ISSN 0027-1314, Moscow University Chemistry Bulletin, 2018, Vol. 73, No. 2, pp. 85–89. © Allerton Press, Inc., 2018.
Original Russian Text © I.I. Nikolskaya, O.V. Beznos, A.I. Eltsov, I.V. Gachok, N.B. Chesnokova, V.P. Varlamov, O.A. Kost, 2018, published in Vestnik Moskovskogo Universiteta, Seriya 2: Khimiya, 2018, No. 2, pp. 170–176.

The Inclusion of Timolol and Lisinopril in Calcium Phosphate Particles Covered by Chitosan: Application in Ophthalmology

I. I. Nikolskaya[a], O. V. Beznos[b], A. I. Eltsov[a], I. V. Gachok[a], N. B. Chesnokova[b], V. P. Varlamov[c], and O. A. Kost[a, *]

[a] *Department of Chemistry, Moscow State University, Moscow, 119991 Russia*
[b] *Helmholtz Moscow Research Institute of Eye Diseases, Ministry of Health, Moscow, 105062 Russia*
[c] *Federal Research Center "Fundamentals of Biotechnology," Russian Academy of Sciences, Moscow, 119071 Russia*
**e-mail: olga.a.kost@gmail.com*

Received November 27, 2017

Abstract—Chitosan-covered calcium phosphate nanoparticles with the mean dynamic radii of 30 to 130 nm and ζ-potential of +22 ± 4 mV containing timolol and lisinopril are prepared and characterized. These particles are formed by the amorphous phase represented by amorphous calcium phosphate $Ca_x(PO_4)_y \cdot zH_2O$ and the crystalline phase represented by hydrated calcium hydrophoshate (brushite) $CaHPO_4 \cdot 2H_2O$. The experiments in vivo demonstrated that the inclusion of timolol into calcium phosphate particles covered with chitosan substantially prolonged its effect on the intraocular pressure.

Keywords: calcium phosphate particles, phase composition, chitosan, timolol, lisinopril, intraocular pressure
DOI: 10.3103/S0027131418020116

The bioavailability of drugs to the anterior chamber of the eye administered by topical instillations is only 1–5% [1]. Therefore, increasing the penetration of ophthalmic drugs into the internal eye by enhancing their corneal permeation and sustained action is an urgent task in ophthalmology. For this purpose, drug delivery systems with cyclodextrins [2] and promoters (capric acid) [3], as well as the inclusion of drugs in mucoadhesive polymer gels [4], liposomes [5], hybrid vesicles based on surfactants, oils or lecithin [6, 7], and drug inclusion in nanoparticles [8–10], have been proposed.

Among nanoparticles the focus is on calcium phosphate particles (CaPh-particles) since they have undeniable benefits over other organic and inorganic carriers: complete biocompatibility, nontoxicity, nonimmunogenicity, and easy preparation in an aqueous medium in a neutral pH [11]. Furthermore, calcium ions and phosphate ions belong to the structural components of the human body.

At present, CaPh-particles are potentially useful in medicine for the transfection of cells [12–14], elaboration of effective vaccines [15], and inclusion of photosensitizers for photoxicity therapy against malignant cells [16]. CaPh-particles can also be used as vehicles for delivering medications. The preparation of cellobiose-coated CaPh-particles containing dopamine receptor antagonists [17], β-blocker timolol [18], an angiotensin-converting enzyme inhibitor (lisinopril) [19], and an antioxidant enzyme (superoxide dismutase, EC 1.15.1.1) [20] has been described. In in vivo tests, these drugs were more effective in decreasing intraocular pressure (IOP) and reducing inflammation of the eye than their aqueous forms. However, cellobiose-coated particles had a negative surface charge, which complicated their penetration through the negatively charged mucin layer of the tear film [21]. It is assumed that the action of drugs in these systems can be enhanced using particles with a positive ζ-potential.

