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ON THE BIOLOGICAL ACTIVITY OF SILVER NANOPARTICLES

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Abstract. Silver nanoparticles (SNPs) were synthesized by reduction of silver ions to elementary silver in the system of β-cyclodextrin clathrates. SNPs were characterized by optical spectroscopy, microscopy (AFM, TEM), and X-ray diffractometry. Toxicity of the SNPs was tested in viruses, bacteria, yeast, cultured human cell lines, mollusks, and mammals. The particles displayed high antimicrobial activity and low toxicity in eukaryotic cells. The invertebrates were found to be more sensitive to SNPs, than mammals. Silver from SNPs was transported (apparently as Ag(I)) into the cells of prokaryotes and eukaryotes, bound to Cu(I) transport proteins. Silver was unevenly distributed between cell organelles; it was inserted to active centers of cuproenzymes and impaired their activity. The prospects to use these SNPs as antimicrobial agents, which enhance the action of antibiotics, as inhibitors of copper transport to growing tumors, and as a tool for studying copper metabolism are discussed.

1. Introduction

Antimicrobial properties of silver are well known from ancient times. However, the widespread use of silver is limited because in biological media silver ions produce poorly soluble salts. Silver nanoparticles (SNPs) are novel and promising functional materials, due to their antibacterial, antifungal, antiviral, and anti-inflammatory activity [1]. SNPs are chemically stable; particle size and concentration can be easily controlled. It is known that bacteria are much more sensitive to SNPs as compared to mammals, and SNPs can enhance the efficiency of antibiotics [2]. SNPs are successfully used for medical textiles, cardiovascular and bone implants, medical catheters, antimicrobial treatments *etc.* [3].

There are good reasons to believe that SNPs may be used as a tool for fundamental studies of copper metabolism and biochemistry of copper-lowering drugs. Unlike silver, which has no known function in cells, copper is an essential trace element for virtually all living organisms. Due to its ability to change oxidation state (Cu(I)↔Cu(II)) in biologically-relevant conditions, copper is a cofactor of enzymes, which catalyze redox reactions [4]. Both copper deficiency and copper excess cause the development of severe diseases (neoplastic, neurodegenerative, and cardiovascular) [5, 6]. Safe copper transport in cells is performed by special proteins, which bind and pass copper to each other in reduced state Cu(I). As Cu(I)

and Ag(I) are isoelectronic and have similar ionic radii, copper-transporting proteins can bind silver and involve it into copper metabolic pathways [7, 8]. Copper routes in cells and organisms are not sufficiently understood at present. One of the reasons is the complexity of direct copper tracing, as copper is always present in the cells, its concentrations are low, radioactive isotopes have impractically short half-life decay time, while stable isotope enrichment techniques require expensive unique equipment. Tracing silver transport in organisms and cells may help to elucidate copper transport routes.

Hundreds of different new SNP species are fabricated and characterized each year; their size varies from 1 to 100 nm in at least one dimension [9–11]. SNP properties depend on size and method of fabrication (physical methods, chemical or biological synthesis). Usually, only antibacterial properties of SNPs are assessed. Taking into account the ideas stated above, a thorough testing of SNP biological properties should include the studies of their toxicity and the rate of involvement of silver into copper metabolism. The present work focuses on these biological effects of novel SNP samples, synthesized by us.

2. Objects and methods of characterization

2.1. Biomaterials and bio-objects. Biological objects were represented by bacterial virus (bacteriophage ϕ80 vir), bacterial cells (*E. coli* strain K802; *E. coli* strain BL21(DE3)/NdCTR1 expressing the fragment of human copper/silver-binding protein), unicellular eukaryotes (baker's yeast *S. cerevisiae*, haploid strain,), cultured human cells HepG2 (hepatoblastoma origin) and HEK293 (human embryo kidney origin), freshwater snails *Planorbarius corneus* (great ramshorn), Wistar laboratory rats, and C57BL laboratory mice. SNPs used in the present work were prepared as described below.

