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Characterization and antimicrobial activity of silver nanoparticles prepared by a thermal decomposition technique

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Abstract Recently, there has been an increasing need of efficient synthetic protocols using eco-friendly conditions including low costs and green chemicals for production of metal nanoparticles. In this work, silver nanoparticles (silver NPs) with average particle size about 10 nm were synthesized by using a thermal decomposition technique. Unlike the colloidal chemistry method, the thermal decomposition method developed has advantages such as the high crystallinity, single-reaction synthesis, and easy dispersion ability of the synthesized NPs in organic solvents. In a modified synthesis process, we used sodium oleate as a capping agent to modify the surface of silver NPs because the oleate has a C_{18} tail with a double bond in the middle, therefore, forming a kink which is to be effective for aggregative stability. Importantly, the as-synthesized silver NPs have demonstrated strong antimicrobial effects against various bacteria and fungi strains. Electron microscopic studies reveal physical insights into the interaction and bactericidal mechanism between the prepared silver NPs and tested bacteria in question. The observed excellent antibacterial and antifungal activity of the silver NPs make them ideal for disinfection and biomedicine applications.

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1 Introduction

Over the decades, silver and its compounds have been used extensively in many bactericidal applications [1]. As an antimicrobial agent, the silver was widely applied in wound treatment, medical devices, water purification, air treatment, cosmetics, aqueous paints, clothing, etc. [2]. Along with the rapid development of nanotechnology, special attention has been focused on the production of silver nanoparticles (silver NPs) because of their exhibited stronger antimicrobial activity and wider range of green applications [3]. Besides their antimicrobial features, the silver NPs also possess other outstanding properties. The silver NPs exhibit strong optical features, i.e., surface plasmon resonance; in which that property made this material suitable for biological sensing and imaging [3]. Due to their high electrical conductivity, the silver NPs are also applied in conductive inks, adhesives, and pastes for a variety of advanced electronic devices [4, 5].

Up to now, many different methods are known for production of the silver NPs [6–10]. Most of them are chemical methods, because they have the advantage of producing the NPs with a distinct size distribution and uniformity [6, 7]. However, chemical methods have drawbacks of particle aggregation and low stability of the silver NPs in solution. The instability of the silver NPs upon application can be problematic since aggregation decreases the specific surface lowering the antimicrobial and catalytic activity [8]. To overcome these issues, the capping and stabilizing agents [9, 10], e.g., polyvinylpyrrolidone (PVP), were used to prevent aggregation and agglomeration of the NPs; however, these reagents showed a toxic effect to the environment and human health. To avoid the use of solvents and toxic reagents, there is an increasing need to eco-friendly approaches for the production of NPs, referred as "green synthesis." Among the existing chemical methods, the photochemical production of the silver NPs offers excellent opportunities to develop "green chemistry" synthesis routes because of its flexibility, low-cost, and reproducible synthesis [11–13]. In a recent work [14], we have produced the finely-dispersed colloidal silver NPs by using a modified photochemical method (named as the modified Tollens technique). With the use of simultaneous UV irradiation during the reduction process, the formation of silver NPs colloid with controllable diameter and narrow size distribution could be achieved. It was found that the developed colloidal solutions of silver NPs could exist in the form of very stable aqueous dispersion up to several months and exhibited a noticeable antibacterial activity [15]. Particularly, it was realized that an advanced synthetic technique and greater stability of fine silver NP's dispersions resulted in the significant enhancement of their antibacterial activity. The observed advantages of aqueous dispersions of the silver NPs made them ideal for green medicinal, microbiological, and other applications [14, 15]. However, the colloidal chemistry method could not be scaled up because of its long synthesis time and particle agglomeration during storage process. In addition, the use of the colloidal chemistry method for preparation of the silver NPs is difficult to obtain finely dispersed silver NPs due to the complex synthesis conditions [16–20]. Therefore, further development of synthetic routes is necessary to produce the highly stable silver NPs with lower costs and shorter reaction time for various antimicrobial applications.

In the present work, a versatile and effective technique for the preparation of silver NPs via a modified thermal decomposition method is demonstrated. The silver NPs with narrow size distribution and high crystallinity are obtained. Besides, these silver NPs can be easily dispersed in organic solvents. We also have demonstrated that the prepared silver NPs possess excellent bactericidal and fungicidal activity against tested microorganisms. Various bacterial and fungal strains as a standard model have been chosen to demonstrate the antibacterial and antifungal effects of the prepared silver NPs. The minimum inhibitory concentrations (MIC) of the silver NPs against the growth of *Escherichia coli* and *Staphylococcus aureus* bacteria were found ~5.21 mg/L and ~6.58 mg/L, respectively. The MIC of the silver NPs against the growth of *Pichia pastoris* fungi was revealed ~3.16 mg/L. Some physical insights into the interaction and bactericidal mechanism of the silver NPs were also provided by adapting electron microscopic analyses.

