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Biodirected synthesis of Miconazole-conjugated bacterial silver nanoparticles and their application as antifungal agents and drug delivery vehicles

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ABSTRACT

The recent strategy to improve the efficacy of drugs is to combine them with metal nanoparticles for the control of microbial infections. Considering this fact, we developed a low cost and eco-friendly method for silver nanoparticles synthesis using the cell free supernatant of *Delftia* sp. strain KCM-006 and their application as antifungal agents and as a drug carrier. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) analysis revealed the formation of spherical and monodispersed silver nanoparticles with an average size of 9.8 nm. The synthesized nanoparticles were found to be photoluminescent, highly stable and crystalline in nature having a zeta potential of −31 mV. The silver nanoparticles exhibited very good antifungal activity against various pathogenic *Candida* strains. Furthermore, the efficacy of nanoparticles was increased by conjugating the antifungal drug Miconazole to silver nanoparticles which exhibited significant fungicidal activity, inhibition of ergosterol biosynthesis and biofilm inhibition by increasing ROS levels. In addition, the cell viability and immunocytochemistry analysis against different normal cell lines including Chinese hamster ovary cells (CHO), human lung cell line (MRC5) and human vascular endothelial cells (HUVEC) demonstrated that these nanoparticles were non-toxic up to a concentration of 20 μM. In conclusion, these results suggest that the synthesized nanoparticles find application as both antifungal agents and drug delivery vehicles. This is a first report on the preparation of silver nanoparticles using culture supernatant from *Delftia* sp. and also on the conjugation of Miconazole, an antifungal drug, to the bacterial silver nanoparticles.

1. Introduction

Metal nanoparticles have attracted much attention in various fields of science and technology including biomedical sciences due to their unique physical and chemical properties dependent on size and shape [1]. In general, their synthesis involves external reducing agents and toxic chemicals, which limits their application in therapeutics. A renewed interest has been therefore, generated on the adoption of safe, cost-effective, non-toxic and greener approaches for nanoparticle synthesis using biomaterials from microbes, fungi, and plants either to eliminate or minimize the use and generation of hazardous substances. Extracellular synthesis of nanoparticles is considered advantageous due to the ease in preparation, recovery and cost-effectiveness. Among the several metal nanoparticles, silver nanoparticles are known to exhibit a broad spectrum of biocidal activity towards many bacteria, fungi and viruses [2,3]. From a therapeutic application perspective, these biosynthesized silver nanoparticles find use as antimicrobial [4], anti-inflammatory [5], anti-angiogenic [6] and antiviral [7] agents.

Candida is one of the main opportunistic fungal pathogens in humans which cause frequent mucosal infections and life-threatening systemic disorders [8]. *Candida* causes significant morbidity and mortality in immunocompromised patients and organ transplant recipients [9]. In general, *Candida* infections begin with the adherence and colonization of yeast cells on a biotic host surface to form microbial communities, the so-called "biofilms" [10]. These biofilms are enclosed in self produced extracellular polymeric substances (EPS) that support survival of the organism

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under unfavorable conditions [11]. *Candida* forms biofilms on almost all medical devices and implants such as stents, shunts, lens and various types of catheters [12].

Miconazole is an imidazole group antifungal drug which inhibits 14-α-demethylase, a key cytochrome P-450 enzyme necessary to convert lanosterol to ergosterol. The reduced levels of ergosterol and the accumulated toxic sterol intermediates cause the leakage of intracellular contents resulting in growth inhibition [13]. It exhibited very good antifungal activity as compared to fluconazole against different *Candida* cells and also inhibited the biofilm formation [14]. It was earlier observed that AgNPs causes the biofilm formation and accumulation of the intracellular ROS resulting in the mitochondrial fragmentation and cell death [15]. We hypothesized that the use of biocompatible b-AgNP in combination with Miconazole at low concentrations could successfully inhibit the *Candida* growth.

In the present study, we developed a biodirected synthetic approach using culture supernatant derived from *Delftia* sp. strain KCM-006 for the preparation of silver nanoparticles (b-AgNPs) without the addition of any reducing agent. The synthesized nanoparticles showed excellent stability at room temperature and also in different biological fluids, and also acted as antifungal agents and as a drug carrier. The biocompatibility and/or toxicological studies of these b-AgNPs were evaluated in different normal cell lines. The antifungal drug, Miconazole, was conjugated to the b-AgNPs and the resulting drug delivery system exhibited a significant antifungal activity. Further, the effect of drug conjugates was evaluated on biofilm formation, ROS accumulation and the total cellular sterol levels was measured against four different *Candida* strains.

2. Experimental details

2.1. Medium and fermentation conditions

Delftia sp. strain KCM-006, previously isolated in our laboratory from oil contaminated soil sample collected at a depth of 10 m from the Manuguru Coal Mines, Khammam, Andhra Pradesh, India, was cultured aerobically in modified glycerol peptone broth containing (in g/L): glycerol, 30; peptone, 5 and meat extract, 5. Fermentation studies were carried out at 35 °C in 1000 mL Erlenmeyer flasks containing 250 mL of modified glycerol peptone broth (adjusted to different pH values ranging from 6 to 9) with agitation at 150 rpm for 72 h in Innova 43R shaker (Eppendorf, Hauppauge, NY, USA). The fermented medium was later subjected to centrifugation (Sorvall RC5C Plus, Kendro Lab Products, Ashville, NC, USA) at 10,400 × *g* for 20 min to obtain a cell-free supernatant.

