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PREPARATION OF SILVER NANOPARTICLES VIA CHEMICAL REDUCTION AND THEIR ANTIMICROBIAL ACTIVITY

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A simple and economic method of synthesis of silver colloid nanoparticles with controlled size is presented. By reduction of $[Ag(NH_3)_2]^+$ complex in sodium dodecylsulfate (SDS) micellar solution with three various reducing agents (hydrazine, formalin and ascorbic acid) the nanoparticles were produced with size below 20 nm. The average size, size distribution, morphology, and structure of particles were determined by dynamic light scattering (DLS), scanning electron microscopy (SEM), and UV/Visible absorption spectrophotometry. The influence of the reducing agent on the size of silver particles, fraction of metallic silver and their antimicrobial properties is discussed. In particular, the reduction of silver complex by hydrazine resulted in silver nanoparticles with size below 20nm. They showed high activity against Gram-positive and Gram-negative bacteria (lab isolated strains), and clinical isolated strains including highly multiresistant strains such as Staphylococcus epidermidis, Staphylococcus aureus and Pseudomonas aeruginosa.

keywords: silver, nanoparticles, antimicrobial properties, MIC.

1. INTRODUCTION

Development of new, effective and low cost antimicrobial agents has been an object

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of research activity of many groups due to build-up of resistance of microbial organisms to traditional antibiotics. It is well known that silver-based compounds are highly toxic to microorganisms. Silver (Ag) has been known for its antibacterial activity since the times of ancient Greece (Silver et al., 1996). It is currently used to control bacterial growth in a variety of applications, including dental work, catheters, and burn wounds (Crabtree, et al., 2003, Catauro, et al., 2004). Recently, silver nanoparticles (Sondi, et al., 2004, Morones, et al., 2005, Baker, et al., 2005). as well as various silver-based compounds containing ionic silver (Butkus, et al. 2003, Chen, et al., 2005) exhibiting antimicrobial activity, have been synthesized. Silver-containing materials and coatings with antimicrobial activity can be used: in medicine to reduce infections in hospitals, in burn treatment, as well as to prevent bacteria colonization on prostheses, catheters, vascular grafts, dental materials, stainless steel materials (Bosetti, et al., 2002, Gauger, et al., 2003, Gosheger, et al., 2004, Rupp, et all., 2004, Strathmann, et al., 2004, Ohashi, et al., 2004, Ulkur, et al., 2005, Parikh, et al., 2005,). Fibers containing silver nanoparticles can be used to eliminate microorganisms on textile fabrics (Yuranova, et al., 2003, Jeong, et al., 2005) . Silver nanoparticles also exhibit a potent cytoprotective activity toward HIV infected cells (Sun, et al., 2005). Reducing the particle size of materials is an efficient tool for improving their bioactivity. Therefore, it is a role of nanotechnology to help overcoming the limitations in the size of efficient particles, which can contribute to the positive change in the public awareness regarding that science in general (Mirkin, et al., 2000). Numerous methods of preparation silver nanoparticles have been developed (Matijevic, 1993, Gutierrez , et al., 1993, Nickel, et al., 2000, Leopold, et al., 2003, Khanna, et al., 2003, Sondi, et al., 2003). The most widespread method of synthesis of silver nanoparticles is based on the chemical reduction of a silver salt solution by a reducing agent. In polysaccharide method, Ag nanoparticles are prepared using water as an environmentally benign solvent and polysaccharides as a capping agent, or in some cases, as both a reducing and a capping agent (Raveendran, et al., 2003). In Tollens method, a synthesis using a Tollens process was used to form silver particles with controlled size in a one-step process. The basic reaction in this process involves the reduction of a silver ammoniacal solution using saccharides (Yin, et al., 2002, Saito, et al., 2003). In that way films with colloidal silver particles ranging from 50 to 200 nm, or silver hydrosols with particles in the order of 20-50 nm, were obtained. Ag nanoparticles can be successfully synthesized by irradiation. For example, laser irradiation (Nd3+-YAG 500nm) of an aqueous solution of Ag salt and surfactant can fabricate Ag NPs with a well-defined shape and size distribution (Abid, et al., 2002). No chemical reducing agent is required in this method. Silver nanoparticles can be also formed by biological method, i.e., extracts from bio-organisms may act both as reducing and capping agents. Reduction of Ag^+ ions was done by combinations of biomolecules found in these extracts such as enzymes/proteins, amino acids,

