

Antibacterial activity of silver nanoparticles against field and reference strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis* and multiple-drug-resistant tuberculosis strains

This paper (No. 24092018-00127-EN) has been peer-reviewed, accepted, edited, and corrected by authors. It has not yet been formatted for printing. It will be published in December 2018 in issue 37 (3) of the *Scientific and Technical Review*

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Summary

Tuberculosis (TB) is an endemic disease in animals and humans in Egypt. This study aims to investigate the antimycobacterial activity of silver nanoparticles (AgNPs) by determining the minimal inhibitory concentration (MIC) of AgNPs, using the microplate Alamar blue assay. The AgNPs were chemically synthesised and their form and size were characterised by ultraviolet-visible absorption spectrophotometry, transmission electron microscopy and X-ray diffraction. The reference strains of *Mycobacterium bovis* and

Mycobacterium tuberculosis H37Rv, and one multiple-drug-resistant (MDR) strain of *M. tuberculosis* were tested, as well as clinical isolates of *M. bovis* and *M. tuberculosis*. The AgNPs were tetrahedral with a few spherical particles and an average particle size of 50 nm. The mycobacterial strains were varied with MICs of AgNPs. Both reference strains of *M. tuberculosis* and *M. bovis*, in addition to the MDR strain of *M. tuberculosis*, were successfully inhibited by AgNPs at MICs of 1 µg/ml, 4 µg/ml and 16 µg/ml, respectively, whereas clinical isolates of *M. bovis* and *M. tuberculosis* were inhibited at MIC values of 4–32 µg/ml and 1–16 µg/ml, respectively. The AgNPs showed an *in vitro* chemotherapeutic effect against *Mycobacterium* spp. Thus, they can be used to treat TB not only in humans but also in animals, and may be useful in TB prevention and control strategies worldwide.

Keywords

Egypt – Microplate Alamar blue assay – *Mycobacterium bovis* – *Mycobacterium tuberculosis* – Silver nanoparticles – Tuberculosis.

Introduction

Both *Mycobacterium tuberculosis* and *Mycobacterium bovis* cause tuberculosis (TB), the most serious infectious disease in humans and animals, with a higher mortality rate than any other infectious disease. Tuberculosis was responsible for 1.7 million deaths in 2016 alone, and 10.4 million people fell ill with the disease in the same year, according to the World Health Organization (WHO). Meanwhile, bovine TB remains a worldwide problem, with more than 50 million cattle estimated to be infected (1, 2).

Bovine TB due to infection with *M. bovis* was reported in 128 countries between 2005 and 2008, according to the World Organisation for Animal Health (OIE) (3). *M. bovis* is zoonotic in nature but its representation among human TB cases was negligible in most countries. For example, it comprised 4.3% of clinical isolates recovered from 31 patients examined in Mexico. Human exposure is

attributed to the consumption of unpasteurised milk or contaminated dairy products (3, 4).

The prolonged duration of tuberculosis treatment and the number and severity of the side effects of antituberculous drugs (including ototoxicity and nephrotoxicity) can lead to low adherence to treatment among patients (5). Moreover, the use or abuse of these drugs over the years has caused the emergence of multiple-drug-resistant (MDR) strains of TB, leading to the urgent need to develop new effective agents (6).

The increasing rates of MDR *M. tuberculosis* and *M. bovis* (7, 8) represent a severe threat to TB control and a growing public health problem, especially in developing countries (9). Nanoparticles have attracted great interest as potential antibacterial drugs (10). Silver has been used since ancient times as an antimicrobial agent but, with the discovery and progress of antibiotics, medical applications of silver have declined (11, 12).

The antimicrobial effects of silver can be increased by controlling its particle size at the nano level, where the physical, chemical and biological properties of such silver nanoparticles (AgNPs) change considerably, due to their surface-to-volume ratio (13). The AgNPs of between 10 nm to 100 nm have demonstrated strong bactericidal potential against MDR bacteria (14). The key element of their antimicrobial effect is due to silver's high affinity for sulphur and phosphorus, which are abundant in the bacterial cell membrane proteins. Silver nanoparticles can react with sulphur-containing amino acids inside or outside the cell membrane, affecting bacterial cell viability. Moreover, silver ions (particularly Ag^+) released from AgNPs can interact with phosphorus moieties in DNA, resulting in inactivation of DNA replication, or with sulphur-containing proteins, leading to the inhibition of their enzymatic functions (6, 15).

