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Chemical and biological metal nanoparticles as antimycobacterial agents: A comparative study

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ABSTRACT

Resistance among mycobacteria leading to multidrug-resistant and extensively drug-resistant tuberculosis is a major threat. However, nanotechnology has provided new insights in drug delivery and medicine development. This is the first comparative report to determine the activity of chemically and biologically synthesised silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) against mycobacteria. Screening data revealed the high mycobactericidal efficiency of AgNPs, with minimum inhibitory concentrations (MICs) of <3 μg/mL, whereas no such activity was exhibited by AuNPs at concentrations up to 100 μg/mL. Moreover, in vitro and ex vivo THP-1 infection model assays showed greater efficacy of chemical AgNPs compared with biogenic AgNPs to inhibit active and dormant stage mycobacterial growth. Up to 40% cytotoxicity against human cell lines was observed at a AgNP concentration of 10× MIC (30 μg/mL) after 48 h. AgNPs were shown to have more specificity towards mycobacteria than towards other Gram-negative and Gram-positive pathogenic bacteria. The selectivity index was found to be in the range of 11–23, indicating the potential of these nanoparticles for use in developing new therapeutics for tuberculosis.

1. Introduction

Tuberculosis (TB), caused by the bacillus *Mycobacterium tuberculosis*, is a major global health issue ranked as the second leading cause of death from an infectious disease worldwide. Although most people recover from primary TB infection without further evidence of the disease, the infection may stay dormant for years and in some cases it can reactivate [1]. A combination of four first-line drugs (isoniazid, rifampicin, ethambutol and pyrazinamide) is included in the treatment regimen [2]. However, multidrug-resistant (MDR)-TB and extensively drug-resistant (XDR)-TB require second-line anti-TB drugs, which are more expensive and have greater toxicity and side effects. Indiscriminate use of antibiotics, improper treatment regimens and failure to ensure completeness of the treatment course in TB patients causes the development of antibiotic-resistant TB strains [3]. Resistance to conventional antibiotics threatens the progress made in TB control worldwide, prompting the need to seek alternative strategies, one of which may be nanomaterials.

Recently, metal nanoparticles have introduced new paradigms in medicine, where easy synthesis, controlled morphology, upscale production and reduced cost with increased sensitivity and specificity constitute an interesting alternative. These nanoparticles exhibit unique physical, chemical, electrical, optical, mechanical, magnetic, thermal and dielectric properties compared with the bulk counterparts, depending on their composition and morphology [4]. Metal nanoparticles are also shown to possess catalytic, free radical scavenging and tumour suppression activities [5,6]. Silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) have been reported for temperature- and morphology-dependent antimicrobial activities against bacterial and fungal pathogens [7,8]. These nanoparticles can be synthesised using either chemical reducing agents such as glycerol, sodium borohydride etc., or through biological systems involving micro-organisms and plants [9–11]. It is often emphasised that bio-nanoparticles are safe, less toxic, biocompatible and eco-friendly compared with chemically synthesised nanoparticles [12]. However, green nanoparticle preparation employing non-toxic biocompatible chemical reducing and stabilising agents, such as trisodium citrate, starch, β-D-glucose, aminocellulose etc., may eliminate the toxicity risks. Therefore, in

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pursuit of developing alternate and novel antitubercular agents, we have directed our efforts to screen these nanoparticles, synthesised through biological and chemical approaches, for their feasibility as antimycobacterial agents.

2. Materials and methods

2.1. Nanoparticles

Two AgNPs (S1 and S11) and two AuNPs (G1 and G11) were screened for antimycobacterial activity (Table 1). S1 and G1 were bacteriogenic metal nanoparticles synthesised using the environmental bacterium *Acinetobacter* sp. [4,13]. S11 and G11 samples, chemically synthesised through reduction using 1% trisodium citrate, were provided by Gayatri Salunkhe (Institute of Bioinformatics and Biotechnology, Savitribai Phule Pune University, Pune, India) [11].

2.2. Mycobacterial cultures and growth conditions

Standard cultures of *M. tuberculosis* H37Ra (ATCC 25177), denoted further as MTB, and *Mycobacterium bovis* BCG (ATCC 35743) were procured from the American Type Culture Collection (Manassas, VA). MTB and *M. bovis* BCG were grown in a defined *Mycobacterium phlei* medium [14] and in 50 mM sodium nitrate-containing Dubos medium (Difco, Detroit, MI), respectively. These were maintained as glycerol stocks at −70 °C. Prior to inoculation for experiments, 50 μL of glycerol stock was pre-inoculated in the corresponding medium to obtain metabolically active mycobacteria. For each experiment, cultures were grown to log phase [optical density at 595 nm (OD_{595}) = 1] under aerobic conditions at 37 °C and 150 rpm. As mycobacteria grow in visibly aggregated clumps in the culture medium, cultures were sonicated for 2 min using a water-bath sonicator (Ultrasonic, Freeport, IL) to obtain viable dispersed cells. This step was introduced to reproducibly inoculate mycobacterial bacilli in fresh medium for carrying out the experiments.

