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Journal of Colloid and Interface Science 275 (2004) 177–182
JOURNAL OF Colloid and Interface Science
www.elsevier.com/locate/jcis

Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria

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Received 3 October 2003; accepted 5 February 2004

Available online 18 March 2004

Abstract

The antimicrobial activity of silver nanoparticles against *E. coli* was investigated as a model for Gram-negative bacteria. Bacteriological tests were performed in Luria–Bertani (LB) medium on solid agar plates and in liquid systems supplemented with different concentrations of nanosized silver particles. These particles were shown to be an effective bactericide. Scanning and transmission electron microscopy (SEM and TEM) were used to study the biocidal action of this nanoscale material. The results confirmed that the treated *E. coli* cells were damaged, showing formation of "pits" in the cell wall of the bacteria, while the silver nanoparticles were found to accumulate in the bacterial membrane. A membrane with such a morphology exhibits a significant increase in permeability, resulting in death of the cell. These nontoxic nanomaterials, which can be prepared in a simple and cost-effective manner, may be suitable for the formulation of new types of bactericidal materials.

Keywords: Bacteria; Bactericide; *E. coli*; Nanoparticles; Silver

1. Introduction

Nanosized inorganic particles, of either simple or composite nature, display unique physical and chemical properties and represent an increasingly important material in the development of novel nanodevices which can be used in numerous physical, biological, biomedical, and pharmaceutical applications [1–8]. A number of recent achievements offer the possibility of generating new types of nanostructured materials with designed surface and structural properties [9–13].

The preparation of uniform nanosized drug particles with specific requirements in terms of size, shape, and physical and chemical properties is of great interest in the formulation of new pharmaceutical products [5,6,10,14,15]. Resistance of bacteria to bactericides and antibiotics has increased in recent years due to the development of resistant strains. Some antimicrobial agents are extremely irritant and toxic and there is much interest in finding ways to formulate new types of safe and cost-effective biocidal materials. Previous studies have shown that antimicrobial formulations in the form of nanoparticles could be used as effective bactericidal materials [16,17]. Recently, Klabunde and co-workers demonstrated that highly reactive metal oxide nanoparticles exhibit excellent biocidal action against Gram-positive and Gram-negative bacteria [18]. Thus, the preparation, characterization, surface modification, and functionalization of nanosized inorganic particles opens the possibility of formulation of a new generation of bactericidal materials.

It is well known that silver ions and silver-based compounds are highly toxic to microorganisms [19,20] showing strong biocidal effects on as many as 16 species of bacteria including *E. coli* [21]. Thus, silver ions, as an antibacterial component, have been used in the formulation of dental resin composites [22–24] and ion exchange fibers [25] and in coatings of medical devices [26–29]. Recently, Tiller and co-workers showed that hybrids of silver nanoparticles with amphiphilic hyperbranched macromolecules exhibit effective antimicrobial surface coatings [30].

Our previous study has shown that stable and highly concentrated aqueous dispersions of silver nanoparticles of narrow size distribution can be simply prepared by reducing silver ions with ascorbic acid in the presence of Daxad 19

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0021-9797/$ – see front matter
doi:10.1016/j.jcis.2004.02.012

(the sodium salt of a high-molecular-weight naphthalene sulfonate formaldehyde condensate) as stabilizing agent [31]. Here we investigate the biocidal action of these particles against *E. coli*. The ultimate goal was to study the interaction between bacteria and silver nanoparticles by means of SEM and TEM microscopy. To our knowledge, the antibacterial activity of silver ions is well known and has been studied in detail [19–21], while the antibacterial activity of nontoxic elementary silver, in the form of nanoparticles, has not been reported in the literature.

2. Materials and methods

2.1. Materials

Escherichia coli strain B was purchased from Presque Isle Cultures, PA. The components of the Luria–Bertani (LB) medium used in growing and maintaining the bacterial cultures were supplied by Difco Laboratories.

Silver nitrate and ascorbic acid were obtained from Aldrich Chemicals. The Daxad 19 surfactant was supplied by Hampshire Chemicals.

2.2. Preparation of silver nanoparticles

The silver nanoparticles were precipitated following the procedure described in our previous paper [31]. However, slight modifications were made in order to obtain smaller particles with higher specific surface area and narrower size distribution.

