

Preparation of Stabilized Silver Nanoparticles and Study of Their Antimicrobial and Cytotoxic Activity on the Human Hepatoma HepG2 Cell Line

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Abstract—Silver nanoparticles were synthesized using arabinogalactan and sodium dioctyl sulfosuccinate to reduce and stabilize them. The average hydrodynamic size of the nanoparticles, determined by photon correlation spectroscopy, was 30 nm, and the zeta potential was -34.04 ± 1.54 mV. According to the electron diffraction method, silver in the sol sample is in metallic form. The preparation of silver nanoparticles showed antibacterial activity against opportunistic Gram-negative (*Escherichia coli*) and Gram-positive (*Bacillus subtilis* and *B. coagulans*) bacteria. Silver nanoparticles also had antifungal activity against strains of phytopathogenic fungi of the genus *Fusarium sporotrichioides* and *F. solani*. A study of the cytotoxic activity of silver nanoparticles was made on human hepatoma cells of the HepG2 line. The inhibitory effect of silver nanoparticles on the metabolic activity and viability of tumor cells has been demonstrated. The average relative EC₅₀ values for silver nanoparticles were 1.5 ± 0.4 µg/ml and 41.2 ± 3.9 µg/mL. The preparation of stabilized silver nanoparticles can find application in medicine, as a potential antimicrobial and antitumor agent, as well as in agriculture as a means of suppressing growth of phytopathogenic fungi.

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INTRODUCTION

In recent years, new possibilities of using metal nanostructured materials with a number of unique properties have been actively studied in medicine [1]. Particularly widely developed are methods for the synthesis and biomedical use of silver nanoparticles, which, due to their small size, high specific surface area, and penetrating ability, can easily penetrate both bacterial and tumor cells [2]. A number of authors have demonstrated the antimicrobial, antifungal, and antiviral effects of silver nanoparticles [3–8]. Silver nanoparticles penetrate target cells after interacting with corresponding macromolecules localized on the cell surface. Moreover, the efficiency of nanoparticle entry into a cell is largely determined by their size and the nature of stabilizing agents, which ensure interaction of particles with the cell membrane [9]. Since the synthesis method and stabilizers used play an important role in manifesting the activity and unique properties of nanoparticles, it seems important when developing new or modified synthesis methods to study the physicochemical properties and biological activity of the obtained nanoparticles.

An important task in modern medicine is applying achievements in nanobiotechnology when developing new effective antitumor drugs [10–12]. Silver nanoparticles have particularly broad prospects for medical use, because they exhibit not only a pronounced bactericidal [13–15], but also antitumor action [16–18].

One of the most promising ways to obtain silver nanoparticles with antitumor activity is the use of green synthesis along with purified natural nontoxic organic substances and biopolymers as stabilizers and reducing agents of Ag⁺ ions. Matrix isolation employing such polymers as carboxymethyl cellulose, chitosan, gelatin, etc., is frequently used as an effective method for stabilizing of metal nanoparticles [19–21]. Polysaccharides can also act as reducing agents and stabilizers in silver nanoparticle synthesis [22]. One promising biopolymer that can be used to reduce metal nanoparticles from silver salts and stabilize them is arabinogalactan, a naturally derived nontoxic water-soluble biopolymer [23, 24].

Currently, silver nanoparticles with antitumor activity against Ehrlich ascites carcinoma cells are obtained by green synthesis using algae [25]. The anti-

tumor activity of silver nanoparticles obtained by green synthesis was also demonstrated *in vitro* and *in vivo* in relation to Dalton lymphoma cells [26]. Nanoparticles obtained by chemical reduction of AgNO₃ using sodium citrate also showed dose-dependent antiproliferative activity with respect to A549 lung carcinoma cells [27].

In relation to the liver's key role in the body, an important task of nanobiotechnology is to develop drugs for treating oncological liver diseases, in particular hepatocellular carcinoma, which is a primary liver cancer that can occur as a result of such factors as cirrhosis [28], viral hepatitis [29], and use of products containing aflatoxin [30]. Possible metastasis to other parts of the liver and tissues significantly increases the malignancy of hepatocellular carcinoma. At later stages, liver cancer can cause internal bleeding, ascites, and liver failure [31]. Earlier, green synthesis was used to obtain silver nanoparticles that suppress proliferation of liver tumor cells [25]. In this study, silver nanoparticles were obtained using arabinogalactan combined with sodium dioctyl sulfosuccinate, and their antimicrobial and cytotoxic activity against human hepatoma cells of the HepG2 line was investigated.

