

Rational design of silver nanoparticles with reduced toxicity and enhanced antimicrobial activity

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Abstract

*This work presents a rapid, eco-friendly and scalable method for the synthesis of silver nanoparticles (Ag NPs) using an electrochemical technique. Data modeling based on the toxicity and antimicrobial activity of Ag NPs was performed in order to obtain a scientific design of Ag NPs. According to the mathematical model, Ag NPs with size ranging from 65 to 95 nm and functionalized with chitosan polymer on their surface were prepared. Ultraviolet–visible spectroscopy and transmission electron microscopy confirmed the Ag NPs formation. The prepared Ag NPs were characterized for size, size distribution and zeta potential using the dynamic light scattering method. Surfactants and polymers were used for the stabilization of Ag NPs. Their antimicrobial activity was evaluated using the minimum inhibitory concentration (MIC) method against reference microbial strains, i.e., *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Candida albicans*. The cytotoxicity studies revealed a good biocompatibility with normal cells, probably due to the presence of the protective polymeric coat. The proposed method allows a facile synthesis of large amounts of Ag NPs with good dispersion, high stability, antimicrobial effectiveness and reduced toxicity.*

Keywords: silver nanoparticles, chitosan, antibacterial activity, response surface method.

1. Introduction

In the last decades, metallic nanoparticles have attracted much attention for biomedical applications, such as bioimaging, drug delivery or antibacterial agents. Among these, silver nanoparticles (Ag NPs) have been extensively studied, due to their unique electrical, chemical, optical and antimicrobial properties significantly different from the bulk silver material. A large variety of Ag NPs were developed during the last 50 years for various industrial applications, such as catalysis [1], drug delivery [2], electronics [3], photonics [4], biosensing [5], non-linear optical materials [6] and conductive nanocomposites [7]. Medical and environmental applications, based on their antibacterial activity were intensively studied, such as water purification [8], textiles [9-10], food packaging [11], wound dressings [12], biocides [13], etc. Due to their large number of active sites on surface, suitable for their antibacterial interactions, Ag NPs provide bactericidal properties and became among the most studied theme in the microbiological nanoscience.

Even though there are many commercially available products containing Ag NPs, with various applications, such as water filtering, food packing, antiseptics and anti-stain clothes [14],

the mechanism of action is still under debate [15]. Also, the scientific link between the physico-chemical properties of the Ag NPs and their antibacterial effects has not been elucidated.

Although the existing literature reports for many decades the use of colloidal Ag dispersions in combating various types of bacterial infections, there are no systematic studies available about the influence of the various characteristics of nanoparticles on their properties related to the biomedical and cosmetic uses [16]. There are only disparate results, recorded on different types of particles, obtained under very different conditions, from electrochemical synthesis, to hydro or solve-thermal chemical synthesis or to biotechnological synthesis [17] and tested in various ways regarding aggregation, concentrations, release of silver ions, etc. The only general conclusion formulated after the analysis of the existing data is an expected one, that the size and surface functionalization are the main two characteristics of the nanoparticles with significant impact on their interaction with the live cells and on the antibacterial activity [18]. Unfortunately, the available data on the toxicity of nanoparticles are not systematized; it was only in 2015 that a project was launched to develop a NanoE-Tox database. The products currently manufactured were not scientific designed; they were based only on generic information in the literature on the existence of an antibacterial activity of both all Ag NPs and Ag ions.

Due to their industrial use, the synthesis of Ag NPs was intensively studied and many methods have been proposed. Commonly used methodologies for the preparation of Ag NPs consist in a large variety, from physical methods such as laser ablation [19], gamma irradiation [20] and electrodeposition, to chemical and biotechnological ones such as, sonoelectrochemical synthesis [21], microwave processing, chemical reduction, photochemical method, thermal decomposition, biosynthesis [22], green synthesis assisted by natural compounds [23], etc. Among them, electrochemical synthesis of Ag NPs is one of the simple, cost effective and frequently used as industrial scale method.