In a typical experiment, the silver hydrosols were prepared by adding, under agitation, 10 cm^3 of an aqueous 1 mol dm^{-3} ascorbic acid solution at a flow rate of 3 $cm^3\,min^{-1}$ into 90 cm^3 of an aqueous solution containing 5 wt% of Daxad 19 and 0.33 mol dm^{-3} $AgNO_3$. The reacting solutions were agitated with a stirrer at 900 rpm at room temperature.

To remove the surfactant and excess silver ions, the resulting silver precipitate was washed five times with deionized water. Finally, the nanosize silver was obtained as a dried powder by freeze drying and kept for future experiments. The obtained powder was fully redispersed in deionized water by sonication and therefore aqueous dispersions of silver nanoparticles at the desired concentrations were easily made.

2.3. Bactericidal test

To examine the bactericidal effect of silver nanoparticles on Gram-negative bacteria, approximately 10^5 colony-forming units (CFU) of *E. coli* strain B were cultured on LB agar plates supplemented with nanosized silver particles in concentrations of 10 to 100 μg cm^{-3}. Silver-free LB plates cultured under the same conditions were used as a control. The plates were incubated for 24 h at 37 °C and the numbers of colonies were counted. The counts on the three plates corresponding to a particular sample were averaged.

To examine the bacterial growth rate and to determine the growth curve in the presence of silver nanoparticles, *E. coli* bacteria were grown in 100 cm^3 of liquid LB medium supplemented with 10, 50, and 100 μg of these particles per cm^3 of medium. Growth rates and bacterial concentrations were determined by measuring optical density (OD) at 600 nm each 30 min (OD of 0.1 corresponds to a concentration of 10^8 cells per cm^3).

2.4. Characterization

The optical properties of the silver hydrosols and the optical density of the bacterial cultures in liquid LB medium supplemented with these particles were evaluated in 10 mm optical path length quartz cuvettes using a Perkin–Elmer Lambda 6 UV–vis spectrophotometer. The size and the morphology of the silver nanoparticles were examined by transmission electron microscopy (TEM). The sizing of the samples was carried out from transmission electron micrographs using the software Image Tool for Windows (Version 2.0), while data were analyzed by means of the software Microcal Origin 6.0.

Specific surface area (SSA) measurements of the dry powder of silver nanoparticles were made by single-point nitrogen adsorption, using a Micromeritics FlowSorb II 23000 instrument.

The size and morphology of the bacteria were examined by scanning electron microscopy (SEM) and by the Voyager X-ray microanalysis and digital imaging system, while the qualitative chemical composition of the bacterial membranes was assayed by EDAX. Prior to SEM analysis, untreated (native) and treated (10^7 CFU in 100 cm^3 of LB supplemented with 50 μg cm^{-3} of silver nanoparticles for 4 h) bacterial cells were deposited on a Millipore filter and washed with deionized water. In addition, TEM was used as a complementary technique to examine sections of the treated bacteria, using standard procedures for fixing and embedding sensitive biological samples, which are described elsewhere [18,32].

3. Results

Highly concentrated and stable silver hydrosol was obtained under the conditions described in Section 2. Transmission electron micrography shows silver to be nanosized and well dispersed (Fig. 1). The absorption spectrum of this sample displayed in Fig. 2 shows a well-defined plasmon band at 405 nm, characteristic of nanosized silver. The corresponding particle size distribution histogram of the obtained nanoparticles is given in the upper right corner of Fig. 2. In comparison to our previous investigation [31], these particles are slightly smaller, with a modal diameter of 12 nm. The

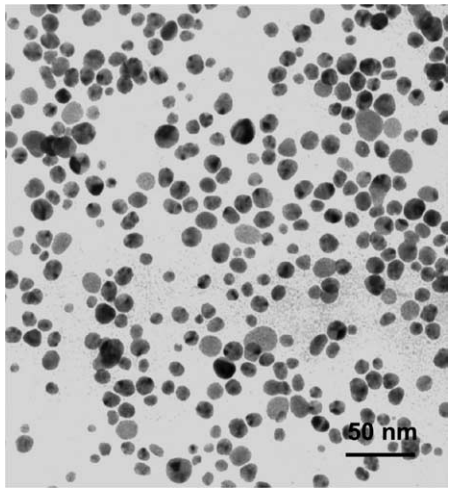

Fig. 1. Transmission electron micrograph of the silver nanoparticles used in this work.

