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Synthesis of Cysteine Capped Silver Nanoparticles by Electrochemically Active Biofilm and their Antibacterial Activities

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Cysteine capped silver nanoparticles (Cys-AgNPs) have been synthesized by employing electrochemically active biofilm (EAB), $AgNO_3$ as precursor and sodium acetate as electron donor in aqueous solution at 30 °C. Cys-AgNPs of 5-10 nm were synthesized and characterized by UV-Vis, FT-IR, XRD and TEM. Capping of the silver nanoparticles with cysteine provides stability to nanoparticles by a thiolate bond between the amino acid and the nanoparticle surface and hydrogen bonding among the Cys-AgNPs. In addition, the antibacterial effects of as-synthesized Cys-AgNPs have been tested against two pathogenic bacteria *Escherichia coli* (O157:H7) and *Pseudomonas aeruginosa* (PAO1). The results demonstrate that the as-synthesized Cys-AgNPs can proficiently inhibit the growth and multiplication of *E. coli* and *P. aeruginosa*.

Key Words : Cys-AgNPs, Silver colloidal nanoparticles, (L)-Cysteine, Electrochemically active biofilm (EAB), Hydrogen bonding

Introduction

Nanoparticles are widely used for targeted drug delivery and for other biomedical application.[1,2] Among the metal nanoparticles, silver nanoparticles gaining much interest because of their antibacterial[1,3] and antifungal[4] response. In general, to synthesize AgNPs, metallic silver has been engineered into ultrafine particles by several methods, including wet chemical reaction, spark discharging, electrochemical reduction, solution irradiation and cryochemical synthesis.[3-5] Synthesis of reliable, green chemistry approach for nanoparticles is an important aspect of nanotechnology. Hence, there is a growing need to develop an environmentally benign nanoparticle synthetic procedure that does not use toxic chemicals and should be environment friendly.[4] Another important facet of nanotechnology is the development of new route to synthesize metal nanoparticles, especially AgNPs, which is toxicity free, is a great challenge. Several syntheses for AgNPs were reported, but in most of the cases chances of AgNPs contamination either with some chemicals[1,2,6] or with by-products of micro-organisms was involved.[7,8] Recently, few green synthesis for AgNPs have been reported but in most of these reports strong reducing agents such as $NaBH_4$ were used which further may contaminate the nanoparticles.[3,9]

Another important factor related with the nanoparticles is the stability of the nanoparticles in the colloidal solution and after isolation of nanoparticles from colloidal solution. There are several reports to synthesize the naked AgNPs in colloidal solutions but stability and their interaction with biosystem remains the main concern.[1,10] In order to overcome these shortcomings, new methodologies were developed to synthesize AgNPs by employing different capping as well as stabilizing agents.[10,11] Among the capped/coated silver nanoparticles cysteine capped AgNPs is gaining much attention because of their selective biological and sensing activities towards microorganisms and amino acids.[10-12]

Hence, there is a need to develop a protocol in which the approach should be green, nanoparticles should not get contaminated and the synthesized nanoparticles should be extracellular and stable. Recently, our group has reported the electrochemically active biofilm mediated synthesis of AgNPs in aqueous solution, in which the synthesized AgNPs were extracellular, stable and pure.[13] Inspiring with this work we extended our work to synthesize the cysteine capped AgNPs mediated by electrochemically active biofilm with enhanced activity and explore its antibacterial activity on *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). To the best of our knowledge, synthesis of cysteine capped silver nanoparticles (Cys-AgNPs) by employing electrochemically active biofilm (EAB) is not yet documented and it is a novel and astute approach. However, synthesis of Cys-AgNPs through other approaches are well known and established.[10,11,14,15]

Experimental

Materials. (L)-Cysteine, $AgNO_3$ and sodium acetate (Duksan Pure Chemicals Co. Ltd. South Korea) was used as received. Carbon paper (without wet proof, Fuel Cell Earth LLC), agar (Becton, Dickinson and Company, Sparks, USA) and paper disc (8 mm diameter, Tokyo Roshi Kaisha, Tokyo, Japan) were used as such. De-ionized (DI) water was prepared by using PURE ROUP 30 water purification system.

