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Antibacterial effect of silver nanoparticles on *Staphylococcus aureus*

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Abstract The antibacterial activity and mechanism of silver nanoparticles (Ag-NPs) on *Staphylococcus aureus* ATCC 6538P were investigated in this study. The experiment results showed the minimum bactericidal concentration (MBC) of Ag-NPs to *S. aureus* was 20 μg/ml. Moreover, when bacteria cells were exposed to 50 μg/ml Ag-NPs for 6 h, the cell DNA was condensed to a tension state and could have lost their replicating abilities. When *S. aureus* cells were exposed to 50 μg/ml Ag-NPs for 12 h, the cell wall was breakdown, resulting in the release of the cellular contents into the surrounding environments, and finally became collapsed. And Ag-NPs could reduce the enzymatic activity of respiratory chain dehydrogenase. Furthermore, the proteomic analysis showed that the expression abundance of some proteins was changed in the treated bacterial cell with Ag-NPs, formate acetyltransferase increased 5.3-fold in expression abundance, aerobic glycerol-3-phosphate dehydrogenase decreased 6.5-fold, ABC transporter ATP-binding protein decreased 6.2-fold, and recombinase A protein decreased 4.9-fold.

Keywords Silver nanoparticles (Ag-NPs) · *Staphylococcus aureus* · Antibacterial effect

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Introduction

Silver nanoparticles (Ag-NPs) represent a new generation of antimicrobials. Ag-NPs have a very broad range of antimicrobial activity and kill both Gram-negative and Gram-positive bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus mutans*, and *Staphylococcus epidermidis* (Sondi and Salopek-Sondi 2004; Yoon et al. 2007; Kim et al. 2007; Lee et al. 2008; Jung et al. 2008; Yamanaka et al. 2005; Espinosa-Cristóbal et al. 2009; Cho et al. 2005). Moreover, Ag-NPs have strong antifungal activity on *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Trichophyton mentagrophytes* (Kim et al. 2008, 2009). Ag-NPs also have antiviral activity against human immunodeficiency virus-1 (HIV-1) (Lara et al. 2010), hepatitis B virus (Lu et al. 2008), herpes simplex (Baram-Pinto et al. 2009), monkeypox (Rogers et al. 2008), and respiratory syncytial virus (Sun et al. 2005). The antimicrobial activity of Ag-NPs appears significantly high. Silver is more toxic element to

microorganisms than many other metals in the following sequence: Ag > Hg > Cu > Cd > Cr > Pb > Co > Au > Zn > Fe > Mn > Mo > Sn (Zhao and Stevens 1998). And Ag-NPs exert more efficient than silver ions and other silver salts in mediating their antimicrobial activity (Lok et al. 2006; Rai et al. 2009). Silver exhibits low toxicity to mammalian cells (Zhao and Stevens 1998). And silver has a lower propensity to induce microbial resistance than many other antimicrobial materials (Kim et al. 2007; Silver 2003; Silver et al. 2006; Franke et al. 2001). As a result, Ag-NPs have been applied to a wide range of products, the most important current use is as antimicrobial agents to prevent infection, such as in burn and traumatic wound dressings, diabetic ulcers, coating of catheters, dental works, scaffold, and medical devices (Rai et al. 2009; Law et al. 2008; Silver et al. 2006; Kim et al. 2007; Thomas et al. 2007; Kim and Kim 2006). Ag-NPs are also used in hygienic products including water purification systems, linings of washing machine, dishwashers, refrigerators, and toilet seats (Silver et al. 2006; Rai et al. 2009).

Although Ag-NPs have been widely used as microbial agents in many products their antimicrobial mechanism is still unclear. Several studies on the antibacterial act of Ag-NPs on Gram-negative bacteria have been carried out previously (Li et al. 2010), but the act on the Gram-positive bacteria is almost unknown. Because the cell structures between Gram-negative bacteria and positive bacteria are much different, it could be conferred the acting mechanism of Ag-NPs on Gram-negative bacteria and positive bacteria may be dissimilar. We chose the Gram-positive bacteria *S. aureus* to study the action mode of Ag-NPs on bacteria in this study. A proteome approach together with biochemical analysis was applied to investigate the mode of antibacterial action. Our results revealed that Ag-NPs damage the structure of bacterial cell membrane of *S. aureus*, inhibit the cell respiration, and enhance or reduce the expression abundance of some enzymes.