EXPERIMENTAL

Silver nanoparticles were synthesized using silver nitrate, ammonium hydroxide (27%), sodium dioctyl sulfosuccinate (Aerosol-OT, or bis (2-ethylhexyl) sulfosuccinate, sodium salt) (Labtex, Russia), and arabinogalactan (Fluka).

To obtain an ammonia complex of silver oxide, an ammonium hydroxide solution was added to an aqueous solution of silver nitrate. An ammonia complex of silver oxide was added to an aqueous solution of arabinogalactan containing sodium dioctyl sulfosuccinate with vigorous stirring; the mixture was kept for 40 min at 75°C, after which it was cooled to room temperature.

Silver nanoparticles were studied with a LEO 912 AB OMEGA transmission electron microscope (Carl Zeiss, Germany) at an accelerating voltage of 100 kV. To prepare samples, a drop of silver sol was deposited on copper grids with a diameter of 3.05 mm coated with a thin polymer substrate film and dried at room temperature. Electron diffraction of nanoparticles was recorded from a small area (a circle ~1 μm in diameter).

The size distribution of silver nanoparticles was determined by processing the obtained micrographs with the UTHSCSA Image Tool 3.00 analysis program.

The hydrodynamic size of silver nanoparticles was determined by the dynamic light scattering method, and the electrokinetic potential was determined by electrophoretic light scattering with a Photocor compact Z analyzer (Russia).

The antibacterial and antifungal activity of silver nanoparticles was studied by the agar well diffusion method [32]. To determine antibacterial activity, we used test strains of conditionally pathogenic Gram-positive bacteria *Bacillus subtilis* ATCC 6633, *B. coagulans* 429 and Gram-negative bacteria *Escherichia coli* ATCC 8739. To assess the antifungal activity of silver nanoparticles, strains of phytopathogenic micromycetes were used: *Fusarium sporotrichioides* Sherb. T11 VKPM F-902 and *F. solani* VKPM F-890.

Mueller–Hinton agar medium was used for bacterial cultivation; for fungi, Sabouraud dextrose agar and yeast malt agar. Three hundred microliters of sols with a silver concentration of 200 μg/mL were added to wells with a diameter of 6 mm. In experiments with bacteria, standard discs with streptomycin (10 μg/disc) were used as a control; in experiments with fungi with amphotericin B (40 μg/disc). Sterile water was used as a negative control.

Bacteria strains *E. coli* and *Bacillus* were incubated at 37°C for 24 h; mushroom strains *F. sporotrichioides* and *F. solani*, at 28°C for 3 days. The diameter of the inhibition zone was measured in millimeters.

The minimum inhibitory concentrations (MIC) of silver nanoparticles were determined on 1-day-old cultures of *E. coli*, *B. subtilis*, and *B. coagulans* using the serial dilution method in a liquid medium (Mueller–Hinton broth) in 96-well plates. The silver nanoparticle preparation was diluted in a culture medium to concentrations from 100 to 5 μg/mL in increments of 5 μg/mL (the final volume in the well was 200 μL). With each dilution, 50 μL of test strain suspension was inoculated with a density of 0.5 according to the McFarland turbidity standard. The control for measuring MIC consisted of a microbial culture control (positive) and sterile medium control (negative). Test cultures with the added preparation were incubated for 24 h at 37°C. The lowest concentration inhibiting visible growth of microorganisms was considered as the MIC value. The standard antibiotic streptomycin was used as the control.

The effect of silver nanoparticles on tumor cells was assayed on the human hepatoma cell line HepG2 ATCC HB-8065. Cells were cultured in DMEM/F-12 medium (Gibco) supplemented with 5% FBS (Gibco), 100 u/mL penicillin, and 100 μg/mL streptomycin (Gibco) in an incubator in a 5% CO₂ atmosphere at 37°C.

