Effect of Ultrasound Treatment on Chitosan-Silver Nanoparticles Antimicrobial Activity

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***Abstract*—Nanoparticles have good prospects as an alternative to antibiotics. Achieving the stability of nanoparticle solutions over time is one of the important tasks for researchers as well. Preparation of new antimicrobial release systems based on Ag NP and biological polymers will contribute to create effective antimicrobial agents.**

In current paper we evaluate antibacterial activity of chitosan-Ag NPs treated by ultrasound against Gram-positive and Gram-negative microorganisms.

Ag NPs were synthesized *via* polyol method. 2% chitosan solution used to produce polymer-Ag NPs composite.

X-ray diffraction (XRD) technique, scanning electron microscopy (SEM), and UV-Vis spectroscopy were used for NPs characterization. The antimicrobial activity of the Ag NPs and Ag NP/chitosan solution was tested against *S. aureus, S. pyogenes, E. coli, .K. pneumonia, P. aeruginosa, P. vulgaris* and *Candida spp*.

Pure Ag NPs demonstrate high antimicrobial activity against all microorganisms. The ultrasonic treatment of nanoparticles makes them more active in relation to gram-negative bacteria but sonicated composition of the Ag NPs and 2 % chitosan gel express the highest antimicrobial activity against all types of microorganisms.

Keywords—silver nanoparticles, antibacterial activity, chitosan, ultrasound.

I. Introduction

Multidrug resistant bacteria resulted increasing morbidity and mortality past decades. Antimicrobial resistance is a threat to all branches of medical and public health practice [[1]]. Skin injuries are a universal aspect of medical care, with approximately 300 million chronic and 100 million traumatic wound patients worldwide **Ошибка! Источник ссылки не найден.**. Both Gram-positive (such as *Staphylococcus spp.* and *Streptococcus* spp.) and Gram-negative bacteria (such as *E. coli* and *Klebsiella spp.*) can cause wound infections [**Ошибка! Источник ссылки не найден.**]. Wound dressing and wound healing are very important components of reducing morbidity and mortality of wound related burden [**Ошибка! Источник ссылки не найден.**]. It is complicated with rise of multi-resistant microorganisms amount. New antimicrobial agents elaboration and introduction into the practice is a way to cope with drug resistance [**Ошибка! Источник ссылки не найден.**, **Ошибка! Источник ссылки не найден.**].

Alternative drugs with antibacterial properties actively investigated last time are metallic nanoparticles (NPs). Among metallic nanoparticles, silver ones (AgNP) have attracted much attention due to their potential as antimicrobial agent; they are widely applied in many biological and medical fields such as biosensors, wound healing, treat burns and cancer therapeutics. Physical and chemical properties of AgNPs including surface chemistry, size, size distribution, shape, particle morphology, particle composition, coating/capping, agglomeration, dissolution rate, particle reactivity in solution, efficiency of ion release, and finally type of reducing agents used for synthesis are crucial factors for determination of cytotoxicity [5, **Ошибка! Источник ссылки не найден.**]. Moreover, various types of NPs exhibit different antibacterial effects, thus comprehensive analysis of NPs has significant meaning to study their potential antibacterial mechanisms [**Ошибка! Источник ссылки не найден.**]. Recent investigations have shown the immobilization and/or hybridization of NPs can enhance and improve the antimicrobial activity of the nanomaterials against a wide range of multi-resistant strains of pathogenic microorganisms [**Ошибка! Источник ссылки не найден.**]. There are a lot of attempts to increase the Ag NPs stability against agglomeration [16] .

The aim or our study was evaluation of antibacterial activity of chitosan-Ag NPs treated by ultrasound against Gram-positive and Gram-negative microorganisms.

II. Materials and Methods

A. Materials

Silver nitrate ($AgNO_3$) (impurity > 99,9%, p.a., Sigma-Aldrich), polyvinylpyrrolidone (PVP — K25, MW 24000, Sigma-Aldrich), ethylene glycol (EG) (impurity > 99,9%, Sigma-Aldrich), isopropyl alcohol (99% pure, p.a., Sigma-Aldrich) were used as raw materials. Distilled water from electric distiller DE 20 was used throughout the experiments.

In our work, chitosan (degree of deacetylation 82% (80-90%) and molecular weight 300 kDa was purchased from

YuDa Chemicals, Qindao, PRC, "Bioprogress", Moscow, and used without further purification. All other reagents were of analytical grade. 2 % solutions of chitosan in acetic acid was used in experiment.

Nutrient broth and nutrient agar were purchased from Hi Media India.

