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Role of reactive oxygen species in the antibacterial mechanism of silver nanoparticles on *Escherichia coli* O157:H7

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Abstract In this study, the conditions and mechanism of antibacterial activity of hydrophilic polymer coated silver nanoparticles (AgNPs) against *E. coli* O157:H7 (CMCC44828) as model pathogen was studied. The AgNPs were coated with amphiphilic polymer that introduced carboxyl groups on the surface to make it water-soluble. The AgNPs were exposed to various treatment conditions of pH and temperature before these were combined with the *E. coli*. The mechanism of the antibacterial activity was studied through the formation of reactive oxygen species (ROS) that was later suppressed with antioxidant to establish correlation with the AgNPs antimicrobial activity. Studies were carried out at both anaerobic and aerobic conditions. The results indicated that 5 mg/L AgNPs inhibited ~50% of the growth of 10^6 colony forming units per milliliter (cfu/mL) *E. coli* cells in liquid Luria–Bertani (LB) medium. This dose-dependent antimicrobial activity was higher at increased temperature (37°C) but was lower when the AgNPs were treated with acid at pH 2 before exposure to the bacteria. It was also established that the conditions of higher antimicrobial effect generated more ROS that was dependent on the presence of oxygen. The antibacterial activity was suppressed in the presence of an antioxidant.

Keywords Silver · Nanoparticle · Antibacterial mechanism · Reactive oxygen species · *Escherichia coli*

Introduction

In recent years, a rapid increase in microorganisms that are resistant to conventional antibiotics had been observed (Neu 1992). Just like a super bacteria, currently the most prominent infectious disease is resistant to all the antibiotics available. Pathogens have evolved to become drug resistant through many generations which have been caused by chromosomal changes or the exchange of genetic material via plasmids and transposons (Neu 1992). These pathogens have been able to produce drug degrading enzymes or to modify antibiotics-specific binding sites to resists the effects of antibiotics (Bryan 1988). These problems led to the resurgence of the use of silver-based antiseptics, including the use of silver

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nanoparticles (AgNPs). AgNPs possess low toxicity to human cells, effective broad-spectrum activity against bacteria and a far lesser probability to cause microorganism resistance than conventional antibiotics (Jones et al. 2004; Zhao and Stevens 1998).

Due to its strong antimicrobial properties, AgNPs have found a variety of applications including water purification and wound dressing to disinfect microorganisms (Gong et al. 2007; Jain and Pradeep 2005; Tian et al. 2007). AgNPs can be coated on common polyurethane foams through its interaction with the nitrogen atom of the polyurethane (Jain and Pradeep 2005). Once incorporated, the AgNPs are stable on the foams and cannot be washed away with soap and water. The AgNPs modified foam was tested with water at a flow rate of 0.5 L/min, in which contact time was about 1 s. The output count of *E. coli* was nil even when the input water had a bacterial load of 10^5 colony forming units per milliliter (cfu/mL) (Jain and Pradeep 2005). In another study, Fe_3O_4 with attached silver nanoparticles, making the nanoparticles easily removed with a magnetic field, had been used for the treatment of water (Gong et al. 2007). This system involving AgNPs in Fe_3O_4 prevented bacterial contamination of the water. In another report, AgNPs in wound dressing showed better wound healing capacity, better cosmetic appearance, and scar-less healing in an animal model (Tian et al. 2007).

