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Cysteine-induced hormesis effect of silver nanoparticles

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The toxicity of silver nanoparticles (AgNPs) is widely exploited, but their hormesis effect has, so far, received little attention. This study reports the hormesis effect at low AgNPs concentrations of 0.34 mg L^{-1}, with a 29.9% increase in bacterial viability compared with the control. Cysteine can induce a hormesis effect at a higher concentration. 12.5 mg L^{-1} cysteine induced a hormesis effect in the AgNP concentration range of 1.7–5.1 mg L^{-1}. Results suggest that this cysteine-induced hormesis effect is concentration-dependent; the concentration that make sulfuration rate (n_s/n_{Ag}) of 6.15 shows strong excitation to cells.

1. Introduction

As the most widely commercialized nanomaterial, silver nanoparticle (AgNP) is increasingly being used in a variety of consumer products and medical devices.[1,2] The widespread use of AgNPs has increased the likelihood of accidental or incidental releases to the environment. This raises the need to assess the potential impact of these nanoparticles on the ecosystem. Thus far, numerous studies have focused on its toxicity mechanism.[3–8] However, there is an ongoing debate as to whether the toxicity is specifically related to the nanoparticles or whether it is due to the effect of dissolved Ag^+ released from nanosilver. Ionic silver (Ag^+) released from AgNP inhibits respiratory enzymes and induces oxidative stress through the generation of a reactive oxygen species (ROS); this has been the widely accepted toxicity mechanism.[9–12] Although some research points towards the non-negligible particle-specific antibacterial activity of AgNP, there is contrasting evidence demonstrating that Ag^+ ions are not toxic to some cell types such as HepG2 cells (Kawata *et al.*).[13] This suggests that both nanoparticles and ions are responsible for the toxicity; the toxic effects of AgNPs on Lolium multiflorum were more than those of silver ions.[14] Ag^+ has a pivotal role in AgNP toxicity, either by itself or in combination with a particle-specific effect. Xiu *et al.* demonstrated the negligible particle-specific antibacterial activity of silver nanoparticles and attributed its toxicity merely to Ag^+.[15]

The presence of low doses of Ag^+ can activate the repair mechanism of cells against the toxicant, and this repair process may sometimes overcompensate for the exposure.[16] It has been reported that Ag^+ concentration of 3–7.9 μg L^{-1} may enhance bacterial fitness and hinder antimicrobial applications.[13] Keeping in mind the Ag^+ contribution in AgNP toxicity, AgNP may produce similar hormesis effects *via* Ag^+. For example, AgNP at 1.56–6.25 mg L^{-1} activates human skin carcinoma cell line A431 and at 0.78–6.25 mg L^{-1} activates human fibrosarcoma cell line HT-1080.[17] In the present study, we investigated how cysteine affects AgNP toxicity to varying extents. We show that cysteine influences the hormesis effect of AgNP in a concentration-dependent manner; the concentration yielding a sulfuration rate (n_s/n_{Ag}) of 6.15 can significantly influence the toxicity profile of AgNPs and produce the strongest hormesis effect.

2. Methods

2.1. Preparation and characterization of AgNP suspensions

Polyvinylpyrrolidone (PVP) coated AgNPs (PVP-AgNPs) were synthesized according to the literature[18] with slight modifications. Briefly, 5 mL of 20 mM silver nitrate was reduced using 12 mL of 15 mM sodium borohydride (>99% purity, Sigma Aldrich) for 3 min in the presence of 0.3% PVP10 (MW 10,000, Sigma Aldrich) in an ice bath. The solution was then stirred continuously at room temperature for 1 h. All particle suspensions were purified by diafiltration using a 1 kDa regenerated cellulose membrane to remove excess PVP and

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Ag^+. The resulting AgNP solution was stored at 4 °C in the dark until used.

