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QUANTITATIVE ASSESSMENT OF FUNGICIDAL AND BACTERICIDAL ACTIVITY OF *NANOSTRUCTURAL SILVER PARTICLES*

Ключевые слова: нанотехнология, фунгициды, биоциды, полистирол, наноструктурные частицы серебра.

В работе показано, что введение наночастиц (НЧ) серебра в многотоннажные полимеры : полистирол (ПС) и сополимер стирола с акрилонитрилом (САН) придает им фунгицидные свойства. Проведено количественное описание процесса роста микроскопических грибов и бактерий в присутствии разных концентраций НЧ серебра с целью дальнейшего прогноза. Получено, что добавка наночастиц серебра существенно подавляет рост и на начальной и на стационарной стадиях роста микроскопических грибов Aspergillus niger и Penicillum chrysogenum. Ингибирование роста бактерий проявляется в увеличении периода индукции (лаг-фазы).

Keywords: nanotechnology; biotechnology; fungicides; biocides; silver nanoparticles.

It is shown that injection of silver nanoparticles (NPs) into large-tonnage polymers, such as polystyrene (PS) and styrene copolymer with acrylonitrile (SAN), imparts fungicide properties to them. For the purpose of further forecasting, the process of microscopic fungi and bacteria growth in the presence of different silver NP concentrations has been described quantitatively. It is shown that addition of silver NPs significantly suppresses growth of Aspergillus niger and Penicillium chrysogenum microscopic fungi both at the initial, and stationary stages of their growth. Inhibition of bacterial growth manifests itself in increased induction period (the lag-phase).

Introduction

It is common knowledge that silver ions are highly toxic for both microorganisms and bacteria, for instance, *E. coli* [1, 2]. It has been shown [3] that silver NPs inhibit microbial growth, suppressing it by the free-radical mechanism, that gives an opportunity to use them in medicine and antimicrobial control systems. Meckling et al. [4] have shown that silver NP compounds with hyper-branched amphiphilic macromolecules are highly efficient antimicrobial agents, too.

Silver NPs (1-2 nm) hybrids with modified highly branched amphiphilic polyethylene imines effectively adhere to polar surfaces imparting them the antimicrobial property. It is well known that colloidal silver manifests an antimicrobial property, but its particles adheres poorly to the surface. The authors have synthesized hybrids of silver particles and modified highly branched amphiphilic polyethylene imines that gives an antimicrobial coating. Obtaining of silver NPs and the effect of medium components on their formation in a composite solvent of methyl cellosolve - butyl acetate - toluene and in solution of methyl metacrylate copolymer with metacrylic acid has been considered in [5]. Using the atomic-force microscopy method, it is shown that the particles sized within 50...500 nm are formed in the silver trifluoroacetate - composite solution system. The authors have shown that butyl acetate and toluene additions increase stability of silver ion-methyl cellosolve complexes. Copolymer molecules in the solution prevent enlargement of silver NPs thus decelerating their precipitation [5]. Alongside with that, despite numerous studies performed in this field, the mechanism of silver NPs action is not unanimous. The most researchers associate microorganism growth suppression with free radicals formation on the silver NP surface which attack the microorganism cell membrane and destroy it completely [4]. In the opinion of another group of authors, the inhibiting action mechanism of nano-sized silver particles may conclude in the electrostatic effect of attraction between negatively charged cell membrane of the microorganism and positively charged silver NP [6 - 8]. On the contrary, Sondi and Salopek [8] point out that antimicrobial silver NP activity for gram-negative bacteria depends on their concentration and is associated with formation of "plagues" on the bacterial cell membrane that change permeability of the cell membrane and cause the cell death due to accelerated effluence of lipopolysaccharide and protein molecules from the membrane [9, 10]. Antimicrobial activity of silver NPs is also associated [3, 11] with the impact of radicals on cellular membranes shown by the ESR method. Electron microscopic analysis has clearly shown that silver NPs accumulate in the membrane, because some of them successfully permeate into the cell.

