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Biocidal Action of Copolymers Based on Aliphatic Diamines and Guanidine Hydrochloride

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ABSTRACT: Copolymers of polyguanidines with various lengths of hydrocarbon radicals were obtained by polycondensation. The biocidal action of water-soluble, guanidine-containing copolymers on the pathogenic microorganisms *Escherichia coli* and *Bacillus cereus* by the method of agar diffusion was evaluated. The results indicate that the ratio of hydrophobic methylene groups to ionogen guanidine groups influenced the copolymer biocidal action. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40319.

KEYWORDS: biocide action; polyguanidine; copolymer; hydrophilic-hydrophobic balance; hydrogel

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INTRODUCTION

Recently, there have been wide applications of cationic biocide polymers[1–3] based on polyguanidines,[4] especially, poly(hexamethylene guanidine hydrochloride) (PHMG) derivatives.[5] Polar guanidine groups, which have their own inherent physiological activity, impart these polymers with high bactericidal activity.[6] Because of this polymeric nature, the bactericidal activity of PHMG salts is more effective than that of chlorhexidine[7] and other preparations of these genus. At the same time, PHMG salts have less toxicity,[8] a low corrosive activity, and a long storage duration without a loss of bactericidal properties and can make a film that protects the surface from microorganism attack over a long period of time after treatment.[9] Annually, tens of bactericidal pharmaceuticals are phased out because of the adaptation of microorganisms to unfavorable factors, including the pharmacological intervention of antibacterial agents. Because of this connection, there always exists the necessity to improve the properties of biocidal preparations.[10,11] The most topical lines of investigation in the creation of a new antibacterial agent are not only increasing their bactericidal activity but also negotiating the microorganism's drug resistance and increasing the bactericidal effect duration after the surface has been treated by the polymer. In addition to the decrease in toxic action, allergenic action and environmental safety are necessary. The structure of polyguanidines[12] opens a variety of abilities to regulate its biocide activity and toxic action by means of the chemical modification of macromolecules.[13,14] By means of the modification of the length and configuration of hydrocarbon radicals and by the creation of copolymers, it is possible to control the hydrophilic–hydrophobic balance of macromolecules. It gives one a chance to enlarge the quantity of biocide polyguanidines and to improve their antibacterial action.

EXPERIMENTAL

Materials

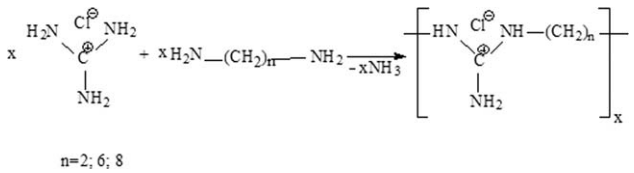
The chemicals guanidine hydrochloride (GndCl; Acros Organics, 98%) and 1,8-diaminooktane (OMDA; Acros Organics, 98%) were used as received. Hexamethylenediamine and ethylenediamine were purified by distillation at 200 and 117°C, respectively.

Synthesis of the Polymers

An amount of 5 g of GndCl and a certain proportion of 1,6-hexamethylenediamine (HMDA), OMDA, or 1,2-ethylenediamine (EDA) were put into a polycondensation test tube. Then, the mixture was heated to 165°C. The reaction was carried out in a thermostatic oil bath under atmospheric pressure for 9 h.[15,16]

Characterization

Polyguanidine copolymers are cationic polyelectrolytes that can split into polycations and low-molecular anions; therefore, in aqueous solutions, the polyelectrolyte effect is observed.[12] The intrinsic viscosities of copolymer samples were studied with a capillary hanging level Ubbelohde viscometer. An amount of 0.25 g of each sample was dissolved in 0.3*N* NaCl solutions. Then, the solution was put into a viscometer flask. The thermostat temperature was stabilized at 25°C for at least 20 min before each measurement. All of the measurements were made at least three times. The intrinsic viscosities of the polymers



Scheme 1. Schematic of reaction between hexamethylenediamine and guanidine hydrochloride for water soluble samples of polymers obtained.

were determined by extrapolation of the reduced viscosity to infinite dilution. In the presence of a hydrogel, the reaction product was washed by distilled water to purge away the soluble part of polymer.

