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Enhanced antibacterial effect of silver nanoparticles obtained by electrochemical synthesis in poly(amide-hydroxyurethane) media

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Abstract In the present study, we report enhanced antimicrobial properties of 29 and 23 nm silver nanoparticles (Ag NPs) obtained by electrochemical synthesis in poly (amide-hydroxyurethane) media. Antibacterial activity assessed by disk diffusion method indicates that silver nanoparticles produced inhibition zones for both *Escherichia coli* and *Staphylococcus aureus* depending on silver concentration. The bacterial growth curve performed in the presence of silver nanoparticles showed a stronger antibacterial effect at lower concentrations than those described in the earlier reports. The effect was both dose and size dependent and was more pronounced against Gram negative bacteria than Gram positive one. The smallest Ag NPs used had a bactericidal effect resulting in killing *E. coli* cells. Scanning electron microscopy analysis indicated major damage and morphology changes of the silver nanoparticles treated bacterial cells. The major mechanism responsible for the antibacterial effect probably consists in clusters formation and nanoparticles anchorage to the bacterial cell surface.

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1 Introduction

Due to the outbreak of the infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance, the pharmaceutical companies and the researchers are in a continuous quest for new antibacterial agents [1]. In the present scenario, nanoscale materials have emerged up as novel antimicrobial agents owing to their unique chemical and physical properties [2]. Over the past few decades, inorganic nanoparticles (NPs), whose structures exhibit significantly novel and improved physical, chemical and biological properties due to their nanoscale size, have elicited much interest [3]. In fact, nanotechnology helps in overcoming the limitations of size and can change the outlook of the world regarding science. Furthermore, nanomaterials can be modified for better efficiency in order to facilitate their applications in different fields such as bioscience and medicine.

Nanoparticles are clusters of atoms in the size range of 1–100 nm. The use of NPs is gaining impetus in the present century as they posses defined chemical, optical and mechanical properties. The NPs are most promising as they show good antibacterial properties due to their high surface-to-volume ratio that radical modify/enhance the chemical reactivity [4]. Different types of NPs like copper, zinc, titanium, magnesium, gold and silver have come up, but metallic silver NPs have proved to be most effective as antimicrobial agents with good efficiency against bacteria, viruses and other eukaryotic microorganisms [1].

The antibacterial activity of silver species has been well known since ancient times [5] and it has been demonstrated that in low concentrations silver is non toxic to human cells [3]. The current investigations support that use of silver ion or metallic silver as well as silver NPs can be exploited in medicine for burn treatment, dental materials, coating

stainless steel materials, as well as for textile fabrics, water treatment, sunscreen lotions, etc. [6]. Silver NPs are substantially more effective than silver ions [7], enhanced antibacterial properties of silver NPs being demonstrated both in vitro and in vivo [1]. It is a well known that Ag ions and Ag-based compounds are highly toxic to microorganisms, showing strong antibacterial effects on as many as 12 species of bacteria including *Escherichia coli* and *Staphylococcus aureus* [2, 8–10].

The actual bactericide mechanism of silver NPs is not fully understood. Some researchers support the idea that silver is highly reactive, as it binds to tissue proteins and brings structural changes in the bacterial cell wall and nuclear membrane leading to cell distortion and death [11]. Silver also binds to bacterial DNA and RNA by denaturing and inhibits bacterial replication [12]. Morones et al. (2005) defined the antibacterial activity of silver NPs against some Gram negative bacteria and suggested that silver NPs attach to the surface of the cell membrane, disturb its function, penetrate bacteria, and release silver ions [8].

The size dependent interaction of silver NPs with bacteria has been reported by previous studies [8, 9]. However, little is known about the correlation between the shape of silver NPs and their biological activity [3].

Some authors reported that silver NPs used as drug disinfectant have some risks as the exposure to silver can cause agyrosis and argyria; also in high concentrations are toxic to mammalian cells [4]. For these reasons, in this study we investigated the antimicrobial effects of low concentrations of Ag NPs against representative microorganisms of public health concern. Here, we report that different Ag NPs sizes and concentrations can be applied effectively in the control of microorganisms and the prevention of deleterious infections. Our results support the hypothesis that Ag NPs can be prepared in a simple and cost-effective manner and are suitable for formulation of new types of bactericidal materials.

