hydroxyl radical production to any great extent. However, cells treated with a combination of nano-Ags and chloramphenicol showed increased hydroxyl radical formation. This result indicated that the generation of hydroxyl radicals might be an important cause of the synergism seen.

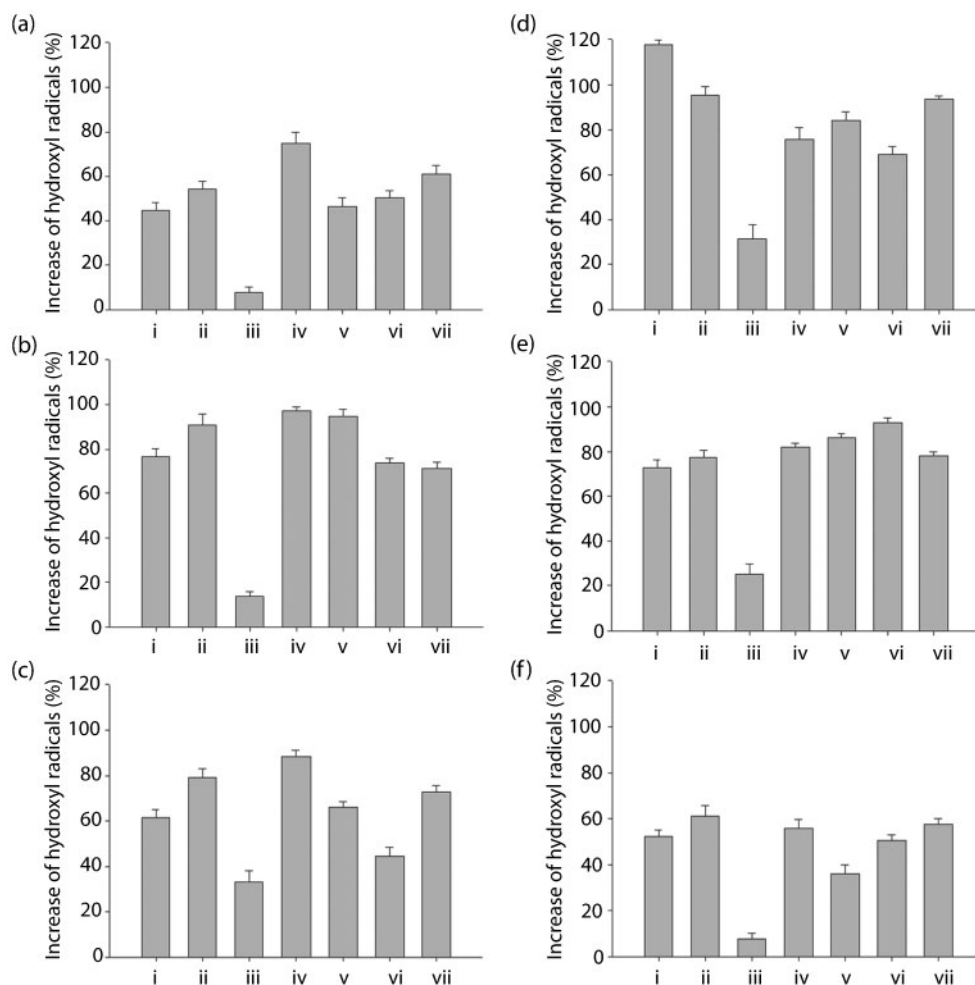


Fig. 4. Hydroxyl radical production, determined by the HPF staining method, after addition of nano-Ags alone or in combination with other conventional antibiotics. (a) *Enterococcus faecium*, (b) *Staphylococcus aureus*, (c) *Streptococcus mutans*, (d) *E. coli* O157, (e) *E. coli* ATCC 25922, (f) *P. aeruginosa*. Treatments were: (i) nano-Ags alone, (ii) ampicillin, (iii) chloramphenicol, (iv) kanamycin, (v) nano-Ags + ampicillin, (vi) nano-Ags + chloramphenicol, (vii) nano-Ags + kanamycin.

In conclusion, this study has presented evidence of the antibacterial effects of nano-Ags and their synergistic capacity against various representative pathogenic bacteria. Additionally, we conducted a biofilm inhibition assay, hydroxyl radical formation measurement and an antibacterial assay with detergents or ATPase inhibitors to determine the characteristics and possible mechanisms of the synergistic effects. These results suggest that nano-Ags have potential as an adjuvant for the treatment of infectious diseases. Furthermore, the generation of hydroxyl radicals and the dysfunction of important protective factors were demonstrated in the antibacterial effects of nano-Ags and their synergism. Thus, our findings support the claim that nano-Ags have considerable effective antibacterial activity and deserve further investigation for clinical application.

ACKNOWLEDGEMENTS

This work was supported by the Korea government (MEST) under a National Research Foundation of Korea (NRF) grant (no. 2012-0000639)

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