The synthesized water-soluble copolymers were investigated by IR spectroscopy with the help of Varian Excalibur 3100 FTIR spectrometer with Pike MIRacle attenuated total reflectance. The comparison of spectra was made by a Bio-Rad Laboratories IR Search Master 6.5 program.

Gel Permeation Chromatography Analysis

Gel permeation chromatography analysis was realized by an Agilent 1200 liquid chromatograph with refractometric and spectrophotometric detectors and a thermostatically controlled column. The analysis conditions included a 7.5 × 300 mm^2 steel column filled by 8 μm PL Aquagel-OH MIXED. The eluent was a 0.06*M* $LiNO_3$ aqueous solution, the temperature was 25°C, and the eluent rate of consumption was 1.0 mL/min. The wavelength of the spectrophotometric detector was 254 nm, and the sample volume was 20 mkL. Chain-length distribution was determined by a calibration diagram plotting in a mass range from 106 to 58,400. Calibration was carried out by poly(ethylene oxide) standard sample, which came with an Agilent PN:5064–8252 gel permeation chromatography system for water-soluble polymer calibration.

Agar Ditch Diffusion Method

Agarized nutrient medium specific for the growth and development of microorganisms under consideration were poured into Petri dishes. Previously, *Bacillus cereus* or *Escherichia coli* was sowed into Petri dishes by the method of a solid bacterial lawn. The depth layer in the Petri dish was 4.0 mm. For inoculums preparation, the 18–20 h agarinic culture of the investigated microorganisms was used. Then, ditches were made 8.0 ± 0,1 mm in diameter. Then, 0.1 mL of an aqueous solution of the polymer (concentration = 10 g/L) under consideration was placed into the ditch. The incubation of Petri dishes was carried out at 37°C in a thermostat for 24 h. Biocidal action was determined by the measurement of zones of growth inhibition of test organisms around ditches.[17]

RESULTS AND DISCUSSION

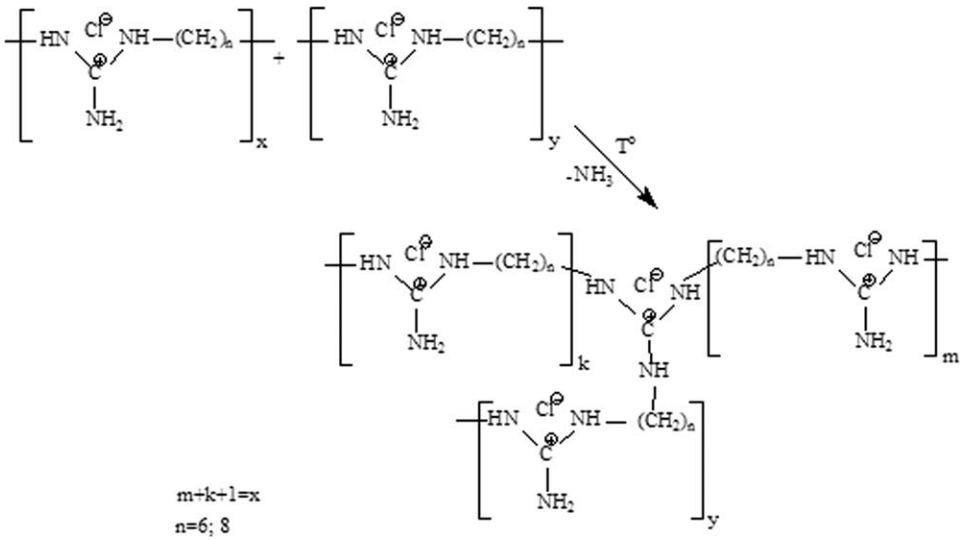
Copolymers with EDA, HMDA, and 1,8-octamethylenediamine (OMDA) as bifunctional agents and GndCl as a trifunctional agent were synthesized. Water-soluble samples of polymers were obtained according to Scheme 1.[12]

The crosslinked polymer was synthesized by the interaction of diamine excess over the third free amino group according to Scheme 2.

