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Biologically synthesized silver nanoparticles enhances antibiotic activity against Gram-negative bacteria

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ABSTRACT

Here we report a simple, fast, cost-effective, and nonpolluting approach for synthesis of silver nanoparticles (AgNPs) using leaf extract of *Typha angustifolia*. We demonstrate the dose-dependent antibacterial activity of AgNPs and different antibiotics against *Escherichia coli* and *Klebsiella pneumoniae*. Furthermore, we demonstrate the efficacy of AgNPs in combination with various broad-spectrum antibiotics against *E. coli* and *K. pneumoniae*. The results show that combinations of antibiotics and AgNPs show significant antimicrobial effects at sub-lethal concentrations of the antibiotics. These data suggest that combinations of antibiotics and AgNPs can be used therapeutically for the treatment of infectious diseases.

Introduction

Recently, nanotechnology has emerged as one of the fastest growing areas of science and technology. Nanoparticles have generated much interest in academia as well as industry because they bridge the gap between bulk materials and atomic or molecular structures [1]. Owing to their unique properties, nanomaterials are increasingly being used in commercial applications in a variety of fields, including optics, electronics, magnetics, mechanics, catalysis, energy science, nanobiotechnology, and nanomedicine, particularly as antimicrobial agents for diagnostic purposes [1]. In addition, silver nanoparticles (AgNPs) are extensively used for the production of clothing, catheters, electric home appliances, and biomedical implants [2]. Because of their well-known antiseptic activities, silver compounds are used in clinical settings to prevent skin infections, such as in the treatment of burns (*e.g.*, silver sulfadiazine) and as coatings on various surfaces such as catheters [2,3]. Furthermore, nanoparticles possess dimensions below the critical wavelength of light. This renders them transparent, a property that makes them very useful for applications in cosmetics, coatings, packaging, and diagnostics [4]. Because of the high demand, one trillion dollars' worth of nanotechnology-based products is expected on the market by the year 2015 [5].

The most widely used methods for synthesis of metallic nanoparticles are traditional physical and chemical methods. Conventional physical methods tend to yield low amounts of nanoparticles, while chemical methods are often toxic, consume a lot of energy, and require the use of stabilizing agents such as sodium dodecyl benzyl sulfate or polyvinyl pyrrolidone (PVP) to prevent agglomeration of the nanoparticles [4,6]. Therefore, a cost-effective, simple, rapid, high-yield, and environmentally friendly approach for the synthesis of metallic nanoparticles is needed. Among several possible approaches, biological synthesis of nanoparticles is particularly promising owing to the ready availability of resources including viruses, bacteria, fungi, algae, plants, and plant products [4].

Recently, several microorganisms have been exploited for synthesis of silver and gold nanoparticles. For example, a silver-resistant bacterial strain isolated from silver mines, *Pseudomonas stutzeri* AG259, accumulates AgNPs within the periplasmic space [7,8]. Parikh et al. [9] found that *Morganella* sp. RP-42 produced extracellular crystalline AgNPs of 20 ± 5 nm when exposed to silver nitrate. *Lactobacillus* spp. produced microscopic gold, silver, and gold–silver alloy crystals of well-defined morphology when exposed to high concentrations of metal ions [10]. *Bacillus licheniformis* produces

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AgNPs with an average size of 50 nm both intracellularly [11] and extracellularly [12]. Sweeney et al. [13] demonstrated that *Escherichia coli* spontaneously formed cadmium sulfide semiconductor nanocrystals when incubated with cadmium chloride and sodium sulfide. *E. coli* also produces AgNPs with an average size of 50 nm [14]. Kowshik et al. [15] demonstrated that MKY3, a silver-tolerant yeast species, produced AgNPs ranging in size from 2 to 5 nm, and Mukherjee et al. [16] described the synthesis of intracellular AgNPs using the fungus *Verticillium* sp. In addition, plants have been used in the synthesis of nanoparticles. Shankar et al. [17] reported the extracellular synthesis of AgNPs by reduction of aqueous Ag^+ ions using extract of geranium leaves, and extract from lemongrass (*Cymbopogon flexuosus*) was used to synthesize triangular gold nanoprisms [18]. Interestingly, the synthesis of AgNPs using plant extracts is fairly rapid compared with synthesis using bacteria or fungi [17]. However, few plants have been exploited for the synthesis of silver or gold nanoparticles. Therefore, we attempted to use a previously unexplored species, *Typha angustifolia*, for AgNP synthesis. *T. angustifolia* is a monocot found in tropical and temperate regions of the world in marshes and wetlands of various depths. It is a common plant of wetlands and is an unexploited taxon that can be used as a source of food, medicines, and fibers as well as for the synthesis of nanomaterials. Londonkar et al. [19] reported that crude aqueous extracts of the aerial part of *T. angustifolia* plants contained alkaloids, tannins, steroids, phenols, saponins, and flavonoids. Based on the presence of these compounds, we expected that the proteins, polysaccharides, or secondary metabolites of *T. angustifolia* leaf extracts would reduce Ag^+ ions to the Ag^0 state, resulting in the formation of silver nanoparticles. Recently, Singhal et al. [20] synthesized AgNPs using *Ocimum sanctum* leaf extract and found that these nanoparticles showed significant antibacterial activity against *E. coli* and *Staphylococcus aureus*. Although several studies have demonstrated the antibacterial activity of AgNPs, studies of the combination of AgNPs and antibiotics are warranted.

