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Synthesis and characterization of pullulan-mediated silver nanoparticles and its antimicrobial activities

Paulraj Kanmani, Seung Taik Lim*

School of Life Sciences and Biotechnology, Korea University, 5-1 Anam-dong, Sungbuk-ku, Seoul 136-701, South Korea

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ABSTRACT

Synthesis of silver nanoparticles was achieved using pullulan as both a reducing and stabilizing agent. The effect of pullulan and silver nitrate amounts on the synthesis of silver nanoparticles (AgNPs) was investigated. The formation of nanoparticles was first screened by measuring the surface plasmon resonance peak at 420–430 nm using UV–vis spectroscopy. The morphology of the synthesized AgNPs was determined using TEM, which indicated that the AgNPs varied in shape and polydispersed with an average size of 2–30 nm. The presence of elemental silver and the crystalline structure of the AgNPs were confirmed by EDX and XRD analyses. The possible functional groups of pullulan responsible for the reduction and stabilization of AgNPs were evaluated using FT-IR. The pullulan-reduced AgNPs showed excellent antibacterial, antifungal, and antibiofilm activity against food and multidrug resistant bacterial and fungal pathogens. The results showed that pullulan could be used as a reducing as well as a capping agent for synthesizing AgNPs which had potent antimicrobial activity.

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1. Introduction

For the last decades, nanotechnology is a skyrocketing multidisciplinary field of research that interweaves physics, chemistry, bionanoscience, and materials science. Recently, nanobiotechnology is being becoming as an emerging and remarkable technology for the production of novel functional materials such as nano-sized particles. Nanoparticles (NPs) have been widely used in various real world industrial, biomedical, and scientific applications. Nowadays, several types of metal nanoparticles (MNPs) such as gold, silver, zinc, and copper have been successfully synthesized with various anticipated applications that includes medicine and diagnosis, nutrition and neutraceuticals, and electronics and optics (Duncan, 2011; Sharma, Yngard, & Lin, 2009). Among them, silver nanoparticles (AgNPs) have attracted special attention because of their widespread physical, chemical, and biological nature, unique particle size (<100 nm), and tunable surface plasmon resonance (Sharma et al., 2009). Various physical and chemical methods have been utilized for the production of silver, gold, and other nanoparticles successfully (Bankura et al., 2012; Wei et al., 2012). Most commonly, chemical reduction methods are being used by researchers for the production nanoscale metal particles.

Chemical reducing agents such as sodium borohydride (Moura, Mattoso, & Zucolotto, 2012), trisodium citrate (Hebeish, Hashem, Abd El-Hady, & Sharaf, 2013), sodium hydroxide (Bankura et al., 2012), N,N-dimethyl formamide (Guari et al., 2003), 2-mercaptobenzimidazole (Stiger, Gorer, Craft, & Penner, 1999), sodium dodecyl sulfate (Kora & Arunachalam, 2011), *etc.*, have been employed for the production and stabilization of NPs and also found to show certain toxicological effects in the medical research field. Some other methods such as gamma ray and solar irradiation (Wei et al., 2012; Yoksan & Chirachanchai, 2010), sono chemical deposition (Pol et al., 2002), electrochemical methods (Zhu, Liu, Palchik, Kottypin, & Gedanken, 2000), UV photo reduction (Kora & Arunachalam, 2011) and microwave assisted (Peng, Yang, & Xiong, 2013) have also been utilized for synthesis of NPs. However, those reducing agents and physical processes are quite expensive and hazardous. Thus, stupendous efforts are being made to fabricate AgNPs using eco-friendly, biocompatible, and safe biological resources or processes. Biological materials such as bacteria, fungus, yeasts, plant extracts, actinomycetes, and some biomolecules have been stated as safe to synthesize of MNPs on extracellular and intercellular level (Bankura et al., 2012; Narayanan & Sakthivel, 2011). Some studies have reported on extracellular and intracellular synthesis of MNPs using the fungi *Cylindrocladium floridanum* (Narayanan & Sakthivel, 2011); *Alternaria alternate* (Gajbhiye, Kesharwani, Ingle, Gade, & Rai, 2009); bacteria such as *Bacillus amyloliquefaciens* (Wei et al., 2012) and *Bacillus licheniformis* (Kalishwaralal, BarathManiKanth, Ram Kumar Pandian, Deepak, & Gurunathan, 2010); and plants such as plant *Hibiscus cannabinus* (Bindhu & Umadevi, 2013) and *Cocos nucifera* coir extract (Roopan et al., 2013).

* Corresponding author. Tel.: +82 2 3290 3435; fax: +82 2 921 0557.
E-mail address: limst@korea.ac.kr (S.T. Lim).

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Recently, there is a growing interest in the fabrication of novel AgNPs with potent antimicrobial nature and new functional attributes using polysaccharides such as chitosan (Yoksan & Chirachanchai, 2010), starch (Mohanty et al., 2012), agar (Shukla, Singh, Reddy, & Jha, 2012), dextran (Bankura et al., 2012), hydrolyzed casein (Radzig et al., 2013), and guar gum (Pandey, Goswami, & Nanda, 2012). However, there is no report on microbial polysaccharide such as pullulan as a reducing and stabilizing agent for the production of metal nanoparticles. Pullulan is a microbial linear homopolysaccharide consisting of repeating units of glucose that is often termed a biopolymer of α-1, 6 glycosidic linked maltotriose units (Mona & Ajay Kumar, 2004; Wu, Zhong, Li, Shoemaker, & Xia, 2013). Pullulan is highly water soluble and is synthesized extracellularly by the polymorphic fungus *Aureobasidium pullulans*. It has various potential applications especially in the food and pharmaceutical industries. Due to its outstanding adhesive and film forming properties, pullulan has been used to make compression moldings, fibers, drug delivery carrier materials, and edible film (Mona & Ajay Kumar, 2004; Wu et al., 2013).