Materials and methods

Chemical reagents, organism, mediums, and cultivation

The Ag-NP solutions and iodonitrotetrazolium chloride reagent were the same as we used in Li et al. (2010). The *S. aureus* ATCC6538P strain was purchased from American Type Culture Collection (ATCC). Mueller–Hinton medium (purchased from Guangdong Huankai Microbial Sci. and Tech. Co., Ltd, Guangzhou, China; final pH 7.3) contains: 2.0 g beef extract, 1.5 g soluble starch, and 17.5 g acid hydrolysate of casein. Mueller–Hinton medium was used for aerobic cultivation at 37°C with shaking at 150 rpm. Growth curves of *S. aureus* exposed and unexposed to Ag-NP were determined based on the absorbing value of optical density (OD) at 600 nm.

Minimum inhibitory concentration and minimum bactericidal concentration of Ag-NPs

To examine the minimum inhibitory concentration of Ag-NPs and the growth curves of *S. aureus* exposed to Ag-NPs, medium, Ag-NPs, and *S. aureus* cells were added separately to 20 ml cultures, resulting in final concentrations of Ag-NPs of 0to 20 μg/ml and 10^7cfu/ml *S. aureus* cells. The incubating and determining methods were according with our previous study (Li et al. 2010).

Action of Ag-NPs on *S. aureus* cell structure

Medium, 50 μg/ml Ag-NPs, and 10^8cfu/ml cells were added to 10 ml cultures. Control experiment was conducted in absence of Ag-NPs. The cultures were incubated at 37°C with shaking at 150 rpm for 6 h. The cultures were centrifuged and the supernatants were discarded for preparation of ultrathin sections of cells. Finally, the internal structure of cells was observed in the ultrathin sections by transmission electron microscopy (TEM, Hitachi H-7650). In addition, cultures incubated for 12 h were loaded on copper screen directly for observing their external structure by TEM.

Effect of Ag-NPs on enzymatic activity of respiratory chain dehydrogenase

Medium, 10 and 50 μg/ml Ag-NPs and 10^8cfu/ml *S. aureus* were added into 10 ml cultures. Experiments were conducted in absence of Ag-NPs as the control. The *S. aureus* cells were boiled for 20 min to inactivate the enzymes completely as a negative control, while the cells were not boiled, and their enzymes maintained native activity as the positive

control. Dehydrogenase activity was determined according to the iodonitrotetrazolium chloride method (Iturriaga et al. 2001; Kim et al. 1994, 2009) with slight modification as our previous study (Li et al. 2010).

Proteomic analysis of *S. aureus* cells exposed to Ag-NPs

Phosphate-buffered saline, 20 μg/ml Ag-NPs, and 10^9cfu/ml *S. aureus* cells were added to 50 ml cultures. Control experiment was conducted in absence of Ag-NPs. The cultures were incubated at 37°C with shaking at 150 rpm for 2 h. The cultures were centrifuged and the supernatants were discarded. Cells collected by centrifugation were suspended in lysis buffer (8 M urea, 4% CHAPS, 20 mM dithiothreitol). The lysates were clarified by centrifugation and the supernatants were stored at −80°C before analysis by two-dimensional electrophoresis.

Two-dimensional electrophoresis was performed with the IPGphor isoelectric focusing and electrophoresis system (Bio-Rad, USA). Total proteins lysates of *S. aureus* were mixed with rehydration solution containing 0.2% Bio-Lyte ampholyte, 8 M urea, 4% CHAPS, 1 mg/ml dithiothreitol, trace bromophenol blue. Isoelectric focusing was performed using a PROTEAN isoelectric focusing system and the second-dimension SDS-PAGE was performed in a PROTEAN II electrophoresis system. All gels were visualized by silver staining and scanned with Image Scanner (Bio-Rad, USA). Protein spots on the image files were detected and quantified using Image Master 2D Elite 5.0 (GE, USA). Experiments were performed twice and protein spots exhibiting consistent differences between samples were excised from the gels. After in-gel digestion, the resulting peptides were identified by MALDI-TOF MS.

