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AN INTERVENTION OF SILVER FROM NANOPARTICLES IN MURINE COPPER TURNOVER

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Abstract. Silver nanoparticles (SNP) were fabricated by method of chemical reduction of silver ions to Ag(0) in aqueous solution in the presence of surfactant micelles. Hydrazine hydrate was used as a reducing agent. Solution of SNP had brown-yellow color; median linear size of SNP was approximately 35 nm: and they showed absorption maximum at 420 nm. Toxicity of the SNP was tested in *E. coli* K802 cells. The particles displayed antimicrobial activity. Effect of SNP on mammalian copper metabolism was tested in mice. Atomic silver was found in blood serum, it was taken up by hepatocytes, inserted to active centers of ceruloplasmin, secreted to blood, and excreted through bile and urine. After cancellation of the SNP injections, silver concentration decreased in extracellular fluids. It is likely that SNP were corroded to form Ag(I), which integrated to copper turnover. The effects of silver intervention in copper metabolism of mammals as well as using SNP to trace copper transfer are discussed.

1. Introduction

Silver nanoparticles (SNP) are modern functional materials, which are widely used in many branches of human activity [1]. SNP are fabricated by various methods based on chemical, physical or biological synthesis [2]. All the known SNP possess antimicrobial properties irrespective of the method of their fabrication. Some of SNP also demonstrate antitumor activity *in vivo* and *in vitro* [3]. These properties cause the high demand for SNP synthesis and characterization. Despite the shortage of studies of SNP impact on human health it is generally believed that SNP toxicity in mammals is low. However, commercialization of nanoparticles might offer possible risks once they are liberated in the environment, raising the probability of potential influence of SNP on mammals. So, it is not sufficient to evaluate perspective SNP only from the points of low fabrication costs and antimicrobial properties; potential risks of influence on humans should also be studied.

At present, there are no conventional assays to evaluate SNP toxicity in mammals. It has been shown in many studies that SNP exhibit toxicity towards the cultured human cells of various origins. SNP are absorbed by the cells, accumulated in phagolysosomes and induce apoptosis [4]. However, cultured cells may not be viewed as a relevant model for evaluating SNP toxicity in humans. Using of zebrafish (*Danio rerio*) as an established animal model system for SNP toxicity assay is currently growing. Adult zebrafish and its developing embryos is a valuable model for nanoparticle toxicity assessment. Different types of parameters

(hatching achievement rate, developmental malformation of organs, damage in gill and skin, abnormal behavior (movement impairment), immunotoxicity, genotoxicity or gene expression, neurotoxicity, endocrine system disruption, reproduction toxicity and finally mortality) can be studied using zebrafish [5]. However, while the data from these investigations have important theoretical significance, they cannot be easily extrapolated to humans as fishes and mammals are phylogenetically distant groups and have many habitat-specific differences in their metabolism. There are also attempts to study acute SNP toxicity in laboratory rodents. SNP are applied by intravenous or intranasal injections in extreme concentrations. In these studies it has been shown that the toxicity of intravenously injected SNP is inversely related to their size. In mice, 40 nm particles did not exhibit toxicity while 10 nm SNP were highly toxicity [6]. However, 100 nm particles caused neurodegenerative changes after intranasal applications [7]. These studies indicate the potential toxicity of SNP, but they still are a poor model of typical human exposure to SNP in practical applications.