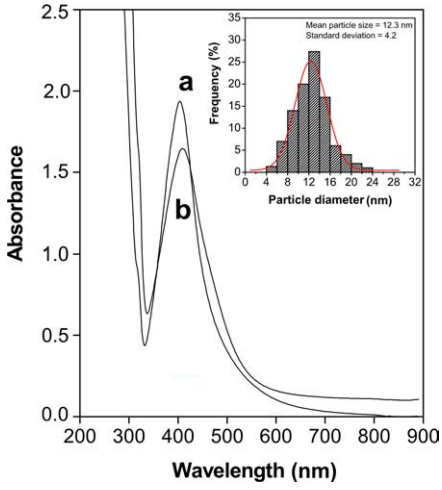

Fig. 2. Absorption spectra of silver hydrosols recorded immediately after the precipitation process (a) and after freeze-drying and redispersion in deionized water (b). The inserted figure displays the particle-size-distribution histogram of the silver nanoparticles, evaluated from the corresponding TEM micrograph.

specific surface area (SSA) of a dried powder of this precipitate was 158 $m^2\,g^{-1}$. After freeze drying, the obtained powder was readily redispersed by mild ultrasonic agitation in deionized water. Indeed, absorption spectrum b in Fig. 2 confirms the colloidal stability and uniformity of the silver hydrosol obtained after this process.

Antibacterial tests were performed against the Gram-negative bacterium *E. coli*, strain B, on LB agar plates containing different concentrations of nanoparticles. Fig. 3 shows the number of bacterial colonies grown on LB plates as a function of the concentration of silver nanoparticles when approximately 10^5 CFU were applied to the plates. The presence of these particles at a concentration of 10 $\mu g\,cm^{-3}$ inhibited bacterial growth by 70%. The size of bacterial colonies grown on plates with more than 20 $\mu g\,cm^{-3}$ of nanoparticles was significantly reduced and

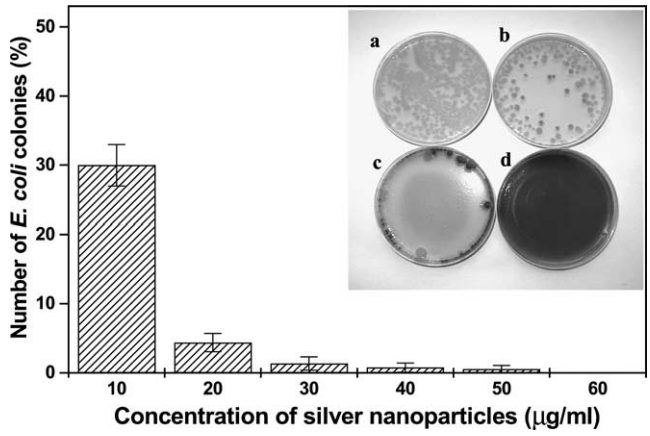

Fig. 3. Number of *E. coli* colonies as a function of the concentration of silver nanoparticles in LB agar plates expressed as a percentage of the number of colonies grown on silver-free control plates. The photograph inserted in the upper right corner shows LB plates containing different concentrations of silver nanoparticles: (a) 0, (b) 10, (c) 20, and (d) 50 $\mu g\,cm^{-3}$.

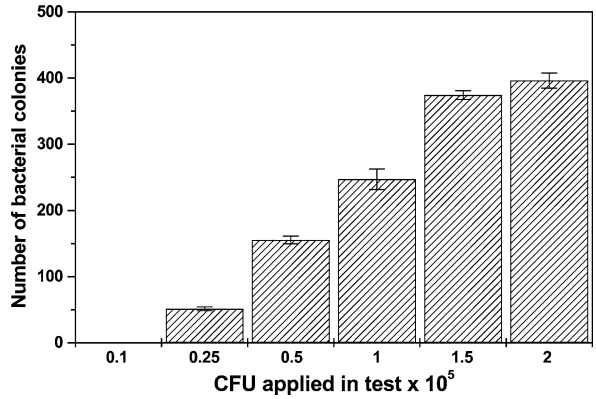

Fig. 4. Number of *E. coli* colonies grown up on LB agar plates containing 20 $\mu g\,cm^{-3}$ of silver nanoparticles as a function of the number of colony-forming units (CFU) applied in the experiments.

the colonies were mostly located at the edges of the agar plates. A concentration of 50–60 $\mu g\,cm^{-3}$ caused 100% inhibition of bacterial growth. As expected, the inhibition of bacterial growth depends on the number of cells applied in the test. Fig. 4 displays the relation between CFU and the number of bacterial colonies grown in the presence of 20 $\mu g\,cm^{-3}$ of silver nanoparticles. If 10^4 CFU were applied to the plate, a concentration of nanoparticles of 20 $\mu g\,cm^{-3}$ completely prevented bacterial growth.

