

## Research Article

# The Mode of Action of Silver and Silver Halides Nanoparticles against *Saccharomyces cerevisiae* Cells

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Silver and silver halides nanoparticles (NPs) (Ag, AgCl, AgBr, and AgI) capped with two different stabilizers (sodium citrate and nonionic surfactant Tween 80) were obtained via sodium borohydride reduction of silver nitrate in an aqueous solution. The effect of the biocidal action of as-prepared synthesized materials 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<sup>+</sup> efflux from the cells depending on concentration of silver. The results clearly indicate that the silver ions either remained in the dispersion of silver NPs and silver halides NPs after their synthesis or were generated afterwards by dissolving silver and silver halides particles playing a major part in the cytotoxic activity of NPs against yeast cells. It was also supposed that this activity most likely does not relate to the damage of cell membrane.

## 1. Introduction

Since the beginning of the antibiotic era in the early 1930s and the introduction of sulphonamides and penicillins 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 around the World War II [1]. However the interest in silver has never entirely disappeared and nowadays it reappeared again with the development of nanosilver containing materials and composites mainly due to the rediscovery of high antibacterial activity of colloidal silver particles. At the moment silver in the form of NPs has the highest degree of commercialization among all nanomaterials [2] and became a promising alternative to traditionally used antimicrobial agents in the treatment of

delayed wounds [3], burns [4], ulcers, bladder inflammation [5], and other diseases.

Unfortunately, uncontrolled production and thoughtless internal use of silver colloids in the treatment of diseases like influenza, hepatitis, pneumonia, bronchitis, and even HIV-infection in the 1990s forced USA Food and Drug Administration to bring in a ban on “claiming any therapeutic or preventive value for the product, noting that colloidal silver was being marketed for numerous diseases without evidence of safety or effectiveness” in August 1999. As a result, colloidal silver is now seen only as a dietary supplement in the US and is no longer listed in the British National Formulary under products for wound management; also, according to Australian Therapeutic Goods Administration “there are no legitimate medical uses for colloidal silver and no evidence

to support its marketing claims.” Obviously these restrictions are related to possible side effects in humans treated with nanosilver containing medications [6]. Keeping in mind that there is no intelligible point in internal use of nanosilver it should be noted that 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 [9]. At the same time it is well known that argyria is not a serious disease and has only negative cosmetic effects.

In our opinion the aforementioned situation was not only caused by uncontrolled silver internal uses but also by the lack of clear scientific information about colloidal silver action on living cells. Furthermore, there is insufficient number of works [10, 11] aiming at the systematical study of the silver NPs action against living cells—neither bacterial nor mammalian. Mechanisms of antibacterial action of silver are poorly researched.

This paper is another step in the process of understanding of the mode of the silver NP's action on living cells.

## 2. Experimental Part

**2.1. Chemicals.** Polyoxyethylene(20)sorbitan monooleate (Tween 80) was purchased from Acros Organics and used without further purification and 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), and sodium chloride (99+%, Sigma-Aldrich) were of analytical grade and used without further purification. All aqueous solutions were prepared with doubly distilled water.

**2.1.1. Synthesis of Tween 80 Stabilized Silver Halides NPs.** The preparation of Tween 80 stabilized colloidal silver halides was performed as follows. Fifty milliliters of  $\text{AgNO}_3$  (0.0157 g,  $9.2 \times 10^{-5}$  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% ( $5 \times 10^{-4}$  g mL<sup>-1</sup>) total concentration in the reaction mixture. Then 100 mL of sodium halide ( $9.2 \times 10^{-5}$  mol) water solution was added dropwise with intense stirring.

