Synthesis of silver nanoparticles with antimicrobial and anti-adherence activities against multidrug-resistant isolates from Acinetobacter baumannii

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Abstract

Objectives: The spread of multidrug-resistant pathogens poses a major health threat. Silver nanoparticles represent a new-class of antimicrobial agents. The aim of this study is the microbial synthesis of silver nanoparticles and the evaluation of their antimicrobial and antibiofilm activities.

Methods: Silver nanoparticles were synthesized using cell free supernatants of Acinetobacter baumannii. Silver nanoparticles were characterized by particle size analysis and transmission electron microscopy (TEM), and the antimicrobial and antibiofilm activities of the synthesized silver nanoparticles were assessed.

Results: The silver nanoparticle synthesis was monitored primarily by the conversion of the pale yellow colour of the bacteria free supernatants into a dark brown colour. Silver nanoparticles had uniform spherical shape, with particle sizes ranging from 37 to 168 nm and a zeta potential of -11.7 mV. Acinetobacter silver nanoparticles were effective against multidrug-resistant Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae with minimal inhibitory concentrations of 3.1, 1.56 and 3.1 mg/ml, respectively. Moreover, acinetobacter silver nanoparticles significantly reduced the attachment activities of E. coli, P. aeruginosa and K. pneumoniae by 66.6%, 86.5% and 75%, respectively.

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Conclusion: Silver nanoparticles, synthesized from Acinetobacter baumannii, inhibited microbial growth and eradicated biofilm assembly by multidrug-resistant isolates that were derived from uropathogenic infection. These results suggested the possibility of using silver nanoparticles as effective antimicrobial and antibiofilm agents against infections caused by resistant isolates.

Keywords: Acinetobacter baumannii; Anti biofilm; Antimicrobial activity; Escherichia coli; Silver nanoparticles

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Introduction

The widespread incidence of multidrug-resistant pathogens constitutes a major clinical problem. Additionally, complications associated with bacterial infection arise due to irreversible bacterial adhesion to either living or nonliving surfaces, especially in deep-seated surfaces of medical devices such as catheters. Adhesion of bacteria begins with the formation of extracellular biopolymers which result in the formation of a biofilm. Embedded microbes inside the formed biofilm matrix are characterized by high growth rates and elevated resistance to conventional antimicrobial therapy. Different antimicrobial agents and delivery strategies for existing antibiotics have been explored to counteract the developed resistance.

Nanotechnology is a new strategy in the antimicrobial field that is being developed to put an end to resistant microbes. In general, nanoparticle forms of such metals as platinum, silver, copper, and gold have antimicrobial activities against various pathogenic bacteria and fungi. Specifically, silver nanoparticles have promising applications in nanotechnology and nanomedicine and have been approved for their bactericidal activity against broad spectrum bacterial infections as well as for their fungicidal effects against pathogenic fungi such as Aspergillus and Candida. Additionally, silver nanoparticles possess antiviral effects against HIV-1. These nanosize particles also assist in wound healing and treat the associated infections. Recently, silver nanoparticles have been shown to eliminate microorganisms on textile fabrics and to inhibit biofilm formation. Additionally, silver nanoparticles have been reported to be biocompatible drug delivery nanocarriers, and soft-tissue friendly dental implants both in vitro and in vivo.

Several chemical and physical methods were developed for the preparation of silver nanoparticles. The natural green synthesis approach is an eco-friendly and cost-effective biosynthesis method for silver nanoparticles. This biological method of production is usually accomplished using bacteria, fungi or plant extracts. The bacterial enzymes and metabolites ensure rapid and consistent reduction of silver ions to their element form. Interestingly, the use of microorganism extract has resulted in an easy method to synthesize nanoparticles with characteristic shapes, size and morphology.

Therefore, in this study, we assessed the antimicrobial and anti-biofilm activities of silver nanoparticles synthesized using nonpathogenic Acinetobacter baumannii bacterial extract. We evaluated the synthesized nanoparticles using transmission electron microscopy (TEM), and analysed their particle size and zeta potential. We evaluated the antimicrobial and adhesive activities of the nanoparticles against multidrug-resistant Gram-negative isolates from urinary tract infections.