2 Materials and methods

2.1 Materials

The two bacterial strains—*E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were obtained from Microbiologics Inc., USA. LB nutrient broth (Roth, Germany) supplemented with the required antibiotics was used for growing and maintaining the bacterial cultures as per supplier's protocol. All the chemicals were of the highest purity available (Sigma-Aldrich, Germany). These reagents were used as received without any further purification. The solutions were prepared with Milli-Q water (18.2 MΩ cm).

2.2 Silver NPs synthesis

The synthesis of Ag-PAmHU coated NPs was performed in a simple two-electrode cell using an Amel 549 potentiostat/galvanostat. A coiled silver wire was used as working electrode (WE) (170 mm long and 1.2 mm in diameter) and a coiled platinum wire as counter electrode (CE) (140 mm long and 1 mm diameter); the distance between electrodes was 2.5 mm. An aqueous solution obtained from $AgClO_4$ (10^{-4} M) and poly(amide-hydroxyurethane)-PAmHU polymer (0.1% w/w) was used as supporting electrolyte; pH was adjusted to 10 with a carbonate and bicarbonate mixture solution. The silver perchlorate was obtained in laboratory from silver nitrate solution treated with sodium carbonate and perchloric acid according to Tang et al. [13]. PAmHU was obtained using a three steps reaction as follows:

1 Preparation of β-hydroxy-β'-aminodiethylurethane by the addition in aqueous solution of ethylene carbonate (EC) and ethylene diamide (ED) in a molar ratio of 1:1 at 40–50°C; β-hydroxy-β'-aminodiethylurethane is separated by filtration and dried under vacuum at room temperature (RT) for 24 h.
2 Neutralization of β-hydroxy-β'-aminodiethylurethane with acrylic acid (AA) (molar ratio 1:1). The β-hydroxy-β'-acrylammoniumdiethylurethane monomer produced in this reaction is further polymerized by the addition of potassium peroxydisulfate as initiator at 23–32°C obtaining poly(acrylohydroxyurethane) (PAcHU). The resulted PAcHU is separated at RT by precipitation with methanol or ethanol;
3 Condensation of $COO^{-}H_3N^{+}$ from the PAcHU precursor with the formation of the amide group and obtaining poly(amide-hydroxyurethane) (PAmHU)—a crystalline polymer, very tough and soluble in water. The PAmHU polymer has a tunable solubility, depending on various stimuli such as concentration, temperature, etc. [14]. In aqueous solution ($c_{PAmHU} < 1\%$ w/w) a micelle to coil mechanism for phase separation can be induced, this feature permitting the NPs synthesis by restricted reaction room method.

Nanoparticles electrosynthesis method combine the tunability of PAmHU solubility with the electrochemical reduction of $[M^{n+}]$ metal ions in a galvanostatic process, the H^{+} proton source being the hydrogen releasing by cathode electrolysis process. The electrosynthesis of Ag NPs was performed in galvanostatic regime, at room temperature, under strong stirring and nitrogen atmosphere. Current densities used in the synthesis of Ag NPs were 6.25 and 1.56 mA/cm^2. Samples obtained using a 6.25 mA/cm^2 current were named Ag I and samples obtained using a 1.56 mA/cm^2 current were named Ag II. The optimal time

for synthesis was 15 min [15]. In order to adjust the pH at 7, the electrochemical synthesis product was dialysed against deionised water at room temperature for 10 h, using a cellulose membrane with 12000 Da MWCO (Sigma-Aldrich).

