SYNTHESIS AND ANTIBACTERIAL EFFECT OF SILVER NANOPARTICLES WITH DIFFERENT SIZES

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Abstract: Silver nanoparticles have well-known antibacterial properties, and is widely used in daily life as various medical and general products. In comparison with silver ion, there is serious lacking of information concerning the biological effects of silver nanoparticles. In this study, we observed significant effects of poly (amidehydroxyurethane) (PAmHU) capped Ag nanoparticles obtained by electrochemical synthesis against *Escherichia coli* and *Staphylococcus aureus*. Our results suggest that silver nanoparticles can be prepared in a simple and cost-effective manner and are suitable for formulation of new types of bactericidal materials.

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. A number of recent achievements offer the possibility of generating new types of nanostructured materials with designed surface and structural properties (Sondi and Salopek-Sondi, 2004). Due to the increasing bacterial resistance to classic antibiotics, the investigations on the antibacterial activity of silver nanoparticles have increased (Panacek *et al*., 2006).

The antibacterial activity of silver species has been well known since ancient times (Shrivastava *et al*., 2007) and it has been demonstrated that, in low concentrations, silver is non toxic to human cells (Pal *et al*., 2007). In fact, it is well known that Ag ions and Ag-based compounds are highly toxic to microorganisms, showing strong biocidal effects on as many as 12 species of bacteria including *E. coli* (Zhao *et al*., 1998); for this reason silver-based compounds have been used extensively in many bactericidal applications (Morones *et al*., 2005). It is worth mentioning some examples such as inorganic composites with a slow silver release rate that are currently used as preservatives in a variety of products; another current application includes new compounds composed of silica gel microspheres, which contain a silver thiosulfate complex, that are mixed into plastics for longlasting antibacterial protection. Silver compounds have also been used to control bacterial growth in the medical field with a variety of applications, including dental work, catheters, and burn wounds (Catauro *et al*., 2004). Recently, Mecking and co-workers showed that hybrids of Ag nanoparticles with amphiphilic hyperbranched macromolecules exhibited effective antimicrobial surface coating agents (Aymonier *et al*., 2002).

The actual bactericide mechanism of silver nanoparticles is not well known (Martınez-Castanon *et al*., 2008). Some researchers support the idea that silver species release Ag+ ions and they interact with the thiol groups in bacteria proteins, affecting the replication of DNA (Marini *et al*., 2007). It has also been reported that Ag+ ions uncouple the respiratory chain from oxidative phosphorylation or collapse the proton-motive force across the cytoplasmic membrane (Holt and Bard, 2005). Silver nanoparticles interactions with bacteria are dependent on the size and shape of the nanoparticles (Morones *et al.*, 2005). Reducing the particle size of materials is an efficient and reliable tool for improving their biocompatibility. In fact, nanotechnology helps in overcoming the limitations of size and can change the outlook of the world regarding science (Mirkin and Taton, 2000). Furthermore, nanomaterials can be modified for better efficiency to facilitate their applications in different fields such as bioscience and medicine.

In this study our research team has investigated the antimicrobial effects of poly (amidehydroxyurethane) (PAmHU) capped Ag nanoparticles obtained by electrochemical synthesis against representative microorganisms of public concern. Here, we report that PAmHU capped Ag nanoparticles can be applied effectively in the control of microorganisms and the prevention of deleterious infections. Our results suggest that Ag nanoparticles can be prepared in a simple and cost-effective manner and are suitable for formulation of new types of bactericidal materials.