A positively charged biocompatible and biodegradable biopolymer chitosan can be used as a coating agent for CaPh-particles. In addition, the body absorbs chitosan degradation products such as glucosamine or N-acetylglucosamine [22]. Chitosan has mucoadhesive properties and the ability to enhance the penetration of large molecules through the mucous surfaces [23]. Furthermore, chitosan nanoparticles can overcome physiological barriers, including the corneal barrier [24]. A chitosan coating on particles broadens the potential of using these particles in ophthalmology [25–27].

The purpose of this study is to prepare and characterize chitosan-coated CaPh-particles loaded with ophthalmic drugs that reduce IOP (the β-blocker timolol and angiotensin-converting enzyme (EC 3.4.15.1)

Abbreviations: calcium phosphate nanoparticles (CaPh-particles), intraocular pressure (IOP), tripolyphosphate (TPP).

inhibitor lisinopril), and evaluate the efficacy of timolol-loaded particles in tests in vivo.

MATERIALS AND METHODS

Dietary chitosan (OOO Bioprogress, Russia) was used. The degree of chitosan deacetylation was assessed by conductometric titration [28]. The average viscosity molecular weight was estimated by the viscosimetry technique [29]. The viscosity of chitosan solutions was measured using an Ubbelohde type viscometer with a capillary diameter of 0.5 mm at 30°C. The solvent was a solution containing acetic acid and sodium acetate at concentrations of 0.2 and 0.1 M, respectively.

CaPh-particles were prepared according to the technique [18] using equimolar amounts of Na_2HPO_4 and $CaCl_2$ (12.5 mM), at pH 6.8 and +4°C.

Timolol ((S)-1-(tert-butylamino)-3-[(4-morpholin-4-yl-1,2,5-thiadiazol-3-yl)oxy]propan-2-ol; Diafarm, Russia) and lisinopril ([N^2-[(1S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline; Sigma, United States) were incorporated during the preparation of the CaPh-particles, followed by a chitosan coating. For this, the required amount of the drug was added to the original solution of Na_2HPO_4 and sodium citrate (pH 6.8) to reach a certain final concentration (1% lisinopril or 0.5% timolol).

To coat the particles with chitosan, an aqueous chitosan solution of 1 mg/mL was added to the initial suspension containing CaPh-particles at room temperature under constant vigorous stirring at the volume ratio between the initial suspension and chitosan solution of 2 : 1. Afterwards, 1 mg/mL of an aqueous solution of sodium tripolyphosphate (TPP) manufactured by Acros (United States) was added by drops, with the chitosan-to-TPP mass ratios ranging from 1 : 1 to 1 : 0.09. The chitosan solution was preliminarily passed through a Millipore filter with a pore diameter of 0.45 μm, and a TPP solution was passed via a 0.2 μm pore filter. After the addition of TPP, the mixture was left to stir overnight at room temperature.

The phase composition of the CaPh-particles was analyzed by spectral X-ray microanalysis. For this, 20 mL of the particle suspension was rinsed several times from the excess salts by filtration on Microcon-30 kDa filters followed by dilution with 0.1 M NaCl and particle lyophilization. The specimens were placed in a vacuum chamber of the LEO Supra scanning electron microscope (Carl Zeiss, Germany) equipped with an X-Max energy dispersive X-ray spectrometer (Oxford Instruments, United Kingdom) and irradiated with a focused electron beam with an energy of at least 20 keV.

The size and surface charge (ζ-potential) of the particles were measured by a dynamic light scattering technique using the Zetasizer Nano ZS multitasking instrument (Malvern Instrument, United Kingdom) in specialized cuvettes at a cell temperature of 25°C. Before the analysis, the particle suspension was filtered through Millipore filters with a pore diameter of 0.45 μm. A nonfiltered sample was also analyzed. The results were processed using Zetasizer v. 7.03 (estimated using instrumental data and statistical analysis).