**2.1.2. Synthesis of Citrate Stabilized Silver NPs.** The preparation of citrate stabilized Ag-NPs was performed as follows. 50 mL of  $\text{AgNO}_3$  (0.0063 g,  $3.7 \times 10^{-5}$  mol) aqueous solution was added dropwise to 50 mL sodium citrate solution with vigorous stirring. Then two 50 mL solutions with different concentration of  $\text{NaBH}_4$  were added step by step (0.0008 g,  $2.1 \times 10^{-5}$  mol and 0.0015 g,  $4 \times 10^{-5}$  mol, resp.). The quantity of sodium citrate was adjusted to reach  $3 \times 10^{-4}$  mol L<sup>-1</sup> total concentration of citrate in the reaction mixture. Reduction process was carried out at room temperature.

**2.1.3. Synthesis of Silver Halide NPs.** Different solutions were made before silver halide synthesis:

- (a) 17 mg (0,0001 mol) of  $\text{AgNO}_3$  was dissolved in 50 mL of water;
- (b) 58.5 mg (0,0001 mol) of  $\text{NaCl}$  was dissolved in 50 mL of water;
- (c) 103 mg (0,0001 mol) of  $\text{NaBr}$  was dissolved in 50 mL of water;
- (d) 150 mg (0,0001 mol) of  $\text{NaI}$  was dissolved in 50 mL of water;
- (e) 20 mg of sodium citrate was dissolved in 100 mL of water;
- (f) 2.3 mg of sodium 3-mercaptopropanesulfonate was dissolved in 100 mL of water;
- (g) 100 mg of polyoxyethylene(80)sorbitan monooleate (Tween 80) was dissolved in 100 mL of water.

The synthesis was carried out in a three-necked flask with a mechanical stirrer and two dropping funnels. 50 mL of silver nitrate solution and 50 mL of sodium halide solution were slowly (1 mL per minute) and simultaneously dropped into 100 mL of stabilizer solution with vigorous stirring. After that the final dispersion was stirred about 60 min and as-prepared solution was held in a dark under argon.

**2.2. 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 distribution was 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,  $\lambda = 633$  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  $\text{Ag}^+$ -ion selected electrode (<<ELIS-131Ag>>, Russia) in the thermostatic cell (3.0 mL) containing 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  $\text{AgNO}_3$  solution. The electrode ESR-10101/4,2 (Russia) was used as a comparative electrode.

**2.2.1. Microbiological Tests.** *Saccharomyces cerevisiae* VKM Y-1173 cells were cultivated in the shaken flasks with Reader medium [12] with 2% glucose at 29°C until the culture has reached the logarithmic phase of growth [13]. 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  $(0.9\text{--}1.2) \times 10^9$  cells/mL throughout the experiment (3-4 hours) at 0°C.

To determine *S. cerevisiae* survival rate after silver nitrate and citrate stabilized silver NPs treatment the primary cell suspension was dissolved in distilled water (1:100). The studied agents (ionic form of silver ( $\text{Ag}^+$ ): 0.01 M aqueous solution of  $\text{AgNO}_3$  in concentrations from 0.75 to 18  $\mu\text{M}$ ) were used. Following silver NPs were used: negatively charged citrate stabilized Ag-NPs in different concentrations from 1.8 to 18  $\mu\text{M}$  were added to the cell suspension ( $\sim 3 \cdot 10^7$  cells/mL) containing 0.5 mL 0.01 M citrate buffer (pH 6.0) and incubated during 30 min at 30°C. The suspension of nontreated 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 and then the colony quantity was accounted.

For registration of the  $\text{K}^+$  efflux from *S. cerevisiae* cells the ion selected electrodes (<<Orion>>, USA) and (<<ELIS-121K>>, Russia) [13] were used. The measurements were conducted in the thermostatic cell (3 mL) at 20°C with agitation. Cell suspension (60  $\mu\text{M}$ ) was added to measurement medium with 0.01 M citrate buffer (pH 6.0) to obtain the cell concentration  $\sim 3 \times 10^7$  cells/mL. The common level of *S. cerevisiae* intracellular  $\text{K}^+$  was determined by addition of 20  $\mu\text{M}$   $\text{Ag}^+$  to cell suspension or after heat treatment at 70°C during 15 min in the water bath. The  $\text{K}^+$  levels determined by the both methods were practically equal. Common  $\text{K}^+$  level determined from native yeast cells was taken as 100% in the experiments aimed at investigating the effects of nanosilver contained agents.

