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SEM and confocal laser scanning microscopic studies on the antibacterial activity of silver nanocolloid prepared by green synthesis against Escherichia coli

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Abstract

The advent of nanotechnology profoundly transformed the biomedical sciences and greatly enhanced the role of nanoparticles with various applications for solving various problems that threaten human life. Among the metallic nanoparticles extensively used in nanobiomedicines, silver nanoparticles are remarkable due to their unique chemical and physical properties. In this work, the antibacterial activity of silver nanocolloid as a potent killing agent prepared by the green synthesis method with gum rosin (as an economically viable reducing and stabilizing agent) against the Escherichia coli with SEM and confocal laser scanning microscopic (CLSM) were discussed. Ag nanoparticles with a 15-30 nm diameter were observed in TEM and DLS analysis. The obtained results indicated that treatment with Ag nanoparticles induced a morphological change of E. coli from rod shape to spherical morphology due to the cell lysis and the untreated E. coli gave an elongated rod shape.

1. Introduction

The antimicrobial properties of silver nanocolloids have been known since ancient times and remained an important antimicrobial agent until the discovery of antibiotics [1]. Silver was used to prevent the growth of bacteria in drinking water by the ancient Egyptians and Persians [2]. The development of nanotechnology and the emergence of strains of microorganisms resistant to drugs and the has brought renewed interest in silver, particularly in silver nanoparticles [3]. Silver nanoparticles appear to be effective, even against drug-resistant strains of microbe. It is clear that the small size of silver nanoparticles (1 nm to 100 nm) with controllable morphology and monodispersity provides a greater surface area that makes it a very efficient antimicrobial agent [4]. Although silver nanoparticles may be prepared by physical and chemical means, there is great interest in synthesizing silver nanoparticles by using plant extracts as a reducing agent to make biogenic silver nanoparticles [5]. The obtained Ag nanoparticles may be capped with compounds extracted from the plants to make stabilized Ag nanoparticles with the biologically derived compounds coating or modifying the surface of the silver nanoparticles, rendering them more stable physically and chemically [6, 7]. As a synergistic effect, when the compounds extracted from the plant also exhibit antibacterial or therapeutic properties, the biogenic silver nanoparticles may exhibit a synergistic effect, being more effective than either the silver nanoparticles or plant extract used separately [1, 7, 8]. There are some limitations to using plants for the synthesis of nanoparticles. for example, monodisperse Ag nanoparticles with reproducibility based on their properties are used in any field of applied science with significant results [9-13]. However, it is tough to maintain the monodispersity of Ag nanoparticles using plant extract as a green reducing agent [14-17]. Therefore, due to these drawbacks, it is very important to search for the invention of a biological route and direction that can play a key role in the synthesis of monodispersed Ag nanoparticles with tunable size and morphology [18-20]. Thus, a method of preparing green silver nanoparticles based on the colloidal systems to solve the problems mentioned above is desired [21].

In continuing our previous report, the colloidal silver nanoparticles as antibacterial material were prepared based on the new combination of green microemulsion synthesis with gum rosin as reducing and stabilizing agents [22]. The silver nanocolloid was characterized by UV-Vis, DLS, Zeta potential, TEM, and ICP-OES. Ag nanoparticles with a diameter of 15-30 nm were observed in TEM and DLS analysis. A negative zeta potential value of −20.54 mV proved the stability of the rosin-stabilized silver nanoparticles. The Ag nanocolloid has shown an antibacterial activity against model organisms, a gram-negative Escherichia coli NCIM 2931 in Mueller–Hinton (MH) medium, which is hitherto unattempted up to now. The current work focuses on the antibacterial activity study against Escherichia coli bacteria with scanning electron microscopy (SEM) and confocal laser scanning microscopic (CLSM) to approve the antibacterial performance of gum rosin -capped silver nanoparticles. Consequently, we have presented on how silver nanoparticles enhance the antibacterial activity with a simple mechanism.

