

Testing and Evaluation of Nanoparticle Efficacy on E. Coli, and Bacillus Anthracis Spores

Mark Sloan*, Stan Farnsworth**

* Conceptual MindWorks, Inc. 8262 Hawks Road Brooks City-Base, TX 78235-5147

phone: (210) 536-1388, Mark.Sloan.ctr@brooks.af.mil

** Nanotechnologies, Inc. 1908 Kramer Ln, Austin, TX 78758

phone: (512) 491 9500, Stan.Farnsworth@nanoscale.com

ABSTRACT

Fast, effective, antimicrobial and antispore methods against biological warfare agents, which include the spores from Bacillus anthracis, are urgently required in the biological warfare agent neutralization arena. Metal nanoparticles, which have been previously shown to have antimicrobial effects against vegetative microbes, show great promise in having likewise efficacy against the more resistant spores. In this study, the suitability of commercial and developmental dry-powder nanoparticles against Escherichia coli, and Bacillus anthracis spores was evaluated. Testing included various metal oxides and metals in nanopowder form, dispersed in water. Nanoparticles with average particle sizes equal to or less than 25nm composed of silver, as well as silver in combination with iron, and nickel, were found to have significant reduction of E. coli and B. anthracis bacteria and spores, in some cases in excess of an 8-log reduction in bacterial count. The dispersed dry powders were shown to efficiently kill vegetative bacterial species. In addition to the specific composition of the nanoparticle, the testing demonstrated that the particle size is a critical parameter for efficacy. Based on these tests, such materials may have application as countermeasures to biowarfare agents.

Keywords:

Anthrax, nanoparticle, silver, antimicrobial

1. INTRODUCTION

Silver-ions and silver compounds show a toxic effect on some bacteria, viruses, algae and fungi because of the oligodynamic effect which is typical for heavy metals. However, little is known about the efficacy of silver nanoparticles to bacterial organisms [5]. Preliminary studies have shown nano-sized silver particles to have antimicrobial effects on Escherichia coli. [1,7,10]. The possibility of silver nanoparticles having antimicrobial properties against Bacillus anthracis spores has now piqued interest in the biological warfare agents research arena.

The purpose of this study was to evaluate the in-vitro antibacterial activity of various metal nanoparticles, with an emphasis on silver, against Escherichia coli and Bacillus anthracis spores. We theorized that the elemental composition of nanoparticles is critical for killing microorganisms.

Previous studies have shown that silver, in the form of powder, ions and nanoparticles [7] can be efficacious against bacteria. To test this we examined the efficacy of different nanoparticle compositions composed of pure metal and mixtures of metals or metal oxides.

We also test the role of primary particle size on the efficacy of the materials. Smaller particles have higher specific surface area, and so are better able to interact with bacterial surface structure. Smaller particle size will also more easily facilitate particle uptake by microorganisms, and so possibly contribute to the fatality mechanism. For microbe/nanoparticle interaction to take place it also becomes important that the nanoparticles are dispersed into a suspended, homogenous solution containing a minimum agglomeration size of particles. We therefore theorize that the particle size and dispersion of particles is a critical parameter for efficacy. To test that theory we examined the efficacy of various sized nanoparticles of the same materials to determine if size was a critical factor.

2. EXPERIMENTAL

2.1. Particle preparation

Nanotechnologies, Inc. supplied unaggregated nanoparticle powders of a variety of compositions (Table 1). These materials were produced via plasma process. The particles were characterized as being primarily spherical and unaggregated. The metal nanoparticles were prevented from aggregating and forming sintered structures through the use of primarily graphitic carbon with trace polyaromatics. By preventing the aggregation of the nanoparticles, the particles could be dispersed in water with resulting agglomerate sizes closer to their primary particle sizes (data not shown) and the dispersability was therefore improved. The carbon treatment was found to have no effect on the microbial activity of the particles, as some metals with this treatment were determined to have no activity. Micrographs of silver particles produced with and without carbon clearly show that the addition of the carbon acts to prevent the natural tendency towards aggregation. The following table is a summary of the types of nanoparticles obtained from Nanotechnologies, Inc. for this evaluation. Particle composition was confirmed by XRD and/or ICP. Average particle size was determined per BET.

