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Study of Antimicrobial and Hemolytic Activities of Silver Nanoparticles Prepared by Chemical Reduction

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Abstract—Hydrosols that contain silver nanoparticles with average particle sizes of 10–15 and 20–25 nm have been synthesized using chemical reduction. The antimicrobial activity with respect to the Gram-negative bacteria *Escherichia coli* ML35p and *Pseudomonas aeruginosa* ATCC 27853, the antibiotic-resistant clinical isolate of *Pseudomonas aeruginosa*, and the Gram-positive bacteria *Staphylococci aureus* SG511 and *Listeria monocytogenes* EGD, as well as the hemolytic activity of the synthesized samples, has been investigated. The results obtained have shown that the use of the samples under investigation for the development of new bactericides holds promise.

Key words: silver, nanoparticles, hydrosols, chemical reduction, tannin, sodium boron hydride, antimicrobial activity, hemolytic activity

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INTRODUCTION

It is known that silver in the ionic form and in the form of colloidal particles exhibits a broad spectrum of antimicrobial activity [1–3]. The data available in the literature indicate that silver offers incomparable advantages over all existing antimicrobial and antiviral agents. However, up to now, the mechanism of action of silver on a cell is not understood. Furthermore, in the majority of works concerned with the action of silver on an organism, the authors investigated the action of silver in the form of charged ions. At the same time, silver in the form of nanoparticles should be of primary interest, because it is known that materials in an ultradisperse state possess unusual properties which are not observed for macroparticles and microparticles. Moreover, stabilized silver nanoparticles have a higher stability and can retain their own biological activity for a longer time as compared to ions. In recent years, there have appeared works [4, 5] in which the authors considered the evolution of silver from ions to nanoparticles and discussed the action of different silver preparations on viruses, bacteria, and cells. It was established that the biocidal effect of silver nanoparticles substantially exceeds the action of silver ions Ag^+ taken in the same concentrations. In this respect, the evaluation of the biological activity of silver nanoparticles and its dependence on the particle size is of special interest. In particular, the inhibition of growth of the human immunodeficiency virus by silver nanoparticles exclusively in the size range 1–10 nm was in vitro demonstrated by direct experiments [6].

In this work, we investigated the biological and hemolytic activities of silver nanoparticles prepared by chemical reduction.

SAMPLE PREPARATION AND EXPERIMENTAL TECHNIQUE

Reagents

Silver nitrate ($AgNO_3$, Khimmedsintez, 99.9%), sodium boron hydride ($NaBH_4$, Sigma Aldrich, 99%), tannin ($C_{75}H_{52}O_{46}$, Acros), trisubstituted sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, Acros, 99%), and gelatin (Acros) were used without additional purification. Working solutions were prepared using distilled water. A Muller–Hinton broth (Sigma) was used to cultivate microorganisms.

Synthesis of Silver Hydrosols

Silver nanoparticles were prepared by chemical reduction in an aqueous solution of silver nitrate. Sodium boron hydride and tannin were used as reducing agents, because, according to the data available in literature [7–9], these reducing agents make it possible to prepare silver nanoparticle sols stable in time

Table 1. Synthesis conditions for silver nanoparticles

Sample no.	Synthesis conditions				
	$AgNO_3$ concentration, *M*	Concentration of the reducing agent, *M*	Stabilizer concentration, *M*	Buffer solution	pH
1	0.025	Tannin 0.1	Gelatin 0.25	Boric acid, potassium chloride, sodium hydroxide	9.8
2	0.025	Tannin 0.1	Gelatin 0.25	Boric acid, potassium chloride, sodium hydroxide	7.3
3	0.00025	Sodium boron hydride 0.01	Sodium citrate 0.00025	–	7

with a narrow particle size distribution. In a number of cases, a variation in the reduction conditions allowed us to synthesize almost monodisperse nanoparticles [10]. Gelatin and sodium citrate were used as stabilizers. The synthesis conditions for the samples under investigation are given in Table 1.

The technique for reducing silver nitrate was as follows. A buffer solution with a particular pH value and a solution of the reducing agent and the stabilizer were poured into a reaction vessel at room temperature. Then, a silver nitrate solution with a necessary concentration was introduced with constant stirring at a constant rate.