Bleeding of intercellular substances and coagulation of nano-sized particles on the bacterial surface can be seen on a gating microscope. Works by Klabunde [12] have demonstrated that NPs of active metal oxides show high antibacterial activity and thus it is of interest to study the use of other inorganic NPs as antibacterial materials. Little is known about the biocide effect of precious metal particles. The mechanism of silver ions inhibitor effect is partly known. It is suggested that DNA loses its reproductive ability and cellular proteins become inactive, when treated by silver ions [13]. It is also shown that silver ion bonds with functional groups of proteins cause degradation of proteins [14]. The features of surfactant catalytic action in hydrocarbons and lipids oxidation have been considered [15, 16]. It is shown that hydroperoxides, the primary amphiphilic products of oxidation of lipids, form mixed micelles with surfactants, in which rapid decay of peroxides happen, other polar components (metal-containing compounds, inhibitors, etc.) are accumulated that significantly affects oxidation rate and mechanism. Comparison of surfactant action of different origin have shown that cationic surfactants speed up hydroperoxide decay with free radical formation [18], i.e. are catalysts

of hydroperoxide radical decay. Anionic and nonionic surfactants have no such influence. Hydroperoxides decay into radicals provides degenerated branching of chains and general autocatalytic development of the oxidation process. The mechanism of cationic surfactants catalytic effect on oxidation processes involves acceleration of degenerated branching of chains in the course of hydroperoxides decay. Generated peroxy radicals pass to the volume and may initiate chain oxidation. Hydroperoxides are also formed in living organisms during biochemical processes. In the precence of cationic surfactants, catalytic degradation of lipoperoxide cellular membranes into radicals and further radical reactions with polyene compounds, lipids, proteins and other components in the cell, which cause their irreversible degradation, represent the possible mechanism of bactericide action of cationic surfactants.

It has been shown [19] that silver is sorbed well by a broad range of microorganisms: algae, fungi and bacteria. However, the most works on silver interaction with cells are devoted to its action in the ionic form [20]. Of interest are works on biological action of silver NPs on yeast cells. The interaction between ions and stable nanosized silver clusters synthesized in inverted micelles by the radiation-chemical method have been studied in a wide range of concentrations on Candida utilis and Saccharomyces Cerevisiae yeast cells in aqueous and aqueous-organic solutions [19]. It has been found that the biocide effect of Ag clusters exceeds the effect of silver ions. It is shown that Ag ions have no effect on yeast cell growth, whereas NPs suppress fermentation. It is also shown [21] that the concentration-dependent toxic effect of ions in relation to bacteria and yeasts is associated with the binding of Ag ions to proteins and lipids of cellular membranes and subsequent change of the transmembrane potential up to membrane breakdown of the cell death. The mechanism of silver NP effect of living cells remains unclear. The inhibiting effect of silver NPs on development of a number of microorganisms is shown. Silver NP organization, both outside the cell and in the preplasmatic space (in bacteria) or on the cell wall surface (in yeasts), is another result of silver ions interaction with microorganisms (at silver ions concentration above $45x10^{-6}$ M). Concurrently with investigations of the mechanism of silver or other metals NP formation, the branch of nanoscience and nanotechnology that uses achievements of physics, chemistry, engineering and technics at the nanoscale levels is ever expanding. In the recent decade, the number of researches in the field of nanotechnologies and nanocomposites (polymer - nanoparticles composites) grows exponentially, and special attention is paid to the "structure-properties" interconnection and its application. Degradation and service life of polymers, as well as the NP role in biodegradation of polymers are considered in the presence of NPs (nanocomponents) under various environmental conditions [23, 24].

For research and applied works, there are three main directions: biodegradable polymers based on polyesters of hydroxy carboxylic acids, composite materials based on natural polymers, modification of existing industrial polymers and attribution of novel properties to them. Large-tonnage polymers: PE, PP, PVC and PS without modifications and articles from them can be stored for decades. Sometimes articles require antibacterial properties, but after the end of service life they have to be disposed of. Wide application of biodegradable polymers is embarrassed because of their high cost as compared with traditional polymers.