2.2. Strain culture

Herein, we use *E. coli* strain ATCC 25922 as the model strain in the toxicity study. The *E. coli* purchased from the American Type Culture Collection (US), was maintained on Luria–Bertani (LB) agar slants at 4 °C. Prior to each microbiological experiment, all glass-ware was sterilized at 121 °C for 20 min by an autoclave. A single colony of *E. coli* grown on LB agar was inoculated into 200 mL LB broth (tryptone 2 g, yeast extract 1 g, and NaCl 2 g in 200 mL of ultrapure water at pH of 7.2) at 37 °C overnight, on a rotary shaker at approximately 120 rpm. The resulting bacteria were harvested by centrifugation at 9000*g* for 5 min, washed three times with sodium bicarbonate buffer (2 mM), and resuspended in 20 mL of the same buffer to make a bacteria stock solution.

2.3. Testing of environmental anion effects on AgNP toxicity

Tests of the effects of environmental anions on AgNP toxicity were performed as described previously.[19,20] Briefly, *E. coli* stock solution was diluted to a concentration of OD_{600} = 0.1 with 2 mM sodium bicarbonate buffer, and 1 mL aliquots were added to test tubes. The AgNP stock solution was diluted with the same buffer to obtain different concentrations and added to *E. coli* and cysteine with various concentrations. Samples were incubated for 6 h at 25 °C, serially diluted, and seven 10 μL droplets from each dilution were placed onto LB agar plates. The plates were incubated at 37 °C for 46 h, and colony forming units (CFU) were counted. For Ag^+ toxicity response, a similar process was performed in the same condition except for the substitution of AgNP with $AgNO_3$. All AgNP and its control sample received the same concentration of KNO_3 to control for the addition of NO_3^- using $AgNO_3$.

The final viability ratio was calculated as $N/N_0 \times 100\%$, where N and N_0 are the concentrations of the remaining viable bacteria (CFU mL^{-1}) and the control values, respectively.

2.4. Statistical analysis

Statistical analysis was performed for each of the assays. Tests were performed in triplicate, and each sample was performed in triplicate. A one-way analysis of the variance (one-way ANOVA) was used to compare the differences between groups, and the p-values <0.05 were considered significant. All data plots represent the average from at least three independent experiments. Error bars represent 95% confidence intervals.

3. Results and discussion

3.1. Particle characterization

AgNP preparations were found to be spherical and non-aggregating in deionized water with an average primary particle diameter of 7.59 ± 2.92 nm based on transmission electron microscopy observations (TEM, Fig. 1). Zeta-potential measurements showed values of −22.5 ± 2.3 mV. The size distribution

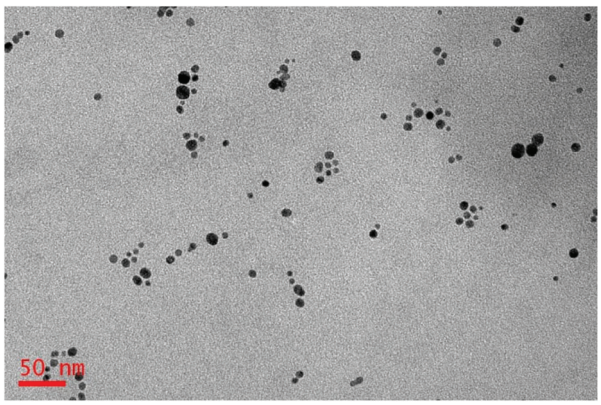

Fig. 1 TEM of AgNP.

was also confirmed by dynamic light scattering (DLS) analysis with an average hydrodynamic diameter distribution of 27.1 ± 2.2 nm. Inconsistent size distribution observed using TEM and DLS resulted from the different measurement principles of the two technologies.[21] The amount of Ag^+ remaining in the filtrate after ultrafiltration centrifugation of AgNP suspensions was measured by inductively coupled plasma mass spectrometry (ICP-MS) and found to be lower than 1% of the lowest concentration of Ag^+ used in this study.