It is known (see, for example, [7, 11, 12]) that the reagent concentrations, temperature, and the pH of the medium strongly affect the size and stability of silver hydrosols. For the silver nitrate–tannin system, it was established [7] that the process of nanoparticle formation most effectively occurs at room temperature and pH 9.8. However, if the prepared hydrosols of silver nanoparticles are considered from the viewpoint of their further application for the solution of the problems in biology and medicine, the pH value of the medium should be close to neutral. In this respect, we carried out the experiments on the reduction of silver nitrate by tannin at pH values of both 9.8 and 7.3 (Table 1, samples *1*, *2*). Sample *3* was synthesized at pH 7. In this case, in order to decrease the average particle sizes, the reagent concentrations were reduced to minimum possible values.

In the case of samples *1* and *2*, the sols were dark brown in color, and sample *3* had a light yellow color. The prepared hydrosols were investigated using transmission electron microscopy, UV absorption spectroscopy, and X-ray diffraction.

METHODS OF INVESTIGATION

The absorption spectra of the samples were investigated on a LEKI SS2109UV spectrophotometer in quartz cells with an optical path length of 10 mm and on a SpectraMax 250 (Molecular Devices) spectrophotometer in 96-well plates in the wavelength range 250–770 nm. In order to remove the reducing agents and other undesirable components of the mixture from the samples, we used the dialysis on a membrane with a nominally intercepted molecular weight of 3.5 kDa with the application of a phosphate salt buffer solution at pH 7.4. In the case of sample *3*, the dialysis did not lead to positive results. Therefore, for this sample, nanoparticles were separated from the chemical reagents with the use of ultracentrifugation at 50000 *g* for 2 h (Optima LE-80K ultracentrifuge, Beckman Coulter). The prepared sediment was resuspended in the phosphate salt buffer solution.

In order to prepare samples for X-ray diffraction analysis and to evaluate the nanoparticle sizes, the synthesized sols were evaporated at a temperature of 110°C. The prepared powder was studied on a Bruker D8-Advance X-ray diffractometer (CuK_α radiation). The average particle size was calculated from the X-ray powder diffraction data with the TOPAS-3 (Bruker) program using the Scherrer formula [13].

The micrographs of the samples were obtained on an EM-125 transmission electron microscope at the accelerating voltage U_{acc} = 75 kV. The nanoparticle size distribution was calculated with the DiaMorf Objective-A.1.6.3 program for image analysis from several micrographs for each sample (at least 100 particles were taken into account).

The antimicrobial activity of the samples was studied by the method of serial dilutions in a culture medium, which is one of the most frequently used

