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Characterization and antibacterial activity of silver nanoparticles prepared with a fungal exopolysaccharide in water

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ABSTRACT

This work demonstrates a simple and feasible approach for the synthesis of silver nanoparticles (AgNPs) in water with a high molecular weight (MW) exopolysaccharide fraction, EPS1 produced by a medicinal fungus Cs-HK1. The formation and properties of AgNPs were evaluated at various temperatures, time periods, and silver nitrate/EPS1 concentrations in water. At suitable conditions (100 °C, 60 min and 10 mM $AgNO_3$ with 1.0 mg/mL EPS1), AgNPs were formed with an average diameter of 50 nm and a narrow size distribution, remaining as a stable dispersion for at least 2 months. EPS1 may be acting as a reducing and stabilizing agent for the formation of AgNPs, which were attached to the hydroxyl groups of EPS1. The AgNPs formed in EPS1 solution exhibited a concentration-dependent inhibition of both Gram-negative and -positive bacteria but a very low cytotoxicity on the RAW264.7 murine macrophage cells. The results demonstrated the feasibility for green synthesis of AgNPs as potential antibacterial agents using natural polysaccharides dissolved in water.

1. Introduction

Silver nanoparticle (AgNP) has attracted enormous interest because of its great potential for wide applications in food, cosmetic, clothing and pharmaceutical industries (Ahamed, Alsalhi, & Siddiqui, 2010; Marambio-Jones & Hoek, 2010; Rai et al., 2014). Many physical (Breitwieser et al., 2013; Pal, Shah, & Devi, 2009) and chemical methods (Guzman, Dille, & Godet, 2012; Raveendran, Fu, & Wallen, 2003) have been exploited to produce AgNPs. Chemical reduction of Ag^+ ions is one of the most common approaches for AgNP synthesis, in which sodium borohydride, sodium citrate and dimethylformamide have been the highly reactive reducing agents (Leung, Wong, & Xie, 2010). However, some of the AgNPs formed with the small molecules as the reducing agents have been found to cause potent cytotoxicity (AshaRani, Low Kah Mun, Hande, & Valiyaveettil, 2009). A possible cause for the strong cytotoxicity of AgNPs is their rapid diffusion into cells (Travan et al., 2009). Alternatively, biopolymers especially natural polysaccharides such as cellulose and chitosan have been applied to produce non-toxic and biocompatible AgNPs (Li, Zhang, Xu, & Zhang, 2011). Modified chitosan has been used to form well-dispersed non-toxic AgNPs in water (Travan et al., 2009).

Microbial exopolysaccharides (EPS) are usually industrially produced by submerged fermentation, which have found wide applications in food and pharmaceutical industry (Lehtovaara & Gu, 2011; Schmid, Meyer, & Sieber, 2011). AgNPs have been synthesized with an EPS extracted from a lactic acid bacterium, showing notable antimicrobial activity (Kanmani & Lim, 2013). Schizophyllan, a linear (1 → 3)-β-D-glucan with strong anticancer and immune-modulatory activities produced by the medicinal fungus *Schizophyllum commune*, has been used to form non-toxic AgNPs with a diameter of 6 nm (Abdel-Mohsen et al., 2014).

Cs-HK1 fungus is a species of *Cordyceps sinensis* (Berk.) Sacc., a valuable medicinal fungus generally known as the Chinese caterpillar fungus, and Cs-HK1 mycelial culture has been applied to liquid fermentation for production of fungal biomass and EPS (Leung, Zhang, & Wu, 2006; Yan, Wang, & Wu, 2014). EPS1 was a partially purified EPS fraction (~2700 kD) isolated from Cs-HK1 mycelial fermentation broth, which was composed mainly of (1 → 3)-β-D-glucan with glucose side chains (Chen, Ding, Wang, Siu, & Wu, 2014) and about 27% (w/w) galactomannan-protein complexes (~50 kDa) (Chen, Siu, Cheung, & Wu, 2014). This study was carried out to evaluate the preparation and properties of AgNPs with EPS1 in an aqueous solution. The AgNPs prepared at various

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conditions were characterized using several instrumental methods and further evaluated for cytotoxicity and antibacterial activities.

2. Materials and methods

2.1. Fungal fermentation and EPS1 isolation

The Cs-HK1 fungus was previously isolated from the fruiting body of a natural *C. sinensis* (Leung et al., 2006). Cs-HK1 mycelial liquid fermentation for EPS production was carried out in shake flasks at 20 °C for a period of 7 days. The fermentation liquid was then centrifuged, and the supernatant medium was collected for EPS isolation by ethanol precipitation. The EPS1 used in this study was isolated from the medium by precipitation with 2/3 volume ratio of ethanol (95% grade) (~40% v/v), followed by dialysis and freeze drying as reported previously (Chen, Siu, et al., 2014). About 3.0 g/L of EPS1 was recovered from the Cs-HK1 fermentation broth, which was relatively high as compared with the EPS yields from mycelial fermentation of other medicinal fungi in the literature (Cui & Zhang, 2011; Lin, 2011; Zhang & Cheung, 2011).

2.2. Synthesis of AgNPs

The synthesis of AgNPs with EPS1 followed a reported procedure with minor modifications (Leung et al., 2010). All the $AgNO_3$ and EPS1 solutions were prepared in distilled water. The $AgNO_3$ was purchased from Sigma-Aldrich (ACS reagent, #209139). The $AgNO_3$ solution (2 mL) at various concentrations was mixed with an equal volume of EPS1 solution at a selected concentration. The reaction mixture was incubated with constant stirring for 10–60 min at 25, 40, 60 or 100 °C in the dark. After completion of the reaction, the solution was stored at room temperature (~25 °C) in the dark before use.