Methods. The as-synthesized Cys-AgNPs were characterized by using UV-Vis-NIR spectrophotometer (VARIAN, Cary 5000) and FT-IR (Excalibur Series, BIORAD, FTS 3000MX). X-ray diffraction (XRD) of powder was measured

by diffractometer (PANalytical, X'Pert-PRO MPD) with Cu Kα radiation (λ = 0.15405 nm). Diffraction peaks of crystalline phases were compared with those of standard compounds reported in the JCPDS data file. The particle size of Cys-AgNPs sample was measured by TEM (Tecnai G2 F20, FEI, USA) operating at an accelerating voltage of 200 kV. Cys-AgNPs were dispersed in ethanol and sonicated for 10 minutes by sonicator (BRANSON 5510) for TEM studies. Selected-area electron diffraction (SAED) images were recorded by the TEM instrument.

Preparation of Electrochemically Active Biofilm (EAB). Electrochemically active biofilm (EAB) on carbon paper were made as reported earlier.[13] In short, carbon paper (2.5 cm × 4.5 cm) were dipped into a mineral salt medium containing sodium acetate as substrate. 10 mL of anaerobic sludge (Biogas plant in Paju, Republic of South Korea) was added under strictly anaerobic conditions by sparging N_2 gas for 5 min. All media, including the bacterial inoculum, were changed at every two days under strict anaerobic conditions. This was repeated for two weeks. The living electrochemically active biofilm formed on the carbon paper was employed for the synthesis of Cys-AgNPs.

Synthesis of Cysteine-AgNPs. 0.01 g of cysteine (0.1 g/L) was dissolved in 100 mL of DI water and 1 mM, 0.0169 g of $AgNO_3$ was added and stirred for 5 minutes. 0.1 g sodium acetate was added as electron donor under strict anaerobic conditions by sparging N_2 gas for 5 min.[16] EAB on carbon paper was hanged and the system was sealed. The whole reaction mixture was left for magnetic stirring. After 8 hours, initial colorless solution changed to yellow color, showing the formation of silver nanoparticles.[12] Finally, UV-visible measurement was made and an absorbance maximum was observed at 397 nm (Fig. 2) which is the characteristics of AgNPs. Finally, the reaction mixture was centrifuged and powder Cys-AgNPs was isolated for further characterization.

Another set of control experiment was performed in which 0.01 g of cysteine (0.1 g/L) was dissolved in 100 mL of DI water, and 1 mM, 0.0169 g of $AgNO_3$ was added. N_2 gas was sparged for 5 min and the reaction flask was sealed and stirred for 8 hrs. No any color change observed, which indicates that cysteine alone cannot reduce the Ag^+ to Ag^0.

Antibacterial Assay. The paper disc diffusion method was used to test the antibacterial activity of Cys-AgNPs.[17] Two pathogenic bacteria, *E. coli* (O157:H7)[18] and *P. aeruginosa* (PAO1) [19] of the sequenced strains were used in this study. Overnight cultures were re-grown to an optical density at 600 nm of 1.0 and about 10^4 cells/plate were spread on LB agar. 20 μL of as-synthesized Cys-AgNPs colloidal solution was placed on the paper disc. Plates were incubated at 37 °C for 18 h and the antibacterial activities of samples were evaluated by measuring a clear zone where inhibition of bacterial growth takes place. Distilled water was used as a positive control. Three independent experiments were performed.

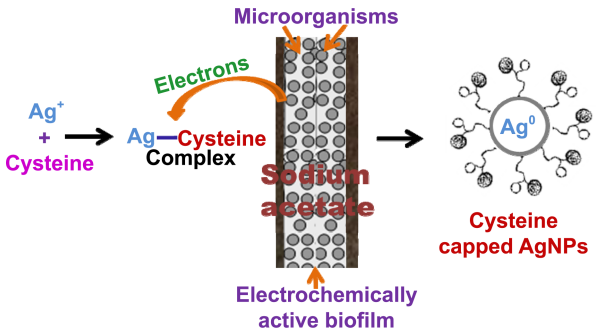

Figure 1. Proposed scheme for the synthesis of cysteine capped silver nanoparticles by Electrochemically Active Biofilm.