When obtaining new materials, it is often desirable, if not particularly enhancement, but at least preservation of their physicochemical and bacterial properties that may be reached by using a nano-additive to the polymer at the processing. The goal of this work was to inject silver NPs into large-tonnage polymers, polystyrene (PS) and styrene copolymer with acrylonitrile (SAN), and to determine antimicrobial and fungicide properties of polymers comprising silver NPs, and quantitatively specify microbiological overgrowth of the polymers for forecasting purposes.

Production of new composite materials provokes continued scientific interest in various types of their degradation and strength. As we have shown in our studies of biostability of polymers and metals [25 - 27], materials are biologically damaged at their contact with the living organisms that causes changes in their performance. In general, the following processes proceed during biodamaging: adsorption of microorganisms on the material surface; growth of microorganisms; material degradation as a result of either specific action (living organisms consume a polymeric material as a nutrition) or the action of metabolic products. Growth and development of microscopic fungi and bacteria on solid surfaces is commonly evaluated by the six-grade scale using the GOST approved methods or by colony diameter increase for a definite kind or set of microscopic fungi. This is because of experimental difficulties in detection on the biomass amounting several micrograms per cm^2 at the initial growth stages [28]. To protect materials against biofouling low-molecular chemical substances, the so-called biocides, are used. The list of substances possessing biocide properties expands continuously. At present, methods of evaluation of fungicide activity of chemical substances based on measurements of fungus colony growth rate on agarized media in the presence of these substances [28] are widely applied. These methods are semiquantitative and subjective and does not allow determination of the influence of biocides on various stages of microorganisms development. Since biofouling of materials develops with time, kinetic methods of investigation are the maximum extent suited to evaluation of biocide efficiency [29, 30]. The goal of this work is in quantitative assessment of growth of GOST approved species of microscopic fungi and bacteria in the presence of multifunctional modifying additive (MMA) of "Akvivon-TM", TU 2499-024-87552538-12 with various silver NP concentrations and evaluation of its influence on physicochemical properties of polystyrene and styrene copolymer with acrylonitrile, as well as on the possibility of adhesion and growth of bacteria and fungi on PS and SAN surfaces.

Experimental

Materials and Methods

Fungicide properties of polymeric samples with

silver NPs applied on polymeric granules from 2% aqueous colloid solution of silver NPs in organic dispersion of «AKVIVON» brand, TU 2499-022-87552538-10, or without them were studied on polymeric disks from polystyrene and styrene copolymer with acrylonitrile. The disks were press moulded at 160° C and then cooled down to 60° C during 40-60 minutes. The samples are 100 μm thick and 50 mm in diameter. Temperature characteristics of the samples, glass transition temperature (T_g), in particular, were determined on DSC Q100 calorimeter by company TA Instruments. Table 1 shows results of direct and repeated melting.

Table 1 - Glass transition temperatures for direct and repeated melting for polystyrene, styrene copolymer with acrylonitrile in the presence and in the absence of silver nanoparticles

Sample name	T_g, °C	T_g, °C, Repeated melting
Styrene copolymer with acrylonitrile, control	110.4	110.6
Styrene copolymer with acrylonitrile with silver NPs, version 1	111.0	110.7
Polystyrene, control	97.9	97.4
Polystyrene with silver NPs, version 2	111.0	110.7

The presence of silver complexes in polymers had no effect on IR-Fourier spectra of all samples, i.e. on temperature and spectral characteristics of samples.

According to GOST 9.049-91, purified polymeric samples were placed into Petri dishes on a solid nutrient medium (the Chapek-Dox medium with agar), contaminated by a suspension of fungi spores in the Chapek-Dox medium and exposed to conditions optimal for development of fungi, and then fungicide properties was evaluated by intensity of fungus growth on the samples and the nutrient medium.

Test conditions: duration 14 days; constant temperature of +29±2°C; relative air humidity over 90%.