2 Materials and methods

2.1 Materials

Silver nitrate ($AgNO_3$, >99 %) was purchased from Shanghai Chemical Reagent Co. Ltd., sodium oleate (>97 %) was purchased from Tokyo Chemical Industries. All chemicals were used as received and without additional purification.

2.2 Synthesis of silver NPs

In the typical synthesis, silver nitrate (10 mmol) was dissolved in deionized water (100 ml) following an addition of sodium oleate (11 mmol). The mixture was stirred vigorously with a magnetic bar for 2 h at 80 °C. The obtained solution was separated by filtration and then washed with deionized water several times to remove remaining sodium nitrate. The obtained silver oleate was dried in the vacuum oven overnight. After drying, the silver-oleate complex of white powder in a flask was slowly heated from room temperature to 330 °C with a heating rate of 2 °C/min and kept at this temperature under N_2 gas blanket. After 1 hour, the reaction solution was cooled to room temperature and then washed with toluene and ethanol for several times to collect silver nanoparticles.

2.3 Physical and chemical measurements

Transmission electron microscope (TEM, JEOL-JEM 1010) was conducted to determine the morphology and distribution of the silver NPs. The samples for TEM characterization were prepared by placing a drop of a colloidal solution on a formvar-coated copper grid, which was dried at room temperature. The composition of the silver NPs was characterized by Energy-dispersive X-ray (EDX, 5410 LV JEOL). The crystalline structure of the silver NPs prepared was analyzed by X-ray diffraction (XRD, Bruker D5005) using CuKα radiation ($\lambda = 0.154$ nm) at a step of 0.02° (2θ) at room temperature. The background was subtracted with the linear interpolation method. The UV–vis absorbance spectra were recorded using a HP 8453 spectrophotometer, and 10 mm path length quartz cuvettes were used for the measurement of the spectra. The silver concentration was determined using a method of atomic absorption spectroscopy (AAS, Shimadzu AA-6300).

2.4 Microbiological assays

2.4.1 Bacterial/fungal strains and media

Various bacterial/fungal strains as a standard model were chosen including the common bacterial strains of Gram-negative *Escherichia coli* (ATCC 43888), Gram-positive *Staphylococcus aureus* (ATCC 43300), and the fungal strain of *Pichia pastoris*. All these strains were provided from the Department of Virology at the National Institute of Hygiene and Epidemiology (NIHE) in Hanoi.

The growth of cell cultures was executed in a Luria–Bertani (LB) medium (1 % tryptone, 0.5 % yeast extract and 1 % NaCl, pH 7). Next, the culture medium containing bacteria was kept in an incubator for 24 h at the temperature 37 °C; then the content of bacterial culture in it was 10^8 CFU/ml, where the CFU is the colony forming unit.

2.4.2 Turbidity assay in LB medium

The dispersions of silver NPs in toluene of various concentrations ranging from 0 mg/L to 10 mg/L were prepared. Then the silver NPs solutions were added to the bacteria suspensions at different silver concentrations, incubated at 37 °C with shaking at 200 rpm for 24 h. The turbidities of bacteria were observed and clear tube demonstrated no bacteria growth. In this method, the control tube will be used to test the growth of bacteria in the LB media without the presence of silver NPs.

2.4.3 Bacterial/yeast growth curve assays in LB medium

For a growth inhibition determination assay, 10^5 cells/ml of each bacteria were inoculated in test tubes containing 5 ml of Luria–Bertani media (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl). The tubes were incubated for 18–30 h with tested bacteria/yeast. In order to determine the growth curves of bacteria/yeast in the presence of the silver NPs, tested bacteria/yeast were grown in 100 ml of liquid LB medium supplemented with different concentrations of the silver NPs at 37 °C, 200 rpm. Control solutions were treated similarly, but without exposure to the silver NPs for a comparative purpose. Growth rates and bacteria/yeast concentrations were determined by measuring optical density (OD) at 600 nm every 60 min. The assays were performed in triplicate.

2.4.4 Standard dilution micromethod assay in agar plates

The standard dilution micromethod was applied in clarifying the antibacterial activity tests on the agar plates [15]. To get a uniform distribution, the nutrient agar medium was heated to 50 °C. Next, 10 ml of each silver NPs solution was added into Petri plates containing 25 ml of nutrient agar medium. Total volume in each Petri plate was kept 35 ml and the mixing solution was solidified with agar after 15 min. After that 100 µl of a suspension of *E. coli* bacteria was pipetted and spread on the surface of agar medium containing silver NPs. The Petri plates were incubated at 37 °C for 24 h in a shaking incubator (150 rpm) to encourage bacterial cell growth. The intensity of bacterial growth on agar plates with silver NPs of variable concentration was monitored by the naked eye and stereo microscope (ZMS800, Nikon). All the tests were compared with the *E. coli* growth intensity on the agar plate in the absence of silver NPs.