Results

Growth of *S. aureus* exposed to Ag-NPs

Growth of *S. aureus* (Fig. 1) was basically same with *E. coli* in our previous study (Li et al. 2010). When exposed to 20 μg/ml Ag-NPs, *S. aureus* cells did not show any growth over 7 days. When exposed to 1.25, 2.5, 5, and 10 μg/ml of Ag-NPs, *S. aureus* cells were lagged to 2, 12, 30, and 96 h to get to the maximum, respectively. The minimum inhibitory concentration (MIC) of Ag-NPs to *S. aureus* was 5 μg/ml because in this incubating condition the bacteria were not growth within 24 h. No growth of *S. aureus* exposed to 20 μg/ml Ag-NPs indicated the minimum bactericidal concentration (MBC) was 20 μg/ml.

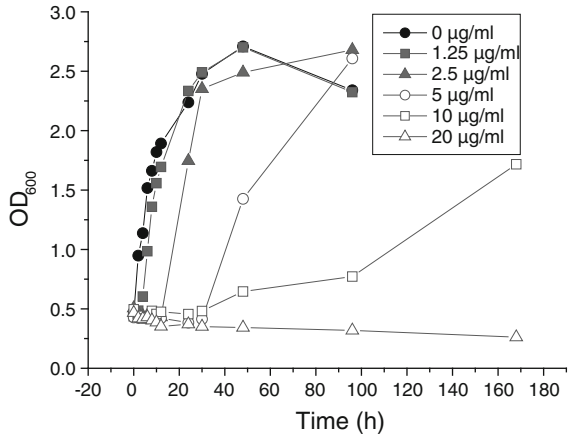

Fig. 1 Growth curves of *S. aureus* cells exposed to different concentrations of Ag-NPs

Effect of Ag-NPs on the structure of *S. aureus* cells

The TEM electron micrographs of *S. aureus* cells treated and untreated with Ag-NPs (Fig. 2a, b) showed the internal structure of native *S. aureus* cells. It was common to find homogeneous electron density in the cytoplasm of native *S. aureus* cells and there was an apparent nuclear region in the center of the cell which was a remarkable electron-light region. Moreover, some materials like string were distributed randomly in the electron-light region which was DNA of *S. aureus* cells. After treated with Ag-NPs for 6 h, the region was shrunk to an electron-light dot and even disappeared (Fig. 2d, e). This may indicate after *S. aureus* cells exposed to Ag-NPs the DNA were condensed.

The TEM micrograph (Fig. 2c) showed the external morphological features of native *S. aureus* cells. Apparently, the surface of untreated *S. aureus* cells was smooth and retained their coccal morphology. In contrast, *S. aureus* cells treated with Ag-NPs for 12 h appeared to undergo lysis (Fig. 2f), their cell wall was breakdown, resulting in the release of their