polysaccharides, and vitamins (Shankar, et al., 2002, Gardea-Torresdey , et al., 2003, Jagadeesh, et al., 2004, Collera-Zuniga, et al. 2005, Shankar , et al., 2005, Richardson , et al., 2006, Li, et al., 2007, Vigneshwaran, et al., 2007, Xie, et al., 2007, Wu, et al., 2008, Sharma, et al., 2009).

The aim of our work was to develop simple and effective method of synthesis of silver nanoparticles with well-defined size and to verify their antimicrobial activity on the series of Gram-positive and Gram-negative bacteria (standard strains and clinical isolated strains including highly multiresistant strains such as *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*). Our work was concentrated on the chemical reduction method and the effect of reducing agents, hydrazine monohydrate, formalin and ascorbic acid, on the size and antimicrobial activity.

2. MATERIALS AND METHODS

2.1. Materials

Silver nitrate (pure p.a.), ammonia (25% w/w aqueous solution pure p.a.), ascorbic acid -AAC (pure p.a.) and formalin -F (36-38% pure p.a.) were purchased from POCH Gliwice Poland. Sodium dodecyl sulfate –SDS (>90%) and hydrazine monohydrate –H (purum, ≥98.0%) were purchased from Sigma-Aldrich. All materials were used without further purification. Distilled water used in all experiments was obtained with the three-stage Millipore Direct-Q 3UV purification system.

Table 1. The components of Ag $3d_{5/2}$ core excitation for the studied systems.

System	Component A		Component B	
	BE (eV)	Ratio (%)	BE (eV)	Ratio (%)
Ag/formalin	368.2	81.1	369.3	18.9
Ag/hydrazine monohydrate	368.2	90.2	369.8	9.8
Ag/ascorbic acid	368.0	78.0	369.3	22.0

Microorganisms used in this study were as follows: Gram-positive bacteria: *Staphylococcus aureus* ATCC 4163, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *Enterococcus hirae* ATCC 10541, *Micrococcus luteus* ATCC 9341, *Micrococcus luteus* ATCC 10240 and clinical isolates of *Staphylococcus epidermidis* and *Staphylococcus aureus* (MSSA and MRSA strains), Gram-negative rods: *Escherichia coli* ATCC 10538, *Escherichia coli* ATCC 25922, *Escherichia coli*

NCTC 8196, *Proteus vulgaris* NCTC 4635, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* NCTC 6749, *Pseudomonas aeruginosa* ATCC 27853, *Bordetella bronchiseptica* ATCC 4617 and hospital isolates of *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Bacterial strains were obtained from the collection of the Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

Table 2. Antimicrobial activity of Ag nanoparticles suspensions and antibacterial drug - ciprofloxacin against standard bacterial strains: Minimal Inhibitory Concenteration (MIC). μg/ml)

