SELENIUM NANOPARTICLES AND EVALUATION OF THEIR ANTIMICROBIAL ACTIVITY ON BACTERIAL ISOLATES OBTAINED FROM CLINICAL SPECIMENS

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Abstract

Our main objective was to determine the antimicrobial effects of selenium nanoparticles (SeNPs). For the testing of their influence we exploited isolates obtained from swabs from patients (*n* = 25) with hard-to-heal skin infections. Using mass spectrometry we identified 49 various bacterial strains. Antimicrobial properties of SeNPs were tested using measurement of inhibition zones; determination of growth curves and measurement of possible toxicity by inhibition of nucleic acid replication. Clinical isolates were exposed to the constant concentration of SeNPs (100 μg/mL). In almost all of bacterial strains we observed inhibition zones higher than 5 mm, which are the legislatively given as the lowest value for confirmation of effectivity of tested antimicrobial agents. The most sensitive strains were further exposed to the concentration range of selenium nanoparticles (0; 1; 2; 4; 8; 16; 32 and 48 μg/mL). Growth curves exhibited inhibition effects caused even by the lowest applied concentration (1 μg/mL) in all of tested bacterial isolates. In *Escherichia fergusonii*, *Pseudomonas aeruginosa* and *Streptococcus agalactiae*, application of 64 μg/mL of SeNPs resulted in total growth inhibition. We confirmed the antimicrobial effects of SeNPs, which may be used in the future for the elimination of hard-to-heal bacterial infections.

Keywords: Nanoparticles; antimicrobial; selenium; bacterial isolates

1. INTRODUCTION

Bacterial resistance to antibiotic therapy is a major public health concern worldwide. Bacteria replicate and mutate quickly, rapidly developing resistance to many commonly used antibiotics. Among the most common resistant strains belongs CA-MRSA (community methicillin-resistant *S. aureus*), which frequently appears between groups of people such as athletes or soldiers and HA-MRSA (nosocomial (hospital) methicillin-resistant *S. aureus*), causing bacterial infections and increased antibiotic resistance [1].

Elimination of occurrence of infectious diseases is carried out mostly by using a range of antibacterial components non-toxic to the human body. The use of antibiotics in clinical practice meets a range of complications. Among them, the emergence of bacterial resistance, and their potential toxicity, for non-targeted tissues and organs should be mentioned.

Nanoparticles possess high catalytic ability due to the large surface and the ability to generate reactive oxygen species causing the high reactivity, which thus results in higher toxicity towards bacteria [2]. Thanks to above mentioned properties, nanoparticles exhibit higher retention in the body, so it is easier to achieve the desired distribution effect.

The most frequently used metal for the formation of nanoparticles with antimicrobial properties is undoubtedly silver. The mechanism of action of silver nanoparticles is still unclear. However, the possibilities of silver nanoparticles effect based on enzyme inhibition, alteration of the membrane integrity, penetration into the bacterial cytoplasm and accumulation in the periplasmic space or in the formation of reactive oxygen species are proposed. It was confirmed that composition of the cell wall and the plasma membrane plays an important role in the penetration of nanoparticles into the cell. In G^+ bacteria is the transmittance significantly slower than in the case of G^- bacteria [3].

More recent option from the perspective of nanotechnologies and bacterial infections are selenium nanoparticles (SeNPs). SeNPs were investigated for various medical applications and as a potential material for orthopedic implants [4]. Currently, studies which indicate precisely the ability of the selenium compounds to inhibit bacterial growth and formation of bacterial biofilms are also available [4].

The aim of this study was to prove the SeNPs effectivity in elimination of bacterial infections. For this purposes, clinical isolates of bacteria acquired from the patients with serious and hard-to-heal bacterial infections was used. The effect of tested nanoparticles was also monitored in connection with the elimination of prokaryotic cells and without affecting the eukaryotic cells.

2. MATERIALS AND METHODS

2.1. Chemicals, preparation of deionized water and pH measurement

Chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise.

2.2. Synthesis and characterization of SeNPs

One hundred mg of chitosan was dissolved in 9 ml of water. Then 0.1 mL acetic acid was added with 1 mL of $Na_2SeO_3.5H_2O$ (0.263 g/50 mL) solution. After that solution was mixed for 1 hour. Then mercaptopropionic acid - MPA (10 mL) was added. The solution was stirred for 1 hour. Necessary p*H* 7 was reached with 1 M NaOH (1.4 mL), and the color of the samples was pale orange. The samples were stirred vigorously for 3 hours at room temperature. Then samples were left at 60 °C for 24 hours on magnetic stirrer.