Materials and Methods

Bacterial strains and cultivation conditions

The bacterial isolate Acinetobacter baumannii (non-pathogenic) was provided by Prof. Abd El-Rahman, faculty of pharmacy, microbiology department, El-Azhar University. Klebsiella pneumoniae, Pseudomonas aeruginosa, and Escherichia coli isolates were purified from urine samples collected from Mansoura University Hospitals. The isolates were identified using the automated identification system, VITEK 2 (bioMerieux, Marcy l’Etoile, France). Antibiotic susceptibility of the isolates was also determined according to clinical laboratory standards. Antibiotic susceptibility was assessed and compared to different classes of antimicrobial agents such as ampicillin (AP), amoxicillin/clavulanic acid (AUG), amikacin (AK), aztreonam (ATM), cefoxitin (FOX), ceftazidime (CAZ), ciprofloxacin (CIP), cotrimoxazole (TS), piperacillin (PRL), gentamicin (GM) and imipenem (IMI).

All bacterial isolates were cultivated in Luria–Bertani (LB) broth containing: yeast extract 0.5%, tryptone 1% and sodium chloride 1% with pH 6.8. A stock solution of AgNO3 10 mM in sterile distilled water was prepared and stored at 4 °C. Amoxicillin/clavulanic acid (Glaxo SmithKline, UK) was used as a positive control.

Synthesis of silver nanoparticles

Luria–Bertani medium in Erlemeyer flasks (50 ml) were inoculated with freshly grown inoculums of A. baumannii. The culture flasks were incubated for 24 h at 37 °C. The cultures were centrifuged at 6.000 × g (Hettich Zentrifugen, Germany) and the supernatants were filtered twice using syringe filters (0.45 μm) (Macherey–Nagel, US). The cell-free filtrates were mixed with AgNO3 to a final concentration of 1 mM. The silver nanoparticles thus obtained were confirmed through observed colour change, changes in the surface plasmon resonance, and visible light spectroscopy measurements measured between 200 and 800 nm (Evolution 201 UV–visible spectrophotometer, Thermo Scientific). The control samples containing AgNO3 1 mM without bacterial cultures were also prepared.

Characterization of the prepared silver nanoparticles

Transmission electron microscopy (TEM)

The synthesis of silver nanoparticles was monitored visually by colour change after the addition of AgNO3. The formed silver nanoparticles were analysed using TEM (JEM-2100, JEOL, UK). Briefly, the carbon grid was mounted with one drop of bacterial filtrates containing silver nanoparticles. Silver nanoparticles were photographed at a magnification power of 60.000–80.000 and size scale range of 50–200 nm.
Particle size analysis and zeta potential measurement

The particle size distribution was assessed using a computer controlled particle size analyser (Nano ZS90, Malvern, UK). The average particle size, size distribution, and zeta potentials were calculated using a Java image tool.

Antibacterial activity of the prepared silver nanoparticles

The minimum inhibitory concentration (MIC) of the prepared silver nanoparticles from *A. baumannii* was quantified using the microtiter plate assay method.15 Twofold serial dilutions of the obtained particles were prepared. Each well was inoculated with 5 × 10⁶ CFU of the test organisms (*E. coli*, *P. aeruginosa*, and *K. pneumoniae*). The antibacterial activity of amoxicillin/clavulanic acid was estimated and used as a positive control. The microtiter plates were incubated at 37 °C for 18 h. The minimal inhibitory concentration was assigned to the lowest concentration that inhibited visible microbial growth.

Effect of silver nanoparticles on bacterial attachment

The effects of the synthesised silver nanoparticles on microbial adhesion and biofilm formation were evaluated using the microtiter plate method.17 Briefly, 100 μl of growth medium containing silver nanoparticles (1/4 MIC) was transferred to each well. Overnight cultures of the test bacteria were diluted, and 10 μl samples were added to the corresponding wells to obtain a final concentration of 5 × 10⁶ CFU/well and the plates were incubated for 24 h. Microbial controls without silver nanoparticles were cultured under the same conditions.

On the second day, the wells were aspirated and the attached cells were washed twice using 150 μl of sterile saline to remove the free ‘planktonic’ bacteria. The adherent biofilm in each well was fixed with methanol for 15 min. The wells were stained with crystal violet (1%, w/v). The wells were washed with distilled water and dried completely. Finally, glacial acetic acid (33%) was added to each well to dissolve the adherent bacteria and the OD at 490 nm was measured using a microtitre ELISA reader (Microplate reader, Perlong Medical Equipment Co., Ltd., China). Percentage inhibition of biofilm formation was calculated as follows: [(OD₄₉₀ control untreated sessile cells − OD₄₉₀ cells treated with Ag nanoparticles)/OD₄₉₀ control untreated sessile cells].

