

Synthesis of a Bioactive Composition of Chitosan–Selenium Nanoparticles

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Abstract— A biologically active composition of chitosan-selenium nanoparticles has been developed. Selenium nanoparticles are characterized by a clear bimodal size distribution: 2–3 and ~37 nm. The main active centers of complexation with nanoparticles are the amino and hydroxyl groups of chitosan. In experiments on culturing fibroblasts of the hTERT BJ-5ta cell line on sample films, high biocompatibility of the composition was shown. It was shown that the composition of chitosan-selenium nanoparticles has a corrective effect on the oxidative processes of the body, reducing the activity of free-radical oxidation in the blood of animals. This opens up prospects for the use of this complex in the composition of antioxidant and adaptogenic drugs.

Keywords: nanocomposite, chitosan, selenium nanoparticles, biocompatibility, antioxidant properties

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INTRODUCTION

Selenium is an essential element of the antioxidant defense system of the human body, possessing immunomodulatory properties, participating in the regulation of thyroid hormone metabolism, and having an anticarcinogenic effect [1–6]. Selenium is included in selenoproteins, which have a wide range of biological effects, including antioxidant and anti-inflammatory effects. There are 25 selenoprotein genes in the human genome. Selenium is found as selenocysteine in various antioxidant enzymes such as glutathione peroxidase, thioredoxin reductase, and selenoprotein P, which is involved in selenium transport. Selenium works as the redox center of all these proteins and is essential for their biochemical activity.

Low levels of selenium in the body can lead to an increased risk of mortality, decreased immunity, and cognitive functions [7]. In humans, chronic selenium deficiency is manifested by cardiomyopathy, which can provoke angina attacks and myocardial infarction. Often, thyroid dysfunction is associated not with iodine deficiency, but with selenium deficiency in the body. Recently, suggestions have been made about the correlation of selenium deficiency with the severity of the course or mortality from COVID-19 [8, 9]. Selenium enters the food chain through plants consumed by humans and animals. Plants absorb selenium directly from the soil; therefore, its consumption by

the population largely depends on the geographic region [10, 11]. The lack of selenium in the body is a risk factor for the development of various pathologies. Selenium compounds protect cell membranes from the effects of free radicals and prevent their generation, reducing the risk of tumors and the development of heart and blood vessel disease. It is important to note that, although selenium is an indispensable element for the functioning of many biological processes in humans, it belongs to trace elements and the concentration interval between its functionality and toxicity is very narrow [12, 13].

There is growing interest in this microelement, which is used in the treatment of many diseases, including cancer [14]. Existing selenium preparations are presented mainly in the form of oxyanions – selenate or selenite (selenomethionine, selenocysteine, methylselenocysteine and sodium selenite), which are toxic at high concentrations. It should be emphasized that the biological activity of selenium depends on its chemical form and structure. For example, elemental selenium is insoluble in water and has long been considered biologically inert. At present, selenium in nanostructural form is being intensively studied, which is reflected in many modern studies [15–19]. Compared to organic and inorganic forms of selenium, selenium nanoparticles (NPs) exhibit lower toxicity and superior antioxidant, immu-

nomodulatory, bactericidal, and antitumor activity. The main advantage of selenium NPs, in comparison with other forms, is much lower toxicity, which makes it possible to use them in doses that significantly exceed the daily requirement. In addition, nanoselenium has the so-called size effect, which is manifested in the fact that smaller particles are biologically more active, accumulate better in tissues [20, 21]. Active work is underway to synthesize and study the biological effect of nanoselenium in various carrier matrices. As a matrix for NPs, bovine serum albumin [19], carrageenan [22], a food supplement—gum arabic [22, 23], and the polysaccharide sclerotia of the edible fungus *Pleurotus tuber-terreus* [24] are used.

The problem of the penetration of selenium NPs from the intestinal cavity into the blood during oral administration, their targeted delivery to various organs, and prolonged-release remain urgent. One of the most promising polymers for creating such a biologically active composition capable of controlled targeted delivery of NPs into the internal environment of the body and their prolonged release is the polysaccharide chitosan, due to its nontoxicity, transport, and other polyfunctional properties [25–27]. It can be assumed that the combination of antioxidant properties of selenium and chitosan NPs will make it possible to create highly effective drugs with a wide spectrum of action, as well as to develop drugs for oral administration with effective immunomodulatory and antioxidant effects.

This work aimed to synthesize biocompatible selenium NPs stabilized by chitosan and to evaluate the effect of nanocomposites, when administered orally, on free radical processes in the body of experimental animals.