B. Synthesis of silver NPs

The synthesis method used in the present study is the so-called polyol method, which is well suited for the preparation of nano-sized silver. The synthesis of spherical silver nanoparticles by the polyol method has been reported in various investigations [7, 8, 9, 10]. In a typical procedure, 3.4 g of PVP was dissolved in 20 ml ethylene glycol and heated up to 155 °C in an oil bath, until it turned from colorless to light yellow. Then, 0.34 g of $AgNO_3$ in another 20 ml ethylene glycol was added drop wise into the above solution, and the reaction allowed to proceed for 0.5-1 h at this temperature. A brown colloidal dispersion was formed which indicated the formation of silver nanoparticles. The colloidal dispersion was cooled down to room temperature and mixed with a certain amount of isopropyl alcohol to allow for the generation of brown precipitate The precipitate was collected after centrifugation (10000 rpm for 30 min) and washed with isopropyl alcohol for three times, followed by drying at 50 °C for 2 h in a vacuum dryer.

Ag NPs were exposed to an ultrasound (ultrasonic dispergator UZDN-A (SELMI, Ukraine) at the following electrophysical characteristics: frequency 22±1,65 kHz, time 1 min.

C. Ag NPs characterization

In a X-ray diffraction investigation of synthesized materials were carried out on the automated diffractometer DRON 4-07 (NPP "Burevestnik", St. Petersburg) connected to the computer-aided experiment control and data processing system. The Ni-filtered CuK_α radiation (wave length 0.154 nm) was used with a conventional Bragg–Brentano ϑ-2ϑ geometry (2ϑ is the Bragg's angle). The samples were measured in the continuous registration mode (at the speed of 1.0 ◦/min) within the 2ϑ-angle range from 30° to 120°. All data processing procedures were carried out with the use of the program package DIFWIN-1 ("Etalon PTC" Ltd, Russia). Phase analysis was carried out by comparing the diffraction patterns from the investigated samples and the reference data JCPDS.

The morphology of the as-synthesized Ag nanoparticles was examined by transmission electron microscope (TEM). Samples for TEM were prepared by deposition isopropyl alcohol solution of Ag NPs on a copper grid covered with a thin carbon film with the subsequent drying in air at room temperature. TEM analyses were performed using "PEM-125K" (Ukraine).

UV-Vis measurements were performed using LI-722 Microprocessor Single Beam Visible Spectrophotometer (Lasany®). EDS elemental analysis was carried out on a JEOL JSM–6390LV scanning microscope with X-ray detector INCA 350 (Jeol, Japan). Concentration of Ag in the aqua solution was determined by the method of inductively-coupled plasma atomic spectrometry (ICP-AES) using an iCAP 6300 Duo spectrometer (Thermo Scientific Corporation, USA).

D. Antibacterial assessment

The antimicrobial activity of Ag NPs was examined against clinical strains of *S. aureus, S. pyogenes, E. coli, K. pneumoniae, P. vulgaris, E. faecalis and C. albicans.* All isolates were stored at Microbiology Lab, Sumy State University, Ukraine. Microorganisms were routinely cultivated overnight in nutrient broth at 37 °C. Then the cultures were diluted with cultivation media to the turbidity equivalent to McFarland 0.5 standard ($1.5x10^8$ CFU/ml). It was used as an inoculum. The minimum inhibitory concentration (MIC) of Ag NPs was measured by tube serial dilution method according to the international recommendations provided by the Clinical and Laboratory Standarts Institute (CLSI). At the beginning, seven concentrations of Ag NPs were prepared (from 100 μg/ml to 1.56 μg/ml). Then, 100 μl of microbial suspension was inoculated into each tubes. The tubes containing growth medium and tested samples without inoculums were used as controls. Then tubes were incubated aerobically at 37 °C for 24 h. The tube with the lowest concentration of the AgNPs that completely inhibits visual growth of bacteria (no turbidity) was considered as the MIC. All the measures were triplicate**.**

MIC of Ag NPs was used as the starting point for *in vitro* time-kill tests. *In vitro* static time-kill studies were conducted with glass tubes containing 2-ml volumes of nutrient broth with logarithmically growing cultures. Starting inoculum of microorganisms was 1×10^6 CFU/ml. In the test, we used the concentrations of Ag NPs equivalent to 1 MIC. In 15, 30 min, 1, 3, 6, and 24 h of incubation 100-μl aliquots from tubes were spotted onto plates with solid media. After that, plates were incubated at 37 ◦C for 24 h. Viable organisms were counted in $\log_{10}$. Time-kill studies were conducted in duplicate; tests were combined, and mean values were reported.

III. RESULTS AND DISCUSSION

In recent years, there has been a growing interest in researching and developing of new antimicrobial agents to combat microbial resistance [**Ошибка! Источник ссылки не найден.**]. Silver had attracted much attention for a long time due to its antimicrobial activity and now there is a huge increasing of the interest to Ag NPs. The stability of silver nanoparticles against agglomeration is considered the most important factor for their antibacterial efficacy and to reach this purpose we synthesized Ag NPs by use polyol method [10].