In the present work a facile method to produce Ag NPs with enhanced performances (high antibacterial efficiency and reduced toxicity) is presented, based on a rational design of size and surface functionalization of the nanoparticles. An analysis of existing data on various Ag NPs with different sizes and stabilizing agents led to a range of optimal size and zeta potential to ensure the suitable balance between the antibacterial activity and the toxicity effect on normal cells.

2. Materials and methods

2.1. Reagents

Chitosan (high molecular weight) BioChemika (CAS No: 9012-76-4) was purchased from Fluka Chemie GmbH. Hexadecyltrimethyl ammonium bromide (CTABr; 99% purity) was purchased from Sigma. Silver bars (99.999% purity) with 80 mm height and 2 mm diameter were used as electrodes and were kindly donated by SC Sonnenkreuz SRL, Brasov, Romania. All other chemicals used in this study were of analytical grade. Deionized water was used in Ag NPs synthesis and distilled water was used in all other experiments.

For the theoretical model response surface method (RSM) was employed, using Design Expert 10.0 program (StateEase Inc., USA). The experiment design used was Historical-Data.

2.2. Synthesis of Ag NPs

The synthesis of Ag NPs was performed using the electrochemical method. The instrument used was a laboratory model Colloid Master 100 (Colloidmaster, Isselburg, Germany) Two silver bars were connected as electrodes to the voltage source and placed in a 250 mL glass beaker with deionized water. In the modified synthesis method, solutions of various concentrations of stabilizing agents (surfactant or polymer) were used to replace water.

The voltage supplied by the electrochemical device to the silver electrodes was maintain for a period of time ranging from 1 hour to 4 hours, at room temperature, in the dark, without stirring.

2.3. Characterization of nanoparticles

The optical properties of the prepared Ag NPs were characterized by using UV-Vis spectroscopy. The measurements were performed on a JascoW530 spectrophotometer. Particle size distribution and electrokinetic potential were measured through Dynamic Light Scattering (DLS) technique, with a Zetasizer Nano ZS (Malvern Instruments Ltd.) device. The Ag NP morphology was investigated by transmission electron microscopy (TEM) using a Tecnai 20 instrument (FEI, Nederland); operating at 200 kV. FTIR spectra of polymer stabilized samples were recorded in the range 400-4000 cm^{-1} , measured using a Tensor 37 spectrometer from Bruker equipped with ATR module.

2.4. The microbial growth inhibitory activity

The minimal inhibitory concentrations (MIC, ppm) were determined by binary serial microdilutions assay performed in 96-well microtiter plates (Limban et al., 2011) against the following ATCC (*American Type Culture Collection*) reference microbial strains: *Escherichia coli* 25922, *Pseudomonas aeruginosa* 27853, *Staphylococcus aureus* 25923, *Enterococcus faecalis* 29212 and *Candida albicans* 10231. The test cultures were maintained in nutrient agar slant at 4°C. The working cultures were obtained by subculturing in nutrient agar. Microdilutions of the tested compounds ranging from 0.1 to 100 ppm were performed in 200 μL volume of nutrient broth and each well was inoculated with 20 μL microbial suspension of 0.5 McFarland density obtained from the corresponding working cultures. After incubation at 37 °C for 24 hours, the microbial growth was assessed by measuring the optical density of the liquid cultures obtained in the presence of compounds at 620 nm. Wells containing broth inoculated with microbial strains and wells with uninoculated broth were used as positive growth controls and as negative controls, respectively. The experiments were performed in duplicate.