The efficacy of including lisinopril and timolol in CaPh-particles was evaluated as follows. The lisinopril and timolol-loaded particles were concentrated from a solution by filtration using a Microcon-centrifugal filter with a membrane of 30 kDa by centrifugation at 9000 *g* for 5 min. The amount of unbound drugs to the particles in solution passed through the membrane was assessed. The concentration of timolol was measured from the optical density of its solutions at $\lambda = 295$ nm. The molar absorptivity of timolol equal to $\varepsilon_{295} = 6300\ M^{-1}\ cm^{-1}$ was estimated in an independent assay. The concentration of lisinopril was assayed using the procedure [30] based on the reaction of the interaction of the free amino group of the inhibitor with *ortho*-phthalaldehyde and N-acetyl-L-cysteine with the formation of a chromophore compound with the maximum absorption at a wavelength of 340 nm. The molar absorptivity of chromophore was estimated in an independent assay and was found to be $\varepsilon_{340} = 6650\ M^{-1}\ cm^{-1}$. All measurements were performed using the Tecan Infinite M200 microplate reader (Switzerland).

The times of lisinopril and timolol desorption from the particles was estimated in vitro as follows. Each of six Microcon-30 kDa membranes were loaded with 0.5 mL of a CaPh-particle suspension in a 0.15 M NaCl solution and the particles were precipitated by centrifugation at 9000 *g* for 5 min. Afterwards, the volume was simultaneously brought to the initial volume (0.5 mL) using a 0.15 M NaCl solution; the suspensions were incubated for various time periods ranging from 5 to 60 min at room temperature, and each solution was then recentrifuged. The amount of the desorbed drug was assessed in filtrates, as indicated above.

The stability of the particles was evaluated from the lack of changes in their size and surface charge during storage in a physiological saline solution (4°C), in a freeze-dried state, and when concentrated on filters.

For the experiments in vivo, the prepared chitosan-coated timolol-loaded CaPh-particles were concentrated by 10 times using Microcon-30 kDa filter membranes. The efficacy of the physiological action of timolol in the particles was compared with the efficacy of the timolol solution in a 0.15 M NaCl solution. The values of the changes in the intraocular pressure (IOP) and duration of the drugs' action on healthy Chinchilla rabbits weighing 2–2.5 kg were estimated. The animals were divided into two groups of three rabbits (six eyes in each group). The experimental group received single instillations of 30 μL of 0.5% timolol included in the chitosan CaPh-particles in each eye; the control animals received single instillations of 30 μL of 0.5% timolol in a physiological saline solution in each eye. IOP was measured using Maklakov's stan-

dard technique (with a Maklakov tonometer) after a preliminary two-fold instillation of a 0.4% inocaine local anesthetic solution. IOP was measured in all rabbits daily for two weeks before the experiment for the animals to get used to the procedure and to measure the range of normal IOP values. The significance of the results was assessed using the Mann–Whitney U-test.

RESULTS AND DISCUSSION

CaPh-particles incorporating timolol and lisinopril but without a chitosan coating carry a negative ζ-potential of –7 mV. A particle coating with cationic chitosan is achieved by ionic gelation using oppositely charged macromolecules, for example, the TPP cross-linking anionic agent [31]. In each case, the chitosan-to-TPP mass ratio is adjusted based on the type and characteristics of chitosan, since this ratio influences both the ζ-potential and the size of the chitosan/TPP nanoparticles.

The average viscosity molecular weight (85 kDa) and the degree of deacetylation (89%) were measured for dietary chitosan. The effects of the chitosan-to-TPP mass ratio (1 : 1 to 1 : 0.09) at a fixed concentration of salts on the characteristics of the fabricated nanoparticles are presented in Table 1. It is seen that the ζ-potential values are negative when TPP is absent or low and particle aggregation occurs when TPP is in excess. Therefore, the optimal chitosan-to-TPP mass ratio was chosen for further study equal to 1 : 0.14, which facilitated the formation of particles with a positive ζ-potential of +22 mV.

The phase composition of the CaPh-particles was analyzed by spectral X-ray microanalysis. Prior to the microanalysis, scanning electron microscopy images were produced and some areas of these images were subjected to spectral analysis (Fig. 1). An analysis of the data revealed that the CaPh-particles were formed by the amorphous phase with a Ca : P ratio of approximately 1.3 ± 0.1 represented by amorphous calcium phosphate $Ca_x(PO_4)_y \cdot zH_2O$ and the crystalline phase with a Ca : P ratio of ~1 represented by hydrated calcium hydrophoshate (brushite, $CaHPO_4 \cdot 2H_2O$).