Ag NPs were prepared by the reduction of $AgNO_3$ and were characterized by XRD, TEM, UV-Vis spectroscopy. The obtained result is reflected in the fig. 1. It shows that the synthesized silver nanoparticles are substantially spherical and distributed in the interval 25-60 nm. The average diameter of Ag NPs is 45 nm.

To further validate the Ag NPs, the samples were analyzed in SEM–EDS and the results are shown in Figure 1 b). The strong peak at 3 keV confirms the presence of silver (91,2 %). Such elements as C (5,58 %), N (2,08 %), O (1,14 %) show the presence of polyvinylpyrrolidone $(C_6H_9NO)_n$, which was used as a stabilizer.

Figure 1 c) shows the X- ray diffraction pattern (XRD) of as prepared silver nanoparticles synthesized using polyol method. There are seven distinct diffraction peaks at 38.15°, 44.32°, 64.55°, 77.52°, 81.55°, 110.47° and 115,17°. The peaks are consistent with the data on the standard card (JCPDS file No. 3-921), which are corresponding to the face-centered cubic Ag plans of (111), (200), (220), (311), (222), (331) and (420). The calculated lattice parameter is a = 0,408 nm and unit cell volume V = 0,068 x 10^{-27} m^3. There is a small amount of noise may be caused by the amount of coated with a certain amount of PVP. No peaks from other phases were detected, indicating high purity of the Ag NPs: PVP capped layer on Ag nanoparticles prevents Ag nanocores from oxidation.

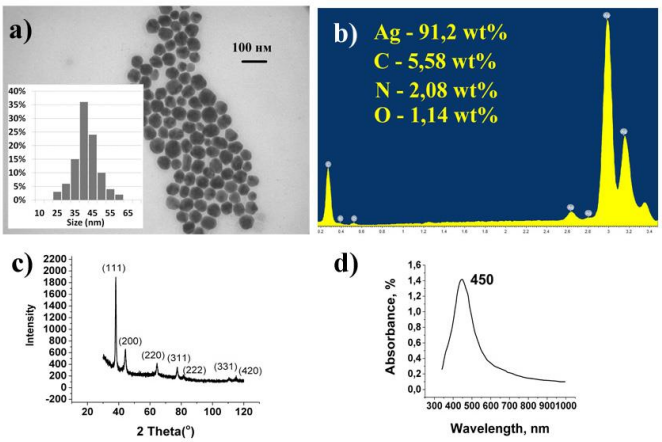

Fig. 1. a) TEM images of PVP capped Ag NPs; b) scanning electron microscopy – energy dispersive spectrum of Ag NPs; c) XRD pattern of Ag NPs; d) absorption spectra of silver nanoparticles.

As we can see from the Figure 1 d), the UV–visible spectra of silver nanoparticles dispersed in water revealed the broad surface plasmon resonance at 450 nm agrees with a size distribution due to the partial aggregation of silver nanoparticles. The surface plasmon resonance band are depended by shape, size, composition, morphology of the prepared NPs. Presence of such peak assigned to a surface plasmon resonance of silver nanoparticles was previously reported [14].

A. Assessment of Ag NPs antimicrobial activity

At the beginning the antibacterial activity of Ag NPs had been evaluated against Gram-positive bacteria (*S. aureus, S. pyogenes*), Gram-negative bacteria (*E. coli, .K. pneumonia, P. aeruginosa, P. vulgaris*) and fungi (*Candida spp*.). It was found that all tested species except *E. coli* that was less sensitive were inhibited with Ag NPs at concentration 25 μg/ml. The obtained values of MIC demonstrate, that silver nanoparticles prepared by the described method have high antibacterial and antifungal activities that are comparable with previously published result **Ошибка! Источник ссылки не найден.**. Based on the results obtained from MIC we can do the suggestion that AgNPs demonstrate similar antibacterial activity against gram-positive and gram-negative microorganisms.

Antibacterial and antifungal activities of polyvinylpyrrolidone, which was used as a stabilizer, in appropriate concentrations as blank samples were tested. It was proved that it does not exhibit any antibacterial or antifungal effects in the used concentrations.

Whole assess of the Ag NPs antimicrobial effectiveness assumes the use of dynamic interpretation of drug-bacteria interactions. Time-kill curves can follow microbial killing and growth as a function of both time and antibiotic concentration [13]. Time-kill determines how fast an antimicrobial material can kill the bacteria. Due to this we used pharmacokinetic-pharmacodynamic models based on time-kill curves. For all seven species, the killing kinetics of Ag NPs was time-dependent. Kill-kinetics study demonstrated that AgNPs at dosage level 1 MIC reduced gradually to the inoculums size of most isolates by >3 $\log_{10}$ CFU/ml at 24 h (Fig. 3). Whereas the concentration of the *S. aureus* inoculums reached 0 $\log_{10}$ in one hour, the concentration of the P. vulgaris achieved 2 $\log_{10}$ inoculum size in 24 hours (fig.2).