2.5 Ultrathin sectioning sample preparation of bacteria cells

In order to obtain further understanding of the bactericidal and interaction mechanism of the silver NPs, the ultrathin sectioning technique was carried out to observe the ultrastructural changes of bacterial cells destroyed by action of the silver NPs. After the strain of bacteria (i.e., *S. aureus*) were exposed to the silver NPs, the samples were collected and fixed by 2.5 % glutaraldehyde in cacodylate buffer (0.1 M) for 30 min at room temperature; washing the fixed samples by cacodylate buffer three times for 10 min each, then transferred to 1 % OsO_4/cacodylate buffer for 1 h. The samples were then dehydrated by using a series of alcohol with 50, 70, 80, 90, and 100 % (two times ×5 min), and then propylene oxide (three times ×5 min). The samples were infiltrated and finally embedded in Epon 812 at 60 °C for 24 h. The polymerized samples were sectioned into ultrathin slices 60–90 nm in thickness, and placed on the collodion-coated copper grids (300 meshes). The analyses of ultrastructural changes of interior of the bacteria cells were conducted by transmission electron microscopy (TEM, JEM 1010, JEOL).

3 Results and discussion

3.1 Characterization of silver NPs

The overall synthetic procedure of the silver NPs is depicted in Fig. 1. Firstly, the silver-oleate precursors were prepared from the reaction of the silver nitrate ($AgNO_3$) and sodium oleate ($C_{17}H_{33}COONa$). Next, the thermal decomposition of the silver-oleate complex was conducted to produce the silver NPs. When dried silver-oleate complex was heated above 200 °C, the silver-oleate complex was dissociated into free silver cations and oleate ions. At a higher temperature (>300 °C, the decomposition of oleate ions occurred, which in-situ generated some products such as CO, CO_2, H_2, water, ketones, esters, and other hydrocarbons [21]. Among them, C, CO, and H_2 are responsible for the reduction of

Ag^+ cation to Ag^0 silver nanoparticles with average particle sizes about 10 nm [22].

This thermal decomposition method offers some advantages such as the high crystallinity of the synthesized NPs and their high dispersion ability in various organic solvents (e.g., hexane and toluene) due to the oleate ligand capped on their surface. In our work, the surface of silver NPs was modified by the sodium oleate because the oleate has a C_{18} tail with a double bond in the middle, therefore, forming a kink. Such kinks were found to be necessary for effectively aggregative stability. Indeed the stearic acid [$CH_3(CH_2)_{16}COOH$] without double-bond in its C_{18} tail could not stabilize suspensions [23]. Previous experimental results also indicated that the sodium oleate agent has been effectively used for synthesis of stable nanocrystals such as copper and silver nanoparticles (Cu, Ag) and iron oxide nanocrystals (Fe_3O_4, γ-Fe_2O_3) from thermolysis of metal-oleate complexes [24–27].

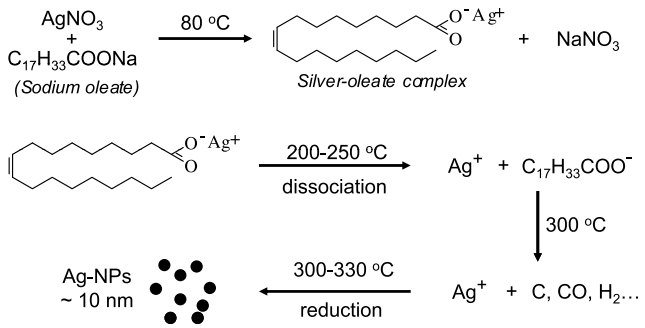

Fig. 1 Illustration for synthesis of silver NPs by using a thermal decomposition method. The silver-oleate precursors were prepared from the reaction of the silver nitrate and sodium oleate. The thermal decomposition of the silver-oleate complex produced the silver NPs with average particle size about 10 nm

The typical TEM image of as-prepared silver NPs is shown in Fig. 2a, which indicates that the silver NPs developed were well formed. It is clear that no aggregates of the silver NPs were observed through TEM investigation; this presents the uniformity of the particle size. Figure 2b shows the EDX spectrum of the as-synthesized silver NPs excited by an electron beam (200 kV). It is obvious that only peaks for the elements of silver atoms were observed. These results indicate that the prepared silver NPs have high purity. The inset of Fig. 2a also shows the histogram of the size distribution of the silver NPs obtained from TEM image. The average size of the silver NPs is about 9–10 nm with a relatively narrow distribution. This result is in good agreement with the calculation of the average particle size obtained from XRD analysis, which is shown in Fig. 3a. The sharp diffraction peaks of XRD pattern revealed a crystalline structure of silver NPs. Three broad diffraction peaks observed at 2 theta values of 38.2°, 44.5°, and 64.5° were assigned to 111, 200, and 220 planes of face-centered cubic silver, respectively [JCPDS pdf 04-0783]. By using the Scherrer formula, the average particle size of as-prepared silver NPs calculated for the strongest diffraction peak at 111 plane is about 10 nm. Obviously, there is no impurity peak in the XRD pattern. This fact confirmed that the synthesized NPs consists of pure silver with high crystallinity. The UV-vis spectrum of the as-synthesized silver NPs dispersion in toluene is shown in Fig. 3b. As observed from Fig. 3b, an optical absorption band with a maximum at 420 nm was found, which is a typi-