Such interactions are possible as the result of the attack of the diamine amino group on the positive charge of the guanidine carbon atom. Copolymer formation arises from a transamination reaction and is realized as a nucleophilic substitution mechanism.[12,18]

The chemical composition and ratio of initial monomers for the synthesized copolymers and hydrogels as well as their intrinsic viscosities, molecular weights, and polydispersity indices of water-soluble polymer samples under synthesis conditions are shown in Table I.

A gradual decrease in the EDA molar ratio in the reaction mixture led to an increase in the intrinsic viscosity for sample groups 1–3 and 12 and 4–6 and 12. The increasing OMDA content in copolymer samples 4–9 were also accompanied by an increase in the intrinsic viscosity values. Samples 9 and 11, which had a high content of OMDA, lost their water solubility



Scheme 2. Schematic of reaction between hexamethylenediamine and guanidine hydrochloride for cross-linked polymer obtained.

Table I. Chemical Composition, Ratio of the Initial Monomers, and Molecular Weight Characteristics of the Copolymers Synthesized at 165°C for 5 h

	Monomers molar ratio						
Number	EDA	HMDA	OMDA	GndCl	$[\eta]$ (dL/g)	M_w (Da)	M_w/M_n
1	0.75	0.25	—	1.00	0.014	1030	1.16
2	0.50	0.50	—	1.00	0.020	1150	1.22
3	0.25	0.75	—	1.00	0.027	1300	1.25
4	0.75	—	0.25	1.00	0.017	1070	1.12
5	0.50	—	0.50	1.00	0.025	1250	1.17
6	0.25	—	0.75	1.00	0.028	1340	1.19
7	—	0.75	0.25	1.00	0.032	1400	1.23
8	—	0.50	0.50	1.00	0.037	1510	1.34
9[a]	—	0.25	0.75	1.00	—	—	
10	—	1.00	—	1.00	0.021	1160	1.26
11[a]	—	—	1.00	1.00	—	—	
12	1.00	—	—	1.00	0.012	1010	1.14
13[b]	—	1.30	—	1.00	—	—	
14[b]	—	—	1.30	1.00	—	—	

$[\eta]$, intrinsic viscosity; M_w, weight-average molecular weight; M_n, number-average molecular weight.
[a] Insoluble in water.
[b] Hydrogel.

as a result of the rise in the hydrophobicity. A change in the intrinsic viscosities took place because of the changes in the macromolecule size and its conformational mobility; this greatly depended on the length of methylene fragments in diamine. Therefore, the differences in the intrinsic viscosities of the synthesized copolymer samples were not in correlation with the degree of polycondensation. The molecular weight and polydispersity index of the polymers (Table I) were received by the gel permeation chromatography method. Notwithstanding the fact that the gel permeation chromatography method cannot determine the molecular weight with high accuracy, this method is convenient for the comparison of the molecular weight of polymers that are similar in structure. The findings were in accordance with the intrinsic viscosities of the samples under consideration and represent negligible differences in molecular weights (± 25%).

In view of their low intrinsic viscosities, these produced copolymers represented an oligomer reaction product. Nevertheless, in the scientific literature, polyguanidines are generally referred to as polymers.

FTIR Spectral Analysis of the Copolymer Formation

The copolymer spectra had similar combinations of pass bands and PHMG. Figure 1 shows the IR spectrum of PHMG. Two bands at 3270 and 3160 cm^{-1} were related to the valence oscillations of amine groups. The bands character did not relate it to the guanidine group or to the diamine group as a result of their mutual overlap. The bands at 2930 and 2855 cm^{-1} were assigned to the valence asymmetric and symmetric oscillations of CH_2 groups, respectively. The characteristic bands of the deformation oscillations of the CH_2 groups were observed at 1470 cm^{-1}. The band at 1630 cm^{-1} was indicated as a characteristic peak of guanidine salts.[19]

As shown in Figure 2, with increasing copolymer CH_2 group content, the intensity of the valence oscillations increased.