MATHERIALS AND METHODS

Synthesis of silver nanoparticles

The synthesis of silver nanoparticles was performed in a simple two-electrode cell by using an Amel 549 potentiostat/galvanostat. A coiled silver wire was used as working electrode (*WE*) (170 mm long and 1.2 mm diameter) and a coiled platinum wire as counter electrode (*CE*) (140 mm long and 1mm diameter). The supporting electrolyte was an aqueous solution of $AgClO_4$ (10^{-4}M) and PAmHU (0.1% *w/w*) adjusted at pH 10 with sodium carbonate and bicarbonate mixture solution. The synthesis and biocompatible properties of PAmHU water soluble polymer were

reported in (Apostu and Melnig, 2006; Melnig and Ciobanu, 2005; Melnig *et al.*, 2006). The water used was Milli-Q with an 18.2 MΩ resistance. The electrosynthesis of silver nanoparticles was carried out in the galvanostatic regime for current densities of 6.25 mA/cm^2 (samples named as I) and 1.56 mA/cm^2 (samples named as II), at room temperature, under strong stirring and nitrogen atmosphere. The optimum time for synthesis was 15 minutes (Obreja *et al.*, 2008).

The typical electrochemical electrode processes are:

$$\textit{Anode}: \quad Ag \rightarrow Ag^+ + e^- (\textit{oxidation})$$
$$HO^- \rightarrow HO^{\cdot} + e^-$$
$$2\,HO^{\cdot} \rightarrow H_2O + [O]$$
$$Ag + [O] \rightarrow AgO$$
$$\textit{Catode}: \quad Ag^+ + e^- \rightarrow Ag^0 (\textit{reduction})$$
$$H_2O + e^- \rightarrow 1/2H_2 + OH^- (\textit{reduction})$$

The obtained solutions were dialyzed against pure water at room temperature for 10 hours using a *Sigma D6191-25EA dialysis tubing cellulose membrane* with pore size 12000 Da MWCO. The silver concentration was determined by atomic absorption spectrophotometry (AAS) with a Perkin Elmer 3300 spectrometer.

The studies of micrograph morphology by Transmission Electron Microscopy (TEM) were done on a Phylips CM100 microscope. The average size of PAmHU coated nanoparticles was determined by visual comparison of TEM micrographs against a standard scale of micrographies using NIS Elements Basic Research imaging software (to determine their distribution it was used the same statistical program).

The stability of colloidal solutions is defined according to the average value of Zeta potential (AZP) (Riddick, 1968). The Zeta potential of nanoparticles suspension was evaluated with a Malvern zetasizer nano ZS at room temperature.

Antibacterial activity of silver nanoparticles

Two bacterial strains, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were subjected to this analysis.

The effect of silver nanoparticles on Gram-negative and Gram-positive bacteria was investigated by culturing the organisms on LB agar plates supplemented with silver nanoparticles (I and II) at concentration of 60 μg/ml. An inoculum with 0,211 optical density for *Escherichia coli* and 0,231 for *Staphylococcus aureus* determined at 600 nm was used. Plates without silver nanoparticles were used as controls (only PAmHU was added). Plates were incubated for 24 h at 37^0 C and the number of colonies was counted. The counts on 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 a study was performed using Luria Bertani (LB) liquid medium. Inoculations were given from fresh colonies on agar plates into 20 ml LB medium supplemented with 5 and 10 μg/ml of silver nanoparticles (I and II), till optical densities (at 600 nm) of 0,028 for *Escherichia coli* and 0,026 for *Staphylococcus aureus* were reached. Controls LB were used without nanoparticles (only PAmHU was added). All flasks were incubated on an orbital shaker at 190 rpm at 37^0 C for 24 hours. Growth rate was determined by measuring optical density at 600 nm at regular intervals, using a Beckman Coulter DU 730 Life Sciences spectrophotometer.

