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# Synthesis and structural characterization of silver nanoparticles using bacterial exopolysaccharide and its antimicrobial activity against food and multidrug resistant pathogens

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ABSTRACT

A green, simple, and effective approach was performed to synthesize potent silver nanoparticles (SNPs) using bacterial exopolysaccharide as both a reducing and stabilizing agent. The synthesized SNPs were characterized using UV-vis spectroscopy, transmission electron microscopy, energy dispersive X-ray analysis, X-ray diffraction, and Fourier-transform-infrared spectra analyses. The SNPs varied in shape and were multidispersed with a mean diameter of 10 nm ranging from 2 to 15 nm and were stable up to 2 months at room temperature. The antimicrobial activity of the SNPs was analyzed against bacterial and fungal pathogens using the agar well diffusion method. Dose dependent inhibition was observed for all bacterial pathogens. The multidrug resistant pathogens *P. aeruginosa* and *K. pneumonia* were found to be more susceptible to the SNPs than the food borne pathogen *L. monocytogenes*. The fungi *Aspergillus* spp. exhibited a maximum zone of inhibition compared to that of *Penicillum* spp. These results suggest that exopolysaccharide-stabilized SNPs can be used as an antimicrobial agent for various biomedical applications.



## 1. Introduction

Nanotechnology is a promising interdisciplinary field of science that offers novel nanoscale materials with anticipated applications in the medical, electrical, mechanical, catalysis, photonics, molecular computing, and structural material fields [1,2]. In the past two decades, an increasing and widening spectrum of nanoscale material applications have been observed in various areas and considerable attention is being paid to the use of nanoparticles in various real world systems. Due to their small size (1–100 nm), nanoparticles have been shown quite challenging unique physicochemical and biological functional properties when compared to macrosized particles. Among the noble metallic nanoparticles, silver nanoparticles (SNPs) have received the greatest attention due to their wide spectrum of antimicrobial activity towards many Gram positive and Gram negative bacteria, fungi, and viruses [3]. Moreover, SNPs have been used in various applications including dental, medical therapeutics and diagnosis, food packaging, catheters, textiles, and coatings [4,5]). Silver has more advantages compared to other antimicrobial agents because of its broad spectrum of inhibitory activity against diverse bacteria, fungi, and viruses [2]. In addition, SNPs have little chance of drug resistance, which overcomes the multidrug resistance problems [5]. Drug resistance is one of the most serious, threatening, and widespread problems in all developing countries [6]. Therefore, silver has emerged as an effective antiseptic agent to reduce multidrug bacterial resistance. Despite that many antibiotics act against pathogens, silver or silver-based products remain most popular and effective antimicrobial agents to kill the bacterial pathogens.

The mechanistic action of SNPs is not fully understood but they may bind with external proteins to create pores, interfere with DNA replication or form reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anions, and hydroxyl radicals [2,7]. Moreover, SNPs can disturb biofilm formation [8]. Biofilm formation by bacterial pathogens is crucial for chronic and recurrent infections, because of the capacity of biofilms to attach and persist on solid surfaces such as medical and dwelling devices [9]. Thus, many studies have attempted to identify the exact bacterial and fungal killing mechanism of silver or SNPs. Polysaccharides are increasingly being used to synthesize silver nanoparticles because they have numerous advantages compared to synthetic polymers and chemical agents. In particular, macromolecular chain of these biopolymers contains many hydroxyl groups that strongly associate with metal ions leading to greater control of shape, size and particle dispersion. Moreover, polysaccharides have other valuable functions such as mucoadhesion, which provides a greater neutral coating with low surface energy and restricts non-specific protein

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receptor recognition [10]. In addition, polysaccharides are non-toxic, biocompatible, easily biodegradable, and abundantly present in natural sources. Therefore, many researchers have been used various natural biopolymers to reducing and stabilizing the metal nanoparticles. Yoksan and Chirachanchai [4] used soluble starch as a stabilizing agent and chitosan as a reducing agent in an aqueous solution of $AgNO_3$ to produce SNPs. Mohanty et al. [11] used soluble starch as a reducing and stabilizing agent for the production of silver nanoparticles. Chitosan is another important natural polysaccharide that has been used to reduce $AgNO_3$ to silver ions and, subsequently, to stabilize SNPs [12]. Natural polysaccharides such as sago starch, calcium alginate, agar, bacterial cellulose, and dextran have also been used to fabricate SNPs [13,3,14–16].