We think that the studies of SNP intervention in mammalian copper metabolism are among the most adequate estimates of SNP toxicity. Copper is a trace element, which is essential for all aerobic organisms. Its role as a cofactor of vitally important enzymes determines its biological significance [8]. On the contrary, silver is abiogenic (non-essential) element: up to now no biological process was described that would require silver. However, Ag(I), which is the only stable ion of silver in aqueous systems, is isoelectronic to Cu(I) and has a similar ionic radius. It is known that SNP corrode in biological media with the formation of Ag(I) [9]. Copper in the enzymes is tightly bound by preorganized coordination spheres, which are adapted to Cu(I)↔Cu(II) transitions of the copper ion in the catalytic redox cycle. In mammals, copper cofactors of cuproenzymes (catalytic copper) comprise the largest fraction of cellular copper. The turnover of copper in this fraction and the consequences of its disorders are well studied [10]. Another biological function of copper was discovered recently: copper takes part in cell signaling, regulation of proliferation, apoptosis, neovascularization and neurotransmission (regulatory copper) [11]. Localization of regulatory copper, its redox states, metabolic cycles, or the specific binding proteins are mostly unknown. The consequences of disturbances in the homeodynamics of the regulatory copper have not been studied either. Vital function of copper is combined with high toxicity of 'free' copper ions, which provoke formation of reactive oxygen species (ROS) and oxidative stress [10]. The conflict between the essential and toxic properties of copper is resolved by dedicated systems of copper transport in the cell and the extracellular fluids [10]. The proteins of this system 'pack' copper ions and safely transport them in Cu(I) state. Ag(I) ions, which are similar to Cu(I) ions, are mistakenly recognized by many copper transporters and intervene in the copper metabolism [12]. The intervention may result in decrease of cuproenzyme activity (Ag(I) may displace copper in active center but it cannot oxidize), disturbance in gene expression, cell cycle progression, tumor growth etc. (Ag(I) may substitute for Cu(I) in regulatory processes). The above considerations indicate that the specification of SNP should include the evaluation of SNP influence on mammalian copper metabolism.

In the present work new SNP species were fabricated by chemical synthesis and characterized by various methods. Antibacterial activity of SNP and ability of SNP to intervene in copper turnover in the body of laboratory mice were evaluated. The emergence of silver in blood serum, bile and urine was used as the main criterion for evaluation of SNP intervention in the copper turnover.

2. Objects and methods of characterization

2.1. Fabrication and characterization of SNP. Sigma Aldrich chemicals: silver nitrate ($AgNO_3$, CAS№ 7761-88-8), oleic acid (CAS№ 112-80-1), potassium hydroxide (CAS№

1310-58-3), hydrazine hydrate ($N_2H_4 \cdot H_2O$, CAS№ 302-01-2), and distilled water prepared by distiller DE-10 (EMO, Saint-Petersburg Russia) were used.

SNP were fabricated by method of chemical reduction of silver ions [Ag(I) → Ag(0)] in aqueous solution in the presence of surfactant micelles. During chemical reaction silver ions were reduced to atomic state, after that created atoms were agglomerated and crystallized to SNP. Micelles of potassium oleate absorbed on the particle surface and prevented nanoparticle agglomeration. Synthesis of SNP included several stages. At first, potassium hydroxide (0.84 g) was dissolved in 200 ml distilled water. Then 0.9 ml of oleic acid was added to prepared alkaline solution. The molar ratio of oleic acid and potassium hydroxide was 1:1. Mixture was blended with 500 rpm by magnetic stirrer US-1500A ULAB to complete dissolution of oleic acid and potassium oleate was synthesized. Surfactant foam was formed onto solution surface . Next, 1 ml of 1M aqueous $AgNO_3$ and 0.03 ml of $N_2H_4 \cdot H_2O$ were added to micellar solution. The molar ratio of $AgNO_3$ and N_2H_4 was 1:1. During the synthesis the solution changed color from uncolored to brown-yellow. Stirring was processed for about 5 minutes. Then the solution was maintained in dark box during a day.

Linear sizes of SNP were measured by Laser Diffraction Particle Size Analyzer, Shimadzu SALD-2300, Japan. UV/vis absorption spectra were made by spectrophotometer, Promecolab PE-5400 UV, Russia. Studies of nanoparticle structure were performed with X-ray diffraction (XRD), Shimadzu XRD 7000, Japan.