Statistical analyses

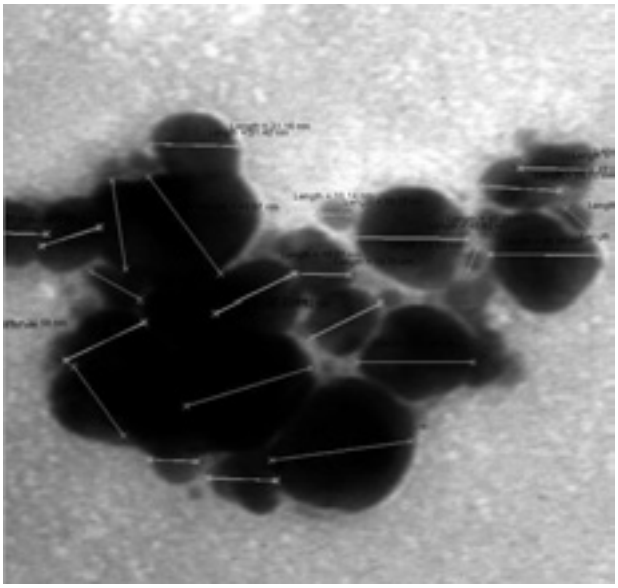
Data were statistical analyzed using ANOVA test (two-way with replication). Values are mean ± SEM. *F* values for $p<0.05$ are statistically regarded.

RESULTS AND DISCUSSIONS

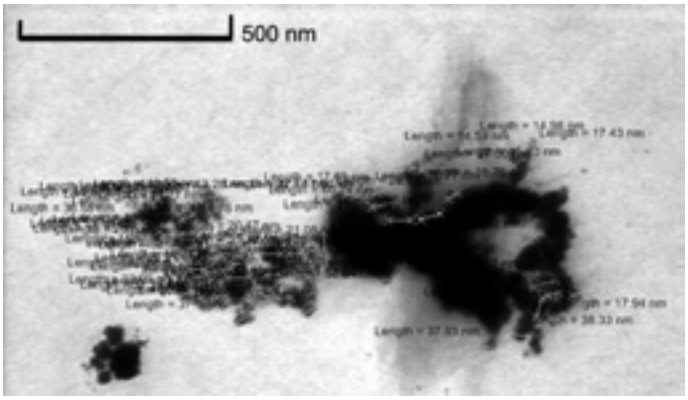
Characterization of silver nanoparticles

The dialyzed against pure water stock solutions of silver nanoparticles with concentration of 63.95 μg/ml (type I) and 88.55 μg/ml (type II) were obtained. For evaluation of antibacterial effect, the stock solutions were diluted with deionized water.

The TEM micrographs of PAmHU caped silver nanoparticles suspensions are presented in Fig. 1. As shown in bar chart distribution of dimensions from Fig. 1 the mean size of particles is 29 nm with a standard deviation of 10.57 nm (type I) and 23 nm with a standard deviation of 10.12 nm (type II).



(a)



(b)

Fig. 1 - TEM micrographs and bar charts of size distributions of PAmHU caped silver nanoparticles synthesised at 6.25 (a) and 1.56 (b) mA/cm^2 current density.

In Table 1 are presented the stability conditions of colloidal solutions according to the average value of Zeta potential (Riddick, 1968). It is worthy to mention that the stability conditions defined in Table 1 were established for suspension of spherical particles model, in which the affinity of the particle surface with water creates a structured surface water layer. This structure changes considerably the properties of the particle – water – polymer from those in the bulk. The electrostatic field, in combination with the thermal motion of the ions, creates a counter-charge, and thus it screens the electric surface charge. The net electric charge in this screening diffuse layer is equal in magnitude to the net surface charge, but has the opposite polarity.

Table 1. The colloidal solutions stability – Zeta potential relation (Riddick, 1968).

Stability	Average Zeta potential (mV)
Extreme to very good stability	–100 to –60 mV
Reasonable stability	–60 to –40 mV
Moderate stability	–40 to –30 mV
Threshold of light dispersion	–30 to –15 mV
Threshold of agglomeration	–15 to –10 mV
Strong agglomeration & precipitation	–5 to +5 mV

Comparing the average values of Zeta potential of samples with those from Table 1, we can conclude that nanoparticles colloidal solutions present a moderate stability (AZP = -33.2 mV) that allow a uniform activity in bulk of microorganisms cultures (Fig. 2).

