Antibacterial Activity of Ag⁺ Ion-Containing Silver Nanoparticles Prepared Using the Alcohol Reduction Method

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Abstract: The reduction of Ag⁺ ions in presence of ethyl alcohol as reducer and polymeric protective agent poly(N-vinylpyrrolidone) (PVP) occurs at 70 °C. When no other additives are present in the system, the slow reduction leads to silver nanoparticles being obtained. When the reduction is performed in the presence of higher PVP contents, the mean diameter of the silver ion-containing Ag nanoparticles decreased and a narrow particle size distribution was obtained. The antimicrobial activities of silver ions and Ag⁺ ion-containing silver nanoparticles against Gram-negative Escherichia coli (E. coli) and Gram-positive Staphylococcus aureus (S. aureus) were investigated. These particles exhibited bactericidal activity against E. coli and S. aureus, with the silver ion-containing Ag nanoparticles having greater bactericidal activity against E. coli compared with S. aureus.

Keywords: bactericidal activity, silver, nanoparticle, PVP, E. coli, S. aureus

Introduction

Metal colloids are widely employed because of their optical properties, catalytic activities, and magnetic properties [1,2]. Many studies have been reported for metal nanoparticles of silver, gold, and copper colloids [3-8]. Silver sol was prepared by alcohol reduction in the presence of the polymeric protecting agent poly(N-vinylpyrrolidone) (PVP) [9-11].

Elemental silver and silver salts have been used for decades as antimicrobial agents in curative and preventive health care [11]. Silver ions and silver complexes as antimicrobial agents usually modify microbial activities [12-15]. The various silver complexes are interesting because the antimicrobial activity and other desirable properties can be changed by the type of ligands coordinated to silver ion; e.g., the silver imidazolate. However, triphenylphosphine adduct silver imidazolate has no antimicrobial activity [16,17]. Some forms of silver ions may be hazardous to the environment and protection may be limited in use. Recent advanced research on metal nanoparticles appears to revive the use of reactive metal oxide and silver nanoparticles in various biomedical applications [18-20]. Silver nanoparticles have some advantage over silver salts because they are more stable against dissolution and diffusion to the surface of materials to be protected. Silver salts and silver complexes have been studied in detail, but the antimicrobial activities of silver ion and silver ion-containing Ag nanoparticles have not been reported yet, as far as we know.

In this study, silver nanoparticles of narrow size distribution were prepared by reducing silver ions with ethanol in the presence of PVP as stabilizing agent. The sizes of the Ag nanoparticles were controlled by the reaction temperature and the PVP content. The particle sizes were observed using a particle size analyzer and TEM. We investigated silver ions, nano silver, and Ag⁺ ion-containing silver nanoparticles for their bactericidal effects against E. coli and S. aureus.

Experimental

Materials

Silver nitrate (99.99 %), poly(N-vinylpyrrolidone) (PVP, Mₙ = 40,000), sodium thiosulfate, and sodium sulfide were purchased from Aldrich Co. Absolute ethyl al-
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Table 1. Strains and Media Used for Antimicrobial Tests

<table>
<thead>
<tr>
<th>Microorganism tested</th>
<th>Media used</th>
<th>Incubation Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O157:H7 ATCC 43894</td>
<td>Tryptic soy broth &amp; agar (Difco)</td>
<td>37</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 25923</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Alcohol was purchased from Merck (HPLC grade). For the preparation of the mixture solution, deionized water was used.

Preparation of Metal Colloids

The silver sol was prepared by reduction of Ag\(^+\) ions using absolute ethyl alcohol (as solvent and reducing agent for AgNO\(_3\)) and PVP (as protective agent). All reagents were used without any further purification. PVP (0.6 %, \(w/v\)) was dissolved ethyl alcohol and deionized water (1 mL) under nitrogen atmosphere; silver nitrate (0.849 g, \(5 \times 10^{-2}\) M) was added at room temperature and the resultant mixture was stirred at 70 °C for 6 h and 48 h in a thermostatic water bath, respectively. After this period, the mixture was cooled at 0 °C in an ice bath. Complete reduction of Ag\(^+\) to Ag\(^0\) was evidenced by extraction of the colloid with an aqueous solution of sodium thiosulfate and subsequent addition of sodium sulfide to probe for any remaining Ag\(^+\).