Many studies had reported that AgNPs showed efficient antibacterial activity compared with their bulk counterpart due to their extremely high surface-to-volume ratio that provides better contact with microorganisms (Morones et al. 2005). However, the antibacterial mechanism has only been partially understood. There are several approaches to explain the antibacterial activity of AgNPs. Some transmission electron microscopic studies had revealed that the majority of AgNPs were localized on the cell membranes that appeared severely damaged as shown by the presence of numerous pits and gaps (Dror-Ehre et al. 2009; Li et al. 2010; Sondi and Salopek-Sondi 2004). AgNPs were predicted to damage the permeability of bacterial membranes causing efflux of reducing sugars and proteins as well as the depletion of the levels of intracellular adenosine triphosphate (ATP) (Li et al. 2010; Lok et al. 2006). Moreover, AgNPs have the ability to dissipate the proton motive force of bacteria (Lok et al. 2006). Elimination of bacterial proton motive force results in cell death. AgNPs also destabilize the outer membrane causing the collapse of the plasma membrane potential (Kim et al. 2009). In addition, the expressions of several cell envelope proteins were found to be stimulated after a short exposure to AgNPs (Lok et al. 2006). When AgNPs enter the bacterial cell, they not only attack the respiratory chain by interacting with thiol groups of essential enzymes that lead to their inactivation, but also dephosphorylate peptides on tyrosine residues that could influence bacterial signal transduction which eventually leads to cell death (Shrivastava et al. 2007). All these studies had focused on the description of damage and mode of action of AgNPs on exposed bacteria, but the primary reason for the induced antimicrobial effects remains unclear.

In this paper, we report the results of our preliminary studies on the significant antimicrobial activities of highly stable polymer coated AgNPs. We examined the antibacterial activity of these AgNPs against Gram-negative bacteria at various environmental conditions. *E. coli* O157:H7 (CMCC44828), a pathogenic strain of Gram-negative bacteria, was tested as the indicator strain. Influences of acid or alkaline treatment and incubation temperature on the antibacterial activity of AgNPs against *E. coli* was studied. We determined the effect of oxygen and an antioxidant on the mechanism of antibacterial activity of AgNPs to confirm the participation of reactive oxygen species (ROS). Results from these studies suggested that the oxidative damage caused by ROS might be responsible for the antibacterial activity of AgNPs.

Materials and methods

Microorganisms and culture conditions

The indicator strain of *E. coli* O157:H7 (CMCC44828) was purchased from the National Center for Medical Culture Collections (CMCC) and cultured in our laboratory. The LB medium was purchased from Hangzhou Bai-si Biotechnology Co., Ltd containing 10 g tryptone, 5 g yeast extract and 10 g NaCl. The LB medium was used for aerobic cultivation at 37°C with shaking at 160 rpm to the bacterial logarithmic phase. A 99 mL of fresh LB medium and 1 mL of the above

bacterial suspension were mixed to a final bacterial concentration of 10^5–10^6 cfu/mL.

Synthesis and characterization of AgNPs

The hydrophobic core silver nanoparticles (core AgNPs) were synthesized using a modification of a previously published method (Yamamoto et al. 2006). Briefly, long-chain alkyl carboxylate was used as stabilizer to avoid the aggregation of silver nanoparticles and to control particle size, and bis(amine)silver(I) carboxylate was used as a mild reducing agent for the intermediate to produce the nanoparticles at a low temperature. The core size was measured using transmission electron microscopy (TEM). After synthesis and characterization of the core AgNPs, the particles were coated with amphiphilic polymers to convert to a water soluble form (AgNPs), which at the same time provided reactive carboxyl groups on the particles' surface for bioconjugation. The hydrodynamic size that was a result of the polymer around the core AgNPs surrounded by water molecules in the colloidal dispersion was measured using dynamic light scattering scan (Zetatrac Ultra 151, MicroTrac, Inc, USA). This is reported in terms of the diameter of the hydrophilic polymer coated AgNPs.

Assay for evaluating the antibacterial activity of AgNPs

To measure the antibacterial activity of AgNPs without the impact of any factors, bacteria was grown in one liter of LB medium supplemented with 2.5, 5, 7.5, 10 and 12.5 mg of AgNPs. Bacteria that was grown in LB without AgNPs was used as control. The bacterial culture was carried out under aerobic condition at 37°C without shaking for 5 h, after which the number of cells was established using the conventional plate count method. This method involved serial 10-fold dilutions with saline plated on plate count agar. The cell concentration was determined by the number of colonies that appeared on the LB agar medium after incubation at 37°C for 16 h. Percent cell mortality was calculated after growth in the presence of AgNPs in comparison with the cell number in the control.