2.3. Characterization of AgNPs

The UV–Vis spectra of AgNPs and EPS1 in water were measured from 300 to 600 nm on a HEWLETT Packard 8453 spectrophotometer against pure EPS1 solution as the blank. Dynamic light scattering (DLS) measurement was performed of AgNPs at 25 °C on a Malvern Zetasizer Nano (Malvern Instruments Ltd., UK) at 632.8 nm and 90° scattering angle. The particle size, morphology and selected-area electron diffraction (SAED) of AgNPs were observed by field emission electron microscope (JEOL model JEM-2100F). TEM samples were prepared by placing a drop of sample solution on the carbon coated copper grids and vacuum dried. Silver content was determined by ICP-OES (Agilent Technologies 700 Series). Standard solutions were prepared by 0–2.0 mM silver nitrate solution. Infrared (IR) spectra were measured at room temperature from 4000 to 500 cm^{-1} at 4 cm^{-1} resolution with baseline corrected on a Perkin-Elmer 1600 instrument.

2.4. Cytotoxicity assay

The murine macrophage cell line RAW264.7 was employed for the cytotoxicity assay. The RAW264.7 cells were maintained in DMEM medium containing 10% FBS (v/v), 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin) (Gibco BRL, U.S.A.). Cells were cultured in a humidified incubator at 37 °C with 5% CO_2. For the assay, 0.5×10^4 of RAW264.7 cells were grown in DMEM medium containing the AgNPs/EPS1 samples at chosen concentrations. Cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method (Mosmann, 1983). The morphology of cells was observed by a microscope (Olympus IX51, Japan).

2.5. Antibacterial assay

Escherichia coli (Gram-negative) and *Staphylococcus aureus* (Gram-positive) were used in the antibacterial tests. The *in vitro* antibacterial activity was conducted in 96-well microplates using the broth micro-dilution procedure according to the Clinical and Laboratory Standards Institute guidelines (Jorgensen & Hindler, 2007). Four to five colonies from overnight cultures of the test bacteria on a tryptone (Fluka, Analytical, Sigma-Aldrich Co., USA) agar plate were inoculated in 10 mL of Luria-Bertani broth at 37 °C for 4–6 h. The bacterial suspension was inoculated into a 96-well microplate (final concentration of ~10^5 CFU/mL) containing 100 μL of serial dilutions of the tested samples. After incubation for 12 h at 37 °C, the absorbance at 600 nm (A_{600}) was recorded to calculate the percentage of bacteria cell inhibition with respect to the untreated control, using a microplate reader (Chan et al., 2013). The assay of each sample was performed in triplicate.

3. Results and discussion

3.1. Optical properties of AgNPs

Fig. 1 shows the UV–Vis spectra of $AgNO_3$ and EPS1 mixture solutions in water prepared at various concentrations, temperatures and time periods. When Ag ion is reduced to Ag atom in a solution, the colorless solution is turned into yellowish brown. An absorption band is an indication of AgNP formation in the solution (Xu et al., 2014), which can be detected by the surface plasmon resonance (SPR) band using UV–Vis spectroscopy (Kanmani and Lim, 2013). In this study, the AgNPs formed in EPS1 solution showed a strong SPR peak at about 410 nm (Fig. 1). The formation of AgNPs was due probably to the reduction of Ag^+ ions into Ag atoms by the EPS1 added to the $AgNO_3$ solution.

The absorption intensity of $AgNO_3$-EPS1 mixture solution initially increased and then decreased with the increase of EPS1 concentration from 0.01 mg/mL to 2.0 mg/mL (Fig. 1A), indicating the increase or decrease in the concentration of AgNPs formed. The similar trend of UV–Vis absorption change has also been reported previously (Wu, Zhang, & Zhang, 2012), due probably to the formation of large particles or clusters by the prepared AgNPs. Fig. 1B shows the UV–Vis absorption of AgNPs prepared with 1.0 mg/mL EPS1 and various concentrations of $AgNO_3$. Relatively high absorption intensities attained at 4 mM and 6 mM $AgNO_3$ but with notable background noise, which was due probably to the poor stability of AgNPs formed (Wei, Sun, Qian, Ye, & Ma, 2009). The most symmetrical absorption peak was observed with 10 mM $AgNO_3$, indicating more uniform AgNPs (Wu et al., 2012). SPR absorption of AgNPs is sensitive to variations in physical properties and aggregation of particles in solution (Liu, Lee, Kim, & Kim, 2007). Chemical modification of the particle surface may be effective to attain uniform particle size and avoid aggregation. Fig. 1C shows the UV–Vis absorption spectra of AgNPs prepared at various temperatures (and a fixed reaction time of 20 min), showing no absorption band until the temperature was increased to 100 °C. In our preliminary experiments (data not shown), absorption band was also observed at 80 °C with a longer reaction time of 40 min. The results suggested that the effect of temperature on the formation of AgNPs in the EPS1 solution was mainly attributed to its influence on the reaction rate of Ag ions reduction. Similarly, formation of AgNPs using sophorolipids as reducing and capping agent was not observed until the temperature was increased to 90 °C (Kasture et al., 2008). Fig. 1D shows the UV–Vis spectra of AgNPs prepared in EPS1 solution for different periods of time, exhibiting a stronger absorbance (more AgNPs formed) with longer time of heat treatment. The experimental results altogether suggested that the most

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