Compound / Strain	H	F	Ciprofloxacin
***Staphylococcus aureus* ATCC 4163**	**20**	**80**	**0,5**
***Staphylococcus aureus* ATCC 25923**	**20**	**80**	**0,5**
***Staphylococcus aureus* ATCC 6538**	**20**	**80**	**0,5**
***Staphylococcus aureus* ATCC 29213**	**20**	**80**	**0,5**
***Staphylococcus epidermidis* ATCC 12228**	**10**	**40**	**0,5**
***Bacillus subtilis* ATCC 6633**	**20**	**80**	**<0,125**
***Bacillus cereus* ATCC 11778**	**20**	**80**	**1**
***Enterococcus hirae* ATCC 10541**	**80**	**80**	**4**
***Micrococcus luteus* ATCC 9341**	**5**	**40**	**2**
***Micrococcus luteus* ATCC 10240**	**5**	**40**	**1**
***Escherichia coli* ATCC 10538**	**10**	**40**	**<0125**
***Escherichia coli* ATCC 25922**	**10**	**40**	**<0,125**
***Escherichia coli* NCTC 8196**	**10**	**40**	**<0,125**
***Proteus vulgaris* NCTC 4635**	**10**	**40**	**<0,125**
***Pseudomonas aeruginosa* ATCC 15442**	**10**	**40**	**0,5**
***Pseudomonas aeruginosa* NCTC 6749**	**10**	**40**	**0,5**
***Pseudomonas aeruginosa* ATCC 27853**	**10**	**40**	**1**
***Bordetella bronchiseptica* ATCC 4617**	**10**	**40**	**1**

2.2. Synthesis of silver nanoparticles.

Colloidal silver particles were synthesized by the reduction of $[Ag(NH_3)_2]^+$ complex with hydrazine monohydrate, formalin and ascorbic acid in micellar solution of sodium dodecyl sulfate - SDS. The initial concentrations of the reaction components were 10^{-2} mol/L for $AgNO_3$ and 0.1 mol/L for SDS. SDS used in our experiments was of technical grade (90%), so the ammonia was added to obtain clear and transparent solution of $[Ag(NH_3)_2]^+$, to prevent AgCl precipitation. Reducing agent (hydrazine monohydrate, formaline, ascorbic acid) was added to the reaction system in one step during stirring at 200 rpm, in ratio 1:1 (without excess of reducer). Reduction was

initiated in several minutes after addition of the reducing agent. Reactions were performed at room temperature (20-25 °C).

2.3. Characterization of silver nanoparticles

Size (hydrodynamic diameter) of silver nanoparticles was determined by DLS (Dynamic Light Scattering) using Zetasizer Nano Series from Malvern Instruments with the detection angle of 173^{o} in optically homogeneous square polystyrene cells. All measurements were performed at 25^{o}C. Each value was obtained as average from three runs with at least 10 measurements. The zeta potential of silver nanoparticles was measured by the microelectrophoretic method using Malvern Zetasizer Nano ZS apparatus. Each value was obtained as an average from three subsequent runs of the instrument with at least 20 measurements. Microscopic SEM (scanning electron microscope) observations of silver nanoparticles were performed with a JEOL JSM-7500F, Field Emission Scanning Electron Microscope. UV/VIS absorption spectra of the silver colloids were acquired by using Analytik Jena AG - SPECORD® 40 spectrophotometer. The chemical composition of the particles was determined by X-ray Photoelectron Spectroscopy (XPS). Prior to analysis, the drop of suspension containing particle was deposited on the pure Au foil surface, dried at room temperature in low vacuum then transferred to the analysis chamber. The measurements were performed in the ultrahigh vacuum ($3 \cdot 10^{-10}$ mbar) system equipped with hemispherical analyzer (SES R 4000, Gammadata Scienta).

Table 3. Antimicrobial activity of Ag nanoparticles suspension H against clinical strains of *taphylococcus epidermidis* and *Staphylococcus aureus* (MRSA – methicillin-resistant, MSSA – methicillin-susceptible): Minimal Inhibitory Concentration (MIC, μg/ml)

S.epidrmidis	**MIC**	***S. aureus* (MRSA)**	**MIC**	***S.aureus* (MSSA)**	**MIC**
311/08	**2,5**	**275/08**	**10**	**256/08**	**10**
315/08	**2,5**	**277/08**	**10**	**261/08**	**10**
316/08	**2,5**	**306/08**	**10**	**267/08**	**10**
317/08	**2,5**	**307/08**	**10**	**268/09**	**10**
318/08	**2,5**	**309/08**	**10**	**269/08**	**5**
340/09	**2,5**	**329/08**	**10**	**338/08**	**10**
341/09	**5**	**355/09**	**5**	**339/08**	**10**
342/09	**5**	**356/09**	**10**	**367/09**	**10**
343/09	**5**	**357/09**	**10**	**369/09**	**10**
377/09	**5**	**376/09**	**10**	**372/09**	**10**