The effect of silver nanoparticles on the metabolic activity of HepG2 cells was analyzed by the colorimetric MTT test (Sigma-Aldrich) [33] in accordance with ISO 10993-5:2009 [34]. A HepG2 culture was inoculated in a 96-well plate in an amount of 15 × 10³ cells per well and left for 24 h for adhesion. Then, silver nanoparticles were added to the well at final concentrations of 0.5–66.7 μg/mL. As a control, a cell culture was used without the test substance. All measurements were carried out in seven replicates (*n* = 7). After 48 h

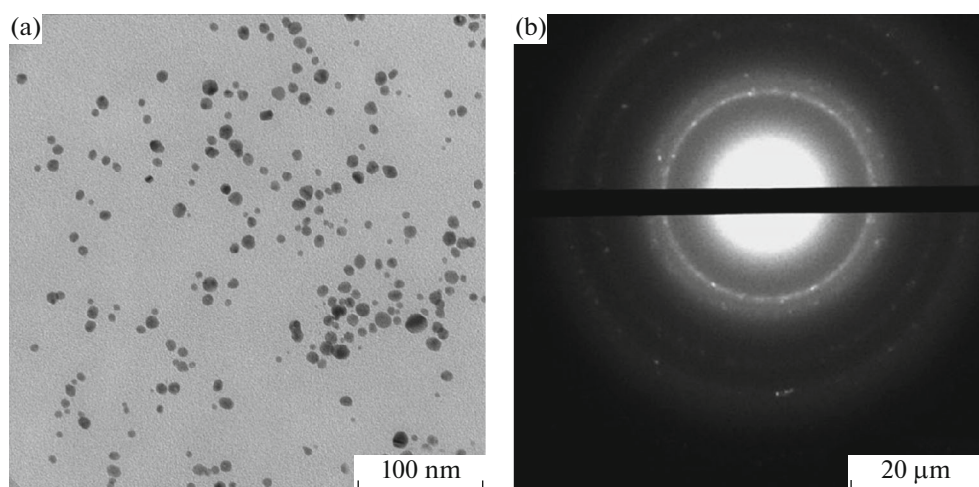


Fig. 1. Micrograph (a) and electron diffraction pattern (b) of sol of silver nanoparticles obtained by transmission electron microscopy.

of cultivation, images of cells were taken to assess their morphology in phase contrast using an TE 2000-U Eclipse (Nikon) inverted microscope, followed by MTT analysis. The culture medium was changed with the addition of 0.48 mm of MTT reagent and incubated for 4 h. The background value was taken into account by adding a reagent to the medium ($n = 7$). Then the supernatant was removed and the resulting formazan crystals were dissolved in 150 μL of DMSO (Sigma). Absorption was measured at a wavelength of 540 nm on a Multiskan FC microplate photometer (Thermo).

Analysis of the effect of silver nanoparticles on the cell index (CI) of HepG2 cell cultures were evaluated in real time using an iCELLigence System RTCA cell analyzer. The device measures the microelectrode impedance, the value of which depends on the number, size, and shape of cells [35]. To evaluate CI in real time, HepG2 cells were inoculated in an 8-well plate (E-Plate L8) in an amount of 30×10^3 cells per well in 600 μL of medium. After 24 h, at the beginning of the exponential growth phase, silver nanoparticles were added to the wells in final concentrations of 0.5–66.7 $\mu\text{g}/\text{mL}$. The background value was taken into account by adding nanoparticles to wells with nutrient medium, but without cells. Cell cultures without addition of silver nanoparticles were used as the control. The analysis was carried out for 96 h.

The effect of silver nanoparticles on cell viability was assayed by staining DNA with fluorescent dyes EthD-1 (Thermo Fisher) and Hoechst 33342 (Sigma-Aldrich) [36]. For analysis, HepG2 cells were inoculated in a 96-well plate in an amount of 15×10^3 cells per well. After 24 h, silver nanoparticles (0.5–66.7 $\mu\text{g}/\text{mL}$) were added and incubated for 24 h. As a control, cells were used without addition of the test substance. Then, staining with EthD-1 and Hoechst

33342 was carried out according to the manufacturer's directions. Cells were fixed with 4% paraformaldehyde in PBS. Fluorescence images were obtained using the EVOS FL Auto Imaging System (Invitrogen).