2.5. In vitro biocompatibility

Ag NPs were tested for the evaluation of their *in vitro* biocompatibility on the normal dermal fibroblast cell line CCD-1070 Sk (CRL-2091) obtained from the ATCC. The fibroblast cells were cultured in Minimum Essential Medium (MEM), pH 7.4 containing 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g sodium bicarbonate, 1% antibiotic mix (penicillin, streptomycin, amphotericin), supplemented with 10% foetal bovine serum in flasks of 75 cm^2 and maintained in 95% humidity and 5% CO_2 . For the evaluation of Ag NPs (bare Ag NPs, Ag NPs+chitosan, Ag NPs+Na citrate) effects on cell viability, dermal fibroblasts were seeded at a density of 5×10^4 cells/mL in 24-well plates and then, treated with different concentrations of nanoparticles between 0.5 and 2.5 ppm for 24 hours. Assessment of cell viability was performed with the MTT colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent as previously described [24].

3. Results and discussions

The use of Ag NPs in commercial products is accompanied by a rising public concern on the lack of rigorous surveillance of their environmental impact and long-term effects on human health. Thus, it has become imperative to develop models in order to estimate the toxicity mechanisms of nanoparticles based on correlations between their physico-chemical properties and biological activity, for example quantitative (nano) structure-activity relationships, QSAR / QNAR. All these models, however, require good experimental toxicity data; in contrast, most of the results published in the literature, even for the same biological species, are very variable [25]. In the absence of rigorous characterization of the nanoparticles used in the toxicity studies, very different data are obtained, that cannot support a predictive model.

Response surface method (RSM) was applied on some data from recent papers [26-27] regarding various samples of Ag NPs. The number of design factors was two, size and surface potential. The historical data design of RSM was used, since support an unlimited number of design factors and import of existing data is very easy. The predicted response was selected based on the specific characteristic measured in the study to evidence the biological effect of Ag NPs (for example antibacterial efficiency, reduced glutathione-lowering effect, etc.) The terms were selected based on the P-value with 95% confidence level and the results were analyzed using analysis of variance (ANOVA) program incorporated in Design Expert Software. Three-dimensional plots and their respective contour plots were obtained based on the effect exerted by the levels of the two factors. The optimum region for the response was identified based on the main parameters in the overlay plot. The nanoparticles prepared under various conditions with different sizes and surface potential have been selected (data from references [26-27]). Nanoparticles with sizes ranging from 10 to 90 nm, without ligand functionalization on surface, were selected and investigated. The obtained results are shown in Figure 1.