The dynamics of bacterial growth was also monitored in liquid LB medium supplemented with 10^7 *E. coli* cells and with 10, 50, and 100 $\mu g\,cm^{-3}$ of silver nanoparticles. At all these concentrations, the nanoparticles caused a growth delay of *E. coli*; increasing the concentration of nanoparticles increased this growth delay (Fig. 5).

SEM microscopy was used to evaluate the surface morphology of both the native (Fig. 6a) and the treated *E. coli* (Fig. 6b) in LB medium. The treated bacterial cells were significantly changed and showed major damage, which was characterized by the formation of "pits" in their cell walls.

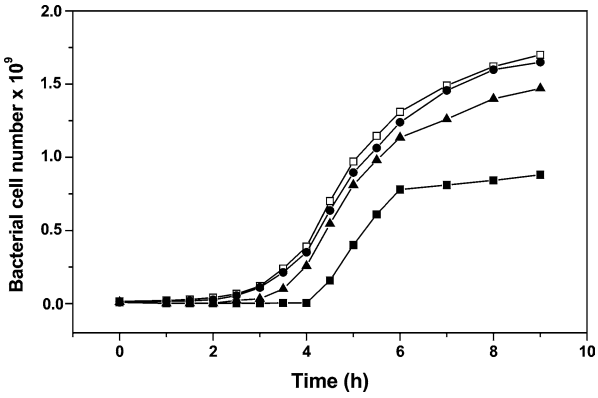

Fig. 5. Growth curves of *E. coli* in LB medium inoculated with 10^7 CFU of bacteria in the presence of different concentrations of silver nanoparticles: (□) 0, (●) 10, (▲) 50, and (■) 100 μg cm^{-3}.

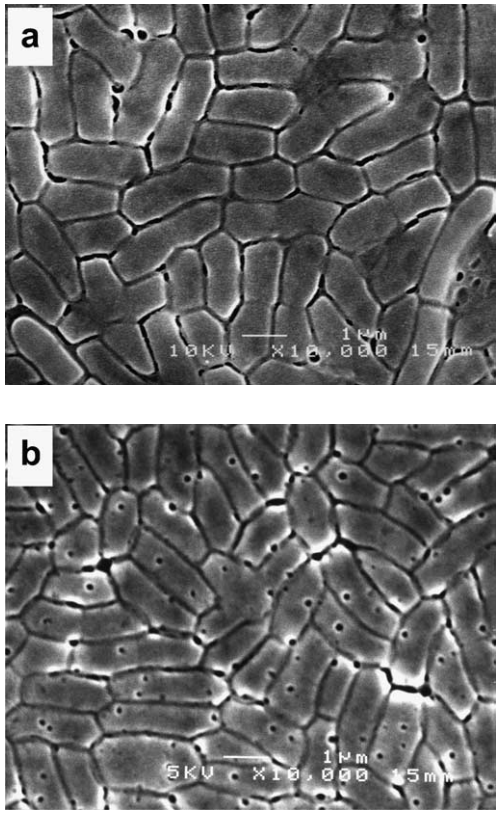

Fig. 6. Scanning electron micrographs of native *E. coli* cells (a) and cells treated with 50 μg cm^{-3} of silver nanoparticles in liquid LB medium for 4 h (b).

The EDAX qualitative chemical analysis of these samples showed that silver nanoparticles were incorporated into the membrane of the treated bacterial cells (Fig. 7, spectrum b). Indeed, TEM analysis clearly showed that the nanoparticles were accumulated in the membrane, while some of them successfully penetrated into the cells (Fig. 8). In addition, leaking of intracellular substances and coagulation of nanosized particles at the bacterial surface are also seen in the same TEM micrograph.