3.2. Cysteine concentration-dependent hormesis effect of AgNP

Fig. 2 shows the toxicity profile of AgNP in the absence and presence of cysteine. AgNP was toxic to *E. coli* cells in 2 mM $NaHCO_3$ buffer solution, except for the concentration of 0.34 mg L^{-1}. Here, we considered 100% viability of the control group. The viability ratio at 0.34 mg L^{-1} was calculated at about 129.9% using the equation $N/N_0 \times 100\%$; therefore, AgNP at a concentration of 0.34 mg L^{-1} resulted in a 29.9%

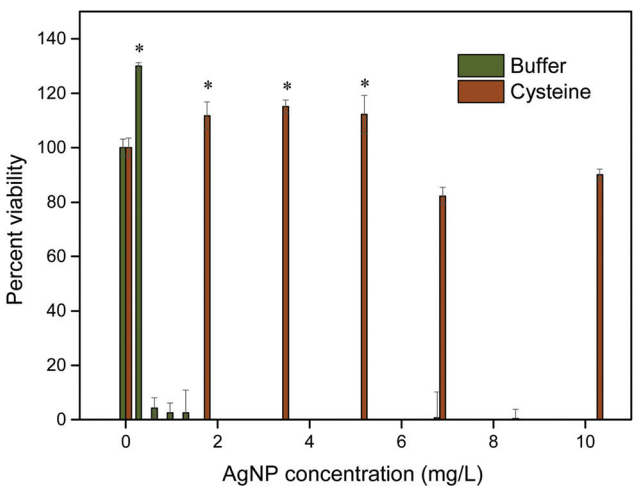

Fig. 2 AgNP toxicity to *E. coli* without cysteine and with 12.5 mg L^{-1} of cysteine. A significant stimulatory effect suggestive of hormesis was observed, as indicated by the asterisk.

increase in bacterial viability compared with the control. In the presence of 12.5 mg L^{-1} cysteine, survival of *E. coli* was stimulated by AgNP administered at various concentrations (1.7, 3.4, 5.1 mg L^{-1}). The concentration that initially stimulated survival (0.34 mg L^{-1} AgNP without cysteine) eventually becomes toxic with the addition of cysteine. Based on these results, we speculate that cysteine may induce hormesis effect of AgNP in *E. coli*, although the dose may be toxic initially. The Ag^+ released from AgNPs has been shown to exert toxic effects in cells.[7] To determine the specific contribution of environmental anions to AgNP toxicity, released Ag^+ concentrations were measured based on various quantities of AgNPs, with the addition of 12.5 mg L^{-1} cysteine (Fig. 3). Ag^+ concentration in 0.34 mg L^{-1} AgNP solution (the concentration at which the survival of resting *E. coli* cells increases is called the stimulation point) without cysteine was 1.42 μg L^{-1}. In the presence of 12.5 mg L^{-1} cysteine, concentration of Ag^+ was 1.01, 1.56, and 3.04 μg L^{-1} corresponding to AgNP concentrations of 1.7, 3.4, and 5.1 mg L^{-1}, respectively. The results indicate that when the released Ag^+ concentration is in the range of 1.01–3.04 μg L^{-1}, *E. coli* survival is stimulated. Cysteine caused a hormesis effect by controlling the final Ag^+ concentration released from AgNPs.

To further exploit the toxicity mechanism, the toxic effects of Ag^+ at various concentrations on *E. coli* survival were evaluated (Fig. 4). Using the viability of *E. coli* with 0 μg L^{-1} as the control, the viability of *E. coli* at Ag^+ concentration 2.0 μg L^{-1} was higher than the control, whereas it was lower when Ag^+ concentrations ≤1.0 μg L^{-1} and ≥5.0 μg L^{-1}. Hence, Ag^+ at a concentration of 2.0 μg L^{-1} showed a stimulatory effect, whereas Ag^+ concentrations ≤1.0 μg L^{-1} and ≥5.0 μg L^{-1} exhibited toxic effects on *E. coli*. Fig. 4 indicates that Ag^+ in the solution produced the same results as AgNPs in the presence of cysteine. These results show that the final concentration of Ag^+ in solution determines the primary toxic effects of AgNPs in cells. We speculate that the sulfide (2SH) group of