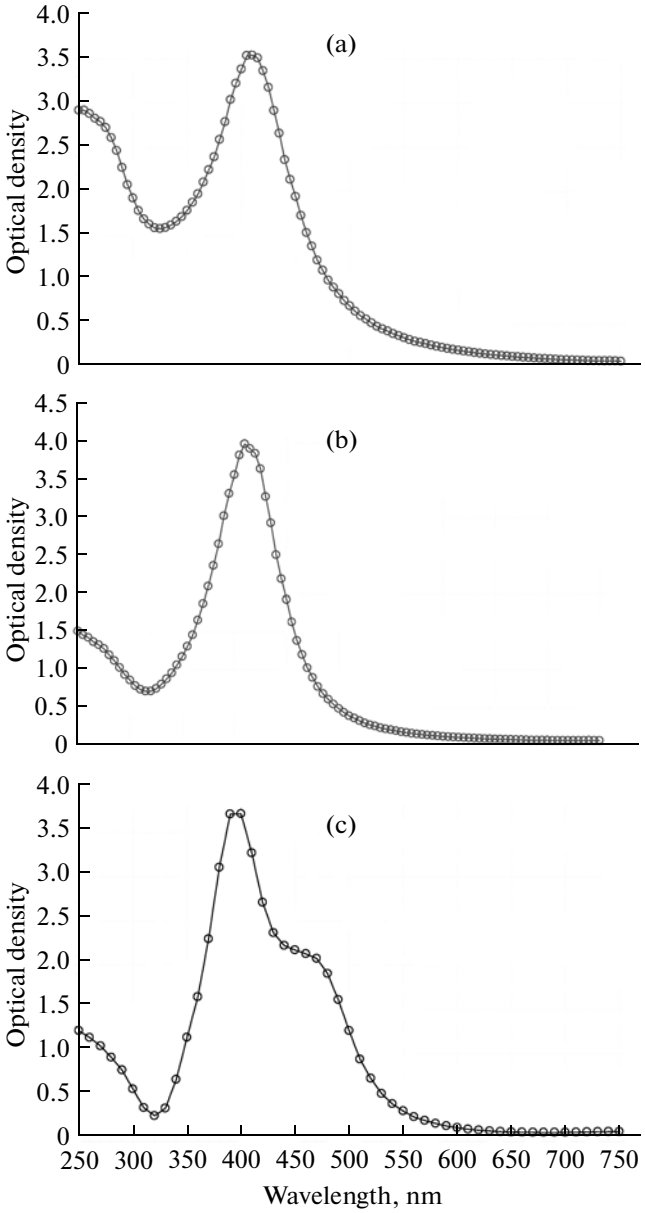

Fig. 1. Absorption spectra of samples (a) *1*, (b) *2*, and (c) *3*.

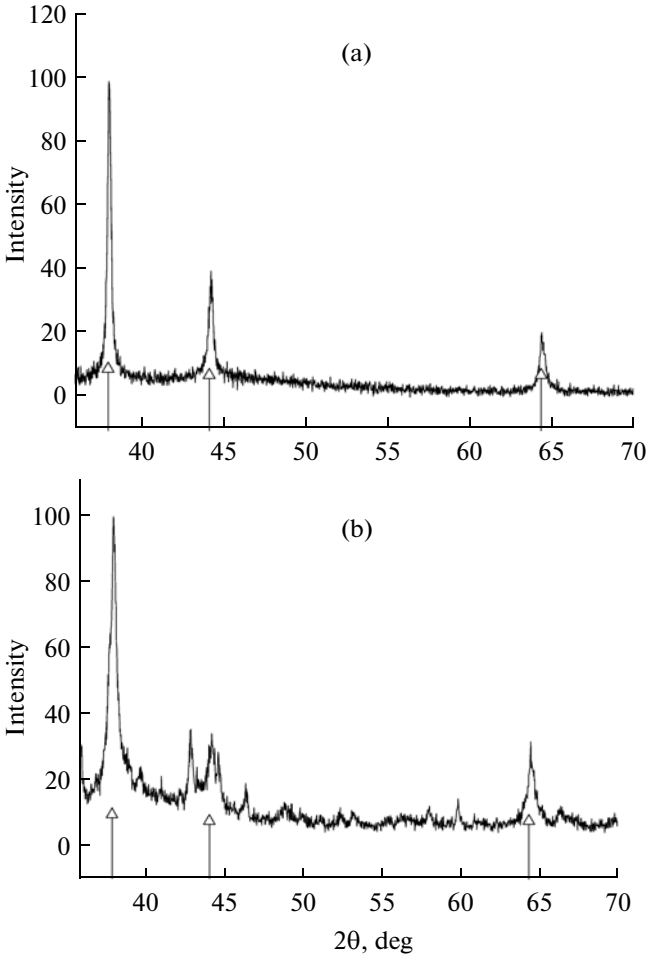

Fig. 2. X-ray diffraction patterns of the dried hydrosols of samples (a) *1* and (b) *3*. Δ indicates Ag.

methods in microbiology for evaluating the antimicrobial activity of different compounds. We used the method modified by Tossi et al. [14].

The activity was studied with respect to the Gram-negative bacteria *Escherichia coli* ML35p and *Pseudomonas aeruginosa* ATCC 27853, the antibiotic-resistant clinical isolate of *Pseudomonas aeruginosa*, and the Gram-positive bacteria *Staphylococci aureus* SG511 and *Listeria monocytogenes* EGD.

The hemolytic activity of silver nanoparticles with respect to human erythrocytes was determined using the technique described in [15].