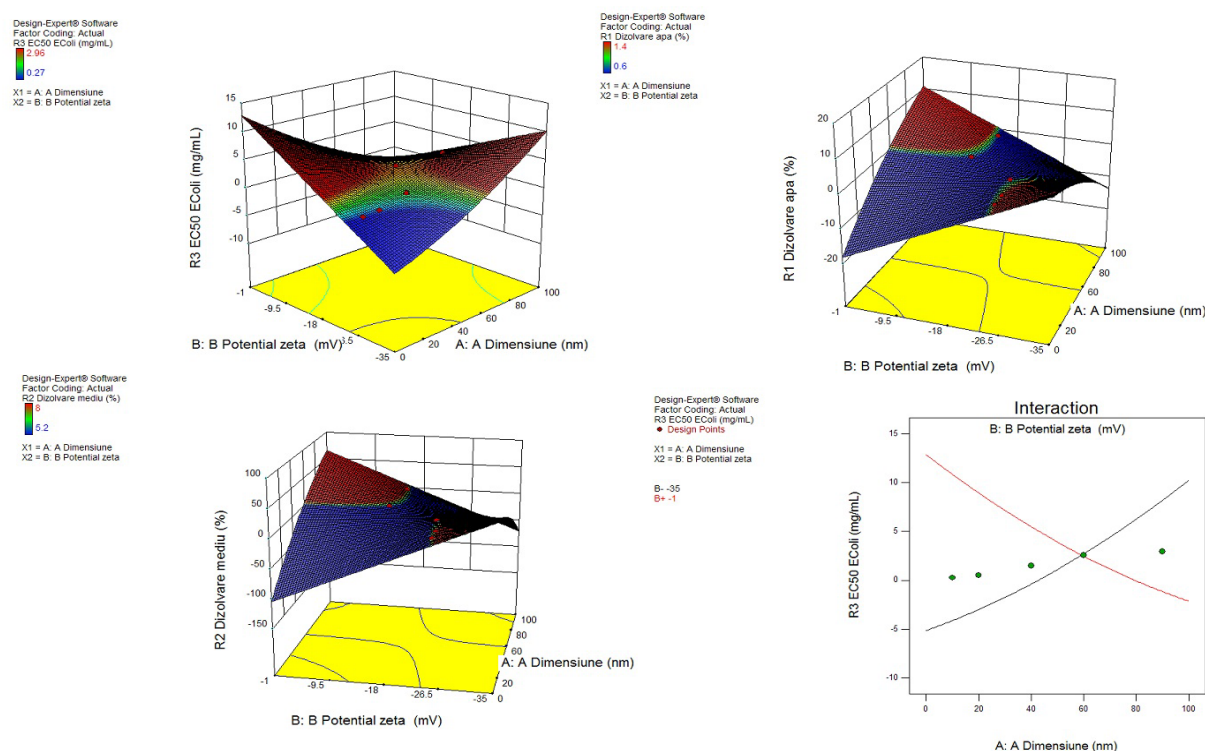


Figure 1. Plots obtained from RSM applied on the hystorical data analysis on Ag NPs with different sizes and zeta potentials.

The Ag NPs showed antimicrobial activity against the test strain of *E. coli*. It was found that biological activity correlated much better with the dissolution capacity in the culture medium of the bacteria. Model optimization suggested that the Ag NPs with the diameter of 60 nm and surface potential up to -35 mV exhibit the best antibacterial activity against *E. coli* ATCC 25922. In order to evaluate other factors that influence the biological activity and toxicity properties, cytotoxicity data of several Ag NPs with different sizes and coatings (surface functionalities) (data from reference [27]) were analyzed. Cytotoxicity data for HepG2 cell cultures, expressed by the IC50 (inhibitory concentration 50) value and reduced glutathione (GSH) decrease as a marker of oxidative stress, were used for modeling. Two particle types of 10 nm and 75 nm diameter, respectively, were introduced into modeling, both of them being

functionalized with two of the usual stabilizing agents, citric acid and PVP (polyvinyl pyrrolidone). The analysis of influence of various factors indicated that a more positive superficial potential was correlating with a toxicity increase (low values of IC₅₀), whereas the tendency of aggregation generating particles between 70 and 135 of nm did not influence the reduced glutathione-lowering effect. The induced effect, however, was very strong in the case of Ag NPs coated with PVP polymer. The toxicity decreased spectacularly with a few orders of magnitude. The effect was not detected in the case of colloidal dispersion of Ag in the culture medium, but only if the internalization of nanoparticles was taken into account.

Based on these preliminary data, Ag NPs with the size in range of 60 – 90 nm and with positive zeta potential obtained by using chitosan polymer for coating were proposed as effective antibacterial material, with a reduced toxicity for normal cells, in order to increase the biocompatibility and to reduce the negative impact on the environment.

To prepare the nanoparticles, a simple, eco-friendly method was used. The Ag NPs were obtained using electrochemical method, in polymeric chitosan solution for both size reduction and surface functionalization.

The optical characterization of Ag NPs was performed within the wavelength range of 300–800 nm to confirm Ag NP formation. The UV-VIS spectra are presented in Figure 2 for samples of Ag NPs prepared in distilled water and polymeric solution.