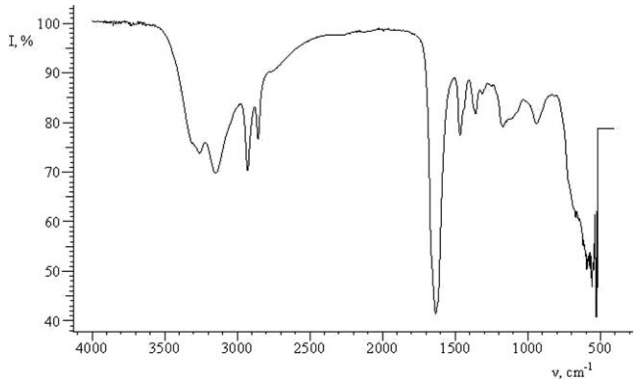


Figure 1. IR-spectrum of PGMG sample 10.

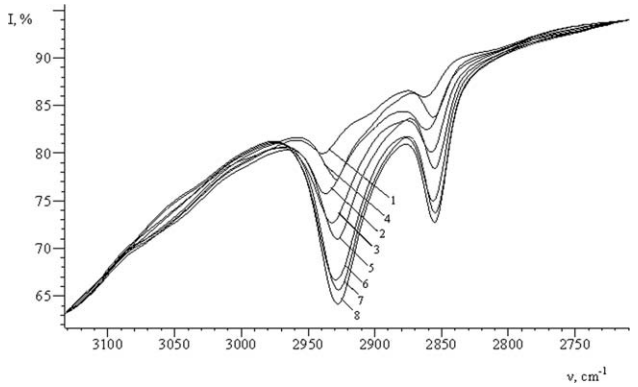


Figure 2. Copolymer CH_2 group content versus the intensity of the valence oscillations.

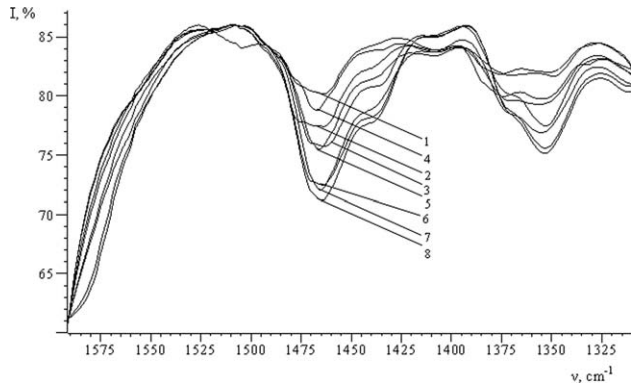


Figure 3. Deformation oscillations of the CH_2 groups.

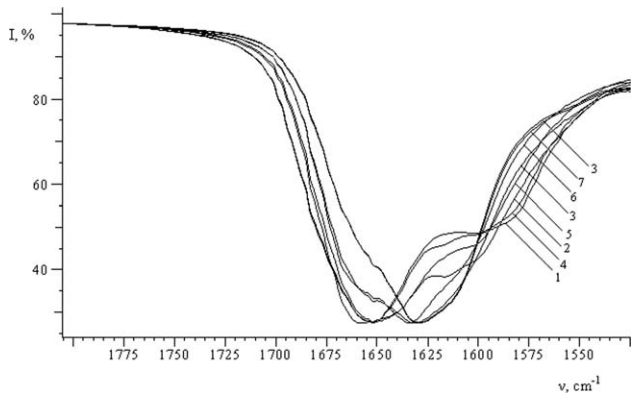


Figure 4. Region of copolymers methylene groups valence asymmetric and symmetric oscillations.