The unmonochromatized Mg Kα X-ray source of incident energy of 1253.6 eV was applied to generate core excitation. The spectrometer was calibrated according to ISO 15472:2001. The energy resolution of the system, measured as a full width at half maximum (FWHM) for Ag $3d_{5/2}$ excitation line, was 0.95 eV. The spectra were processed by CasaXPS 2.3.12 program. The calibration was performed for carbon C 1s core excitation at electron binding energy (BE) of 285 eV taken as an internal standard. In the spectra, the background was approximated by a Shirley profile. The spectra deconvolution into a minimum number of the components was done by application of the Voigt-type line shapes (70:30 Gaussian/Lorentzian product). The analysis depth of 9 nm allowed sampling of the particle volume.

2.4. Antimicrobial properties

To eliminate influence of SDS and some by-products of reduction reaction on antimicrobial properties, silver colloid nanoparticles were dialyzed 4 times using distilled water. Reference solution (water taken after last dialysis) didn't show antimicrobial activities. During dialysis of silver nanoparticles reduced by ascorbic acid aggregation process was observed. Suspension of silver nanoparticles were tested in vitro for their antibacterial activity using standard and clinical strains of Gram-positive and Gram-negative bacteria.

Table 4. Antimicrobial activity of Ag nanoparticles suspension H against clinical strains o *Pseudomonas aeruginosa and Escherichia coli*: Minimal Inhibitory Concenteration (MsIC, μg/ml)

P. aeruginosa	MIC	E. Coli	MIC
6m	5	ML1	2.5
7m	5	ML3	2.5
10m	5	ML5	2.5
11m	5	ML6	2.5
12m	5	ML8	2.5
16m	5	ML9	2.5
18m	10	ML12	2.5
22m	10	ML15	2.5
23m	5	ML16	2.5
31m	5	ML17	5

Clinical strains were isolated from different biological materials of patients hospitalized in one of the Warsaw Medical University Hospitals. Minimal Inhibitory Concentrations (MIC) were established by the twofold serial agar dilution technique using Mueller–Hinton II agar medium (Becton Dickinson), according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2006). Concentrations of silver

nanoparticles suspensions reduced by hydrazine monohydrate (H) and by formalin (F) in solid medium ranged from 80 to 1,25µg/ml, concentrations of silver suspension reduced by ascorbic acid (AAC) ranged from 8 to 1,25µg/ml. The final inoculum of bacterial strain was 10^4 CFU/mL (colony forming units per mL), except the final inoculum for *E. hirae* ATCC 10541, which was 10^5 CFU/mL. Minimal inhibitory concentrations were read after 18 h of incubation at 35 °C.

3. RESULTS AND DISCUSSION

Figure 1 demonstrates examples of size distributions of silver suspensions obtained using different reducing agents.

