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Preparation of gold nanoparticles by novel bacterial exopolysaccharide for antibiotic delivery

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ABSTRACT

Aim: To develop potent drug delivery agents to treat multidrug resistant (MDR) bacterial infections using gold nanoparticles (AuNPs) prepared by bacterial exopolysaccharide (EPS) being a reducing and stabilizing agent.
Methods: Gold nanoparticles were prepared by EPS, extracted from *Lactobacillus plantarum*. AuNPs were characterized by UV–visible spectrophotometer, transmission electron microscope (TEM), zeta potential and Fourier transform infrared spectroscopy. Antibiotics used for functionalization of AuNPs were levofloxacin, cefotaxime, ceftriaxone and ciprofloxacin. The resulted functionalized AuNPs were tested against various MDR bacteria by employing different assays such as well diffusion assay, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time killing assay.
Key findings: The nanostructures exhibited excellent bactericidal activity and reduced MIC and MBC against MDR Gram positive and Gram negative bacteria compared to free drugs. *Escherichia coli* was the most susceptible MDR bacteria followed by *Klebsiella pneumoniae* and *Staphylococcus aureus*. TEM results revealed that the bactericidal activity of nanostructures could be mediated by penetration, loss of cytoplasmic contents and cell lysis.
Significance: Antibiotic functionalized nanostructures were more potent than free drugs and could be used as potent drug delivery vehicles.

1. Introduction

In clinical biology, the emergence of multidrug resistant (MDR) microorganisms plays a significant role in the spreading of diseases, poses a severe threat to the public health [1] and eventually leads to loss of economy. According to World Health Organization (WHO), about 480,000 new cases of MDR-tuberculosis have been identified in 100 different countries [2]. The antibiotic resistance proportion is gradually increasing in common bacterial infections such as pneumonia and urinary tract infections. Resistance to antibiotics is frequently found in hospital acquired infections caused by methicillin resistant *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*) species [3,4]. The patients infected from MRD bacteria are generally in a serious condition and death may occur due to failure in disease eradication and impaired immunity [5]. Several new antimicrobials against these bacteria are in development, but unfortunately, the strains are rapidly acquiring resistance to those antimicrobials. Additionally, decreased production and approval of new types of antimicrobials has contributed greatly for this harsh situation [6].

Nanoparticles are an important type of antimicrobial agent tested against various diseases caused by MDR bacteria [7,8]. They have the capacity to inhibit bacterial growth by the accumulation in the cell and damaging the capsule around the cells [9]. The most common examples of antimicrobial agents are silver and chitosan nanoparticles. Silver nanoparticles were used in several applications; however, they have potential cellular toxicity which minimizes in vivo applications in human and other animals. In contrast, gold nanoparticles (AuNPs) are generally weak antimicrobials but they are very good contrasting and less cytotoxic agents, and have been used as target drug delivery system [10], medical imaging [11] and as diagnostic agents for various diseases [12]. The high surface to volume ratio of AuNPs allows incorporation of various antibiotics and other ligands, enabling multivalency on surface of nanoparticles enhancing interaction against target bacteria [13]. The antibiotic molecules can be attached via either non-covalent [14] or covalent interactions [15] and both methods have been reported for enhanced antimicrobial effects comparing free drugs [16].

Although several chemical reducing agents and physical methods (γ radiations, microwave assisted, etc.) have been employed for preparation and stabilization of nanoparticles, they have shown some toxic effects on animals and environment. Hence, these techniques are now being slowly replaced by environment friendly biological approaches such as bacteria and plant extracts which are known to reduce inorganic materials for several scientific applications [17]. In the present work, AuNPs were synthesized by green technique using *Lactobacillus plantarum* (*L. plantarum*) exopolysaccharide (EPS). The beneficial

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property of *Lactobacillus* and its extracellular products such as EPS was utilized in the present research work for AuNPs preparation. A novel EPS was isolated and characterized by Fourier transform infrared (FTIR) spectroscopy and high-performance liquid chromatography (HPLC). Nanoparticles were characterized by transmission electron microscope (TEM), FTIR, zeta potential (ζ) and energy dispersive X-ray spectroscopy (EDX). AuNPs was functionalized with various antibiotics and their antimicrobial activity was tested against MDR bacteria.

2. Materials and methods

Gold (III) chloride trihydrate (PubChem CID: 28133) was purchased from Sigma chemicals, USA. Ciprofloxacin hydrochloride (PubChem CID: 62999), levofloxacin hydrate (PubChem CID: 3033924), cefotaxime sodium (PubChem CID: 10695961) and ceftriaxone sodium (PubChem CID: 2379441) were procured from HiMedia Laboratory, India. Pathogenic bacteria such as *K. pneumoniae*, *S. aureus* and *E. coli* and were procured from K.S. Hegde Medical Academy, Mangalore, India. *E. coli* DH5α was purchased from Genei, India. All the cultures were maintained in Luria broth (LB broth).