The particle size was analyzed by dynamic light scattering for the solutions of unfiltered particles and the solutions filtered through 0.45 μm filters (Fig. 2). It was found that nanoparticles (30–130 nm) are mostly formed with a chitosan coating, while particles with a larger radius of up to 230 nm were formed when the CaPh-particles were coated with cellobiose in a previous paper [19].

The β-blocker timolol (316 Da) and angiotensin-converting enzyme inhibitor lisinopril (405 Da, which also reduces IOP), which are widely used ophthalmic drugs, were incorporated in the CaPh-particles [32]. As shown, the incorporation of drugs did not change the size and very weakly changed the ζ-potential of the particles. The efficacy of including lisinopril in the CaPh-particles was 7.5 times higher than that of including timolol (Table 2), which indicates a greater affinity of lisinopril for the CaPh-particles. A higher (2-fold) efficacy of including lisinopril rather than timolol was also observed in a previous paper [19],

Table 1. Effects of chitosan-to-TPP mass ratio on characteristics of CaPh-particles

Chitosan-to-TPP ratio	*r*, nm	ζ, mV
1 : 1	>1000	–
1 : 0.25	>1000	–
1 : 0.14	**70 ± 40**	**+22 ± 4**
1 : 0.09	44 ± 15	–6 ± 4
Without TPP	40 ± 15	–6 ± 4

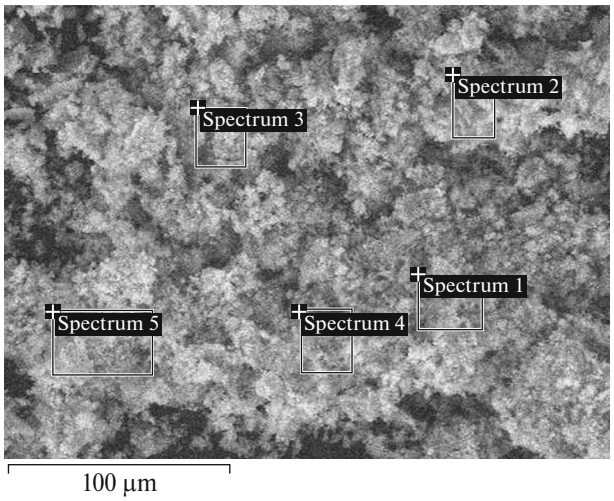

Fig. 1. Scanning electron microscopy images with different resolutions (regions in frames were assessed by X-ray spectral microanalysis).

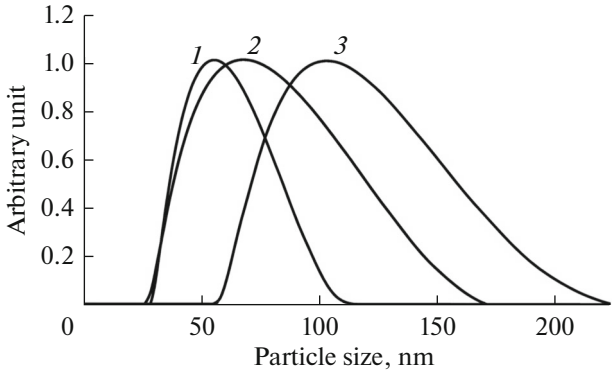

Fig. 2. Distribution of chitosan-coated and cellobiose-coated CaPh-particles according to particle size based on dynamic light scattering ((*1*) chitosan CaPh-particles, 0.45 μm filter; (*2*) chitosan CaPh-particles, nonfiltered sample; (*3*) cellobiose CaPh-particles, nonfiltered sample).

Table 2. Characteristics of chitosan-coated CaPh-particles with incorporated drugs

CaPh-particle	*r*, nm	ζ, mV	Drug inclusion, %
CaPh-Chitosan/TPP	70 ± 40	+22 ± 4	–
CaPh/Lisinopril-Chitosan/TPP	65 ± 40	+17 ± 4	75 ± 9
CaPh/Timolol-Chitosan/TPP	70 ± 40	+16 ± 4	10 ± 4

when lisinopril and timolol were incorporated in cellobiose-coated CaPh-particles.

