The silver ions contribution into the cytotoxic activity of silver and silver halides nanoparticles

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Abstract. The biocidal action of silver nanoparticles capped with sodium citrate and silver halides nanoparticles capped with non-ionic surfactant polyoxyethylene(20)sorbitan monooleate (Tween 80®) against yeast cells Saccharomyces cerevisiae was compared to the effect produced by silver nitrate and studied through the measurement of cell loss and kinetics of K+ efflux from the cells. The cytotoxicity of the obtained colloids was strongly correlated with silver ion content in the dispersions. The results clearly indicated that silver and silver halides nanoparticles destroyed yeast cells through the intermediate producing of silver ions either by dissolving of salts or by oxidation of silver.

1. Introduction
At the beginning of the antibiotic era in the early 1930s the application of silver-based compounds in medical practice was of rare occurrence and the use of silver salts in the treatment of patients strictly diminished [1]. However the interest in silver has never entirely disappeared and nowadays it revived again with the development of nanosilver containing materials and composites mainly due to the rediscovery of high antibacterial activity of colloidal silver particles [2, 3]. The increased interest in nanosilver during the recent 10 years is mainly explained by the appearance and spread of pathogenic microorganisms with multiple drug resistance, including the resistance to antibiotics of the last generation. At the present time silver in the form of nanoparticles (NPs) has the highest degree of commercialization among all nanomaterials and became a promising alternative to traditionally used antimicrobial agents in the treatment of delayed wounds [4-6], burns [7, 8], ulcers, bladder inflammation and other diseases.

External use of silver-based antiseptic agents is known to be safe enough. In order to be toxic externally silver needs to be absorbed into the body in sufficient amounts [7, 8]. The most common change seen in the skin in response to percutaneous silver absorption is argyria – blue or bluish-grey skin discoloration [11]. At the same time it is well known that argyria is not serious disease and has only negative cosmetic effects.

Nevertheless in order to prevent possible side-effects in humans treated with nanosilver containing medications we need to obtain more accurate scientific information about colloidal silver action on...
living cells. Nowadays there is a lack of fundamental works aiming at the systematical study of the silver NPs action against living cells – neither bacterial nor mammalian.

This article is another step in the process of understanding of the mode of the silver NP's action on living cells. Three main concepts of antibacterial and antiviral action of nanosilver are commonly accepted and widely discussed. On the one hand, silver NPs themselves can penetrate through cell membrane and interact with organelles, thus leading to disorder of the cell functioning. This mechanism was well-proven with respect to antibacterial and antiviral activity of nanosilver [12, 13]. On the other hand, silver NPs can easily generate Ag⁺-ions via oxidation of surface silver atoms by different oxidants dissolved in cytoplasm. So, the silver particles act as a buffer which maintains concentration of Ag⁺ in the surrounding media at the approximately constant level. According to the third concept the bactericidal action of silver NPs is determined by the intermediates formed during the silver oxidation such as peroxides and free radicals.

Actually, the observed antibacterial activity of the nanosilver is a superposition of those three ways. The contribution of each way depends on many factors; that's why the detailed investigation of the mechanism of nanosilver action is necessary for the rational design of silver NPs-based pharmaceutical compositions. The aim of present research was to determine the contribution of silver NPs themselves and silver ions of NPs' origin into the activity of silver and silver halides nanoparticles against *Saccharomyces cerevisiae* cells.

2. Chemicals

Polyoxyethylene(20)sorbitan monooleate (Tween-80®) was purchased from Acros Organics and used without further purification, silver nitrate (99.9+%, Sigma-Aldrich), sodium citrate dihydrate (99+% , Sigma-Aldrich), sodium borohydride (Lancaster, 98+%), sodium bromide (99+%, Sigma-Aldrich), sodium iodide (99.9+%, Sigma-Aldrich), sodium chloride (99+%, Sigma-Aldrich), were of analytical grade and used without further purification. All aqueous solutions were prepared with doubly distilled water.

3. Synthesis of Tween-80® stabilized silver halides NPs

The preparation of Tween-80® stabilized colloidal silver halides was performed as follows. Fifty milliliters of AgNO₃ (0.0157 g, 9.2×10⁻⁵ mol) water solution was added dropwise to 50 ml of Tween-80® aqueous solution with vigorous stirring. The mixture was kept stirred for 15 min. The quantity of Tween-80® was adjusted to 0.05% (5×10⁻⁴ g ml⁻¹) total concentration in the reaction mixture. Then 100 ml of sodium halide (9.2 × 10⁻⁵ mol) water solution was added dropwise with intense stirring.

