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Inhibition of Staphylococcus Bacteria by Ag Nanoparticles Under Plasmon Resonance

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The antimicrobial activity of colloids of Ag nanoparticles (NPs) with a diameter of 90 and 7 nm in a quercetin shell, obtained by thermal reduction and chemical reduction of NaBH4, was studied. Colloids exhibited antimicrobial activity against a test culture of S. aureus. At the same time, NPs of smaller sizes showed antimicrobial activity more effectively (at lower concentrations of the solution). The additional irradiation of the colloid solutions covered by quercetin Ag NPs with a diameter of 7 nm on the S. aureus test culture was also studied. The SF26 spectrophotometer was used as a radiation source to determine the effect on S. Aureus under LSPR conditions of NPs. The SF26 spectrophotometer can manually select the wavelength and radiation intensity. It allows us to irradiate the maximum volume of solutions. The sample was irradiated with light with a wavelength of 380 nm, corresponding to the resonance frequency of NPs in the solution. The transmission band was ±15 nm. The control sample was stored in the absence of external irradiation (in the dark) and under the same conditions as the irradiated one. The samples were irradiated for 110 minutes. Then, the irradiated and control samples were sown on Petri dishes with a solid nutrient medium and incubated in a thermostat at 37 °C for 24 hours. After that, we compared the seeds of irradiated and control samples. A relative decrease in the growth of the population of S. aureus bacteria in the culture of irradiated samples was found. The enhancing effect of additional external irradiation under the conditions of surface plasmon resonance of NPs was determined (by 33 percent). The authors associate this effect of irradiation with the physical (field) interactions of NPs and bacteria.

Keywords: Nanoparticles, Plasmon Resonance, Antimicrobial activity.

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1. INTRODUCTION

The influence of nanoparticles onbio-objects, such as viruses and microbes – bacteria and fungi has been intensively studied during the last decades [1-3]. Partially, it was discovered that the influence of nanoparticles on viruses and bacteria often led to the inhibition of infection activities [4] up to the destruction of viral [5] and bacteria [6] envelopes. The wide applications of nanoparticles (NPs) in various fields of medico-biological applications are based on the unique characteristics of the nanoparticles, such as their shape, charge, size, high ratio of surface area to mass, high reactivity, and unique optical properties [7]. Silver nanoparticles have broad-spectrum antimicrobial potential and can act at low concentrations, which reduces potential toxicity to humans and the environment. Therefore, nanoparticles are attractive for use in the medical industry, pharmaceuticals, packaging materials and other industries directed to fight against infectious diseases [8, 9].

There are numerous mechanisms of the antiviral and antimicrobial actions of nanoparticles. One can point out two different mechanisms of antiviral and antimicrobial activity of nanoparticles - chemical and physical [7, 10]. One needs to emphasize that both of these (chemical and physical) mechanisms are based on the local-field enhancement effect. This effect leads to the activation of chemical hetero-catalytic reactions at the nanoparticle surface on one side and the emergence of hotspots in the local field on the surface of viruses and microbes, which cause the ponderomotive forces mechanically acting on the envelopes of viruses and microbes, on the other side [10]. Ponderomotive forces can be evaluated by means of the formula

$$F_i(\boldsymbol{R}) = \left(P_j(\boldsymbol{R}) \cdot \frac{\partial}{\partial x_j}\right) E_i(\boldsymbol{R}), \qquad (1.1)$$

with $P_j(\mathbf{R})$ local dipole moment induced in the microbe envelope and $E_i(\mathbf{R})$ local electric field (in the hot spot) acting on the envelope. It is easy to understand that the value of ponderomotive force strongly depends on the inhomogeneity of the field. Both characteristics $P_j(\mathbf{R})$ and $E_i(\mathbf{R})$ are frequency-dependent. Thus, the acting on the system by an external electrodynamic field can cause the enhancement of hot spots of the field and dipole moments on the microbial envelope. As a result, this action leads to the inhibition of the infection activity of microbes. Because the nanoparticle at the surface of the microbe envelope can be considered as a nanoantenna, the irradiation of the system by the light of resonant frequency can cause an increasing local-field enhancement effect. That can lead to an increase in the antimicrobial action of nanoparticles. As it was mentioned above, the local-field enhancement effect can improve the chemical interaction between the nanoparticles and molecules-receptors of the microbial envelope. This effect can be considered in the frame of nano-catalysis phenomena [11].