Results and Discussion

EAB is well known biogenic system that provide electrons by decomposing sodium acetate.[13,16] After successful formation of EAB on carbon paper, we exploited it for the synthesis of cysteine capped silver nanoparticles. Figure 1 shows the overall mechanism for the synthesis of Cys-AgNPs by EAB. In the first step, Ag ions form complexes with the cysteine by thiolate bonding (Ag-SH) which arrest the fast reduction of Ag ions.[10,15] It is well known that presence of cysteine decreases the rate of formation of silver nanoparticles.[20] In the second step, EAB in presence of sodium acetate produces electrons, which were used for the slow reduction of Ag ions. It ultimately leads to the formation of cysteine capped silver nanoparticles which are apparent from the yellow color of the colloidal solution (Fig. 2).[12]

UV-visible spectroscopic measurement of the dispersed aqueous solution was recorded (Fig. 2) and appearance of absorbance maxima at 397 nm, which is typically ascribed to the plasmon resonance band of AgNPs. Similar UV-vis spectra for Cys-AgNPs have been already reported.[15] According to Mie theory, metal nanoparticles like Ag shows a surface plasmon band within the range of 400-450 nm.[21] The absorbance spectra of as-synthesised Cys-AgNPs were measured after 5 days and the spectra remained same which indicates that the as-synthesized Cys-AgNPs were very stable for long time in the solution.

FT-IR spectra of the isolated Cys-AgNPs powder in KBr

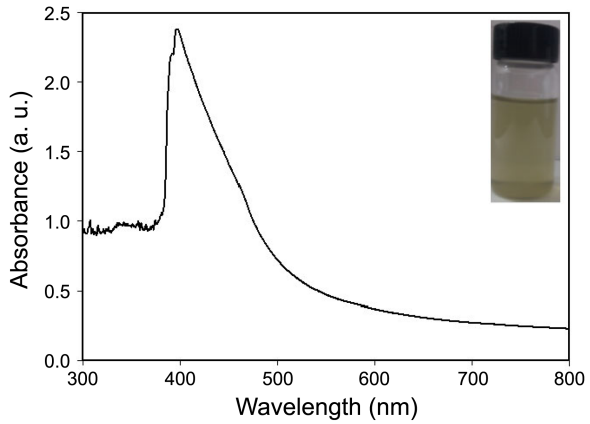

Figure 2. UV-visible spectra of cysteine capped silver nanoparticles.

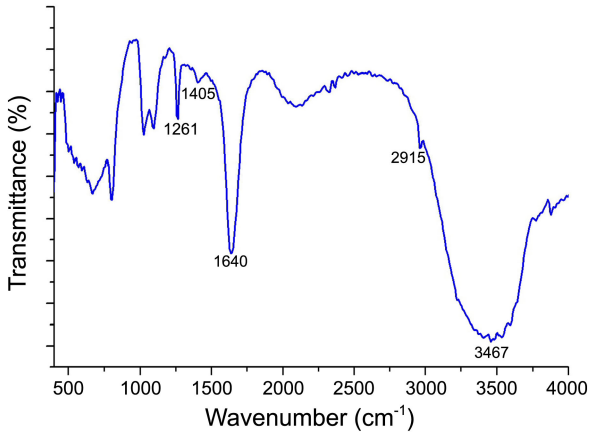

Figure 3. FT-IR spectra of cysteine capped silver nanoparticles.

were recorded as shown in Figure 3. The prominent S-H vibrational band of free cysteine in general observed at *ca.* 2555 cm^{-1} which is not observed in the above spectra. It disappears on coordination of these molecules with colloidal silver.[15] This is the strong evidence of surface binding of cysteine to the silver particles *via* a thiolate linkage. Murray and coworkers studied such behavior on alkanethiol modification of gold nanoparticles.[22] Further indication for the presence of surface bound cysteine is provided by FT-IR measurements of the Cys-AgNPs in which carboxylate stretch vibration of the cysteine molecules is observed at *ca.* 1640 cm^{-1}. It is well-known from studies on Langmuir-Blodgett films of metal salts of fatty acids that the position of this band is dependent on the nature of the bound metal cation.[10,15] The position of the carboxylate stretch vibration at 1640 cm^{-1} in the silver colloidal solution suggests that some interaction of the acid group with other cysteine molecules, possibly through hydrogen bonding. This view is strengthened when one considers that the carbonyl stretch vibration of the acid group in free cysteine molecules occurs at 1650 cm^{-1}.[15]