E. coli cells were grown in liquid nutrient medium based on bovine serum hydrolyzate (Samson-Med, St. Petersburg) at 37 °C. At the end of exponential growth phase, cells were collected by centrifugation and resuspended in water. Yeast cells were initially grown for 24 h in complete yeast medium YEPD (glucose, peptone, yeast extract). The cell suspension was then diluted with water to concentration 10^7 cells/mL (about 10-fold). Bacteriophage ϕ80 vir lysate of *E. coli* cells was also diluted with water (1:100). 50 μL of cell or phage suspension were mixed with 950 μL of the respective nanoparticle solution and incubated for various time intervals at 25 °C prior assessments. After the treatment the cells were titrated by successive 10-fold dilution method to assess cell viability by colony forming ability on agar plates. Bacteriophages were titrated similarly and their viability was assessed by plaque-forming ability in *E. coli* cell culture in soft agar. *E. coli* colonies and ϕ80 plaques were formed overnight at 37 °C. Plates with yeast cells were incubated for 4-5 days at 30 °C.

The HepG2 and HEK293 cell lines were maintained in DMEM/F12 (Biolot, Russia) with amphotericin B (3.1 μg), penicillin G (100 units), and streptomycin (100 μg) per 1 mL of medium supplemented with 10 % fetal bovine serum (Sigma). Cells were seeded in 96-well microtiter plates at 5000 cells/0.120 mL/well. When the cells reached 70 – 100 % of the well capacity, SNPs were added in various concentrations, and then the cells were incubated for 24 h at 37 °C in the 5 % CO_2 atmosphere. The viability of the cells was assessed by MTT [12]. The concentration of generated formazan was determined at 560 and 670 nm by CLARIOstar fluorimeter (BMG LABTECH, Germany). Subcellular fractions were isolated by differential centrifugation [13].

Snails *P. corneus* were held in water, which contained various concentrations of SNPs, for 24 h. Phenoloxidase activity of hemolymph, corresponding to copper-containing tyrosinsase, was measured in the presence of L-DOPA [14]. Mice and rats received 4 intraperitoneal injections of SNP solutions in Na/K/P_i buffer, pH 7.4, physiological ionic strength (PBS) daily. A group of rats received 4 intranasal injections daily. Blood serum oxidase activity was detected by assay-in-gel. The gels were stained with *o*-dianisidine,

specific chromogenic substrate for ceruloplasmin [13]. Ceruloplasmin is a blue copper (ferr)oxidase, which is synthesized in liver and secreted to bloodstream, accounts for up to 95 % of blood serum copper and serves as a major copper transporter / copper source for various organs [15].

2.2. Silver nanoparticles (SNPs). Silver nanoparticles were fabricated by the modified reducing method of ions to zero oxidation state with subsequent crystallization. Synthesis was performed in aqueous solution using β-cyclodextrin as a capping agent, silver nitrate as a source of silver ions and flavonoids as a reducing agent. Crystallization was carried out in the system of molecular ensembles of β-cyclodextrin clathrates to protect nanoparticles from agglomeration and other destructive factors. SNP samples were characterized by UV/Vis absorption (Shimadzu UV 1800), atomic force microscopy (AFM) (SOLVER P47), transmission electron microscopy (TEM) (FEI Tecnai G2 F20 TMP 2013), and X-ray diffractometry (Shimadzu XRD 7000).

Silver concentration in tissues was measured by flameless atomic absorption spectrometry (FAAS) with electrothermal atomization and Zeeman correction of nonselective absorption on a ZeeNit P650 spectrometer (Jena, Germany) with automatic sampling duplication. Tissue samples were homogenized in PBS, and then three volumes of pure concentrated HNO_3 were added to the homogenate to dissolve. Data are expressed as means ± standard deviation. All statistical analysis was performed by using one-way ANOVA followed by Bonferroni post hoc test.

The concentrations of initial components were varied to produce SNP with different sizes. The ratios of all reactants to silver concentration were kept constant in all experiments. The composition of the reaction mixtures is given in Table 1.

Table 1. Composition of reaction mixture for nanoparticle synthesis.

SNP	β-cyclodextrin (stabilizer)	Flavonoid (reducer)	NaOH, 1.0 M	$AgNO_3$, 3.0 M (Ag source)	[Ag]
	mg/L	mg/L	ml/L	ml/L	mg/L
1	230	180	1.8	0.3	108
2	1130	910	8	1.7	540
3	5680	4560	45	8.3	2700