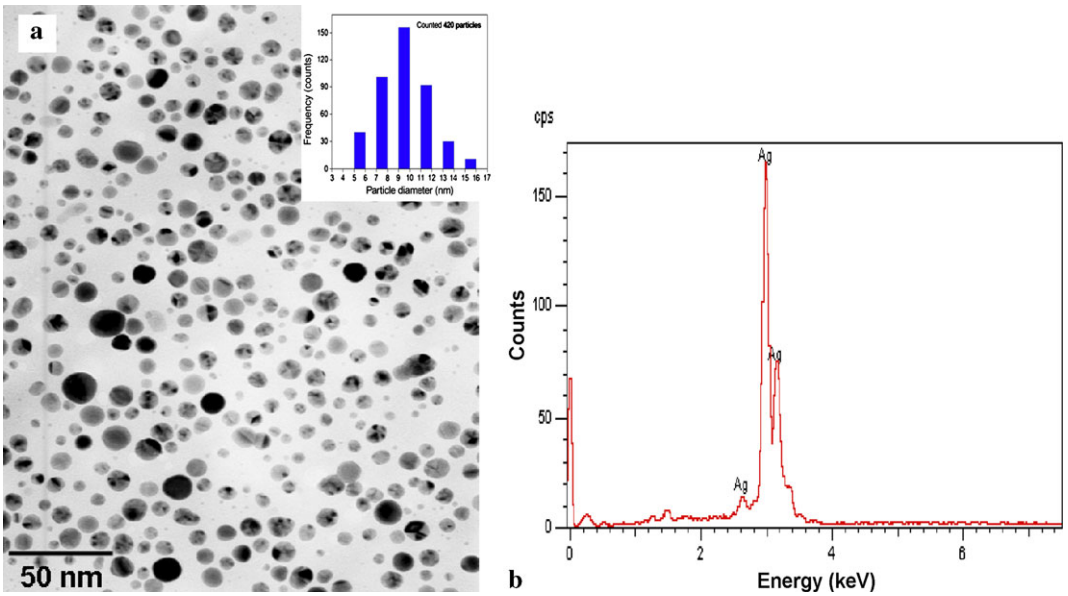

Fig. 2 (**a**) Transmission electron microscopy image and size distribution of the as-prepared silver NPs; (**b**) An energy-dispersive X-ray spectrum of prepared silver NPs

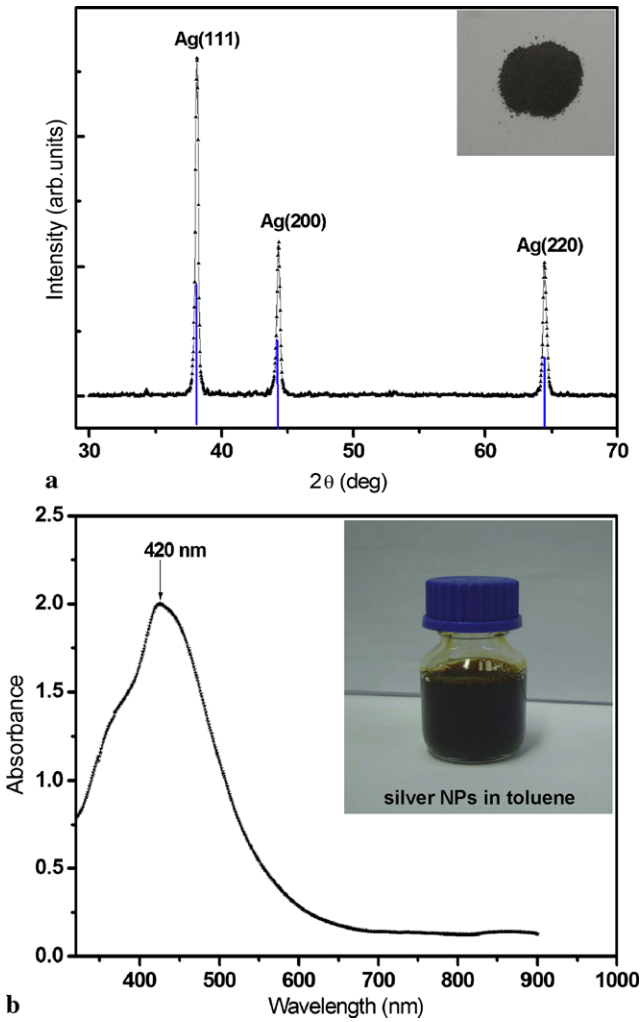

Fig. 3 (**a**) X-ray diffraction pattern of the silver NPs. Inset is a photograph showing a paper containing as-synthesized silver NPs. (**b**) UV-vis spectrum of silver NPs in toluene

cal feature of the absorption of metallic silver NPs due to the surface plasmon resonance (SPR), indicating the presence of silver NPs in the solution.