Nanoparticle Product (Composition - Size)	Average Particle Size (nm)
Al ₂ O ₃ -27	27
Fe ₃ O ₄ -30	30
TiO ₂ -20	20
Fe-15-ST2	15
Fe-25-ST2	25
Ni-10-ST2	10
Ni-15-ST2	15
Ag-10-ST2	10
Ag-25-ST2	25
Ag-35-ST3	35
NiO-23	23
NiO-23	23
NiO-24	24
Ag/Fe-15-ST2	13
Ag/Fe-30-ST2	31
Ag/Ni-15-ST2	15
Ag/Ni-25-ST2	25
Ag/NiO-35	35

Table 1: Nanoparticles used in efficacy testing

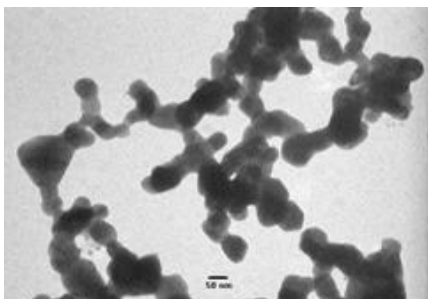


Figure 1: TEM of silver produced without use of carbon as a particle buffer to prevent formation of sintered aggregates.

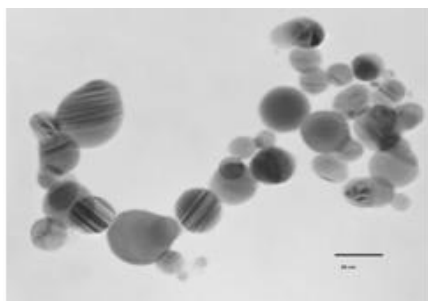


Figure 2: TEM of silver formed with use of carbon to prevent formation of sintered aggregates.

The nanoparticles were carefully weighed for use using a L-series Acculab scale inside a vacuum safety hood and transferred into 15 ml glass scintillation vials (Research Products International Corp.). Particles were suspended in

sterile, distilled water at desired concentrations of 100- and 500µg/mL. All nanoparticle solutions were prepared using a Branson sonicator for 2 minutes, implementing a duty cycle of 30 and an output of 10-30 to break up the looser agglomerate structures.

2.2. Cultures and media

Escherichia coli. (TOP10 strain) was obtained from Invitrogen. The components of the Luria-Bertani (LB) medium and the Tryptic Soy Broth (TSB) medium were obtained from Sigma. The Bacillus anthracis spores were Sterne strain and obtained from Colorado Veterinary Products (19102).

2.3. E. coli preparation and exposure

A single colony of E. coli was picked from a streak plate and inoculated into a 5mL snap-cap tube containing 3mL of LB media. Tubes were shaken at 250 rpm, in a 37°C incubator. After overnight incubation, 5mL snap-cap tubes containing 3mL of LB media were inoculated with 300µL of E. coli culture. The diluted E. coli cell cultures were treated with 100 µg/mL and 500 µg/mL concentrations of nanoparticles in deionized water. In every experiment, three diluted E. coli cultures were used as negative controls for log reduction comparison (No treatment culture). Also, three diluted E. coli cultures were inoculated with 50-100 µg/mL of Kanamycin to be used as positive controls. All E. coli cultures were incubated in the Innova 4300 incubator at 37° C and shaken at 200 RPM for the various time period exposures. After nanoparticle treatment, all E. coli cultures were diluted according to their cell density. The last 3 dilutions were plated 3 times each on LB plates and incubated at 37° C overnight. Next morning, all colony-forming units (CFUs) on each plate were counted to find out nanoparticle killing efficacy. The average of triplicate CFU counts of the same dilution was obtained per sample. Once the average was found, it was multiply by the dilution and conversion factors to get CFU/ml. The CFU/ml number was then converted to a log number. The log of the negative control group minus the log of treatment group was equal to the log reduction number indicated. The same mathematical procedure and established microbiological quantitative assay [2] was applied to all samples.

2.4. Growing and harvesting of B. anthracis spores

Bacillus anthracis spores were prepared using the Sterne strain anthrax spores vaccine. A volume of 0.5mL of the vaccine was extracted from vial using a sterile 1cc hypodermic syringe (Fisher: 14-823-2F) and insulin needle (Fisher: 14-826AA). The vaccine was then placed into a 5mL snap-cap tube containing 3mL of TSB. After inoculation, the tube was inverted several times to insure a proper mix and placed in a 37°C incubator for 2 hours. Once incubated, a cotton-tipped swab was dipped into inoculum and thoroughly streaked onto the top of a 5% sheep blood agar plate (REMEL: 1202) using the raster pattern. This procedure was repeated for 10 plates. The plates were then placed in a 37°C incubator for four days.

Upon complete sporulation of the culture, the spores were gently scraped off of the agar plate using a cell scraper (Fisher: 08-771-1B) and placed into a 50mL Falcon tube containing 35mL of chilled ddH₂O. Contents of the tube were poured into a 500mL sterile, disposable bottle (Fisher: 09-761-10) and the tube was rinsed thoroughly to remove any remaining spores and poured into the bottle. The bottle was then placed in 4°C overnight. The filtrate was then heat treated at 60 °C for 20 minutes to kill any vegetative bacteria. After the suspension was allowed to cool, it was then filtered twice using a 500mL filter apparatus (Fisher: 09-761-5), modified to have 2 sterile 70mm Whatman filter coupons. The collected suspension was then centrifuged using 10-50mL Falcon tubes for 10 min at 3000 rpm. The supernatant was discarded properly and all pellets were washed and collected into 1-50mL Falcon tube using ddH₂O with a total volume of 13mL. The spores were then re-suspended and poured into a vacutainer tube (Fisher: 02-685-C) and stored in 4°C. A titer of the stock was performed to determine the concentration (spores/mL). Samples of spores exposed to nanoparticles were plated on growth agar plates with subsequent culture forming units (CFUs) being counted to assess the efficacy of the nanoparticles.