Three species of SNPs (SNP1-SNP3) were synthesized. Initially, β-cyclodextrin was dissolved in aqueous sodium hydroxide, after that flavonoid was added, and the system was thoroughly mixed to reach homogeneous state. Then silver nitrate was added and the thorough mixing was repeated. The reaction system developed color after adding silver nitrate (SNP1 – yellowish-orange, SNP2 – reddish-brown, SNP3 – dark brown). Black precipitate was also formed in reaction system 3. The actual yield of silver nanoparticles was determined by Beer-Lambert-Bouguer law:

$$D = \varepsilon \cdot C \cdot l, \quad (1)$$

where D – optical density, ε - molar absorptivity, C – molar concentration of silver, l – optical pathway. SNP samples were characterized by UV/vis absorption spectroscopy, electron and atomic force microscopy, FAAS as well as X-ray diffraction method. The results are summarized in Table 2. They demonstrate that SNP2 sample is the most suitable for biological testing by the concentration, stability, homogeneity and size of nanoparticles. The properties of SNP sample are demonstrated in Fig. 1.

Table 2. Selected parameters of the synthesized SNP.

SNP	SNP1	SNP2	SNP3
Absorption band maximum, nm	400	330	330
Optical density at the maximum absorption wavelength	1.7	1.8	2.2
Concentration of silver nanoparticles, mg/L	100	500	476
Dilution factor for assessment of biological properties	1/10	1/50	1/30
Nanoparticle yield, %	100	100	17
SNP size, TEM, nm	15	25	20, 50
SNP size, AFM, nm	-	57	211
Content of crystalline silver, X-Ray-diffractometry data, counts	-	2500	700
Silver concentration as determined by FAAS, mg/L	141	382	200

