

Inhibition of *E. coli* and *S. aureus* with selenium nanoparticles synthesized by pulsed laser ablation in deionized water

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Abstract: Nosocomial diseases are mainly caused by two common pathogens, *Escherichia coli* and *Staphylococcus aureus*, which are becoming more and more resistant to conventional antibiotics. Therefore, it is becoming increasingly necessary to find other alternative treatments than commonly utilized drugs. A promising strategy is to use nanomaterials such as selenium nanoparticles. However, the ability to produce nanoparticles free of any contamination is very challenging, especially for nano-medical applications. This paper reports the successful synthesis of pure selenium nanoparticles by laser ablation in water and determines the minimal concentration required for ~50% inhibition of either *E. coli* or *S. aureus* after 24 hours to be at least ~50 ppm. Total inhibition of *E. coli* and *S. aureus* is expected to occur at 107 ± 12 and 79 ± 4 ppm, respectively. In this manner, this study reports for the first time an easy synthesis process for creating pure selenium to inhibit bacterial growth.

Keywords: nosocomial disease, bacteria, antibiotics resistant, cytotoxicity

Introduction

Escherichia coli is a gram-negative bacteria that is commonly present in the intestines of humans and animals, while *Staphylococcus aureus* is a gram-positive bacteria frequently found in the human respiratory tract and on the skin. These bacteria are the two main pathogens responsible of nosocomial diseases,^{1,2} which are also called hospital-acquired infections and defined as infections occurring within 48 hours of hospital admission, 3 days of discharge, or 30 days after an operation. Infections due to bacteria causing nosocomial diseases are difficult to treat, as they are drug resistant to a large group of antibiotics.^{3,4} In the US, two million people are infected each year by nosocomial diseases, of whom, 5% die.⁵ Therefore, it is becoming increasingly necessary to develop new strategies to fight nosocomial infections and the solution may come from nanotechnology, where the particle size is <100 nm, exhibiting a large surface-to-volume ratio and therefore especially adapted to interact with bacteria.⁶ The benefit of using selenium as a therapeutic drug is that selenium is already present in our body as a trace element⁷ and has very interesting biological assets, such as anticancer^{8,9} and antibacterial properties.¹⁰ It belongs to the oxygen family (group 16 in the periodic table); therefore, it does not oxidize in air and is insoluble in water. Pulsed laser ablation in liquids (PLAL) has several advantages compared to the other conventional methods such as wet chemistry routes, physical vapor deposition, or chemical vapor deposition to synthesize selenium.¹¹ Indeed, the surface of the synthesized nanoparticles is absent of unnecessary adducts and byproducts, and the produced nanoparticles

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are easy to collect and store as a colloidal solution, and the setup is simple. Furthermore, it does not require any vacuum chamber or clean room environment.

Only four papers in the literature report the synthesis of selenium nanoparticles by PLAL.^{12–15} The first indication of the antibacterial properties of selenium nanoparticles synthesized by laser ablation was reported by our group using a neodymium-doped yttrium aluminum garnet laser at 355, 532, and 1,064 nm.¹⁵ During that study, we could not decrease the *E. coli* density by a significant percentage due to a low concentration of selenium nanoparticles used in solution (1.35 ppm). Therefore, a new vessel/target combination has been created to increase selenium concentration. The goal of this communication is to report a ~46% decrease in *E. coli* and a ~63% decrease in *S. aureus* densities by using pure selenium nanoparticles synthesized by pulsed laser ablation in deionized (DI) water.