2.2. Biomaterials and bio-objects. Biological objects were represented by bacterial cells (*E. coli* strain K802) and C57BL 7-9 week-old male mice (16-20 g body mass) purchased from nursery "Rappolovo" (Leningradskaya region, Russia). The methods of SNP application are 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. 50 μL of cell suspension were mixed with 950 μL of the SNP 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. *E. coli* colonies were formed overnight at 37 °C, the results were expressed as colony formed units (CFU).

Animals were maintained in polycarbonate cages with wood shavings in a temperature-controlled facility (23 – 25 °C) under a 12:12-h light-dark cycle and 60 % humidity; food and water was provided *ad libitum*. Procedures involving the animals and their care were conducted in conformity with the institutional guidelines, which are in compliance with national (Order N267 of the Ministry of Health of the Russian Federation, June 19, 2003; Guide for the Use of Laboratory Animals, Moscow, 2005) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, December 12, 1987; Guide for the Use of Laboratory Animals, U.S. National Research Council, 1996).

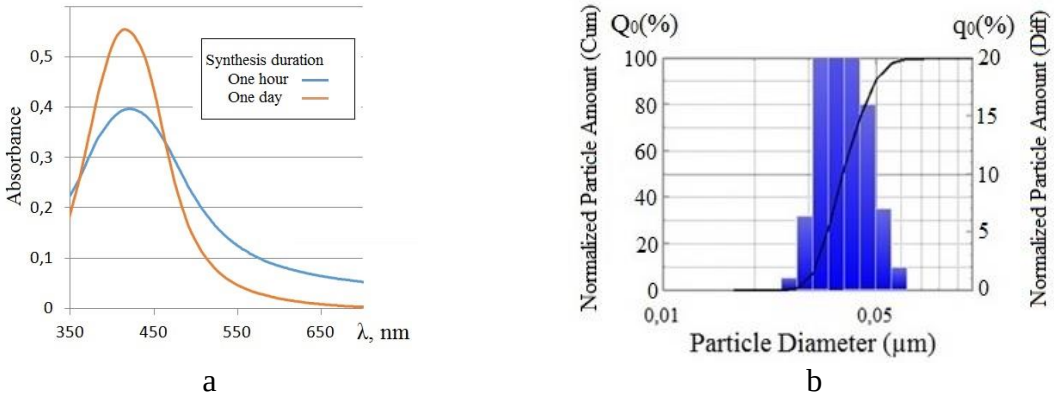
Experiments were carried out on the three groups: mice were treated intraperitoneally with 0.03 μmol SNP per 1 g of body weight daily for 7 days, (Group 1, n = 10) and were assessed on day 7. Mice of group 2 (n = 5) received analogous injection for 7 days, followed by 9 days without treatment and assessed on day 16 of experiment. The intact untreated mice (Group 0, n = 5) were used as a common reference group for groups 1 and 2. The mice were sedated using diethyl ether vapor and were euthanized by cervical dislocation, which was performed by skilled personnel. Blood was collected from the eye vessels. Tissues samples were collected post mortem and stored at -80 °C before using. Serum samples were collected by centrifugation after clot formation. To collect bile samples, gallbladders were carefully separated, placed in Eppendorf type tube, minced with scissors, centrifuged for 15 min at 10000×g, and the supernatant (bile) was collected. Urine was collected during urination.

Specific activity of ceruloplasmin (Cp) was estimated by assay-in-gel methods. After non-denaturizing 8% PAG electrophoresis, the gels were stained with *o*-dianisidine to determine the oxidase activity of Cp [13]. Atomic silver and copper concentration was measured by FAAS with electrothermal atomization and Zeeman correction of non-selective absorption by ZEEnit 650P spectrometer (AnalytikJena, Germany). For measurement of the atomic concentrations the samples were dissolved in pure HNO_3.

One-way ANOVA tests were performed with post hoc Bonferroni correction, and 95% confidence intervals were constructed using Excel software (Microsoft; Redmond, WA). Changes were considered significant at $P < 0.05$ level.

