

Synergistic effect of silver nanoparticles with the cephalixin antibiotic against the test strains

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Abstract

In recent years, medical science has turned to a variety of new products and technologies to halt the spread of infections. The most effective and promising antimicrobial agents being embraced by modern medical science today is silver, which is also one of the oldest. In 1940s breakthroughs in antibiotics such as penicillin, actinomycin and streptomycin, medical science began to shift its attention away from preventative compounds such as antimicrobial silver and focused instead on antibiotic treatments. With increasing drug-resistance and growing concern regarding the over-prescribing of antibiotics, there has been a resurgent interest in the use of antimicrobial silver. Unlike antibiotics, silver appears to be immune to resistance. Thus, the conjugation of antibiotic with AgNP (Silver nanoparticles) would prevent development of resistance by microbes and enhance the antimicrobial property of the antibiotic. This study is based on the synergistic effect of Cephalixin antibiotic with AgNP. The nanoparticles were evaluated for their increased antimicrobial activities with Cephalixin antibiotic against *E.coli* and *S. aureus*. The antibacterial activity of Cephalixin was increased in the presence of AgNPs against test strains. The results showed that the combination of antibiotics with AgNPs have better antimicrobial effects.

Introduction

The word “nano” itself refers to the length scale (one nanometer is one billionth of a meter) that is one thousand times smaller than the micro scale. Viruses and DNA are examples of natural objects on the nanoscale, in contrast a human cell can appear enormous. It is well known that Ag ions and Ag-based compounds have strong antimicrobial effects¹², and many investigators are interested in using other inorganic nanoparticles as antibacterial agents^{9,12,15}. Research in antibacterial material containing various natural and inorganic substances¹⁷ has been intensive. Among metal nanoparticles (Me-NPs), silver nanoparticles (Ag-NPs) have been known to have inhibitory and bactericidal effects⁷. It can be expected that the high specific surface area and high fraction of surface atoms of Ag-NPs will lead to high antimicrobial activity as compared with bulk silver metal⁷. Mecking and co-workers showed that hybrids of Ag nanoparticles with amphiphilic hyperbranched macromolecules exhibited effective antimicrobial surface coating agents³. The biomedical application of silver nanoparticles also attracted increasing interest³, such as antimicrobial activity of silver nanoparticles for wound healing³⁴, and silver nano-coated medical devices¹², etc. Duran and co workers investigated that use of silver ion or metallic silver as well as silver nanoparticles can be exploited in medicine for burn treatment, dental materials, coating stainless steel materials, textile fabrics, water treatment, sunscreen lotions, etc. and possess low toxicity to human cells, high thermal stability and low volatility.

Antimicrobial effects of novel silver nanoparticles

The antibacterial activity of silver species has been well known since ancient times³¹ and it has been demonstrated that, in low concentrations, silver is non toxic to human cells³¹. It is well known that silver ion and silver-based compounds are highly toxic to microorganisms³², showing strong biocidal effect against as many as 16 species of bacteria, including *Escherichia coli*⁵. The antimicrobial activity of Ag nanoparticles was investigated against yeast, *Escherichia coli*, and *Staphylococcus aureus*. Antimicrobial studies has also been observed against methicillin-resistant *S. aureus* followed by methicillin-resistant *Staphylococcus epidermidis* and *Streptococcus pyogenes*, whereas only moderate antimicrobial activity was seen against *Salmonella typhi* and *Klebsiella pneumoniae*²⁷. The antifungal activity of fluconazole was enhanced against the test fungi in the presence of Ag-NPs. Fluconazole in combination with Ag-NPs showed the maximum inhibition against *C.albicans*, which was confirmed from the increase in fold area of inhibition, followed by *P. glomerata* and *Trichoderma sp.*, which showed less increase in the fold area, whereas no significant enhancement of activity was found against *P. herbarum* and *F. semitectum*²⁵.