With observed features of as-prepared silver NPs, the synthetic procedures developed in our present work offer several important advantages over conventional colloidal chemistry methods for the production of silver NPs. The main advantages of the thermal decomposition technique are: (i) this synthesis process allows the silver NPs with average size ∼10 nm to be obtained on a large scale of several grams in a single reaction. When the reactors are set up in parallel, multi-kilograms of the silver NPs can be readily obtained. Therefore, this method is very promising for mass production of the silver NPs for antimicrobial applications; (ii) the synthetic process is environmentally friendly and economical, because it uses nontoxic reagents such as sodium oleate; (iii) the synthetic method is a generalized process that can be used to improve the aggregative stability of silver NP's dispersions in organic solvents. From an application point of view, this stable dispersion feature is very important because a considerable improvement of the aggregative stability results in the enhanced antimicrobial activity of the silver NPs. Based on these mentioned advantages, it is reasoned that the silver-oleate complex would make an effective growth source for the synthesis of the silver NPs with large scale.

3.2 Antibacterial activity of silver NPs

3.2.1 Turbidity and standard dilution assay

First, we studied the antibacterial effect of the as-prepared silver NPs in LB liquid medium against tested bacteria by measuring the microbial viability of bacteria incubated with different concentrations of the silver NPs in the range from 0 mg/L to 6 mg/L. When bacteria (either *E. coli* or *S. aureus*) were cultured in LB liquid media containing the silver NPs for 24 h, the mixture containing only LB broth became turbid (see Figs. 4a and 4b). This suggests that bacteria in such a mixture medium rapidly proliferated. However, the medium containing the silver NPs remained pellucid (Figs. 4a and 4b), indicating few bacteria proliferated. These results clearly showed that the silver NPs prepared could prevent the bacterial growth [28, 29].

To further clarify this, the activity of suppression of bacterial growth by the silver NPs was demonstrated using the antibacterial tests on agar plates (see Figs. 4c and 4d). The bacterial cell colonies grown on the agar-plates were detected by viable cell counts, which were the counted number of colonies that developed after a sample was diluted and spread over the surface of a nutrient medium solidified with agar and contained in a Petri dish. The number of colony-forming units (CFU) reduced significantly with the increase in silver concentration. As observed from Figs. 4c and 4d, in the presence of the prepared silver NPs (2 mg/L), *E. coli* bacterial growth was completely inhibited (see Fig. 4d), whereas the control samples (0 mg/L), those that were not added with silver NPs, showed a drastic growth of bacteria (see Fig. 4c).

3.2.2 Bacterial growth curve assay

Next, the antibacterial performance of the silver NPs was quantitatively investigated by measuring the bacterial growth curves in LB liquid medium [30, 31]. The bacterial proliferation was monitored by measuring the optical density at 600 nm (OD_{600}) as a function of time based on the turbidity of the cell suspension within 18 h. For these experiments, bacteria were grown to an $OD_{600} = 0.1$, and then mixed with various concentrations of the silver NPs. The bacterial growth curves showed a typical dose-dependency antibacterial effect of the prepared silver NPs (see Fig. 5). It can be