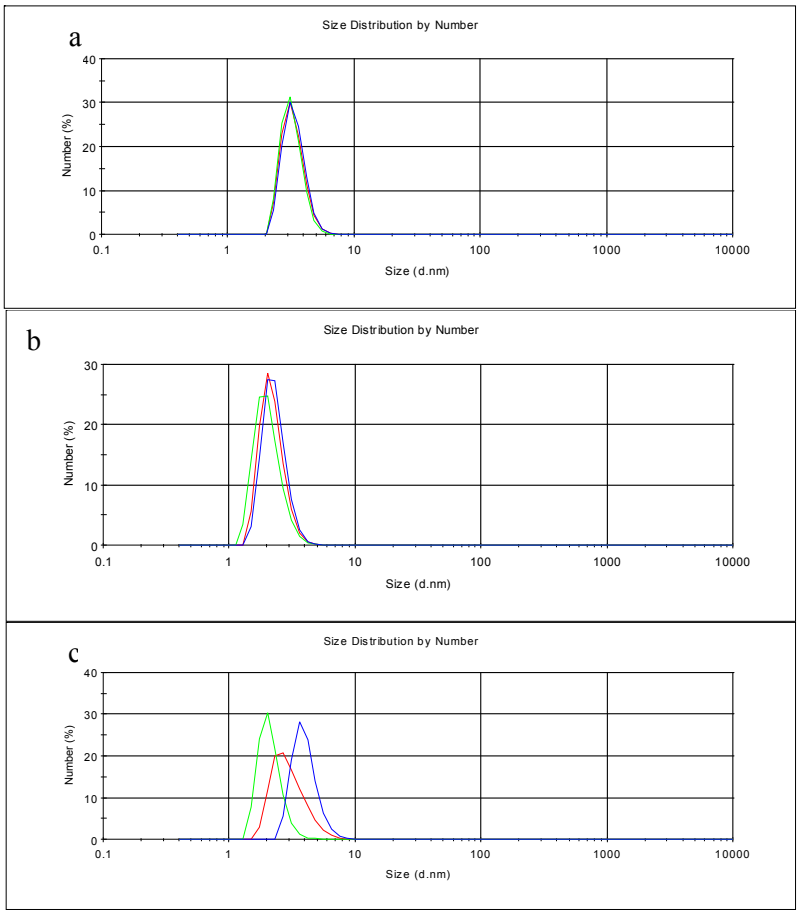

Fig. 1. Size distribution of silver nanoparticles measured by DLS reduced with a) formalin b) hydrazine monohydrate c) ascorbic acid.

One can see that the average size of nanoparticles, 4 nm, was practically not influenced by the type of the reducing agent, hydrazine monohydrate, formaline and ascorbic acid. Therefore, size and stability of the particles was controlled by addition of surfactant - sodium dodecylsulfate (SDS). We found that optimal concentration of SDS in reaction system was ~0.1 mol/L and the average size of nanoparticles, as measured by DLS, was in the range of a few nanometers. SEM micrograph (Fig.2) illustrates silver nanoparticles reduced with hydrazine monohydrate, which were deposited on opposite charged latex particles. The size of the observed particles, below 20nm, is in agreement with the values obtained by DLS. Zeta potential of Ag nanoparticles was negative (c.a. -60mV) due to SDS presence at their surface and slightly increases by c.a. 10mV after dialysis due to removal of excess of SDS.

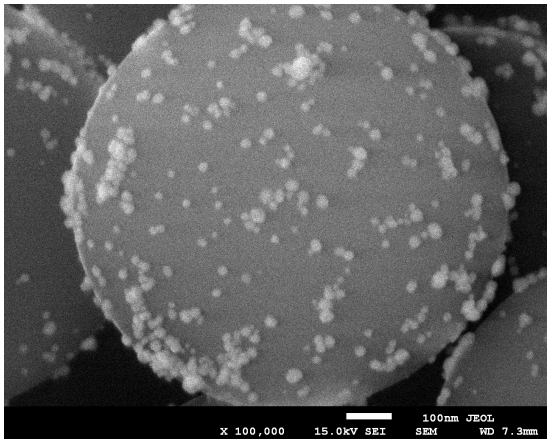

Fig. 2. SEM micrograph of silver nanoparticles reduced with hydrazine monohydrate deposited on oppositely charged Latex particles.

The suspension of 1600 ppm of silver was stable for a year. In the case when ascorbic acid was used as reducer stability of nanoparticles at that concentration was much lower and we observed aggregation in a few minutes after preparation of the Ag suspension. To obtain stable suspension with ascorbic acid we used 10 times lower concentration of $[Ag(NH_3)_2]^+$. In this concentration (160ppm) suspension of silver nanoparticles was stable for a year.