4. Synthesis of citrate-stabilized silver NPs

The preparation of citrate-stabilized Ag NPs was performed as follows. 50 ml of AgNO₃ (0.0063 g, 3.7×10⁻⁵ mol) aqueous solution was added dropwise to 50 ml sodium citrate solution with vigorous stirring. Then two 50 ml solutions with different concentration of NaBH₄ were added step by step (0.0008g, 2.1×10⁻⁵ mol and 0.0015g, 4×10⁻⁵ mol respectively). The quantity of sodium citrate was adjusted to reach 3×10⁻⁵ mol l⁻¹ total concentration of citrate in the reaction mixture. Reduction process was carried out at room temperature.

5. Measurements

The UV-VIS absorbance spectra were recorded using a Shimadzu UV-1800 spectrophotometer (Japan), and 1 mm path length quartz cuvettes were used for the measurement of visible spectra.

The electronic images and diffractograms were made on Leo 912 AB Omega (Leo Ltd., Germany) transmission electron microscope (TEM) operating at 100 kV. The samples for TEM characterization were prepared by placing a drop of a colloidal solution on a formvar-coated copper grid which was dried at room temperature. All size distributions were calculated using Femtoscan Online v. 2.2.91 software (Advanced Technologies Center, Russia).
Zeta potential measurements of the obtained dispersions were carried out on Zetasizer Nano ZS analyzer with integrated 4 mW He-Ne laser, λ=633 nm (Malvern Instruments Ltd). Zeta potential was measured by applying an electric field across the dispersion of silver NPs using the technique of laser Doppler anemometry.

The concentration of silver ions in NPs suspensions was determined by Ag⁺-ion selected electrode («ELIS-131Ag», Russia) in the thermostatic cell (3.0 ml) contained 0.01 M citrate buffer (pH 6.0) at 20°C with agitation. The calibration of electrode was conducted by fractional addition of 0.01 M AgNO₃ solution. The electrode ESR-10101/4,2 (Russia) was used as a comparative electrode.

6. Microbiological tests
Saccharomyces cerevisiae VKM Y-1173 cells were cultivated in the shaken flasks with Reader medium [14] with 2% glucose at 29°C until the culture has reached the logarithmic phase of growth [14]. After that yeast cells were washed with distilled water twice, harvested by centrifugation at 5000 g during 15 min and stored in a dense water suspension (0.9-1.2) × 10⁹ cells/ml throughout the experiment (3-4 hours) at 0°C.

For determination of S. cerevisiae survival rate after silver nitrate and citrate stabilized silver NPs treatment the primary cell suspension dissolved in distilled water (1:100). The studied agents (ionic form of silver (Ag⁺): 0.01 M aqueous solution of AgNO₃ in concentrations from 0.75 to 18 μM was used. Following silver NPs were used: negatively charged citrate stabilized Ag-NPs in different concentrations from 1.8 to 18 μM were added to the cell suspension (~ 3·10⁷ cells/ml) contained 0.5 ml 0.01 M citrate buffer (pH 6.0) and incubated during 30 min at 30°C. The suspension of non-treated cells was used as a control (100% of survival rate) sample. Different dilutions (from 1:10 to 1:1000) of yeast cells (0.1 ml) were inoculated on Petri plates with agar-agar and incubated during 3-5 days at 24-30°C, then the colony quantity was accounted.

For registration of the K⁺ efflux from S. cerevisiae cells the ion selected electrodes («Orion», USA) and («ELIS-121K», Russia) [15] were used. The measurements were conducted in the thermostatic cell (3 ml) at 20°C with agitation. Cell suspension (60 μM) was added to measurement medium with 0.01 M citrate buffer (pH 6.0) to obtain the cell concentration ~ 3·10⁷ cells/ml. The common level of S. cerevisiae intracellular K⁺ was determined by addition of 20 μM Ag⁺ to cell suspension or after heat treatment at 70°C during 15 min in the water bath. The K⁺ level determined by the both methods was practically equal. Common K⁺ level determined from native yeast cells was taken as 100% in the experiments with investigation of effects of nanosilver contained agents.

