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SYNTHESIS OF SILVER NANOPARTICLES USING THE RHAMNOLIPID BIOCOMPLEX OF MICROBIAL ORIGIN

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Silver nanoparticles (AgNPs) have been synthesized by “green synthesis” using the silver nitrate as precursor and surface-active rhamnolipid biocomplex – product of microbial synthesis of the strain *Pseudomonas* sp. PS-17 as reducing agent and stabilizer. Kinetics of AgNPs formation was monitored by UV/vis spectrometry. It was found that the reaction is carried out without the induction period indicating the heterogeneous nature of AgNPs nucleation. Using the transmission electron microscopy it was found that obtained silver sol is consisted of small nanoparticles with diameter 3.6±2.0 nm. Antimicrobial activity of obtained AgNPs against *Agrobacterium tumefaciens* was investigated and it was found that the minimum bactericidal concentration is equal to 0,27 μg/mL.

Keywords: silver nanoparticles, biosurfactants, kinetics, transmission electron microscopy, antimicrobial activity.

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1. Introduction

Over the past few decades, metal nanoparticles less than 100 nm in diameter have made a substantial impact across diverse biomedical applications, such as diagnostic and medical devices, for personalized healthcare practice. In particular, silver nanoparticles (AgNPs) have great potential in a broad range of applications as antimicrobial agents, biomedical device coatings, drug-delivery carriers, imaging probes, diagnostic and optoelectronic platforms, since they have discrete physical and optical properties and biochemical functionality tailored by diverse size- and shape-controlled AgNPs. Silver nanoparticles have been investigated extensively due to their superior physical, chemical, and biological characteristics, and their superiority stems mainly from the size, shape, composition, crystallinity, and the structure of AgNPs compared to their bulk forms [1–5]. Recently in the literature, there have been reports of AgNPs obtaining in different biological

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solutions via the so-called “*green methods*”, for example, using plant extract [6–10] and supernatants obtained from bacterial [11–15] or fungus cultures [16, 17]. Several studies reported the synthesis of AgNPs using green, cost effective, and biocompatible methods without the use of toxic chemicals via biological methods. In this green chemistry approach, several bacteria, including *Pseudomonas stutzeri* AG259 [18], *Lactobacillus* [19], *Bacillus licheniformis* [20], *Escherichia coli* [21], *Brevibacterium casei* [22], plant extracts such as *Allophylus cobbe* [23], *Artemisia princeps* [24], *Typha angustifolia* [25] were used. In addition to these, several biomolecules, such as biopolymers [26], starch [27], fibrinolytic enzyme [28], and aminoacids [29] were used. Biological entities could act simultaneously as reducing and stabilizing agents. Increasing attention to the biological synthesis of AgNPs can be attributed to environmental and economic reasons. Green synthesis of AgNPs with naturally occurring reducing agents could be a promising method to replace more complex physiochemical syntheses since the green synthesis is free from toxic chemicals and hazardous byproducts and instead involves natural capping agents for the stabilization of AgNPs. Use of biological agents seem to provide controlled particle size and shape, which is an important factor for various biomedical applications [30]. The other advantages of biological agents are the availability of a vast array of biological resources, a decreased time requirement, high density, stability, and the ready solubility of prepared nanoparticles in water. Biogenic surfactants are one of promising agents for the development of nanocomposites, they are ecologically safe alternative to toxic chemicals. The most known biosurfactants are rhamnolipids, which represent by themselves the low molecular weight secondary metabolits of bacteria genera *Pseudomonas*. Such biosurfactants were obtained in the Department of Physical Chemistry of Fossil Fuels of the Institute of Physical Organic Chemistry and Coal Chemistry named after L. M. Lytvynenko of the National Academy of Sciences of Ukraine. The strain *Pseudomonas* sp. PS-17 synthesizes extracellular mono- and dirhamnolipids and polysaccharide of alginate nature. Rhamnolipids and polysaccharide form surface-active complex at the ratio 4 : 1, so-called the rhamnolipid biocomplex (RBC) [31].

Recently it was shown that RBC, being inexpensive and effective product, can be used in environmentally friendly technologies, for example as corrosion inhibitors [32], in agriculture for stimulation of plant growth [33], for remediation of contaminated soils [34], production of detergent compositions. From this point of view, biosurfactants of microbial origin, in particular the rhamnolipid biocomplex are attractive precursors for green synthesis of metallic nanoparticles.