2. Experimental

2-1. Materials

Silver nitrate ($AgNO_3$) (≥99.0%) and ethanol (99.0%) were purchased from Merck. Chinese gum rosin was received and used as a reducing and stabilizing agent. Deionized water (18 MΩcm-1) which was produced by the reverse osmosis (RO) water purification process.

2-2. Ag nanocolloid preparation

First, an ethanolic solution of gum rosin (10 ml, 20 mg/L) was prepared and then an aqueous solution of silver nitrate (2 ml, 0.01 M) was added during the 5 min and vigorously stirred for 15 min. After that a clear color change from light yellow to dark brown was observed, confirming the formation of Ag nanoparticles. At the end, 30 ml water was added to the above solution and was characterized with UV-Vis, DLS, Zeta, ICP-OES, and TEM analysis [22].

2-3. Colony observation test

After observation of turbidity in tube culture of Escherichia coli bacteria, to observe colony formation assessment, 100 μL volumes of the Escherichia coli bacteria were aspirated and diluted to 10−5. Dilutions were cultured on the Mueller–Hinton (MH) medium for 16–18 h. Colony-forming units were manually counted.

2-4. SEM analysis for morphological observations of Escherichia coli bacteria

To observe the changes of Escherichia coli bacteria treated with Ag nanoparticles and control sample the scanning electron microscopy (SEM, Zeiss EVO 50, Carl Zeiss Meditech, Oberkochen, Germany) was used. In this study, bacterial cells (1×10^5 CFU/mL) were treated with Ag nanoparticles for 24 h in an orbital shaker (200 rpm) at 37 °C. Then the bacterial cells were harvested by centrifugation and washed in PBS, fixed in a mixture of 2.5% glutaraldehyde plus 1% formaldehyde for 24 h, followed by further fixation in 1% aqueous osmium tetroxide for 1 h. The pellets were then subjected to dehydration with ethanol (95 %). For SEM analysis, 5 µL of each sample was placed on an SEM stub dried in ambient condition, and coated with gold [23].

2-5. CLMS test for discrimination of live/dead cells of Escherichia coli bacteria

To study the antibacterial activity for discrimination of live from dead cells based on the membrane integrity for gum rosin-capped Ag nanoparticles on Escherichia coli bacteria the Confocal Laser Scanning Microscopic (CLSM) (710, Carrel Zeiss, Germany) was used and compared with control sample. In brief, 20 µL of bacteria for both sample (with Ag nanoparticles and control) were placed in glass bases plates. Then, 5 µL of AO/EB (1 mg/mL, 0.3 mg/mL, respectively) were added and left for 5 min. Both samples have been examined immediately under confocal microscopy before leakage of dyes from cells after five minutes using the excitation laser lines at 405/530 and 543/640 nm [24,25].

3. Result and discussion

3-1. Colony formation study

Fig. 1- (a and b) presents the Petri plates that were used for the observation of the colony formation for the silver colloid sample and control sample in the E. coli culture. As can be seen from Fig. 1-(a) spherical gum rosin -capped Ag nanoparticles after 24 h of incubation have the maximum capacity to release Ag^+ ions and it showed no bacteria growth and no bacterial colonies were obtained in the E-coli culture for the concentrations of 60 μg·mL−1of used silver nanocolloid compared to the control sample. So, this concentration is effective as there is no E. coli growth, and they did not recover after treatment. The Ag nanoparticles play a vital role in determining its antibacterial effect by binding ions to –OH groups and internalizing through the cellular membranes leading to exposed atoms and available for redox reactions [26-29]. Also, high accumulation of ROS causes damage to nucleic acid leading to apoptosis [30,31].