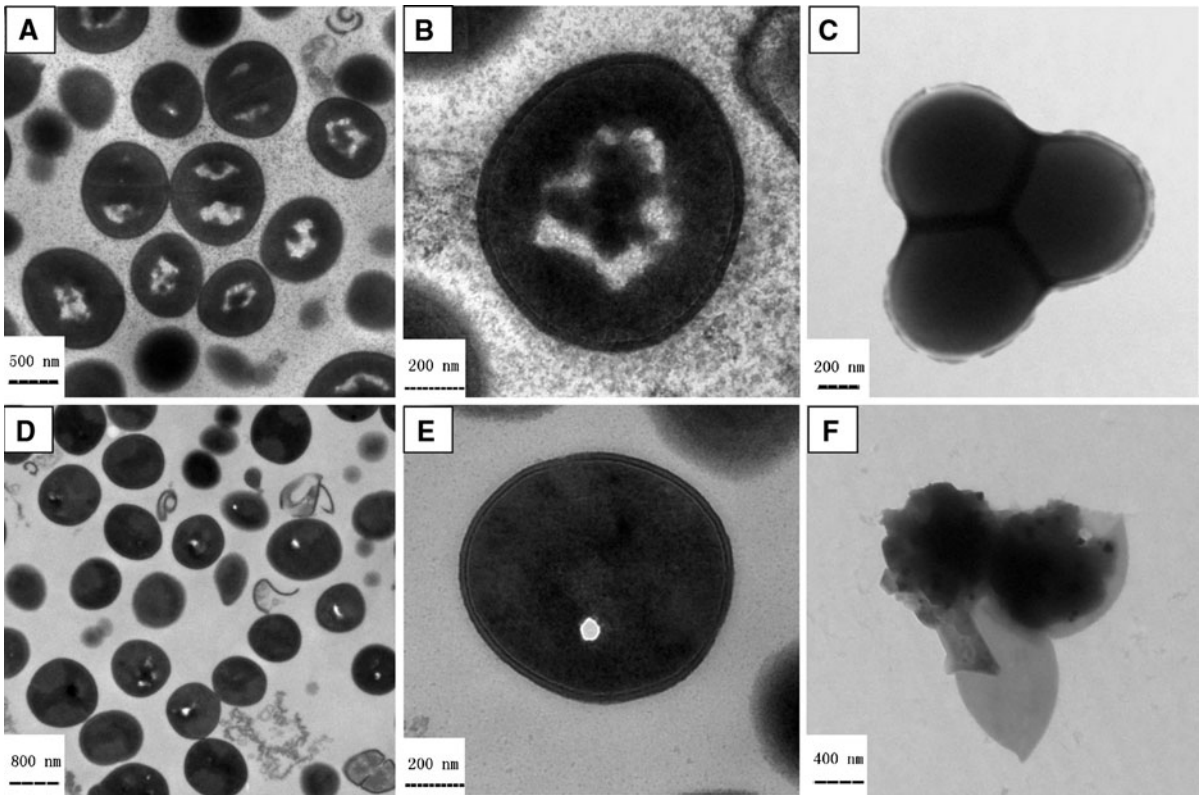

Fig. 2 Action of Ag-NPs on *S. aureus* cells observed by TEM. **a**, **b** the internal structure of native *S. aureus* cells culture for 6 h; **c** the external structure of native *S. aureus* cells culture for 12 h; **d**, **e** the internal structure of *S. aureus* cells treated with 50 μg/ml Ag-NPs for 6 h; **f** the external structure of *S. aureus* cells treated with 50 μg/ml Ag-NPs for 12 h

cellular contents into the surrounding environment, and finally the cytoplasm was emptied.

Effect of Ag-NPs on respiratory chain dehydrogenase

The effect of Ag-NPs on respiration chain dehydrogenase of *S. aureus* (Fig. 3) showed that dehydrogenase activity in positive control cells increased with the incubation. Interestingly, enzymatic activity of cells treated with 5 μg/ml Ag-NPs was almost as high as that of positive control in initial time, subsequently, the activity was reduced, and finally its activity gradually increased. It suggested that 5 μg/ml Ag-NPs did not completely inhibit the 10^8cfu/ml *S. aureus* cells, when the bacteria adapt to the condition for 20 min their respiration enhanced gradually. But enzymatic activity of cells exposed to 50 μg/ml Ag-NPs was always lower than that of cells treated with 5 μg/ml Ag-NPs and it did not show increased over 30 min.

Effect of Ag-NPs on proteome of *S. aureus* cells

Exponentially growing *S. aureus* cells were exposed to 20 μg/ml Ag-NPs for 2 h. The proteomes were then analyzed by two-dimensional electrophoresis followed by silver staining and scanned with Image Scanner (Fig. 4). Some spots in the patterns indicate a decreased or increased amount of protein expression after treated with Ag-NPs compared with controls. Only the proteins with at least fourfold decrease or increase in density ratio were submitted for further analysis. One spot with an increase and three spots with a decrease are marked on Fig. 4 and Table 1. These spots were cut from the gels and analyzed by MALDI-TOF/TOF/MS. Through searching of NCBI database for peptide mass fingerprinting one protein with 5.3-fold increase in abundance of expression was identified with high probability as formate acetyltransferase, and three proteins with a decrease in abundance of expression were identified with high probabilities as aerobic

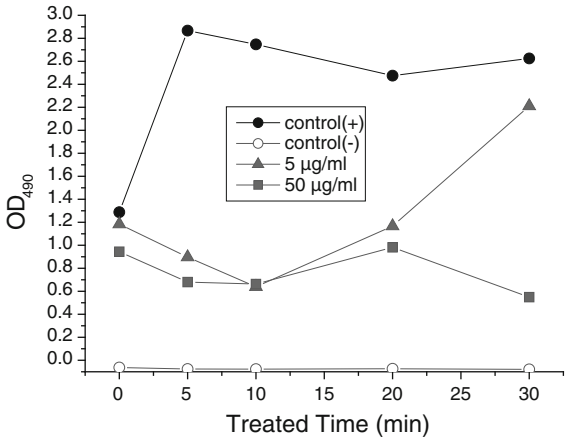

Fig. 3 Effect of Ag-NPs on respiration chain dehydrogenases in *S. aureus* cells. Data are average from duplicate experiments. *Error bars* represent standard deviations of duplicate incubations. The negative and positive controls represent the boiled and not boiled *S. aureus* cells respectively

glycerol-3-phosphate dehydrogenase, ABC transporter ATP-binding protein, and recombinase A protein (Table 1).