Mold fungi species: *Aspergillus niger van Tieghem, Aspergillus terreus Thom, Aspergillus oryzae (Ahlburg), Penicillium funiculosum Thom, Penicillium chrysogenum Thom, Penicillium cyclopium Westling, Paecilomuces varioti Bainier, Chaetomium globosum Kunze, Trichoderma viride Pers. Ex Fr.*

Indices of fungicide properties are growth intensity of mold fungi in samples in points by the six-grade scale, GOST 9.048-89 [31]; the presence of the inhibitor zone (the growth absence zone) in the nutrient medium around the sample. According to GOST 9.049-91 [32], a strong fungicide effect is characterized by the absence fungi growth on the sample (point 0). The absence of growth on the sample and the presence of the inhibitor zone on the nutrient medium around the sample means a strong manifestation of the fungicide effect of silver NPs as a result of diffusion into the nutrient medium. Growth of fungi on the sample that corresponds to point 1, indicates low fungal resistance of the material, whereas points 2...5 growth indicates the absence of the fungicide effect.

Experimental results

The results of the investigation indicate that PS and SAN samples with silver NPs manifest high fungicide properties. At the same time, control samples have no fungicide properties [32].

Electron microscopic analysis of polymer samples after rinsing microbial colonies from their surfaces indicates that adhesion is irreversible, in the presence of silver NPs numerous fragments of microorganisms being observed.

Testing for bactericide properties of silver NPs

For the test organisms, the following microscopic fungi and bacteria were used: *Bacillus mycoides, Micrococcus flavus; E. coli; Aspergillus niger; Penicillium chrysogenum.*

For the biocide, the growth inhibitor of cultures, the modifier MMA "Akvivon-TM", TU 2499-024-87552538-12 was used.

"Akvivon-TM" modifier in concentrations 0.5, 1.0, and 2.0% was injected in agar located in Petri dishes, 20 ml in each. After solidification of agar, holes of 7 mm in diameter were made in it by a drill, where agar block with pure culture were placed.

Pure cultures of microorganisms were cultivated on a solid agar medium MPA, Saburo (day old cultures of bacteria and three day old cultures of fungi were used).

The test was repeated three times. Every day during 6 days, diameter of the microorganisms growth zone was measured. For the control, growth of cultures on the agar medium without "Akvivon-TM" modifier was taken. S-shaped kinetic curves of bacteria and microscopic fungi growth are shown in Figs. 1 - 5.

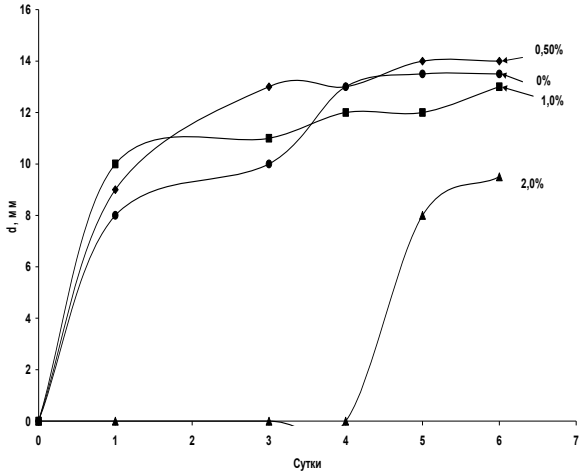

Fig. 1 - *E.coli* growth in the agarized medium containing different concentrations of "Akvivon-TM" MMA

Previously, using a logistic function [33], the method of kinetic curve analysis for microbial culture growth concluded in determination of the growth rate constant K_c in the presence of various concentrations of biocides [34] and its application [35, 36] has been discussed. These constants are virtually independent of the biocide concentration and are calculated from

culture growth rates in the presence of different additive concentrations and in the control (without additive). These rates describe the initial and stationary stages of microbial growth which allow determination of the activity sequence of additive effect on bacteria and fungi growth.