2.3 Silver NPs characterization

Silver concentration was determined by atomic absorption spectrometry (AAS) with a Perkin Elmer 3300 spectrometer. The Ag metal identification and nanophase crystallites structure analysis of lyophilized product obtained by electrochemical synthesis were investigated by Xray diffraction (XRD) performed on a DRON-2 diffractometer, employing nickel-filtered Cu Kα radiation (1.54182 Å) at 25 kV operational voltage. The Ag NPs size and morphology were investigated by a Philips CM100 transmission electron microscope (TEM). The statistic distribution of Ag NPs sizes was performed using NIS Elements Basic Research imaging software (NIS E-Br). The Ag NPs size distribution done by NIS E-Br software was compared with the experimental one obtained by laser granulometry (LG) with Malvern Zetasizer Nano ZS ZEN-3500. The experimental evaluation of Zeta potential dependence with dimensions of PAmHU coated Ag NPs was performed at room temperature also with Malvern Zetasizer Nano ZS ZEN-3500. The stability of silver-coated PAmHU colloidal solutions was defined in accordance with average Zeta potential (AZP) [16].

2.4 Antimicrobial assays

A disk diffusion method [17] was used to assay the susceptibility of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) to different silver NPs sizes and concentrations. A 500 µl sample of overnight bacterial suspension cultured in LB medium (adjusted to an approximately optical density—OD_{600} of 0.700) was plated on a Muller-Hinton agar plate (Roth, Germany). Sterile paper disks (Whatman no. 5, 6 mm diameter) were further impregnated with 25 µl of Ag-PAmHU coated NPs in aqueous solutions at a final content of 0.25–1.50 µg Ag/disk. After 18 h of incubation at 37°C the zones of inhibition were measured.

In order to evaluate the bacterial growth curve in the presence of Ag NPs, an overnight preculture was prepared. 125 µl from the above preculture (adjusted to an approximately OD_{600} of 0.700 for *E. coli* and 0.800 for *S. aureus*) was added in 25 ml LB medium with 1, 5 and 10 µg/ml (final concentrations) of Ag NPs. LB medium supplemented with PAmHU without NPs was used as control. All Falcon tubes were incubated on an orbital shaker at 190 rpm at 37°C for 60 h. Growth rates were determined by measuring optical density at 600 nm at regular intervals, using a Beckman Coulter DU 730 Life Sciences spectrophotometer.

The size and morphology of Ag NPs on the bacteria were examined by scanning electron microscopy (SEM). Prior to SEM analysis, samples containing untreated and treated bacterial cells were deposited on a Millipore filter. The bacterial cells were further fixed in glutaraldehyde 2.5% for 2 h, dehydrated in a stepwise concentration gradient of ethanol solutions (70, 80, 90 and 100%) and acetone. The filters containing bacterial cells were dried overnight with CO_2 in an EMS 850 critical point dryer, sputter-coated with a 30 nm layer of gold (EMS 550X Sputter Coater) and finally examined by SEM (Tescan Vega II SBH) at an acceleration voltage of 27.88 kV.

2.5 Statistical analysis

The Ag NPs characterization analysis and bacterial tests were performed in triplicate, and the results were expressed as means ± the standard errors of the means. The data were statistical analyzed using ANOVA test followed by Tukey's post hoc multiple comparison test using XLSTAT software package version 7.5.2. P values < 0.05 were considered as significant.

3 Results and discussions

3.1 Silver NPs characterization

The characteristic TEM micrographs of Ag I and Ag II NPs-PAmHU systems (Fig. 1) show that the Ag NPs tend to be agglomerated inside the PAmHU matrix. The particles were found to be spherical or polyhedral in shape, having an average size of 28.87 nm (±9.16 nm) for Ag I and 23 nm (±11.13 nm) for Ag II as shown in the diagram of size distribution (Fig. 2). On the other hand the LG analysis revealed that the average size of Ag I is 43.79 nm and respectively 40.68 nm for Ag II due to NPs packing in a thick layer of PAmHU.

All Ag-capped PAmHU colloidal solutions have a moderate stability (APZ within −40 to −30 mV). In Fig. 3 is presented the dependence of Zeta potential for a representative Ag II versus distribution dimension (implicit); APZ = −33.2 mV.