Effect of acid/alkaline treatment on the antibacterial activity of AgNPs

To examine the effect of acid/alkaline treatment on the antibacterial activity, AgNPs were exposed to acid (pH 2.0) or alkali (pH 12.0) at room temperature for 30 min, and then were neutralized by adding 1 M NaOH or 1 M HNO_3. After getting the pH back to 7, the hydrodynamic sizes and zeta potentials were determined with a zeta tracker. These treated AgNPs at a concentration of 5 mg/L were added to bacteria suspension at approximately 10^6 cells per milliliter. Bacterial cultures not exposed to AgNPs were used as controls. The mixture was incubated aerobically at 37°C for 5 h.

Antibacterial activities of AgNPs under different temperatures

To estimate the effect of incubation temperature on the antibacterial activity of AgNPs, a mixture containing fresh LB medium, AgNPs, and bacterial suspension were combined to create a final concentration of AgNPs at 5 mg/L and *E. coli* cells at 10^6 cfu/mL. The mixtures were aerobically cultured at 4, 23, and 37°C for 5 h without shaking. The unexposed bacteria under the same temperature were used as control. Percent cell mortality was calculated with respect to the unexposed bacteria at each temperature.

AgNPs antibacterial mechanism

To understand the mechanism of antibacterial activity, ROS generated by AgNPs under different temperatures were monitored. ROS was measured with an oxidation-sensitive fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA) (Tian et al. 2006). This DCFH-DA passively diffuses through the cell membrane into the cell and is deacetylated by esterases to form non-fluorescent 2,7-dichlorofluorescein (DCFH). The DCFH reacts with ROS to form the fluorescent product 2,7-dichlorofluorescein (DCF), which is trapped inside the cell making the cell fluorescent. For this study, bacteria were cultured to 10^8 cfu/mL, and cells were washed three times with fresh medium. DCFH-DA was mixed with the cultures at a ratio of 1:2000 and the mixture was

shaken for 30 min at 37°C to successfully load the probes into the cells. After loading the probes, the bacteria were pelleted by centrifugation and washed two times to remove the probes outside the cell. The cleaned cells were exposed to AgNPs under the different temperatures as described in the previous section. The fluorescent signal intensity of DCF was detected by fluorescence spectrophotometer at an excitation wavelength of 488 nm and at an emission wavelength of 535 nm.

Effect of oxygen and antioxidants on the antibacterial activity of AgNPs

To confirm the effect of oxygen on the antibacterial activity of AgNPs against *E. coli*, a comparative study was performed. Fresh *E. coli* culture at approximately 10^6 cfu/mL was inoculated in fresh LB medium. AgNPs were added to these mixtures at final concentrations from 3 to 7 mg/L. One set was incubated under aerobic conditions while another was incubated anaerobically.

The antioxidant *N*-acetylcysteine (NAC) was used to investigate the effect of reactive oxygen species (ROS) on the antibacterial activity of AgNPs. To do this, two sets of culture, one with AgNPs alone and another with AgNPs and NAC (10 mM) were prepared containing 5 and 10 mg/L AgNPs. A control containing 10 mM NAC in the presence of *E. coli* alone under similar conditions was used to establish the effect of NAC in the absence of AgNPs.

Statistical analyses

All the tests were performed at least in duplicate. The data gathered were analyzed using the statistical program SigmaPlot 8.02 (SPSS, Inc., Chicago, USA).

Results and discussion

Characterization of silver nanoparticles

Based on the TEM data, the hydrophobic organic solvent soluble core AgNPs were 3 nm in diameter with uniform size and shape. The diameter of the hydrophobic core AgNPs is due only to the core AgNP which is not water soluble. Upon conversion into the hydrophilic polymer coated form, the AgNPs exhibited a hydrodynamic diameter that was approximately 15 nm with a zeta potential that was −28 mV. In Table 1, the hydrodynamic diameter of the hydrophilic polymer coated AgNPs was due to the polymer coating that introduced carboxyl groups that were surrounded by water molecules in the nanoparticle colloidal dispersion. Thus, the water soluble form of the AgNPs is a lot bigger than the hydrophobic core AgNPs. The water soluble AgNPs were yellowish black with significant light scattering.