2.2. Silver nanoparticle synthesis and characterization studies

The silver nanoparticles were synthesized using aqueous silver nitrate at different pH values. The independent reactions were carried out separately with aqueous silver nitrate (10 mL of 10^{-2} M) added to 90 mL of cell-free supernatant cultured at different pH values, i.e. 6.0, 7.0, 8.0 and 9.0, and kept for continuous stirring on a magnetic stirrer at 200 rpm for 14 h at ambient temperature (28 °C). The effect of temperature on the nanoparticle synthesis was studied by performing the reactions at different temperatures, i.e. 35, 45, 55 and 100 °C individually under earlier mentioned conditions using the cell-free supernatant (optimum pH 8.0). Sample aliquots (2 mL) of the reaction mixture withdrawn at periodic intervals were also monitored for the kinetics of bioreduction of silver ions as a function of time by UV–vis spectroscopy using a UV–vis double beam spectrophotometer (Lambda 25, Perkin-Elmer, Shelton, CT, USA). The effect of pH and temperature on nanoparticle formation was examined by recording the UV-vis scan spectra of these sample aliquots from 300 to 800 nm. The synthesized bacterial silver nanoparticles (b-AgNP) were washed with distilled water, centrifuged at 10,000 × *g* for 30 min, concentrated and later stored at 4 °C for further use.

Morphological analysis of b-AgNP and drug-conjugated b-AgNP was carried out using transmission electron microscope (TEM). Reaction mixtures were subjected to ultrasonication and a drop of each reaction mixture was placed over carbon-coated copper grids and the samples were dried prior to the measurements using a 100 W infrared lamp (Philips, Holland). TEM images were acquired on Technai-FE 12 (Philips, Holland) TEM instrument which was operated at an accelerating voltage of 120 kV. The histograms were constructed based on size distribution to determine the average size of nanoparticles. The crystalline structure of nanoparticles was determined by X-ray diffraction analysis. X-ray diffraction (XRD) pattern of nanoparticles was recorded on Bruker AXS D-8 Advance powder X-ray diffractometer (Shimadzu, Japan) operated at a voltage of 40 kV and current of 30 mA using CuKα radiation (λ = 1.5406 Å). The diffractometer was controlled with Data scan software and the scan parameters set were scan rate 1.2° per minute and scan range 2θ = 0–80°. The dried powder of the synthesized nanoparticles was re-dispersed in deionized water by ultrasonication and were subjected to dynamic light scattering (DLS) measurements at 25 °C to determine the hydrodynamic particle size and the nanoparticle charge quantified as zeta potential was determined on a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) instrument equipped with a He–Ne laser operating at 632.8 nm and a scattering detector at 173°. The DLS studies and nanoparticle charge were determined using the same instrument at 25 °C. Polydispersity index (PDI) was also measured to determine the particle size distribution range.

The concentration of silver ions in the aqueous silver nanoparticle solutions was determined by inductively coupled plasma optical emission spectrophotometer (ICP-OES, IRIS Intrepid II XDL, Thermo Jarrel Ash, USA). The concentration of silver ions in the respective nanoparticle sample solutions was calculated from $AgNO_3$ standard graphs (1–50 ppm) plotted based on ICP-OES analysis. Chemically synthesized silver nanoparticles (c-AgNP) prepared by citrate reduction [16] were used as positive reference controls. The photoluminescence spectrum of silver nanoparticles dispersed in water and their appropriate controls was recorded on Infinite M200 (Tecan Trading AG, Switzerland) microtitre plate reader using a non-fluorescent 96-well microtitre plate at an excitation wavelength of 300 nm. The dried silver nanoparticle powder was re-dispersed in water and further the stability of the nanoparticles was monitored by UV–vis spectroscopic analysis at regular intervals over a period of 5 months.

The synthesized b-AgNP and drug-conjugated b-AgNP were centrifuged at 12,000 × *g* for 10 min and the obtained pellet was washed thrice with deionized water to remove proteins and other components present in the solution and the nanoparticles were dried by lyophilization to obtain a powder. The FT-IR spectrum of the dried nanoparticle powder in the form of KBr pellets was recorded on Thermo-Nicolet Nexus 670 spectrometer at a resolution of 4 cm^{-1} in a wavenumber region of 400–4000 cm^{-1}.

2.3. In vitro stability studies of silver nanoparticles

The stability of b-AgNP was studied in different solutions including fetal bovine serum, phosphate buffer, Dulbecco phosphate buffered saline (DPBS), 5% NaCl, 10% NaCl and buffered saline adjusted to pH 6.0, 7.0 and 8.0. Each solution (200 μL) was added to 800 μL of silver nanoparticle solution and incubated at room temperature for 24 h to 10 days. The stability