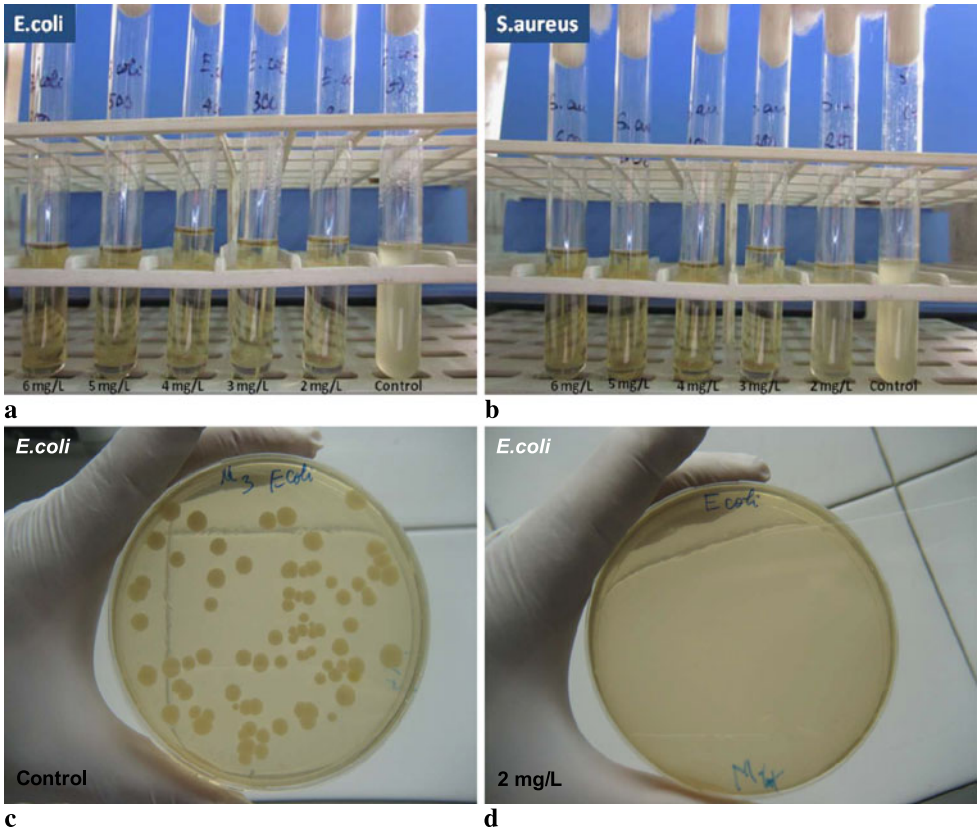

Fig. 4 (**a** and **b**) The turbidity assays were conducted by measuring the microbial viability of both tested bacteria after 24 h of incubation at 37 °C in LB medium loaded with different silver NPs contents (from 0 mg/L to 6 mg/L); (**c** and **d**) Agar plates samples treated (**a**) without silver NPs and (**b**) with silver NPs correspond to *E. coli* bacteria

seen from Fig. 5 that increasing concentration of the silver NPs progressively inhibited the growth of studied bacteria of *E. coli* and *S. aureus*. Compared to the growth curve of the control bacteria, the growth curve in the presence of the silver NPs (3.57 mg/L) showed the lag phase was extended to 4 h for *E. coli* and 8 h for *S. aureus*, respectively. These results revealed strong inhibition of proliferation of *E. coli* and *S. aureus* by the presence of the silver NPs. Noticeably, the growth of both tested bacteria was completely inhibited during the whole 18 h culture period when the concentration of silver NPs was increased. These results suggested that the observed antibacterial activity of the as-prepared silver NPs toward bacteria suspension, which is particularly important for practical applications.

It is noted that the antimicrobial performance of the silver NPs was quantified based on the minimum inhibitory concentration (MIC). The MIC is defined as the concentration of silver NPs required to completely inhibit the bacterial growth. Evaluation of the MIC was conducted by visual inspection of growth/no growth in mixtures containing different concentrations of silver NPs. The lowest concentration of silver that inhibited bacterial growth was taken as the MIC for that particular bacterium. Based on above analysis, in our present case, the MICs of the silver NPs obtained values of 5.21 mg/L for the complete growth inhibition of *E. coli* bacteria (see Fig. 5a) and of 6.58 mg/L for the *S. aureus* bacteria (see Fig. 5b). The variability of the values of the inhibition activity of the silver NPs against studied bacteria can be ascribed to strain differences of the used bacteria. The difference in the sensitivity of the bacterial strains can be observed also in the case of the sensitivity against commonly used antibiotics [31, 32]. It is noted that the MIC values of silver NPs are also dependent on the particles size, shape, and surface modifications of silver NPs prepared as well as biological properties of individual bacterial species [33–36].

3.3 Antifungal activity of silver NPs

The antifungal activity of the prepared silver NPs was studied by means of the yeast growth observation and measurements of the optical density of the sample during the 30 h cultivation. The rate and extent of growth inhibition can be determined from the time-dependency of the recorded growth of the tested yeast. The obtained results of the antifungal activity clearly reveal that the growth of yeasts was inhibited at concentrations as low as 3.16 mg/L (see Fig. 6). The MIC of the silver NPs prepared against *Pichia pastoris* fungi was 3.16 mg/L. The comparison of the antifungal activity of the silver NPs with their antibacterial activity clearly showed that the silver NPs inhibit yeast growth at lower concentrations than in the case of the bacterial growth inhibition. The differences in the minimal bactericidal and fungicidal concentrations of the silver NPs probably