Action of silver nanoparticles

Several studies propose that AgNPs may attach to the surface of the cell membrane disturbing permeability and respiration functions of the cell²². Smaller Ag NPs having the large surface area available for interaction would give more bactericidal effect than the larger AgNPs²². The mechanism of action of silver is linked with its interaction with thiol group compounds found in the respiratory enzymes of bacterial cells. Silver binds to the bacterial cell wall and cell membrane and inhibits the respiration process¹⁸. In case of *E. coli*, silver acts by inhibiting the uptake of phosphate and releasing phosphate, mannitol, succinate,

proline and glutamine from *E. coli* cells⁶¹⁴²⁹³⁰³⁵. Silver nitrate was combined with sulfonamide to form silver sulfadiazine cream, which served as a broad-spectrum antibacterial agent and was used for the treatment of burns. Silver sulfadiazine is effective against bacteria like *E. coli*, *S. aureus*, *Klebsiella sp.*, *Pseudomonas sp.* It also possesses some antifungal and antiviral activities¹¹. Recently, due to the emergence of antibiotic-resistant bacteria and limitations of the use of antibiotics the clinicians have returned to silver wound dressings containing varying level of silver⁸¹³. There lies a strong challenge in preparing nanoparticles of silver stable enough to significantly restrict bacterial growth. The major mechanism through which silver nanoparticles manifested antibacterial properties was by anchoring to and penetrating the bacterial cell wall, and modulating cellular signalling by dephosphorylating putative key peptide substrates on tyrosine residues¹⁰.

The size of the nanoparticle implies that it has a large surface area to come in contact with the bacterial cells and hence, it will have a higher percentage of interaction than bigger particles²¹²⁶. The nanoparticles smaller than 10 nm interact with bacteria and produce electronic effects, which enhance the reactivity of nanoparticles. Thus, it is corroborated that the bactericidal effect of silver nanoparticles is size dependent²⁸. According to Raimondi²⁸ truncated triangular nanoparticles show bacterial inhibition with silver content of 1 µg. While, in case of spherical nanoparticles total silver content of 12.5 µg is needed. The rod shaped particles need a total of 50 to 100 µg of silver content. Thus, the silver nanoparticles with different shapes have different effects on bacterial cell. The strong interaction between negatively charged bacterial wall and HPAMAM-NH₂ macromolecules⁹ can possibly decrease the distance between the Ag NPs and bacteria. This process could facilitate the release of active Ag into the bacteria resulting in a synergistic antibacterial effect of the HPAMAMNH₂/Ag nanocomposites.

In proteomic and biochemical studies, nanomolar concentrations of AgNPs have killed *E. coli* cells within minutes possibly due to immediate dissipation of the proton motive force¹³. Importantly, the determined effective concentration of Ag NPs was at nanomolar levels while Ag⁺ ions were effective at micromolar levels¹³. Ag NPs thus seem to be more efficient than Ag⁺ ions in performing antimicrobial activities. The oxygen can easily oxidize nano-Ag to yield partially oxidized nano-Ag with chemisorbed Ag⁺ ions²⁴. The antibacterial activities of Ag NPs against *E. coli* depended on the chemisorbed Ag⁺ ions (surface oxidation) and particle size.

Synergistic effect

The minimum inhibitory concentrations (MIC) of extracellular biosynthesized AgNPs on gram-positive and gram-negative bacteria were determined by broth dilution method. The observed MIC values for AgNPs were 30, 35, 80, and 65 µg/ml for *E. coli*, *S. typhi*, *S. aureus*, and *M. luteus*, respectively. The combination of these AgNPs with different antibiotics was investigated against gram-positive and gram-negative bacteria using the disk diffusion method. The diameter of the inhibition zone (mm) around the different antibiotic disks with and without AgNPs against test strains was found. The highest percentage of fold increase was found for ampicillin followed by kanamycin, erythromycin and chloramphenicol². Also in the article cited by Shahverdi et al.,³¹ nanoparticles are evaluated for their part in increasing the antimicrobial activities of various antibiotics against *Staphylococcus aureus* and *Escherichia coli*. The antibacterial activities of penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin were increased in the presence of Ag-NPs against both test strains. The highest enhancing effects were observed for vancomycin, amoxicillin, and penicillin G against *S. aureus*.