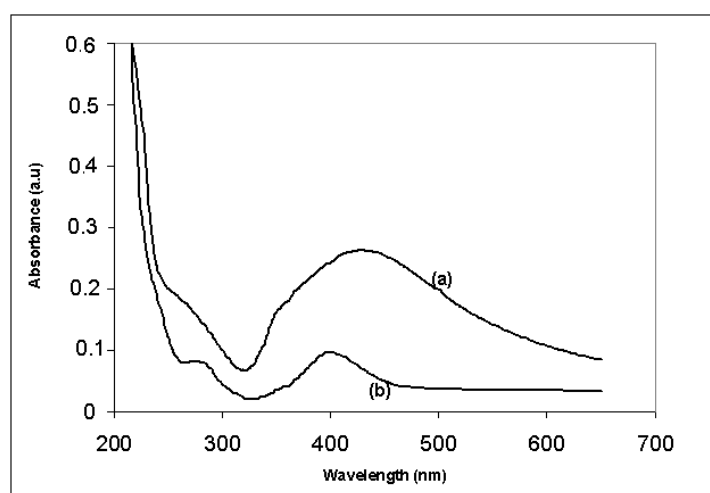


Figure 2. The optical properties of Ag NPs prepared in distilled water (a) and in 0.1% chitosan solution (b).

Formation of Ag NPs in both media was confirmed through the presence of absorption peaks at around 400 nm, corresponding to the characteristic surface plasmon resonance (SPR) signal specific for the Ag NPs. The nanoparticle synthesis in the presence of polymer was associated with formation of smaller particles, thus the absorbance peak became sharp and slightly shifted to smaller wavelength.

The DLS diagram for size distribution exhibited a monomodal distribution, thus the Ag NPs samples prepared with citrate (usual reagent used for particle size reduction) and chitosan as stabilizers did not contain large aggregates. The mean diameter of the particles ranged from 65 to 98 nm, in function of concentration of stabilizer. Zeta potential of Ag NPs prepared in chitosan was positive, ranging from +55 to +78 mV, which denoted a very good stability against aggregation.

The size of the Ag NPs was confirmed also by the TEM images shown in Figure 3.

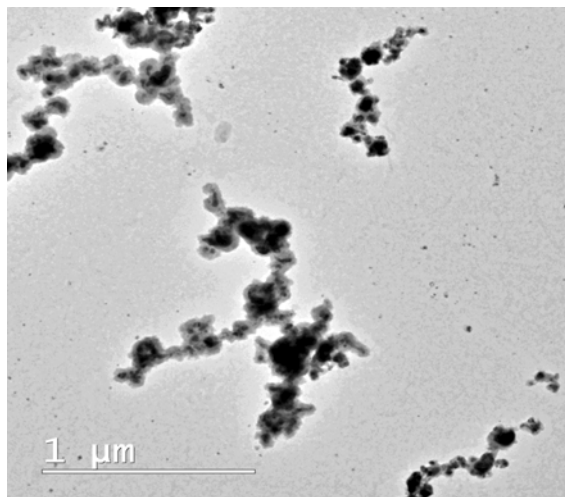


Figure 3. TEM image of Ag NPs prepared in 0.075% chitosan solution.

The shape of the particles was approximately spherical and polymeric shell on the particles could be observed. The FTIR spectrum presented in Figure 4 demonstrated the functionalization of Ag NPs with chitosan.