2. MATERIALS AND PREPARATIONS

2.1 Nanoparticles Preparations

Colloidal solutions of silver nanoparticles were prepared by chemical reduction of $AgNO_3$ silver salt with sodium tetrahydroborate ($NaBH_4$). Then, 0.0228 g

of $NaBH_4$ dissolved in 20 ml of H_2O was added to 100 ml of an aqueous solution of $AgNO_3$ ($1 \cdot 10^{-4}$ M) with intensive stirring. The recovery reaction proceeds according to the scheme described in [12]:

$$4Ag^+ + nBH_4^- + 3nH_2O \rightarrow 4Ag^0 + nH_2BO_3^- + 4nH^+ + 2nH_2$$

The synthesis of nanoparticles was monitored by measuring the optical absorption spectra of the solution. After the appearance in the spectrum of the solution of a stable band of SPR silver nanoparticles (NPs), a solution of quercetin with $pH = 6.5$ (pH was achieved by the addition NaOH) was added. The molar ratio of silver to quercetin was 1:1. Absorption spectra of solutions at the synthesis stages were recorded using a Perkin-Elmer Lambda 35 spectrophotometer.

To prepare silver colloid using the thermal recovery method, an aqueous solution of quercetin and $AgNO_3$ salt in ratios similar to the previous technique was heated to 90 °C with intensive stirring for 5 hours. As a result, a stable plasmon resonance band was formed in the spectrum of the colloid.

Note that quercetin is a flavonoid of plant origin. It has antioxidant properties, has an antispasmodic, antihistaminic, and anti-inflammatory effect, and can also be useful as an ecological reducer of silver ions Ag^+ to Ag^0 in green synthesis [13].

The absorption spectrum of the colloid solution of silver nanoparticles shelled with the flavonoid quercetin contains several bands (Fig. 1): quercetin in the UV region and localized surface plasmon resonance (LSPR) of the silver core in the visible region ($\lambda_{max} = 389$ nm). The absence of a shift in the peak of the LSPR of Ag nanoparticles (NPs) after the addition of the stabilizer to the colloid indicates the preservation of the size of the nanoparticles, and the absence of additional bands or shoulders in the spectrum indicates the absence of agglomeration of NPs.

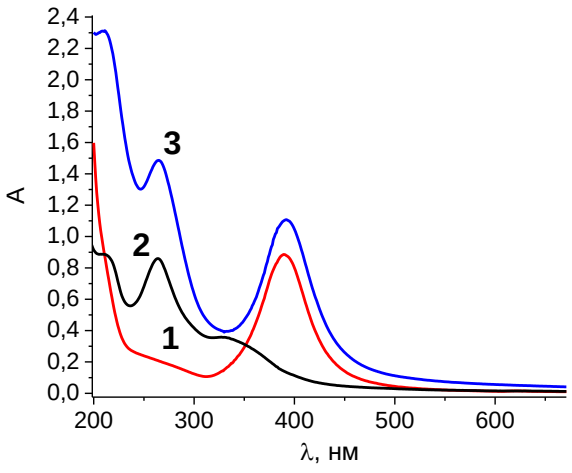

Fig. 1 – Absorption spectra of colloid solution of silver NPs (curve 1), quercetin solution (curve 2) and colloid solution of silver NPs stabilized by quercetin (curve 3) (Ag : Quer = 1 : 1)

The ratio of components silver: quercetin = 1:1 was chosen to obtain nanoparticles with maximum stability. It was experimentally discovered that in the spectra of colloids with a small amount of stabilizer, the additional band (maximum ~ 450 nm) in the long-wavelength range shoulders appeared over time. This additional band is evidence of agglomeration of nanoparticles (Fig. 2). The process of NPs agglomeration in silver colloids is accompanied by a decrease in the bactericidal activity of NPs. Therefore, optimal concentration ratios were established to prevent agglomeration and to ensure colloid stability.

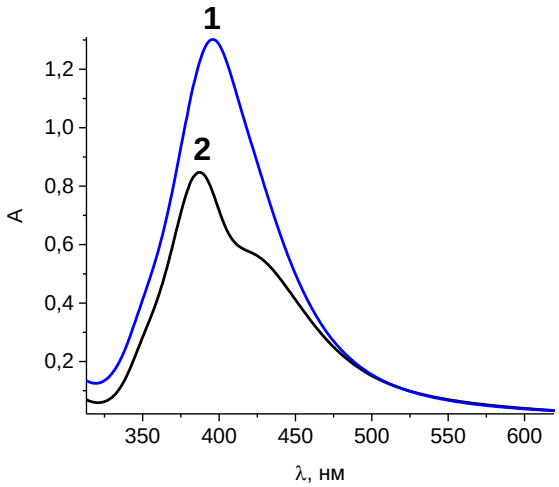

Fig. 2 – Absorption spectra of colloid solution of silver NPs stabilized by quercetin (Ag : Quer = 1 : 0.0025). Curve 1 corresponds to fresh solution, curve 2 corresponds to solution after 500 hours later

Prepared solutions of Ag NPs were used for biological part of experiments.