Materials and Methods

Source of micro organism

Two bacterial strains—namely *Escherichia coli* (ATCC10536) and *Staphylococcus aureus* (ATCC 29737) were obtained from the Culture Collection Center (CAS in Botany, University of Madras, India) and maintained Nutrient agar (HiMedia, Mumbai, India) slant at 27°C respectively. The cultures were confirmed by growing in selective media Eosin Methylene Blue agar and Salt agar respectively.

Maintenance of culture

The culture was maintained by repeated sub culturing on Nutrient agar slants. The 24 hour culture was prepared for each experimental procedure.

Source of Nanoparticles

Silver Nanoparticles used in this study were obtained from research laboratory, Centre for Advanced Studies in Botany, University of Madras, which was synthesized from the fungus *Trichoderma viridae* was utilized for extra cellular biosynthesis of extremely stable AgNPs. The nanoparticles show maximum absorbance at 420 nm on ultraviolet-visible spectra. This proves the presence of pure AgNPs in solution. Antibiotic Cephalexin was obtained commercially and used for the study.

Broth Dilution method

Nutrient broth was prepared and sterilized in an autoclave at 120°C and 15 lbs pressure for 15 minutes. Pure culture of a single microorganism is grown in nutrient broth. The culture was incubated at room temperature over night in a shaker. To the sterile side arm flask 100ml of nutrient broth, 150 µl of micro organism and varying concentrations of nanoparticles / antibiotics were

added. 100ml of nutrient broth inoculated with 150 μ l of organism was taken as positive control and 100ml of nutrient broth alone was taken negative control. Initial optical density reading was at 600 nm and the reading was noted. At 2 hours interval optical density reading was noted. A graph was plotted as OD value at X axis and time at Y axis. From the graph Minimum Inhibitory Concentration (MIC) for nanoparticles / antibiotic was determined.

Pour Plate Technique

After 30 hours, 1ml of sample from each of the side arm flasks was taken and poured onto sterile Petri plates above which 20ml of nutrient agar was poured. This was incubated for 24 hours and the colonies were observed.

Conjugating Cephalexin with Silver Nanoparticles

Cephalexin at different concentration was added to silver nanoparticles and incubated overnight. This concentration of silver nanoparticles is used to treat the *Staphylococcus aureus* and *E. coli*.

Enhancement of Cephalexin Activity

Broth dilution method for finding MIC for *S.aureus*

Cephalexin activity

To the 50ml of Nutrient broth prepared in side arm conical flasks 50 μ g, 100 μ g, 150 μ g, 200 μ g, 250 μ g, and 300 μ g per ml concentration of Cephalexin was added. To measure the bacterial growth rate and to determine the growth curve 0.15ml (1×10^{-8} CFU) over night culture of *S. aureus* was added to the broth containing Cephalexin. The OD was read at 600 nm at 2 hours intervals for 30 hours. MIC was confirmed by pour plate technique.

Silver nanoparticles activity

To the 50ml of Nutrient broth prepared in side arm flasks 50 μ g, 100 μ g, 150 μ g, 200 μ g, 250 μ g and 300 μ g per ml concentration of Silver nanoparticles were added. To this 0.15 ml (1×10^{-8} CFU) over night culture of *S.aureus* was added. The OD was read at 600 nm at 2 hours intervals for 30 hours. MIC was confirmed by pour plate technique.

Cephalexin with silver nanoparticles activity

To the 50 ml of Nutrient broth prepared in side arm flasks 50 μ g, 100 μ g , 150 μ g, 200 μ g, 250 μ g per ml concentration of Cephalexin with 1 μ g/ml Silver nanoparticles were added .To this 0.15 ml (1×10^{-8} CFU) over night culture of *S.aureus* was added. The OD was read at 600 nm at 2 hours intervals for 30 hours. MIC was confirmed by pour plate technique.

The same was repeated with 1×10^{-8} CFU overnight culture of *E.coli*.