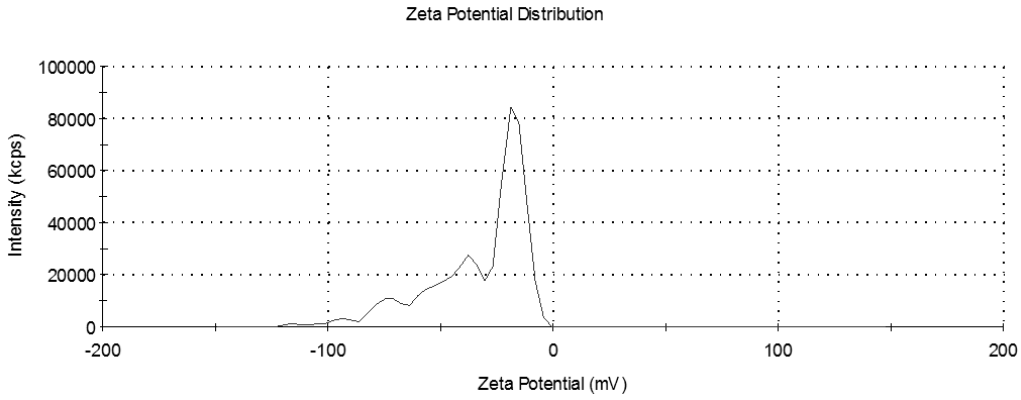


Fig. 2 - Zeta potential for silver nanoparticles type I

Antibacterial activity of silver nanoparticles

Three replicates for each determination were performed.

Inhibitory effects on growth of tested bacteria were evidenced when LB agar plates supplemented with nanoparticles at concentration of 60 μg/ml were used, compared to control (Photo 1-6). A stronger inhibitory effect was evidenced when I and II silver nanoparticles were added to *Escherichia coli* growing medium (Photo 1-3) compared to *Staphylococcus aureus* (Photo 4-6).

Previous studies reported inhibitory effects of silver nanoparticles at lower concentrations: 5, 10, 25 or 35 μg/ml (Kim *et al*., 2007, Shrivastava *et al*., 2007). According to these data a study was performed to determine tested bacteria growth curve in the presence of silver nanoparticles, using LB liquid medium supplemented with 5 and 10 μg/ml of silver nanoparticles (I and II) – Fig. 3-6.

Silver nanoparticles type I (5 μg/ml) significantly prolonged lag phase ($F(4,12)=371,4898$, $p<0.0006$) of *E. coli* with approximately 8 hours compared to control (Fig. 3). Increasing concentration of nanoparticles progressively inhibited the growth of *E. coli*. The concentration of 10 μg/ml silver nanoparticles type I was found to be strongly inhibitory for bacteria, as it took more than 24 h after inoculation to initiate any noticeable growth. The lag

phase was found to be more prolonged than that described in the earlier reports (Sondi and Salopek-Sondi, 2004).