Antimicrobial Activity

The antimicrobial activities of silver ions and Ag\(^+\) ion-containing silver nanoparticles were tested qualitatively using an inhibition zone method. Two different bacteria, *Escherichia coli* O157:H7 ATCC 43894 and *Staphylococcus aureus* ATCC 25923, were used for testing the antimicrobial activities of silver ions, silver nanoparticles, and silver ion-containing nano silver. The bacteria and culture medium are shown Table 1. The LB agar was treated with a high-pressure autoclave at 121 °C and 15 lb/in\(^2\) for 15 min. A filter paper disk was wetted with silver solutions at various concentrations. The LB agar in each Petri dish was inoculated with each bacterium species, and then each paper disk was placed on each Petri dish and pressed gently. The samples were incubated at 37 °C for 48 h. The presence of a clear zone that formed around the disk on the plate medium was recorded as an indication of inhibition against the microbial species. The clear zone on the surface of the test specimen was observed by the naked eye, and compared to ethyl alcohol as a control sample.

Measurements

The particle sizes were observed for their poly-dispersed distribution and diameter range using a particle size analyzer (UPA150); the mean diameter of the volume distribution (MV) equation showed it to be weighted by coarse particles.

\[
MV = \frac{\sum Vd_i}{\sum V_i}
\]

Transmission electron microscopy (TEM) was performed with a Jeol JEM2010. The Ag diameters were measured from 100 particles at three sites selected at random. UV-Visible spectra were measured in 10-mm optical-path-length quartz cuvettes with a Shimadzu 1601 UV-VIS spectrophotometer. X-ray diffraction (XRD) data was collected using a Rigaku D/MAX RINT 2500 with Cu Kα radiation.

Results and Discussion

The variation of the absorbance in the solution containing AgNO\(_3\) and PVP as polymeric protect agent, in terms of the reducing time, is shown in Figure 1. Silver colloids are reduced using ethyl alcohol in the presence of stabilizer, according to

\[
2AgNO_3 + C_2H_5OH \rightarrow 2Ag^0 + 2HNO_3 + CH_3CHO
\]
Figure 2. XRD Patterns of silver nanoparticles; reaction time: 60 min; AgNO$_3$ = 5 × 10$^{-2}$ M; PVP = 0.6 g; (left) before thermal treatment; (right) after thermal treatment.

Figure 3. TEM micrograph of Ag$^+$ ion-containing silver nanoparticles when the reaction time was 60 min; AgNO$_3$ = 5 × 10$^{-2}$ M; PVP = 0.6 g.

The intensity of the surface plasmon band increases during the particle growth process; no distortion of the spectral dependence of the absorbance data was observed, which means that the nanoparticles were growing during the reaction. Addition of sodium sulfide afforded no noticeable precipitate of Na$_2$S, whereas in a comparative, experiment, extracting Ag$^+$ from silver sol for 6 h in the reduction time, 10 % of the remaining Ag$^+$ in a silver colloids was observed. The time need for completion of the reduction was 48 h. The silver colloids were not observed to precipitate silver sulfide. The X-ray diffraction patterns of silver nanoparticles without thermal treatment and the Ag nanocluster at calcined 200 °C for 3 h are presented in Figure 2. The XRD pattern of pure silver ions is known to display peaks at 2θ = 7.9°, 11.4°, 17.8°, 30°, 38°, and 44°. The value of the pure silver lattice constant has been estimated to be a = 4.081, a value that is consistent with a = 4.0862 Å reported by the JCPDS file n°4-0783. This estimation confirmed the hypothesis of particle monocrystallinity [10]. Therefore, silver nanoparticles without thermal treatment contain Ag$^+$ ions and PVP. The hydrophilic amide groups of PVP are bound to the surface of the silver nanoparticles through the strong affinity of N and O atoms for transition metallic clusters, whereas the polyvinyl backbone of PVP forms a hydrophobic domain, which surrounds the silver nanoparticles to prevent agglomeration. PVP serves as both a coordinating agent and a stabilizing agent; it also plays an important role in controlling the size and shape of the silver nanoparticles [11].