3. Results and discussion

3.1. Properties the fabricated silver nanoparticles. 5 mM solution of the fabricated SNP had brown-yellow color. Their UV/vis absorption spectra presented highly symmetric bands of absorption, with peak maxima at 420 nm (Fig. 1a), which indicated the excitation of surface plasmons typical of SNP and proved the formation of SNP. The size of nanoparticles varied from 30 to 55 nm as evidenced by laser difractometry. More than 70% of the particles had the size in the range 35-38 nm. (Fig. 1b).



a b

Fig. 1. Characteristics of SNP. (a) UV/Vis spectra of the SNP. (b) Distribution of the SNP bytheir linear size.

The obtained SNP differ by several properties (Table I) from the SNP species that we have described previously [14].

Table 1. Properties of the SNP fabricated for this study and described earlier.

Parameters		SNP (new)	SNP2 [14]
Reaction mixture composition, mg/l	Solvent	Distilled water	Distilled water
	Silver nitrate	500	540
	Flavonoid	-	910
	Hydrazine hydrate	160	-
	β-cyclodextrin	-	1130
	Potassium oleate	960	-
Synthesis, time		1 day	2 min
Particle size, nm		35	25
Solution color		Brown-yellow	Reddish-brown
Absorption band maximum, nm		420	330

Another reducing agent (hydrazine) was used for fabrication, while potassium oleate foaming agent was used as the stabilizer. The same concentrations of new and previously described SNP possessed different color; this effect was explained by the differences in SNP

median size. New species of nanoparticles was tested for antimicrobial activity and possibility to enter copper metabolic pathways in mouse.

3.2. Toxicity of silver nanoparticles. The new SNP under study displayed time- and dose-dependent antibacterial activity (Fig. 2), which was retarded as compared to silver nitrate. These SNP were generally less toxic to E. coli cells than the previously described SNP2 [14]. So, 24 h treatment of *E. coli* cells with 10 μM of SNP2 reduced the concentration of viable cells down to the detection limit (< 10^2 CFU/ml).

Meanwhile, incubation with new SNP in the same conditions did not reduce the CFU count, which was the same as in the reference (cells incubated with water) even after 48 hours of incubation.

Interestingly, that the bacterial growth curves for both SNP's and $AgNO_3$ were biphasic. In all cases, the survival of the bacteria decreased in 30 minutes, then raised and dropped again through 2 hour. It is possible that this phenomenon is due to the mobilization of a system that protects *E. coli* cells from an excess of copper. *E. coli* cells have a multi-component homeostatic system for the detoxification and efflux of copper ions [16]. This system includes the P-type Cu(I)-ATPase [17], the multi-copper oxidase CueO [18], and the four-part Cus copper proton-driven efflux complex CusCFBA [19]. All of these proteins are able to recognize and bind both copper and silver ions. At high SNP concentrations or prolonged their action of the protective effect system is reduced.

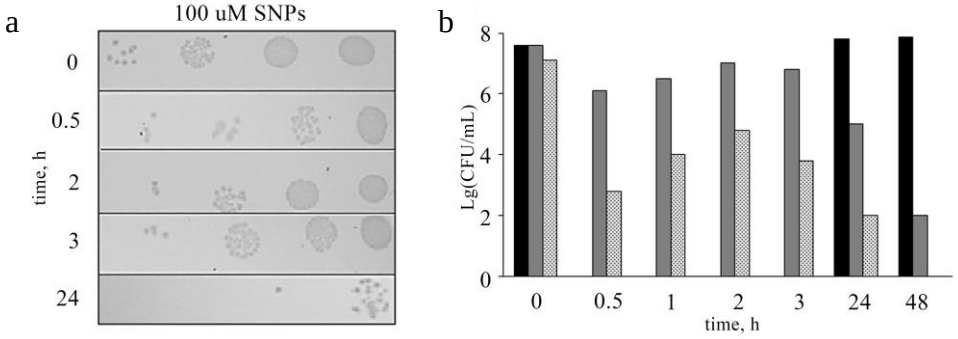


Fig. 2. The dependence of survival of *E. coli* cells on time of treatment with solutions of silver nanoparticles and on their concentrations. (a) Growth of *E. coli* strain K802 on agarose plates. (b) Dependence of CFU of *E. coli* (log scale) on time of treatment with 10 μM (black) and 100 μM (gray) solutions of silver nanoparticles, or 3.5 μM $AgNO_3$ (light).