Exopolysaccharides are long chain polysaccharides containing branched and repeating units of sugar molecules such as glucose, fructose, or rhamnose and *etc*., [17]. Due to their remarkable functional properties, these natural biopolymers have been widely used as viscofying, bioflocculating, stabilizing, gelling, and emulsifying agents in the food industry and do not carry a health risk which might make EPSs a generally recognized as safe (GRAS) substance [18]. Furthermore, EPSs have also been used in the enhancement of rheology, texture, and mouth feel of dairy products [19]. In this study, we demonstrated the bacterial exopolysaccharide mediated synthesis of silver nanoparticles by direct reduction of silver nitrate with EPS. The synthesized SNPs were characterized using UV-vis spectroscopy, transmission electron microscopy (TEM), energy dispersive X-ray (EDX), X-ray diffraction (XRD), and Fourier transform-infrared spectroscopy (FT-IR). Moreover, the antibacterial, antifungal and antibiofilm activities of the SNPs were examined *in vitro*.

## 2. Materials and methods

### 2.1. Materials and microbial strains

Silver nitrate, brain heart infusion broth (BHIB), tryptic soy broth (TSB), potato dextrose agar (PDA), nutrient broth (NB), Muller–Hinton agar, and agar powder were purchased from Duksan Pure Chemicals Co., Ltd (Gyeonggi-do, South Korea). The exopolysaccharide producing lactic acid bacterium *Lactobacillus rhamnosus* GG ATCC 53103, pathogenic strains such as *Escherichia coli* ATCC 35218, *Bacillus cereus* ATCC 10987, the food borne pathogen *Listeria monocytogenes* ATCC 15313, and the multidrug resistant pathogens *Pseudomonas aeruginosa* ATCC 15442 and *Klebsiella pneumonia* ATCC 27736 were obtained from Korean Collection for Type Cultures (KCTC), (Seoul, South Korea). Fungal pathogens such as *Aspergillus* spp. and *Penicillium* spp. were isolated from contaminated foods and characterized in our laboratory. All solutions were made using ultra filtered high purity deionized water.

### 2.2. Extraction of EPS from lactic acid bacteria

EPSs were extracted from lactic acid bacterium according to method of Kanmani et al. [20] with a slight modification. Briefly, EPS producing *L. rhamnosus* GG was cultured in a 2 L conical flask containing 1 L of MRS broth at 37 °C. After 18 h incubation, the cultured broth was heated to 100 °C for 15 min to inactivate the EPS degrading enzymes. Subsequently, the treated culture broth was centrifuged at 6000 rpm for 10 min at 4 °C to separate the probiotic cells and debris. The cell free supernatant (CFS) was collected and mixed with a double volume of ice cold ethanol (95%) and maintained at 4 °C for overnight to complete precipitation of EPS. After centrifugation at 12,000 rpm for 15 min, the precipitated EPS pellet was washed twice with distilled water and recovered by adding 20 ml of water. The concentration of EPS in the solution was quantified by the phenol-sulfuric acid method [21].

### 2.3. Synthesis of SNPs using EPS

The solution of EPS (20 ml) was mixed with a 9 mM aqueous solution of $AgNO_3$ prepared freshly in deionized water under stirring conditions. The mixture was stored at room temperature for 2 months in a dark place. After 10 h incubation, the colorless solution turned yellow which confirmed the formation of SNPs.

### 2.4. Characterization of silver nanoparticles

#### 2.4.1. UV-visible spectroscopy analysis

EPS reduction of $Ag^+$ ions in aqueous solution was monitored for 12 h, 5 days, 10 days and 2 months by measuring the ultraviolet-visible absorbance spectrum of the solution using a UV-vis spectrophotometer (BIOMATE-3S, Thermo Fisher Scientific, Boston, MA, USA) at 300–800 nm.

#### 2.4.2. Transmission electron microscopy and EDX

For the analysis of TEM, a drop of SNP-containing aqueous solution was directly placed onto a carbon-coated copper grid and allowed to air dry completely prior to TEM observations. The TEM images of the SNPs were obtained using a transmission electron microscope (FEI Tecnai G2 F30, Eindhoven, The Netherlands) at an accelerating voltage of 300 kV. The presence of elemental silver in the nanoparticles was analyzed using a TEM equipped with an EDX spectrum.