Results and Discussion

Antibacterial activity of silver nanoparticles

Antibacterial activity of silver nanoparticles has been checked in Nutrient broth were 0.15mL (1×10^8 CFU) of *E. coli* cells and *S. aureus* is supplemented with different concentration nanoparticles and the OD value is checked for every 2hrs. For *E.coli* the minimum inhibitory concentration was found to be 300 μ g/ml and the range is between 300-400 μ g/ml and for *Staphylococcus aureus* the minimum inhibitory concentration was found to be 800 μ g/ml and the range is between 800-900 μ g/ml. This shows that silver nanoparticles has better activity on *E.coli* than *Staphylococcus aureus*.

Minimum inhibitory concentration was confirmed by pour plate technique were no colonies were observed for *E.coli* from the plates starting from the silver nanoparticles concentration of 300 μ g/ml and 800 μ g/ml for *S.aureus* which confirms the MIC of silver nanoparticles for both organisms.

Antibacterial activity of Cephalexin

Antibacterial activity of Cephalexin has been checked in Nutrient broth were 0.15mL (1×10^8 CFU) of *E. coli* and *S.aureus* is supplemented with different concentration of Cephalexin and the OD value is checked at 2 hours interval for 24 hours. For *E.coli* the minimum inhibitory concentration was found to be 400 μ g/ml and the range is between 400-450 μ g/ml and for *Staphylococcus aureus* the minimum inhibitory concentration was found to be 150 μ g/ml and the range is between 150-200 μ g/ml. Minimum inhibitory concentration was confirmed by pour plate technique were no colonies were observed for *E.coli* from the plates starting from the antibiotic concentration of 400 μ g/ml and 150 μ g/ml for *S.aureus* which confirms the MIC of antibiotic cephalexin for both organisms.

Enhancement of antibacterial activity (Cephalexin + nanoparticles)

Cephalexin is conjugated with silver nanoparticles by overnight incubation. Enhancement of antibacterial activity of Cephalexin conjugated nanoparticles has been checked in Nutrient broth were 0.15ml (150μ l) of *E.coli* cells and *S.aureus* is supplemented

with different concentration of Cephalexin conjugated nanoparticle and the OD value is checked for every 2hrs. For *E.coli* the minimum inhibitory concentration was found to be 200 µg/ml and the range is between 200-250 µg/ml for silver, where as for *Staphylococcus aureus* the minimum inhibitory concentration was found to be 100 µg/ml and the range is between 100-150 µg/ml for silver. This shows an enhancement in antibacterial activity on *E.coli* and *Staphylococcus aureus*.

Minimum inhibitory concentration was confirmed by pour plate technique were no colonies were observed for *E.coli* from the plates starting from the antibiotic concentration from 200 µg/ml and 100 µg/ml for *S.aureus* which confirms the MIC of antibiotic cephalaxin conjugated with silver nanoparticles for both organisms.

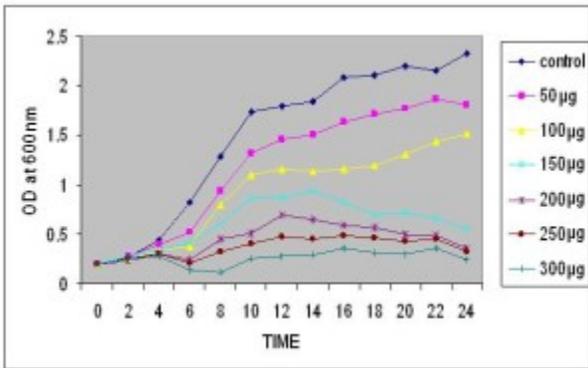


Fig 1(a). Growth curve of *S.aureus* with antibiotic.

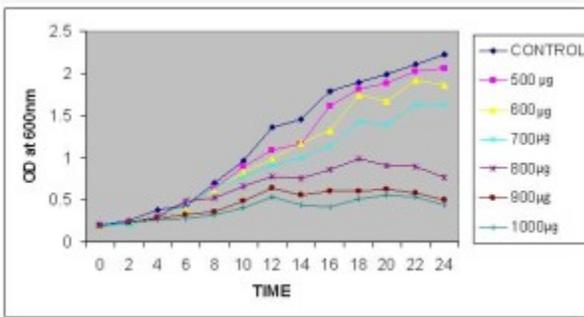


Fig 1(b). Growth curve of *S.aureus* with AgNP.