Statistical analyses

MIC and biofilm values were calculated from the mean of three separate experiments using Microsoft Excel and expressed as the mean ± standard deviation. Statistical analysis was conducted using GraphPad Instate software package (version 3.05), Tukey–Kramer multiple-comparison test and the differences were considered significant when *p* < 0.01.

Results

Biosynthesis of silver nanoparticles

The formation of silver nanoparticles using microbial sources was visually monitored. A change in the colour of the reaction mixtures containing AgNO₃ and bacterial supernatants was considered to be the primary indicator of silver nanoparticles formation (Figure 1). The formation of nanoparticles was also monitored by measuring absorption spectra. A change in the colour of AgNO₃ solution as well as the appearance of an absorption peak at 390 nm (Figure 2), confirms the synthesis of silver nanoparticles.

Transmission electron microscopy

Images of the silver nanoparticles are shown in Figure 3. As shown, the TEM indicated that the silver nanoparticles were dispersed in the supernatant and had a spherical shape.

Particle size analysis and zeta potential

The particle size distribution of the synthesized nanoparticles was 37–168 nm (Figure 4), and the zeta potential was −11.7 mV.

Antibacterial activity of *Acinetobacter* nanoparticles

The isolates *E. coli* (E3), *P. aeruginosa* (P21), and *K. pneumoniae* (K32) were resistant to three classes of antibiotics, that is, β-lactams, aminoglycosides and quinolones (Table 1).

The antimicrobial effects of the silver nanoparticles produced from *A. baumannii* against Gram-negative pathogens *E. coli*, *P. aeruginosa*, and *K. pneumoniae* derived from urinary tract infections were determined (Table 2). The MICs of silver nanoparticles were determined to be 3.125 μg/ml against *E. coli*, 1.56 μg/ml against *P. aeruginosa* and 3.125 μg/ml against *K. pneumoniae* compared to the control AgNO₃ solution (Table 2).

Figure 1: Colour change of cell-free supernatants of *A. baumannii* from pale yellow to dark brown after treatment with AgNO₃.
Silver nanoparticles inhibited biofilm synthesis

Acinetobacter silver nanoparticles eliminated the attachment and the formation of biofilm by the tested pathogenic organisms. Figure 5 shows that low concentrations of silver nanoparticles significantly \((p < 0.01)\) reduced the attachments of \(E.\ coli\), \(K.\ pneumoniae\) and \(P.\ aeruginosa\) by 66.6%, 75% and 86.5%, respectively, compared to the untreated control cultures.

Discussion

Infections due to multidrug-resistant pathogens represent a serious challenge for both hospitals and clinics. Bacterial biofilm formation is a critical mechanism for accumulation, and protection from antimicrobial therapy. Biofilm formation begins when microorganisms colonize medical devices, such as internal catheters, causing chronic infections.\(^3\) Resistant microbes are sometimes controlled by administration of high-doses of antibiotics which results in more adverse effects. Nano-size metallic materials represent a novel antimicrobial approach. Various types of nanoparticles and nanocarriers of antibiotics are efficient in treating infectious pathogens, biofilm formation and bacterial attachment.\(^4\)

Silver ions are reported to be effective against microbial infections and bacterial biofilm formation. Nevertheless, silver ions are characterized by low stability and high toxicity that hinder their clinical use. They are also easily inactivated through precipitation and complexation with salts.\(^18\) Therefore, silver in the nanoparticle format has been developed to overcome these limitations. Silver nanoparticles are characterized by high surface area and are associated with new chemical, physical and mechanical characteristics that are different from the ion or salt forms. The microbial approach for the synthesis of silver nanoparticles represents the optimal preparation method because it is a simple, biocompatible and cost effective method.\(^19\)
In our study, combining cell-free supernatants of *A. baumannii* with a silver nitrate solution resulted in a colour change into dark brown (Figures 1 and 2), which suggests the reduction of Ag ions into silver nanoparticles. This colour change is consistent with a previously reported method for extracellular synthesis of silver nanoparticles using *Bacillus* sp. Similarly, bacterial suspension of *Streptomyces* sp also reduces AgNO₃ to silver nanoparticles with a colour change to brown. Similarly, extracellular synthesis of silver nanoparticles by *Fusarium semitectum* and *Aspergillus fumigatus* have also been reported. In a study by Kannan et al. (2011), the surface plasmon resonance of the biosynthesized AgNPs was observed at 390 nm and indicated small monodispersed particles.