### 3. Discussion

**3.1. 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  $\text{NaBH}_4$  solution. The optimal concentration of citrate anion turned out to be in a good agreement with [14]. The as-prepared silver NPs had an average diameter about 10 nm, were well dispersed and negatively charged, and their zeta potential was  $-29.1 \pm 0.5$  mV at pH = 7.8. Nanosilver dispersion was stable at least 6 month after synthesis and exhibited surface plasmon bands at 401 nm corresponding to noncovalently stabilized Ag NPs (Figures 1 and 2).

Dispersion of silver halides ( $\text{AgCl}$ ,  $\text{AgBr}$ , and  $\text{AgI}$ ) obtained by precipitation from aqueous solutions of silver nitrate in the presence of Tween 80 was characterized using UV-Vis spectroscopy (Figure 3) and transmission electron microscopy (Figures 4, 5, and 6).

The figure 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.

The mechanism of the stabilizing action of Tween 80, apparently, is typical for solubilizing surfactant. In our

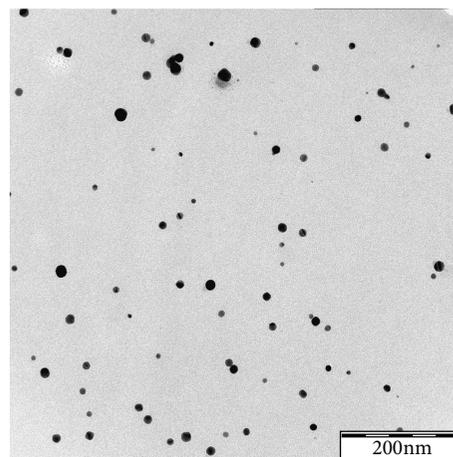


FIGURE 1: TEM image of citrate stabilized silver NPs.

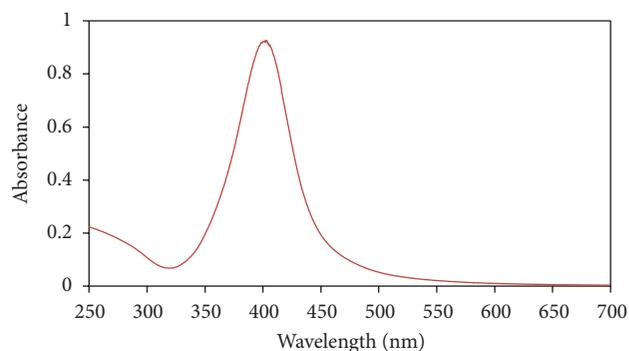


FIGURE 2: UV-visible absorbance spectrum of citrate stabilized silver NPs.

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 allows silver ions go into solution and back.

**3.1.1. Microbiological Activity of Silver Nanoparticles Dispersion.** Nowadays 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, for example, with respect to antibacterial and antiviral activity of nanosilver [15, 16]. On the other hand, silver NPs can easily generate  $\text{Ag}^+$ -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  $\text{Ag}^+$  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, for example, peroxides and free radicals.

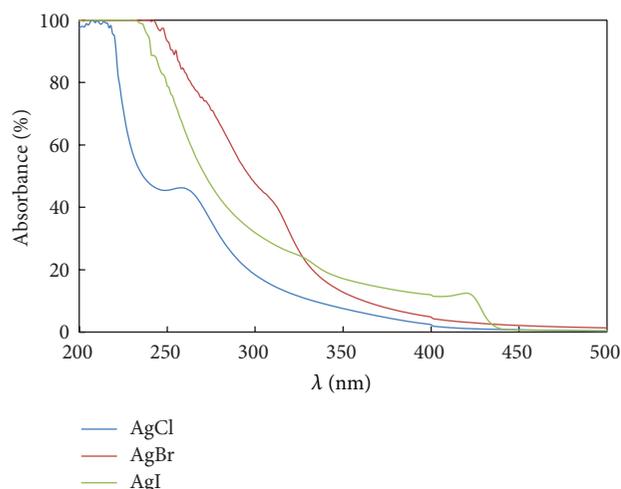


FIGURE 3: UV-visible absorbance spectra of silver halides NPs.