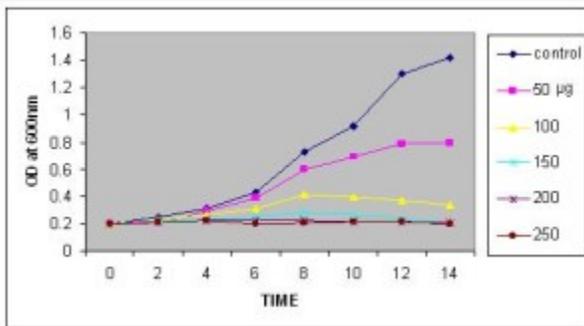


Fig 1(c). Growth curve of *S.aureus* with Cephalexin+AgNP.

Organism	Antibacterial agent	MIC (µg/ml)	Range
<i>Staphylococcus aureus</i>	Cephalexin	150	150-200
	Silver nanoparticles	800	800-900
	Cephalexin conjugated silver nanoparticles	100	100-150

Table 1. Antimicrobial activity of Cephalexin, Silver nanoparticles and Cephalexin conjugated Silver nanoparticles against *Staphylococcus aureus*.

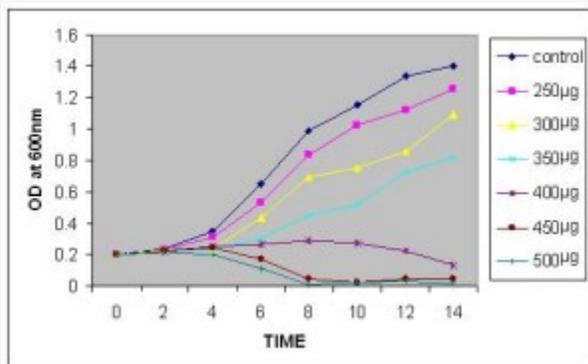


Fig 2(a). Growth curve of *E.coli* with antibiotic.

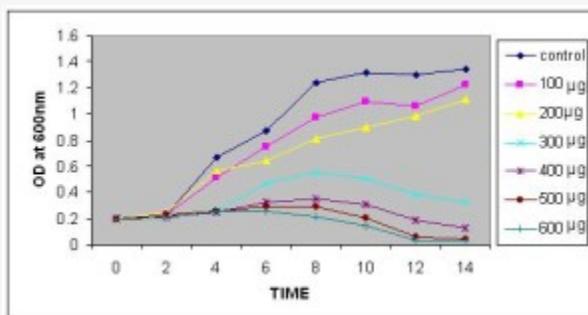


Fig 2(b). Growth curve of *E.coli* with AgNP.

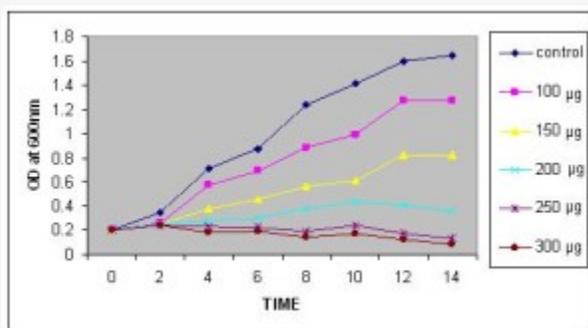


Fig 2(c). Growth curve of *E.coli* with Cephalexin+AgNPs.

Organism	Antibacterial agent	MIC (µg/ml)	Range
<i>Escherichia coli</i>	Cephalexin	400	400-150
	Silver nanoparticles	300	300-400
	Cephalexin conjugated Silver nanoparticles	200	200-250

Table 2. Antimicrobial activity of Cephalexin, Silver nanoparticles and Cephalexin conjugated Silver nanoparticles against *Escherichia coli*.