MATERIALS AND METHODS

We used chitosan with a molecular weight (MW) of 2.0×10^5 Da, the degree of deacetylation 85% (ZAO Bioprogress, Biokombinat, Moscow Region) without additional purification (mass fraction of insoluble substances—0.25%); succinic acid grade “Pure”; sodium selenite grade “Pure” TU 6-09-17-209-88; ascorbic acid; potassium bromide brand “Pure”.

Selenium NPs were obtained by a redox reaction between ascorbic acid and the NP precursor—sodium selenite, in solutions of the stabilizer—chitosan polysaccharide (3 wt %) in 2% succinic acid. The content of sodium selenite in solutions varied from 0.03 to 0.012 mol/L. Ascorbic acid was added in a 1 : 1 molar concentration with sodium selenite.

The production and formation of NPs were monitored spectrophotometrically. The solution was placed under a 1600 mW/m^2 UV lamp at a fixed distance of 15 cm from the lamp at a temperature of 35°C to accelerate the process of NP reduction. Samples of the solution were taken every 30 min, diluted 10 times with dis-

tilled water, and the absorption spectrum of the sample relative to water was recorded on a SHIMADZU UV-1650 PC UV spectrometer. The maximum of the absorption band was observed at 250–280 nm, which corresponds to the absorption of ascorbic acid [28].

The dimensional characteristics of selenium NPs in chitosan solutions were determined by small-angle X-ray scattering at the BiOMUR experimental station at the RSC “Kurchatov Institute”.

The functional groups of chitosan participating in the stabilization of selenium NPs were determined by infrared spectroscopy on an Infracum-FT 801 IR spectrometer in the frequency range $500\text{--}4000 \text{ cm}^{-1}$ with a resolution of 2 cm^{-1} . Samples of the initial chitosan and the composition containing selenium NPs were dried by vacuum to a fixed weight. Then they were crushed and mixed with KBr at a sample/KBr ratio of 1 : 20. The mixture was pressed on an IR hydraulic press at a pressure of 400 kg/cm^2 .

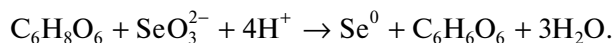
To study the biocompatibility of the material, films were made by pouring onto a lamsan substrate from solutions of chitosan succinate and compositions with selenium NPs based on it. The films were obtained under conditions of uniform solvent evaporation to constant weight at room temperature. Then the samples were washed from acid with an alcoholic alkali solution and the pH value was brought to neutral by repeated washing with water. The films were studied for adhesion, cytotoxicity, and cell growth on their surface during the cultivation of human fibroblasts of the hTERT BJ-5ta cell line. Films of material were placed in the wells of a 6-well cell culture plate and filled with $500 \mu\text{L}$ of DMEM medium. The cells were seeded on the surface of the film with a density of $1.6 \times 10^5/\text{cm}^2$ and cultured for 24 hours. Cell visualization and assessment of their viability were assessed by fluorescence microscopy. A 2×10^{-4} wt % solution of acridine orange in phosphate buffer was used as a dye for staining fibroblasts. This dye selectively interacts with DNA and RNA located in the nucleus and mitochondria of the cell by intercalation or electrostatic attraction, respectively. This allows you to assess the general state of cells - activity, proliferation, and apoptosis. Microsampling of the films was carried out on an Olympus IX71 inverter microscope (Japan/Germany) using a “green” filter (emission 510–555 nm, excitation 460–495 nm), which allows visualizing the green color of the nucleus of living cells.

The antioxidant properties of the composition of chitosan-selenium NPs were studied in vivo in experimental animals—nonlinear white rats. The animals were divided into 4 groups of 3 animals each. The first group included intact animals, the second group received a solution of chitosan succinate orally, the third group received a solution of chitosan succinate containing selenium in the ionic form at a concentration 3 times higher than the LD_{50} for rats [13], the fourth group received selenium NPs stabilized by chi-

tosan into the same concentration as for the third group. The evaluation of free radical activity was carried out according to the content of lipid oxidation products (LOPs) in the blood plasma of experimental animals—diene and triene conjugates (DC, TC), Schiff bases (SB) 3 days after daily oral administration of solutions. The assessment method is based on the determination of the content of LOPs in the blood by the absorption of a monochromatic light flux by a lipid extract in the ultraviolet region of the spectrum. The amount of DC, TC, and SB are extracted in heptane-isopropanol fractions. Since in heptane mainly neutral lipids are extracted, and in isopropanol—phospholipids, so the heptane fraction indicates LOPs activity in neutral lipids, and the isopropanol fraction—in phospholipids. To 0.1 mL of blood plasma add 8 mL of the heptane-isopropanol mixture in a 1 : 1 ratio, shake for 15 minutes, and centrifuge at 6000 rpm for 10 minutes. Next, the lipid extract is transferred into a clean test tube, and 5 mL of the heptane-isopropanol mixture in a ratio of 3 : 7 is added, after which 2 mL of 0.01 N aqueous solution of hydrochloric acid are added to the test tube to dilute the phases and remove non-lipid impurities. After phase separation, the (upper) heptane extract is transferred into a clean test tube, and 1 g of calcined sodium chloride is added to the lower one to dehydrate the isopropanol extract, which is transferred into a clean test tube. Measurement of optical densities (E) is performed on a spectrophotometer. Each phase is scored against the corresponding control at wavelengths 220 nm (absorption of isolated double bonds), 232 nm (absorption of DC), 278 nm (absorption of MC), and 400 nm (absorption of SB). The content of DC, TC, and SB is estimated by the relative values of E_{232}/E_{220} , E_{278}/E_{220} , E_{400}/E_{220} and expressed in relative units.