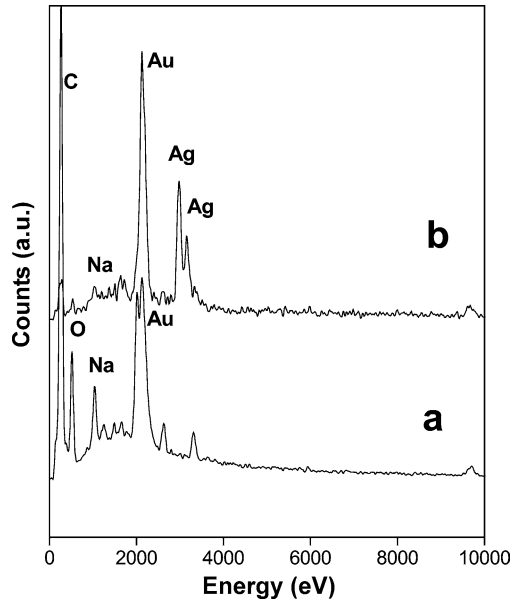

Fig. 7. EDAX spectra of native *E. coli* (a) and *E. coli* treated with 50 μg cm^{-3} of silver nanoparticles in liquid LB medium for 4 h (b).

4. Discussion

Since Klabunde and co-workers demonstrated that reactive metal oxide nanoparticles show excellent bactericidal effects [18], it is of great interest to investigate the use of other inorganic nanoparticles as antibacterial materials. Much less is known about the biocidal effects of noble-metal particles. It has been known for a long time that silver ions and silver compounds are highly toxic to most bacteria [19–21], while just a few rare strains are silver-resistant [33–35]. Recently it was shown that highly concentrated and nonhazardous nanosized silver particles can easily be prepared in a cost-effective manner [31] and tested as a new type of bactericidal nanomaterial.

In this study, the application of silver nanoparticles as an antimicrobial agent was investigated by growing *E. coli* on agar plates and in liquid LB medium, both supplemented with silver nanoparticles. There are distinct differences between these two methods. When nanoparticles were present on LB agar plates, they could completely inhibit bacterial growth (Fig. 4). However, inhibition depends on the concentration of the silver nanoparticles as well as on the CFU of bacteria used in the experiments. Since the high CFU applied in this study are rarely found in real-life systems, it appears that these particles could have an excellent biocidal effect and effectiveness in reducing bacterial growth for practical applications such as the formulation of various biocidal materials.

In contrast, silver nanoparticles in liquid medium, even at high concentrations, caused only growth delay of *E. coli* (Fig. 5). The concentration of the nanoparticles gradually decreases, allowing resumed growth of bacterial cells. This process is governed by the interaction of these particles with intracellular substances of the destroyed cells, causing their

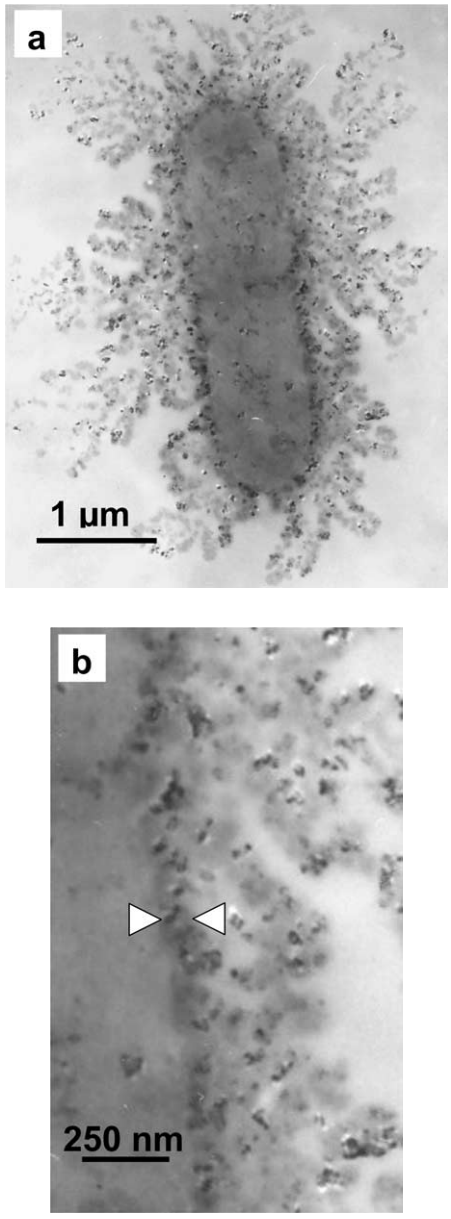

Fig. 8. Transmission electron micrograph of *E. coli* cell treated with 50 µg cm^{-3} of silver nanoparticles in liquid LB medium for 1 h (a) and enlarged view of the membrane of this cell (b).