3. RESULTS AND DISCUSSION

3.1. Microscopy

Scanning electron micrographs of nanoparticles (Figure 3) revealed that they were generally spherical, with some clumping being observed.

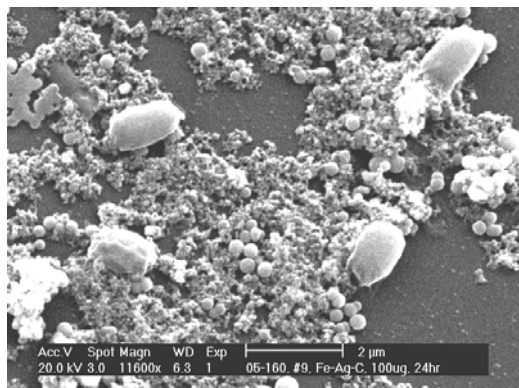


Figure 3: Micrograph of Bacillus anthracis spores exposed to Fe-Ag-C nanoparticles. Note general spherical nature of nanoparticles, with some agglomeration.

Evidence of cellular destruction by the nanoparticles on *E. coli* was observed in many of the silver nanoparticle treatments, as seen in Figure 4.

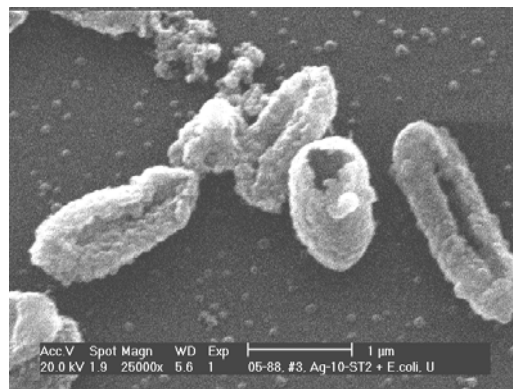


Figure 4: Apparent cellular damage of *E. coli* treated with Ag-10-ST2 nanoparticles.

Some micrographs seemed to suggest that nanoparticles had somehow been internalized by the *B.a.* spores, as shown in Figure 5.

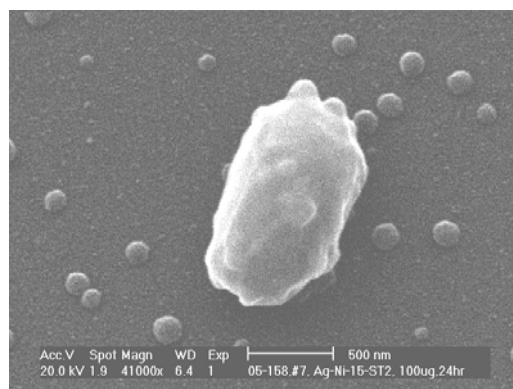


Figure 5: Apparent internalization of nanoparticles inside *B.a.* spores

Silver nanoparticles that exhibited high efficacy against *E. coli* were observed coating the entire bacteria (Figure 6.)

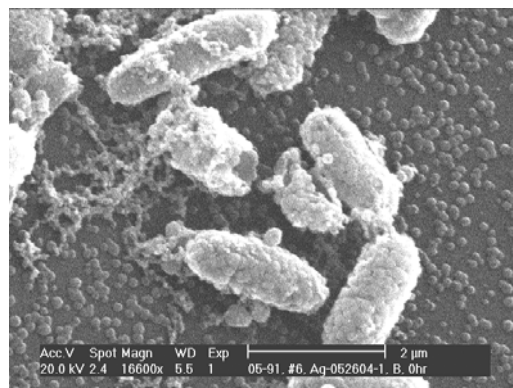


Figure 6: Complete or near complete coating of *E. coli* by silver nanoparticles was regularly observed.

3.2. Nanoparticle efficacy on *E. coli*.

The killing efficacy of the nanoparticles was based on the number of colony forming units (CFU) counted after

exposure. Only plates that contained 0-300 CFUs were counted. Only nanoparticles of either silver or silver compounds that were $\leq 25\text{nm}$ were able to significantly reduce *E. coli* bacteria cell numbers equivalent to reduction values observed in the Kanamycin treatment groups (data not shown).