A blood stabilized by heparin was centrifuged at 300 *g* and a temperature of 4°C for 10 min. The phosphate salt buffer solution was added to the sediment, and the system was centrifuged under the same conditions. The procedure of washing erythrocytes was repeated three times. A sample 280 μl in volume was taken from the prepared sediment, and the volume of the suspension was increased to 10 ml by the cooled phosphate salt buffer solution. The erythrocyte suspension (90 μl of the suspension per 10 μl of the preparation) was added to the diluted preparations. In order to obtain positive (100% lysis of erythrocytes) and negative (0% lysis of erythrocytes) controls, instead of the preparations to be investigated, 10% of Triton X-100 or phosphate salt buffer solution, respectively, were added to the samples. After completion of incubation, the reaction was terminated by adding the cooled phosphate salt buffer solution and the samples were centrifuged at 3000 *g* for 4 min. The supernatant was taken and introduced into cells of the 96-well plate (Costar, Corning).

The optical density of the samples was measured on a SpectraMax 250 spectrophotometer at the wave-

length $\lambda = 540$ nm (OD_{540}). The percentage of hemolysis was calculated from the formula

$$\text{Hemolysis (\%)} = \frac{OD_{540}\text{ (sample)} - OD_{540}\text{ (0\% lysis)}}{OD_{540}\text{ (100\% lysis)} - OD_{540}\text{ (0\% lysis)}} \times 100\%.$$

In the experiments, we used the nanoparticle suspensions diluted to the same degree at which the inhibition of growth of microorganisms was observed in the study of the antimicrobial activity of microorganisms (conventionally, this degree of dilution was designated as the minimum inhibiting concentration). We also investigated the samples whose concentrations exceeded the minimum inhibiting concentration by a factor of two and four.

RESULTS AND DISCUSSION

The optical absorption spectra of the samples under investigation are shown in Fig. 1. As can be seen from Fig. 1, the spectra of all samples contain a broad diffuse band with the absorption maximum at $\lambda_{max} =$ 400 nm, which indicates the presence of silver particles with sizes of approximately 10 nm [10, 16, 17]. Moreover, the absorption spectrum of sample *3* exhibits a shoulder shifted toward the long-wavelength range, which is characteristic of the samples synthesized with a concentration of sodium citrate lower than 0.8×10^{-4} *M* [18] and is a consequence either of the particle aggregation or the formation of particles with a nonspherical shape. The spectral characteristics of the samples remained unchanged for two months from the instant of synthesis, which suggests the stability of the prepared sols.

The X-ray diffraction patterns of samples *1* and *2* are depicted in Fig. 2. For sample *3*, we failed to prepare the amount sufficient for performing the X-ray powder diffraction analysis. According to the X-ray powder diffraction data for samples *1* and *2*, they contain metallic silver. The results of the calculations of the particle sizes in the samples under investigation from the X-ray powder diffraction data are presented in Table 1.

The micrographs of the samples, from which it can be seen that the prepared nanoparticles have a spherical shape, are displayed in Fig. 3. For samples *2* and *3*, the particles undergo partial aggregation, which can be associated with the influence of the pH of the medium for sample *2* and with the low concentration of the stabilizing agent (sodium citrate) for sample *3*.

The histograms, which reflect the particle size distribution according to the electron microscopy data, are also presented in Fig. 3. The average particle sizes for each sample are listed in Table 2. The particle sizes determined from the X-ray diffraction and electron microscopy data are in good agreement.

Table 2. Average sizes of silver nanoparticles in the samples under investigation

Sample no.	Average particle size, nm	
	According to the transmission electron microscopy data	According to the X-ray diffraction data
1	10–15	20 ± 3
2	15–30	25 ± 3
3	10–15	–

It follows from Table 2 that silver nanoparticles have average particle sizes of 10–15 nm for samples *1* and *3* and average particle sizes of 15–30 nm for sample *2*.

Table 3 presents the results of the study of the biological activity of samples. It is evident from Table 3 that the prepared samples showed a high antibiotic activity with respect to both the Gram-negative and Gram-positive microorganisms, including the strains that are resistant to traditional antibiotics. Although the activity of sample *3* is considerably lower than the activity of samples *1* and *2*, taking into account that, for the synthesis of this sample the lower concentration of $AgNO_3$ was used (by a factor of 100), we can assume that, in terms of the number of particles in the sample, all three samples have approximately identical activity.