Materials and methods

Materials

Selenium pellets (Se, diameter <5 mm, purity $\geq 99.99\%$ based on trace metals analysis) and sodium hydroxide (NaOH, American Chemical Society reagent, $\geq 97.0\%$, pellets) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Synthesis of selenium nanoparticles

The selenium nanoparticles were produced by irradiating pure selenium pellet placed at the bottom of a 1.5 mL microcentrifuge tube filled with 0.5 mL of DI water. The laser used for the irradiation was the neodymium-doped yttrium aluminum garnet NT342B (EKSPLA, Vilnius, Lithuania) with a pulse duration of 3.6 ns and a 20 Hz repetition rate. Each pulse, having a top hat profile, delivers ~20 mJ/pulse. The laser beam was focused on the surface of the selenium pellet, which corresponds to a fluence of 2.5 J/cm². The irradiation time was fixed at 15 minutes, and the ultraviolet wavelength was chosen to be at 355 nm to produce a more stable colloidal solution compared to visible or infrared wavelengths.¹⁵ The conical shape of the cuvette helps to reduce the amount of water required in the vessel compared to flat squared cuvettes used previously,¹⁵ while at the same time, maintaining enough height of water above the target and preventing evaporation during irradiation.

Concentration analysis

The selenium concentration was determined by dissolving 1 mL of the colloidal solution into 10 M NaOH and then diluting it 100× before being analyzed by inductively coupled plasma mass spectrometry (ICP-MS, Bruker Aurora M90;

Bruker Corporation, Billerica, MA, USA). Previously, it has been reported by Van Overschelde and Guisbiers¹⁶ that the production of nanoparticles was larger for a bulk target compared to a powder target.

Morphology

The size distribution and zeta potential were determined after decantation by dynamic light scattering (DLS, Zetasizer Nano ZS from Malvern Instruments, Malvern, UK) at 25°C. Transmission electronic microscope (TEM, JEOL 1230, Tokyo, Japan at 120 kV) images were taken to determine the size and shape of the selenium nanoparticles. Energy dispersive X-ray analysis was done using the scanning electron microscope (SEM, Hitachi STEM S5500; Hitachi Ltd., Tokyo, Japan) at 30 kV.

Cytotoxicity assay

A stock solution of selenium nanoparticles was diluted to the desired concentrations ranging from 50 to 1 ppm in growth medium and subsequently added into 96-well plates containing ARPE-19 cells (5×10^4 cells/well). Microtiter plates were incubated at 37°C in a 5% CO₂ air humidified atmosphere for 24 hours. Assessment of cell viability was carried out using a CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega Corporation, Fitchburg, WI, USA).

E. coli and *S. aureus* culture and treatment

Bacterial cell lines of biofilm-producing *E. coli* and *S. aureus* were obtained in freeze-dried form from the American Type Culture Collection (8739 and 25923, respectively; ATCC, Manassas, VA, USA). The cells were propagated in 30 mg/mL of tryptic soy broth (TSB) (MP Biomedicals, Solon, OH, USA). Once the second passage of bacteria reached its stationary phase, the second passage was frozen in one part TSB and one part 50% glycerol (Sigma-Aldrich). All experiments were conducted from this frozen stock. One day before bacterial seeding, a sterile 10 μ L loop was used to withdraw bacteria from the frozen stock and streaked onto a TSB agar plate and incubated at 37°C for 16 hours. Bacteria from a single colony were then collected using a sterile loop and inoculated in a test tube containing 3 mL of TSB overnight. The test tube was agitated in a shaking incubator at 37°C, 250 rpm to achieve a bacterial solution at the exponential phase of growth.

Antibacterial tests

The antibacterial test was performed on *E. coli* and *S. aureus* by using the broth dilution method.¹⁷ Various concentrations of selenium nanoparticles and bacteria were added to the plate and incubated for either 4, 8, or 24 hours. The survival bacteria

