Silver Nanoparticles Induced Cell Apoptosis, Membrane Damage of *Azotobacter vinelandii* and *Nitrosomonas europaea* via Generation of Reactive Oxygen Species

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Abstract

Silver nanoparticles (AgNPs) is widely used as an antibacterial agent, but the specific antibacterial mechanism is still conflicting. This study aimed to investigate the size dependent inhibition of AgNPs and the relationship between inhibition and reactive oxygen species (ROS). *Azotobactervinelandii* and *Nitrosomonaseuropaea* were exposed to AgNPs with different particles size (10 nm and 50 nm). The ROS production was measured and the results showed that the generation of ROS related to the particle size and concentrations of AgNPs. At 10 mg/L of 10 nm Ag particles, the apoptosis rate of *A. vinelandii* and *N. europaea* were 20.23% and 1.87% respectively. Additionally, the necrosis rate of *A. vinelandii* and *N. europaea* reached to 15.20% and 42.20% respectively. Furthermore, transmission electron microscopy images also indicated that AgNPs caused severely bacterial cell membrane damage. Together these data suggested that the toxicity of AgNPs depends on its particle size and overproduction of ROS.

Keywords Silver nanoparticles · Reactive oxygen species · *Azotobactervinelandii* · *Nitrosomonaseuropaea* · Apoptosis

With the rapid development of nanotechnology, metal silver has been prepared into nanosilver (AgNPs) by different ways or means, and it has become one of the commonly used antibacterial agents (Gruen et al. 2018). AgNPs have a particle size distribution between 1 and 100 nm (Fabrega et al. 2011), which exhibit unique antibacterial activity that unmatched by other silver-loaded inorganic materials, and kill pathogenic microorganisms including bacteria, fungi and mycoplasma effectively (Li et al. 2010). Yang et al. (2013) have demonstrated that AgNPs inhibited growth of *Pseudomonas stutzeri, Azotobacter vinelandii,* and *Nitrosomonas europaea* Li et al. (2010) revealed that AgNPs kill *Escherichia coli* by damaging the structure of cell membrane.

Ahmed et al. (2018) confirmed this finding, and reported that AgNPs-induced reactive oxygen species (ROS) mediated destruction of cell membrane, growth and biofilms of *Escherichia coli, Pseudomonasaeruginosa* and *Staphylococcusaureus*. Some studies have indicated that AgNPs-induced ROS played an antibacterial role by destroying bacterial cell membrane, leading to bacterial apoptosis death (Dwyer et al. 2012; Schaumann et al. 2015). Quinteros et al. (2018) found that ROS induced by AgNPs caused oxidative stress, leading to modification of the membrane potential and lipid peroxidation of *Staphylococcus aureus* and *Escherichia coli*. Bao et al. (2015) reported that the presence of AgNPs caused apoptosis and DNA damage in *E. coli* in a dose dependent manner. Numerous researchers have revealed that AgNPs exert toxicity both in concentration and size dependent manner (Gliga et al. 2014; Wang et al. 2017b). AgNPs with small particle sizes and large specific surface area can bind to the cell membrane fully. Additionally, due to the large number of active sites, smaller AgNPs can produce more ROS, thus showing stronger antibacterial activity (Choi and Hu 2008; Riaz Ahmed et al. 2017).

The toxicity of AgNPs to various bacteria are widely reported. ROS are generally considered to be one of the most important antibacterial mechanisms of AgNPs (Duran

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et al. 2016). Long et al. (2017) believed that AgNPs played a bactericidal role by releasing Ag^+ and interacting with the cell membrane to generate ROS in *E. coli* cells. In fact the Ag^+ release was also one of the reasons for ROS production (Beer et al. 2012). Excessive ROS accumulation will disturb the normal oxidative-antioxidant level in cells, induce oxidative stress and lead to apoptosis (Duran et al. 2016). Moreover, collapsing cell membrane/wall is also a pronounced toxic effect of AgNPs (Shi et al. 2018). Numerous studies have explored the antimicrobial effectiveness of AgNPs against various bacteria, viruses, and fungi (Tang and Zheng 2018), very limited reports were available on N-cycling bacteria. Herein, we extend this work to research interactions of AgNPs with *Azotobactervinelandii* and *N. europaea*. These bacteria are commonly present in both natural and engineered systems (Yang et al. 2013). The aim of this study was to investigate the toxicity of AgNPs with a specific focus on size and oxidative stress effects, and to explore the mechanisms of toxicity. The results showed that AgNPs-induced ROS played a key role in bacterial apoptosis and membrane damage.