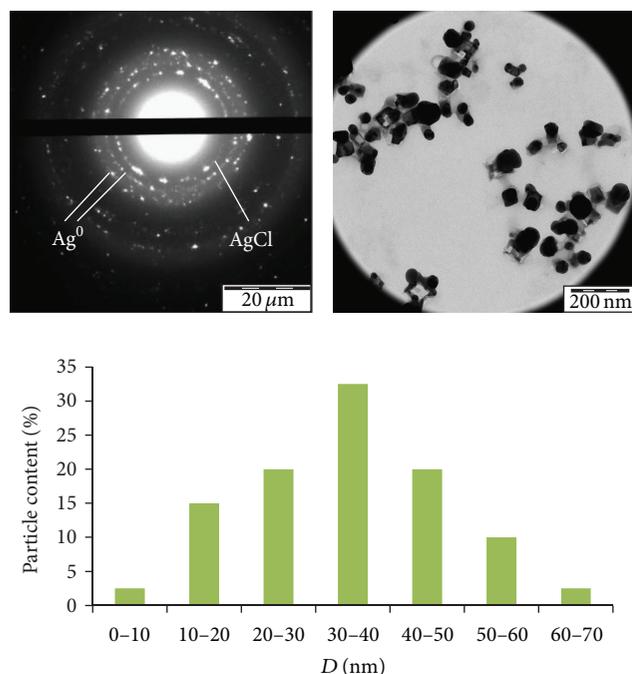


FIGURE 4: Electron micrographs, electron microdiffraction data, and histograms of the size distribution of silver chloride nanoparticles stabilized with Tween 80.

Actually, the observed antibacterial activity of the nanosilver is a superposition of those three ways. The contribution of each way depends on many factors, which is the reason 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.

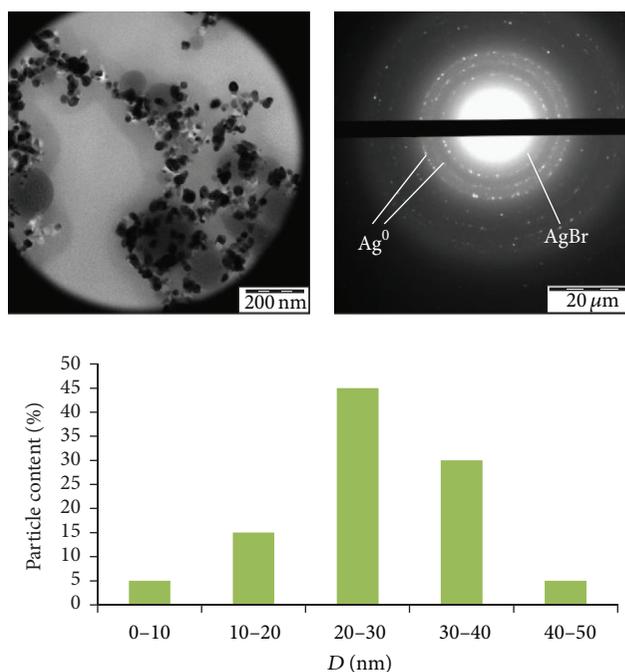


FIGURE 5: Electron micrographs, electron microdiffraction data, and histograms of the size distribution of silver bromide nanoparticles stabilized with Tween 80.

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 there between Figures 7 and 8. The rates of  $K^+$  efflux and cell loss increase when the 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 do not relate completely to damage of cell membrane.

