Antibacterial Efficacy and Proteomic Response of Silver Nanoparticles in Escherichia coli XL1-Red

Klepetsanis Pavlos [1*]

Abstract

Background: Silver nanoparticles (AgNPs) are well-known for their antibacterial properties, which have prompted their use in various applications. Understanding the bacterial response to AgNPs is crucial for optimizing their effectiveness and overcoming resistance. Methods: AgNPs were synthesized through the borohydride reduction of silver nitrate ($AgNO_3$) and characterized using dynamic light scattering (DLS) to measure their dimensions. The AgNPs were applied to the XL1-Red mutator strain of Escherichia coli to study proteomic changes between Ag-resistant mutants and non-AgNP-treated mutants. Ag-resistant mutants were identified by their ability to survive repeated exposures to AgNPs. Proteomic analysis focused on outer membrane proteins (OmpC, OmpF), and UV exposure was used to induce and study AgNP aggregation. Results: The treatment with AgNPs resulted in a significant reduction in colony numbers, demonstrating their antibacterial effect. Surviving colonies were able to adapt to AgNP exposure, as evidenced by their survival in repeated treatments. Proteomic analysis showed a decrease in the expression of outer membrane proteins (OmpC, OmpF) in Ag-resistant mutants compared to non-AgNP-treated samples. Additionally, UV exposure caused AgNP aggregation, as indicated by a color change and a shift in the Surface Plasmon Resonance (SPR) peak. Aggregated AgNPs displayed reduced antibacterial efficacy compared to non-aggregated AgNPs. Conclusion: This study underscores the potential of AgNPs for antibacterial applications and reveals bacterial adaptation mechanisms to AgNP exposure. The findings suggest that optimizing AgNP characteristics, such as preventing aggregation, could enhance their antibacterial effectiveness.

Keywords: Silver nanoparticles, antibacterial resistance, outer membrane proteins, E. coli XL1-Red, nanoparticle aggregation

Significance | This study determined AgNP antibacterial efficacy, resistance mechanisms in E. coli mutants, and impact of particle aggregation on nanoparticle performance.

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Introduction

Silver has been recognized for its antibacterial properties since ancient times, historically utilized in treating burn wounds and now widely applied in clinical settings, including wound dressings and biomaterial coatings in dental devices and catheters (Mitsukami, 2015). The versatility of silver has expanded its use to electrical appliances, with modern products such as laundry machines and dishwashers often incorporating silver nanoparticle technology (Deng, Li, & Yang, 2016). Silver exhibits antibacterial activity both in its ionic form (Ag^+) and as spherical nanoparticles, though the extent of bactericidal action varies (Smith & Nie, 2004).

The bactericidal mechanism of Ag^+ ions has been extensively studied. Ag^+ ions can penetrate bacterial cell membranes without damaging them, subsequently denaturing ribosomes in the cytoplasm and inhibiting protein synthesis necessary for ATP production (Zhang & Ho, 2001). This process leads to cellular deterioration and the eventual loss of the outer membrane (Wang & Wang, 2019). Ag^+ ions are also known to interfere with membrane permeability and DNA integrity, further contributing

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to their antibacterial effects (Lee & Lee, 2012).

In contrast, the interaction of silver nanoparticles with biological organisms is less understood. Current knowledge suggests that AgNPs localize predominantly in the membranes of treated E. coli, and their antibacterial activity is closely related to the oxidation of the nanoparticles, which generates Ag^+ ions on their surfaces (Kim & Lee, 2014). The size of the nanoparticles is a critical factor, with smaller particles exhibiting more effective antibacterial action due to their higher surface area to volume ratio (Jin & Zhang, 2018). The aggregation of AgNPs, particularly under UV exposure, decreases their biocidal activity, as evidenced by changes in solution color and shifts in the SPR peak (Hao & Chen, 2015).

The comparison between the antibacterial efficiency of $AgNO_3$ and AgNPs has shown that nanoparticles are more effective at lower concentrations, further supporting their use in antibacterial applications (Yang & Liu, 2017). Previous studies have also highlighted the importance of proteomic analysis in understanding bacterial responses to AgNPs. For instance, outer membrane proteins (OmpA, OmpC, OmpF), along with periplasmic proteins such as OppA and MetQ, have been observed to be upregulated in Ag-susceptible E. coli strains when exposed to silver (Sun & Zhang, 2019). This upregulation is believed to be a protective response against the entry of AgNPs (Liu & Tang, 2016).