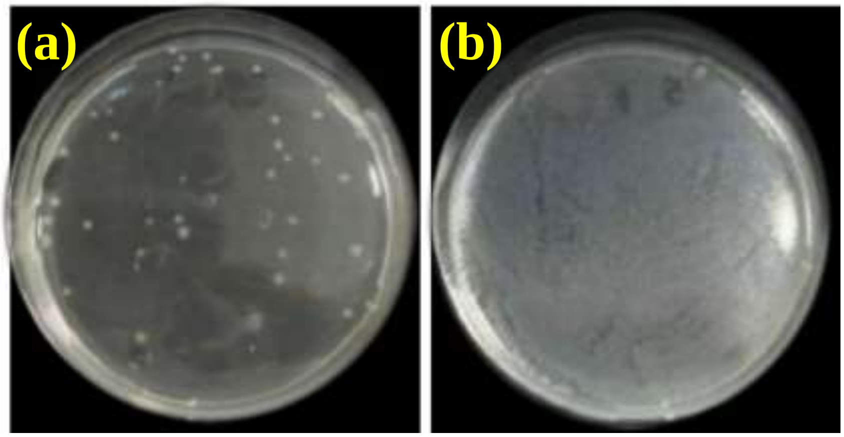

Fig. 1. Photos showing the colonies of E. coli in agar petri dishes (a) control sample and (b) after treatment with 60 µg/ml of the Ag nanocolloid

3-2. CLSM results

Confocal laser scanning microscopic (CLSM) imaging is used for visual differentiation between live and dead cells based on membrane integrity for E. coli (treated with Ag nanoparticles and control sample) with Acridine orange (AO) fluoresces green (stain life cells) and Ethidium bromide (EB) fluoresces red (stain dead cells). Fig. 2 (a and b) represents the distribution of dead/viable bacteria in the gum rosin-capped Ag nanoparticles co-culture tested, whereas in Fig. 2-(a) which corresponds to the control sample (untreated bacteria), only live bacteria appear (green fluorescence). Fig.2-(b) represents the distribution of dead bacteria treated with Ag nanoparticles the viability was about 30% and red fluorescence appeared as a result of dead abundance [32,33].

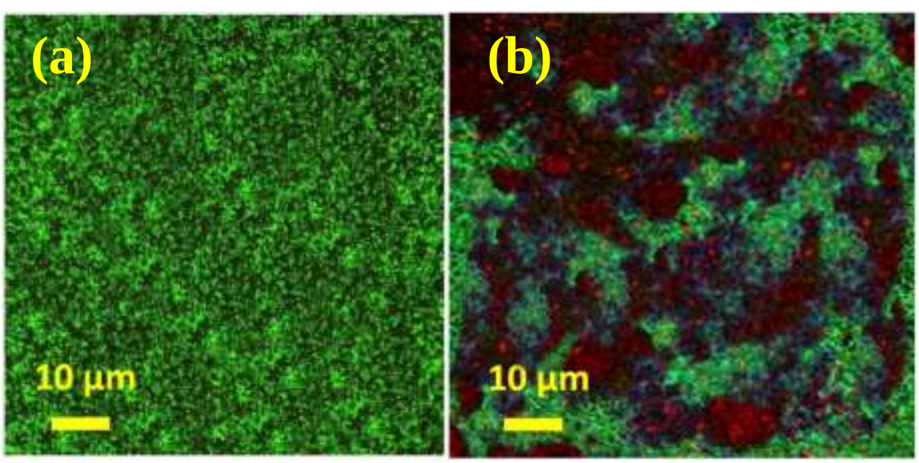

Fig. 2. CLSM images of (a) E. coli (untreated), (b) E. coli (treated with 60 µg/ml stabilized Ag nanoparticles)

3-3. SEM results

The SEM images of control and treated bacterial cells with Ag nanoparticles are shown in Figures 3 (a and b). As can be seen from SEM images, it is indicated that treatment with Ag nanoparticles induced a morphological change of E. coli from rod shape to spherical morphology due to the cell lysis and the untreated E. coli gave an elongated rod shape. The SEM image of the E. coli treated with gum rosin -capped Ag nanoparticles at 60 revealed the shrinkage of the E. coli cell, with the formation of a porous structure and a distorted shape, from the adverse effect of silver nanoparticles [23,34]