7. Results and discussion
NPs characterization
The manner of preparation of citrate-stabilized silver NPs was in the reduction of silver nitrate aqueous solution containing sodium citrate as non-covalent stabilizer by NaBH₄ solution. The optimal concentration of citrate-anion turned out to be in a good agreement with [16]. The as prepared silver NPs had an average diameter about 10 nm (figure 1A), were well dispersed and negatively charged, their zeta potential was -29.1 ± 0.5 mV at pH 7.8. Nanosilver dispersion was stable at least 6 month after synthesis and exhibited surface plasmon bands at 401 nm (figure 1B) corresponding to non-covalently stabilized Ag NPs.

The mechanism of the stabilizing action of Tween-80, apparently, is a classic for solubilizing surfactant. In our technique Tween-80 was used in concentrations one order greater than its own CMC (0.8 mM against CMC Tween-80 of 0.08 mM). Forming around the nanoparticle micelle acts as a wrapper that prevents coagulation, but allow silver ions go into solution and back.

Dispersions of silver halides (AgCl, AgBr, AgI) obtained by precipitation from aqueous solutions of silver nitrate in the presence of Tween-80 were characterized using UV-Vis spectroscopy (figure 2), transmission electron microscopy (figures 3-5).
The figure 2 clearly indicates the offset of the region of absorption to longer wavelengths area passing from silver chloride to bromide and then to iodide, which is consistent with the decrease of photolytic stability of halides in this series.

Figure 1. TEM image of citrate stabilized silver NPs (A), UV-visible absorbance spectrum of citrate stabilized silver NPs (B).

Figure 2. UV-visible absorbance spectra of silver halides NPs.
Figure 3. Histograms of the size distribution (A), electron microdiffraction data (B), electron micrographs (C), of silver chloride nanoparticles stabilized with Tween 80.

Figure 4. Histograms of the size distribution (A), electron micrographs (B), electron microdiffraction data (C) of silver bromide nanoparticles stabilized with Tween 80.

Figure 5. Histograms of the size distribution (A), electron micrographs (B), electron microdiffraction data (C) of silver iodide nanoparticles stabilized with Tween-80.

Microbiological activity of silver nanoparticles dispersions
The cytotoxic activity of silver nanoparticles was characterized by survival rate of yeast cells and by rate of $K^+$ efflux from the cells incubated with nanosilver dispersion. The data obtained by those two techniques are in good correlation therebetween (figures 6, 7). The rate of $K^+$ efflux and cell loss increase when concentration of AgNO$_3$ and Ag NPs increases. At that, the percentage of cell loss always exceeds the $K^+$ efflux at the end of an experiment, thus indicating that some dead cells still contain $K^+$ inside. This effect allows to suppose that cytotoxic activity of silver ions and nanoparticles against yeast cells does not relate completely to damage of cell membrane.

The determined rates of $K^+$ efflux from the cells incubated with Ag-NPs (figure 7A) were approximately 5 times lower as compared to the cells incubated with AgNO$_3$ of the same concentrations (figure 7B).
Figure 6. Survival rate of yeast cells incubated with AgNO$_3$ solutions and silver NPs colloids.

Addition of small amounts (up to the concentration of 50 μM) of NaBH$_4$ into the NPs sol before incubation resulted in dramatic decrease in the K$^+$ efflux (figure 8A). On the contrary, the addition of dilute H$_2$O$_2$ (up to 100 μM in the yeast cells suspension) to the cells incubated with silver NPs led to significant increase in the rate of K$^+$ efflux. It should be noted that NaBH$_4$ and H$_2$O$_2$ themselves in those concentrations did not influence on the cells’ survival rate. Therefore, the additions of NaBH$_4$ and H$_2$O$_2$ does not directly influence on the yeast cell functioning. Those chemicals alter the rate of K$^+$

Figure 7. The rate of K$^+$ efflux from the yeast cells incubated with silver NPs colloids (A) and with AgNO$_3$ solutions (B).
efflux indirectly by modifying the concentration of Ag$^+$-ions due to reduction of Ag$^+$ by NaBH$_4$ or at least partial oxidation of silver particles by H$_2$O$_2$. Since the reduction of Ag$^+$ led to total disappearance of the nanosilver activity it can be concluded that the activity of the studied silver NPs follows only from the activity of silver ions. On the basis of this finding and the obtained data concerning the rate of K$^+$ efflux induced by silver NPs and AgNO$_3$ the concentration of Ag$^+$-ions in the studied dispersion may be estimated as about 15% of the total concentration of silver. Nearly the same value was obtained from the direct measurements of the Ag$^+$ concentration in the synthesized nanosilver dispersion by Ag$^+$-ion selected electrode before the incubation with yeast cells.