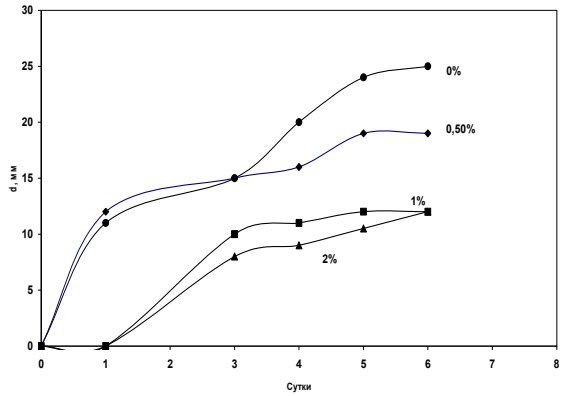

Fig. 2 - *Bacillus mycoides* growth in the agarized medium containing different concentrations of "Akvivon-TM" MMA

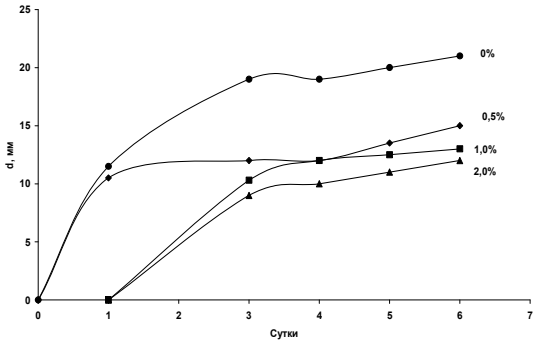

Fig. 3 - *Micrococcus flavus* growth in the agarized medium containing different concentrations of "Akvivon-TM" MMA

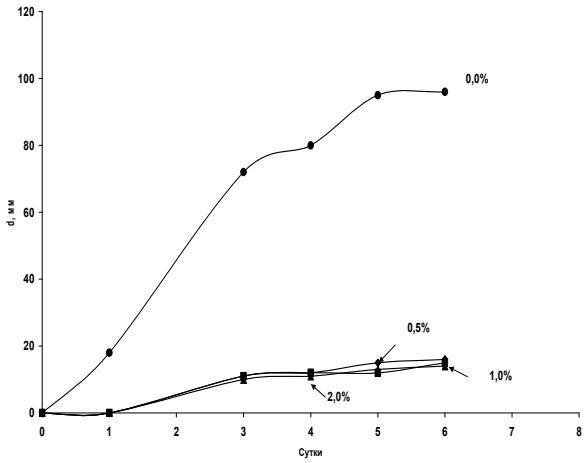

Fig. 4 - *Aspergillus niger* growth in the agarized medium containing different concentrations of "Akvivon-TM" MMA

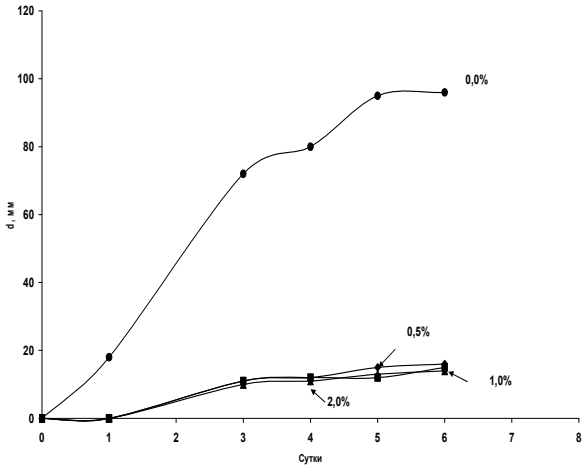

Fig. 5 - *Penicillum chrysogenum* growth in the agarized medium containing different concentrations of "Akvivon-TM" MMA

It is known that the most enzymatic reactions and the effective rate constant of a fungus colony growth in the presence of biocides is described by the following formula:

$$b_i = b_o \bullet K_c / (K_c + C), \quad (1)$$

where b_i is the effective rate constant of a fungus colony growth in the presence of the biocide; b_o is the effective rate constant of a fungus colony growth in the absence of the biocide; C is the biocide concentration; K_c is a constant quantitatively equal to the biocide concentration, at which $b_i = b_o/2$ and can be used for assessment of biocide activity.

The lower K_c values are, the stronger the biocide effect is. As Fig. 6 shows, addition of silver NPs significantly depresses growth of *Aspergillus niger* and *Penicillium chrysogenum* both at the initial and at stationary stages.