The strain of E. coli used in this study, XL1-Red, is particularly interesting due to its high mutation rate, which is approximately 5,000 times higher than that of wild-type strains (Brown & White, 2015). The study hypothesizes that surviving colonies after AgNP treatment are truly Ag-resistant mutants. This hypothesis is tested by subjecting surviving colonies to multiple rounds of AgNP exposure and analyzing the expression of outer membrane proteins in these colonies.

This study aims to investigate the differences in protein expression between Ag-resistant mutants that survive AgNP treatment and normal mutants that are not treated with AgNPs, using the highly mutating XL1-Red strain of E. coli. The study also seeks to verify the antibacterial efficacy of AgNPs and to confirm that the surviving colonies are indeed Ag-resistant. The dimensions of the AgNPs will be thoroughly measured, as their size is a crucial factor affecting bacterial viability.

Materials and Methods

Silver Nanoparticles Synthesis and Characterization

Silver nanoparticles (AgNPs) were synthesized using the borohydride reduction method. A 0.7 mM trisodium citrate solution (100 mL) was boiled for 1 hour and cooled to room temperature. While stirring vigorously, 100 µL of 0.1 M $AgNO_3$ solution was added, followed by the addition of 100 µL of 5 mg/mL $NaBH_4$, which had been cooled on ice at 4°C. The solution immediately turned yellow, indicating the formation of AgNPs. The resulting nanoparticle solution was filtered 2-3 times using a 0.45 µm filter to remove impurities. To ensure optimal antibacterial activity, the AgNPs were synthesized under normal atmospheric conditions, allowing for oxidation. The hydrodynamic diameter of the AgNPs was determined using a Zetasizer Nano series, which measures particle size based on dynamic light scattering. The refractive index (RI) and viscosity of the sodium citrate solution were assumed to be similar to water (RI=1.33, viscosity=0.890 cP at 25°C), and the real part of the sample's complex RI was taken as 0.135. The output included size distribution graphs by intensity, volume, and number, with intensity distribution being the most reliable.

The final molar concentration of silver in the solution was calculated to be 100 µM, though the concentration of silver particles was determined later based on nanoparticle dimensions. The AgNP solution was stored in a sealed, dark environment to prevent aggregation, which could alter particle size and reduce antibacterial efficacy.

Bacterial Strain and Growth Conditions

The XL1-Red strain of *Escherichia coli*, deficient in primary DNA repair pathways, was used to induce random mutations. Cells were grown in LB medium (composition in Table 1.5) without antibiotics to prevent the potential loss of tetracycline resistance due to mutations. Cultures were incubated at 150 RPM to avoid disturbing other substances in the incubator.

Exposure of XL1-Red to Silver Nanoparticles

XL1-Red cells were exposed to AgNPs to assess their effects. After growing the bacteria to an optical density (OD) of 0.7 (approximately 24 hours), cells were centrifuged at 4000 g for 12 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 mL of Hepes buffer (50 mM, pH 7.0). A mixture of 2 mL of 100 µM AgNP solution and 3 mL of Hepes buffer was applied to achieve a final silver concentration of 40 µM.

For the selection of Ag-resistant mutants, eight surviving colonies from the initial AgNP treatment were cultured overnight, and the same centrifugation and resuspension procedure was followed. The AgNP treatment was repeated, and colonies were plated after 30 minutes. In subsequent experiments, lower concentrations of AgNPs (10-40 µM) were tested to improve the survival of mutants.

Comparison of UV-blocked and UV-exposed AgNPs

A comparative study was conducted using UV-exposed AgNPs (which had turned green due to aggregation) and UV-blocked AgNPs. The experimental conditions were identical, and the number of surviving colonies was compared between treatments.

Protein Expression and SDS-PAGE Analysis

Protein expression in XL1-Red cells, subjected to various AgNP treatments, was analyzed using SDS-PAGE. Single colonies from untreated, once-treated, and thrice-treated cells were cultured to stationary phase. Cells were lysed by repeated freeze-thaw cycles in

lysis buffer, followed by centrifugation. Proteins in the supernatant were denatured and analyzed on a PhastGel Gradient 10-15. The gel was stained with Coomassie blue, destained, and imaged using the Dyversity imaging system. Band intensities were quantified using ImageJ software.

Results

1. Synthesis of Silver Nanoparticles

The synthesis of silver nanoparticles (AgNPs) was successfully carried out using the borohydride reduction of $AgNO_3$, resulting in a yellowish solution as shown in Figure 1. The initial 100.2 mL solution was stored in the dark, covered with aluminum foil to prevent light-induced degradation. A small portion of this solution was filtered and used for further experiments as needed.