2.2 Bacteria Preparations

The reference strain of conditionally pathogenic gram-positive microorganisms *Staphylococcus aureus* (*S. aureus*) from American Collection of Type Cultures (ACTC) 6538 was used as test cultures. The *S. aureus* strain was obtained from the Ukrainian collection of microorganisms of the Zabolotny Institute of Microbiology and Virology National Academy of Sciences of Ukraine. This microorganism is a test strain for determining the antimicrobial effect of drugs [14]. At 37 °C, bacteria were grown on meat-peptone agar (MPA) for 24 hours. Antimicrobial activity was determined by the diffusion method in agar on Muller-Hinton medium [15]. To do this, daily cultures of *S. aureus* were washed with a sterile physiological solution, and the suspension was diluted according to the McFarland turbidity standard of 0.5 (~1.5×10^8 CFU/ml). 0.1 ml of the suspension was sown with lawn on cups with Muller-Hinton agar. Holes ($d = 6$ mm) were made on the dried surface, into which 1 ml of the tested agent was introduced. The cups were incubated for 24 hours at a temperature of 37 °C. The indicator of antimicrobial activity was the presence of a clear zone around the well with the tested solutions. The method of serial dilutions in a liquid nutrient medium of MPB was used to establish the minimum inhibitory concentration. Several test tubes were prepared with successive serial dilutions of experimental samples. For sowing, an *S. aureus* suspension was prepared according to the McFarland turbidity standard of 0.5 (~1.5×10^8 CFU/ml), followed

by dilution to 10^5 CFU/ml. 0.2 ml of the suspension of the test strain of the microorganism was added to all test tubes in a row. The test tubes were incubated in a thermostat at 37 °C for 24 hours. The bacteriostatic effect was determined by diluting the tested solutions, where no visible culture growth was observed in the test tube.

From each test tube, where there was no visible culture growth, sowing on Petri dishes with a solid nutrient medium of MPA was done to determine the bactericidal effect. After 24 hours of incubation at a temperature of 37 °C, the dilution of the solution in the test tube, the inoculation from which did not grow, was noted.

3. EXPERIMENTAL STUDIES

The main idea of experimental part of the work is the studies of antibacterial properties of solutions of nanoparticles of different concenrations. The actions of nanoparticles were studied without and with external radiation. The wavelength external light radiation was chosen that correspond to the excitation of localized plasmon resonance in the nanoparticles.

3.1 The Studies of the Effect of LSPR on the Test Strain of *S. aureus*

As a first stage, we performed the optical absorption spectra measurements of colloidal solutions of Ag NPs (see Fig. 2). As a result, the resonant wavelength of the nanoparticles solution was established as 380 nm.

To establish the effect of LSPR on *S. aureus*, a test culture was irradiated with the SF26 spectrophotometer. The SF26 spectrophotometer has the ability to select manually the wavelength and the intensity of irradiation. That was the reason for choosing this non-modern device for experiments where the spectrophotometer is used as a source of radiation with well-defined (manually controlled) characteristics. Such manual control allowed us to irradiate the maximum volume of solutions.

3.2 Experimental Setup and Details of Biologic Measurements

Pre-prepared certain concentrations of colloidal solutions were added to the liquid nutrient medium of MPB, which was inoculated then with S. aureus culture (1.5×10^5 CFU/ml). After 10 min, the samples were irradiated with visible light at a wavelength of 380 nm. A non-irradiated sample served as a control. Irradiation was carried out for 110 minutes, and then test and control samples were sown on Petri dishes. After 24 hours of incubation at a temperature of 37 °C, the growth intensity of the test culture on the plates was noted. It investigated colloidal solutions with silver nanoparticles in the quercetin shell obtained by thermal reduction of $d \sim 90$ nm (sample 8) and chemical reduction of $NaBH_4$, $d \sim 7$ nm (sample 9), as well as control samples of quercetin and $NaBH_4$ (samples 10, 11).

The study of antimicrobial activity was carried out by the well method against gram-positive bacteria S. aureus. Namely, the colloidal solutions of samples 8-11 were dropped onto the surface of the bacteria sample at different points (see Fig. 3).