Several studies have shown not only an increasing number of infections caused by gram-negative bacteria worldwide but also mounting rates of resistance. In a study of 1265 intensive care units in 75 countries, Vincent et al. [21] found that gram-negative bacteria were present in 62% of patients with an infection, while gram-positive bacteria were present in 47% of these patients. Gram-negative bacteria are highly adaptive pathogens that can develop resistance to antibiotics through several mechanisms; this resistance is a serious concern in terms of public health and health care costs [22–26]. Gram-negative bacteria are common causes of intra-abdominal infections, urinary tract infections, nosocomial pneumonia, and bacteremia [27]. Tamma et al. [28] reported that combination antibiotic therapy may have benefits other than the prevention of resistance during definitive treatment. We selected *E. coli* and *Klebsiella pneumoniae* as model gram-negative bacteria because both bacteria cause infections in the abdominal and urinary tracts. In light of the observations described above, we first investigated the extracellular synthesis of AgNPs using leaf extract of *T. angustifolia*. Second, we investigated the antibacterial effect of the prepared AgNPs against *E. coli* and *K. pneumoniae*. Finally, we investigated the effect of combinations of selected antibiotics with AgNPs against *E. coli* and *K. pneumoniae*.

Materials and methods

Reagents, bacterial strains, and culture conditions

Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), silver nitrate, gentamicin, cefotaxime, and meropenem were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich unless otherwise stated. The *E. coli* and *K. pneumoniae* strains used in the present study were from our culture collections.

Bacterial culture and media preparation were carried out according to previously described methods [14]. Briefly, *E. coli* and *K. pneumoniae* were grown aerobically at 37 °C in MHB. The cultures were maintained by streaking the organisms on LB agar plates and subculturing every fortnight. Pure colonies were isolated and stored at −80 °C. Cells were harvested by centrifugation at 6000 rpm for 10 min and resuspended in sterile LB medium to obtain an optical density at 600 nm of 1.0.

Synthesis of AgNPs

T. angustifolia leaves were collected from a marshy area around Coimbatore, Tamilnadu, India, and stored at 4 °C until needed. Twenty grams of leaves were washed thoroughly with double-distilled water and then sliced into fine pieces, approximately 1–5 cm^2, using a sharp stainless steel knife. The finely cut leaves were suspended in 100 mL of sterile distilled water and boiled for 5 min. The resulting mixture was filtered through Whatman filter paper (grade No. 1). The filtered extract was used for the synthesis of AgNPs by adding 10 mL of extract to 100 mL of 1 mM aqueous $AgNO_3$ solution and stirring the mixture at 37 °C for 15 min. The bioreduction of $AgNO_3$ was monitored spectrophotometrically at 420 nm.

Characterization of AgNPs

The synthesized nanoparticles were characterized according to previously described methods [14]. Briefly, the prepared AgNPs were characterized primarily by UV–vis spectroscopy, which has proved to be a very useful technique for the analysis of AgNPs. UV–vis spectra were obtained using a Biochrom (Cambridge, UK) WPA Biowave II UV–vis spectrophotometer. The synthesized AgNPs were freeze-dried, powdered, and analyzed by X-ray diffraction (XRD) spectroscopy. The spectra were produced using an X'Pert-MPD X-ray diffractometer (Philips, the Netherlands) and Cu $K\alpha$ radiation (λ = 1.5405 Å) over an angular range of 10 to 80° at 40 kV and 30 mA. The dried powder was diluted with KBr at a ratio of 1:100 and analyzed by Fourier transform infrared spectroscopy (FTIR) using a Spectrum GX spectrometer (Perkin Elmer Inc., USA) within the range of 500 to 4000 cm^{-1}. The size distribution of the dispersed particles was measured using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK). Transmission electron microscopy (TEM) was used to determine the size and morphology of the AgNPs. A small amount of aqueous dispersion was dropped on copper grids, which were dried and examined in the transmission electron microscope (JEM-1200EX).

Determination of minimum inhibitory concentrations of AgNPs and antibiotics

To determine the minimum inhibitory concentrations (MICs) of AgNPs and antibiotics, bacterial strains were cultured in MHB. Cell suspensions were adjusted to obtain standardized populations by measuring the turbidity with a spectrophotometer (DU530, Beckman, Fullerton, CA, USA). Susceptibility tests were performed by twofold microdilution of the antibiotics and AgNPs in standard broth according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2003). The bacterial strains were grown in MHB to mid-log phase (1×10^6 cells/mL) and diluted in fresh MHB, and 0.1 mL of the diluted cell suspension was dispensed into each well of a 96-well microtiter plate. *E. coli* and *K. pneumoniae* were then exposed to different concentrations of AgNPs or antibiotics. Growth was assayed by monitoring absorbance at 600 nm using a microplate reader (EMax, Molecular Devices, Sunnyvale, CA, USA).