3.2 Effect of silver nanoparticles on bacterial growth

Since Klabunde and co-workers proved that reactive metal oxide NPs present excellent bactericidal effects [18], it is of great interest to investigate the use of other inorganic NPs as antibacterial materials. These inorganic NPs have a distinct advantage over conventional chemical

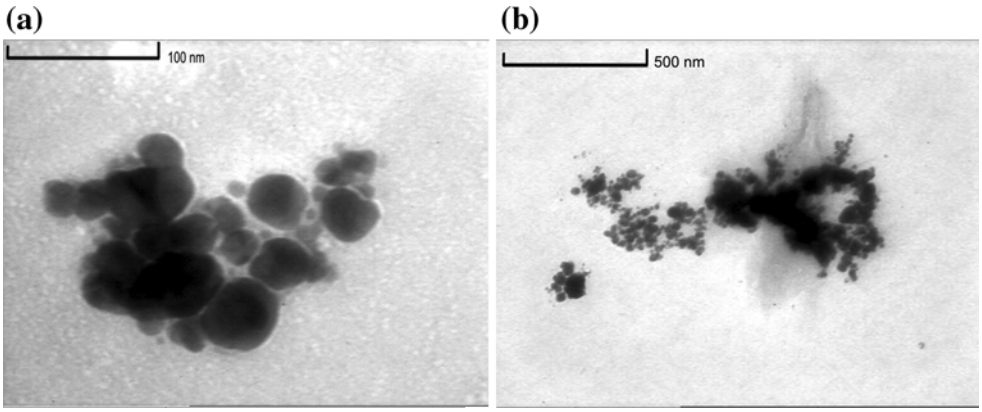

Fig. 1 TEM micrographs of Ag I (**a**) and Ag II (**b**) NPs

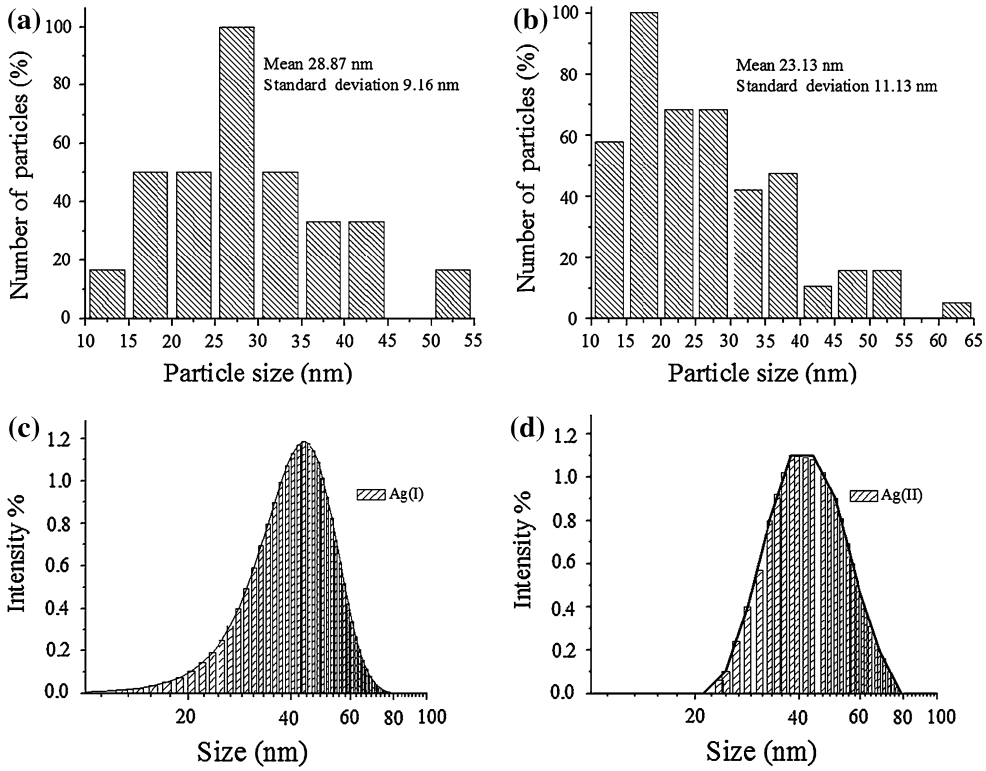

Fig. 2 Size distribution charts of silver NPs obtained from TEM analysis (**a** Ag I, **b** Ag II) and by laser granulometry (**c** Ag I, **d** Ag II)