Materials and Methods

Studies have reported that the toxicity of AgNPs was size dependent, and only AgNPs with diameters in the range of 1–10 nm can interact directly and preferentially with bacteria (Li et al. 2019), thus the 10 nm and 50 nm AgNPs were selected for toxicity testing. The aqueous suspension of polyvinyl pyrrolidone (PVP) capped 10 nm nanosilver (nAg50) were purchased from Nanjing Xianfeng Nanomaterial Technology Co. According to the manufacturer's protocol, this suspension contained 99% silver, an average particle size of 10–15 nm and a nominal concentration of 1000 mg/L. The 50 nm nanosilver (nAg50) suspension was prepared by adding AgNPs powders (50 nm with the purity of 99%; Nanjing Emperor Nano Material Co.) into ultra-pure water. According to inductively coupled plasma-mass spectrometry (ICP-MS), the actual concentrations of nAg10 and nAg50 in bacteria growth medium were lower than nominal stock concentrations (Table S1). The Ag^+ released from nAg10 and nAg50 in both media were less than 0.5% (Table S2), so the toxicity of Ag^+ was not considered in present study. After 4 h of ultrasonic oscillation, the final concentration of nAg50 suspension was 1000 mg/L. The two sizes of AgNPs suspension were stored in the dark at 4°C before use. The morphology of AgNPs was observed by transmission electron microscopy (TEM, H-7650, Hitachi, Japan). The average sizes were about 10.48 ± 0.94 nm and 48.08 ± 3.44 nm for nAg10 and nAg50, respectively. The size of nAg10 was obviously smaller than nAg50 and both AgNPs were spherical in shape; nAg50 were easier to aggregate according to

the TEM image, which less stable (Fig. 1a, b). The tested concentrations of both AgNPs in this study were obtained by adding appropriate amount of AgNPs stock solution into a volume of used medium, so that the final concentrations of AgNPs were 5 mg/L and 10 mg/L for nAg10, 10 mg/L and 20 mg/L for nAg50, respectively. Before the assays, preliminary work about inhibition of nAg10 and nAg50 on bacterial growth were measured and the results showed that the nAg50 with a concentration of 10 mg/L had little effect on the cell viability of *A. vinelandii,* while the nAg10 at a concentration of 10 mg/L have already inhibited the growth of *A. vinelandii* (data were not shown), so the highest doses of nAg10 and nAg50 were set to 10 mg/L and 20 mg/L, respectively.

Azotobacter vinelandii was grown in 1 L of nitrogen-free medium containing 0.5 g yeast, 20.0 g mannitol, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaSO_4 \cdot H_2O$, 1 mL trace elements, containing 22 g/L $ZnSO_4 \cdot 7H_2O$, 5 g/L $MnCl_2 \cdot 4H_2O$, 5 g/L, $FeSO4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 6H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, 7.5 g/L, $Na_2MoO_4 \cdot 2H_2O$ and 60 g/L $EDTA-Na_2$. *N. europaea* was grown in a defined mineral salt medium, the composition could refer to Yang et al. (2013). ROS production can be divided into two pathways. NPs with large surface areas and excess reactive sites can produce ROS, which is exogenous pathway. Endogenous ROS are generated inside the cells (Choi and Hu 2008). In this study, *A. vinelandii* and *N. europaea* were exposed to nAg10 (5 mg/L and 10 mg/L) and nAg50 (10 mg/L and 20 mg/L) for 12 h respectively. It has been reported that glutathione (GSH) could eliminate ROS generation (Long et al. 2017), the additional exposure groups were set to investigate the antagonistic effects of GSH on AgNPs. *A. vinelanii* and *N. europaea* were stimulated with highest concentrations of both AgNPs (10 mg/L for nAg10 and 20 mg/L for nAg50) in the presence of 1 mM GSH for 12 h. Each treatment had three replicates. After the 12 h incubation, the samples were collected by centrifugation and washed 3 times with phosphate buffer saline (PBS). ROS generation was measured through adding the 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and AgNPs-free fresh medium at a volume ratio of 1:2000 and incubated at 30°C for 30 min. The ROS production in *A. vinelandii* and
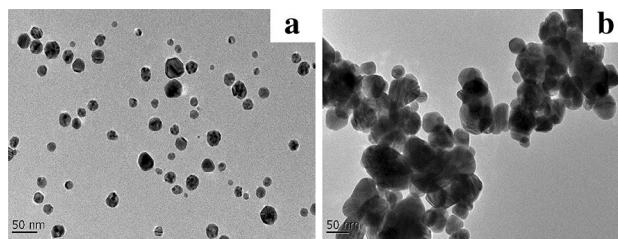