That is why herein we present the use of microbial origin rhamnolipid biocomplex for reducing of Ag^+ ions and stabilization of AgNPs.

2. Experimental section

AgNPs have been synthesized in thermostated glass reactor equipped by magnetic stirrer and Dimroth's fluid-cooled condenser. Silver nitrate (Aldrich, 99 %) was used as a precursor for AgNPs synthesis; rhamnolipid biocomplex (RBC) (microbial origin biosurfactant) was used both as stabilizer and reducing agent. Cultivation of strain *Pseudomonas* sp. PS-17 was carried out on a rotary shaker, 220 rpm, (WL-2000, JV Electronic, Poland), 30 °C, 5 days with an optimized liquid medium using glycerol (30 g/L) as a carbon source [35]. RBC was isolated from the cell-free cultural liquid of the strain *Pseudomonas* sp. PS-17 (from the collection of Department of Physical Chemistry of Fossil Fuels of the Institute of Physical Organic Chemistry and Coal Chemistry named after L. M. Lytvynenko of the National

Academy of Sciences of Ukraine) via acid precipitation (10 % of HCl, pH = 3) and purified by reprecipitation. Concentration of RBC for AgNPs synthesis was equal to 1 g/L.

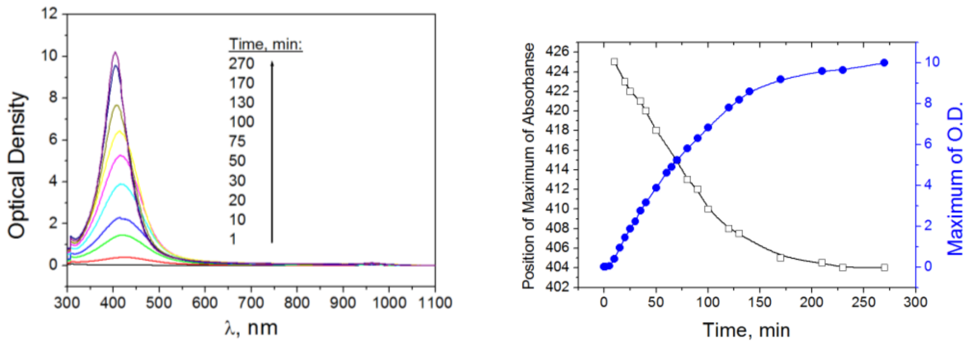
UV/vis spectra of AgNPs sols were recorded using the Uv-mini–1240 UV/vis spectrophotometer (Shimadzu, Japan).

The diameters of obtained AgNPs were determined using transmission electron microscopy (TEM) by comparison of the sizes of individual particles with the scales presented on the images. TEM images of the samples were recorded using a JEM-I230 (JEOL, Japan) with an acceleration voltage of 80 kV. The samples for TEM investigations were prepared by drying of 5 μl of silver sol on the carbon grid at room temperature.

Antimicrobial activity of obtained AgNPs has been evaluated according to their minimum inhibitory concentration (*MIC*). *MIC* is the lowest concentration of antimicrobial agents that inhibits test microorganism phytopathogenic bacteria *Agrobacterium tumefaciens* growth after incubation in a 24-well round-bottom microplate at 37 °C for 20 hours. In order to determine the minimum bactericidal concentration (*MBC*) the subcultures are subcultured on agar plates on medium without inhibitors from each tube from the *MIC* series of studies, after incubation at 37 °C for 20 hours [36].

3. Results and discussion

AgNPs have been synthesized via reduction of $AgNO_3$ (1×10^{-3} mol/L) by RBC (1g/L) at 70 °C. pH of starting reaction mix was 8.2. Kinetics of reaction was monitored using UV/vis spectroscopy (Fig. 1) that is one of proper techniques for investigation of AgNPs formation [37–40].



a *b*

Fig. 1. The change of UV/vis spectra of silver solution during the synthesis (*a*) and dependencies of position of maximum of the surface plasmon absorption band (*squares*) as well as maximum of optical density (*circles*) on time (*b*)

It was found that the reaction proceeds without the induction period (Fig. 1, *b*) as well as some opalescence of reaction mixture after adding of $AgNO_3$ was observed. These facts indicate the heterogeneous nature of nucleation of AgNPs that may be caused by interaction of Ag^+ and acidic group of RBC followed by the precipitation of polymeric balls saturated with silver ions. This is also indicated by a blue shift of the position of the AgNPs solution absorbance maxima during the synthesis that may be caused by strong interaction of small silver particles and / or nuclei of the AgNPs with the acidic groups of RBC [40, 41]; such interaction is decreased with the growth of particles due to decreasing of ratio of ionized surface silver atoms.