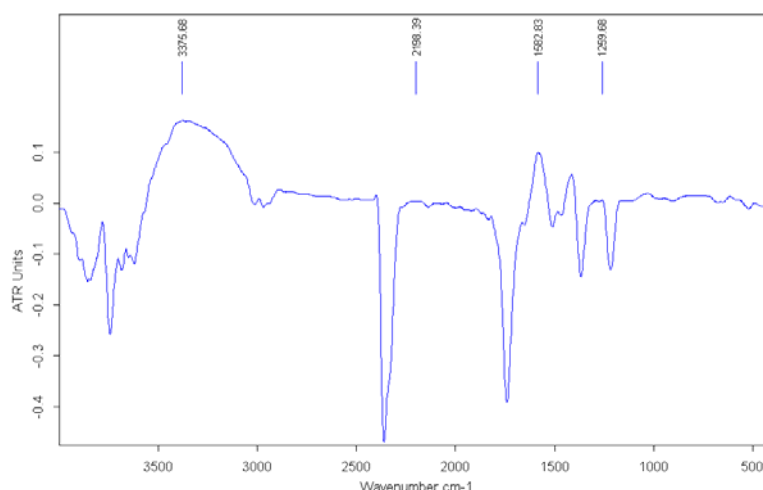


Figure 4. The FTIR spectrum of Ag NPs prepared in 0.2% chitosan solution.

Most of the peaks related to the chitosan chemical structure are insensitive to the presence of the Ag NPs, such as bands at 1650 cm⁻¹ (amide I band characteristic to C=O stretching of *N* acetyl group), 1400 cm⁻¹ (bending vibration of OH group) and 1375 cm⁻¹ (symmetric deformation vibration mode of CH₃), while few of them suffer minor alteration. The peaks in the region 3500 cm⁻¹ specific for the N-H extension vibration of the chitosan is shifted to higher wavelength, and the band at 1557 cm⁻¹ assigned to the amino group shifts to a lower wavelength in the presence of silver nanoparticles. The changes in the FTIR spectrum suggest that the primary amino groups of chitosan molecules were involved in the interaction with the Ag NPs surface during the stabilization process.

The results of the susceptibility tests for the Ag NPs with mean size of 85 nm, and various functionalization are shown in Figure 5. The MIC values revealed a good antimicrobial activity of the analyzed dispersions against all the microbial reference strains, at different concentrations, depending on their surface characteristics.

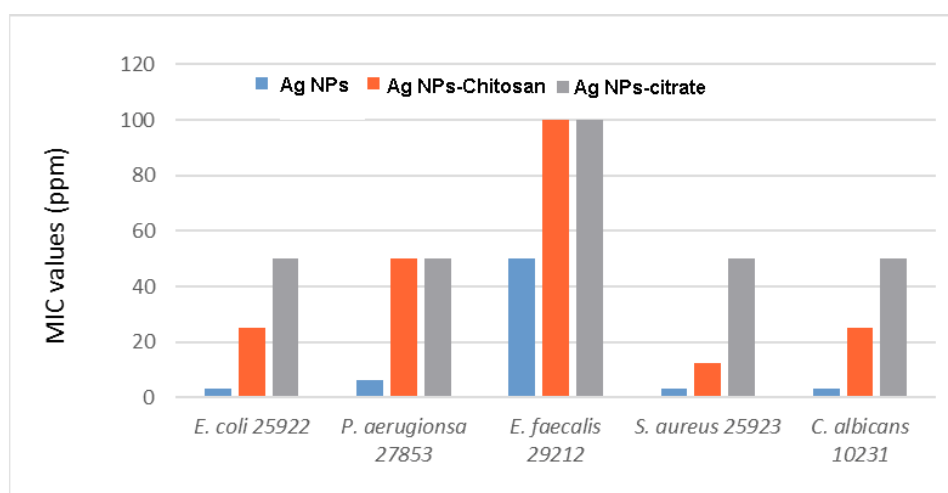


Figure 5. MIC values of the Ag NPs with and without surface functionalization.

The best inhibitory activity (3.125 ppm) of the series were observed for bare Ag NPs against *E. coli* 25922 and *S. aureus* 25923, and for chitosan stabilized Ag NPs against *C. albicans* 10231. As it is expected, the functionalized Ag NPs exhibited a lower antimicrobial activity compared to those of bare Ag NPs, due to the faster release of Ag ions in the last case.

The presence of chitosan polymer adsorbed on the surface of nanoparticles increased the antimicrobial effect compared to the samples stabilized with citrate. For all samples the highest values of MIC were recorded against *E. faecalis* 29212.