Fig. 1 TEM results for two size of AgNPs. **a** nAg10 and **b** nAg50

N. europaea cells was detected at excitation wavelength of 485 nm and emission wavelength of 525 nm using fluorescence measurement (Wang et al. 2017a). Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that binds with high affinity to phosphatidylserine during apoptosis. Fluorescein isothiocyanate (FITC) is a fluorescein with a strong green fluorescence signal. FITC labeled Annexin V is used as a fluorescence probe to detect the apoptosis by flow cytometry (Fakai et al. 2019). Apoptosis assay was determined by adding 300 μL binding buffer solution to the precipitate that has been washed with PBS and centrifuged. 5 μL Annexin V-FITC was added and incubated in the dark for 15 min. Propidium iodide (PI) was added 5 min before the test. The sample was analysed by FACSCalibur flow cytometry (BD Co. USA). After incubation with AgNPs, the *A. vinelandii* and *N. europaea* cells were washed with PBS and collected, fixed with glutaraldehyde for 12 h, and fixed with 1% OsO4 for 1 h. The morphology of the cells was observed under transmission electron microscope (TEM, Model H-7650, Hitachi, Japan) after acetone treatment. More details could refer to Wang et al. (2017c).

Results and Discussion

ROS is the general term of oxygenated compounds including superoxide, hydrogen peroxide and hydroxyl radicals, which can initiate various cellular biological effects (Petrov et al. 2015). ROS production was an indicator of the oxidative stress level in cells (Choi and Hu 2008). As shown in Fig. 2, after treated with both AgNPs for 12 h, the *A. vinelandii* and *N. europaea* intracellular ROS production were significantly increased. Additionally, there were significant differences in ROS generation between the both AgNPs with different concentrations. Suggesting that the ROS production was dose dependent. After the antioxidant GSH was added, the ROS levels in bacteria cells decreased compared with before, indicating that GSH could protect the cells from oxidative damage (Choi et al. 2018). Noteworthily, the nAg10 at a concentration of 10 mg/L caused highest ROS level in both bacteria cells and there were some differences in ROS generation between *A. vinelandii* and *N. europaea* cells treated with nAg10 and nAg50. Indicating the ROS generation was also size dependent and might relate to the bacterial species (Chen et al. 2017). Previous studies have researched toxic effects of AgNPs on various organisms, including aquatic organisms, bacteria and mammals (Burdusel et al. 2018; Franci et al. 2015). The toxicity mechanisms of AgNPs are complex and may result from interactions between several different processes, while the ROS generation and oxidative stress are the most widely accepted mechanism for the toxicity of AgNPs currently (Wang et al. 2017a). When various exogenous or endogenous factors caused ROS
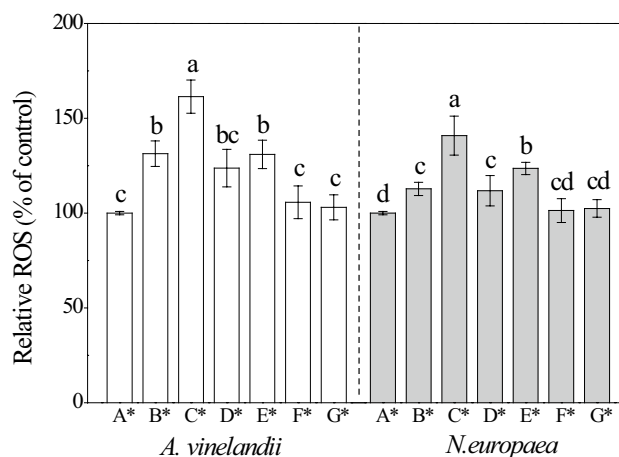