3.3. Intervention of silver nanoparticles in the copper metabolism of mice. The ability of SNP to intervene in mammalian copper metabolism was assessed by measuring copper status indexes (CSI) in blood serum of mice, treated with SNP. The indexes comprised total copper concentration, and the content of holo-ceruloplasmin (Cp, a copper containing blood serum glycoprotein, which is a multicopper ferroxidase facilitating transmembrane iron transport and also an extracellular copper transporter [15]). The indexes were measured in mice that received intraperitoneal injections of SNP for 7 days; just after the last treatment or 9 days later. The concentration of silver in body fluids was also measured. Specifically, silver was measured in bile, this extracellular liquid is known as the main route of normal copper excretion. It is known that normally copper is not excreted with urine. However, in the conditions of failure or overload of bile excretion system (Wilson disease; embryonal type of copper metabolism), a fraction of copper is removed from the body with urine. So, silver concentration was also measured in the urine. The data were processed and converted to relative values (UN percent) for more convenient comparison and display. The data presented in Fig. 3 indicate that injection of SNP caused 10-fold decrease in ceruloplasmin-specific oxidase activity in the blood serum of the treated mice.

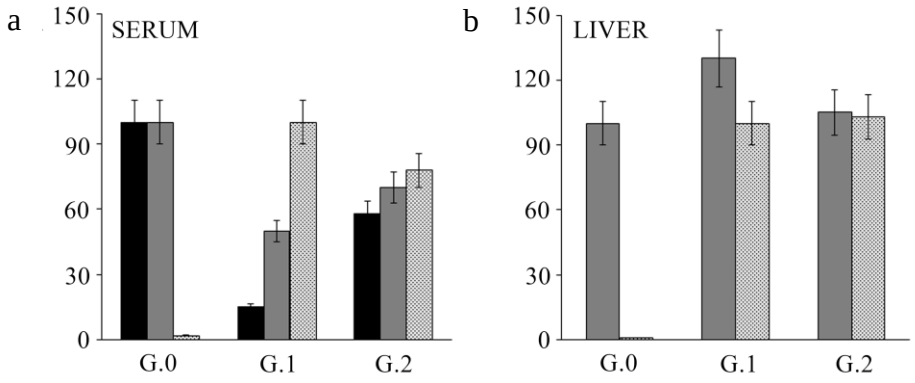


Fig. 3. Dynamics of copper and silver distribution in the body of the mice treated with silver nanopaticles. Concentrations and enzymatic activities are expressed as percent of the respective Group 0 (reference) values. Blood serum: oxidase activity (blue), copper (red) and silver (green); (G.0) – control, Group 1 (G.1) – treated with SNP, 7 injections, and Group 2 (G.2) – treated with SNP, 7 injections, followed by no treatment for 9 days. Liver: copper (red) and silver (green) concentrations in liver tissue of the same murine groups, respectively. Ordinate: %.

Total copper concentration decreased only 2-fold (9.5 μM in control group *versus* 5.0 μM in the mice of Group 1). These results are in good agreement with the modern concept of murine serum copper metabolism: in mice, only half of serum copper is associated with Cp [16]. The data firmly indicate that the injected SNP corrode; silver ions are transported through the bloodstream to the liver, where they are inserted to Cp and impair its catalytic activity. In group 1, blood serum silver concentration comprised 1.7 μM. Assuming that 1 holo-Cp molecule contains 6 – 8 copper atoms, the concentration of missing (displaced) copper is 4.5 μM, and 1.7 μM of silver are bound to Cp, we may estimate that Cp of mice, which received SNP, contains approximately 1 silver atom for every 3 copper atoms. Nine days after the cease of SNP injections (Group 2) oxidase activity increased up to 60 % of its value in the untreated mice (Fig. 3). The concentration of copper increased correspondingly (up to 6.5 μM), while silver concentration decreased (down to 1.4 μM).