The determined rates of  $K^+$  efflux from the cells incubated with Ag-NPs (Figure 8) were approximately 5 times lower as compared to the cells incubated with  $AgNO_3$  of the same concentrations (Figure 9).

Addition of small amounts of  $NaBH_4$  into the NPs sol before incubation resulted in dramatic decrease in the  $K^+$  efflux (Figure 10). On the contrary, the addition of dilute  $H_2O_2$  to the cells incubated with silver NPs led to a significant increase in the rate of  $K^+$  efflux. It should be noted that  $NaBH_4$  and  $H_2O_2$  themselves in the studied concentrations did not affect the cells' survival rate. Therefore, the additions of  $NaBH_4$  and  $H_2O_2$  does not directly affect the yeast cell functioning. Those chemicals alter the rate of  $K^+$  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  $H_2O_2$ . 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 happens only due to the activity of silver ions. On the basis of this

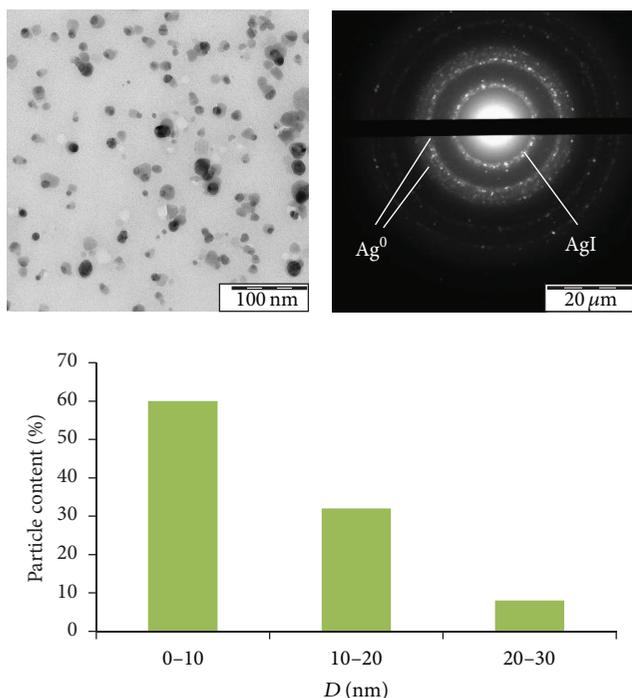


FIGURE 6: Electron micrographs, electron microdiffraction data, and histograms of the size distribution of silver iodide nanoparticles stabilized with Tween 80.

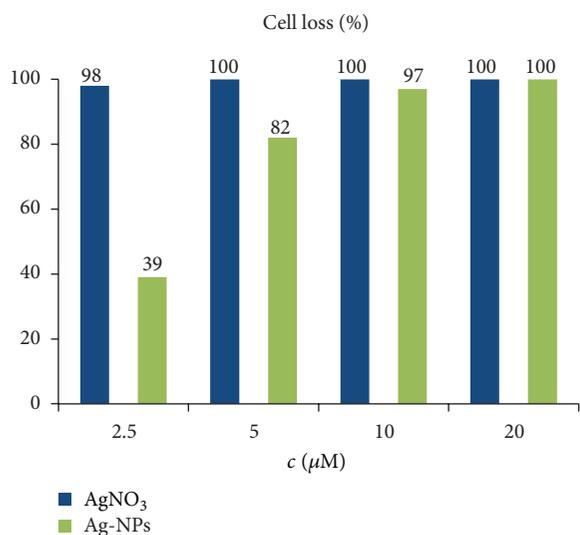


FIGURE 7: Survival rate of yeast cells incubated with AgNO<sub>3</sub> solutions and silver NPs colloids.

finding and the obtained data concerning the rate of K<sup>+</sup> efflux induced by silver NPs and AgNO<sub>3</sub> the concentration of Ag<sup>+</sup>-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<sup>+</sup> concentration in the synthesized nanosilver dispersion by Ag<sup>+</sup>-ion selected electrode before the incubation with yeast cells.