Taking into account the heterogeneous nature of nucleation the kinetic curve of AgNPs formation was fitted using the eq. (1) [42, 43] and the rate constant of growth as well as the overall concentration of nuclei were calculated (Fig. 2).

$$\ln\frac{a}{1-a} = k[\mathrm{Ag}^+]_0 t - \ln\left(\frac{[\mathrm{Ag}^+]_0}{[\mathrm{B}]_0}\right), \qquad (1)$$

here $a = \frac{\text{O.D.}}{\text{O.D.}_{max}}$ is the conversion of reaction; O.D. is the optical density of solution; O.D._{max} is the final optical density of solution; $[\mathrm{B}]_0$ is the concentration of nuclei, i. e. the overall concentration (or number) of silver ions that took part in the nucleation process.

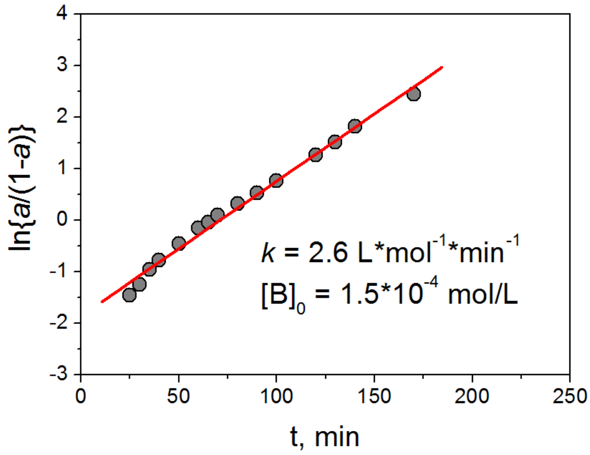
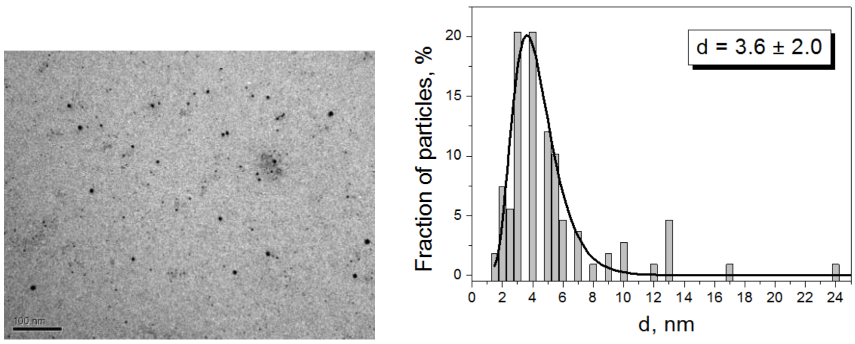


Fig. 2. Linear anamorphose of kinetic curve of the AgNPs formation

Calculated value $[\mathrm{B}]_0$ is near 10 times lower than the starting concentration of Ag^+. It means that the nucleation is started on the heterogeneities existing in the reaction in a form of polymeric balls of the RBC saturated with the silver ions, but the growth of the AgNPs is caused by reduction of solved Ag+ ions via an interaction with these Ag–RBC formations.

Obtained AgNPs were investigated using TEM (Fig. 3) and it was found that synthesized sol is consisted of small particles with mean diameter of 3.6 nm. At the same time, the size distribution of AgNPs is wide and can be fitted by Log-Normal distribution function. Such phenomenon may be caused by heterogeneous nature of the AgNPs nucleation.



a *b*

Fig. 3. TEM-image (*a*) and the size distribution (*b*) of obtained AgNPs

Taking into account the results of TEM investigations it can be assumed that such small AgNPs are formed via the growth process without the aggregation. That is why the number of obtained AgNPs ([AgNPs]) will be equal to the number of nuclei and such value can be used for the calculation of the number (*n*) of silver ions that are in the heterogeneous Ag–RBC precursor (eq. 2) [39].