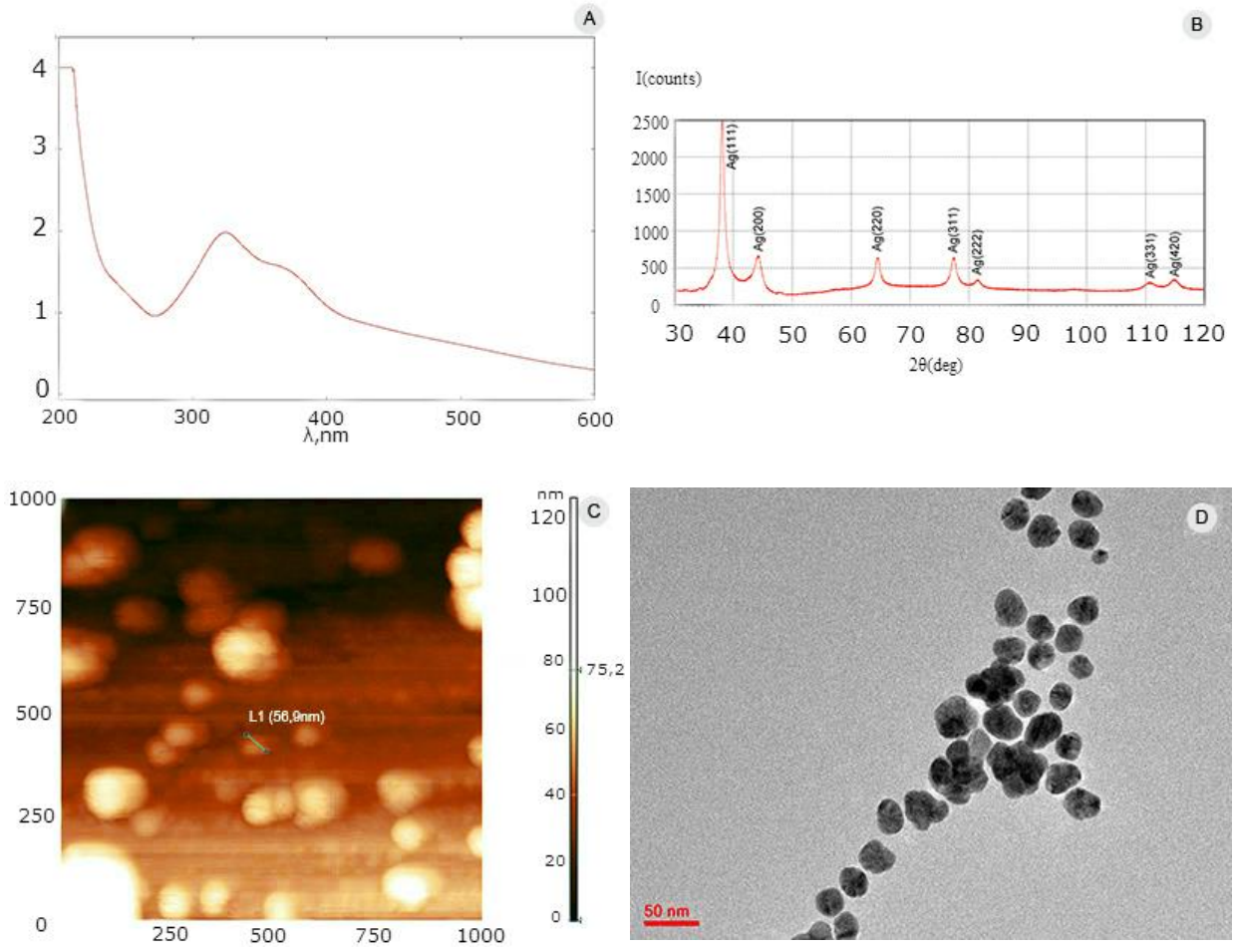

Fig. 1. Characterization of SNP2 sample. (A) UV/Vis spectrum, (B) X-Ray diffractometry 2θ-scan, (C) AFM image of SNPs on the SiO_2 surface, (D) TEM micrograph.

3. Results and discussion: assessment of biological activities of SNP2 sample

Biological activity of SNP2 sample was tested in prokaryotic and eukaryotic unicellular organisms, virulent bacteriophage ϕ80, and cultured human cells. The results are given in Fig. 2.