Discussion

Cell wall of Gram-negative bacteria consists of outer membrane and peptidoglycan layer. The major component of outer membrane is lipopolysaccharide. Gram-negative bacteria are intrinsically resistant to Cephalexin because their outer membrane is impermeable to large glycopeptides molecules. The structure of the cell wall may provide resistance to drug effect. Due to Gram negative bacteria with their extra lipid bilayer, many antibiotics may not reach the sites of action. Therefore any antibiotic drug that is not lipid soluble are enough to traverse the outer lipid bilayer and small enough to traverse the porin channel will have no effect on the microorganism. Alternately, a small drug with suitable solubility profile may pass through the porin channel and exert an antibacterial effect. On the other hand, the cell wall in Gram-positive bacteria is principally composed of a thick layer (~20–80 nm) of peptidoglycan consisting of linear polysaccharide chains cross-linked by short peptides to form a three-dimensional rigid structure⁴. The rigidity and extended cross-linking not only endow the cell walls with fewer anchoring sites for the AgNPs but also make them difficult to penetrate.

E.coli when treated with nanoparticles conjugated Cephalexin, they will bind to the cell wall and destroys the stability of the outer membrane, which makes nanoparticles coated Cephalexin easier to bind to the peptidoglycan structure. Nanoparticles destroy the stability of LPS, allowing increase in permeability of the outer membrane and the peptidoglycan structure and is recognized and captured by Cephalexin immediately. This makes nanoparticles conjugated Cephalexin as an effective antibiotic against gram negative bacteria.

This rigid cross-linking of cell wall of *S.aureus* gave fewer anchoring sites for the AgNPs also making them difficult to penetrate. As the antibacterial activity of cephalexin was found to increase in the presence of AgNPs the above said difficulty was overcome. The increase in synergistic effect may be caused by the bonding reaction between antibiotic and nanosilver. The antibiotic molecules contain many active groups such as hydroxyl and amido groups, which reacts easily with nanosilver by chelation. More recently, Batarseh's research showed that the bactericidal effect was caused by silver (I) chelating, which prevents DNA from unwinding⁵. Cephalexin is conjugated with silver nanoparticles to enhance the antibacterial activity. Conjugation is done here by biological method through overnight incubation. By combining the Cephalexin with Silver nanoparticles, the resistant strains also gets sensitive to Cephalexin. By reducing the concentration, we can also reduce the side effects caused by Cephalexin and at the same time will be cost effective also.

Silver will tend to have a higher affinity to react with phosphorus and sulphur compounds. The membrane of the bacteria is well known to contain many sulphur-containing proteins; these might be preferential sites for the silver nanoparticles. On the other hand, nanoparticles inside the bacteria will also tend to react with other sulphur-containing proteins in the interior of the cell, as well as with phosphorus-containing compounds such as DNA. To conclude, the changes in morphology in the membrane of the bacteria, as well as the possible damage caused by the nanoparticles reacting with the DNA, will affect the bacteria in processes such as the respiratory chain, and cell division, finally causing the death of the cell.

Conclusion

In this current work nanoparticles which was biologically synthesized was used, which is a pure green chemistry and completely toxic free compared to chemical synthesis methods. Minimum inhibition concentration of nanoparticles and Cephalexin antibiotics was found out by broth dilution method. Cephalexin was conjugated with nanoparticles through overnight incubation and its MIC also was found out. Enhancement study of Cephalexin antibiotics along with silver nanoparticles against *E.coli* and *S.aureus* was studied. For *E.coli* the minimum inhibitory concentration was found to be 200 µg/ml and the range is between 200-250 µg/ml for silver. For *Staphylococcus aureus*, the minimum inhibitory concentration was found to be 100 µg/ml and the range is between 100-150 µg/ml for silver. From the above results obtained, we can conclude that a silver nanoparticle plays a vital role in enhancing the antibacterial activity of Cephalexin. When nanoparticles conjugated with Cephalexin, in lower concentrations also it was found to be effective when compared to the individual antibacterial activity of nanoparticles as well as antibiotic. Cephalexin as any other antibiotics has side effects but yet used as a life saving antibiotic when all other antibiotics fail. At the same time the cost of the Cephalexin is more compared to other commercially available antibiotics. By our findings, since the concentration of Cephalexin is reduced, the side effects caused due to the antibiotics can be minimized up to an extend and at the same time cost effective also. By carry out further experiments like animal modeling and various trials, it is possible to use in human also, but it requires vast and extensive studies before human trials.

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