antimicrobial agents [2]. The most important problem caused by the chemical antimicrobial agents is multidrug resistance. An alternative way to overcome the drug resistance of various microorganisms is needed, especially in medical devices, etc. Ag ions and Ag salts have been used for decades as antimicrobial agents in various fields because of their growth-inhibitory capacity against microorganisms [19].

In order to asses the effectiveness of silver NPs as antimicrobial agents, an initial disk diffusion assay was performed against two representative microorganisms: Gram negative bacterium *E. coli* (ATCC 25922) and Gram positive bacterium *S. aureus* (ATCC 25922). The experimental data (Fig. 4) indicates that, regardless of the size, the Ag NPs produced an inhibition zone on the Muller-Hinton agar plates for both tested bacteria. A clear connection between the inhibition zone diameter and silver concentration can be inferred. Similar results were previously reported by Shrivastava et al. [5] using a plate culturing method and incorporating silver NPs in the growth medium.

Although growth on agar plates is a more ready means of distinguishing antimicrobial properties of Ag NPs of different sizes, Sondi and Salopek-Sondi [20] previously reported different effects of Ag NPs depending on growth conditions used. Thereby we also considered employing in our study liquid-growth experiments. Because most of the

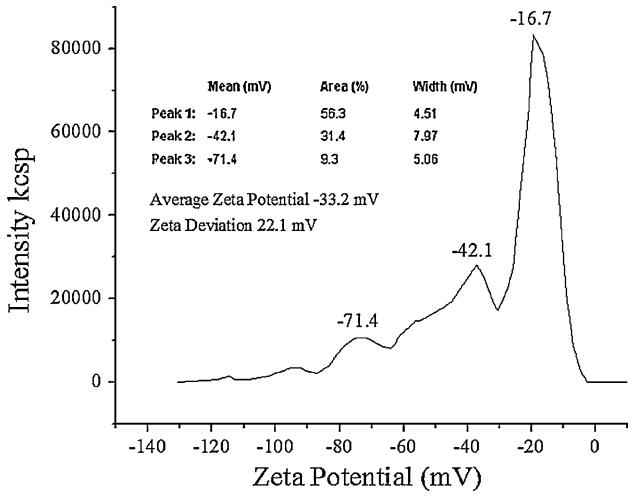

Fig. 3 Zeta potential versus distribution dimension (implicite) for Ag II

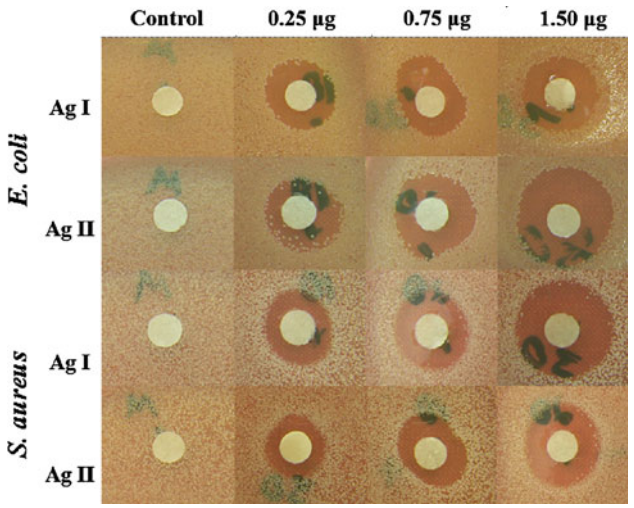

Fig. 4 Disk diffusion assay using Muller-Hinton agar supplemented with 29 nm (Ag I) respectively 23 nm (Ag II) at different concentrations (0.25–1.50 µg Ag/disk)

studies describing antibacterial activity of silver NPs reported in the literature were performed at high concentrations (10, 50, 100 µg/ml [8, 20, 21], further experiments were carried out using LB medium supplemented with low concentrations of Ag NPs: 1, 5 and 10 µg/ml.