Copper concentration in liver did not significantly change throughout the experiment (it varied around 0.08 μmol/g tissue in the groups). Liver is the main site of silver accumulation during SNP treatment [12, 14]. Silver concentration in liver reached 0.3 μmol/g tissue after 7 days of SNP injection and persisted for 9 days after the cease of the injections. At the same time, in the body of the treated mice, silver was present in metabolically active form because silver was found in bile analogously to copper. Silver concentration in bile samples from Group 1 is almost 2 times higher than copper concentration (38.7 μM silver *versus* 23.0 μM copper). After the cease of SNP treatment silver concentrations in bile decrease but their absolute values are still high (17.3 μM in Group 2 *versus* 38.7 μM in Group 1). In bile samples from Group 1, the presence of silver was accompanied by the decrease in copper concentrations (from 23.0 μM in Group 0 to 18.1 μM in Group 1). Copper concentrations in bile samples from Groups 1 and 2 did not differ significantly (10.7 and 10 μM, respectively). In urine samples from Group 1 silver concentration comprised 1.1 μM, it dropped to 0.05 μM after the cease of SNP injections (G. 2).

The presented data firmly showed that silver ions, but not the whole SNP, were present in bloodstream, liver tissue, bile and urine. In liver, silver ions were inserted to Cp, secreted to the bloodstream, recaptured by liver and excreted through bile. So, they were trafficked by copper transporters through the intracellular and extracellular copper transport routes. As silver

from these SNP remains metabolically accessible, it is rapidly excreted and the impaired enzymatic activities are recovered. So, we may suggest that small concentrations of SNP should possess low and transient toxicity in mammals.

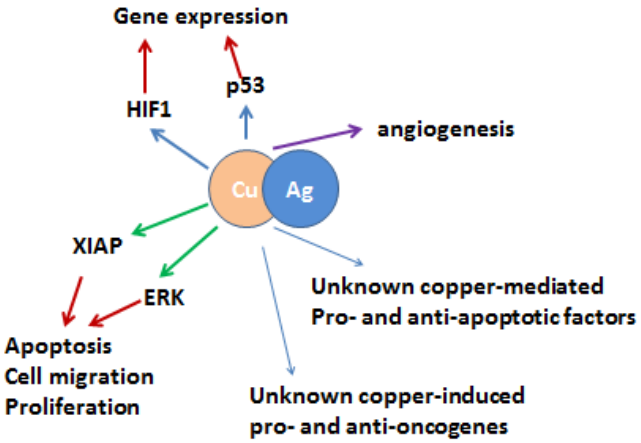


Fig. 4. The potential pathways of silver intervention in the functions of regulatory copper. The diagram displays: HIF1 and p53 –transcription factors, which are related to copper, and regulate the expression of many target genes; ERK and XIAP – members of cell signaling pathways, which control cell proliferation, death and migration; copper dependent processes (e.g. angiogenesis); and hypothetical copper-dependent activators and inhibitors of apoptosis and carcinogenesis.

4. Conclusion

The data on regulatory copper in the mammalian cells are very scarce. It is not known how copper, which was imported into the cell, enters the regulatory pool, so we do not know if silver can get to this pool and disturb copper regulatory functions. A diagram in Fig. 4. shows the relations between the regulatory copper and fundamental cellular processes or events, which can be possibly influenced by silver from the SNP [11]. If these influences do exist, then SNP may prove to be effective against tumor growth and neurodegeneration.

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