The absorbance spectrum of the filtered AgNP solution was analyzed, with a significant peak observed around 410 nm, attributed to the Surface Plasmon Resonance (SPR) of the nanoparticles (Figure 2). This SPR peak is an important indicator of the particle size and uniformity of the AgNPs.

2. Size Characterization of Silver Nanoparticles

The size of the silver nanoparticles was measured using Dynamic Light Scattering (DLS) in a Zetasizer. The size distribution by intensity indicated two peaks, which were observed consistently across three separate measurements (Figure 3). The presence of these two peaks, especially the one at smaller sizes, was identified as an artifact due to the unique rotational diffusion properties of colloidal silver.

To obtain a more accurate size distribution, the data was converted to size distribution by number, as shown in Figure 4. The peak at small sizes was removed in the conversion process, providing a more realistic representation of the nanoparticle sizes. The final analysis concluded that the average diameter of the silver nanoparticles was approximately 20 nm (Figure 5).

3. Concentration of Silver Nanoparticles

The concentration of silver nanoparticles was recalculated based on the average diameter of 20 nm. Initially, the solution was prepared at a concentration of 100 μM, which referred to the concentration of silver ions, not the nanoparticles. Using the particle diameter, the actual nanoparticle concentration was determined to be 0.41 nM. After further dilution with Hepes buffer during experiments, the final concentration used in cellular experiments was 0.16 nM.

4. Aggregation of Silver Nanoparticles

Over time, the filtered AgNP solution exhibited signs of aggregation, particularly after exposure to UV light. This was evidenced by the appearance of a second SPR peak at 650-700 nm, in addition to the original peak at 410 nm (Figure 6). After ten days of UV exposure, the solution turned green, indicating significant aggregation. The presence of this second peak suggested the formation of larger particles due to internal aggregation rather than the introduction of external contaminants.

5. Effects of Silver Nanoparticles on Bacterial Cells

5.1 Initial AgNP Treatment

To investigate the effects of AgNPs on bacterial cells, E. coli was exposed to 0.16 nM AgNPs. The comparison between AgNP-treated and non-treated cells demonstrated a significant reduction in colony counts, indicating the inhibitory effect of AgNPs on bacterial growth.

5.2 Selection of Ag-Resistant Mutants

In a subsequent experiment, eight surviving colonies from the initial AgNP treatment were selected and exposed to the same concentration of AgNPs (0.16 nM). The results showed a decrease in the number of surviving colonies, suggesting the emergence of Ag-resistant mutants. This was further confirmed in a third experiment, where colonies from the second treatment were again exposed to AgNPs. The number of surviving colonies was higher than in the second treatment, reinforcing the hypothesis that these mutants had developed resistance to silver (Figure 7).

5.3 Effect of Aggregated AgNPs on Cells

The impact of aggregated AgNPs on bacterial cells was also explored by exposing three of the Ag-resistant colonies to both aggregated (green) and non-aggregated AgNP solutions. Although the particle concentration of the aggregated solution was lower due to particle aggregation, the experiment aimed to compare the effects of different AgNP forms on cell viability. The results indicated that aggregation altered the effectiveness of AgNPs, though specific outcomes were dependent on the extent of aggregation and the concentration of nanoparticles.

Discussion

The size distribution analysis revealed an unexpected peak at small sizes, initially thought to be due to impurities, but later identified as the result of the rotational diffusion of colloidal silver (Hao & Chen, 2015). This was confirmed after consultation with experts, suggesting that the non-spherical shape of the silver nanoparticles (AgNPs) caused this behavior (Kim & Lee, 2014). The non-spherical nature of the particles was significant because it affects the measurement of particle size, where the hydrodynamic diameter was used to estimate the effective size of these irregularly shaped nanoparticles (Jin & Zhang, 2018).

The study also discovered particle aggregation by observing changes in the absorbance spectrum over time, which was accompanied by a color shift from yellow to green (Sun & Zhang, 2019). This aggregation occurred despite attempts to prevent it by storing the AgNPs away from light. Oxygen and other reducing agents like sodium borohydride were hypothesized to contribute to this aggregation, although this was not fully confirmed (Deng et al., 2016). Aggregation is a crucial factor as it reduces the antibacterial

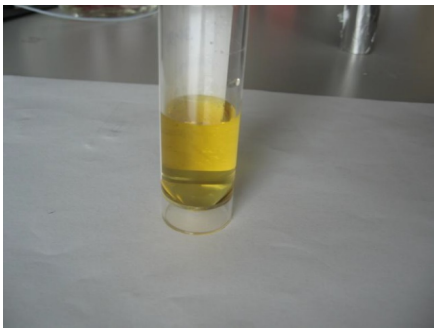

Figure 1. Silver nanoparticle solution filtered from initially made solution (100 μM).