Table 1 Changes of AgNPs after pH-treatment

pH treatment	Hydrodynamic size (nm)	Zeta potential (mV)
Treatment-free	22.18 ± 7.54	−35.82
pH 12.0	21.35 ± 6.53	−28.94

The antibacterial activity of AgNPs against *E. coli*

Antibacterial properties of the AgNPs were tested against *E. coli* in LB mediums. The effect of various concentrations of AgNPs on the growth of 10^6 cells/mL bacteria is shown in Fig. 1a. These results indicated that the number of colonies of *E. coli* that survived was inversely proportional to the concentration of AgNPs. The numbers of live bacteria that were based on the number of colony forming units decreased significantly with an increase in the concentration of AgNPs. Exposure of the bacteria to AgNPs at a concentration of 5 mg/L inhibited bacterial growth by 50%. AgNPs with a concentration of 12.5 mg/L almost totally inhibited bacterial growth.

Although the AgNPS used in these studies was polymer coated, the antibacterial effects of AgNPs against *E. coli* may be a result of the nature of the bacterial cell membrane. *E. coli* has a layer of lipopolysaccharide on the membrane surface that is negatively charged. Some studies have reported that the positive charge on silver ions, Ag^+, and perhaps on AgNPs that resulted in an electrostatic attraction with the negatively charged bacterial cell membrane (Dibrov et al. 2002; Hamouda et al. 2001; Stoimenov et al. 2002). However, in this study, the AgNPs were coated with a polymer that had negatively charged carboxyl groups and yet, still resulted in significant