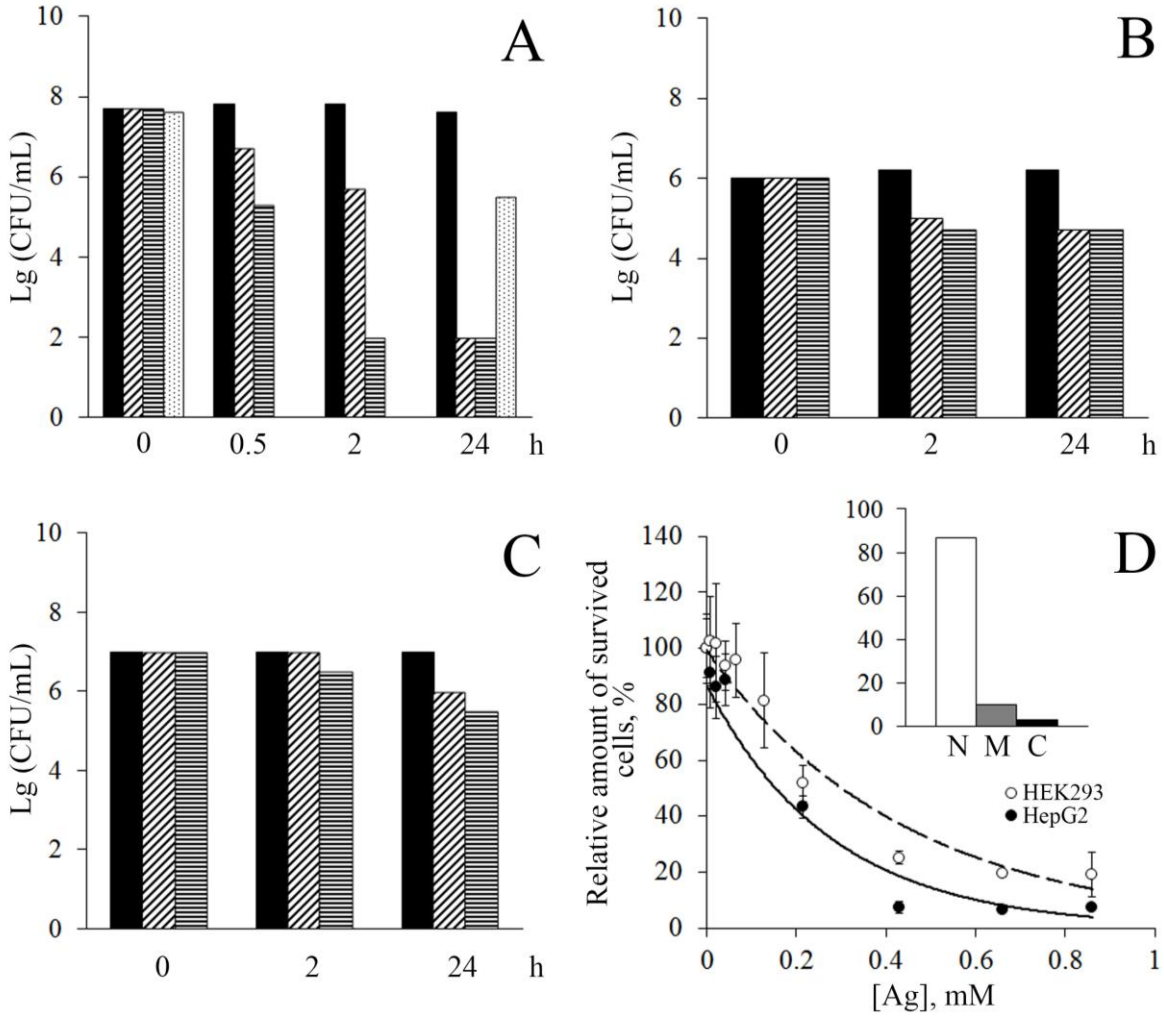

Fig. 2. Biological activity of SNP2: SNP2 reduce colony forming ability of *E. coli* cells (A), yeast *S. cerevisiae* cells (B), infectious properties of bacteriophages ϕ80 vir (C), and viability of human cells (D), inset: distribution of silver in the intracellular fractions; N – nuclei, M – mitochondria, C – cytosol Black bars – water, sparse stroked bars – 10 μM SNP2, dense stroked bars – 100 μM SNP, light stroked bars – *E. coli*/NdCTR1 treated with 100 μM SNP2. Data are expressed as means ± standard deviation.

They indicate that SNP2 suppressed the growth / viability in all the used biological models, both in concentration-dependent and time-dependent manner. However, the sensitivity varied significantly between the cells of different phylogenetic groups. *E. coli* cells practically lost the ability to reproduce after the treatment with 100 μM SNP2 for 2 h. After treatment with 10 μM SNP2 for 24 h colony forming ability of *E. coli* cells was reduced by 6 orders of magnitude. In the same conditions, colony forming ability of yeast cells was reduced by 1.5 orders of magnitude after 2 h of 100 μM treatment, and in 24 h of 10 μM treatment (Figs. 2A and 2B). The higher resistance of eukaryotic cells to high SNP2 concentrations may be explained by more perfect system of copper / silver transport and excretion in eukaryotes [16]. This assumption is indirectly supported by the observation of higher resistance of *E.*

coli/NdCTR1 expressing a cloned fragment of human protein, which can chelate copper and silver (*E. coli*/NdCTR1 are 1000-fold more resistant to SNPs than *E. coli*/K-802) (Fig. 2A). Treatment of viruses with 100 μM of SNP2 for 24 h reduced virulence by approximately 2 orders of magnitude (Fig. 2C). For cultured human cells the observed average half-lethal dose of SNP2 comprised 200 μM, total cell death was observed only at SNP concentration as high as 500 μM (Fig. 2D). Silver was detected in nuclei and mitochondria (Fig. 2D, inset), the cellular organelles which are known to accumulate silver under treatment with silver salts [17]. HepG2 cells were significantly more sensitive to SNP2 treatment than HEK293 cells (Fig. 2D). One of the possible factors is the hepatic origin of HepG2 cells, as hepatic cells transport and accumulate silver more efficiently.

The biological activity of SNP2 was also tested in both mice and rats (as most human tumor models are realized in mice, while copper-associated neurodegenerative disorders are mostly studied in rats). The data on silver distribution in the organs of rats are given in Fig. 3.