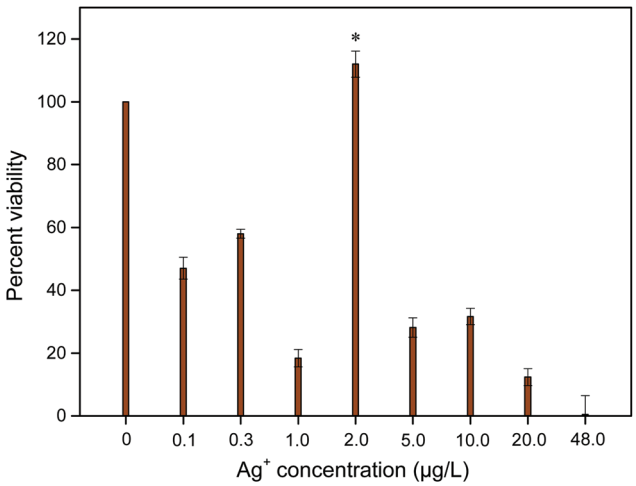

Fig. 4 Ag^+ increased *E. coli* survival. A significant stimulatory effect suggestive of hormesis was observed, as indicated by the asterisk.

cysteine influences Ag^+ release by complexing Ag^+ on the surface of AgNP:[22]

$$2Ag_{(s)} + \frac{1}{2}O_{2(aq)} + 2H^+_{(aq)} \rightleftharpoons 2Ag^+_{(aq)} + 2H_2O_{(l)} \quad (1)$$

$$Ag^+_{(aq)} + HS\text{-}cys_{(aq)} \rightleftharpoons AgS\text{-}cys_{(s)} + H^+_{(aq)} \quad (2)$$

The final Ag^+ concentration determines the toxic effect of AgNP.

Various concentrations of cysteine were employed in AgNP toxicity tests to evaluate *E. coli* survival (Fig. 5). The result indicates that cysteine does not always trigger the hormesis effect of AgNP in cells. The effects of cysteine are different at different concentrations. At 12.5 mg L^{-1} cysteine, evident hormesis was observed in a solution with 1.81 mg L^{-1} AgNP. The sulfuration rate (n_s/n_{Ag}) at the stimulation point (AgNP

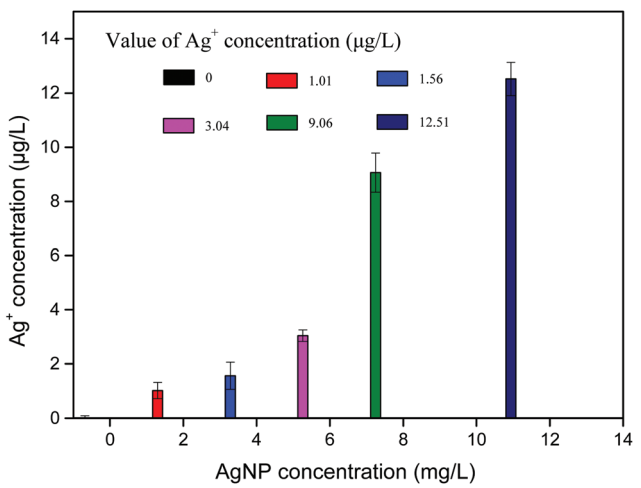

Fig. 3 Released Ag^+ concentrations measured in solution with 12.5 mg L^{-1} cysteine.