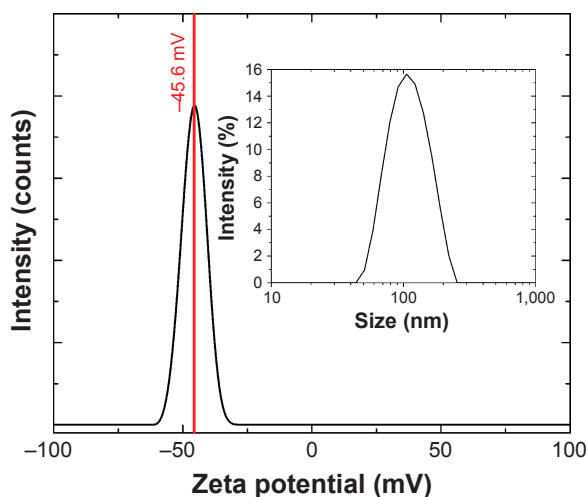


Figure 1 Zeta potential of the selenium nanoparticles synthesized by PLAL in DI water using a ultraviolet wavelength at $\lambda=355$ nm. Inset: size distribution of the selenium nanoparticles determined by DLS, average size = 115 ± 38 nm.

Abbreviations: DI, deionized; DLS, dynamic light scattering; PLAL, pulsed laser ablation in liquids.

numbers were then calculated by measuring the optical density of the bacterial solution at 562 nm using a standard curve correlating optical densities and bacterial concentrations.

Results

Selenium nanoparticles were synthesized by the PLAL of selenium pellets within a 1.5 mL conical microcentrifuge tube filled with 0.5 mL of DI water using a 3.6 ns pulse neodymium-doped yttrium aluminum garnet laser at 20 Hz in the ultraviolet wavelength of 355 nm. This configuration allowed us to produce a 50 ppm solution compared to a previous study where only 1.35 ppm was prepared.¹⁵ The

stability of the colloidal solution was evaluated by measuring its zeta potential, that is, the electric potential surrounding the particle. If the zeta potential is higher than the absolute value of ± 30 mV, then the solution is considered stable, while below this value, it is unstable and tends to flocculate. In this article, the zeta potential of the selenium nanoparticles was measured at approximately -45.6 mV, which is very stable (Figure 1). The average size of the nanoparticle was determined by DLS to be around 115 ± 38 nm (inset, Figure 1). From our TEM analysis, just after synthesis, the nanoparticles were observed to be spherical with a high polydispersity (Figure 2A). The discrepancy observed between TEM and DLS size distributions can be explained as follows: the size measured by DLS (called hydrodynamic diameter) is representative of the size of a hypothetical sphere that diffuses at the same rate as the particles being measured, while the size measured from TEM observations really informed the physical size of the particle. Indeed, the Rayleigh approximation tells us that the scattered intensity is proportional to d^6 where d is the particle diameter and, therefore, the light scattered by the largest particles may hide the light scattered by the smallest ones. As an example, if we consider two particles, one with a diameter ten times larger than the other one, the d^6 factor tell us that the largest particle will scatter 10^6 more light than the smallest one. Moreover, the purity of the selenium nanoparticles produced by PLAL was confirmed by carrying out energy dispersive X-ray analysis (line scans) through those particles (Figure 2B).

Various concentrations of selenium nanoparticles were subjected into *E. coli* and *S. aureus* bacteria plated and incubated for either 4, 8, or 24 hours. The most concentrated