The synthesis of silver nanoparticles was assessed using transmission electron microscopy, particle size measurements, and zeta potential (Figures 3 and 4). The synthesized silver nanoparticles were spherical and had sizes in the range of 37–168 nm. The variation in nanoparticle sizes may be attributed to the formation of particles at different time intervals. A variety of silver nanoparticle sizes, depending on the method of production have been previously reported. Different shapes of silver nanoparticles have been observed however, the particles are predominantly of spherical shape. Silver nanoparticles from *A. fumigatus* have a diameter 15–45 nm and particles from *Streptomyces* sp. have a smaller size range from 15 to 25 nm. Silver nanoparticles synthesized from the white rot fungus *Pycnoporus sanguineus* have diameters between 52.8 and 103.3 nm. Additionally, silver nanoparticles in the present study showed high particle size potential around \(-90^\circ\), indicating that the particles have a low affinity for aggregation.

Additionally, the potential antibacterial activities of the synthesized nanoparticles were evaluated against multidrug-resistant pathogens (Table 2). The tested bacterial isolates were resistance to three classes of antibiotics, that is, β-lactams, aminoglycosides, and quinolones. The MICs of silver nanoparticles against Gram-negative bacteria were very low, which could be due to the small size of the synthesized particles in addition to the large surface area. Silver nanoparticles bind to bacterial cell membranes disturbing their permeability and cellular functions. Therefore, the small silver nanoparticles with large surface areas are more efficient than the larger silver nanoparticles. Silver nanoparticles with a smaller size range between 4 and 32 \(\mu\)g/ml are effective against Gram-positive, Gram-negative, and *Candida* sp.

Similarly, low concentrations of silver nanoparticles (1/4 MIC) significantly inhibited bacterial attachment and biofilm formation of the tested isolates (Figure 4). Likewise, biofilm formation by *P. aeruginosa* and *Staphylococcus*
epidermidis are significantly lowered when treated with silver nanoparticles of size 100 nm.2 Dressings containing silver inhibit microbial adhesion and eliminate microbial infection.2 Chitosan-silver nanoparticles also eliminate foot infection caused due to multi-drug-resistant P. aeruginosa and S. aureus.8

Conclusion

These results suggest that silver nanoparticles may serve as effective anti-microbial and anti-biofilm agents against infections caused by multidrug-resistant isolates, which pose a serious threat to public health worldwide.

Recommendations

An important aspect of future investigation is potentially expanding the application of the prepared nanoparticles by attaching powerful antibiotics to their surfaces. These antibiotics-loaded particles could actively and precisely destroy multi-resistant bacteria.

Ethical approval

All samples were processed according to the Code of Ethics outlined in the World Medical Association Declaration of Helsinki involving the use and handling of human subjects.

Authors’ contributions

MAS and MIS conceived and designed the study. Both MAS and MIS conducted the experiments, provided research materials, and collected, organized, analysed and interpreted data. MIS wrote initial and final drafts of the article, and both authors have reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Conflicts of interest

The authors have no conflict of interest to declare.