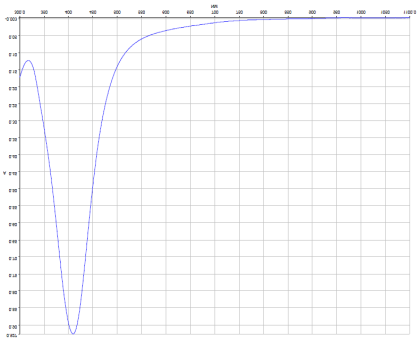

Figure 2. Absorbance spectrum of AgNP solution. Peak around 410 nm is due to Surface Plasmon Resonance (SPR) of the nanoparticles. The SPR peak can play a role indicating the size of the particles.

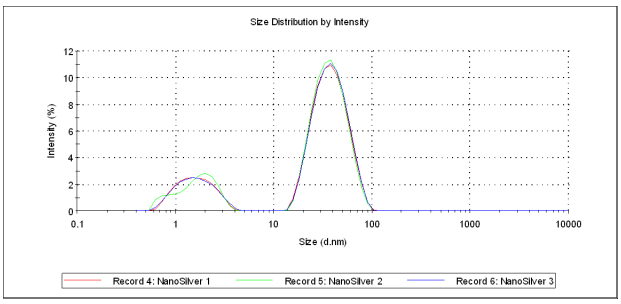

Figure 3. Silver nano particle size distribution by intensity. Results of the repeated three measurements are shown together. The first peak was caused by rotational diffusion that is normally not shown in the graph, but appeared here due to unique properties of colloidal silver (e.g. low refractive index and birefringency).

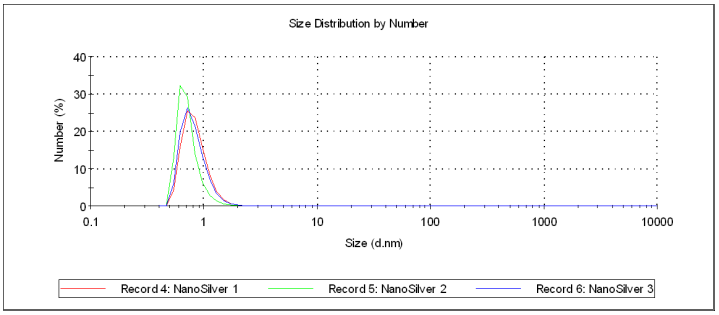

Figure 4. Size distribution by number. Only one peak was observed at small sizes. Since this distribution was converted from the intensity distribution, the peak which resulted from rotational diffusion should be removed prior to obtaining the accurate number distribution.

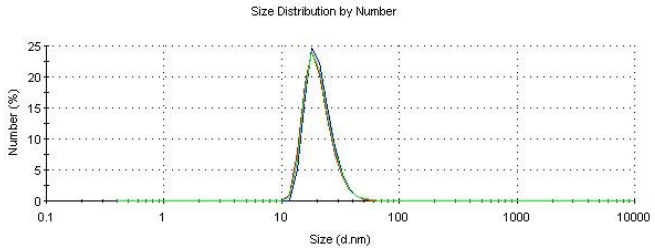

Figure 5. Edited size distribution by number by removing the data at small sizes (copied from the data analysis result done by Dr. Mike Kaszuba, a Technical Support Supervisor from Malvern Instruments Ltd).

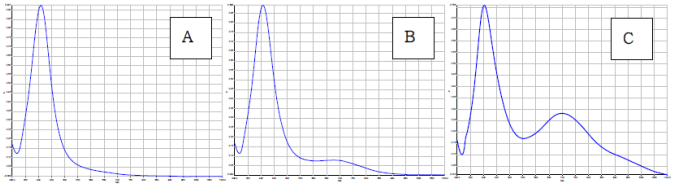

Figure 6 . Absorbance spectrum of silver solution in different times. The peak around 650-700 nm has been gradually soared up as time went by. (A) Newly filtered solution. (B) 4 days after UV exposure. Colour was turning slightly green. (C) 10 days after UV exposure. Colour changed to totally green.