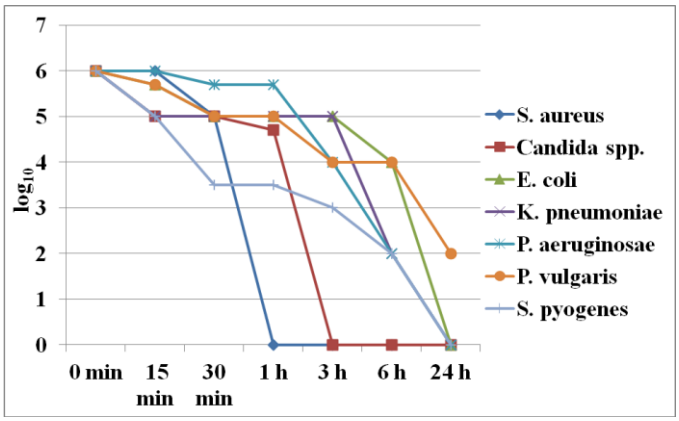

Fig. 2. In vitro time-kill antimicrobial activity of AgNPs.

The pharmacodynamic parameters for the Ag NPs illustrated the various effects that silver nanoparticles have on the growth of different species. It is the strongest antimicrobial actions on *Staphylococcus spp.* and *Candida spp*. Hence the ability to kill bacteria at a fast speed would be clinically important.

In order to improve effectiveness of AgNPs and based on our previous results we did the *in situ* preparation of the chitosan gel/Ag NPs composites by simple mixing of both components. Despite our expectation and other researches date **Ошибка! Источник ссылки не найден.** the combination of the Ag NPs with chitosan gel did not show any improvement of their antimicrobial activities. Probably the polyvinylpyrrolidone used in synthesis of AgNPs covers the particles and deteriorates their incorporation in chitosan gel. It is resulted in lack of antibacterial activity enhancement.

It is known that under ultrasound exposure nanoparticles expressed better antimicrobial activity [11, 12]. In order to

evaluate impact of ultrasound on the Ag NPs antimicrobial efficiency we sonicated the AgNPs before their use. Gram negative bacteria demonstrated higher sensitivity to sonicated Ag NPs. There was also sharp increasing of the Ag NPs/chitosan gel antimicrobial activities (fig. 2). Because the all tested microorganisms minimal inhibitory concentrations were decreased significantly.

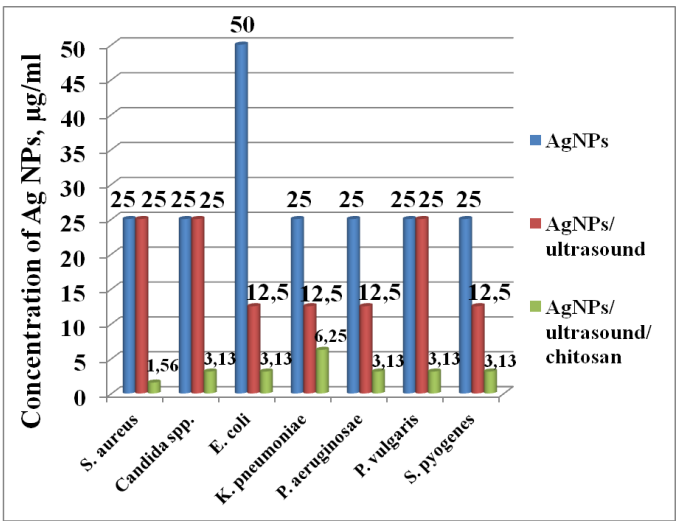

Fig. 3. Minimal inhibitory concentration of the treated and untreated Ag NPs.

This enhancement can be due to two mechanisms. One of them is nanoparticle dissociation, which will increase nanoparticles penetration into cell membranes in the presence of ultrasound treatment. Another one is the increasing of the silver ions Ag^{2+} concentration in the NPs suspensions after sonication, since ultrasonication is a method frequently used to improve the solubility of nanomaterials [12].

IV. Conclusion

The Ag NPs sizes of 25-60 nm possess high antimicrobial activity against gram-positive, gram-negative bacteria and fungi. The use of ultrasound pretreatment decreases the minimal inhibitory concentration of Ag NPs, especially Ag NPs combined with chitosan gel. Consiquently sonicated Ag NPs /chitosan gel is promising system for the future development of topical antimicrobial therapy.

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