The size and ζ-potential of the chitosan-coated CaPh-particles with the inclusion of timolol and lisinopril did not change when stored in a solution at 4°C or in a freeze-died state at –20°C for at least 3 months. In addition, the particles remained stable when concentrated by 20 times.

The desorption time of the incorporated drugs from the nanoparticles after placing the particles into a fresh salt solution was assessed; almost complete (~90%) desorption was observed within a short period of time (about 30 min for lisinopril and 5 min for timolol).

The slower release of lisinopril compared to timolol can indicate that lisinopril was bound more strongly to the CaPh-particles. In addition, the anionic carboxyl groups of lisinopril can interact with the cationic amino groups of chitosan, whereas timolol cannot be involved in such interactions.

The penetration of medications incorporated in the nanoparticles into the internal ocular structures, the efficacy and duration of the action of the drugs can be objectively assessed in the in vivo tests by including IOP-lowering drugs in the particles. IOP can be repeatedly measured in the same animal over the required time periods allowing for the quantitative evaluation of the dynamics of the hypotensive effect. The optimal glaucoma model cannot be reproduced in animals and the available models of ocular hypertension do not reflect the processes occurring in glaucoma. Therefore, ocular normotensive rabbits are used to assess the ocular hypotensive effects of various drugs as the physiological actions of hypotensive drugs on normotensive rabbits are more reflective of the ocular hypotensive drug action on patients with glaucoma [33].

We used chitosan-coated CaPh-particles containing timolol for experiments in vivo. Figure 3 illustrates the reduction of IOP after the instillation of a timolol solution and suspended chitosan CaPh-particles containing timolol in the eyes of rabbits. A slower reduction of IOP under the action of timolol included in the particles than under the action of a timolol solution was observed. This can be associated with the potent mucoadhesive characteristics of chitosan suggesting an increased retention time over the ocular mucosa after instillation [23]. When added by drops, the particles interact with the conjunctival mucosa and a mucin layer of the tear film, where the chitosan coating can help retain particles, leading to slower drug absorption into the internal ocular structures. However, the IOP level was similar 4–5 h after the administration of drugs. Most importantly, the inclu-

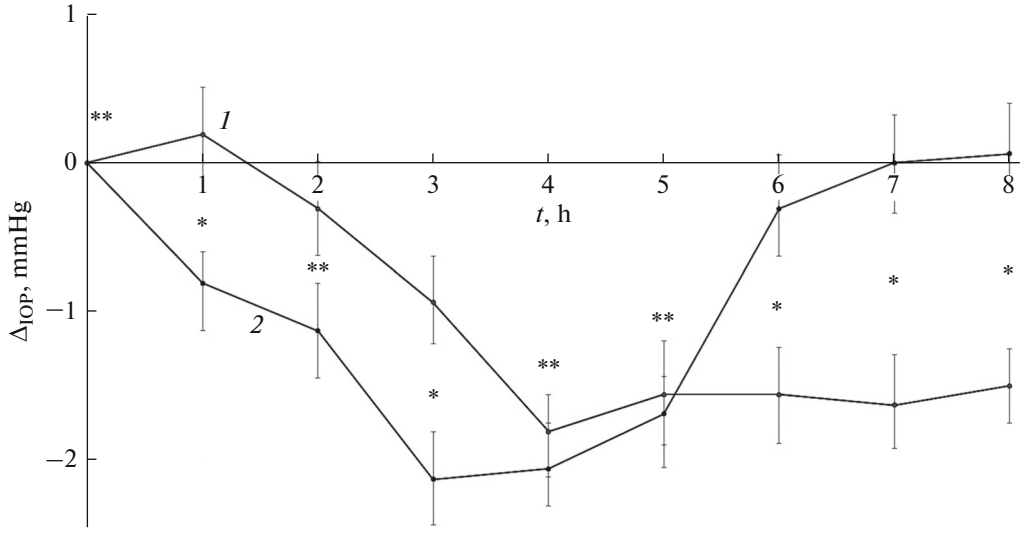

Fig. 3. Average change in IOP after single instillation of timolol included in chitosan-coated CaPh-particles (experiment) and in aqueous solution (control). Asterisk indicates significant differences ($p \le 0.05$) ((*1*) timolol included in chitosan-coated CaPh-particles; (*2*) timolol in solution).