#### 2.4.3. Analysis of the XRD and FT-IR spectra

The phase composition and crystal structure of the SNPs was determined using XRD (Philips XPERT MPD). For this, the dried sample was prepared by placing on the microscopic glass slide and the diffractogram was recorded using Cu-Kα radiation and a nickel monochromator filtering wave at a voltage and current of 40 kV and 30 mA, respectively. The FT-IR spectrum of the EPS-stabilized SNPs were analyzed using FT-IR spectroscopy (JASCO FT-IR 460, Daejon, South Korea) operated at resolution of 4 $cm^{-1}$. For the measurement of FT-IR spectrum, the dried sample was powdered by grinding with KBr pellets and pressed into a mold. The spectrum was recorded at a wave range of 500–4000 $cm^{-1}$.

### 2.5. Antibacterial activity of the SNPs

Antibacterial activity of the EPS-stabilized SNPs was measured using the agar well diffusion method. Bacterial pathogens such as *E. coli*, *K. pneumonia*, *L. monocytogenes*, and *P. aeruginosa* were used as indicator strains for this analysis. These bacteria were aseptically inoculated into appropriate liquid media and incubated at 37 °C. After 16 h, the cells were centrifuged at 6000 rpm for 10 min and then suspended in sterile water. The different cells (1 ml) were added to appropriate agar media (100 ml) prior to plating, and the wells were made using an agar well borer. To these wells, different concentrations of SNPs (80 μl) were added and subsequently incubated at 37 °C for 24 h. Zone of inhibitions were estimated by measuring the diameter of the bacterial growth inhibition zone. Values were averaged from three independent experiments. Moreover, the antibacterial activity of the SNPs was analyzed in liquid medium. To this experiment, the cells of various bacterial pathogens ($10^6$ CFU/ml) were taken in separate Eppendorf tubes containing various concentrations of SNPs and incubated at 37 °C for 4 h. The morphological changes in the treated cells were examined using field emission scanning electron microscopy (FE-SEM, S-4700, Hitachi, Tokyo, Japan) and TEM respectively.

### 2.6. Antifungal activity of EPS stabilized SNPs

The antifungal activity of the SNPs was measured using the agar well diffusion method. To this experiment, two fungal pathogens were isolated from contaminated foods and identified as *Aspergillus* spp. and *Penicillum* spp. These two fungal strains were inoculated in dextrose agar medium (PDA) and incubated at room temperature (25–27 °C) for 4 days. Four day old fungal spores were collected by washing with water and subsequently plated (0.1 ml) on PDA plate. Wells were made using an agar well borer. Various concentrations of SNPs were added to the wells and incubated at room temperature for 4 days. Zone of inhibitions were estimated by measuring the diameter of the fungal growth inhibition zone. The values were averaged from three independent experiments. In addition, both fungal strains were treated with SNPs (100 μg/ml) in a liquid system and the morphological changes in the treated fungal strain were examined using FE-SEM at an accelerating voltage of 5.0 kV.

### 2.7. Antibiofilm activity of the SNPs

Bacterial pathogens such as *E. coli*, *Bacillus cereus*, *L. monocytogenes* and *P. aeruginosa* were inoculated into appropriate liquid medium and incubated at 37 °C for 16 h. The cultures were serially diluted 1:100 in fresh liquid medium containing various concentrations of SNPs and incubated at 37 °C for 24 h under gentle shaking (100 rpm). Polystyrene microtiter plates (96 wells) were used to grow the bacteria. The growth of unattached cells was removed, and the wells were washed twice with sterile water. The attached cells were stained with 0.1% crystal violet. After 10 min of staining period, the crystal violet was removed, and the wells were thoroughly washed twice with sterile water. For the quantification of antibiofilm activity of AgNPs, the cells associated with crystal violet were solubilized with absolute ethanol, and the absorbance was recorded at 600 nm. These experiments were repeated three times with six replicates, and the average values were calculated.

## 3. Results and discussion

### 3.1. UV-vis spectrophotometer

SNPs were synthesized by reducing $Ag^+$ to $Ag^0$ with addition of exopolysaccharide in a solution of silver nitrate followed by storage at room temperature in the dark. The colorless solution was turned