SeNPs were further characterized by dynamic light scattering (Zetasizer Nano ZS90, Malvern instruments, Malvern, UK)and by scanning electron microscopy (MIRA, Tescan, Brno, Czech Republic) [5].

2.3. Preparation of hospital samples and their cultivation

Cohort of patients with bacterial infections

For evaluation, patients with surface or deep wounds were selected. Total of 25 patients in age between 19 and 93 years were enrolled into the clinical study. Most of the tested subjects (n = 17) belonged to age group 70 - 79 years. In all patients were by traumatologists determined the expected duration of treatment depending on the severity and extent of infectious disease, associated diseases complicating the process of treatment and healing of wounds and other factors such as patient age, medications, previous medical history. This time period is given regardless of previous treatment by the patient himself and is determined on the date of admission of the patient to the infectious department. Enrollment of patients into the clinical study was approved by the Ethics Committee of Trauma hospital in Brno.

Collection of wound swabs from patients with bacterial infections

The smears were collected from infected wounds with the agreement of patients. Smear was sampled by rolling motion at the site of skin puncture using a sterile swab sampler. All patients were divided into two subgroups, concerning the infection severity - deep and superficial wound, with respect to diagnosis, detailed description of comorbidities and duration of treatment. The classification was carried out according to Classification of surgical wounds - SSI (surgical site infections) [6]. Infectious wounds were sampled by using disposable tampon swabs, to prepare representative sample in order to collect the maximal amount of present microflora. Tampons were subsequently stored in transport medium (inorganic salts, sodium thioglycolate, 1 % agar, activated charcoal).

Cultivation of clinical specimens

Four types of selective nutrient media (blood agar enriched by 10 % NaCl, Endo agar, blood agar without any other components and blood agar with aminoglycoside antibiotic amikacin) were employed for further

microbiological selection. Petri dishes, containing above mentioned media were subsequently incubated according to conventional protocols, used in other studies to maintain a suitable conditions for growth of all types of bacteria.

2.4. MALDI-TOF MS identification of bacteria

The sample preparation and identification of bacterial strains was carried out following the protocol optimized in our previous study [7].

2.5. Determination of antibacterial properties

Inhibition zones

To determine the antimicrobial effect of SeNPs on bacterial strains, the measurement of the inhibition zones was performed according to [5].

Growth curves

The second procedure for the evaluation of an antimicrobial effect of tested nanoparticles was based on the measurement of an absorbance of tested bacteria. An apparatus Multiskan EX (Thermo Fisher Scientific, Germany) via Ascent Software for Multiskan was used with subsequent analysis in the form of growth curves as was shown in [8].

2.6. Determination of interactions between selenium nanoparticles and Taq DNA polymerase

Polymerase chain reaction and visualization by agarose gel electrophoresis

DNA isolated from bacteriophage λ (48 502 bp) and Taq PCR kit were purchased from New England Biolabs (Ipswitch, MA, USA). Amplification of λ *xis* gene fragment was performed by polymerase chain reaction according to [9].

The amplified product was analysed using agarose gel electrophoresis and the working conditions were as follows: 2% agarose gel (High melt/Medium fragment, Mercury, Sand Diego, CA, USA) with 1× TAE buffer, 60 V and 160 min (Bio-Rad, Hercules, CA, USA). The 100 bp DNA ladder (New England Biolabs, Ispwich, MA, USA) was used as a molecule size marker. Bands were visualized using UV transilluminator at 312 nm (VilberLourmat, Marne-la-Vallée Cedex, France).

3. RESULTS AND DISCUSSION

The surface wounds are often populated by a wide range of bacterial strains which severely complicate the healing process. The most abundant are *Staphylococcus* sp. or family *Enterobacteriaceae*. In this work, cohort of 25 patients was investigated in terms of the bacterial population in the hard-to-heal surface wounds of patients in Trauma Hospital in Brno and possible exploitation of SeNPs in management of these serious health complications.