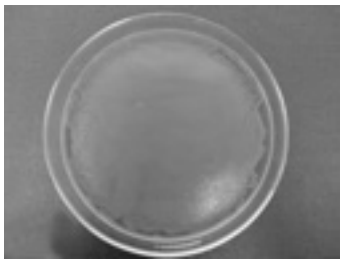


Photo 1 - *Escherichia coli*: Control

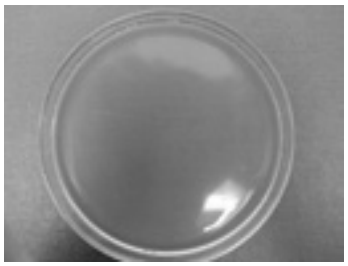


Photo 2 - *Escherichia coli*: Ag I, 60 µg/ml

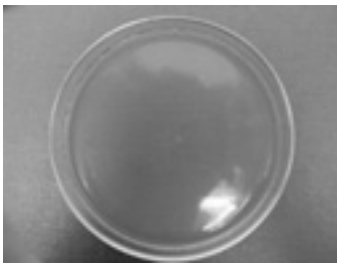


Photo 3- *Escherichia coli*: Ag II, 60 µg/ml

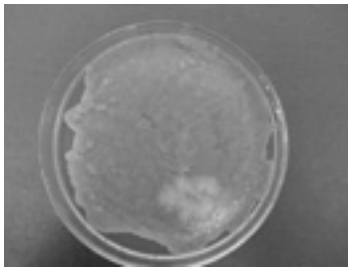


Photo 4 - *Staphylococcus aureus*: Control

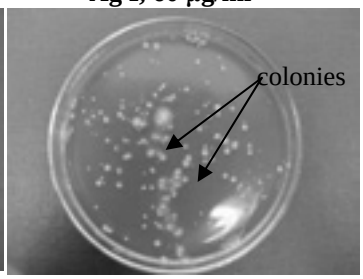


Photo 5- *Staphylococcus aureus*: Ag I, 60 µg/ml

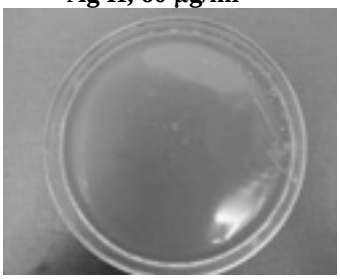


Photo 6- *Staphylococcus aureus*: Ag II, 60 µg/ml

Similar results ($F(4,12)=204,23$, $p<0.0003$) were registered when silver nanoparticles type II were used (Fig. 4, Photo 7).

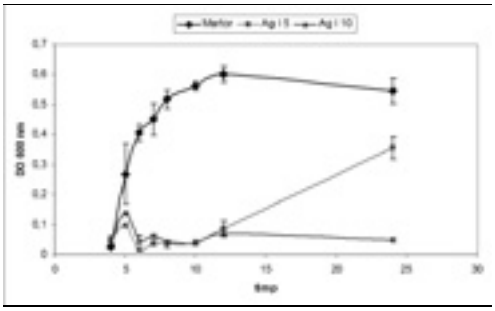


Fig. 3 – *Escherichia coli* dynamic growth curve in LB media supplemented with silver nanoparticles type I (29 nm): 5 µg/ml and 10 µg/ml. Values are means ± SEM, $p<0.0006$ vs. control.

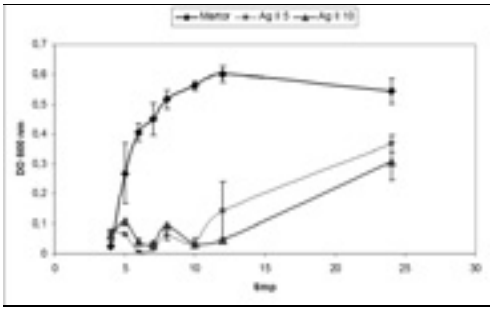


Fig. 4 – *Escherichia coli* dynamic growth curve in LB media supplemented with silver nanoparticles type II (23 nm): 5 µg/ml and 10 µg/ml. Values are means ± SEM, $p<0.0003$ vs. control.

Silver nanoparticles type I and II significantly prolonged lag phase of *Staphylococcus aureus* ($F(2,14)= 391{,}1921$, $p<0.0001$ and $F(2,14)=541{,}0301$, $p<0.0001$), respectively, with approximately 8 hours compared to control (Fig. 5-6, Photo 8).). Consequently, a shorter lag phase was associated with the growth of gram-positive bacteria treated with nanoparticles type II at concentration of 5 μg/ml contrasted with that in the case of other types of silver nanoparticles and the gram-negative strain used.

