Green Synthesis of Silver Nanoparticles by Sinorhizobial Octasaccharide Isolated from *Sinorhizobium meliloti*

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In recent years, noble metal nanocrystals have been the subject of intense research in chemistry and physics due to their nano-size dimensions.[1] Due to their quantum size effects and surface effects, nanocrystals can exhibit novel electronic, magnetic, chemical and structural properties and have the potential for a wide array of significant applications.[2] However, development of straightforward and versatile methods for the preparation of mono-dispersed silver nanocrystals using cheap and nontoxic chemicals, environmentally benign solvents, and renewable materials remains a chellenge.[3] Although many chemical synthesis methods have been used for the synthesis of metallic nanocrystal dispersions, the most commonly applied method involves the use of excess reducing agents such as hydrazine, sodium borohydride ($NaBH_4$) and dimethyl formamide (DMF), and is reported to rely heavily on an organic solvent due to the hydrophobicity of the capping agent.[4] Although some methods are known to use water as a solvent medium for the synthesis of the silver nanocrystals, all of these methods need heat or pressure for preparation of silver nanocrystals.[5] In this study, we utilized water as the environmentally benign solvent for the preparation of nanocrystals under basic condition without heat or pressure. The majority of methods reported to date uses reducing agents such as hydrazine, sodium borohydride ($NaBH_4$), or dimethyl formamide (DMF). All of these are highly toxic chemicals and poisonous materials for environment. In the present method, the sinorhiziobial octasaccharides were used as capping agenta without toxic reducing agents for the generation of Ag nanocrystals, upon the addition of basic aqueous solution. This environmentally benign method is mild, renewable and inexpensive. Succinoglycan, a symbiotically important acidic polysaccharide of *Sinorhizobium meliloti*, is composed of polymerized octasaccharide subunits.[6] Succinoglycan monomer consists of one galactose at the reducing end and seven glucose residues. Analyses with nuclear magnetic resonance (NMR) and mass spectrometry (MS) techniques have confirmed the structural details of the succinoglycan monomer.[7] The succinoglycan monomer has heterogenous and noncarbohydrate substituents (acetyl, pyrvate, and succinate).[8] The authors of the present study recently reported the chiral separation of some flavonoids using sinorhizobial octasaccharides produced by *S. meliloti*, as chiral additives in capillary electrophoresis (CE),[9] as well as the catalyzation of the strecker reaction by microbial oligosaccharides, including the succinoglycan monomer.[10] Because sinorhizobial octasaccharide has potential as an effective catalyst and a chiral selector, sinorhizobial octasaccharide-stabilized Ag nanoparticles will be good candidates to develop chiral recognition processes and new biomimetic catalysts.

An easy and simple approach to the green synthesis of Ag nanoparticles has been developed by employing a novel sinorhizobial octasaccharide to act as both a reducing agent and a capped agent in controlled pH environments. Nanoparticles can be rapidly and easily produced in the proposed system by controlling of the pH condition. We consider that a role of OH^- ion is important to produce Ag nanoparticles because one reducing sugar in the octasaccharide serves one electron to reduce Ag^+ for Ag nanoparticles only under basic condition (Scheme 1). The basic condition was controlled and maintained by adding aqueous NaOH solution to this system. The sinorhizobial octasccharide-capped Ag nanocrystals have been investigated in the system by UV-Visible spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, transmission electron microscopy (TEM) and X-ray diffraction (XRD) techniques. As the sinorhizobial octasaccharide has one galactose as reducing sugar (Figure 1A), the oligosaccharide can effectively reduce Ag^+ ions into Ag^0 upon the addition of a small amount of aqueous sodium hydroxide solution (0.05 M). This was confirmed through both visual observation and accurate UV-Visible absorption spectroscopy. The solution finally turned a clear yellow color after the reduction reaction and showed an intense absorption peak at 400 nm in the UV-Visible absorption spectrum (Figure 1B). Detailed information about the surface of the as-synthesized Ag nanoparticles was supported by the comparison of the FT-IR spectra of pure sinorhizobial octasaccharide and Ag nanoparticles fabricated in aqueous octasaccharide dispersions. The pure sinorhizobial octasaccharide in IR spectrum showed a broad band at 3280 cm^{-1} that originated from the -OH stretch of the sinorhizobial octasaccharide. The absorption at 2870 cm^{-1} is attributed to C-H stretching in the sugar backbone. The absorption peak at 1712 cm^{-1} is assigned to the symmetric stretching of the carboxyl