The objectives of this study were to determine the minimal inhibitory concentration (MIC) of AgNPs against *M. tuberculosis*, *M. bovis* and MDR *M. tuberculosis*, using microplate Alamar blue assay.

Methodology

Mycobacterial strains

The Mycobacteriology Unit of the Veterinary Serum and Vaccine Institute, Abbassia, Cairo, Egypt, supplied one reference strain of *M. bovis* and one of *M. tuberculosis* H37Rv. It also supplied ten clinical isolates for *M. bovis* (with ID numbers from B1 to B10), with two isolates belonging to type SB0268; and ten clinical isolates for *M. tuberculosis* (with ID numbers from H1 to H10), with two isolates belonging to type SB0223.

One clinical isolate of MDR *M. tuberculosis*, diagnosed by the simplified version of the indirect proportion method against 0.2 µg/ml isoniazid, 40 µg/ml rifampicin, 2 µg/ml ethambutol and 4 µg/ml streptomycin, according to standard procedures (16), was supplied by the Abbassia Chest Hospital, Cairo, Egypt.

In addition, the MIC values of isoniazid, rifampicin, ethambutol and streptomycin were diagnosed against the ten clinical isolates of *M. bovis* and *M. tuberculosis* by the simplified version of the indirect proportion method. The MIC for isoniazid was found to be 4–8 µg/ml; for rifampicin, it was 4–16 µg/ml; for ethambutol, 0.5–20 µg/ml; and for streptomycin, 5–20 µg/ml. All strains were maintained on Löwenstein-Jensen medium (Sigma-Aldrich Chemie GmbH, Munich, Germany) and freshly subcultured before evaluation.

Synthesis of silver nanoparticles

The AgNps were manufactured by NanoTech for Photo-Electronics, Al Giza, Egypt, and prepared by the chemical reduction method. They were synthesised using sodium borohydride (NaBH₄) and polyvinylpyrrolidone (PVP) (molecular weight [Mw] = 40,000) as the reducing agent and stabilising agent, respectively. An aqueous solution of trisodium citrate (0.5 ml, 30 millimolar or mM) was poured into a flask (50 ml of deionised water), and an aqueous solution of silver nitrate (AgNO₃) (1 ml, 5 mM) was added. Freshly prepared NaBH₄ aqueous solution (0.5 ml, 50 mM) was quickly

mixed, and the suspension immediately turned a light yellow colour. After 30 s, an aqueous solution of PVP (0.5 ml, 5 mg/ml; Mw = 40,000) was added. After 30 min of the reaction, the suspension changed to a darker yellow colour (17).

Characterisation of silver nanoparticles

The size and morphology of the nanoparticles were analysed with a transmission electron microscope (TEM) (JEOL 1210, from JEOL Ltd, Tokyo, Japan), and the optical absorption features of silver colloids in the UV-visible range of 200 to 800 nm wavelength were measured using an Ocean Optics USB2000+VIS-NIR fibre optic spectrophotometer. The samples for X-ray diffraction analysis were prepared by taking a small amount of solution from the bottle and drying it on a quartz plate.

Preparation of silver nanoparticles

Serial two-fold dilutions of AgNPs were prepared from 0.25 to 256 µg/ml.

Evaluation of the minimal inhibitory concentration of silver nanoparticles, using microplate Alamar blue assay

The bacterial strains were prepared as follows: the bacterial inoculum was withdrawn from a fresh Löwenstein-Jensen tube and added to 5 ml of Middlebrook 7H9 broth, containing 0.1% casitone and 0.5% glycerol and supplemented with oleic acid, albumin, dextrose, and catalase (7H9-S) (Becton Dickinson Microbiology Systems, Franklin Lakes, New Jersey, United States of America [USA]), in a tube containing several glass beads.