Discussion

The growth curves indicated that Ag-NPs could inhibit the growth of *S. aureus* and even kill the cells completely. Ag-NPs prolonged the growth lag phase of *S. aureus*. The effect is similar to *E. coli* (Li et al. 2010), but the MBC of Ag-NPs is different between *E. coli* (10 μg/ml) (Li et al., 2010) and *S. aureus* (20 μg/ml) under the same experimental condition, which implying that Ag-NPs are more toxic to *E. coli* than to *S. aureus*.

In Fig. 2a, b by TEM DNA of bacteria cells condensed after exposed to Ag-NPs for 6 h. It is known only when DNA molecules are in a relaxed state they can accomplish replication effectively. So, DNA molecules of *S. aureus* cells exposed to Ag-NPs could have lost their replicating ability. The result was consistent with the study of antibacterial effects of silver ions on *E. coli* (Feng et al. 2000). This might be one reason for the death of bacteria cells. Subsequently, when the *S. aureus* cells were exposed to Ag-NPs for 12 h, the cell wall breakdown and

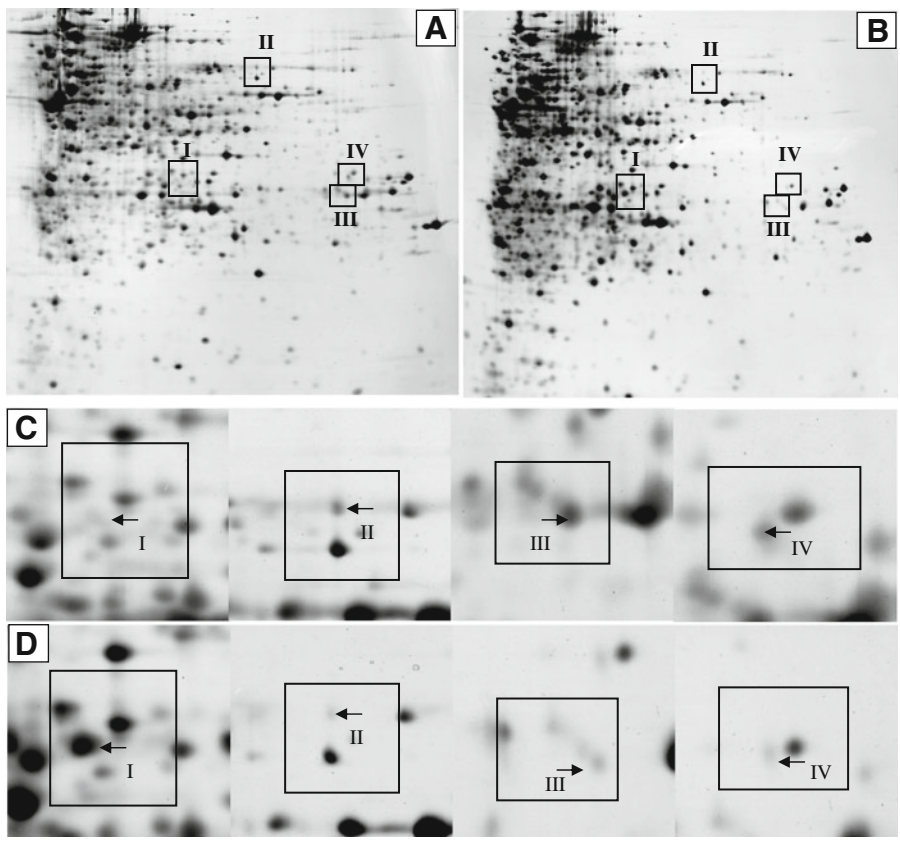

Fig. 4 Action of Ag-NPs on proteomic of *S. aureus* cells. 2D gel images of **a** untreated *S. aureus* cells and **b** *S. aureus* cells treated with Ag-NPs. **c**, **d** Squared areas (I–IV) represent areas of interest containing the Ag-NPs stimulated and weakened protein spots in untreated and treated cells and are enlarged