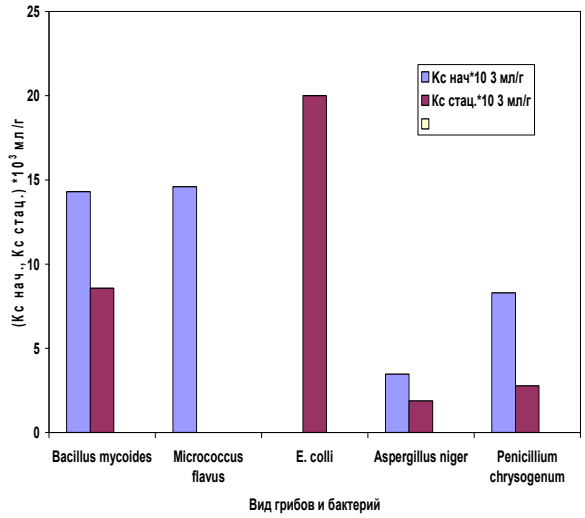

Fig. 6 - Histogram of constants for initial and stationary growth of microscopic fungi and bacteria in the presence of silver NPs

Inhibition of bacterial growth manifests itself in increased induction period (the lag-phase) (Table 2).

Table 2 - Induction periods (days) for microscopic fungi and bacteria growth in the presence of various silver NP concentrations

Test organism	Silver NP concentration			
	0%	0.5%	1%	2%
Bacillus mycoides	0	0	1	1
Micrococcus Flavus	0	0	1	1
E. colli	0	0	0	4
Aspergillus niger	0	1	1	1
Penicillium chrysogenum	0	1	1	1

Addition of silver NPs significantly increases the lag-phase. In the case *E.coli* growth (Fig. 1), the lag-phase duration reaches 4 days at the maximum concentration of 2 % silver NPs (as bacteria growth inhibitor) in agar, whereas for two bacterial cultures, *Bacillus mycoides* (Fig. 2) and *Micrococcus flavus* (Fig. 3), the threshold concentration of the inhibitor is 1.0%. For microscopic fungi *Aspergillus niger* (Fig. 4) and *Penicillum chrysogenum* (Fig. 5), different situation is observed, when growth depression is initiated at 0.5 % of silver NPs, and within the first day, equilibrium growth pattern is reached. First and foremost, these results indicate different mechanisms of inhibitor effect on microorganisms. Fig. 6 shows a histogram of constants fot initial and stationary growth of microscopic fungi and bacteria.

As follows from the results obtained, silver NPs depress the growth of microscopic fungi abruptly. In this case, the ultimate value of fungus biomass decreases by 2.5 - 5 times (Figs. 2, 3)and not concentration dependence of its increase is observed, whereas for agar, bacterial growth is gradually decelerated with addition of small silver NP concentrations. In the case of *E. coli* growth on agar, the inhibiting effect of silver NPs is only observed in the presence of 2% solution of "Akvivon-TM" MMA.

Apparently, the destructive effect of silver NPs has two mechanisms: electrochemical mechanism associated with accumulation of charges on the cellular membrane surface and free-radical mechanism, when free radicals are released. As is known, cation-containing surfactants intensify the decay of hydroperoxides with free radical formation. Hydroperoxides are the primary products of oxidation of many organic substances by molecular oxygen and are formed spontaneously in materials and products. They are also formed in biochemical processes in living organisms. The cooperative action of cationic surfactants and metal compounds (homogeneous catalysts of hydrocarbon oxidation) is synergistic. Radicals produced by peroxide decay diffuse into cellular membranes and destroy it. If the growth of microscopic fungi is decelerated, the free-radical degradation mechanism is predominant, because no concentration dependence is observed, whereas in case of bacteria the electrochemical mechanism is predominant, and the concentration dependence of bacterial growth depression is observed.

Conclusion

Experimental results are present, showing designing and creation of new generation of modifiers with introduced silver NPs to be applied in advanced technologies, various compositions and materials to adhere them new biological, physicochemcial and technical properties. Possible mechanism of silver NP impact on microscopic fungi and bacteria is discussed.

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