For calculation of [AgNPs] the "magic number" concept [44] is acceptable. From the point of view of this concept, 3,6 nm silver nanoparticle represents by itself the cluster, which possess 6 fully filled shells and consist of 923 (*Number of atoms* = $(10N^3 + 15N^2 + 11N+3)/3$, N is the number of shells) silver atoms. That is why the number of AgNPs in solution (or concentration [AgNPs] in term mol/L) can be expressed as $[Ag^+]_0/1000$. Then,

$$n = \frac{[B]_0}{[AgNPs]}, \qquad (2)$$

and calculated value of *n* is close to 150 meaning that polymeric RBC ball is saturated by 150 silver ions.

Obtained AgNPs solution was tested as antimicrobial agent against phytopathogenic bacteria *Agrobacterium tumefaciens* which is the causal agent of crown gall disease (the formation of tumours) in over 140 species of flowering plants (Table). That is why the development of ecological and safe drugs for the plant protection is an actual task.

Antimicrobial activity of biosurfactants and their compositions with AgNPs against *Agrobacterium tumefaciens.*

	RBC		AgNPs*		AgNPs (RBC)	
	MIC	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*
Concentration, μg/mL	10	20	—	1	0,135	0,27

* *MBC* for AgNPs was taken from [45].

It was found that both MIC and MBC of obtained silver sol are in 8 time lower than the ones for pure RBC and minimum bactericidal concentration of AgNPs is close to $2.5\cdot10^{-6}$ mol/L (or 0.27 μg/mL) in terms of Ag. Such low MBC (four times lower than it was observed for commercially produced AgNPs [45]) can be explained by high affinity of RBC to the cell membrane which facilitates the interaction of silver with the micro-organisms.

4. Conclusions

Solution of silver nanoparticles stabilized by microbial origin rhamnolipid bio-complex has been obtained using "green synthesis" technique. Using TEM it was found that obtained solution containing mainly small AgNPs consisted from ca. 1000 Ag atoms. Ag–RBC composition has been tested as antimicrobial preparation and it was observed good efficiency against the test phytopathogenic bacteria *Agrobacterium tumefaciens*. So, synthesized composition can be considered as promising drug for the protection of plants.

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СИНТЕЗ НАНОЧАСТИНОК СРІБЛА З ВИКОРИСТАННЯМ РАМНОЛІПІДНОГО БІОКОМПЛЕКСУ МІКРОБНОГО ПОХОДЖЕННЯ

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Наночастинки срібла, стабілізовані біосурфактантом мікробного походження, отримано відновленням нітрату срібла в присутності рамноліпідного біокомплексу, який синтезований культурою *Pseudomonas* sp. PS–17. Кінетику формування наночастинок срібла досліджено з використанням спектроскопії в УФ/видимому діапазоні. З’ясовано, що процес відбувається без індукційного періоду, що свідчить про гетерогенний характер зародження нової фази. Висловлено припущення, що формування гетерогенностей у реакційному середовищі відбувається завдяки взаємодії іонів срібла з карбонільними групами рамноліпідного біокомплексу, що спричиняє зменшення його розчинності у воді. На основі експериментальних даних розраховано значення константи швидкості росту наночастинок та ефективної концентрації зародків. З використанням трансмісійної електронної мікроскопії визначено середній діаметр отриманих наночастинок, який становить 3,6±2,0 нм. На основі порівняння результатів трансмісійної електронної мікроскопії та кінетичних даних розраховано кількість іонів срібла в зародку. Досліджено антимікробну активність отриманого розчину наночастинок срібла та визначено його високу ефективність проти грам-негативних облігатно-аеробних паличкоподібних ґрунтових бактерій *Agrobacterium tumefaciens*, які є відомим патогеном понад 140 видів рослин і викликають хворобу корончастих галлів. Отже, синтезовану композицію можна розглядати як перспективний препарат для захисту рослин.

Ключові слова: наночастинки срібла, біосурфактанти, кінетика, трансмісійна електронна мікроскопія, антимікробна активність.

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