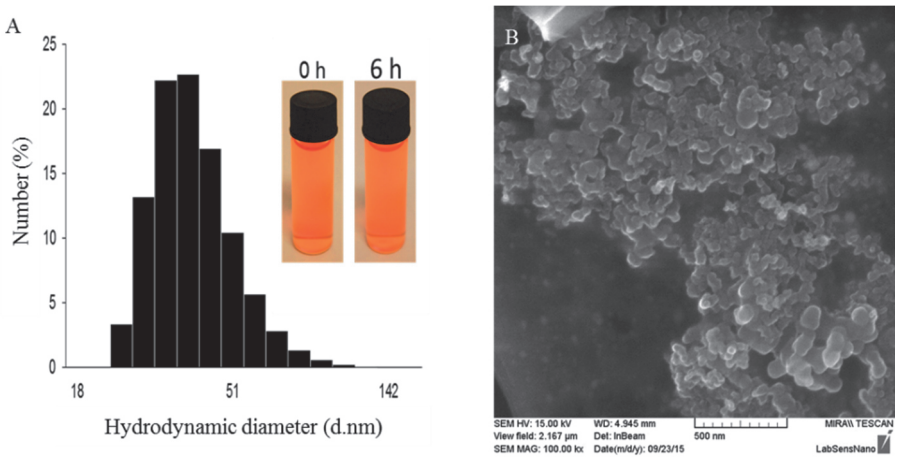

Fig. 1 A) Expression of hydrodynamic diameter of SeNPs and their colloidal stability immediately after synthesis and 6 h after storage in 25 °C. **B)** Micrograph of selenium nanoparticles measured by SEM

Firstly, SeNPs were synthesized. The resulting product exhibited orange color, zeta potential -31.9 mV and perfect colloidal dispersion immediately after synthesis (**Fig. 1A**). Noteworthy, after 6 hours, SeNPs remained stable in dispersion (insert of **Fig. 1A**). Spherical nanoparticles of selenium were also observed under the electron microscopy (**Fig. 1B**).

Using MALDI-TOF it was confirmed that the swabs from subjects were populated by 49 different bacterial strains. Within all subjects the applied antibiotic treatment was provided. With all of these strains measurement of inhibition zones (IZ) after the application of SeNPs (100 µg/mL) was carried out. The IZs after 24 hours of inhibition in 37 °C were determined and the resulting values of IZs ranged between 1 to 14 mm. In accordance with legislation, the effect of selenium nanoparticles can be considered as an effective when forming minimally 5 mm IZ (**Table 1**).

Table 1 Bacterial strains isolated from infectious wounds of patients with serious bacterial infections and identified by MALDI-TOF mass spectrometry.

Bacterial strains	ATBs	Inhibition zone (mm)
Acetobacter aceti	Cyphin	5
Actinocorallia libanotica		6
Acinetobacter genomospecies		6
Aeromonas media		4
Bacillus salarius		3
Candida albicans	Biseptol	2
Candida lmbicalanal	Ceturoxin	6
Candida mesenterica		5
Candida sorbosana	Cyphin; Ceturoxin	5
Candida tropicalis	Ceturoxin; Metronidazol	14
Corynebacterium striatum		3
Enterobacter cloacae	Ceturoxin	9
Enterococcus faecalis	Cyphin; Ceturoxin; Augmentin	4
Escherichia coli	Biseptol	6
Escherichia ferqusonii		9
Filifactor villosum		6
Hafnia alvei	Cyphin	6
Klebsiella oxytoca		2
Klebsiela pneumoniae	Biseptol	4
Lactobacillus kalixensis		6
Lactobacillus parabuchneri	Medoclav; Ciphin	6
Lactobacillus paracasei		6
Lactobacillus satsumensis	Dalacin	6
Lactococcus raffinolactis	Cyphin	4
Morganella morganii	Colomycin; Nitrofurantoin	5
Neisseria gonorrhoae		6
Neisseria weaveri	Medoclav	5
Proteus mirabilis		6
Proteus vulagris	Dalacin	6
Pseudomonas aeruginosa	Cyphin; Ceturoxin; Augmentin; Biseptol	7
Pseudomonas hydrocarboxydans	Biseptol; Ceturoxin	5
Pseudomonas mendocina		6
Pseudomonas nitroreducens		1
Pseudomonas syrinqae	Augmentin	6
Raoutella ornithinolytica	Dalacin	5
Staphylococcus aqalactiae	Medoclav	6
Staphylococcus aureus	Biseptol; Ceturoxin	8
Staphylococcus carnosus		5
Staphylococcus epidermis	Ceturoxin; Medoclav	5
Staphylococcus haemolyticus	Augmentin	5
Staphylococcus hominis	Medoclav	5
Staphylococcus simulans		4
Staphylococcus warneri	Dalacin	5
Stenotrophonas maltophilia		6
Streptococcus agalactiae	Metronidazol; Medoclav	8
Streptococcus ovis	Cyphin; Ceturoxin	6
Streptococcus salvarius	Medoclav	3
Streptomyces avidinii	Ceturoxin	5
Streptomyces lavendulae		4