sion of timolol into the CaPh-particles resulted in a significantly prolonged reduction of IOP. After instillation of an aqueous timolol solution, the IOP almost returned to the initial levels in 6 h, whereas timolol instilled inside the particles was associated with a significant reduction of the IOP for at least 8 h ($p < 0.01$) (Fig. 3). It is suggested that chitosan-coated CaPh-particles carrying timolol have longer retention times on the eye surface compared to low molecular weight timolol. This results in extended periods of drug release from the particles into the internal ocular structures maintaining effective therapeutic concentrations of timolol to reduce the IOP. The IOP level returned to its initial level 24 h after the instillation of timolol included in the chitosan-coated CaPh-particles. Note that the inclusion of timolol in cellobiose-coated CaPh-particles instead of chitosan-coated nanoparticles did not prolong the drug action [18]. Therefore, despite the low percentage of timolol included in the CaPh-particles covered with chitosan and the rapid release of the drug in vitro, this approach yields a significant positive effect on the IOP reduction in vivo.

In this paper, chitosan-coated CaPh-particles containing low molecular weight drugs have been prepared and characterized. The use of chitosan as a coating agent significantly extends the hypotensive action of timolol included in the CaPh-particles versus the actions of timolol in an aqueous solution and when included in cellobiose-coated CaPh-particles. Thus, the high level of efficacy of chitosan-coated CaPh-particles as vehicles for delivering drugs into the internal ocular media can help reduce doses and lower the frequency of drug administration.

ACKNOWLEDGMENTS

The preparation of chitosan was supported by the Russian Science Foundation (project no. 16-14-00046).