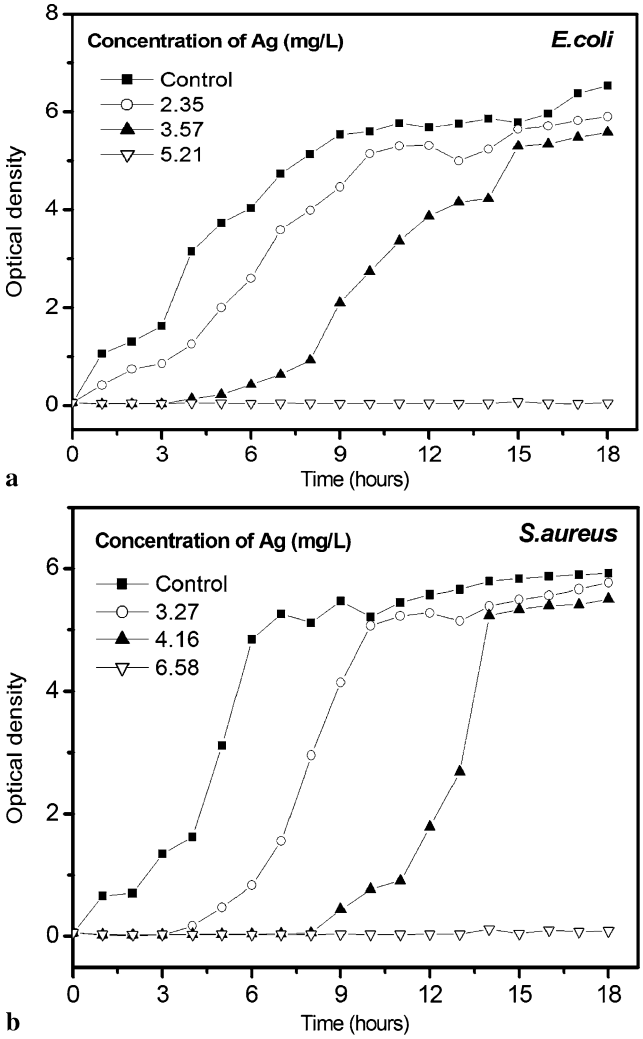

Fig. 5 The time-dependency of the inhibition of bacteria growth of the *E. coli* (**a**) and *S. aureus* (**b**) by the silver NPs. The growth of the bacteria was measured by the optical density at 600 nm wavelength. Each data point represents a minimum of three independent experiments shown with standard error of the mean

result from the differences between the bacterial and yeast cell type. Due to their less complex structure, evolutionarily older prokaryotic types of bacteria are unable to fight the toxic effects of the silver NPs as effectively as the eukaryotic yeast cells that can resist higher concentrations of silver thanks to their better cell organization and structure, and better detoxification system [32–35].

3.4 Ultrastructural and morphological analyses

In order to provide rudimentary insights into the interaction and bactericidal mechanism between the prepared silver NPs and the tested bacteria (i.e., *S. aureus*), the ultrastructural and morphological analyses by using the electron microscopic technique were conducted. The prepared silver NPs was dropped onto the surface of *S. aureus* grown on

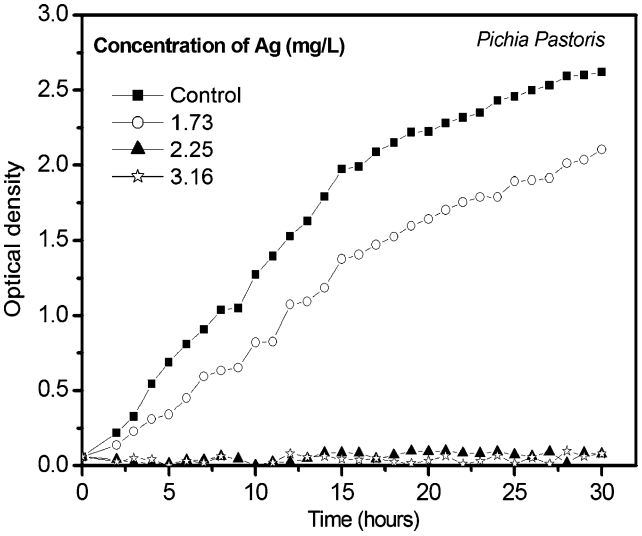

Fig. 6 The time-dependency of the inhibition of the yeast growth of *P. pastoris* by the silver NPs

agar plates. After 15 min, 30 min, and 60 min, *S. aureus* bacterial cells were taken out and underwent the sectioning method for TEM observation. At different magnifications and sections (see Fig. 7), many silver NPs bindings around both the *S. aureus* cell membranes as well as inside the cells were found. As observed, our silver NPs first attached to the surface of the cell membrane, penetrating further inside the bacteria. It should be noted that only silver NPs with sufficiently small diameters penetrated into the cells. The cytoplasm was destroyed as the silver NPs penetrated the cell (Fig. 7). This proves how *S. aureus* bacterial cells can be eliminated by the silver NPs.