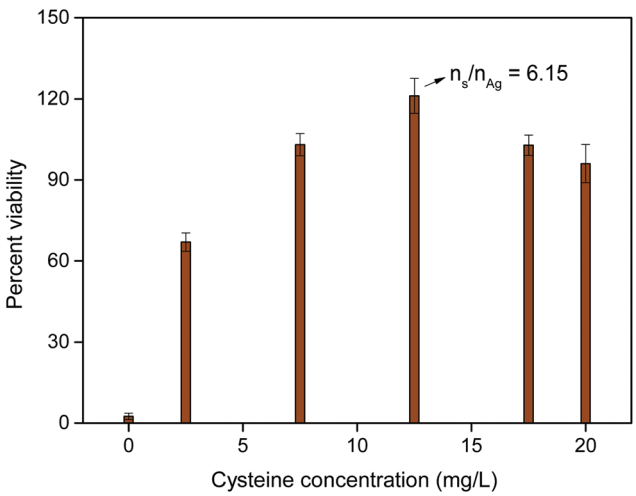

Fig. 5 AgNP toxicity to *E. coli* in the presence of various concentration of cysteine. The AgNP concentration was 1.81 mg L^{-1}. Error bars represent 95% confidence intervals.

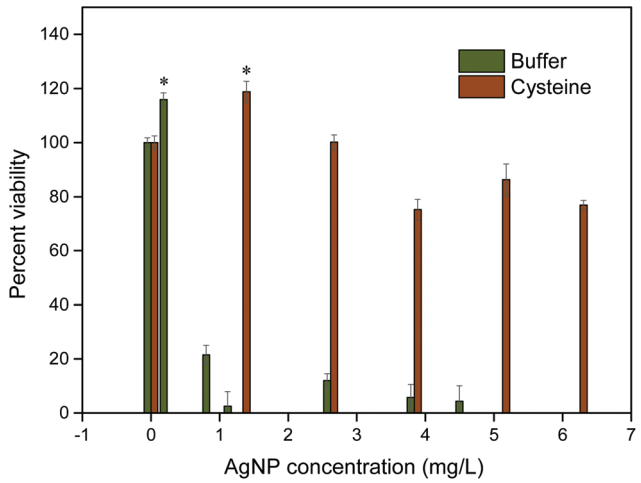

Fig. 6 AgNP toxicity to *Staphylococcus aureus* without cysteine and with 12.5 mg L^{-1} of cysteine. A significant stimulatory effect suggestive of hormesis was observed, as indicated by the asterisk.

concentration was 1.81 mg L^{-1}) was calculated as 6.15 in the solution. The other concentrations inhibited *E. coli* survival, indicating that this particular concentration of cysteine is important to the hormesis effect. As shown in Fig. 2, we can conclude that cysteine and AgNP concentrations simultaneously affect toxicity behavior and cysteine at various concentrations can induce multidimensional AgNP toxicity at the same concentration.

We also tested the effect of PVP on *E. coli* and little effect was observed. In addition, we have tested the effect of cysteine on AgNPs toxicity to *Staphylococcus aureus*, and a similar hormesis effect in low AgNPs concentrations was observed (Fig. 6). This study indicates the hormesis effect of AgNPs was not only suitable for *E. coli* but also for other bacteria.

3.3. Environmental implications

AgNPs have been extensively reported to act as a toxic agent to microbes in the environment by releasing Ag^+.[23–26] This study indicates that cysteine can induce Ag^+ release and induce the hormesis effect of AgNP. The release of AgNPs to the environment may cause changes in microbe population density (some species were stimulated to survive and become a dominant species) in the presence of cysteine, thereby disturbing the ecological balance. A detailed study should be carried out to understand the potential effect of cysteine on AgNP toxicity.

In the past, AgNPs have been widely exploited for their potential antibacterial capacity,[27–31] and considered as a suitable substitute for antibiotics as they do not exhibit the same side effects as antibiotics. When using AgNPs for bacteriostasis (such as in surgery), harmful germs may be stimulated instead of inhibited, owing to the hormesis effect. This study demonstrates that the initial stimulation concentration can become toxic with the addition of cysteine. In real medical applications, cysteine could be added to avoid hormesis in harmful pathogenic bacteria. Therefore, the effect of cysteine on AgNP toxicity should be determined in order to facilitate the effective application of AgNPs for bacteriostasis.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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