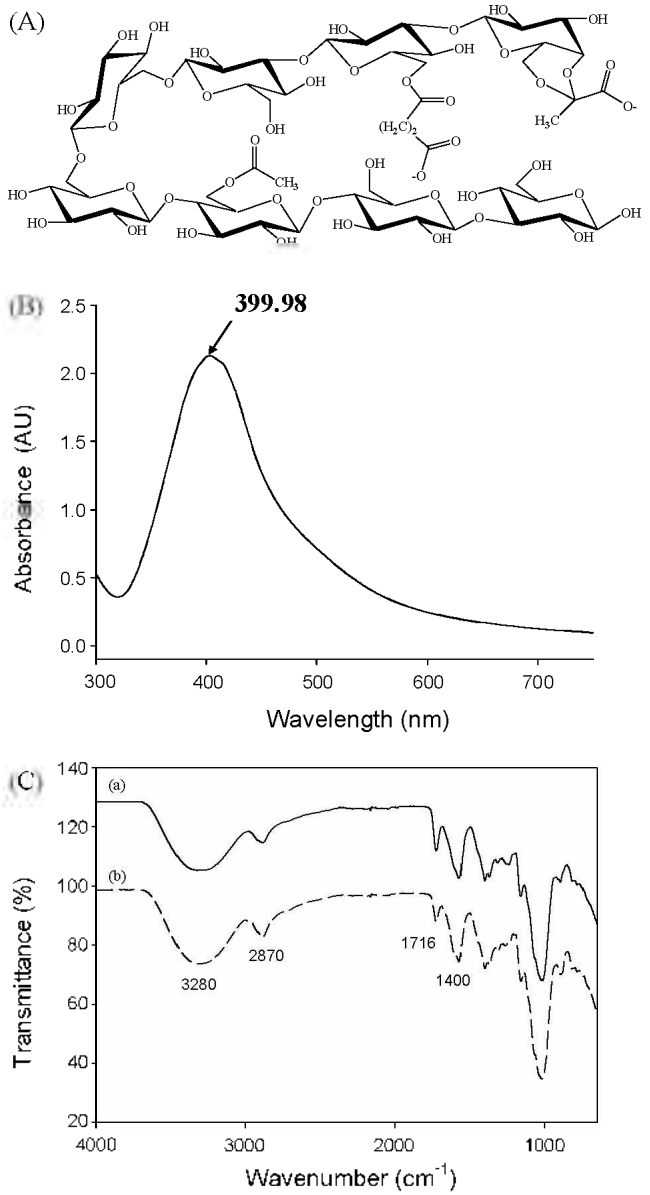
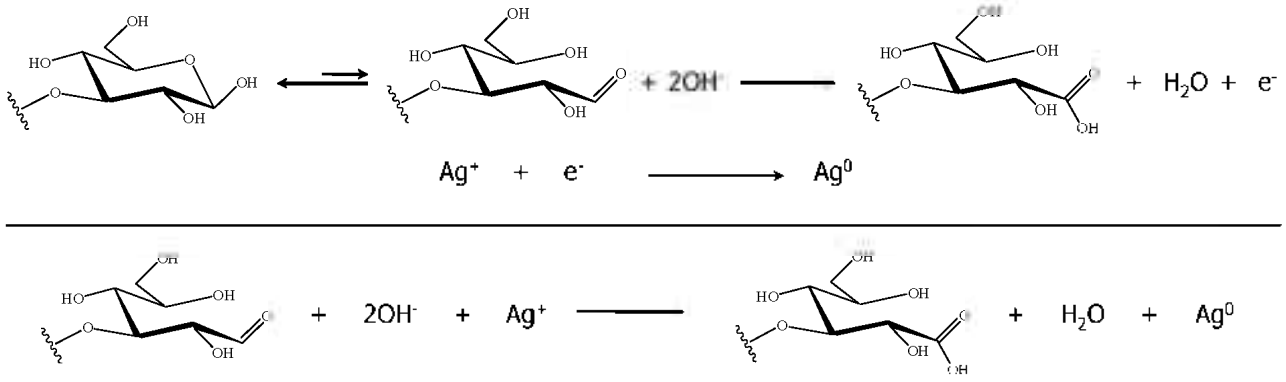


Figure 1. (A) The structures of the sinorhizobial octasaccharide (succinoglycan monomer) involved in this work. (B) UV-Visible spectrum of Ag nanoparticle stabilized with octasaccharide and (C) FT-IR spectra of (a) free sinorhizobial octasaccharide and (b) octasaccharide coated Ag Nanoparticles.