In the present study, the synthesis of pullulan-mediated AgNPs by direct reduction of silver nitrate with pullulan was demonstrated. The synthesized AgNPs were characterized using UV–vis spectroscopy, transmission electron microscopy (TEM), energy-dispersive X-ray spectroscopy (EDX), dynamic light scattering (DLS), X-ray diffraction (XRD), and Fourier transform infrared (FT-IR) spectroscopy. In addition, the antibacterial, antifungal and antibiofilm activity of the pullulan-stabilized AgNPs *in vitro* will be examined.

2. Materials and methods

2.1. Materials and microbial strains

Biopolymer pullulan was procured from Putus Macromolecular Sci & Tech. Ltd. (Wuhan, China). 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. (Gyeonggido, South Korea). Bacterial strains such as *Escherichia coli* ATCC 35218, *Bacillus cereus* ATCC 10987, food borne pathogen *Listeria monocytogenes* ATCC 15313, and the multidrug resistant pathogens *Pseudomonas aeruginosa* ATCC 15442 and *Klebsiella pneumonia* ATCC 27736 were obtained from the Korean Collection for Type Cultures (KCTC) in South Korea. Fungal pathogens such as *Aspergillus* spp. and *Penicillium* spp. were isolated from contaminated food and subsequently characterized in our laboratory. All solutions were made using ultra-filtered high purity deionized water.

2.2. Preparation of pullulan-based silver nanoparticles (AgNPs)

Various concentrations of pullulan (1%, 5%, 10%, and 15%) were mixed with a 9 mM aqueous solution of silver nitrate ($AgNO_3$) prepared freshly in deionized water under magnetic stirring to optimize the pullulan concentration for $AgNO_3$ synthesis. Different concentrations of $AgNO_3$ (1, 3, 5, 7, 9, and 12 mM) in 15% of pullulan solution were compared under the same conditions. Subsequently, the homogeneous solutions were autoclaved at 121 °C for 15 min. The viscous white-colored solutions turned yellow, which confirmed the formation of pullulan-based AgNPs. All solutions were stored at room temperature (22–25 °C) for more than 3 months in a dark place.

2.3. Characterization of AgNPs

The biopolymer reduction of Ag^+ ions in the aqueous solutions were monitored by measuring the ultraviolet–visible absorbance spectrum of the solution using a UV-vis spectrophotometer (BIOMATE-3S, Thermo Fisher Scientific, Boston, MA, USA) in the range of 300–700 nm. For the analysis of TEM, a drop of AgNPs containing aqueous solution was directly placed onto a carbon-coated copper grid and allowed to air dry completely prior to the TEM observations. The TEM images of AgNPs were obtained using a transmission electron microscope (FEI Tecnai G2 F30, Eindhoven, The Netherlands) at an accelerating voltage of 300 kV. EDX analysis was also carried out using the same TEM equipment to confirm the elemental silver present in the NPs. The size of the AgNPs was evaluated using the DLS method (Wyatt Technology Corp, Santa Barbara, CA, USA), which uses laser light diffraction to measure particle size distribution. The phase composition and crystal structure of the AgNPs was determined with XRD (Philips XPERT MPD). A dried sample was prepared by placing it on a 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 pullulan-stabilized AgNPs were analyzed using FT-IR spectroscopy (JASCO FT-IR 460, Daejon, South Korea) operated at a resolution of 4 cm^{-1}. The dried sample was powdered by grinding with KBr pellets and pressed into a mold. The spectrum was recorded between the wavelength ranges of 500–4000 cm^{-1}.

2.4. Antibacterial activity of AgNPs

The antibacterial activity of the pullulan-stabilized AgNPs was measured using the agar well diffusion method. Bacterial pathogens such as *E. coli*, *K. pneumonia*, *P. aeruginosa*, and *L. monocytogenes* were used as indicator strains for this analysis. These bacterial strains were aseptically inoculated into tryptic soy broth (TSB) and brain heart infusion broth (BHIB) broth, and then incubated at 37 °C. After 16 h, the cells (1 ml) were centrifuged at 6000 rpm for 10 min and then the cells were suspended in sterile water (2 ml). Cells from different pathogens (1 ml) were added to tryptic soy agar (TSA) and brain heart infusion agar (BHIA) media (100 ml) prior to plating and wells were made using an agar well borer. Different concentrations of AgNPs (80 μl) were added to these wells, and the plates were incubated at 37 °C for 24 h. Zone of inhibitions were estimated by measuring the diameter of the bacterial growth inhibition zone. The values were averaged from three independent experiments.

Moreover, the antibacterial activity of the AgNPs was analyzed in liquid medium using the plate count method. To this experiment, the cells of various bacterial pathogens (10^6 CFU/ml) were taken in separate Eppendorf tubes containing various concentrations of AgNPs and then incubated at 37 °C for 6 h. At desired time intervals, the relative cell viabilities of various pathogens were estimated by counting bacterial colonies on the plates. Pure pullulan and silver nitrate solutions were used as controls for comparison. All experiments were carried out in triplicate.

2.5. Antifungal activity of AgNPs

The antifungal activity of the AgNPs was measured using the agar well diffusion method. Two fungal pathogens were isolated from contaminated food and identified as *Aspergillus* spp. and *Penicillum* spp. These two fungal strains were inoculated in potato 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 AgNPs were added to the wells and incubated at room temperature for 4 days. Zone of inhibitions were estimated by measuring

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