We observed that the valence asymmetric and symmetric oscillations of the CH_2 groups (for samples 1, 2, and 3) shifted to a lower wave number and appeared at 2940 and 2861 cm^{-1}.

Figure 3 shows that the deformation oscillations of the CH_2 groups did not shift bands. Nevertheless, the intensities of the valence oscillations for copolymers with greater contents of EDA (samples 1 and 4) were greater than those of the other samples.

For all of the copolymers (see Figure 4) obtained with EDA as a monomer, a strong splitting of guanidine salts bands was observed at 1650–1630 cm^{-1}. At that for copolymers with great contents of EDA (samples 1, 2, 4, and 5), a more intensive peak shifted to a lower wave number region. For copolymers 3 and 6, which had 0.25 mol of EDA, the peak shifted to a greater wave-number region. The copolymers without any EDA content (samples 7 and 8) had less evidence of band splitting.

The bands shifted to a low-frequency region, and this was characteristic of copolymers with different contents of EDA and was admittedly due to the fact that the inductive effect weakened with increasing copolymer methylene chain length.

Biocidal Action of the Copolymers

To research the effects of different lengths of hydrocarbon radicals on the biocidal action, a series of copolymers was studied. The alteration of a number of diamine methylene groups led to the formation of copolymers with different hydrophobicities. That may have influenced the copolymer biocidal action in comparison with well-known PHMG.

The quantitative assessment of the biocidal action of water-soluble guanidine containing copolymers was studied by the agar diffusion method. This method was based on the diffusion ability of the material under consideration to penetrate into the agar nutrient medium where the bacterial inoculation of the medium was carried out and to depress microorganism growth.[20]

PHMG had a widespread biocidal effect. It is the major constituent of many antibacterial agents based on polyguanidines[21] and haa a high bactericidal activity toward a number of well-known microorganisms. So to carry out research into the new copolymers' biocidal action, the pathogenic microorganisms *E. coli* and *B. cereus* were chosen as the test cultures, which were the model objects used for microbiology and biotechnology study. The content of the copolymers in the samples under consideration was 1 mg; this is the usual amount for testing guanidine-containing bactericidal preparations.[5,22] The biocidal action of the copolymers to bacterial cultures of *E. coli* and *B. cereus* is shown in Table II.

Table II. Chemical Composition and Biocidal Action of the Copolymers in the *E. coli* and *B. cereus* Bacterial Cultures

Sample[a]	Diamine molar ratio in the copolymer samples		Zone of retardation for *E. coli* (mm)[b]	Zone of retardation for *B. cereus* (mm)[b]
1	HMDA 0.25	EDA 0.75	22.0	14.0
2	HMDA 0.50	EDA 0.50	28.0	16.0
3	HMDA 0.75	EDA 0.25	32.0	23.0
4	OMDA 0.25	EDA 0.75	26.0	15.0
5	OMDA 0.50	EDA 0.50	32.0	22.0
6	OMDA 0.75	EDA 0.25	34.0	28.0
7	OMDA 0.25	HMDA 0.75	33.0	29.0
8	OMDA 0.50	HMDA 0.50	35.0	30.0
10	HMDA 1.00	—	32.0	25.0
12	EDA 1.00	—	Growth	Growth

[a] Number of the sample from Table I.
[b] Ditch diameter = 13 mm.

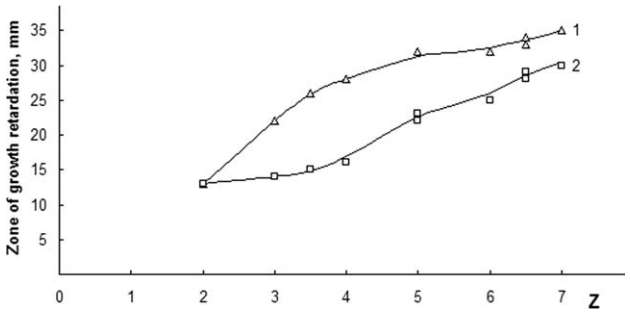


Figure 5. Zone of growth retardation depending on the ratio of hydrophobic methylene groups to ionogen guanidine groups in copolymers: 1 – bacterial culture *E. coli*, 2 – bacterial culture *B. cereus*.