Statistical analysis was performed using GraphPad Prism version 7.00 with one-way analysis of variance (ANOVA) by the a posteriori Dunnett's test and verification of the Gaussian distribution by the Shapiro–Wilk criterion. Calculation of multiphase sigmoidal regression models was done in the DrFit program [37] to search for EC_{50} (concentration 50% reduction in vitality). Results are presented as mean \pm standard error (SEM) or standard deviation (SD).

RESULTS AND DISCUSSION

Synthesis and Characterization of Silver Nanoparticles

Natural polysaccharides can act both as reducing agents and stabilizers during metal nanoparticle synthesis [22, 23]. In this study, to reduce and stabilize silver nanoparticles from silver nitrate solution, we used arabinogalactan polysaccharide in combination with sodium dioctyl sulfosuccinate. At the first stage, an ammonia complex of silver oxide was obtained by adding an ammonium hydroxide solution to an aqueous solution of silver nitrate. Then, an ammonia complex of silver oxide was added to an aqueous solution of arabinogalactan containing sodium dioctyl sulfosuccinate. The resulting mixture was incubated at 75°C for 40 min, followed by cooling to room temperature. The metallic silver content in sol was 0.02 wt %. Based on transmission electron microscopy data, the obtained preparation contained spherical metal silver nanoparticles (Fig. 1).

Figure 2 shows a histogram of the size distribution of nanoparticles obtained from processing of the

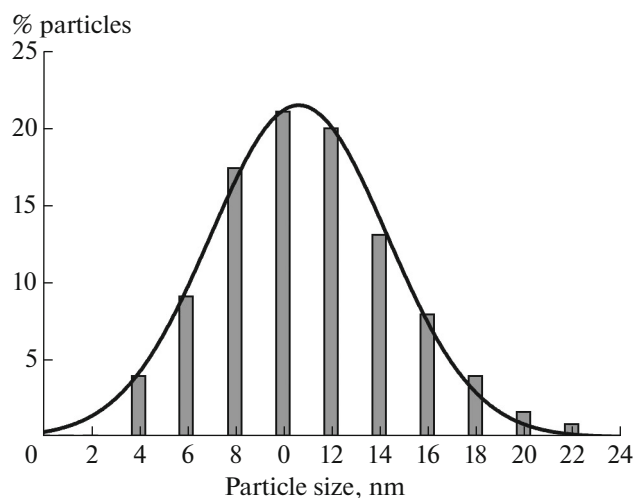


Fig. 2. Size distribution of silver nanoparticles obtained by analyzing TEM-microphotographs using UTHSCSA Image Tool 3.00 software.

microphotographs. The average particle size calculated from the histogram was 11.03 nm. The average hydrodynamic size of nanoparticles determined by photon correlation spectroscopy was above this value, 30 nm, which may be due to the presence of a polymer shell on the nanoparticle surface. The zeta potential of the obtained nanoparticles was -34.04 ± 1.54 mV.

Nanoparticle Antimicrobial Activity

One of the most important problems in modern medicine is congenital and acquired multidrug resistance of microorganisms to the action of traditional antimicrobial agents, such as antibiotics [38].

The absence of multidrug resistance to silver nanoparticles in microorganisms creates wide prospects for effective antimicrobial agents based on them. Silver nanoparticles kill microbial cells by damaging effect their cell walls and membranes, as well as proteins, lipids, and nucleic acids [39–41].

The antibacterial activity of silver sol was studied by diffusion into agar (Table 1). After 24 h of incubation,

the zone of *E. coli* inhibition by silver nanoparticles (27.3 ± 0.5 mm) significantly exceeded the inhibitory zone of streptomycin (17.1 ± 0.3 mm). The zone of *B. coagulans* inhibition also significantly exceeded the control inhibitory zone. In this case, the zone of *B. subtilis* inhibition was comparable to the control (Table 1). The MIC of silver nanoparticles were determined by serial dilutions in a liquid medium. The most effective inhibitory effect of the nanoparticle preparation was exerted on *E. coli* cells (MIC 15 $\mu\text{g}/\text{mL}$). MIC values for the test strains of *B. coagulans* and *B. subtilis* were higher: 45 and 55 $\mu\text{g}/\text{mL}$, respectively. Thus, the strain of Gram-negative bacteria *E. coli* was the most vulnerable to the action of silver nanoparticles; Gram-positive bacteria strains *B. coagulans* and *B. subtilis* showed greater stability.