After seven days of incubation at 37°C, the tube was vortexed for 2 min and allowed to settle for 15 min. The supernatant was transported to another tube and the turbidity adjusted to 1 on a McFarland scale, using a nephelometer. The suspension was further diluted 1:5 in 7H9-S and used as the inoculum for the test.

The microplate Alamar blue (AB) assay was performed as described by Banu *et al.* (18). Briefly, 200 µl of sterile de-ionized water was added to all outer perimeter wells of a sterile, 96-well microtitre plate (Becton Dickinson Labware, Franklin Lakes, New Jersey, USA) to minimise evaporation of the medium in the test wells during incubation. For each strain evaluated, 100 µl of 7H9-S containing each nanoparticle dilution was dispensed into the wells and inoculated with 100 µl of the diluted mycobacterial suspensions. The plates were covered and sealed with parafilm and incubated at 37°C for five days. Then, 25 µl of a freshly prepared 1:1 mixture of AB reagent and 10% polysorbate 80 (Tween 80) was added to the plate and incubated for 24 h. A blue colour in the well was interpreted to mean no bacterial growth and pink was scored as growth. The MIC was determined as the lowest drug concentration which prevented the colour change from blue to pink. Controls included: medium alone; medium plus AB; medium plus AB plus drug dilution; and bacterial cells plus medium plus AB. All manipulations were performed under a biological safety cabinet.

Results

Characterisation of chemically synthesised silver nanoparticles

The AgNPs prepared in this study were manufactured by a chemical reduction method. In Figure 1, the TEM images show the formation of AgNPs with an average size of up to 50 nm. It was also observed that most of the AgNPs were tetrahedral in shape, with a few spherical particles (Fig. 1).

Effects of silver nanoparticles on mycobacterial species

The *in vitro* activity of AgNPs against mycobacterial strains in different concentrations, ranging from 0.25 µg/ml to 256 µg/ml, was assessed by determining the MIC, using the AB assay (Fig. 2). The results showed the effective inhibitory activity of the AgNPs against all tested mycobacterial strains. The MIC of AgNPs against *M. tuberculosis* H37Rv was 1 µg/ml, while it was 4 µg/ml for the

reference *M. bovis* strain. In regard to the clinical isolates of *Mycobacterium* spp. included in this study, the MIC values against clinical isolates of *M. bovis* were in the range of 4 µg/ml to 32 µg/ml, compared with 1 µg/ml to 16 µg/ml for the clinical *M. tuberculosis* strains. In addition, the MIC value of AgNPs against MDR *M. tuberculosis* was 16 µg/ml.

Discussion

Antibacterial agents are very important in the textile industry, water disinfection, medicines, and food packaging. Nanoparticles have potential application as antimicrobial compounds since they can bind to DNA and inhibit DNA unwinding, which leads to cell death (19). Silver nanoparticles naturally interact with the bacterial membrane to disrupt its integrity, and the silver ions bind to the sulphur, oxygen and nitrogen present in essential biological molecules and inhibit bacterial growth (20).

Nanoparticle-based systems have been tested for the prevention, diagnosis and treatment of TB. Such systems may provide a solution for the problems of:

- drug bioavailability
- the need for frequent dosage
- the hydrophobic nature of the cell wall, which reduces binding and penetration of anti-TB drugs, thereby impairing their bactericidal activity
- the appearance of multi-drug resistance.

These are the major obstacles in the control of TB epidemics (21). Nanoparticle-based systems may overcome them by targeting the phagocytic cells infected by intracellular pathogens and acting directly on the cell wall (22).

Although several reports are available on the antibacterial activity of AgNPs against Gram-positive and Gram-negative bacteria, there are few data on the use of AgNPs against *M. bovis* of bovine origin. To

the authors' knowledge, the present study examining the use of AgNPs against bovine TB strains is the first conducted in Egypt.