In order to verify that the trace amounts of sodium boron hydride do not introduce distortions into the results of evaluating the activity of nanoparticles, we determined the activity of this component of the reaction mixture. The results of evaluating the antimicrobial properties of sodium boron hydride are given in Table 4. It can be seen from Table 4 that the concentrations of sodium boron hydride used in this work are considerably lower than its minimum inhibiting concentration; i.e., the presence of this reagent does not introduce substantial distortions into the results of the measurements.

Furthermore, we studied the hemolytic activity of the synthesized nanoparticles with respect to human erythrocytes (Table 5). Hemolysis[1] is a process of destruction of erythrocytes with the escape of hemo-

[1] From the Greek words "haima" (blood) and "lysis" (disintegration, destruction).

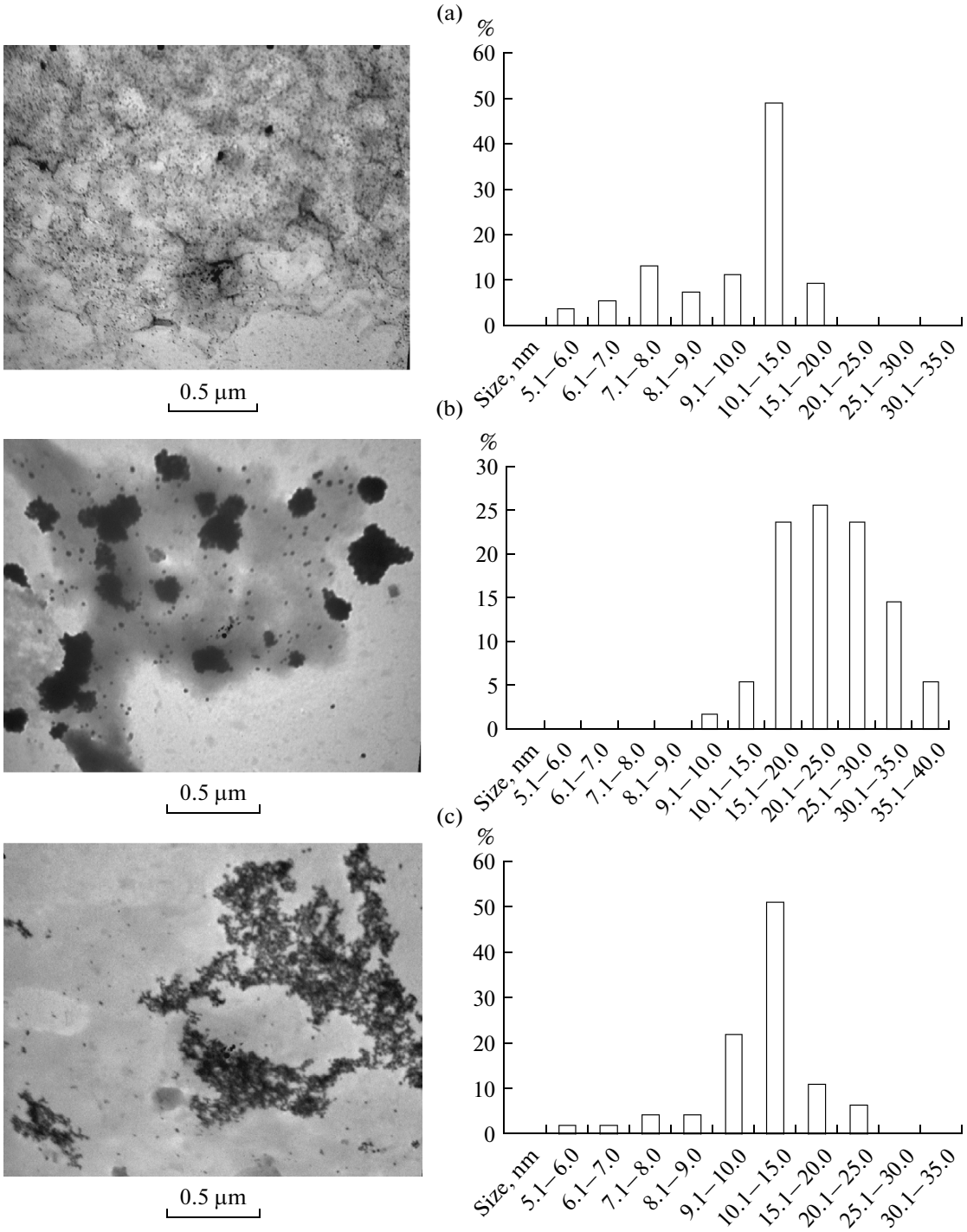

Fig. 3. Micrographs and histograms of particle size distributions for samples (a) *1*, (b) *2*, and (c) *3*.