REFERENCES

1. Achouri, D., Alhanout, K., Piccerelle, Ph., and Andrieu, V., *Drug Dev. Ind. Pharm.*, 2013, vol. 39, no. 11, p. 1599.
2. Saari, K.M., Nelimarkka, L., Ahola, V., Loftsson, T., and Stefansson, E., *Graefe's Arch. Clin. Exp. Ophthalmol.*, 2006, vol. 244, no. 5, p. 620.
3. Sasaki, H., Igarashi, Y., Nagano, T., Nishida, K., and Nakamura, J., *Pharm. Res.*, 1995, vol. 12, no. 8, p. 1146.
4. Ludwig, A., *Adv. Drug Delivery Rev.*, 2005, vol. 57, no. 11, p. 1595.
5. Shen, Y. and Tu, J., *AAPS J.*, 2007, vol. 9, no. 3.
6. Abdelbary, G.A., Amin, M.M., and Zakaria, M.Y., *Drug Delivery*, 2017, vol. 24, no. 1, p. 309.
7. Naguib, S.S., Hathout, R.M., and Mansour, S., *Drug Delivery*, 2017, vol. 24, no. 1, p. 99.
8. Sahoo, S.K., Dilnawaz, F., and Krishnakumar S., *Drug Discovery Today*, 2008, vol. 13, nos. 3–4, p. 144.
9. Ogunjimi, A.T., Melo, S.M., Vargas-Rechia, C.G., Emery, F.S., and Lopez, R.F., *Carbohydr. Polym.*, 2017, vol. 157, p. 1065.
10. Fangueiro, J.F., Veiga, F., Silva, A.M., and Souto, E.B., *Curr. Pharm. Des.*, 2016, vol. 22, no. 9, p. 1135.
11. Bell, S.J.D., Morco, T., and He, Q., US Patent 6355271B1, 2002.
12. Hu, J., Kovtun, A., Tomaszewski, A., Singer, B., Seitz, B., Epple, M., Steuhl, K., Ergun, S., and Fuchsluger, T., *Acta Biomater.*, 2012, vol. 8, no. 3, p. 1156.
13. Xie, Y., Chen, Y., Sun, M., and Ping, Q., *Curr. Pharm. Biotechnol.*, 2013, vol. 14, no. 10, p. 918.
14. Liu, Y., Wang, T., He, F., Liu, Q., Zhang, D., Xiang, S., Su, S., and Zhang, J., *Int. J. Nanomed.*, 2011, vol. 6, p. 721.
15. Viswanathan, K., Gopinath, V.P., and Raj, G.D., *Colloids Surf., B*, 2014, vol. 116, p. 9.
16. Schwiertz, J., Wiehe, A., Grafe, S., Gitter, B., and Epple, M., *Biomaterials*, 2009, vol. 30, no. 19, p. 3324.
17. He, Q., Chu, T.-C., and Potter, D., *J. Ocul. Pharmacol. Ther.*, 2002, vol. 18, no. 6, p. 507.
18. Shimanovskaya, E.V., Beznos, O.V., Klyachko, N.L., Kost, O.A., Nikol'skaya, I.I., Pavlenko, T.A., Chesnokova, N.B., and Kabanov, A.V., *Vestn. Oftal'mol.*, 2012, no. 3, p. 15.
19. Shimanovskaya, E.V., Nikol'skaya, I.I., Binevskii, P.V., Beznos, O.V., Klyachko, N.L., Pavlenko, T.A., Chesnokova, N.B., and Kost, O.A., *Nanotechnol. Russ.*, 2014, vol. 9, nos. 3–4, p. 219.
20. Nikol'skaya, I.I., Beznos, O.V., Galitskii, V.A., Chesnokova, N.B., and Kost, O.A., *Moscow Univ. Chem. Bull. (Engl. Transl.)*, 2016, vol. 71, no. 4, p. 209.
21. Liaw, J., Rojanasakul, Y., and Robinson, J.R., *Int. J. Pharm.*, 1992, vol. 88, p. 111.
22. *Khitozan* (Chitosan), Skryabin, K.G., Mikhailov, S.N., and Varlamov, V.P., Eds., Moscow: Tsentr Bioinzheneriya, Ross. Akad. Nauk, 2013.
23. Menchicchi, B., Fuenzalida, J.P., Bobbili, K.B., Hensel, A., Swamy, M.J., and Goycoolea, F.M., *Biomacromolecules*, 2014, vol. 15, no. 10, p. 3550.
24. Da Silva, S.B., Ferreira, D., Pintado, M., and Sarmento, B., *Int. J. Biol. Macromol.*, 2015, vol. 84, p. 112.
25. De Campos, A.M., Sanchez, A., Gref, R., Calvo, P., and Alonso, M.J., *Eur. J. Pharm. Sci.*, 2003, vol. 20, no. 1, p. 73.
26. Costa, J.R., Silva, N.C., Sarmento, B., and Pintado, M., *Eur. J. Clin. Microbiol. Infect. Dis.*, 2015, vol. 34, no. 6, p. 1255.
27. Nagarwal, R.C., Kumar, R., and Pandit, J.K., *Eur. J. Pharm. Sci.*, 2012, vol. 47, no. 4, p. 678.
28. Raymond, L., Morin, F.G., and Marchessault, R.H., *Carbohydr. Res.*, 1993, vol. 246, p. 331.
29. Wang, W., Bo, S., Li, S., and Qin, W., *Int. J. Biol. Macromol.*, 1991, vol. 13, no. 5, p. 281.
30. Svedas, V., Galaev, I., Borisov, I., and Berezin, I., *Anal. Biochem.*, 1980, vol. 101, no. 1, p. 188.
31. Xu, Y. and Du, Y., *Int. J. Pharm.*, 2003, vol. 250, no. 1, p. 215.
32. Chesnokova, H.B., Kost, O.A., Nikol'skaya, I.I., Beznos, O.V., Davydova, N.N., Stolyarova, E.A., Binevskii, P.A., and Pavlenko, T.A., *Ross. Oftal'mol. Zh.*, 2009, vol. 2, p. 42.
33. Orihashi, M., Shima, Y., Tsuneki, H., and Kimura, I., *Jpn. J. Ophthalmol.*, 2005, vol. 49, no. 6, p. 523.

Translated by M. Novikova