groups and the absorption at 1400 cm^{-1} is possibly due to the bending tendency of symmetric CH_3 groups within the acetyl and pyruvyl groups as substituents. The absorption at 1010 cm^{-1} is attributed to an asymmetric C-O-C stretching band resulting from the sugar backbone (Figure 1C). Though the absorption slightly decreased at 1712 cm^{-1} and broadened at 1400 cm^{-1}, characteristic absorption peaks between the pure octasaccharide and octasaccharide stabilized Ag nanoparticles were not significantly different in the FT-IR spectra (Figure 1C), suggesting the presence of sinohrizobial ocasaccharide as an intrinsic component of the Ag nanoparticles. Although the exact reducing and capping mechanism of sinorhizobial octasaccharide on the formation of Ag nanoparticles and the effect of basic pH environment on stability of Ag nanoparticle dispersions remain to be elucidated, we speculate that OH^- ion is involved in the reaction to produce Ag nanoparticles. The mixed solution of $AgNO_3$ and sinorhizobial octasaccharide was stirred for more than 30 minutes but no color change was observed at pH 5.2 without adding of NaOH solution. The NaOH solution was continuously added to the mixed solution until the pH reached greater than 6.8 where an aldehyde group of reducing sugar oxidized to a carboxyl group by nucleophilic addition of OH^- ion, which reduced Ag^+ to Ag^0 (Scheme 1).

The plentiful hydroxyl groups in the sinorhizobial octasaccharide can promote to form the complex matrix of Ag nanoparticles by the oeatsaccharide.[11] This may suggest that the octasaccharide can act as a capping agent without a reducing agent on the formation of Ag nanoparticles. A typical TEM image of the Ag nanoparticles formed is presented in Figure 2A. The characteristic spherical Ag nanoparticles can be observed with a relatively narrow particle size distribution (5-13 nm range). Multiple lattice fringes with interplanar spacing of 2.33 Å can be observed on high-resolution TEM (Figure 2B). The X-ray diffraction (XRD) pattern of the Ag nanoparticles is displayed in Figure 3. The diffraction peaks of the (111), (200), (220), (311) and (222) lattice planes appear in the XRD pattern of Ag nanoparticles. Thus, the XRD pattern corresponds well to the crystalline planes of the face-centered-cubic (fcc) structured Ag, suggesting the crystalline nature of these Ag nanoparticles. From the Scherrer equation and the XRD data in Figure 3, the average Ag particle size was estimated to be 8.9 nm (SD = 3.3 nm), indicating a relatively high monodispersity of Ag nanoparticles formed in



Scheme 1. The reduction reaction equation for the formation of Ag nanoparticles.

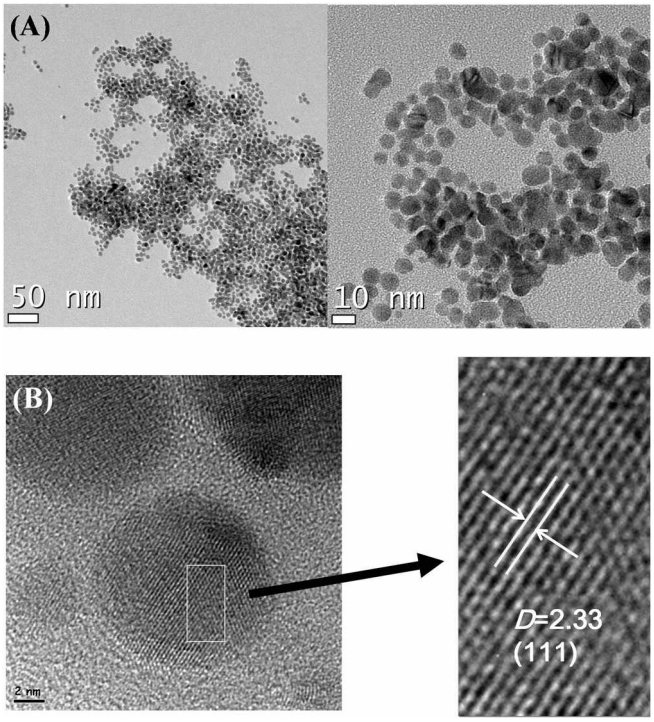


Figure 2. (A) Representative TEM images of the Ag nanoparticles stabilized by 0.03 M sinorhizobial octasaccharide at a pH of 7.0 and (B) High resolution TEM images of Ag nanoparticles formed in the system (the scale bar equal 2 nm).