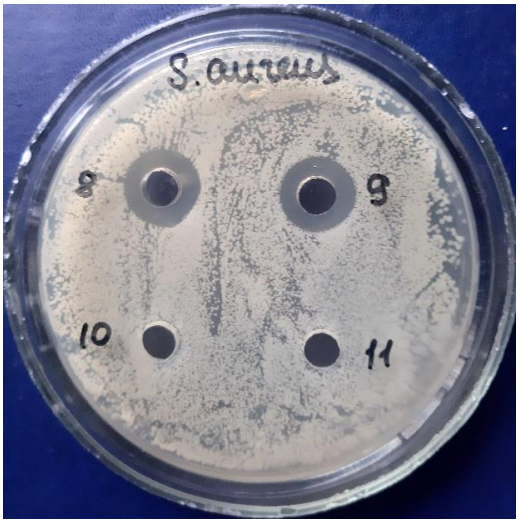

Fig. 3 – Antimicrobial activity of colloidal solutions with silver nanoparticles in the quercetin shell provided by thermal reduction and chemical reduction of $NaBH_4$ against Gram-positive bacteria *S. aureus*

As can be seen from Fig. 3 colloidal solutions obtained by chemical and thermal regeneration show antimicrobial activity against *S. aureus* test culture. Thus, after 24 hours of incubation at 37 °C under the influence of both solutions, clear zones of growth retardation of *S. aureus* bacteria are observed. The diameter of the zone of growth retardation under the action of the colloidal solution with silver nanoparticles in the quercetin shell obtained by thermal reduction is 15.29 mm (sample 8), the solution obtained by chemical reduction of $NaBH_4$ is 15.46 mm (sample 9).

The test solutions contained silver nanoparticles of different diameters, namely 90 nm and 7 nm, but no significant difference and dependence of antimicrobial activity on the specified characteristic was noted. The recorded indicators of their action were within close limits. Control samples had no antimicrobial activity against *S. aureus* bacteria and were characterized by intensive growth of the test culture around the wells (samples 10, 11) (Fig. 3).

One should point out that the method of wells allows only indirectly to conclude the value of the minimum inhibitory concentration (MIC). Therefore, in the future, we determined the MIC, which characterizes the microbiological activity of the test solutions, using the method of serial dilutions (Table 1). To determine the MIC value, certain concentrations of colloid solutions were added to the nutrient medium, which was then inoculated with *S. aureus* culture and after incubation. The presence or absence of visible growth was assessed. The results of the studies shown in Table 1 once more demonstrated that small nanoparticles have a high antibacterial effect. Thus, to study the effect of resonant external irradiation we shoos the preparations of small ($d \sim 7$ nm) nanoparticles.

Table 1 – The minimal inhibit concentrations (dilutions) of colloidal solutions of Ag NPs shelled by quercetin obtained by thermal reduction and chemical reduction of $NaBH_4$ against gram-positive bacteria *S. aureus*

No of the sample	The sample	Incubation time (hours)	Dilution of the solution							
			$1:10^{-1}$	$1:10^{-2}$	$1:10^{-3}$	$1:10^{-4}$	$1:10^{-5}$	$1:10^{-6}$	CG	SC
8	Ag-quercetin ($d \sim 90$ nm)	24	–	–	–	#	#	#	#	#
		48	–	#	#	#	#	#	#	#
9	Ag-quercetin - $NaBH_4$ $d \sim 7$ nm	24	–	–	–	–	#	#	#	#
		48	–	–	#	#	#	#	#	#
10	Quercetin	24	#	#	#	#	#	#	#	#
		48	#	#	#	#	#	#	#	#
11	$NaBH_4$	24	#	#	#	#	#	#	#	#
		48	#	#	#	#	#	#	#	#

The following notations are used here. "–" is the lack of culture growth; "#" is the intensive culture growth, CG – test culture growth control, SC – solvent control (distilled water)

3.3 Resonant Conditions

To determine the effect of external irradiation, the concentration of the colloidal solution was taken to be an order of magnitude higher than the established MIC, namely $1:10^{-5}$. A previously prepared colloidal solution of Ag-quercetin-$NaBH_4$ ($d \sim 7$ nm) at a concentration of $1:10^{-5}$ was added to test tubes with a liquid nutrient medium of MPB and seeded with a test culture of *S. aureus*. After a short time (~10 minutes), when the processes of coagulation of nanoparticles did not play a significant role, the sample was placed in a quartz tube. The test sample was illuminated with light with a wavelength of 380 nm, which corresponds to the resonance frequency of nanoparticles in the solution. The control sample was stored in the absence of external irradiation (in the dark) and under the same conditions as the irradiated sample. The source of irradiation was an incandescent lamp, the light of which was transmitted through the monochromator of the SF-26 spectrophotometer. The bandwidth was ±15 nm. The samples were irradiated for 110 minutes at 5-minute intervals. After alternate exposure, test and control samples were sown on Petri dishes with MPA and incubated in a thermostat at 37 °C for 24 hours. The antibacterial potential and the enhancing effect of light on silver nanoparticles were evaluated by reducing the populations of *S. aureus* bacteria.