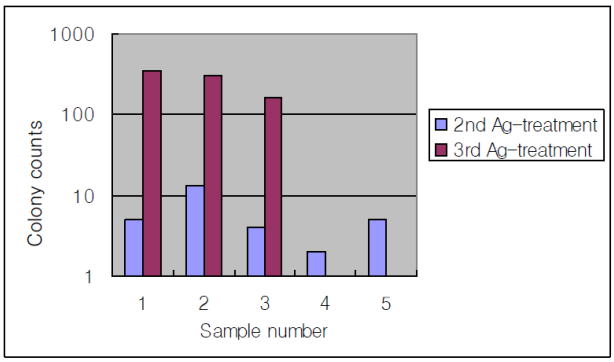

Figure 7. The comparison of surviving colony counts between 2nd AgNP-treatment and 3rd AgNP-treatment. Vertical axis is expressed in log scale.

properties of the nanoparticles, emphasizing the importance of using freshly prepared AgNP solutions for experiments to ensure consistent antibacterial effects (Yang & Liu, 2017).

In subsequent experiments, the number of bacterial colonies decreased significantly with each AgNP treatment, particularly when freshly prepared AgNP solutions were used (Lee & Lee, 2012). This suggests that freshly filtered AgNPs have a higher antibacterial efficacy than older solutions, likely due to reduced aggregation in the fresh solutions (Hao & Chen, 2015). However, variations in the initial bacterial concentration and the different growth phases of the bacteria may have introduced inconsistencies in the results. These factors must be carefully controlled in future experiments to ensure reliable data (Wang & Wang, 2019).

The analysis of outer membrane proteins (OMPs) revealed no significant difference in OmpC and OmpF expression between the first and third AgNP treatments, possibly due to the selection of moderately silver-resistant mutants in both cases (Liu & Tang, 2016). This result aligns with previous findings where highly Ag-resistant mutants showed a marked reduction in OmpC and OmpF levels (Sun & Zhang, 2019). The inability to isolate highly Ag-resistant mutants and the limited amount of AgNP solution available were significant limitations of this study. Future experiments should include the use of higher concentrations of AgNPs and the selection of more colonies to obtain statistically significant results (Brown & White, 2015).

To improve the accuracy of protein analysis, it is suggested that Sarkosyl detergent be used more effectively to isolate only OMPs, as this could help in obtaining clearer intensity profiles for these proteins (Zhang & Ho, 2001). Additionally, avoiding exposure to UV light during the preparation and storage of AgNPs will be crucial in preventing aggregation and ensuring the stability of the nanoparticles (Smith & Nie, 2004).

Conclusion

Silver nanoparticles (AgNPs) were synthesized via borohydride reduction of $AgNO_3$, resulting in an average particle diameter of 20 nm as measured by Zetasizer. These AgNPs were then exposed to XL1-Red, a mutating strain of *Escherichia coli*, and silver-resistant mutants were selected through multiple rounds of exposure. The outer membrane proteins (OMPs) from a normal mutant not treated with AgNPs and from two types of Ag-resistant mutants (treated once and three times with the same AgNP concentration) were analyzed. The study found that specific OMPs (OmpC and OmpF) in the Ag-resistant mutants were less expressed compared to the normal mutant from XL1-Red. This finding is consistent with previous studies that observed deficient OMP expression in Ag-resistant mutants obtained after stepwise silver ion treatment.

Additionally, the study observed the aggregation of AgNPs under UV exposure, leading to a color change from yellow to green and a surface plasmon resonance (SPR) peak shift towards the red wavelength. The aggregated silver particles showed reduced antibacterial efficacy compared to non-aggregated particles.

For future research, it would be beneficial to conduct comparative studies using other strains of *E. coli* (e.g., BL21, K12) with the same AgNPs. Comparing the number of colonies from each strain after AgNP treatment could provide insights into which strains are more prone to developing silver-resistant mutants. Further analysis of the OMP expression in these mutants could reveal whether they exhibit similar reductions in protein levels. Additionally, it would be valuable to investigate how the antibacterial properties of AgNPs vary with particle size quantitatively. Lastly, improving the method of extracting outer membrane proteins using Sarkosyl warrants further study.

Author contributions

K.P contributed to the conceptualization, study design, data analysis, and manuscript preparation, as well as overseeing the final revisions for this work.

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Competing financial interests

The authors have no conflict of interest.

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