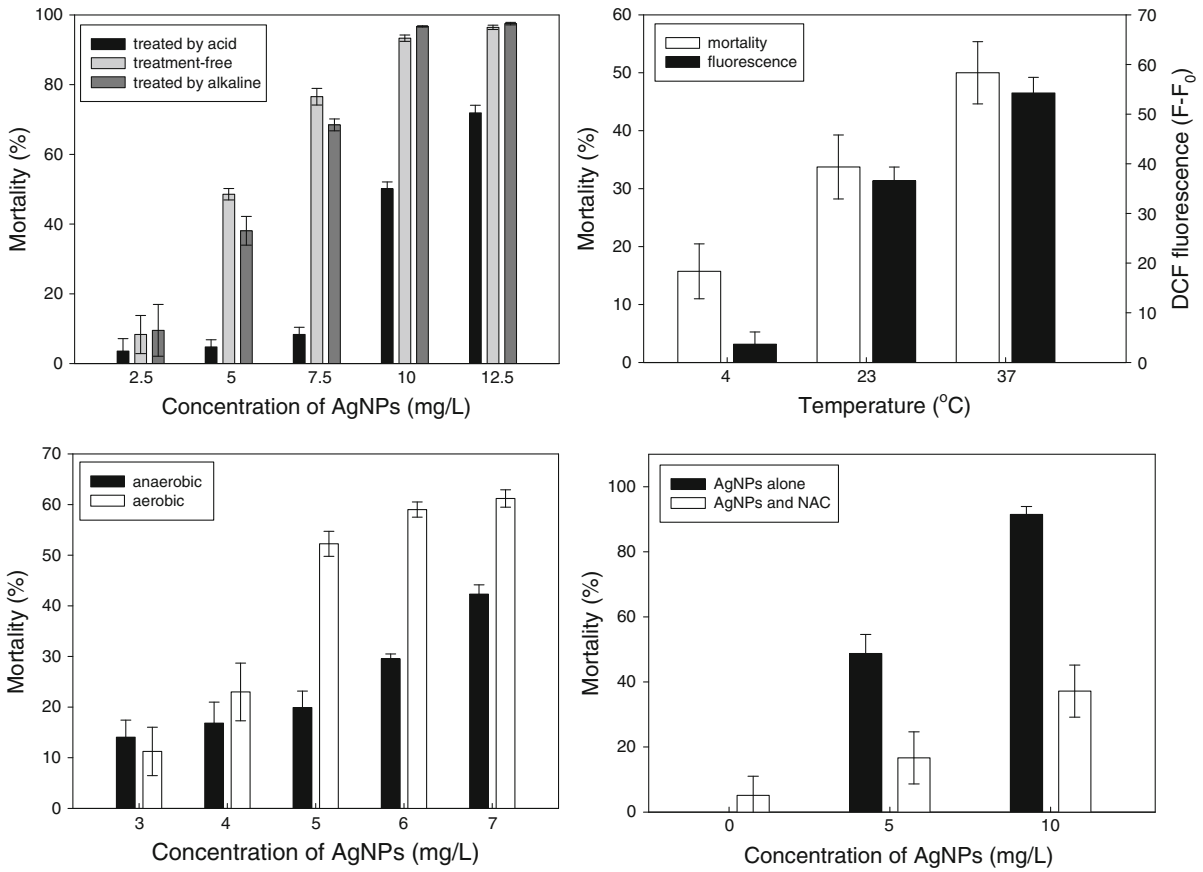

Fig. 1 The antibacterial activity of AgNPs as a function of **a** pH, **b** temperature, **c** Oxygen, and **d** antioxidant NAC

antibacterial effect. From these observations, it may be inferred that the remarkable antibacterial activity was not mediated by the electrostatic attraction between the AgNPs and the bacterial cell membrane. Other hypotheses widely believe that silver nanoparticles are incorporated in the cell membrane, which causes leakage of intracellular substances and eventually causes cell death (Cho et al. 2005; Jain and Pradeep 2005; Sondi and Salopek-Sondi 2004; Kumar et al. 2004) while some of the silver nanoparticles also penetrate into the cells.

Effect of acid/alkaline treatment on the antibacterial activity of AgNPs

The expanding application of nanoparticles can lead to their use in acid or alkali conditions. Some of these pH conditions could lead to the destruction of the surface coating on AgNPs and may lead to aggregation making the particles bigger. Thus, we tested the effect of extreme acid and alkali exposure of the AgNPs on their antimicrobial effects.

To evaluate the effect of pH on the antibacterial activity of AgNPs, we treated these at an acidic pH of 2 and a basic pH of 12. Upon exposure to acidic conditions, monodisperse AgNPs immediately flocculated and formed precipitates at pH 2.0. The zeta potential was measured before and after pH treatment shown on Table 1. The zeta potential values of AgNPs indicated that the original nanoparticles were negatively charged. When exposed to acidic pH, the negative charges on the AgNPs surface were neutralized and caused aggregation. Exposure to alkaline pH did not result in significant changes in hydrodynamic size or zeta potential and there was no aggregation observed.

The results shown in Fig. 1a indicated the effect of antibacterial properties of AgNPs against *E. coli* treated with acid or alkali. The alkali treatment showed no significant change on the antibacterial

activity that was still comparable with the effect of the untreated AgNPs at 5 mg/L that killed nearly 50% of the bacteria population. On the other hand, the antibacterial activity of AgNPs treated with acid solution at a pH of 2 showed an obvious decrease in the antimicrobial activity, down to below 10% at 5 mg/L. This may be explained in terms of possible damage on the surface functional group at low-pH environment, leading to the aggregations of the nanoparticles. The aggregates may have been too large to penetrate the cell membrane to exert antibacterial effect. These observations indicated that the nanoparticles size and surface charge were necessary for the AgNPs antibacterial activity; changes on these properties decreased the antibacterial property.

Antibacterial activities of AgNPs at various temperatures

Microbes grow at various conditions and most pathogens grow at human body temperatures. Thus, we studied the effect of various temperatures on the antibacterial activities of AgNPs.