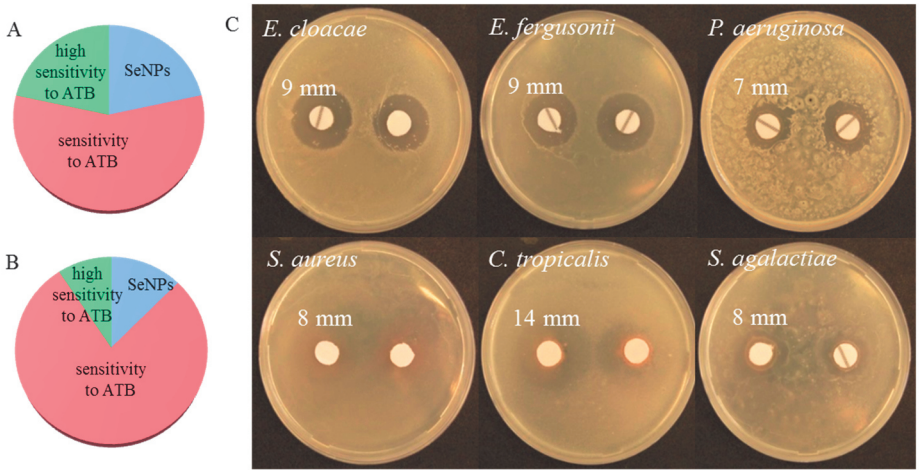

Fig. 2 All identified clinical isolates were divided into **A)** G^+ and **B)** G^- bacteria as an isolates sensitive to ATBs, high sensitive to ATBs or sensitive only to SeNPs. **C)** The most sensitive bacterial strains to the effect of 100 µg/mL SeNPs. Measurement of inhibition zone sizes after 24 hours of incubation in 37 °C

All identified clinical isolates were divided according to their sensitivity or high sensitivity to antibiotics and sensitivity to selenium nanoparticles. All these strains were further subdivided into G^+ (**Fig. 2A**) and G^- bacterial strains (**Fig. 2B**). For other measurements we selected six most sensitive strains (*Enterobacter cloacae* (IZ = 9 mm), *Escherichia fergusonii* (IZ = 9 mm), *Pseudomonas aeruginosa* (IZ = 7 mm), *Staphylococcus aureus* (IZ = 8 mm), *Candida tropicalis* (IZ = 14 mm) and *Streptococcus agalactiae* (IZ = 8 mm)) to SeNPs effects.

Selected bacterial strains were further exposed to SeNPs (0; 1; 2; 4; 8; 16; 32 and 64 µg/mL). Growth curves (**Fig. 3 B, C, F**) show the significant inhibition effect of SeNPs with increasing applied concentrations. The highest decrease of bacterial growth was observed in *E. coli*, *P. aeruginosa* and *S. agalactiae*, when the highest concentration of SeNPs (64 µg/mL) caused almost total inhibition of these bacteria