Currently, the effect of silver nanoparticles on phytopathogens has not been adequately studied [42, 43]. The cytotoxic activity of silver nanoparticles in relation to fungal cultures largely depends on the nature of the stabilizing agents and species affiliation of the fungi [44]. An in vitro study on the phytopathogenic fungi cultures *Rhizoctonia solani*, *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Pythium aphanidermatum*, etc. demonstrated the antifungal activity of silver nanoparticles [45], which indicates their potential for controlling phytopathogens in agriculture.

To assay antifungal activity, sol was used at an initial concentration of 200 $\mu\text{g}/\text{mL}$. The antifungal effect of silver nanoparticles was manifested toward both test strains of phytopathogenic fungi, and the diameter of the inhibition zone of *F. sporotrichioides* slightly exceeded (17.7 ± 0.8 mm) the diameter of the inhibition zone of *F. solani* (15.6 ± 0.4 mm) (Table 1).

Suppression of phytopathogenic fungi of the genus *Fusarium* indicates the potential for agricultural use of silver nanoparticles stabilized in arabinogalactan and sodium dioctyl sulfosuccinate.

Assessment of Cytotoxicity of Nanoparticles

MTT analysis. The results of assessing the cytotoxicity of silver nanoparticles by MTT analysis showed a

Table 1. Antimicrobial activity of silver nanoparticles against certain strains of bacteria and phytopathogenic fungi

Test organism	Inhibition zone, mm		
	Silver nanoparticles 200 $\mu\text{g}/\text{mL}$	Streptomycin 10 $\mu\text{g}/\text{disc}$ (control)	Amphotericin B 40 $\mu\text{g}/\text{disc}$ (control)
<i>B. subtilis</i> ATCC 6633	19.4 ± 0.7	18.6 ± 0.6	
<i>B. coagulans</i> 429	23.3 ± 0.5	16.7 ± 0.4	
<i>E. coli</i> ATCC 8739	27.3 ± 0.5	17.1 ± 0.3	
<i>F. sporotrichioides</i> Sherb. T11 VKPM F-902	17.7 ± 0.8		13.0 ± 0.2
<i>F. solani</i> VKPM F-890	15.6 ± 0.4		14.3 ± 0.3

two-phase decrease in the metabolic activity of cells during cultivation with different concentrations of silver nanoparticles (0.5–66.7 $\mu\text{g}/\text{mL}$) over the course of 48 h (Fig. 3a). The minimum studied concentrations (0.5 and 1 $\mu\text{g}/\text{mL}$) had no statistically significant cytotoxic effect on cells ($p > 0.05$) with respect to the control group. In the nanoparticle concentration range of 2.0–33.4 $\mu\text{g}/\text{mL}$, a decrease in cell metabolic activity of $75.7 \pm 2.1\%$ on average was observed ($p < 0.0001$) compared to the control. The maximum decrease in cell metabolic activity (up to 31.6%) was observed at a nanoparticle concentration of 66.7 $\mu\text{g}/\text{mL}$. The relative EC_{50} values were 1.8 $\mu\text{g}/\text{mL}$ ($\text{EC}_{50[1]}$) and 41.4 $\mu\text{g}/\text{mL}$ ($\text{EC}_{50[2]}$).

In phase-contrast microscopy images of the HepG2 cell culture (Fig. 4a) obtained after 48 h of cultivation with different concentrations of silver nanoparticles, were observed a change in the cell morphology and a decrease in cell density with increasing nanoparticle concentration.

Cell index analysis. Figure 3b shows the real-time CI measurements of HepG2 cells at a silver nanoparticle concentration in the culture medium of 0.5–66.7 $\mu\text{g}/\text{mL}$. At minimal silver nanoparticle concentrations (0.5 and 1 $\mu\text{g}/\text{mL}$), a cytotoxic effect was not observed ($p > 0.05$). At concentrations of 4.2–33.4 $\mu\text{g}/\text{mL}$, the action of silver nanoparticles led to a significant decrease in CI ($p < 0.0001$), which may be associated with suppression of cell proliferation, a change in morphology, or death. At a silver nanoparticle concentration of 66.7 $\mu\text{g}/\text{mL}$, a sharp decrease in CI was observed ($p < 0.0001$) caused by cell death. The relative EC_{50} values calculated based on the areas under the curve according to CI data (Fig. 3c) were 1.2 $\mu\text{g}/\text{mL}$ ($\text{EC}_{50[1]}$) and 46.9 $\mu\text{g}/\text{mL}$ ($\text{EC}_{50[2]}$).