In Figure 6, the biocompatibility of Ag NPs is presented as a function of surface functionalization and dose.

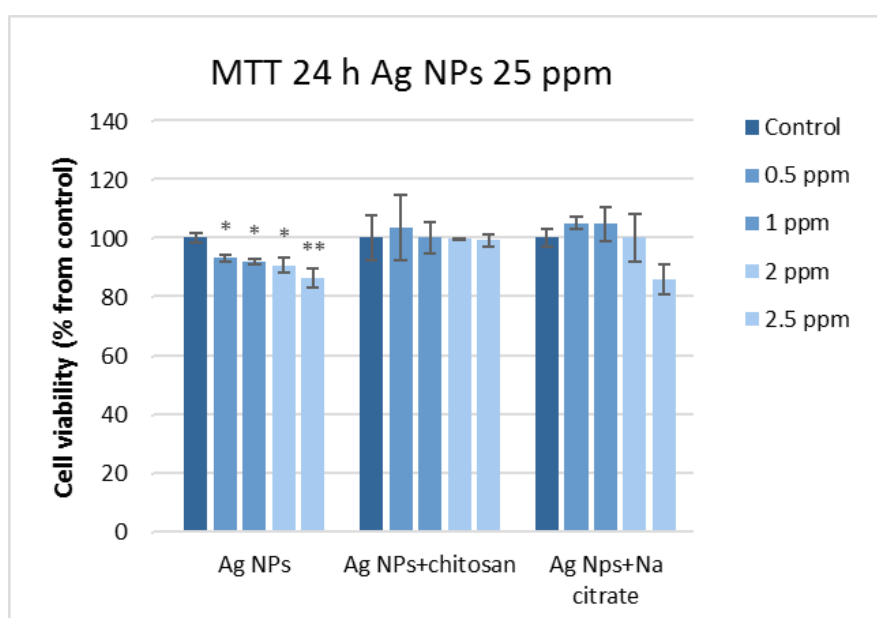


Figure 6. Viability of human dermal fibroblast cells exposed to different concentrations of Ag NPs after 24 hours. Cells untreated were used as controls. Data are expressed as average \pm SD (n=3).

The viability analysis (MTT test) revealed that after 24 hours this parameter decreased in a concentration dependent manner in the cells treated with bare Ag NPs. The dose of 2.5

ppm generated a diminished viability compared to control. When cells were treated with Ag NPs stabilized with chitosan no significant change was noticed for all concentration tested. Also in the case of Na citrate-stabilized particles doses between 0.5 and 2 ppm did not influence cellular viability of dermal fibroblasts whereas the one of 2.5 ppm generated a decrease by 14%.

4. Conclusions

An inexpensive electrochemical synthesis is proposed for the preparation of large quantities of Ag NPs, using polymeric solution as stabilizer and surface functionalization. The parameters of the preparation method were tuned in order to obtain metallic nanoparticles with size and zeta potential in the specific ranges, obtained from theoretical modelling of the correlation of antibacterial activity with the physico-chemical properties. Highly stable Ag NPs with spherical shape, as revealed by TEM images were prepared using various concentration of chitosan as polyelectrolyte stabilizer. The absorption spectra confirmed the formation of Ag NPs and the FTIR spectrum showed that the polymer is attached to the surface of nanoparticles.

The antimicrobial activity of the chitosan-decorated Ag NPs was also investigated against both Gram-positive and Gram-negative bacteria and fungal strains. The samples stabilized with polymer exhibited significant microbicidal activity, despite their rather high size. At higher concentration, the Ag NPs stabilized with chitosan showed better microbicidal effect against *E. coli* 25922, *S. aureus* 25923, and *C. albicans* 10231 compared to *P. aeruginosa* 27853 and *E. faecalis* 29212. The Ag NPs toxicity analysis revealed that surface polymeric chitosan coating increased the cell viability compared to bare Ag NPs.

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