The phase of the as-synthesized Cys-AgNPs was determined by X-ray diffraction with Cu Kα radiation. The X-ray diffraction pattern (Fig. 4) confirmed the formation of crystalline Cys-AgNPs. XRD pattern shows the four prominent diffraction peaks in the 2θ range 30-80° at 38°,

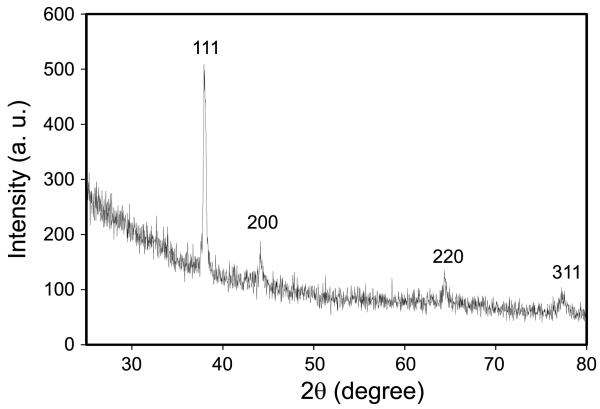

Figure 4. XRD spectra of cysteine capped silver nanoparticles.

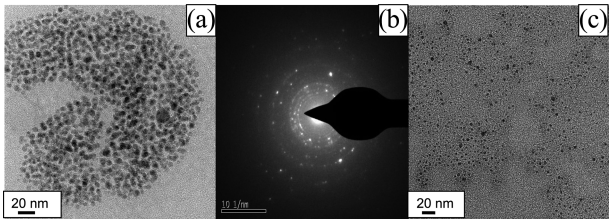

Figure 5. (a) TEM micrograph of as-synthesized Cys-AgNPs. (b) SAED of as-synthesized Cys-AgNPs. (c) TEM of silver nanoparticles (without capping) as reported earlier.[13]

46.33°, 64.58° and 77.24° corresponding to 111, 200, 220 and 311 planes of polycrystalline face-centered cubic (fcc) silver, respectively (JCPDS file no. 04-0783). From the spectra it is also clear that no any impurity peaks were detected which reveals that the as-synthesized Cys-AgNPs were of high purity. The average of all the Cys-AgNPs peaks in the XRD spectra was used to calculate the average crystallite size by using the Scherrer equation and the full width at half maximum (fwhm).[23] It shows that the average size of the Cys-AgNPs was ~10 nm which are concomitant with the TEM values.

Direct evidence for the state of aggregation of the Cys-AgNPs is determined by TEM at low magnification as shown in Figure 5(a). It illustrates that the size of as-synthesized Cys-AgNPs are in 5-10 nm range and monodispersed. Selected-area electron diffraction (SAED) is shown in Figure 5(b) which clearly shows well resolved lattice fringes and diffraction cycles and is indicative of a highly crystalline nature of Cys-AgNPs. It further confirmed the characteristic crystal planes of Cys-AgNPs which are concomitant with the XRD (Fig. 4) studies. Figure 5(c) shows the TEM image of naked AgNPs (without cap), determined at low magnification, synthesized by our group previously.[13] In comparison to Figure 5(a), it shows that the AgNPs are monodispersed, discrete, in the range of 2-5 nm and are not aggregated. From Figure 5(a) it is clear that Cys-AgNPs were slightly aggregated in comparison to the naked AgNPs (Fig. 5(c)) where AgNPs were without capping. This slight aggregation is because of cross-linked structure *via* hydrogen bonding which provides stability to the AgNPs.[10,15,24]

From the above scheme, characterization techniques and discussion, it was concluded that the as-synthesized Cys-AgNPs was well formed, crystalline in nature and pure. The amine and carboxylic acid functional groups of cysteine on the surface of the colloidal silver nanoparticles form hydrogen bonds which provide cross-linked structure to the colloidal silver nanoparticles which further stabilizes the AgNPs in aqueous solution.[24] The AgNPs are well capped with cysteine as a capping and stabilizing agent. It further reveals that EAB might be a better tool to synthesize cysteine capped silver nanoparticles without any chemical contamination.