Synthesized guanidine-containing copolymers 1–8 and 10 showed different bactericidal activities to the studied bacterial cultures. Biocidal activity increased for samples 1–3 and 7 with increasing HMDA content, and the content of OMDA increased for samples 4–6. The increasing EDA in the monomer content led to a decrease in the copolymer biocidal activity. Sample 12, which had only EDA in its content, did not inhibit the growth of the microorganisms.

It is known the ability of polymers to interact with the cellular membrane of bacterial cells is determined in the main by the presence of positively charged groups in macromolecules and by the presence of negative charges on the cell surfaces because of phosphate lipid groups.[6,23] When the polymer has contact with the cell at first, electrostatic interaction takes place, and this leads to the introduction of positively charged fragments of the macromolecule to the lipid membrane monolayer.[24] As the result of these actions, the membrane permeability changes, and then, the membrane integrity breaks, and this is completed by the cell destruction.[25,26]

All of the copolymers obtained contained in their composition positively charged guanidine groups. However, all of the investigated samples had different microorganism biocidal actions. To determine the copolymer biocidal action depending on the monomer composition, the value Z was introduced as the ratio of hydrophobic methylene groups to ionogen guanidine groups:

$$Z=(\upsilon_1 n_1+\upsilon_2 n_2)/\upsilon_3$$

where υ_1 and υ_2 are the molar ratios of the diamines, n_i is the number of CH_2 groups in each respective diamine, and υ_3 is the molar ratio of GndCl.

The received dependences (see Figure 5) showed the growth of the biological activity of the polymer samples with increasing macromolecule hydrophobicity. Most probably, this fact was the result of the better sorption of polymer with greater hydrophobicity to a lipid layer of cell membrane. The evident growth of both organisms' biocidal action was observed up to $Z = 5$ when hydrophobicity increased, whereas further increases in the hydrophobicity had a more significant influence on the Gram-positive bacteria *B. cereus* only. Thus, it may have interfered not only with electrostatic interaction of the polymer's positively charged groups with the negatively charged lipid phosphate groups of the cell surface but also with the sorption of macromolecular chain hydrophobic fragments to the membrane lipid layer, which determined the first stage of interaction among the cell and the bactericidal polymer.

The biocidal action of the hydrogels (samples 13 and 14, Table I) was investigated by inoculation on the medium by a deep method, that is, by the mixture of the hydrogel sample with nutrient medium.[26] Hydrogels were synthesized at a 0.3 molar excess of HMDA.[16] It was determined that the hydrogel presence in agar did not influence the growth of the test cultures; that is, the hydrogels did not have biocidal action.

CONCLUSIONS

Copolymers based on the bifunctional monomers EDA, HMDA, and 1,8-octamethylenediamine and trifunctional guanidine chlorides were synthesized. The dependence of the ratio of hydrophobic methylene groups to ionogen guanidine groups and the biocidal action of the synthesized copolymers to *E. coli* and *B. cereus* microorganisms was determined. The sorption of the hydrophobic fragments of the macromolecule chains to the lipid layer of the cell membranes made its contribution to interaction among the cell and the bactericidal polymer.