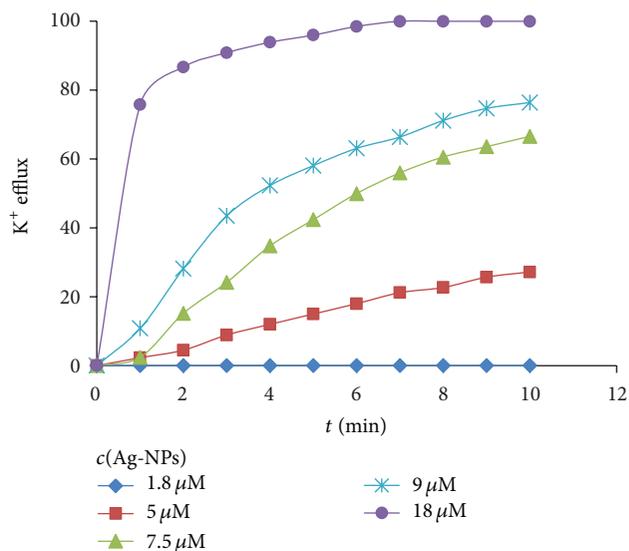


FIGURE 8: The rate of K<sup>+</sup> efflux from the yeast cells incubated with silver NPs colloids.

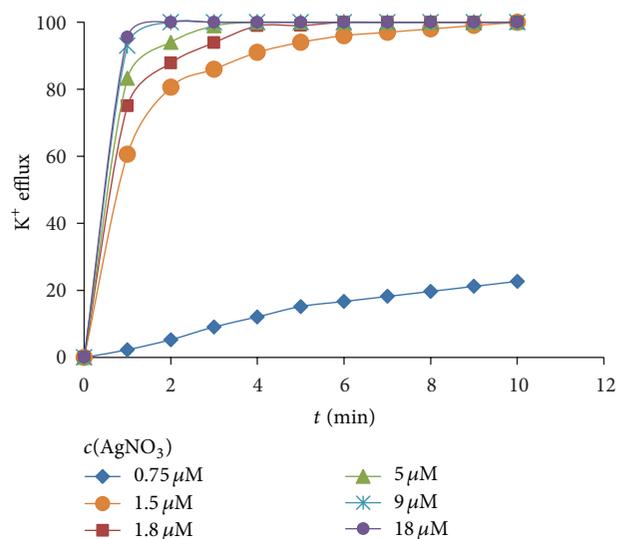


FIGURE 9: The rate of K<sup>+</sup> efflux from the yeast cells incubated with AgNO<sub>3</sub> solutions.

Short time after the reduction of AgNO<sub>3</sub> with the excess of strong reducing agents like sodium borohydride silver ions in dispersion of as obtained silver nanoparticles are of course absent. But then metallic silver nanoparticles inevitably generate Ag<sup>+</sup> ions during the contact of resulting dispersion with oxygen or by the action of reactive oxygen species generated in living cells. To estimate the rate of oxidation of silver nanoparticles we used the sensitive spectrophotometric technique using 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

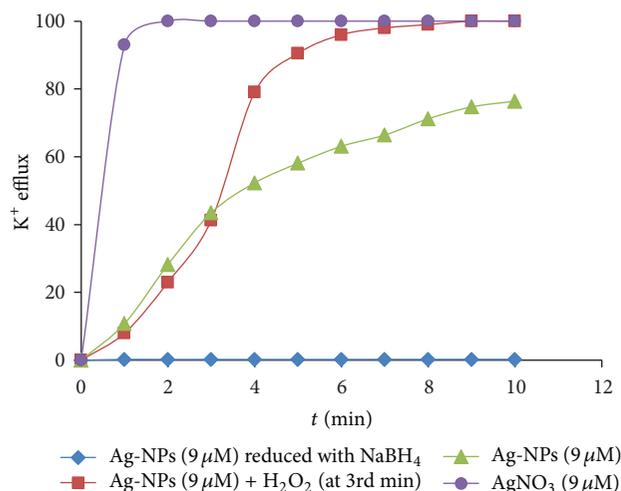


FIGURE 10: The rate of  $K^+$  efflux from the yeast cells incubated with silver NPs colloids treated with  $\text{NaBH}_4$  and  $\text{H}_2\text{O}_2$  and the corresponding curve for the yeast cells incubated with  $\text{AgNO}_3$  is shown for comparison.