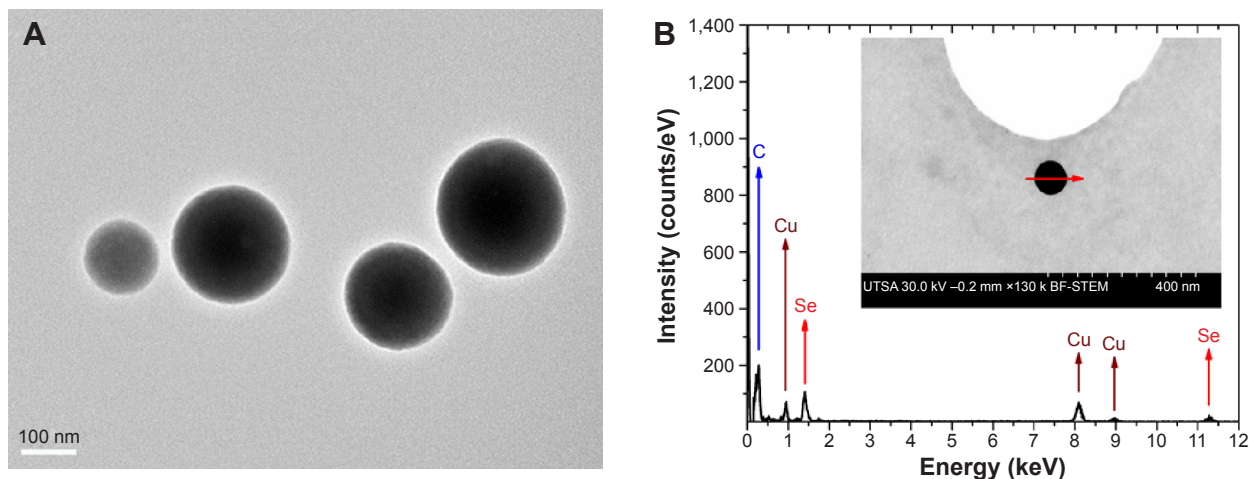


Figure 2 Advanced TEM characterization.

Notes: (A) TEM image of Se nanoparticles synthesized by PLAL in DI water using a ultraviolet wavelength at $\lambda=355$ nm. (B) EDX line scan spectrum clearly showing the pure nature of the selenium nanoparticles produced. The carbon and copper signals come from the grid. Inset: SEM image of the selenium nanoparticle analyzed by EDX.

Abbreviations: DI, deionized; DLS, dynamic light scattering; EDX, energy dispersive X-ray; PLAL, pulsed laser ablation in liquids; SEM, scanning electron microscope; TEM, transmission electronic microscope.

selenium sample (50 ppm) exhibited the highest inhibition rate with 46% and 63% of *E. coli* and *S. aureus* growth inhibition after 24 hours, respectively (Figure 3). To survive, bacteria have evolved a sophisticated and complex cell envelope that protects them, but allows for the selective passage of nutrients from the outside and waste products from the inside. A possible mechanism toward inhibiting *E. coli* bacteria is that selenium nanoparticles attach by chemisorption¹⁸ and penetrate the outer membrane that contains lipopolysaccharides, linked by a covalent bond to the cell's peptidoglycan by Braun's lipoprotein.¹⁹ The canonical biosynthetic pathway of lipoproteins in the *E. coli* bacteria involves three enzymes, including prelipoprotein diacylglyceryl transferase, prolipoprotein signal peptidase, and apolipoprotein *N*-acyltransferase, which

have been shown to play an essential role in the survival of *E. coli*.²⁰ Therefore, selenium nanoparticles inhibit *E. coli* by modifying the role of these enzymatic conveyors. By contrast, in gram-positive bacteria, such as *S. aureus*, the cell wall structure is different, ie, it has a thicker peptidoglycan membrane without any outer lipopolysaccharide membrane. Consequently, selenium penetrates much more easily into the *S. aureus* bacteria by chemisorption, where the lipoproteins involved are of the diacyl and triacyl forms.²⁰ Therefore, it can be concluded that the cell wall and its polysaccharide components constitute a barrier that reduces the penetration of selenium nanoparticles into the cell interior. Indeed, from Figure 3A, the total inhibition of *E. coli* and *S. aureus* is expected to occur at 107 ± 12 and 79 ± 4 ppm, respectively. It is confirmed that selenium nanoparticles can inhibit both