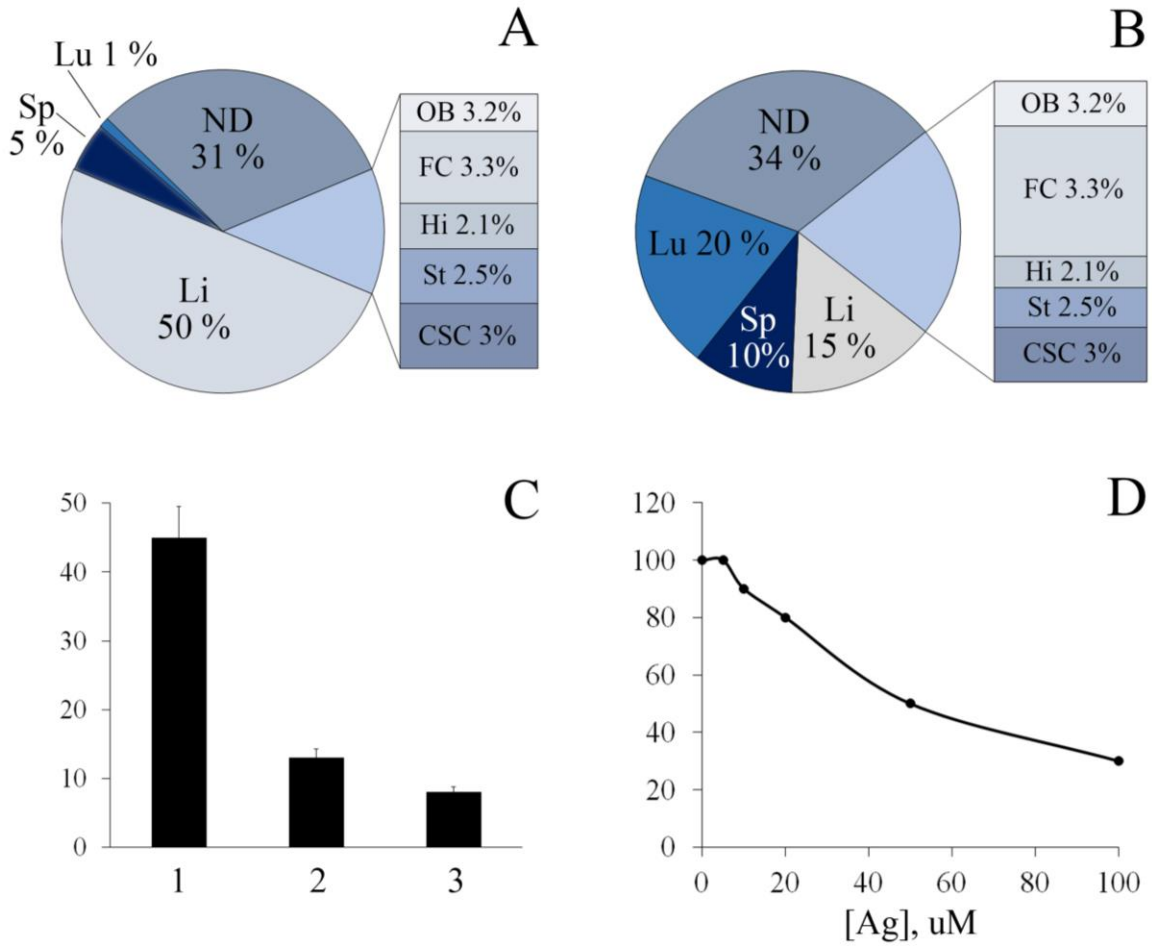

Fig. 3. Distribution of silver in organs of rats, treated by SNP2 intraperitoneally (A) or intranasal (B). ND – not determined, Li – liver, Lu – lungs, Sp – spleen, exploded sector – brain, brain regions: OB – olfactory bulbs, FC – frontal cortex, Hi – hippocampus, St – striatum, CSC – cervical spinal cord. (C) Serum oxidase activity in mice treated by SNP2: (1) - control, (2) - 2-fold, and (3) – 4-fold injections of SNP2. (D) Tyrosinase activity of *P. corneus* hemolymph, %.

SNP2 were applied for 4 days with 1 intraperitoneal injection per day (cumulative dose 400 μg/kg body mass; Fig. 3A) or as analogous intranasal injections (cumulative dose 160 μg/kg body mass; Fig. 3B). It can be seen that after intraperitoneal SNP injection the major fraction of silver was detected in liver. After intranasal SNP application silver

concentration in liver was 4 times lower, while in lungs silver content was 20 times higher as compared to intraperitoneal injection. Intranasal application also produced larger silver concentrations in brain regions selected for the study. The investigated application of SNP2 did not affect oxidase activity of blood serum in rats. We have shown previously that longer periods (1 month in rats, 1 week in mice) of exposure to silver ions are required to reach the significant decrease of blood serum oxidase activity [18]. In mice treated intraperitoneally by SNP2 in the same dosage (4 times in 24 h, 400 μg/kg in total), 25 % of the cumulative dose was detected in liver and oxidase activity of blood serum decreased by a factor of 9 (Fig. 3C). It is definite evidence that silver from SNP2 was put into copper metabolism, transported to liver, inserted to secreted ceruloplasmin, and inhibited its activity. It also indicated than SNP injection was a more efficient copper-lowering method than Ag-diet. No toxic effects (weight loss, digestion problems, and behavioral changes) of SNP2 in mammals were observed. For comparison, toxic effects of SNP2 were tested in freshwater snail *P. corneus*, which is widely spread in our region and is an intermediate host of parasites (trematodes) posing danger to livestock and humans. Groups of 5 snails were kept for 24 h in water with 5, 10, 20, 50 or 100 μM SNP2. Snails that were kept with 50 or 100 μM SNP2 displayed swelling of the mantle and the decrease in phenoloxidase activity of hemolymph by a factor of 2 (Fig. 3D).