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References

1. Rai MK, Deshmukh SD, Ingle AP, Gade AK. Silver nanoparticles: the powerful nanoweapon against multidrug-resistant bacteria. J Appl Microbiol 2012; 112: 841–852.
2. Percival SL, Bowler PG, Dolman J. Antimicrobial activity of silver-containing dressings on wound microorganisms using an in vitro biofilm model. Int Wound J 2007; 4: 186–191.
3. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002; 15: 167–193.
4. Huh AJ, Kwon YJ. “Nanoantibiotics”: a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. J Control Release 2011; 156: 128–145.
5. Gajbhiye M, Kesharwani J, Ingle A, Gade A, Rai M. Fungus-mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole. Nanomedicine 2009; 5: 382–386.
6. Sun RW, Chen R, Chung NP, Ho CM, Lin CL, Che CM. Silver nanoparticles fabricated in Hepes buffer exhibit cytoprotective activities towards HIV-1 infected cells. Chem Commun (Camb) 2005; 40: 5059–5061.
7. Tian J, Wong KK, Ho CM, Lok CN, Wu YW, Che CM, et al. Topical delivery of silver nanoparticles promotes wound healing. Chem Med Chem 2007; 2: 129–136.
8. El-Naggar MY, Gohar YM, Sorour MA, Waheed MG. Hydrogel dressing with a nanoformula against methicillin-resistant S. aureus and P. aeruginosa diabetic foot bacteria. J Microbiol Biotechnol 2015; 25(8).
9. Gurunathan S, Han J, Kwon D, Kim J. Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. Nanoscale Res Lett 2014; 9: 373.
10. Siegel J, Polivková M, Kasáškova NS, Kolská Z, Švorčík V. Properties of silver nanostructure-coated PTFE and its biocompatibility. Nanoscale Res Lett 2013; 8: 388.
11. Benyettou F, Rezgui R, Ravaux F, Jaber T, Blumer K, Jouidi M, et al. Synthesis of silver nanoparticles for the dual delivery of doxorubicin and alendronate to cancer cells. J Mater Chem B 2015; 3: 7237–7245.
12. Lee PC, Meisel D. Adsorption and surface-enhanced Raman of dyes on silver and gold sols. J Phys Chem 1982; 86: 3391–3395.
13. Tanoue Y, Sugawa K, Yamamuro T, Akiyama T. Densely arranged two-dimensional silver nanoparticle assemblies with optical uniformity over vast areas as excellent surface-enhanced Raman scattering substrates. Phys Chem Chem Phys 2013; 15: 15802–15805.
14. Iravani S, Korbekandi H, Mirmohammadi SV, Zolfaghari B. Synthesis of silver nanoparticles: chemical, physical and biological methods. Res Pharm Sci 2014; 9: 385–406.
15. CLSI Clinical Laboratory Standards Institute. Performance standards of antimicrobial susceptibility testing; twenty second informational supplement. CLSI document M100-S22. Wayne, PA 2012.
16. Saeb A, Alshammari A, Al-Brahim H, Al-Rubeaan K. Production of silver nanoparticles with strong and stable antimicrobial activity against highly pathogenic and multidrug resistant bacteria. Sci World J 2014; 2014704708.
17. El-Mowafy SA, Shaaban MI, Abd El-Galil KH. Sodium ascorbate as a quorum sensing inhibitor of Pseudomonas aeruginosa. J Appl Microbiol 2014; 117: 1388–1399.
18. Mohanty S, Mishra S, Jena P, Jacob B, Sarkar B, Sonawane A. An investigation on the antibacterial, cytotoxic, and antibiofilm efficacy of starch-stabilized silver nanoparticles. Nanomedicine 2012; 8: 916–924.
19. Whitesides GM. Nanoscience, nanotechnology and chemistry. Small 2005; 1: 172–179.
20. Wang C, Kim YJ, Singh P, Mathiyalagan R, Jin Y, Yang DC. Green synthesis of silver nanoparticles by Bacillus methylotrophicus, and their antimicrobial activity. Artif Cells Nanomed Biotechnol 2015; 6: 1–6.
21. Alani F, Moo-Young M, Anderson W. Biosynthesis of silver nanoparticles by a new strain of Streptomyces sp. compared with Aspergillus fumigatus. World J Microbiol Biotechnol 2012; 28: 1081–1086.
nanoparticles using *Bacillus subtilis* and *Catharanthus roseus* (L.) G. Don. *Colloids Surf B Biointerfaces* 2011; 1; 86: 378–383.

23. Chan YS, Don MM. Biosynthesis and structural characterization of Ag nanoparticles from white rot fungi. *Mater Sci Eng C Mater Biol Appl* 2013; 33: 282–288.

24. Kumar CG, Mamidyala SK. Extracellular synthesis of silver nanoparticles using culture supernatant of *Pseudomonas aeruginosa*. *Colloids Surf B Biointerfaces* 2011; 84: 462–466.

25. Chandrakanth KR, Ashajyothi C, Oli AK, Prabhorajeshwar C. Potential bactericidal effect of silver nanoparticles synthesized from *Enterococcus* species. *Orient J Chem* 2014; 30: 1253–1262.

26. Kalishwaralal K, BarathManiKanth S, Pandian SR, Deepak V, Gurunathan S. Silver nanoparticles impede the biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. *Colloids Surf B Biointerfaces* 2010; 79: 340–344.

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