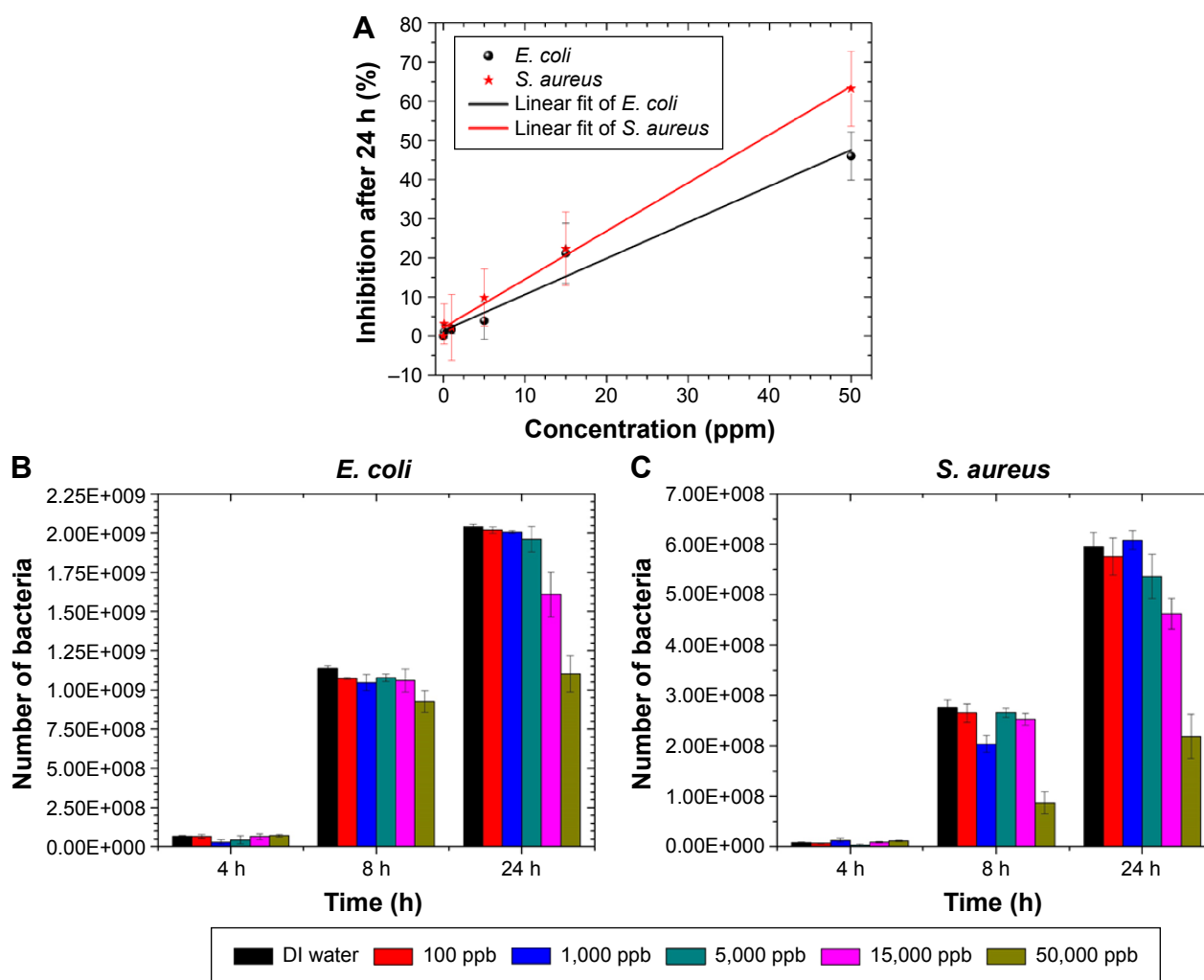


Figure 3 Inhibition tests.

Notes: (A) Inhibition after 24 hours of *E. coli* and *S. aureus* by using different concentrations of selenium nanoparticles. (B) Number of bacteria after 4, 8, and 24 hours incubation with *E. coli*. (C) Number of bacteria after 4, 8, and 24 hours incubation with *S. aureus*. The control group is in DI water. Values are the mean \pm standard deviation of the mean; N=3.