In our present case, the possible mechanism for bactericidal action of as-prepared silver NPs is proposed according to the morphological and structural changes found in the bacterial cells. The bactericidal action of our silver NPs were proposed to divide in three stages [36–39]: (i) the silver NPs with sufficiently small diameters attach to surface of the cell membrane and drastically disturb its proper function like permeability and respiration; (ii) then the silver NPs are able to be penetrated inside the bacteria and caused further damage by possibly interacting with sulfur- and phosphorus-containing compounds and lose their activity. (iii) Inside a bacterium, the silver NPs can interact with DNA, the latter thus loses its ability to replicate, which may lead to the cell death; finally, the silver NPs release silver ions Ag^+, which will have an additional contribution to the antibacterial activity of the silver NPs [40, 41].

Here, it should be noted that the bactericidal action of silver NPs (Ag^0) and silver ion (Ag^+) is much different under the view of structural changes. In the case of the silver ions Ag^+, the antimicrobial action of silver ions is linked with interactions with thiol group compounds found in the respiratory enzymes of bacterial cells. The silver ions enter into

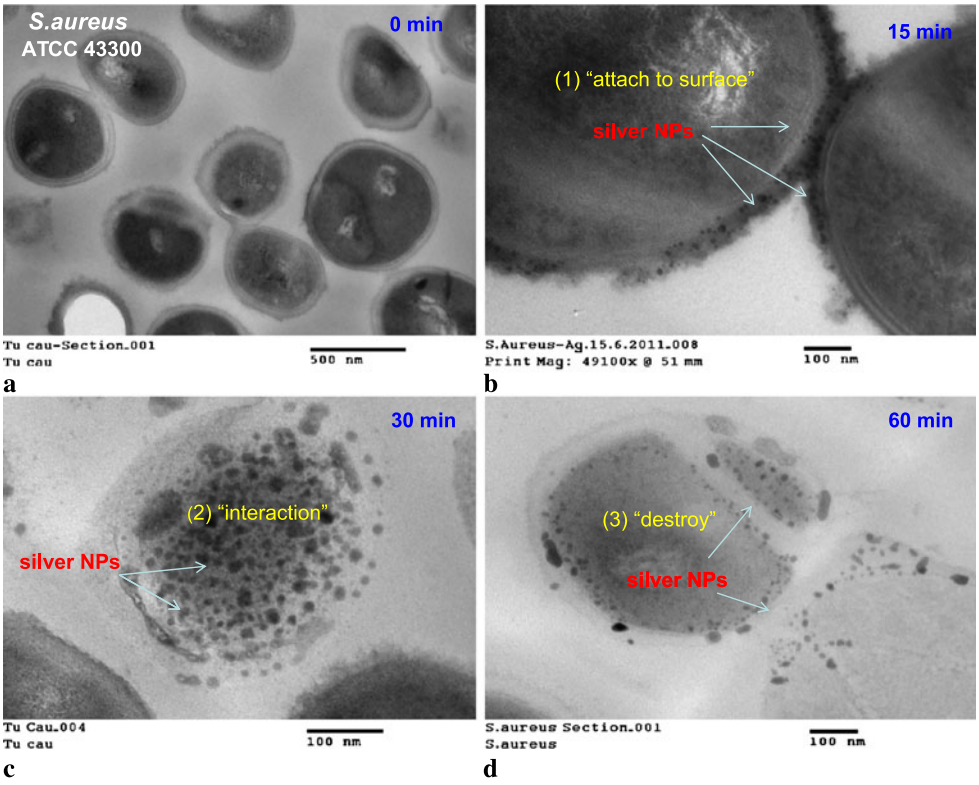

Fig. 7 The cross-sectional TEM images showing different stages of interaction of prepared silver NPs with *S. aureus* bacteria after (**a**) 0 min, (**b**) 15 min, (**c**) 30 min, and (**d**) 60 min

the bacterial cells by penetrating through the cell wall and consequently turn the DNA into condensed form, which reacts with thiol group proteins and results in cell death. The silver ions also interface with the replication process [42]. The changes took place in the cell membrane morphology, producing a significant increase in their permeability. This affects the proper transport through the plasma membrane, leaving the bacterial cells incapable of properly regulating transport through the plasma membrane, resulting eventually to cell death [43]. As shown in Fig. 7, in addition to being fixed to the cell membrane, the silver NPs are capable of penetrating through it to be distributed inside a bacterium. After interacting with the *S. aureus* bacteria, the silver NPs adhered to the cell wall of the bacteria and penetrated the cell membrane, resulting in the inhibition of bacterial cell growth and multiplication.

4 Conclusions

In this work, a versatile technique for synthesis of the silver NPs via a thermal decomposition method was presented. The silver NPs with average size about 10 nm were obtained in a single reaction and without a further size-sorting process. In addition, the as-prepared silver NPs possessed uniform dispersion of particles size, high crystallinity, and easy dispersion ability in various organic solvents. Moreover, the excellent antibacterial and antifungal activities of the silver NPs prepared were demonstrated by turbidity assay, standard dilution assay and growth curve assay in both LB liquid and agar-plate medium. The generation of stable and efficient silver NPs offers an advanced perspective in the field of disinfection and biomedicine.

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