This study revealed that mycobacterial spp. differed in their susceptibility to the antibacterial action of AgNPs. The MIC of AgNPs was effective against the reference strain of *M. bovis* (at 4 µg/ml) and the reference strain of *M. tuberculosis* H37Rv (at 1 µg/ml), but a higher MIC (16 µg/ml) was recorded with MDR *M. tuberculosis*. This may be related to the higher level of antibiotic resistance in the MDR strain, in comparison to the other strains. Moreover, it is interesting to note that a higher concentration of AgNPs was needed against a few clinical isolates of *M. bovis* (MIC ≈ 32 µg/ml) and *M. tuberculosis* (MIC ≈ 16 µg/ml). These findings are supported by Seth *et al.* (23), who observed that a complete inhibition of growth in the MDR clinical isolates of *M. tuberculosis* required a higher concentration of AgNPs.

Over all, the MIC of AgNPs against clinical *M. bovis* isolates ($n = 10$) ranged from 4 µg/ml to 32 µg/ml. The effects of AgNPs against the clinical and reference isolates of *M. bovis* investigated in this study are in accord with those found in previous studies (19, 24), which reported that AgNPs display excellent antibacterial potential against the Gram-negative bacterium *Escherichia coli*, and Gram-positive bacterium *M. bovis* Bacillus Calmette-Guérin (BCG).

However, the bactericidal efficacy of AgNPs depends on several factors, including particle size, concentration of released silver ions (Ag^+) and surface area. Small-diameter AgNPs can release more silver ions and a larger surface area is more effective in inhibiting the growth of the bacteria examined (25, 26).

This study revealed that AgNPs at an average size of 50 nm showed inhibitory activity against all mycobacterial strains tested. This finding is within the range reported by Mahanty *et al.* and Bose and Chatterjee (27, 28).

In previous studies (6), biologically synthesised spherical-shaped AgNPs were evaluated against clinical isolates of MDR

M. tuberculosis, using microplate Alamar blue assay. All clinical isolates were inhibited within the MIC range of 6.25 to 12.5 µg/ml of AgNPs. The MIC of AgNPs from the *Artemisia pallens* plant showed significant antimycobacterial activity against *M. tuberculosis*, *M. pheli* and *M. avium* at 230 µg/ml, which is much higher than in the findings of this study. Moreover, it was reported that AgNPs with an average size of between 90 nm and 118 nm exhibited excellent antimicrobial activity against all tested Gram-positive and Gram-negative bacterial strains (29).

In contrast to the findings of Banu and Rathod (6), the current authors observed a higher MIC of AgNPs against MDR *M. tuberculosis* (16 µg/ml) by microplate Alamar blue assay, in comparison to *M. tuberculosis* H73Rv. However, some clinical *M. bovis* isolates had higher MICs. This may be attributable to the fact that tested strains may have been collected from different geographical regions and may have been more resistant.

A recent *in vivo* experiment was performed on 65 white mice, which were infected with a two-week culture of the virulent clinical strain of *M. tuberculosis*. This strain is resistant to traditional anti-TB drugs. The animals were treated with nanoparticles in combination with isoniazid and their survival rate was 95% (30).

Further studies are needed to examine the feasibility of using AgNPs *in vivo* against *M. bovis* of animal origin. In addition, large-scale molecular surveillance is needed to determine if bovine *M. bovis* has MDR properties in Egypt.

Reports on bovine TB therapy are both scarce and controversial. Theoretically, animal TB can be treated with the same anti-TB drugs used to treat humans. This requires careful management of multiple drugs for a long time. In practice, treating TB in cattle by the same anti-TB drugs used for humans does not appear practicable. Therefore, it is encouraging that, in this study, AgNPs appear to be a viable bactericidal agent for controlling bovine TB infection. However, there is a need for further study to investigate how AgNPs can be used to

improve the prevention and control of tuberculosis in both animals and humans.