![Graph](image)

**Figure 8.** The rate of K$^+$ efflux from the yeast cells incubated with silver NPs colloids treated with NaBH$_4$ (50 μM) and H$_2$O$_2$ (100 μM), the corresponding curve for the yeast cells incubated with AgNO$_3$ is shown for comparison (A), the rate of K$^+$ efflux from the yeast cells incubated with silver halides colloids with silver concentration of 1.5 μM (B).

Short time after the reduction of AgNO$_3$ with the excess of strong reducing agents like sodium borohydride silver ions in dispersions of as obtained silver nanoparticles are of course absent. But after that oxygen or reactive oxygen species generated in living cells start to oxidize silver and produce Ag$^+$ ions. To estimate the rate of oxidation of silver nanoparticles we used well known and sensitive spectrophotometric technique with 1,10-phenanthroline and bromopyrogallol red. These organic substances formed an intensely blue ternary complex with ionic silver contained in silver NPs sol which then easily registered at the wavelength of 635 nm. Since the absorption spectrum of silver nanoparticles has a maximum at 405-415 nm, the presence of silver nanoparticles did not interfere with the spectrophotometric determination of ion Ag$^+$ at 635 nm. Intense bubbling of air for 30 minutes through silver nanoparticles dispersion does not resulted in the appearance of the absorption bands in the spectrum of the ternary complex, while adding sufficient amounts of hydrogen peroxide leads to a rapid (within 3 minutes) quantitative oxidation of nanoparticles with the formation of silver ions, which leads to a significant increase in activity against living cells.
Microbiological activity of silver halides dispersions

Taking into account that the biocidal activity of silver NPs against the *Saccharomyces cerevisiae* cells is mainly results from the silver ions action it could be assumed that slightly-soluble silver salts which can generate silver ions when slowly dissolving may provide prolonged biocidal action as strong as the action of silver nanoparticles. To provide a sufficiently intense and at the same time permanent and long-term release of silver ions, it is promising to use nanodispersed silver salts as a biocidal agents. To further understand the mode of action of poorly soluble silver salts dispersions on living cells, we also studied the effect of silver halides on the K\(^+\) efflux rate from *Saccharomyces cerevisiae* cells. Figure 8B shows the dependence of the release of K\(^+\) from the cells by incubation time with dispersion of silver halide.

Comparing the data from figure 7 it can be seen that the efficiency of biocidal effect of silver halide substantially exceeds the cytotoxic activity of silver nanoparticles and comparable to that of silver nitrate solution of the same concentration. However, at the initial stage of incubation the rate of release of potassium ions from the cells under the influence of nanoparticles of silver halides is several times less than the rate of release of potassium ions by the action of silver nitrate. This delay is due apparently to the slow dissolution of silver halides in citrate buffer. The solubility of silver halides in 0.01 M citrate buffer (pH 6.0), which can be calculated taking into account the possibility of the formation of complexes in solution \([AgHal]^3, [AgHal]^2\) и \([AgCit]^2\) (Cit – citrate ion), equals 2.5 mM for AgCl, 133 μM for AgBr, 1.7 μM for AgI. Thus, the NPs dispersions with a silver halide concentration of 1.5 μM should be dissolved in a buffer solution. The rate of silver halides dissolution, as shown in [17] correlates with the silver salt solubility in the solution of complexing agent. This fact is in a good agreement with the observed decrease in the efflux rate of potassium ions from *Saccharomyces cerevisiae* cells in a row of AgCl-AgBr-AgI.

8. Conclusions

The discussion about the mechanism of the cytotoxic activity of the dispersions of silver nanoparticles lasts for a long time and reliable data concerning the nature of main active agent are hard to obtain. The results of the present work clearly indicate that the silver ions either remained in the dispersion of silver nanoparticles after their synthesis or generated afterwards by oxidizing silver particles make the crucial contribution into the cytotoxic activity of citrate-stabilized silver NPs against yeast cells. It was also shown that this activity most likely does not relate to damage of cell membrane. The same way the cytotoxic effect of silver halide NPs can be explained because of its attributing to generating Ag\(^+\) ions through NPs dissolution.

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