2.1. Bacteria isolation and 16S rRNA sequencing

L. plantarum was isolated from marine fish *Sardinella longiceps* obtained from a local fish market, Mangalore, India. Briefly, serially diluted gut homogenate was transferred to deMan, Rogosa and Sharpe (MRS) plate and incubated for 48 h at 37 °C under aerobic condition. The colonies obtained were tested for Gram staining and catalase assay. The strains belonging to Gram positive and catalase negative were selected and used for EPS production test. The bacterial EPS which exhibited good reducing power for AuNPs synthesis was used for the present study. The strain was characterized using 16S rRNA sequencing protocol by employing universal primer of 27F (5′-AGAGTT TGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). Detailed DNA sequencing protocols employed in the present study along with results are given in the supplementary information 1.

2.2. EPS isolation, purification and characterization

L. plantarum was grown in MRS broth with 2% (w/v) sucrose on a rotary shaker at 37 °C for 48 h. The culture was centrifuged at 7500 rpm for 10 min at 4 °C. The EPS was then precipitated from the supernatant by the addition of double volume of chilled ethanol, centrifuged at 10,000 rpm for 10 min and washed twice with absolute ethanol. Purification of EPS was performed by employing the modified method of Bales et al. [18] using phenyl sepharose CL-4B column (1.5 × 15 cm). Presence of carbohydrate in each fraction was determined colorimetrically [19]. The fractions containing carbohydrates were pooled and lyophilized (Labtech® Freeze Dryer, LFD-5508, Daihan Labtech Co. Ltd., Korea) for 48 h and stored at room temperature.

FTIR spectra were recorded using a Shimadzu FTIR-8300 spectrometer in 4000–400 cm^{-1} frequency range using the KBr pellet method. A total of 250 scans were employed to obtain the spectra. Monosaccharide analysis was done by HPLC coupled with RI detector [20].

2.3. Preparation and characterization of AuNPs and functionalized AuNPs (FAuNPs)

Green synthesis of AuNPs using EPS was followed according to the modified method of Kim et al. [21]. EPS (1%, 5 ml) was added slowly to a boiling $HAuCl_4 \cdot XH_2O$ solution (1 mM) and was stirred for 1 h. The nanoparticles were purified from excess EPS by repeated centrifugation at 20,000 rpm for 30 min at 4 °C. Final pellet obtained was resuspended in deionized water and stored at 4 °C in dark bottles.

Functionalization of antibiotics on to the surface of AuNPs was done using third generation antibiotics (levofloxacin, ceftriaxone and cefotaxime) and a second generation antibiotic, ciprofloxacin. The chemical structure of the antibiotics and protocols employed for AuNPs functionalization and characterization is given in the supplementary information 2. The concentration of the drug conjugated over AuNPs was analyzed by HPLC; using C18 reverse phase column and gradient elution. The final drug concentration of 5 μg/ml was adjusted and stored in darkness at 4 °C.

2.4. Antimicrobial assays

2.4.1. Preliminary test using agar well diffusion assay

Inhibitory activity of AuNPs alone and FAuNPs was evaluated against various MDR bacteria by well diffusion method. Approximately 10^5–10^6 colony forming unit (CFU)/ml of the indicator bacteria were inoculated on LB agar plate, and then 9 wells (5 mm) were made with a sterile cork bore. 30 μl free drug and gold conjugated drug at 5 μg/ml was added to the wells and incubated at 37 °C for 24 h. After incubation, the plates were observed for the presence of zone of inhibition and the results were expressed as mean ± SE inhibitor zone in mm.

2.4.2. Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The effect of free antibiotics and FAuNPs on pathogenic bacterial growth was determined by a broth dilution method. The initial concentration of antibiotics used in this study was 10 μg/ml. One hundred microliters of LB broth was distributed to all the wells of microtiter plates and 100 μl of free drug and FAuNPs individually was added to the wells of the first column and mixed. Then 100 μl of mixed sample from the first column was transferred to the wells of the second column and similar transfer was repeated so that each wells of the column received half of the drug concentration of the previous column. Finally, 10 μl of the freshly prepared indicator bacteria were added and incubated for overnight at 37 °C. At the end of the incubation, the results were recorded by observing visual sign of bacterial growth and the lowest concentration of the test samples that inhibited the bacterial growth was considered as MIC.

To determine the MBC, 10 μl of the sample from MIC microtiter plate was directly transferred to LB agar plates and incubated for 24 h at 37 °C. The bacterial colony count was performed and the results were expressed as mean ± SE CFU/ml.

2.4.3. Effect of FAuNPs on bacterial growth kinetics

The rate of bacterial growth inhibition by FAuNPs was tested by the plate count method. Briefly, overnight grown indicator bacterial cultures were centrifuged and the absorption was adjusted to 0.1 at 600 nm. For each bacterium, the final concentration of the FAuNPs was maintained at their respective MBC. Samples were withdrawn at every 1 h time intervals for 24 h and inoculated in LB agar plates. The CFU of each bacterium was determined and the results obtained were plotted as the mean CFU/ml over various time intervals.

2.5. Statistical analysis

For the growth curve analysis, four independent experiments were maintained for each bacterium tested. All the other experiments were conducted in triplicates. Statistical analysis was performed using one sample *t*-test in GraphPad Prism (version 5.01).

3. Results

3.1. Bacteria isolation and characterization

From the MRS plate with different dilution, several colonies were picked up based on distinctive morphological characteristics. Of these, 10 colonies were further selected based on Gram positive and catalase negative tests and presumed to be lactic acid bacteria (LAB). The EPS