Conclusion

This study shows that AgNPs are an effective agent against both clinical and reference mycobacterial strains of TB. However, further studies are needed to evaluate their efficacy against a greater number of isolates *in vitro*. Moreover, additional large-scale studies are warranted to assess the potential efficacy of AgNPs *in vivo*, so that their pathway can become more fully understood.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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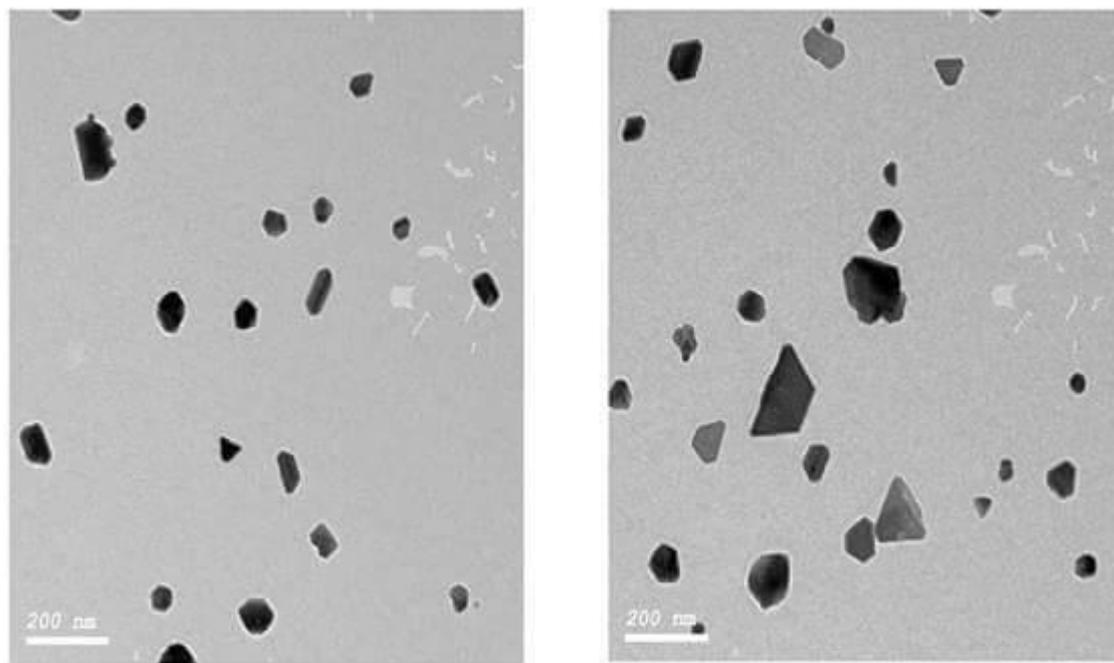


Fig. 1

Transmission electron microscope micrographs of prepared silver nanoparticles

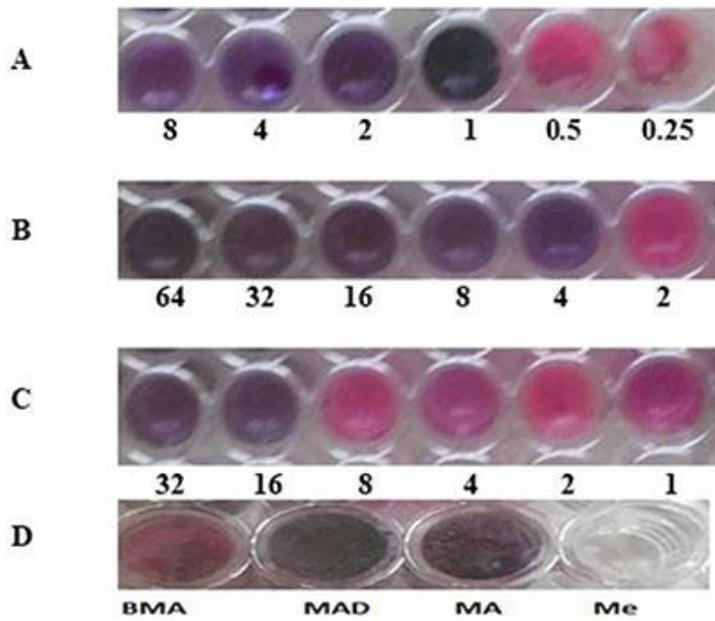


Fig. 2

The minimal inhibitory concentration of silver nanoparticles (µg/ml) against *Mycobacterium tuberculosis* H37Rv (A), reference *M. bovis* (B), and multiple-drug-resistant *M. tuberculosis* (C), using the Alamar blue assay; and controls (D)

AB: Alamar blue
 BMA: Bacterial cells plus medium plus AB
 MA: Medium plus AB
 MAD: Medium plus AB plus drug dilution
 Me: Medium alone