did not interfere with the spectrophotometric determination of ion  $\text{Ag}^+$  at 635 nm. Intense bubbling of air for 30 minutes through silver nanoparticles dispersion does not result in 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 formation of silver ions, which leads to a significant increase in activity against living cells.

**3.1.2. Microbiological Activity of Silver Halides Dispersion.** Taking into account that the biocidal activity of silver NPs against the *Saccharomyces cerevisiae* cells is mainly a result of 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. The usage of nanodispersed silver salts as biocidal agents has a big potential as a way to provide a sufficiently intense and at the same time permanent and long-term release of silver ions. To further understand the mode of action of poorly soluble silver salts dispersion on living cells, we also studied the effect of silver halides on the  $K^+$  efflux rate from *Saccharomyces cerevisiae* cells. Figure 11 shows the dependence of the release of  $K^+$  from the cells by incubation time with dispersion of silver halide.

Comparing the data from Figure 8 it can be seen that the efficiency of biocidal effect of silver halide substantially exceeds the cytotoxic activity of silver nanoparticles and is 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 due to the action of silver nitrate. This delay is apparently due to the slow dissolution of silver halides in citrate buffer. The solubility of

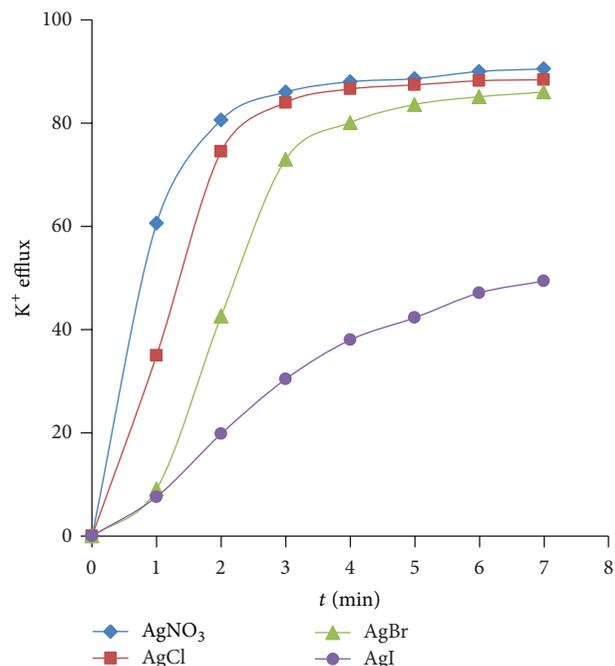


FIGURE 11: The rate of  $K^+$  efflux from the yeast cells incubated with silver halides colloids with silver concentration of  $1.5 \mu\text{M}$ .

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  $[\text{AgHal}]^0$ ,  $[\text{AgHal}_2]^-$  и  $[\text{AgCit}]^{2-}$  (Cit—citrate ion), equals 2.5 mM for AgCl, 133  $\mu\text{M}$  for AgBr, and 1.7  $\mu\text{M}$  for AgI. Thus, the NPs dispersion with a silver halide concentration of  $1.5 \mu\text{M}$  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.

## 4. Conclusions

The discussion about the mechanism of the cytotoxic activity of the dispersion 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 which either remained in the dispersion of silver nanoparticles after their synthesis or were generated afterwards by oxidizing silver particles make the crucial contribution to 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 with the generation of  $\text{Ag}^+$  ions through NPs dissolution.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of the paper.

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