2.3. Primary screening

Nanoparticles were screened for their inhibitory activity against active (8 days incubation) and dormant (12 days incubation) mycobacteria at concentrations of 0.1, 0.3, 1, 3, 10, 30 and 100 μg/mL. Activity against MTB was determined through the XTT reduction menadione assay (XRMA) reading absorbance at 470 nm as per the protocol described by Singh et al. [15]. The nitrate reductase (NR) assay was performed to estimate inhibition of *M. bovis* BCG by nanoparticles [14]. Absorbance for the NR assay was measured at 540 nm. Percentage inhibition was calculated using the following formula:

$$\%\,\text{inhibition} = \left[\frac{(\text{control} - \text{NP})}{(\text{control} - \text{blank})}\right] \times 100$$

where 'control' is the activity of mycobacteria without nanoparticles, 'NP' is the activity of mycobacteria in the presence of nanoparticles and 'blank' is the activity of the culture medium without mycobacteria.

The experiment was performed in triplicate and the quantitative value was expressed as the mean ± standard deviation (S.D.).

Table 1
Morphology of metal nanoparticles used for primary screening.

Sample	Metal nanoparticle	Synthesis[a]	Shape	Size (nm)
S1	AgNP	Biological	Spherical	8–12
S11	AgNP	Chemical	Spherical-oval	1–5
G1	AuNP	Biological	Polyhedral	10–30
G11	AuNP	Chemical	Aggregated	5–15

AgNP, silver nanoparticle; AuNP, gold nanoparticle.
[a] Biological synthesis of nanoparticles was mediated through *Acinetobacter* sp., and chemical synthesis was achieved through reduction using 1% trisodium citrate.

2.4. Determination of the minimum inhibitory concentration (MIC) and 50% inhibitory concentration (IC_{50})

Depending upon primary screening, active nanoparticles were further evaluated to estimate their MIC and IC_{50} against mycobacteria through a dose–response assay over the concentration range of 0.02–2.56 μg/mL. The dose–response curve was plotted using OriginPro software (OriginLab Corp., Northampton, MA). The MIC and IC_{50} were taken as the lowest concentration of nanoparticle exhibiting growth inhibition of ≥90% and 50%, respectively, relative to the growth control without nanoparticles. The standard antitubercular drug rifampicin was used as a positive control. All experiments were performed in triplicate.

2.4.1. In vitro assay

In vitro activity against MTB and *M. bovis* BCG at active (8 days) and dormant (12 days) stages was performed using the XRMA and NR assay, respectively, as described in Section 2.3.

2.4.2. Ex vivo infection model assay

The human acute monocytic cell line THP-1 was purchased from the National Centre for Cell Science (NCCS) (Pune, India). THP-1 cells were cultured in RPMI 1640 medium (HiMedia, Mumbai, India) supplemented with 10% fetal bovine serum (FBS) (Gibco, Bangalore, India), 1 mM sodium pyruvate (HiMedia), 1% non-essential amino acids (HiMedia), 1% glutamine (HiMedia), 50 mg/mL ampicillin (Sigma Chemical Co., St Louis, MO) and 50 mg/mL gentamicin (Sigma Chemical Co.), and were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity in a CO_2 incubator (Thermo Fisher Scientific, Waltham, MA). For the infection model study, 3×10^5 THP-1 cells/mL were passaged in complete RPMI medium containing 100 nM/mL phorbol myristate acetate (Sigma Chemical Co.) in 96-well microtitre plates (Tarsons, Kolkata, India) and were plated for differentiation to macrophages. After 24 h, these were infected with log-phase MTB (OD_{595} = 1) at a multiplicity of infection (MOI) of 100 for 12 h. Fresh Minimum Essential Medium (MEM) (HiMedia) containing 50 mM sodium nitrate (HiMedia) was added to the plate after thorough washing with phosphate-buffered saline (PBS) (pH 7.2). Infected cells were then treated with nanoparticles at different doses. Plates were further incubated for 8 days (active) and 12 days (dormant) followed by estimation of nanoparticle activity through NR assay as described in Section 2.3. The experiment was performed in triplicate. IC_{50} and MIC values were calculated from their dose–response curves plotted using Origin8 software (OriginLab Corp.).

2.5. Cytotoxicity assay

2.5.1. Cell lines (THP-1, A549, PANC-1 and HeLa)

The effect of nanoparticles on cell growth was determined in a panel of human tumour cells including the acute monocytic leukaemia cell line THP-1 (NCCS, Pune, India) as well as lung adenocarcinoma A549, cervix adenocarcinoma HeLa and pancreas adenocarcinoma PANC-1 obtained from The European Collection of Cell Cultures (ECACC) (Salisbury, UK). THP-1 cells were maintained in RPMI 1640 without phenol red; A549 and PANC-1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (HiMedia); and HeLa cells were cultured in Eagle's Minimum Essential Medium (EMEM) (HiMedia). All media used were supplemented with 10% FBS and 0.68 mg/mL gentamicin. Cell lines were

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