Assessment of cell viability. The effect of silver nanoparticles on cell viability was assayed after 24 h of cultivation with the studied silver nanoparticle concentrations, followed by DNA staining with EthD-1 and Hoechst 33342. EthD-1 is an indicator of dead cells, which has a high affinity for DNA and low penetrability through cell membranes. Hoechst 33342, by contrast, can easily penetrate cell membranes and bind to DNA. This dye stains the nuclei of both living and dead cells. As can be seen from the results of fluorescence microscopy (Fig. 4b), with an increase in silver nanoparticle concentration, the number of dead cells (stained with EthD-1) increases with respect to the total number of cells (stained with Hoechst 33342). Cell density is also reduced with respect to the control, which confirms the cytotoxic effect of silver nanoparticles on tumor cells.

The results of analyzing the cytotoxic effect of the preparation on the HepG2 cell culture showed that silver nanoparticles exhibit a two-phase toxic effect in

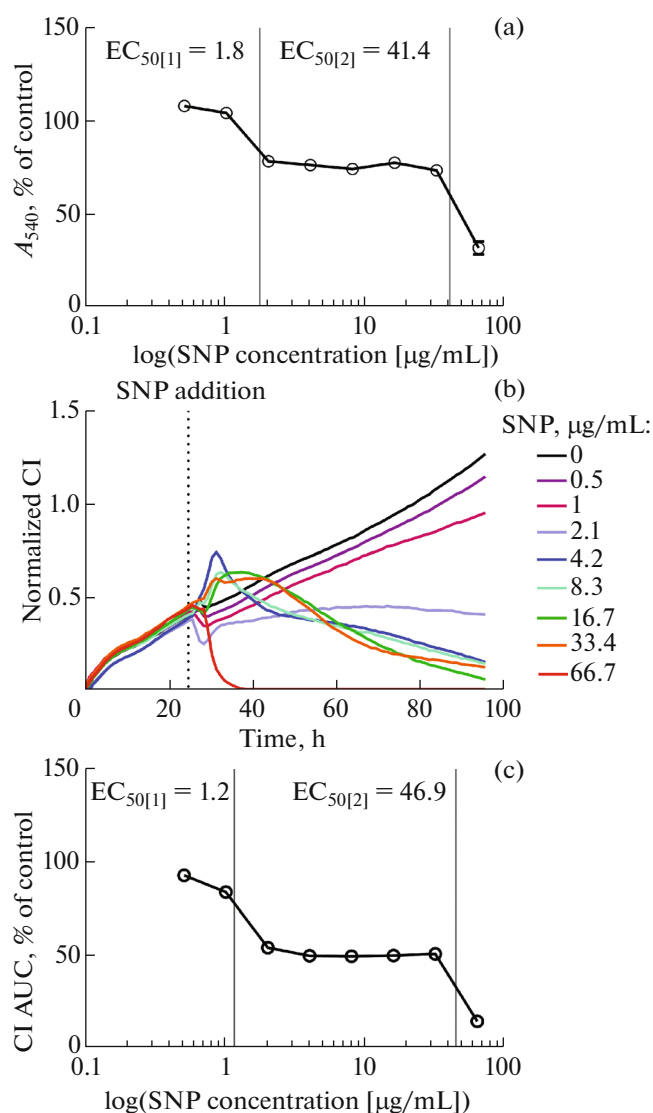


Fig. 3. (Color online) EC_{50} estimate for silver nanoparticles (AgNPs) on HepG2 cells: (a) graph of MTT analysis data on cell metabolic activity during cultivation with nanoparticles (0.5–66.7 $\mu\text{g}/\text{mL}$) for 48 h; $\text{EC}_{50[1]} = 1.8 \mu\text{g}/\text{mL}$, $\text{EC}_{50[2]} = 41.4 \mu\text{g}/\text{mL}$. Data are presented as mean \pm SEM; (b) curves of dependence of cell index (CI) of HepG2 on incubation time obtained from real-time analysis with addition of nanoparticles (0.5–66.7 $\mu\text{g}/\text{mL}$) 24 h after start of cell cultivation (dashed line); (c) graph of the area under curve (AUC), based on CI values of HepG2 cells during cultivation with studied AgNP concentrations; $\text{EC}_{50[1]} = 1.2 \mu\text{g}/\text{mL}$, $\text{EC}_{50[2]} = 46.9 \mu\text{g}/\text{mL}$.