Fig. 2 ROS generation and GSH scavenging in *A. vinelandii* and *N. europaea* cells after nAg10 and nAg50 treatment for 12 h. A*: control; B*: 5 mg/L nAg10; C*: 10 mg/L nAg10; D*: 10 mg/L nAg50; E*: 20 mg/L nAg50; F*: 10 mg/L nAg10 with 1 mM GSH; G*:20 mg/L nAg50 with 1 mM GSH. *Lower letters* (*a*, *b*, *c* and *d*) above the bars indicate that the data points are significantly different at $p < 0.05$ (n = 3)

overproduction which exceeds the ability of the intracellular antioxidant defense system to clear, oxidative stress occurs (Barcinska et al. 2018). Excessive ROS has been confirmed in multifarious cell models. Mao et al. (2018) have found that AgNPs could activate a series of ROS-mediated cytotoxic pathways to *Drosophila melanogaster*. Yan et al. (2018) explored the effects of AgNPs on *Pseudomonasaeruginosa* using proteomics methods, and have indicated that disruption of bacterial cell membrane and ROS production were the main mechanisms of AgNPs antibacterial activity. GSH acts as ROS scavenger could combine with ROS to form glutathione disulfide (GSSG), which maintains a dynamic balance between oxidation and antioxidant levels (Choi et al. 2018). However, if ROS is overproduced, GSH will be depleted and the body's natural antioxidant mechanism will be destroyed. Excessive ROS also cause lipid peroxidation, resulting in cells death (Zapór 2016).

Apoptosis was detected by Annexin V FITC/PI double staining (Table 1). Compare with control, the apoptosis rate was significantly increased in nAg10 and nAg50 groups. Additionally, *A. vinelandii* and *N. europaea* apoptotic rate were different when exposed to the same AgNPs. As shown in Table 1, the apoptotic rate and necrosis rate of *A. vinelandii* were 20.23% and 15.20% while the *N. europaea* were 1.87% and 42.20% with treatment of nAg10 at a concentration of 10 mg/L. Obviously, *N. europaea* has a higher rate of necrosis than apoptosis, whereas *A. vinelandii* has the opposite result. The difference in these results probably due *N. europaea* was more sensitive to external stimuli than *A. vinelandii*. Yang et al. (2013) have showed that the minimum inhibitory concentration (MIC) of AgNPs on *N. europaea*

**Table 1** The cell apoptosis rates of *A. vinelandii* and *N. europaea* treated with AgNPs for 12 h (n=3)

Bacteria	Treatments	(mg/L)	Rate of living cells (%)	Apoptosis rate (%)	Necrosis rate (%)
A. vinelandii	Control	0	96.20 ± 1.08	2.56 ± 0.01	1.22 ± 0.01
	nAg10	5	86.20 ± 0.97	8.41 ± 0.04	5.43 ± 0.02
		10	64.60 ± 1.02	20.23 ± 0.12	15.20 ± 0.06
	nAg50	10	95.00 ± 1.80	3.14 ± 0.01	1.83 ± 0.01
		20	93.50 ± 1.23	3.42 ± 0.01	3.07 ± 0.02
N. europaea	Control	0	98.10 ± 1.82	0.49 ± 0.01	1.43 ± 0.01
	nAg10	5	80.70 ± 1.46	0.90 ± 0.01	18.40 ± 0.07
		10	55.90 ± 0.94	1.87 ± 0.02	42.20 ± 0.16
	nAg50	10	89.40 ± 1.01	0.61 ± 0.01	9.95 ± 0.09
		20	56.80 ± 1.04	1.11 ± 0.01	42.10 ± 0.14

was 4 mg/L, while 12 mg/L for *A. vinelandii*. The bacterial apoptosis has been reported gradually in recent years. Dwyer et al. (2012) found that antibiotic-induced bacterial death showed physiological and biochemical markers similar to apoptosis. Moreover, Hakansson et al. (Hakansson et al. 2011) demonstrated that apoptosis may be present in most bacteria which depend on different agonist. Since ROS have been considered to be an activator to inducing apoptosis (Huang et al. 2010), and AgNPs can stimulate bacteria to produce ROS (Kim et al. 2014), so whether AgNPs could inhibit bacterial growth by inducing bacterial apoptosis was explored. As shown in Table 1, the proportion of apoptotic bacteria increased after the addition of AgNPs. Consistent with Bao et al. (2015) research that AgNPs can induce apoptosis in the bacteria cells.