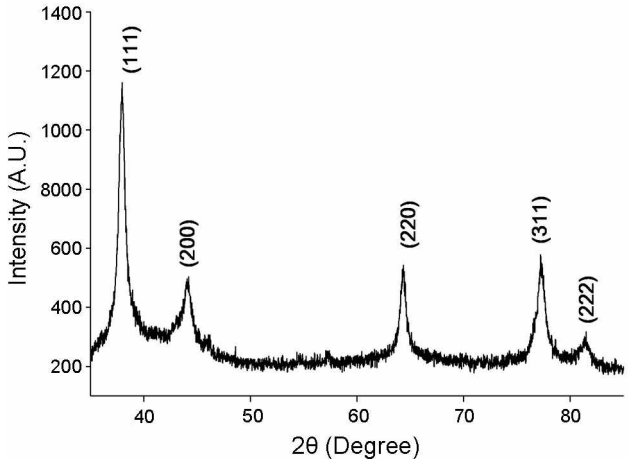


Figure 3. XRD (X-Ray Diffraction) pattern of Ag nanoparticles formed in the system.

the system.[12] In summary, through a facile and simple approach to the synthesis of relatively monodisperse Ag nanoparticles (average diameter = 8.9 nm, SD = 3.3 nm), octasaccharide-stabilized Ag nanoparticles can be synthesized by using sinorhizobial octasaccharide as both reducing agent and capping agent in controlled pH environments. Because sinorhizobial octasaccharide have biologically compatible characteristics and offer the potential for novel function, it is very important to explore the pharmaceutical, biomedical, and biosensor applications of octasaccharide-stabilized metal nanoparticles.

Experimental Section

Chemicals. $AgNO_3$ and NaOH were purchased from Aldrich and dissolved with deionized water.

Preparation of Sinorhizobial Octsaccharide. Cells were removed by centrifugation (13,000 g for 10 min) and supernatants were concentrated 5-fold by rotary evaporation. Next, high-molecular-weight (HMW) succinoglycan was precipitated from concentrated supernatants by adding 3 volumes of ice-cold ethanol. HMW exopolysaccharide was then removed from the concentrated supernatants by centrifugation (12,000 g for 10 min). The supernatant was once more concentrated 5-fold by rotary evaporation and low-molecular-weight (LMW) exopolysaccharide was obtained by adding 7 volumes of ice-cold ethanol and subjecting the mixture to centrifugation. The precipitate was dissolved in distilled water and samples were applied to a Bio-Gel P6 column (1 × 120 cm) that was eluted at room temperature with 0.5% AcOH at a rate of 20 mL/h. Fractions (1 mL) were collected and assayed for carbohydrate content. Material eluting in the position expected for the sinorhizobial octasaccharides from LMW exopolysaccharide was pooled and then applied to a column (2 × 45 cm) of Bio-Gel P4 to separate linear octasaccharides at a rate of 30 mL/h. A fraction of the monomer was pooled, concentrated, and subsequently desalted using a Sephadex G-15 column (1 × 49 cm). The monomer was separated into three fractions using a DEAE Sephadex column according to succinate moiety. Those octasaccharides were further fractionated on a column (1.5 × 48 cm) of DEAE Sephadex that was pre-equilibrated with 5 mM KCl in a 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (10 mM; pH 7.0). The sample was loaded onto the column and eluted with KCl linear gradients using 400 mL of 5-250 mM KCl. Fractions (4 mL) were collected and assayed by the phenol-sulfuric acid method. Peaks were pooled, thoroughly dialyzed against water with a SpectraPor dialysis membrane tube having a molecular-weight cutoff of 1000 and lyophilized. Then the purified sinorhizobial octasaccharides were then confirmed using a Brucker 500 MHz NMR spectroscope.

Preparation of Nanoparticles. The Ag nanocrystals were synthesized by the reduction of Ag^+ ions in the aqueous octasaccharide dispersions. An aqueous solution of $AgNO_3$ (0.1 M; 40 mL aliquot) was added to an aqueous solution of octasaccharide (0.03 M; 5 mL). The mixed solution was stirred for more than 30 minutes and no color change was observed (pH ≈ 5.2). Approximately 0.6 mL of aqueous solution NaOH (0.05M) was continuously added dropwise until there was no further change in solution color (pH ≈ 6.8).

Preparation of FT-IR Samples. The aqueous Ag nanoparticles dispersion was centrifuged for about 20 minutes at a speed of 8000 rpm. The performance was repeated three times to disperse the highly concentrated Ag nanoparticles. Afterwards, freeze drying under vacuum was applied overnight to obtain very dry composite Ag nanoparticles. A pellet for FT-IR analysis was obtained by grinding 2 mg of Ag nanoparticles with a KBr and pressing them in a mold. The FT-IR spectrum of the Ag nanoparticles was obtained using a JASCO FT-IR-300E spectrometer (USA).

Morphology and Crystallinity Studies. Sample grids for TEM measurements were prepared by placing a drop of aqueous Ag nanoparticle dispersion onto the copper grids and subsequently evaporating the water naturally over night under ambient conditions. The morphology of the Ag nanoparticles was determined using an FEI Tecnai F20 TEM at an operating voltage of 200 kV. The crystallinity of the Ag nanoparticles was studied by XRD (Philips XPERT MPD diffractometer) with $Cu_{k\alpha}$ radiation (40 kV and 20 mA) and ED techniques.

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