Abbreviations: DI, deionized; *E. coli*, *Escherichia coli*; h, hours; *S. aureus*, *Staphylococcus aureus*.

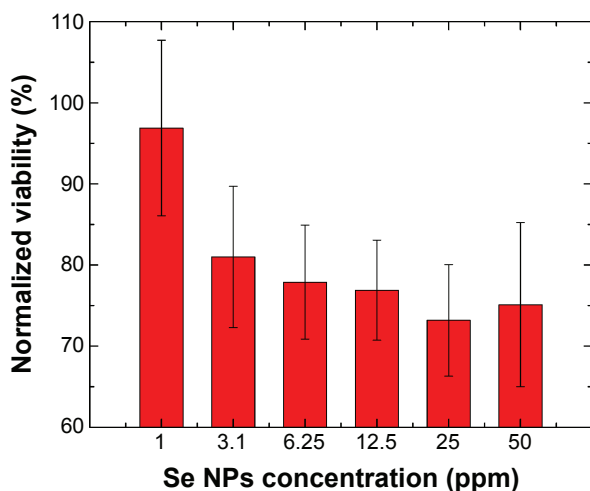


Figure 4 Cytotoxicity of selenium nanoparticles synthesized by pulsed laser ablation in DI water (time pulse ~3.6 ns, 20 Hz, 15 min irradiation time) on ARPE-19 cells. More than 70% of the cells are still alive with 50 ppm of Se nanoparticles. Values are the mean \pm standard deviation of the mean; N=3.

Abbreviations: DI, deionized; min, minutes; NPs, nanoparticles; Se, selenium.

gram-negative and gram-positive bacteria with a higher efficiency against gram-positive bacteria (slope_{-*E. coli*} = 0.92 ± 0.08 and slope_{-*S. aureus*} = 1.23 ± 0.04). Finally, the minimal concentration required to ~50% bacterial inhibition (*E. coli* or *S. aureus*) after 24 hours should be at a minimum 50 ppm (Figure 3B and 3C).

Discussion

Our results agree with a recent study made by Tran et al²¹ stipulating that selenium nanoparticles affect *S. aureus* in a more efficient way than *E. coli*. However, disagreement arises with the claim that selenium nanoparticles do not show any significant inhibitory effect on *E. coli* where we obtained 46% inhibition after 24 hours of incubation. The difference may be due to the selenium synthesis process, since their selenium nanoparticles were produced by a chemical reduction of sodium selenite, whereas we produced selenium nanoparticles by PLAL in DI water. Therefore, the surface contamination that may happen during chemical synthesis could affect their surface and consequently their efficiency.

Apart from selenium, silver is also reported to have excellent antibacterial properties. However, silver is not present naturally in the human body, and the toxicity of silver nanoparticles toward bacteria and human cells is similar;²² while selenium being a trace element found naturally in the body has been demonstrated to be much less toxic. Indeed, our cytotoxicity tests on ARPE-19 cells demonstrate that viable cells decrease slightly by increasing the dose of Se nanoparticles, but >70% of the cells were still

alive at concentrations ~50 ppm (Figure 4). This result is in agreement with another study where a cytotoxicity test on 3T3 fibroblasts demonstrated that >70% of cells were still alive at a concentration as high as ~128 ppm.²¹

Conclusion

Based on the results of bacteria assays after 4, 8, and 24 hours, selenium nanoparticles significantly decreased the number of *E. coli* and *S. aureus* bacteria. This study showed that selenium nanoparticles, synthesized by pulsed laser ablation in DI water, can be used efficiently as an antibacterial therapeutic. Most importantly, these results demonstrate that decreasing the density of bacteria can be achieved without the use of antibiotics. Nevertheless, further work is still in progress to confirm those results by using the disc diffusion method.

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Disclosure

The authors report no conflicts of interest in this work.

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