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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

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.

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.

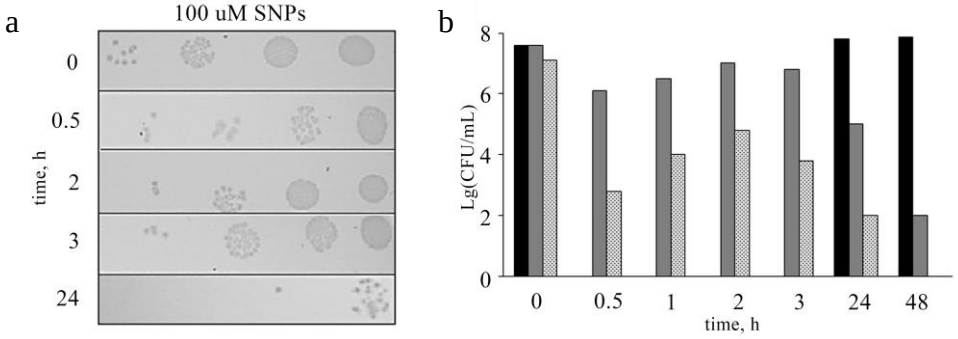

Fig. 2. The dependence of survival of *E. coli* cells on time of treatment with solutions of silver nanoparticles and on their concentrations. (a) Growth of *E. coli* strain K802 on agarose plates. (b) Dependence of CFU of *E. coli* (log scale) on time of treatment with 10 μM (black) and 100 μM (gray)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

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.

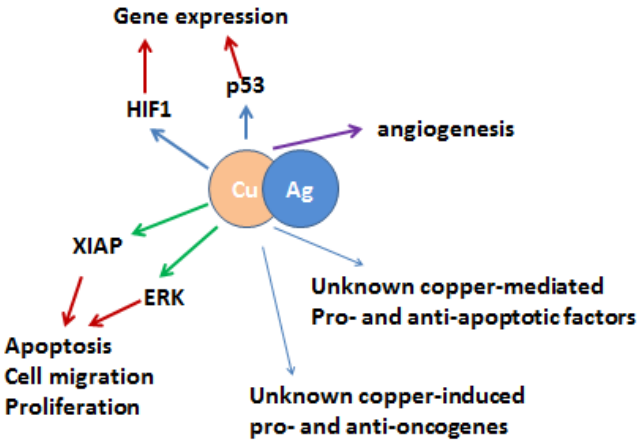

Fig. 4. The potential pathways of silver intervention in the functions of regulatory copper. The diagram displays: HIF1 and p53 –transcription factors, which are related to copper, and regulate the expression of many target genes; ERK and XIAP – members of cell signaling pathways, which control cell 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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