4. Conclusions

1). The SNP2 are promising antibacterial agents, as they display high toxicity in bacteria with low toxicity in free-living unicellular eukaryotes, cultured human cells and mammals. Considering this fact, SNP2 is a suitable agent for studying synergistic action of antibiotics and silver nanoparticles.

2). In mammals and freshwater mollusks silver atoms from SNP2 enter copper metabolic pathways, bind to copper transport proteins, cross cell membranes, are transferred to lumina of cellular organelles, and are inserted to enzymes.

3). In rats, silver distribution is tissue-specific and depends on the method of SNP application. Silver is predominantly accumulated in liver after intraperitoneal SNP injection, while after intranasal injection silver is mostly accumulated in lungs and brain.

4). *P. corneus* (living in North-West Russia) display higher sensitivity to SNP2 as compared to mammals and, thus they may be a useful model for studying potential ecological impacts of wide economical use of silver nanoparticles in this region.

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References

[1] G. Franci, A. Falanga, S. Galdiero, L. Palomba, M. Rai, G. Morelli, M. Galdiero // *Molecules* **20(5)** (2015) 8856.

[2] S.Z. Naqvi, U. Kiran, M.I. Ali, A. Jamal, A. Hameed, S. Ahmed, N. Ali // *International Journal Nanomedicine* **8** (2013) 3187.

[3] L. Ge, Q. Li, M. Wang, J. Ouyang, X. Li, M.M. Xing // *International Journal Nanomedicine* **9** (2014) 2399.

[4] K.D. Karlin // *Science* **261(5122) (**1993) 701.

[5] E. Gaggelli, H. Kozlowski, D. Valensin, G.Valensin // *Chemical Review* **106(6)** (2006) 1995.

[6] H. Kozlowski, P. Kolkowska, J. Watly, K. Krzywoszynska, S. Potocki // *Current Medicinal Chemistry* **21(33)** (2014) 3721.

[7] A.C. Havelaar, I.L. de Gast, S. Snijders, C.E. Beerens, G.M. Mancini, F.W. Verheijen // *FEBS Letters* **436(2) (**1998) 223.
[8] J. Bertinato, L. Cheung, R. Hoque, L.J. Plouffe // *Journal Trace Elements Medicine and Biology* **24(3)** (2010) 178.
[9] A. Mohamed, M.M. Xing // *International Journal Burns Trauma* **2(1)** (2012) 29.
[10] J. Chen, J. Ouyang, J. Kong, W. Zhong, M.M. Xing // *ACS Applied Materials and Interfaces* **5(8)** (2013) 3108.
[11] Y. Tian, J. Chen, F. Zahtabi, R. Keijzer, M. Xing // *Pediatric Pulmonology* **48(11)** (2013) 1098.
[12] T.L. Riss, R.A. Moravec, A.L. Niles // *Methods in Molecular Biology* **740(1)** (2011) 103.
[13] Y.A. Zatulovskaia, E.Y. Ilyechova, L.V. Puchkova // *PLoS One* **10(10)** (2015) e0140797.
[14] A. Laughton, M. Siva-Jothy // *Apidologie* **42(2) (**2011) 140.
[15] J. Healy, K. Tipton // *The Journal of Neural Transmission* **114(6)** (2007) 777.
[16] T. Nevitt, H. Öhrvik, D.J. Thiele // *Biochimica et Biophysica Acta (BBA) - General Subjects* **1823(9)** (2012) 1580.
[17] E.A. Zatulovskiy, A.N. Skvortsov, P. Rusconi, E.Y. Ilyechova, P.S. Babich, N.V. Tsymbalenko, M. Broggini, L.V. Puchkova // *Journal of Inorganic Biochemistry* **116 (**2012) 88.
[18] E. Ilyechova, A. Skvortsov, E. Zatulovsky, N. Tsymbalenko, M. Broggini, L. Puchkova // *Journal Trace Elements Medicine and Biology* **25(1) (**2011) 27.