In our study, PAmHU coated Ag NPs showed antimicrobial activity against both Gram positive and Gram negative tested bacteria (Fig. 5). Nevertheless, distinct reactions between the two microorganisms could be observed, results being in line with previous reports by several authors [2, 5, 20]. The inhibitory effect of Ag NPs was mild in the case of *S. aureus* as compared with *E. coli* and was determined only by Ag II. Ag I had no significant influence on *S. aureus* growth regardless of tested concentrations. Both types of used NPs significantly inhibited the development of *E. coli* cells ($P < 0.0001$). Our results suggest that the antimicrobial effects of Ag NPs could be associated with characteristics of certain bacterial species. The lower efficacy of the Ag NPs against *S. aureus* may derive from the differences in cell wall structure and permeability compared with *E. coli*. In most Gram positive bacteria, the cell wall consists of many layers of peptidoglycan, forming a thick, rigid, structure. By contrast, Gram negative cell walls contain only a thin layer of peptidoglycan [22]. Therefore the antibacterial effect of Ag NPs must be associated with the peptidoglycan layer [2].

We have to mention that previous studies [5, 20] reported low antibacterial activity of 10–15 nm sized Ag NPs against *S. aureus* at 25 µg/ml. Despite the fact that bigger Ag NPs (23 nm) were used in our experiments, a stronger bactericidal effect against *S. aureus* was observed at concentrations as low as 5 µg/ml.

The dynamics of bacterial growth also revealed that increasing concentrations of NPs progressively inhibited the growth of *E. coli* and *S. aureus* (Fig. 5). Although the lowest concentration used (1 µg/ml) did not had any significant effects, Tukey's test showed that higher concentrations (5 and 10 µg/ml) significantly inhibited ($P < 0.0001$) the growth of both tested bacteria. Ag NPs used in our experiments caused a growth delay of *E. coli* and *S. aureus* up to 10 h. Similar effects were obtain also by Shrivastava et al. [5] using 25 µg/ml Ag NPs and by Sondi and Salopek-Sondi [20] using 50 and 100 µg/ml Ag NPs final concentration in the growth medium. In our study the lag phase was found to be more prolonged although lower concentrations of Ag NPs (5, 10 µg/ml) were used. This could be explained by the greater stability of the PAmHU coated NPs used in our experiments. Moreover, it is necessary to emphasize that Ag II have bactericidal effects resulting not only in inhibition of bacterial growth but also in killing *E. coli* cells at a concentration of 10 µg/ml. This irreversible inhibition of bacterial growth is desirable to prevent bacterial colonization of silver-containing medical devices, such as catheters [23], where bacteria-killing activity is required [24].

The antimicrobial effect can also be correlated with particles size: Ag II (23 nm) proved a more pronounced antimicrobial activity compared with Ag I (29 nm) on both tested bacteria, as it can be seen in Figure 5. This higher antibacterial activity of smaller sized NP is probably due to their bigger surface area and therefore their greatest contact with bacterial cell [25]. Some authors argue that for solid systems Ag^+ released from the surface of Ag NPs are responsible for their antibacterial activity [26]. In the case of aqueous systems (as liquid culture media) the results found by Lok et al. [25] show that the antibacterial activity of Ag^+ is low at the concentrations levels reached by releasing, and the presence of NPs is vital. This proves the