In the UV/VIS absorption spectra (Fig. 3) of silver particles, synthesized using various reducing agents, narrow surface plasmon absorption peaks at the wavelengths 390-420 nm were observed. That confirms the nanocrystalline character of the particles (Schneider, et al., 1994) and the low degree of their polydispersity.

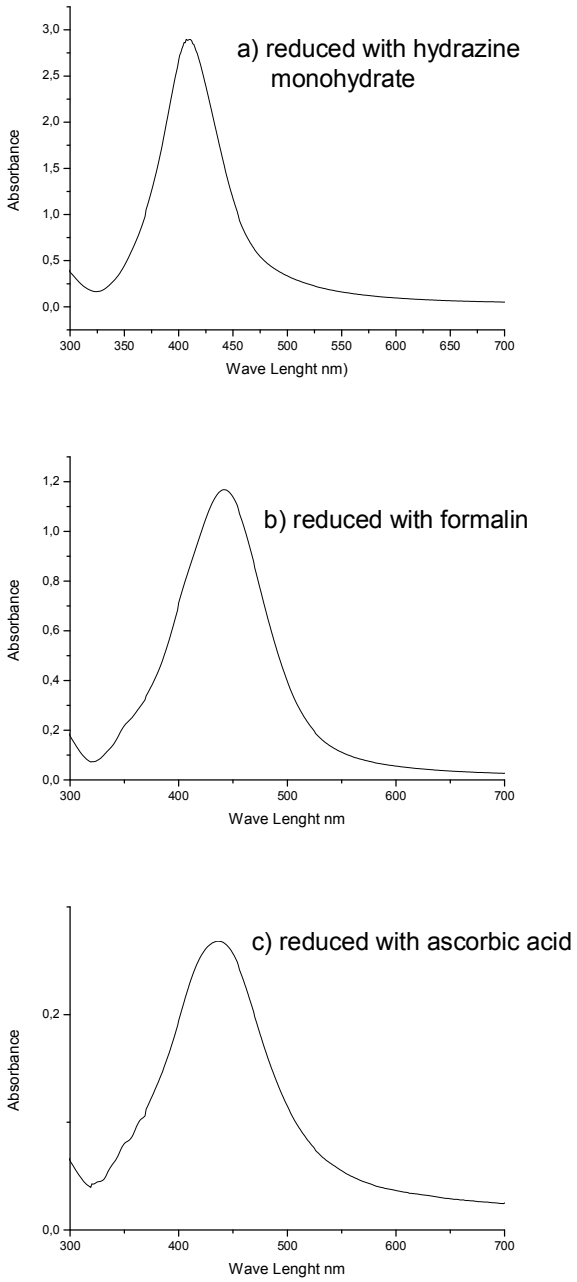

Fig. 3. Absorption spectra of Ag nanoparticle suspension reduced with a) hydrazine monohydrate b) formalin c) ascorbic acid.

Colloids containing particles larger than 100 nm, possess a uniform light absorption at longer wavelengths, a characteristic feature of metal particles of such size. The XPS measurements of the dried Ag suspensions allowed determining of the electronic states of silver in the obtained suspensions. The sample XPS spectrum of Ag 3d core excitation for Ag particles synthesized with the use of hydrazine is shown in Figure 4. This spectrum was deconvoluted into 2 components, where the component A at BE of 368.2 eV was assigned to metallic silver (Moulder, et al., 1992). The other fitting component (B) showed unexpectedly high BE of 369.8 eV that can be related either to the presence of Ag nanoparticles (Shin, et al., 2004) or to silver bonded to organic species [46]. However, the most probable state can be assigned to the peak B is silver immersed in or covered by an organic matrix (Majid, et al., 2003, Shin, et al., 2004, Lim, et al., 2006). The XPS spectra obtained for other systems (Table 1) showed also