4. RESULTS AND DISCUSSIONS

It was established that external irradiation in the conditions of surface plasmon resonance enhances the effect of silver nanoparticles on the growth of the *S. aureus* test culture. From Fig. 4, it can be seen that during the irradiation of the test sample, there is a decrease in the population of bacteria by 33 % compared to the control.

The obtained results indicate that the viability of bacteria depends on the morphology and plasmonic properties of silver nanoparticles. In addition, it was established that additional irradiation of silver colloids in the mode of plasmon resonance leads to an increase in their bactericidal effect. Also, the bactericidal effect of silver nanoparticles can be controlled by changing the concentration and exposure time.

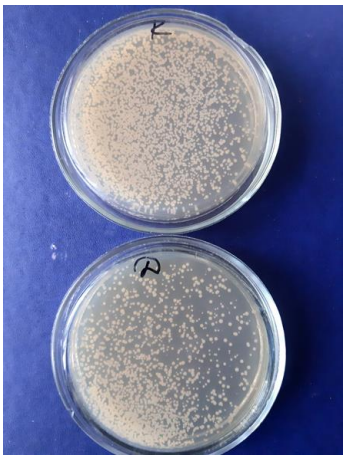

Fig. 4 – Antimicrobial activity of colloidal solutions with silver nanoparticles in the quercetin shell provided by thermal reduction and chemical reduction of $NaBH_4$ against Gram-positive bacteria *S. aureus*

We obtained some unexpected results when using the standard point of view that the chemical interactions form the antibacterial properties of nanoparticles. Indeed, the external visible light irradiation should not affect the chemical interaction between the atoms of silver at the surface of the nanoparticle and the membrane of bacteria. Experimental results shown in Fig. 4 demonstrate the effect of external irradiation. The irradiation at the wavelength corresponding to the resonant conditions can act to the formation of local-field distribution at the surface of the bacteria. Thus, we can guess that the physical action of the nanoparticles plays a role in the antibacterial properties of nanoparticles.

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Пригнічення бактерій Staphylococcus за допомогою наночастинок Ag під плазмонним резонансом

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Досліджено антимікробну активність колоїдних розчинів наночастинок срібла діаметром 90 і 7 нм в оболонці кверцетину, отриманих термічним відновленням і хімічним відновленням NaBH4. Було показано, що колоїди виявляють антимікробну дію проти тестової культури S. aureus. У той же час наночастинки менших розмірів виявляли антимікробну активність ефективніше (при менших концентраціях розчину). Було також досліджено вплив додаткового опромінення розчину наночастинок срібла діаметром 7 нм в оболонці кверцитину натестову культуру S. Aureus.Спектрофотометр SF26 використали в якості джерела випромінювання для встановлення впливу на S. Aureus в умовах LSPR наночастинок. Спектрофотометр SF26 має можливість ручного вибору довжини хвилі та інтенсивності випромінювання, що дозволило нам опромінити максимальний об'єм розчинів. Зразок опромінювали світлом з довжиною хвилі 380 нм, що відповідає резонансній частоті наночастинок у розчині. Смуга пропускання становила ±15 нм. Контрольну пробу зберігали за відсутності зовнішнього опромінення (у темряві) і в тих же умовах, що й опромінену. Зразки опромінювали протягом 110 хвилин. Після цього опромінені та контрольні зразки висівали на чашки Петрі з твердим живильним середовищем та інкубували в термостаті при 37 °C протягом 24 годин. Висіви опромінених і контрольних зразків порівнювали. Було виявлено відносне зменшення росту популяції бактерій S. Aureus у висіві опромінених зразків. Встановлено ефект посилення дії додаткового зовнішнього опромінення в умовах поверхневого плазмонного резонансу наночастинок (на 33 відсотки). Цей ефект зовнішнього освітлення автори пов'язують з фізичними (польовими) взаємодіями наночастинок і бактерій.

Ключові слова: Наночастинки, Плазмонний резонанс, Антимікробна активність.