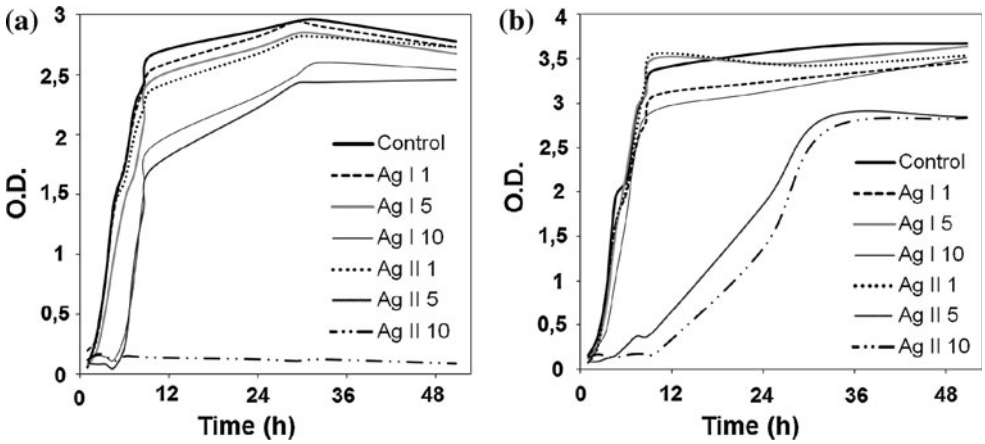

Fig. 5 Bacterial dynamic growth curve in LB media at different sizes and concentrations of silver NPs. **a** *E. coli*; **b** *S. aureus*

idea that the greatest the surface area, the greatest the antibacterial activity [27]. Although structural and morphological changes induced by silver NPs are frequently reported [1], the mechanism of the bactericidal effect of Ag NPs is partially known. Our study as well as several other reports shows that Ag NPs attach to the cell surface and may disturb the permeability and respiration functions [28].

In order to demonstrate that the Ag NPs used in this study have a direct contact with bacteria, as well as a potential for inducing changes in bacterial cell morphology, SEM microscopy was employed. We observed a significant number of NPs that have been released from the PAmHU matrix and clusters of NPs both types anchored on the surface of bacteria membrane. The Ag NPs treated bacterial cells showed major damage and their morphology was significantly changed (Fig. 6). Clusters of NPs were found to anchor to the bacterial cell wall (Fig. 7), possibly at sites that are rich in negatively charged functional groups.

The overall surface of the bacteria is negatively charged at biological pH values, due to the dissociation of functional groups (carboxylic and other) in the membrane [18]. In view of the strongly electronegative character of the surfaces of cells, their capacity for binding anionic NPs-polymer aggregates is unexpected. Nevertheless, the NPs-PAmHU aggregates are heterogeneous mixtures of