RESULTS AND DISCUSSION

Synthesis of selenium NPs in chitosan solutions. We used the method of chemical synthesis of selenium NPs with their simultaneous stabilization with the polysaccharide chitosan. The main advantage of this method is the ease of synthesis, and ascorbic acid used as a reducing agent and chitosan as a stabilizer are non-toxic and biocompatible components. Selenium NPs are formed by the following reaction:



During the reaction, ascorbic acid is converted to dehydroascorbic acid. The progress of the reaction was monitored spectrophotometrically by the change — the appearance and decrease — of the absorption band characteristic of ascorbic acid in the wavelength range of 250–280 nm (Fig. 1) [28]. It was shown that the peak of the absorption band corresponding to ascorbic acid completely disappears after 2.5 hours. In this case, during the reduction of selenium ions and the

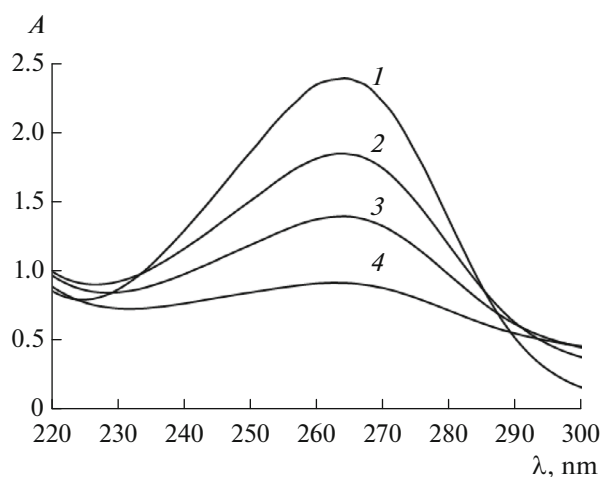
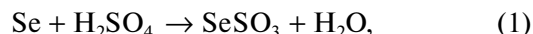


Fig. 1. Typical absorption bands of ascorbic acid during the formation of selenium NPs in a chitosan solution: 1—0 min, 2—30 min, 3—60 min, 4—90 min.

formation of Se NPs, the color of the solution changes from pale yellow to bright red.

Confirmation that ascorbic acid is consumed for the reduction of selenium ions is the result of a “blind” experiment in the absence of sodium selenite in the solution. In the latter case, no change in the absorption bands of ascorbic acid in chitosan solutions with time was observed.

At the qualitative level, the formation of selenium NPs was confirmed as follows. When cold concentrated sulfuric acid is added to a dilute solution of chitosan bonded with selenium NPs, a green color appears, indicating the formation of the SeSO_3 salt. When the resulting solution is diluted, a red selenium precipitate is formed due to the reactions:



Determination of the dimensional characteristics of selenium NPs. The dimensional characteristics of selenium NPs were determined by small-angle X-ray scattering (Fig. 2).

In Fig. 2a the dots represent the experimental SAXS from the samples and the difference curve from them, from which the distribution $D_V(R)$ of Se NPs by volume was calculated as a function of their radii, R . In Fig. 2b the points “ $D_V(R)$ ” represent the found distribution it corresponds to the fitting model “fit mod I (q)” — the red line on the scattering curves in Fig. 2b. The red line in Fig. 2b fit $D_V(R)_{\text{mod}}$ is a fitting model when the found distribution “ $D_V(R)$ ” is decomposed into the sum of Gaussian components. Clear bimodal size separation of synthesized selenium NPs stabilized by chitosan is observed. During the synthesis, NPs with sizes of 2–3 nm (33.4 wt %) and ~ 37 nm (66.6 wt %) are formed.