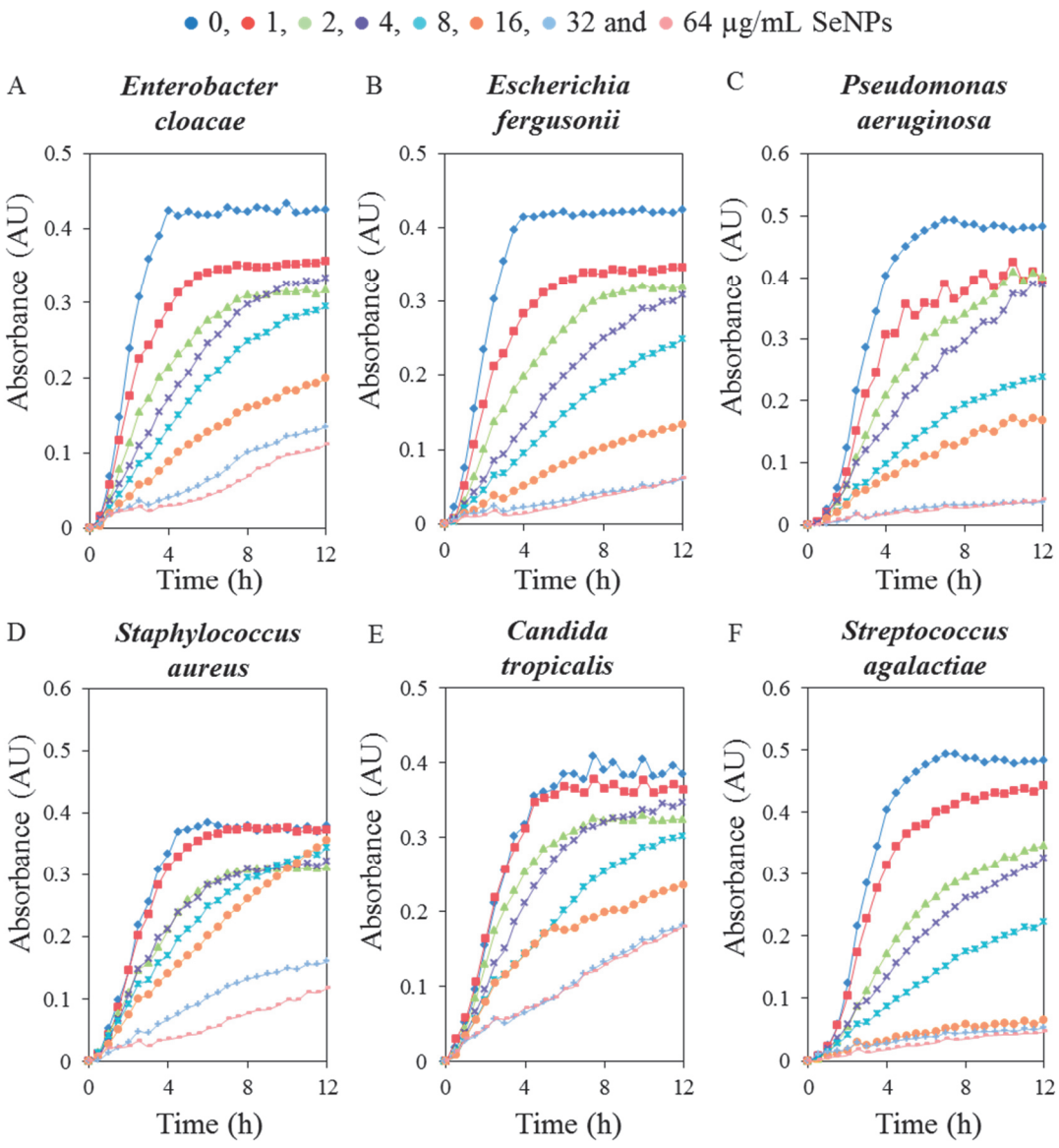

Fig. 3 Bacterial strains exposed to SeNPs in concentration range 0; 1; 2; 4; 8; 16; 32 and 64 µg/mL. Measurements were carried out for a 12 hours in 37 °C during constant shaking. Growth curves are made from the absorbance values determined every half an hour of measurement

Polymerase activity assay was performed by addition of SeNPs to PCR mixture. Visualization of PCR products revealed the downward trend of fluorescence with increasing concentrations of SeNPs. Thus it can be stated that SeNPs can inhibit the polymerase activity of the enzyme (**Fig. 4**). Polymerase is involved in DNA replication, which catalyzes the polymerization of DNA strand. If the activity of the polymerase is disturbed,

there cannot take place the cell division. We assume that effect of SeNPs on inhibition of the replication of the nucleic acid of bacteria occurs only in prokaryotic cells.

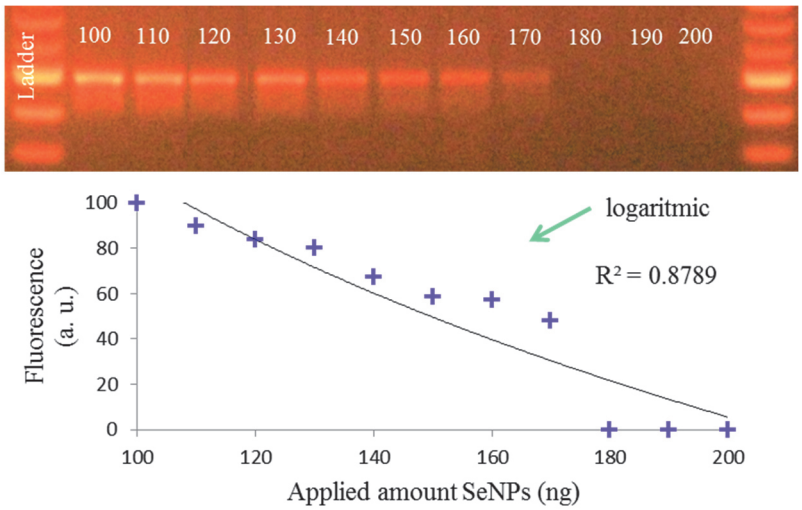

Fig. 4 Inhibition of polymerase activity by 100 - 200 ng of selenium nanoparticles evaluated by polymerase chain reaction with ethidium bromide detection on 2% agarose gel after 160 min separation at 60 V