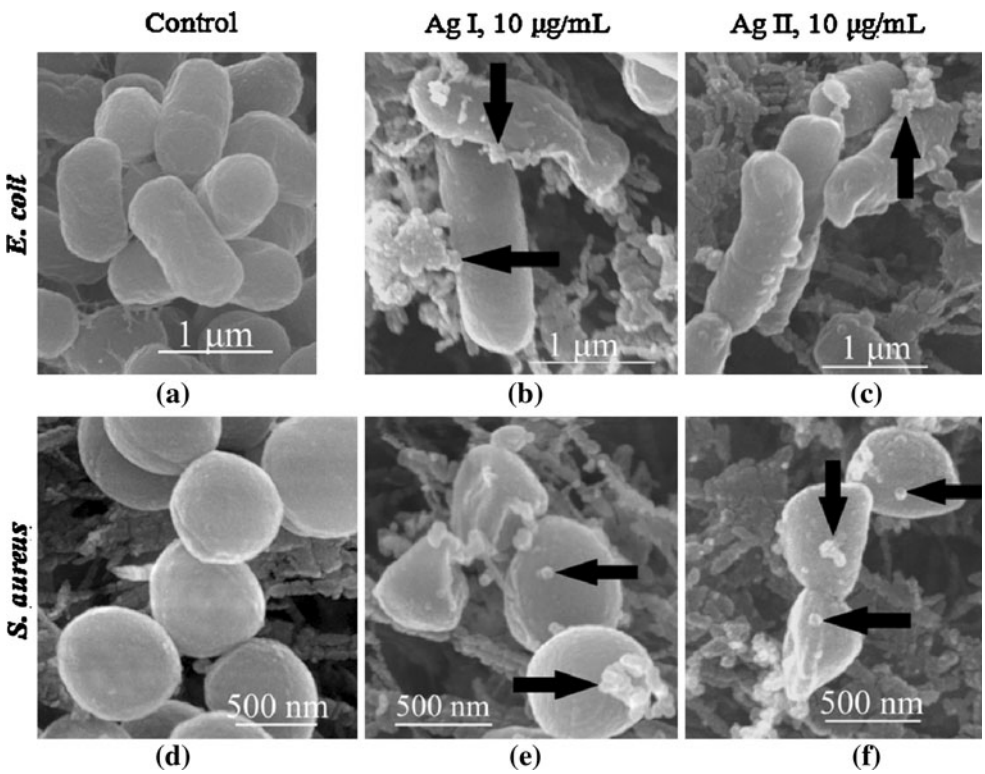

Fig. 6 SEM micrographs showing the effects of silver NPs (Ag I—29 nm, Ag II—23 nm) on *E. coli* and *S. aureus* cells morphology (*arrows* indicate the NPs attached on cell wall surface)

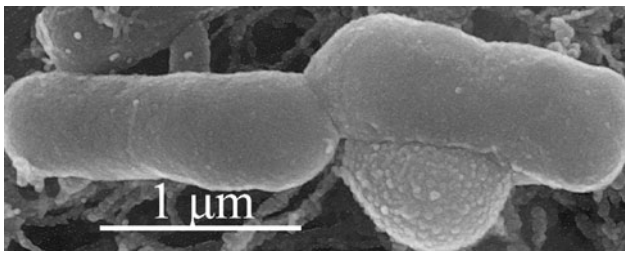

Fig. 7 SEM micrograph of *E. coli* cells and NPs aggregates

various NPs differing widely in size, degree of PAmHU-NPs coated, and fractions of charged surface functional groups. As result charged functional groups from cell membrane exhibits any degree of selectivity for different fractions of polymer surface functional groups. The highly electronegative potential of the three different fractions of PAmHU coated NPs (see Fig. 3) cause a transfer of charge (electrons) and therefore a high reactivity at the interface PAmHU/bacteria membrane. As a result a Donnan equilibrium is restored in the contact area; partial fixed charge may become positive (due to the electron transfer) and therefore favoring the formation of channels with high mobility for the silver ions transport.

The Gram negative bacteria have a layer of lipopolysaccharide at the exterior, exposing more negative charges on their cell wall then Gram positive bacteria [22]. This can explain our results and the observed fact that the growth of *E. coli* was more profoundly affected by the silver NPs than *S. aureus*. These NPs clusters probably induce formation of perforations in the wall and membrane of the cell, which could result in cell lysis [5]. It is also possible that Ag NPs not only interact with the cell surface, but can also penetrate inside the bacteria [8]. The damage to cell may be caused by interaction of Ag NPs with phosphorous- and sulfur-containing compounds such as DNA [19]. Silver tends to have a high affinity for such compounds. Ag^{+} strongly interact with the available –SH groups of the biomolecules to inactivate the bacteria. Such interactions in the cell membrane would prevent DNA replications, which would lead to bacterial death [29].

4 Conclusions

Silver NPs used in this study were synthesized in a simple and cost-effective manner and were found to have a stronger antibacterial effect at lower concentrations than those described in the earlier reports [5, 20]. The effect was dose dependent and was more pronounced against Gram negative bacteria than Gram positive ones. Moreover, the effect was size dependent, the smallest Ag NPs used having bactericidal effects resulting in killing *E. coli* cells.

The major mechanism through which silver NPs manifested antibacterial properties was by forming clusters and anchoring to the bacterial cell surface. However, further investigations are necessary for a better understanding of interaction between silver NPs and bacteria components in order to explain the action mechanisms of this nanomaterial. Also, before proposing the use of Ag NPs as antimicrobial agent it is necessary to verify if the bacteria develop resistance towards NPs and to examine cytotoxicity of NPs towards human cells before proposing their therapeutic use [30].

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