coagulation and removal from the liquid system. SEM microscopy shows macroscopic aggregates composed of nanosized silver particles and dead bacterial cells (Fig. 9). Obviously, these particles have only a limited use as biocidal materials in liquid systems because of their low colloidal stability.

The mechanism of inhibitory action of silver ions on microorganisms is partially known. It is believed that DNA loses its replication ability and cellular proteins become inactivated on Ag^+ treatment [36]. In addition, it was also shown that Ag^+ binds to functional groups of proteins, resulting in protein denaturation [21]. The obvious question is how nanosize silver particles act as biocidal material against

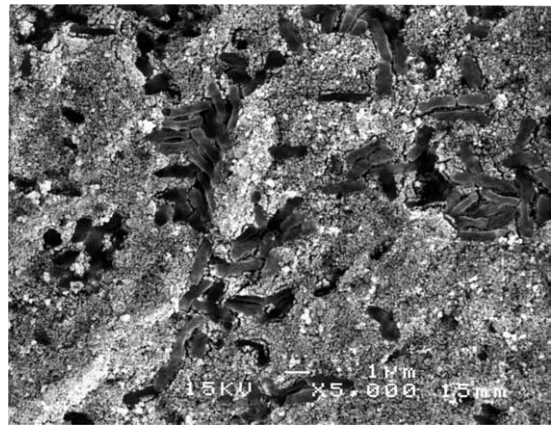

Fig. 9. SEM micrograph of bacteria and nanoparticle aggregates.

E. coli. There are reports in the literature that show that electrostatic attraction between negatively charged bacterial cells and positively charged nanoparticles is crucial for the activity of nanoparticles as bactericidal materials [18,37]. However, silver particles used in this study are negatively charged [31]. While the mechanism of the interaction between these particles and the constituents of the outer membrane of *E. coli* is unfortunately still unresolved, it would appear that, despite their negative surface charge, they somehow interact with "building elements" of the bacterial membrane, causing structural changes and degradation and finally, cell death. Indeed, the TEM analysis (Fig. 8) and the existence of elementary silver in the membranes of treated bacteria, detected by EDAX (Fig. 7), confirm the incorporation of silver nanoparticles into the membrane structure. This observation is crucial for explaining the antibacterial mode of these particles. It is clear that treated bacteria also show significant changes in and damage to membranes, which are recognized by the formation of "pits" on their surfaces (Fig. 6b). A similar effect was described by Klabunde and co-workers [18] when *E. coli* bacteria were treated with highly reactive metal oxide nanoparticles. A bacterial membrane with this morphology exhibits a significant increase in permeability, leaving the bacterial cells incapable of properly regulating transport through the plasma membrane and, finally, causing cell death. It is well known that the outer membrane of *E. coli* cells is predominantly constructed from tightly packed lipopolysaccharide (LPS) molecules, which provide an effective permeability barrier [38–40]. Recently, Amro and co-workers [41] have shown that metal depletion may cause the formation of irregular-shaped pits in the outer membrane and changed membrane permeability, which is caused by progressive release of LPS molecules and membrane proteins. We may speculate that a similar mechanism causes the degradation of the membrane structure of *E. coli* during treatment with silver nanoparticles. Extensive investigations directed to better understanding of interaction between silver nanoparticles and bacterial components should shed light on the mode of action of this nanomaterial as a biocidal material.

Finally, this study shows that silver nanoparticles have excellent antibacterial activity against *E. coli*. This work, following previous research [18], integrates nanotechnology and bacteriology, leading to possible advances in the formulation of new types of bactericides. However, future studies on the biocidal influence of this nanomaterial on other Gram-positive and Gram-negative bacteria are necessary in order to fully evaluate its possible use as a new bactericidal material.

Acknowledgments

This work was supported by the Ministry of Science and Technology of the Republic of Croatia, Grants 0098126 and 0098080.

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