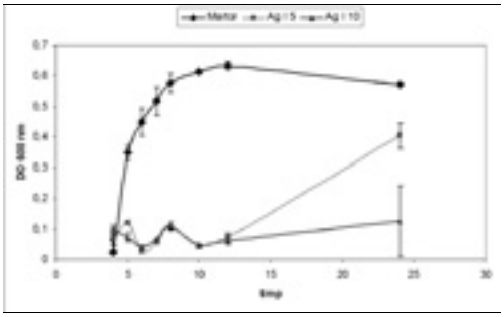


Fig. 5 – *Staphylococcus aureus* dynamic growth curve in LB media supplemented with silver nanoparticles type I (29 nm): 5 μg/ml and 10 μg/ml. Values are means ± SEM, p<0.0001 vs. control.

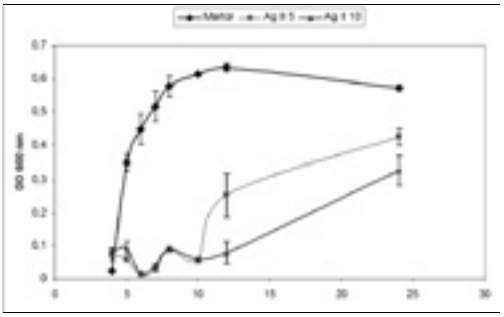


Fig. 6 – *Staphylococcus aureus* dynamic growth curve in LB media supplemented with silver nanoparticles type II (23 nm): 5 μg/ml and 10 μg/ml. Values are means ± SEM, p<0.0001 vs. control.

It is noticeable that whatever the size of silver nanoparticles tested, longer lag phase is not dependent on the concentration used for both bacteria test. However, exponential phase evolution is different depending on the concentration used, meaning the presence of higher concentrations (10 μg/ml) is accompanied by the decrease in number of cells in relation to the use of lower concentrations of nanoparticles in the environment (5 μg/ml) - Fig. 3-6.

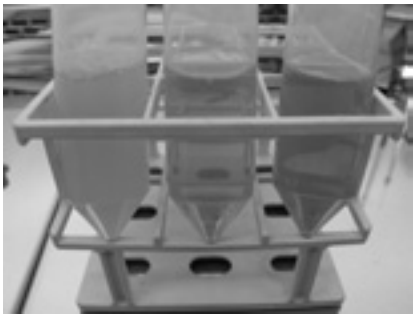


Photo 7 - *Escherichia coli*: LB medium supplemented with silver nanoparticles type II left control, middle 5 μg/ml, right 10 μg/ml

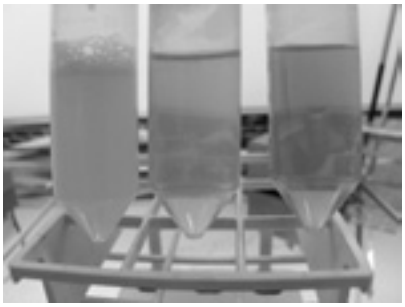


Photo 8 - *Staphylococcus aureus*: LB medium supplemented with silver nanoparticles type II left control, middle 5 μg/ml, right 10 μg/ml

CONCLUSIONS

The PAmHU capped silver nanoparticles, obtained by electrosynthesis, with the mean size of particles of 23 nm (I) and 29 nm (II) were found to have stronger antibacterial potency than those described in the earlier reports.

The inhibitory effect was pronounced against both Gram-negative and Gram-positive bacteria.

Whatever the size of silver nanoparticles tested, longer lag phase is not dependent on the concentration used.

As a novelty, we have shown that silver nanoparticles with sizes of 29 nm at a concentration of 10 μg/ml inhibit the growth of bacteria *Escherichia coli* and subsequent phase lag.

However, further studies must be conducted to verify if the bacteria develop resistance towards the nanoparticles and to examine cytotoxicity of nanoparticles towards human cells before proposing their therapeutic use (Braydich-Stolle *et al*., 2005).

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