Figure 1b shows that the antibacterial activity of AgNPs was proportional to the incubation temperature. At 4°C, no obvious antibacterial effect was achieved ($P > 0.05$). This is a significant observation because cooling of biological samples is usually performed to prevent contamination as well as degradation. It seemed like the AgNPs did not affect the lag-phase of the bacteria. On the other hand, at the maximum temperature tested, 37°C ($P < 0.01$), the antibacterial activity was found to be most effective. More than 50% of the bacteria population was killed which was a lot higher than the other temperatures. It is possible that at higher temperature, the cell membrane were leaky allowing entry of the AgNPS which resulted in higher mortality. This will be in agreement with studies that state that transport into the plasma membrane is essential for AgNPs to inhibit bacterial growth (Shrivastava et al. 2007).

AgNPs antibacterial mechanism

Some studies state that attachment to and transport into the plasma membrane was essential for AgNPs to inhibit the growth of bacteria (Shrivastava et al. 2007). Higher incubation temperature resulted in enhanced membrane fluidity that made the transport of AgNPs easier. It has also been reported that metal nanoparticles induced a significant rise in ROS in cell lines that elicited toxic effects related to oxidative stress (Gurr et al. 2005; Limbach et al. 2007; Olmedo et al. 2005). Reactive oxygen species were possibly produced when respiratory enzymes were inhibited through the interaction of silver ions with the thiol group of the enzymes (Matsumura et al. 2003). As one of the critical factors affecting oxidative stress, ROS could react directly with DNA and proteins or could react with lipids to generate malondialdehyde that may subsequently react with DNA, proteins and/or other lipids (Mats and Scnchez-Jimnez 2000). An earlier study reported that ROS in *E. coli* primarily resulted from the autoxidation of NADH dehydrogenase II in the respiratory chain which was accelerated at high temperatures (Messner and Imlay 1999).

To understand the mechanism of antibacterial activity, ROS generated in the presence of AgNPs under different temperatures were monitored with oxidation-sensitive fluorescent probe DCFH-DA that passively diffuses through the cell membrane into the cell. This probe is deacetylated by esterase to form non-fluorescent DCFH that in turn reacts with ROS to form the fluorescent product DC that is trapped inside the cell making the cell fluorescent. Thus, the ROS level is directly proportional with the cell fluorescence intensity at 480 nm. The ROS level is in turn correlated with the antibacterial activity of the AgNPs that is due to its damaging effect to the bacterial cell membrane. When the AgNPs affect the ‘cell membrane that leads to ROS formation, DCFH-DA inside the cell reacts with the ROS forming the fluorescent by-product.

The ROS level was analyzed in the original bacteria in comparison with that in AgNPs-exposed bacteria ($F–F_0$) using the cell fluorescence intensity. As shown in Fig. 1b, the fluorescence intensity increased at higher temperature, which indicated an increased ROS level. As seen in the previous section, higher temperatures resulted in higher bacterial mortality. Thus, it may be inferred that the mechanism of AgNPs antibacterial action was through increased ROS formation which was an indication of the increased antibacterial activity of AgNPs at higher temperatures.

Effect of oxygen and antioxidants on the mechanism of AgNPs antibacterial activity

Based on the literature, reactive oxygen species (ROS) including superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and highly toxic hydroxyl radical ($^{\bullet}OH$) are generated during aerobic metabolism according to the following reactions (Neal 2008):

$$Ag + hv \rightarrow Ag(h^+ + e^-)$$
$$e^- + O_2 \rightarrow {}^{\bullet}O_2^-$$
$${}^{\bullet}O_2^- + 2H^+ + e^- \rightarrow H_2O_2$$
$$H_2O_2 + {}^{\bullet}O_2^- \rightarrow {}^{\bullet}OH + OH^- + O_2$$
$$H^+ + H_2O \rightarrow {}^{\bullet}OH + H^+$$