Figure 4. Particle size distribution of silver ions containing Ag nanoparticles, measured using a particle size analyzer; AgNO$_3$ = 5 × 10$^{-2}$ M.
Table 2. Antimicrobial Effect of Ag⁺ Ion-Containing Silver Nanoparticle Solutions Treated with Various Concentrations

<table>
<thead>
<tr>
<th>Concentration (µg cm⁻³)</th>
<th>Control</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 0157:H7 ATCC 43894</td>
<td>8ᵃ)</td>
<td>9</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>S. aureus ATCC 25923</td>
<td>8</td>
<td>10</td>
<td>11</td>
<td>13</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

ᵃ) clear zone diameter (mm)

Table 3. Antimicrobial Effect of Silver Nanoparticles, Ag⁺ Ion-Containing Nano-Ag, and Silver Ions at 50 µg cm⁻³

<table>
<thead>
<tr>
<th></th>
<th>Ag-ions</th>
<th>Nano-silver</th>
<th>Ag⁺ ion-containing nano-Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 0517:H7 ATCC 43894</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>S. aureus ATCC 25923</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

+, Clear zone of 8 – 12 mm; ++, clear zone of 12 – 16 mm; ++++, clear zone of 16 – 20 mm

A TEM micrograph and the particle size distribution are shown in Figures 3 and 4. The Ag particles obtained by direct mixing of AgNO₃ and PVP with ethyl alcohol solution had spherical shape. The possibility of controlling the final size and size distribution of metal particles in the nanometer range is the aim of most modern nanochemistry. The particle size distribution was polydisperse with particle sizes ranging from 6 to 50 nm. The mean diameter of Ag particles was 12 nm.

When a disk of repellent is placed in a Petri dish of semisolid agar, bacteria will swim away from the repellent, creating a clear zone around the disk. Usually they sense repellents only at higher concentrations. Considering the growing interest in silver ions containing antimicrobial agents, we decided to test the antibacterial activity of silver ions, silver ion-containing Ag nanoparticles, and pure Ag nanoparticles against E. coli and S. aureus. As the bacteria grew to form a confluent lawn, the extent of growth inhibition could be measured as the extent of the clear zone surrounding the disk. Bacterial inhibition tests against E. coli and S. aureus are shown in Figures 5 and 6. Clear zone diameter of the bacterial inhibition zone was correlated to antibiotic activity of silver particles in Tables 2 and 3. These data are consistent with previously reported studies in which silver ions had effective antimicrobial properties at concentrations of 1 ppm or less [15]. The mechanism of inhibition of silver ions on microorganisms is partially known. It is believed that DNA loses its replication ability and cellular proteins become inactivated on silver ion treatment. Higher concentrations of Ag⁺ ions have been shown to interact with cytoplasmic components and nucleic acids [21]. In addition, micromolar levels of Ag⁺ ions are known to uncouple respiratory electron transport from oxidative phosphorylation; concentrations of AgNO₃ as low as 1 µM are found to inhibit bacterial growth [22]. The clear zone diameter increased as the concentration of silver ion-containing Ag nanoparticles increased, due to bactericide of Ag⁺. These results may indicate the ability of silver nanoparticles to make silver ions more “antimicrobially active”. PVP is homopolymer whose individual structural units contain a hydrophobic vinyl group and a hydrophilic cyclic amide group. On the other hand, PVP is positively charged in aqueous solution because of protonation of the amide nitrogen atoms. The nitrate anions associate with the amphipphilic PVP remaining in
the silver sol. The resulting silver ion-containing nano-Ag particles are stabilized by the capping effect of the PVP. Silver ion-containing nano-Ag is a Ag/PVP nanocomposite that releases silver ions very slowly. The Ag⁺ ion-containing silver nanoparticles are good antibiotic agents against E. coli when compared with S. aureus. It has a thicker wall compared to E. coli, a typical Gram-negative bacteria [23]. Silver nanoparticles act as bactericidal materials against E. coli and S. aureus. It is postulated that the incorporation of silver nanoparticles into the membrane structure occurs. Clear zone diameter between Ag⁺ ion-containing silver nanoparticles and Ag⁺ ions against S. aureus suggest a bacteria membrane exhibiting permeability, leaving the bacterial cells incapable of properly regulating transport through the membrane and causing cell death. These results suggest that S. aureus may have a stronger defense system against silver nanoparticles, even though almost the same phenomenon was shown as a clear zone in the low concentrations of nano-sized particle-treated cells.

**Conclusion**

We used ethyl alcohol as a reducing agent for silver nitrate and PVP as a protective agent. Higher PVP contents decreased the mean diameter and improved the monodispersity of the Ag⁺ ion-containing Ag and Ag nanoparticles. Three different types of silver ions, Ag⁺ ion-containing Ag, and Ag nanoparticles were tested for their antimicrobial activity. Silver nanoparticles exhibited bactericidal activities against E. coli and S. aureus. Ag⁺ ion-containing nano-Ag particles exhibited good antibiotic agent relative to Ag⁺ ions against E. coli. The bactericidal activities depended on the ability to permeability and the penetration rate against the bacteria cell wall.

**References**