4. CONCLUSION

It was shown that nanoparticles of silver, and silver compounded with metals such as iron and nickel have strong efficacy with *E. coli*, and *Bacillus anthracis* spores in an idealized water dispersion. Nickel oxide was also seen to have efficacy, though to a lesser extent.

The efficacy is seen to decrease as the primary particle size increases. The efficacy can therefore be said to be proportional with the specific surface area of the nanoparticles. This is seen consistently with silver nanoparticles, as well as silver/metal mixtures.

This work suggests the possibility for unique biological warfare countermeasure systems based on the use of nanoparticle silver or silver mixtures as the active component.

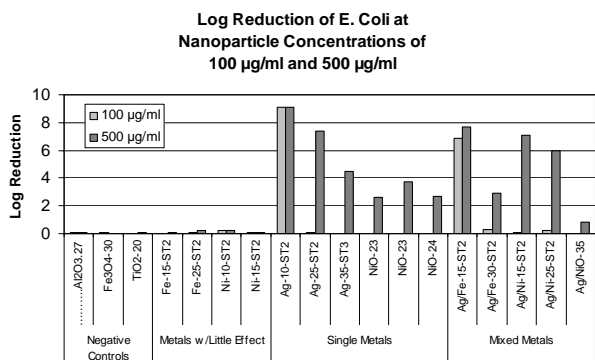


Figure 7: Log Reduction of *E. coli* at nanoparticle concentrations of 100 µg/mL and 500 µg/mL.

3.3. Nanoparticle efficacy on *B. anthracis* spores

Most of the nanoparticles that significantly reduced *Bacillus anthracis* spore growth did so at both 100 µg/mL and 500 µg/mL concentrations. A possible explanation of the killing mechanism would be the analogy of WWII limpet mines on enemy ships. What might be happening is that the silver nanoparticles first become attached to spores, but exhibit no effect on the dormant spore. When spores exposed to these nanoparticle concentrations are plated, a meaningful amount of nanoparticles are also transferred to the blood agar plates affixed to the spores. When the spores with the silver nanoparticles in close proximity germinate into vegetative cells on the blood agar plates, the emerging bacteria are then neutralized by the nanoparticles. The particles with the larger average particle sizes tended to have significantly less efficacy at the 100 µg/mL concentration.

REFERENCES

- [1] Berger, T. J. et al. "Electrically generated silver ions: quantitative effects on bacterial and mammalian cells 23." *Antimicrob. Agents Chemother.* 9.2 (1976): 357-58.
- [2] DeVries, T. A. and M. A. Hamilton. "Estimating the Antimicrobial Log Reduction: Part 1. Quantitative Assays." *Quantitative Microbiology* 1.1 (1999): 29-45.
- [3] Gupta, A. and S. Silver. "Silver as a biocide: will resistance become a problem?" *Nat Biotechnol.* 16.10 (1998): 888.
- [4] Ho, K. C. et al. "Using biofunctionalized nanoparticles to probe pathogenic bacteria." *Anal. Chem.* 76.24 (2004): 7162-68.
- [5] Sawai, J. "Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay." *Journal of Microbiological Methods* 54.2 (2003): 177-82.
- [6] Silver, S. "Bacterial silver resistance: molecular biology and uses and misuses of silver compounds." *FEMS Microbiol. Rev.* 27.2-3 (2003): 341-53.
- [7] Sondi, I. and B. Salopek-Sondi. "Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria." *J Colloid Interface Sci* 275.1 (2004): 177-82.
- [8] Thomas, S. and P. McCubbin. "A comparison of the antimicrobial effects of four silver-containing dressings on three organisms." *J Wound Care* 12.3 (2003): 101-07.
- [9] Yacaman, et al, "The bactericidal effect of silver nanoparticles", *Nanotechnology*, Vol 16, pp2346 (2005)
- [10] Zhao, X. et al. "A rapid bioassay for single bacterial cell quantitation using bioconjugated nanoparticles." *Proc. Natl. Acad. Sci. U.S.A.* 101.42 (2004): 15027-32.

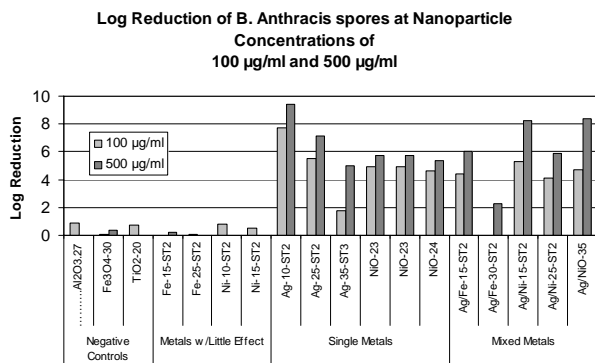


Figure 8: Log Reduction of *Bacillus anthracis* spores at nanoparticle concentrations of 100 µg/mL and 500 µg/mL.