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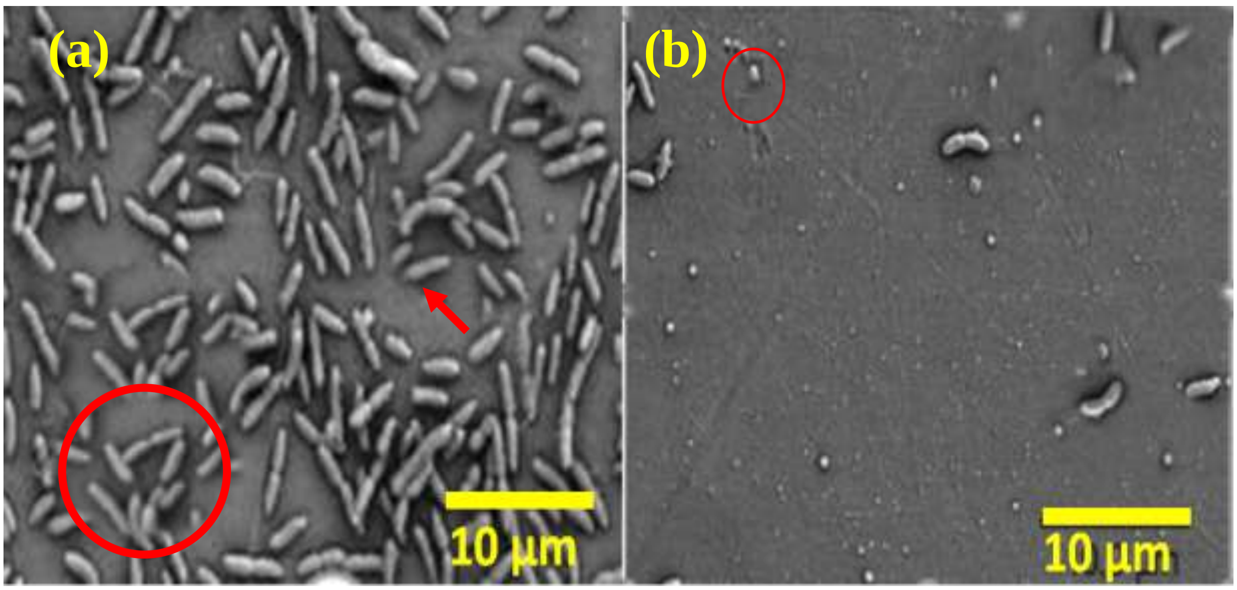

Fig. 3. SEM images of (a) E. coli (untreated), (b) E. coli (treated with 60 μg/ml stabilized Ag nanoparticles)

3-4. Proposed mechanism

The exact mechanism of the antibacterial actions of silver antibacterial has not been entirely clarified. A variety of the antibacterial effects have been proposed. (1) release of silver ions and adhere to or pass through cell wall and cytoplasmic membrane. (2) denature ribosomes done by silver ions and synthesis of protein inhibited. (3) silver ions deactivate respiratory enzyme on cytoplasmic membrane and consequently ATP production is terminated. (4) Membrane disruption by reactive oxygen species reactive oxygen species (ROS) produced by the broken electron transport chain. (5) Interference of deoxyribonucleic acid (DNA) replication: silver and reactive oxygen species bind to deoxyribonucleic acid and prevent its replication and cell multiplication. 6) membrane denaturation with accumulated silver nanoparticles in the pits of cell wall. (7) Perforation of membrane due to the silver nanoparticles move across cytoplasmic membrane and finally release organelles from cell [35, 36].

4. Conclusion

In summary, a silver nanocolloid was successfully synthesized via a green route- using gum rosin without any environmentally harmful by-products. Furthermore, economical Ag nanocolloid was assessed for the subsequent anti-bacterial properties against Escherichia coli. Finally, the morphological observations were made after and before the Ag nanocolloid treatment to study microorganisms by SEM and CLSM analysis. The obtained results indicated that treatment with Ag nanoparticles induced a morphological change of E. coli from rod shape to spherical morphology due to the cell lysis with 70% of red fluorescence appeared as a result of dead abundance. Hence, Ag nanocolloid and their potent antibacterial agents can be applied in several antibacterial applications.

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