Antibacterial Activity. An antibacterial test against *E. coli* and *P. aeruginosa* was used to compare colonies on agar plates in the presence or absence of the as-synthesized Cys-AgNPs. Approximately 10^4 colony-forming units (cfu)

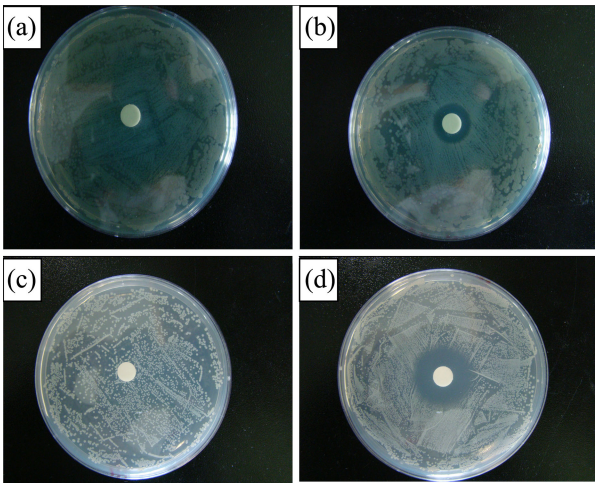

Figure 6. Antibacterial activity of as-synthesized Cys-AgNPs. (a) Controlled *P. aeruginosa* samples. (b) *P. aeruginosa* with Cys-AgNPs showing growth inhibition. (c) Controlled *E. coli* samples. (d) E. coli with Cys-AgNPs showing growth inhibition.

of *E. coli* and *P. aeruginosa* were cultured on LB agar plates as control and LB agar plates supplemented with 20 μL of as-synthesized Cys-AgNP. After incubation at 37 °C for 18 h, bacterial colony crowding was observed on the control plates in the absence of any additives (Fig. 6(a) & 6(c)) whereas, growth of bacteria in the presence of Cys-AgNPs has inhibited (Fig. 6(b) & 6(d)). The presence of the Cys-AgNPs inhibited bacterial growth by approximately 80%, indicating that the as-synthesized Cys-AgNP has a good antibacterial effect (Fig. 6(b) & 6(d)). Zone of inhibition of as-synthesized Cys-AgNPs against pathogenic bacteria shows that in case of *P. aeruginosa*, it was ~4 mm and in case of *E. coli*, it was ~3 mm. The as-synthesized Cys-AgNPs shows much better bacterial inhibition as compared to without capped (naked) AgNPs.[25]

It is necessary to emphasize here that the as-synthesized Cys-AgNPs have bactericidal effects not only by inhibiting the bacterial growth but also by killing the bacteria. Cysteine enhances the role of antibacterial effect of AgNPs by interacting with the biosystems.[11] Although the detailed and exact mechanism of the antibacterial effect of silver nanoparticles is still unclear, there are some clues in the literature that silver nanoparticles can interact with sulfur-containing proteins from cell membrane and phosphorus-containing compounds in cells, attacking the respiratory chain, with cell division leading to cell death.[25,26]

Conclusion

We have reported synthesis and surface modification of colloidal silver nanoparticles with the cysteine by EAB, which is a novel, simple, inexpensive, controlled and green protocol. EAB is used as a reducing agent. The presence of amine and carboxylic acid functional groups on the surface of the colloidal particles leads to formation of hydrogen bond and thereby cross-linking of the colloidal silver nanoparticles which further stabilizes the AgNPs in aqueous solution. Antibacterial experiments demonstrated that the as-synthesized Cys-AgNPs shows excellent antibacterial activity for *E. coli* and *P. aeruginosa* by inhibiting the growth and multiplication of the bacteria. The Cys-AgNPs synthesized by this clean biochemical route without using any additives are expected to find potential applications as antibacterial agents and in other medical and biotechnological fields. This novel approach could be used to synthesize other metal nanoparticles as well and it is under investigation in our laboratory.

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