Table 1 List of identified *S. aureus* proteins whose expressions were stimulated or weakened by Ag-NPs

Protein no.	Density ratio	Protein description	NCBI accession no.	Mr (kDa)	PI
I	+5.28565	Formate acetyltransferase	gi161508484	85.3731	5.27
II	−6.45941	Aerobic glycerol-3- phosphate dehydrogenase	gi122063487	62.3789	6.57
III	−6.18394	ABC transporter ATP -binding protein	gi221140322	29.3783	7.05
IV	−4.89835	Recombinase A protein	gi87126813	34.8569	5.04

cytolysis happened, resulting in the release of their cellular contents into the environment, and finally the cytoplasm was emptied.

The respiratory chain dehydrogenase was to study the effect of Ag-NPs on enzymes and proteins. Respiratory chain dehydrogenase was inhibited by Ag-NPs, and the higher of concentration of Ag-NPs, the lower of the activity of the enzyme. The same phenomenon occurred in the *E. coli* cells exposed to Ag-NPs (Li et al. 2010). Ag-NPs could destroy the respiratory chain dehydrogenase and accordingly inhibited the respiration to interfere with normal growth and metabolism of bacteria cells.

The proteome results showed formate acetyltransferase increased 5.3-fold in expression abundance, and aerobic glycerol-3-phosphate dehydrogenase decreased 6.5-fold, ABC transporter ATP-binding protein decreased 6.2-fold, recombinase A protein decreased 4.9-fold. Formate acetyltransferase also known as pyruvate formate-lyase is a typical signature under anaerobic conditions in *S. aureus* and *E. coli*. Generally, aerobically grown cells contained a basal level of pyruvate formate-lyase which was derepressed 5- to 10-fold under anaerobiosis (Hecker et al. 2009; Fuchs et al. 2007). The enhanced expression of the formate acetyltransferase implied an anaerobic condition occurred in which oxygen cannot be used as terminal electron acceptor when *S. aureus* cells were exposed to Ag-NPs. Moreover, the aerobic glycerol-3-phosphate dehydrogenase is an enzyme, expressed under aerobic conditions, which converts glycerol-3-phophate to dihydroxyacetone. While another enzyme, anaerobic glycerol-3-phosphate dehydrogenase is expressed under anaerobic conditions converted glycerol-3-phophate to dihydroxyacetone. The reduced expression of aerobic glycerol-3-phosphate dehydrogenase also implied an anaerobic fermentation of *S. aureus* cells. ABC transporters are integral membrane proteins that carry diverse substrates across lipid bilayers (Hollenstein et al. 2007). In bacteria, ABC transporters catalyze the uptake of essential nutrients or the extrusion of toxic substances, thus contributing to drug and antibiotic resistance. ABC transporters are divided into two subtypes on the basis of the direction of the transport reaction. ABC importers, require a binding protein (ABC transporter ATP-binding protein) that delivers captured substrate to the internal face of the transporter. ABC exporters, by contrast, recruit their substrates directly from the cytoplasm or from the inner leaflet of the lipid bilayer (Hollenstein et al. 2007). Accordingly, the less expression of ABC transporter ATP-binding protein may disturb the natural activity of ABC importers from absorbing nutrition. Recombinase A protein (RecA) is essential for the repair of both plasmid and chromosomal DNA in bacteria and is considered to be related to the SOS-response in response to DNA damage or DNA replication handicap brought by ultraviolet light, certain chemicals, or other mutagens (Cox 2007). The experiment result of proteome suggested that Ag-NPs may do harm to the RecA or correlative process such as transcription, translation and post-translational modification of recA gene. As a result, the reduced RecA cannot do enough to repair the damaged DNA or lead to SOS-response and this may accelerate the death course of bacteria cells.

In conclusion, the above experiments demonstrated a possible antibacterial process of Ag-NPs on *S. aureus* cells: Ag-NPs over passed cell wall and acted with cell membrane to cripple the relative enzymes and interfered with normal metabolism of cells, subsequently, Ag-NPs entered into bacteria cells and condensed DNA to prevent DNA from replicating and cells from reproducing. Simultaneously, Ag-NPs continuously act with cell wall and cell membrane to destroy them, finally, the bacteria cells were pulled down drastically.

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