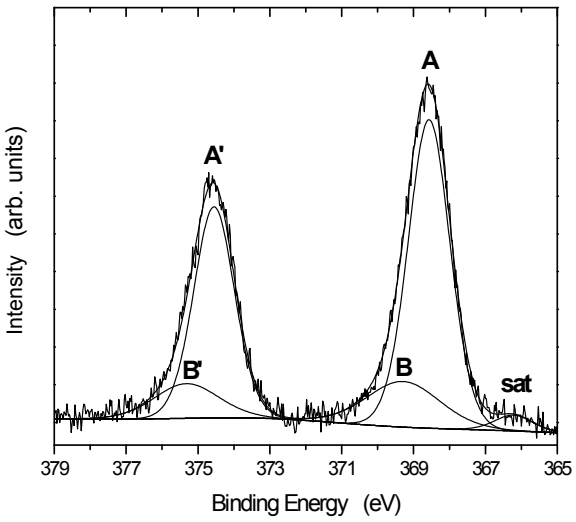

Fig. 4. The Ag 3d core excitation spectrum for Ag Hydrazine monohydrate sample.

two components of the Ag 3d core excitation. The XPS analysis suggests that hydrazine monohydrate used in the particle synthesis results in the lowest amount of the organic-bonded silver fraction. The reduction by ascorbic acid and formaline produces twice as organic-bonded silver as in the case of hydrazine monohydrate. The results of antibacterial tests (MIC) are summarized in tables 2-4. These results demonstrate that silver nanoparticles reduced with hydrazine monohydrate and formalin (suspension H and F) were active against Gram-positive and Gram-negative bacteria. Very good activity was observed for suspension H, MICs values of standard bacterial strains were

between 5 and 20 μg/ml. Suspension F showed good activity (MICs 40-80 μg/ml). On the other hand silver suspension reduced with ascorbic acid (concentration of silver 10 times lower) was inactive against tested strains due to aggregation process during dialysis. The next step of antimicrobial test was the evaluation of activity of silver nanoparticles H against clinical isolates of Gram-positive and Gram-negative bacteria: *Staphylococcus epidermidis*, methicillin-resistant and methicillin-susceptible *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Eschericha coli.* Species of these bacteria are a biofilm-forming important human pathogens, responsible for a many nosocomial infections. Silver suspension H exhibit very high activity against hospital strains of *Staphylococcus epidermidis* and *Escherichia coli* (MICs values between 2,5 and 5 μg/ml), and good activity against *Staphylococcus aureus,* both methicillin-susceptible and methicillin-resistant strains, and *Pseudomonas aeruginosa* (MICs 5-10 μg/ml).

4. CONCLUSION

Simple and effective method of synthesis of silver nanoparticles with well-defined size and antimicrobial activity was demonstrated. The method was based on the chemical reduction of silver nitrate by reducing agents, hydrazine monohydrate (H), formalin (F) and ascorbic acid (AAC). Size and stability of obtained silver nanoparticles was controlled by presence of surfactant - sodium dodecylsulfate (SDS). The average size of nanoparticles as measured by DLS and SEM micrograph was below 20nm. The UV/VIS absorption spectras of silver particles confirmed the nanocrystalline character of the particles synthesized using various reducing agents. The XPS analysis showed that hydrazine monohydrate used in the particle synthesis results in the lowest amount of the organic-bonded silver fraction. Antimicrobial tests demonstrated that silver nanoparticles reduced with hydrazine monohydrate and formalin were active against Gram-positive and Gram-negative bacteria. The best activity was observed for suspension reduced with hydrazine monohydrate; MICs values of standard bacterial strains were between 5 and 20 μg/ml. On the other hand silver suspension reduced with ascorbic acid (AAC) was inactive against tested strains probably due to aggregation process during dialysis. Nanoparticles reduced with hydrazine monohydrate also showed high activity against clinical isolated strains, included highly multiresistant strains such as *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The received silver antimicrobial agents can be proposed as an alternative strategy for reducing bacterial adhesion and to prevent bacterial bio-film formation.

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