REFERENCES

1. Findlay, B.; Zhanel, G. G.; Schweizer, F. *Antimicrob. Agents Chemother.* **2010**, *54*, 4049.
2. Albert, M.; Feiertag, P.; Hayn, G.; Saf, R.; Honig, H. *Biomacromolecules* **2003**, *4*, 1811.
3. Kenawy, E. R.; Worley, S. D.; Broughton, R. *Biomacromolecules* **2007**, *8*, 1359.
4. Zhang, Y. M.; Jiang, J. M.; Chen, Y. M. *Polymer* **1999**, *40*, 6189.
5. Oulé, M. K.; Azinwi, R.; Bernier, A.-M.; Kablan, T.; Maupertuis, A.-M.; Mauler, S.; Nevry, R. K.; Dembélé, K.; Forbes, L.; Diop, L. *J. Med. Microbiol.* **2008**, *57*, 1523.
6. Bera, S.; Zhanel, G. G.; Schweizer, F. *J. Antimicrob. Chemother.* **2010**, *65*, 1224.
7. Gilbert, P.; Moore, L. E. *J. Appl. Microbiol.* **2005**, *99*, 703.
8. Wei, D.; Zhou, R.; Guan, Y.; Zheng, A.; Zhang, Y. *J. Appl. Polym. Sci.* **2013**, *127*, 666.
9. Vointseva, I. I.; Gembitskii, P. A. Polyguanidines: Disinfectant and Polyfunctioning Agents to Composite Materials; LKM: Moscow, **2009**; p 81.
10. Zaikov, G. E.; Malkanduev, Y. A.; Khashirova, S. Y.; Esmurziev, A. M.; Martynenko, A. I.; Sivova, L. I.; Sivov, N. A. *J. Appl. Polym. Sci.* **2004**, *91*, 439.
11. Gusev, M. V.; Mineeva, L. A. Microbiology, 4th ed.; Academia: Moscow, **2003**; p 463.
12. Stel'Mah, S. A.; Bazaron, L. U.; Mognonov, D. M. *Russ. J. Appl. Chem.* **2010**, *83*, 342.
13. Saczewski, F.; Balewski, L. *Expert Opin. Ther. Pat.* **2009**, *19*, 1417.
14. Berlinck, R. G. S.; Burtoloso, A. C. B.; Kossuga, M. H. *Nat. Prod. Rep.* **2008**, *25*, 919.
15. Khokhlov, A. R.; Pavlova, S.-S. A.; Timofeeva, G. I.; Stoljarova, E. V.; Bazaron, L. U. *Polymer* **1994**, *35*, 1769.

16. Grigor'eva, M. N.; Stel'Makh, S. A.; Bazaron, L. U.; Mognonov, D. M. *Russ. J. Appl. Chem.* **2011**, *84*, 724.
17. Navashin, S. M.; Fomina, I. P. Rational Antibiotic Therapy; Medicine: Moscow, **1982**; p 496.
18. Stel'makh, S. A.; Grigor'eva, M. N.; Bazaron, L. U.; Mognonov, D. M. *J. Mater. Sci. Eng.* **2012**, *10*, 421.
19. Goto, N.; Nakanishi, K.; Ohashi, M. *Bull. Chem. Soc. Jpn.* **1957**, *30*, 723.
20. Pimenova, M. N.; Grechushkina, N. N.; Netrusov, A. I.; Semenova, E. V. In A Guide to Microbiology Practical Training; Egorov, N. S., Ed.; MStU: Moscow, **1995**; p 224.
21. Berlinck, R. G. S.; Burtoloso, A. C. B.; Kossuga, M. H. *Nat. Prod. Rep.* **2008**, *25*, 919.
22. Pekar, S. S.; Khashirova, S. Y.; Sapaev, K. K.; Malkanduev, Y. A. *Plast. Masses* **2008**, *5*, 53.
23. Sivets, N.; Pankratov, O.; Mazolevskyi, D.; MKrasilnikov, A.; Ryabtseva, N. *Med. Bull.* **2009**, *12*. 898.
24. Ikeda, T. *High Perform. Biomater.* **1991**, *7*, 743.
25. Davies, A.; Bentley, M.; Field, B. S. *J. Appl. Microbiol.* **2008**, *31*, 448.
26. Trifonova, T. A.; Sahno, O. N. Microorganism's Ecology; VStU: Vladimir, **2005**; p 76.