To further examine the toxicity of AgNPs-induced ROS on bacteria, we investigated the bacterial morphogenesis under AgNPs exposure. TEM images showed that nAg10 and nAg50 significantly damaged cell morphology compared with the control and the leakage of intracellular contents was observed. Furthermore, AgNPs also caused large blank areas in cells center (Fig. 3, CDF). As shown in Fig. 3e, f, blurred substances next to the cell wall were considered to be caused by the broken and dissolution of bacteria. AgNPs were found to attach to the cell surface after exposing to bacteria (Choi et al. 2018). Studies have shown that after passing through the bacterial cell wall and reaching the cell membrane, the free radicals on the silver surface would attack the membrane protein, and oxidize with the unsaturated fatty acid in cell membrane, causing the oxidative damage, thereby interfering the fluidity and stability of the membrane (Ahmed et al. 2018; Wang et al. 2016). Dasgupta and Ramalingam (2016) have reported that ruptures in cell membrane were caused by generation of reactive oxygen species. Long et al. (2017) observed *E. coli* cell morphology which treated with AgNPs and 1 mM glutathione (GSH) or without GSH, and they found that the bacteria bodies with GSH were as smooth and intact as the control. Together these findings, suggested that collapse in cell structure mainly due to the oxidative damage.

Fig. 3 Transmission electron microscope (TEM) images of *A. vinelandii* (upper) and *N. europaea* (lower) exposure to AgNPs for 12 h. **a** control cells without AgNPs added, **c** nAg10 at a concentration of 10 mg/L and **e** nAg50 at a concentration of 20 mg/L; **b** control cells without AgNPs added, **d** nAg10 at a concentration of 10 mg/L and **f** nAg50 at a concentration of 20 mg/L
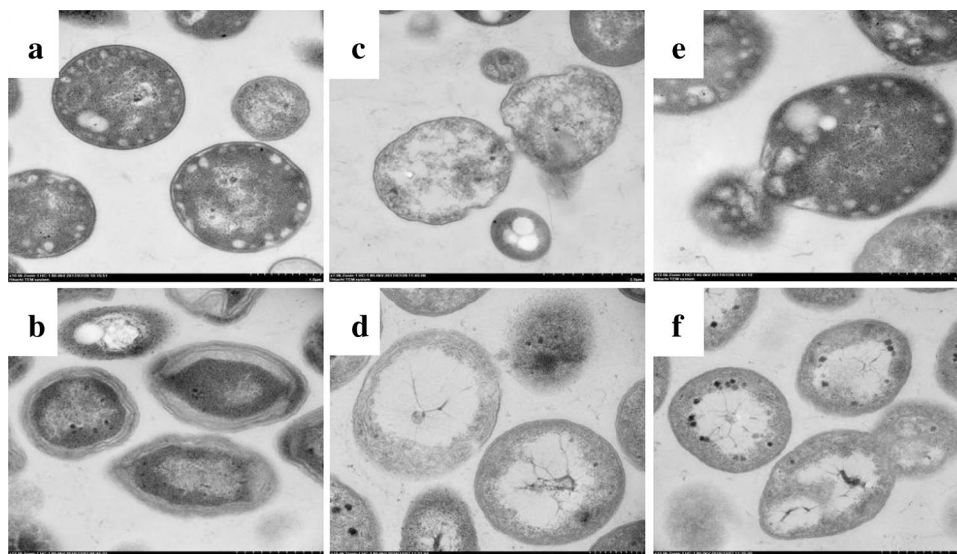
In present study, we observed the inhibition of AgNPs on *A. vinelandii* and *N. europaea*. ROS and oxidative stress elicited a wide variety of cellular responses including cell membrane damage and apoptosis. Furthermore, the bactericidal of AgNPs was size dependent. These results provided a valuable knowledge for further investigations on the toxicity of AgNPs to bacteria.

Compliance with Ethical Standards

Conflict of interest We declare that we have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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