relation to tumor cells. Silver nanoparticles have two relative half-maximum effective concentrations with a cytotoxic effect ($\pm\text{SD}$): $\text{EC}_{50[1]} = 1.5 \pm 0.4 \mu\text{g}/\text{mL}$ and $\text{EC}_{50[2]} = 41.2 \pm 3.9 \mu\text{g}/\text{mL}$. The action of nanoparticles can lead to a decrease in cell metabolic activity, inhibition of cell proliferation, and a change in cell morphology and viability.

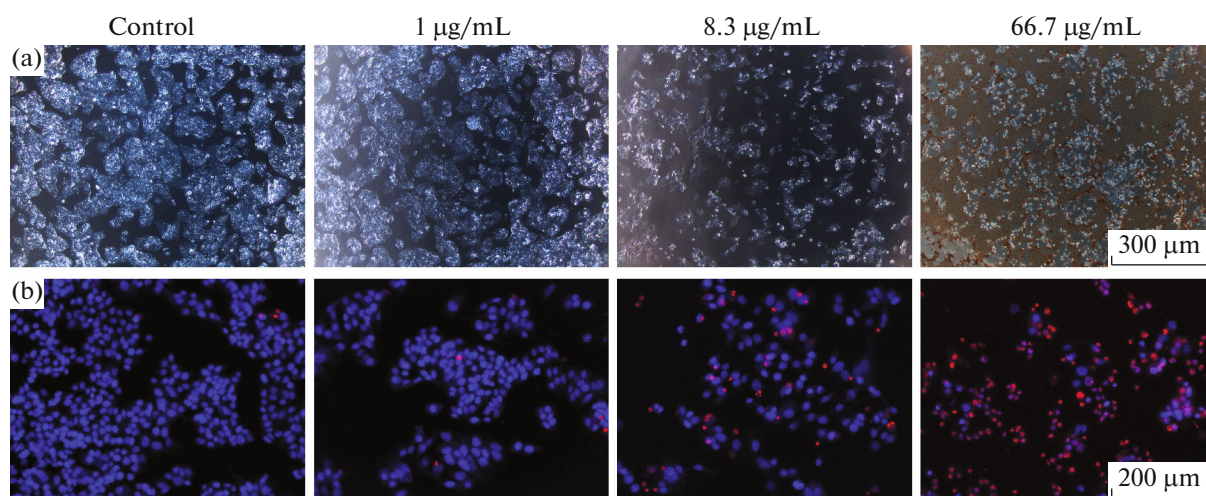


Fig. 4. (Color online) Phase contrast micrographs of HepG2 cells after 48 h of cultivation with different silver nanoparticle concentrations (1.0, 8.3, 66.7 $\mu\text{g/mL}$) (a); fluorescence images of HepG2 cells stained with EthD-1 and Hoechst 33342, when cultured with studied silver nanoparticles concentrations for 24 h (b). Magnification $\times 100$ (a) and $\times 200$ (b).

CONCLUSIONS

Silver nanoparticles stabilized with arabinogalactan in combination with sodium dioctyl sulfosuccinate were obtained with an average size of 30 nm and a zeta potential of -34.04 ± 1.54 mV. The stability of silver sols results from electrostatic repulsion of nanoparticles from each other [46]. An additional contribution to the stability of silver sols comes from steric stabilization provided by arabinogalactan, which is an easily accessible natural nontoxic polymer conveniently and safely used in silver nanoparticle synthesis [23, 24]. The silver nanoparticle preparation showed antibacterial activity against opportunistic Gram-positive and Gram-negative bacteria, fungicidal activity against phytopathogenic fungi, and cytotoxic activity against human tumor cells in vitro. The multidirectional biological effects of silver nanoparticles stabilized by biopolymers indicate their wide potential in biomedical applications.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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