globin in the environment. The wide use of a number of antibacterial preparations is limited as a result of their high hemolytic activity. The interaction of some peptide antibiotics with the erythrocyte membrane can cause its damage, which leads to the escape of hemoglobin from the cell. At a particular concentration of an antibiotic, the degree of damage is so high that the structural integrity of the lipid layer is lost. As a consequence, when developing new pharmaceutical preparations, the test on the hemolytic activity is a necessary component, together with the check of their antibacterial activity. It follows from Table 5 that all

Table 3. Hemolysis of human erythrocytes (%) after their incubation with the silver nanoparticle suspension at given concentrations

Nanoparticle concentration	Hemolysis of erythrocytes, %					
	1	*2*	*3*	*1* after dialysis	*2* after dialysis	*3* after centrifugation
Minimum inhibiting concentration	0.2 ± 0.1	0.6 ± 0.2	0.9 ± 0.3	0.1 ± 0.05	0.5 ± 0.2	0.8 ± 0.3
Minimum inhibiting concentration × 2	0.3 ± 0.2	0.4 ± 0.1	0.8 ± 0.3	0.4 ± 0.2	1.0 ± 0.4	0.8 ± 0.3
Minimum inhibiting concentration × 4	0.3 ± 0.1	1.1 ± 0.4	1.5 ± 0.4	0.9 ± 0.3	1.0 ± 0.5	0.9 ± 0.4

Table 4. Minimum inhibiting concentrations of the samples. The results are presented as the dilution of the samples by a factor of 2 (1/2), 4 (1/4), 8 (1/8), etc.; in this case, the highest dilution of the preparation at which the inhibition of growth of microorganisms was observed was taken as the minimum inhibiting concentration

Microorganism	Minimum inhibiting concentration of the silver nanoparticle samples					
	1	*2*	*3*	*1* after dialysis	*2* after dialysis	*3* after centrifugation
Escherichia coli ML35p	1/128	1/128	1/2	1/128	1/64	1/2
Pseudomonas aeruginosa ATCC 27853	1/64	1/64	1	1/64	1/32	1/2
Antibiotic-resistant clinical isolate of *Pseudomonas aeruginosa*	1/64	1/64	1	1/64	1/32	1
Staphylococcus aureus SG511	1/128	1/128	1/2	1/128	1/64	1/2
Listeria monocytogenes EGD	1/64	1/64	1/2	1/64	1/32	1/2

Table 5. Minimum inhibiting concentrations of sodium boron hydride

	Microorganism			
	Escherichia coli ML35p	*Pseudomonas aeruginosa* ATCC 27853	*Staphylococcus aureus* SG511	*Listeria monocytogenes* EGD
Minimum inhibiting concentration of $NaBH_4$, *M*	0.2	0.2	0.4	0.2

three samples of nanoparticles under investigation demonstrate a low hemolytic activity over the entire range of concentrations, including the concentration that is higher than the antimicrobial concentration by a factor of four.

CONCLUSIONS

The antimicrobial and hemolytic activities of the samples of silver nanoparticles with average particle sizes of 10–15 and 20–25 nm prepared by chemical reduction of silver nitrate by tannin and sodium boron hydride have been studied. It has been demonstrated that the samples under investigation are characterized by a high antibiotic activity with respect to both Gram-negative and Gram-positive microorganisms, including the strains resistant to traditional antibiotics irrespective of the size of nanoparticles and the method of their synthesis. At the same time, the hemolytic activity of the synthesized samples is low over the entire range of concentrations, including the concentration that exceeds the antimicrobial concentration by a factor of four.

The obtained characteristics of the samples under investigation make promising their further use for the development of new antibacterial preparations.

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