To further elucidate the mechanism of AgNPs antimicrobial activity, various levels of reactive oxygen species were recorded. A comparative study was carried out to verify the role of oxygen in the antibacterial activity of AgNPs. Figure 1c shows the effect of oxygen on antibacterial activities when 3–7 mg/L AgNPs were added to the LB medium containing 10^6 cfu/mL of *E. coli* that were conducted in aerobic and anaerobic conditions, respectively. It was reported that inactivation of *E. coli* was associated with the ratio between the number of nanoparticles and the initial bacterial cell count (Dror-Ehre et al. 2009). Identical population level was essential to establish antibacterial activity of AgNPs under aerobic and anaerobic conditions. Being a facultative anaerobe, *E. coli* grew to similar level in both aerobic and anaerobic condition in the absence of AgNPs.

Based on Fig. 1c, it was apparent that AgNPs exhibited antibacterial effect under both aerobic and anaerobic conditions. The antibacterial activity of AgNPs in aerobic condition was comparatively stronger than that in anaerobic condition ($P < 0.01$). It may be inferred that due to the lack of oxygen during anaerobic incubation, there was less ROS generated by AgNPs that maybe responsible for the lower antibacterial activity. This observation was in line with the bactericidal activity of silver zeolite wherein microbial inactivation decreased in the absence of oxygen (Matsumura et al. 2003).

To confirm the involvement of ROS in the antibacterial mechanism of AgNPs, we evaluated the antibacterial activity of AgNPs in the presence of NAC. NAC is an effective antioxidant containing a mercapto group, which could prevent the oxidative damage induced by AgNPs. The results of the effect of NAC on the antibacterial activity of AgNPs are shown in Fig. 1d. The NAC test in its own that was used as the control, did not show any lethal effect on *E. coli* at the working concentration of 10 mM ($P > 0.1$). AgNPs alone showed strong antibacterial activity that killed ~50% of the bacteria at a concentration of 5 mg/L and killed almost 99% of the bacteria at 10 mg/L. However, mortalities decreased to 20 and 40% when 10 mM NAC was added to AgNPs at 5 and 10 mg/L, respectively. These results demonstrated that the antibacterial activity of AgNPs was attenuated in the presence of antioxidant ($P < 0.01$). It has been reported that NAC not only reduce the concentration of ROS in the culture medium as a ROS scavenger, but also induces the production of glutathione which is an important cellular antioxidant (Lovric et al. 2005). Thus, the presence of NAC as ROS scavenger may have been responsible for the reduced antimicrobial activity of AgNPs. It was also possible that NAC induced the production of glutathione in our studies but this remains to be tested in the future.

From these series of studies, the idea that ROS were responsible for the antibacterial activity of AgNPs was supported by the fact that oxygen significantly affected the antimicrobial activity. When oxygen was transformed to ROS in a series of reactions in the presence of AgNPs and NAC that acted as a ROS scavenger, the antibacterial activity was significantly reduced. This was also supported by the lower AgNPs antimicrobial activity in anaerobic conditions. Thus, these results indirectly proved that ROS played a very important role in the antibacterial mechanism of AgNPs antibacterial activity.

Conclusion

We established that the antibacterial activity of hydrophilic polymer coated AgNPs is affected by temperature and its exposure to acid conditions. Higher temperatures up to 37°C (we did not study higher than this) resulted in higher bacterial mortality in the presence of AgNPs. Higher bacterial mortality was also observed at aerobic conditions than at anaerobic conditions. Through the monitoring of ROS levels at conditions of maximum antibacterial activity and the use of ROS scavenger, NAC, we established that ROS were responsible for the

antibacterial activity of AgNPs that was supported by the significant effect of oxygen. When oxygen was transformed to ROS in a series of reactions in the presence of AgNPs and NAC that acted as a ROS scavenger, the antibacterial activity was significantly reduced. This was also supported by the lower AgNPs antimicrobial activity in anaerobic conditions. Thus, the combined results of the studies conducted indirectly proved that ROS played a very important role in the mechanism of AgNPs antibacterial activity.

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