4. CONCLUSION

SeNPs were tested for their antimicrobial effect on clinical isolates. Using the method of growth curves we confirmed the inhibition effect caused even by the lowest applied concentration (1 µg/mL) in all tested bacterial isolates. In *E. fergusonii*, *P. aeruginosa* and *S. agalactiae* application of 64 µg/mL of SeNPs resulted in total inhibition. Synthesized nanoparticles are thus the suitable as an alternative to the antibiotic drugs applicable for the treatment of hard-to-heal skin bacterial infections, with perfect stability and toxicological properties.

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REFERENCES

[1] OGATA K., NARIMATSU H., SUZUKI M., HIGUCHI W., YAMAMOTO T., and TANIGUCHI H., Molecular epidemiological study of community-acquired methicillin-resistant staphylococcus aureus (CA-MRSA) - an examination of commercially distributed meat as a possible vehicle for CA-MRSA. Journal of UOEH, Vol. 36, No. 3, 2014, pp. 179-190.

[2] CHOI O., DENG K.K., KIM N.J., ROSS L., SURAMPALLI R.Y., and HU Z.Q., The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. Water Res., Vol. 42, No. 12, 2008, pp. 3066-3074.

[3] GRIGOR'EVA A., SARANINA I., TIKUNOVA N., SAFONOV A., TIMOSHENKO N., REBROV A., and RYABCHIKOVA E., Fine mechanisms of the interaction of silver nanoparticles with the cells of Salmonella typhimurium and Staphylococcus aureus. Biometals, Vol. 26, No. 3, 2013, pp. 479-488.

[4] WANG Q. and WEBSTER T.J., Nanostructured selenium for preventing biofilm formation on polycarbonate medical devices. Journal of Biomedical Materials Research Part A, Vol. 100A, No. 12, 2012, pp. 3205-3210.

[5] RICHTERA L., CHUDOBOVA D., CIHALOVA K., KREMPLOVA M., MILOSAVLJEVIC V., KOPEL P., BLAZKOVA I., HYNEK D., ADAM V., and KIZEK R., The Composites of Graphene Oxide with Metal or Semimetal Nanoparticles and Their Effect on Pathogenic Microorganisms. Materials, Vol. 8, No. 6, 2015, pp. 2994-3011.

[6] EISENBERG D., Surgical Site Infections: Time to Modify the Wound Classification System? Journal of Surgical Research, Vol. 175, No. 1, 2012, pp. 54-55.

[7] CHUDOBOVA D., DOSTALOVA S., RUTTKAY-NEDECKY B., GURAN R., RODRIGO M.A.M., TMEJOVA K., KRIZKOVA S., ZITKA O., ADAM V., and KIZEK R., The effect of metal ions on Staphylococcus aureus revealed by biochemical and mass spectrometric analyses. Microbiological Research, Vol. 170, No., 2015, pp. 147-156.

[8] CHUDOBOVA D., NEJDL L., GUMULEC J., KRYSTOFOVA O., RODRIGO M.A.M., KYNICKY J., RUTTKAY-NEDECKY B., KOPEL P., BABULA P., ADAM V., and KIZEK R., Complexes of Silver(I) Ions and Silver Phosphate Nanoparticles with Hyaluronic Acid and/or Chitosan as Promising Antimicrobial Agents for Vascular Grafts. International Journal of Molecular Sciences, Vol. 14, No. 7, 2013, pp. 13592-13614.

[9] SMERKOVA K., DOSTALOVA S., VACULOVICOVA M., KYNICKY J., TRNKOVA L., KRALIK M., ADAM V., HUBALEK J., PROVAZNIK I., and KIZEK R., Investigation of interaction between magnetic silica particles and lambda phage DNA fragment. Journal of Pharmaceutical and Biomedical Analysis, Vol. 86, No., 2013, pp. 65-72.