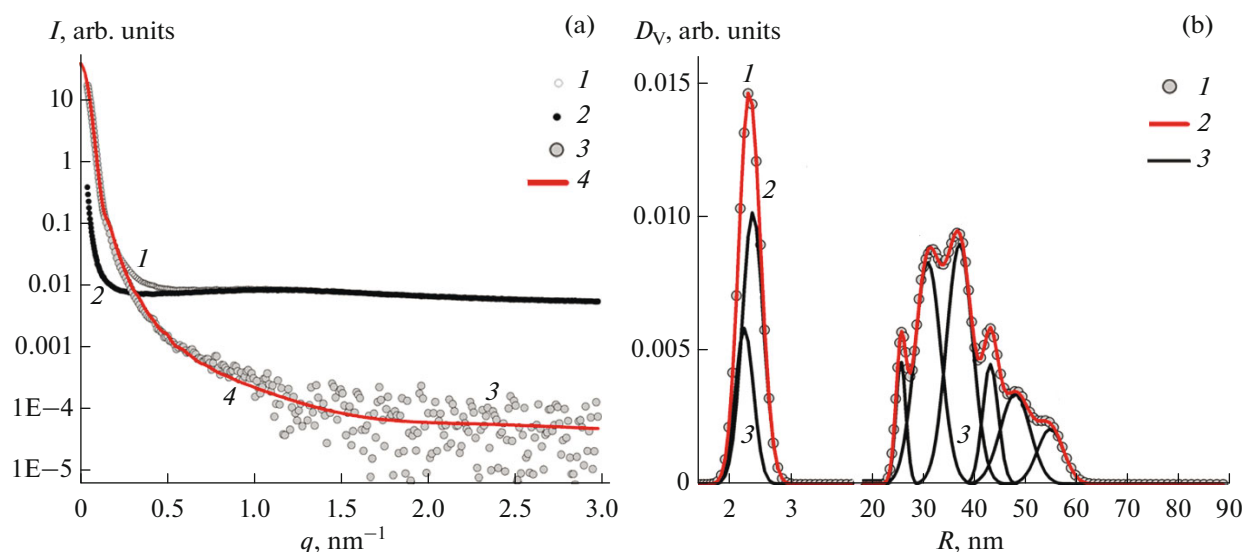


Fig. 2. (a) Experimental SAXS curves for chitosan solution containing (1) and not containing (2) selenium NPs, differential curve (3), and size distribution curve of selenium NPs (4). (b) Calculated size distributions of selenium NPs in chitosan solution: (1) distribution (D_V) of selenium NPs corresponds to curve (4) in Fig. 2a; (2) curve of the sum of expansion of D_V into Gaussian components (3).

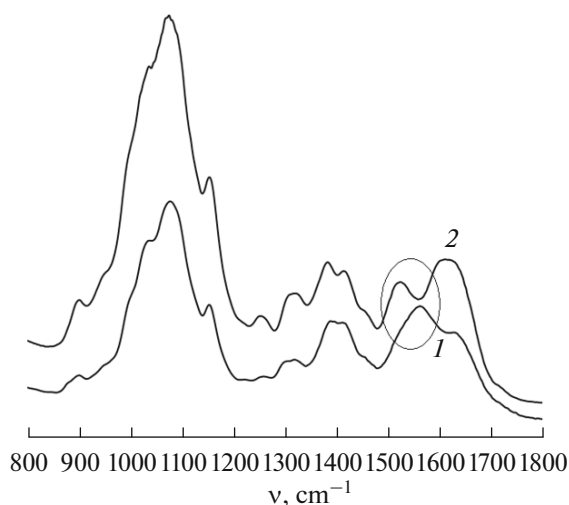


Fig. 3. IR spectra of chitosan (1) and chitosan complexed with selenium NPs (2).

By IR spectrometry, we determined which functional groups of chitosan are involved in the stabilization of selenium NPs. Figure 3 shows a characteristic shift of chitosan bands from 1570 to 1536 cm^{-1} corresponding to stretching vibrations of NH amino groups of the polysaccharide, and also observed a shift of bands in the region of 3400 cm^{-1} , corresponding to vibrations of hydroxyl groups of the polymer, indicating their participation in the stabilization of NPs of selenium.

Thus, the main active centre of complexes formation with selenium NPs are amino and hydroxyl groups of the polysaccharide.

Investigation of the biocompatibility of the composition. In vitro studies of the biocompatibility of films of

samples were carried out for adhesion and proliferation of fibroblast cells as precursors of connective tissue after 24 hours of incubation on the surface of the films. Figure 4b shows a uniform distribution of fibroblasts, their growth, and division on the surface of the film of the composite chitosan – selenium NPs, which indicates a high degree of biocompatibility of this sample. At the same time, in the sample containing ionic selenium at the same concentration (Fig. 4a), the cells died after their cultivation and are not visible.

Study of antioxidant properties. It is known that the increased production of free oxygen radicals and the accumulation of lipid peroxidation products (LOPs) cause several negative effects in the membranes of

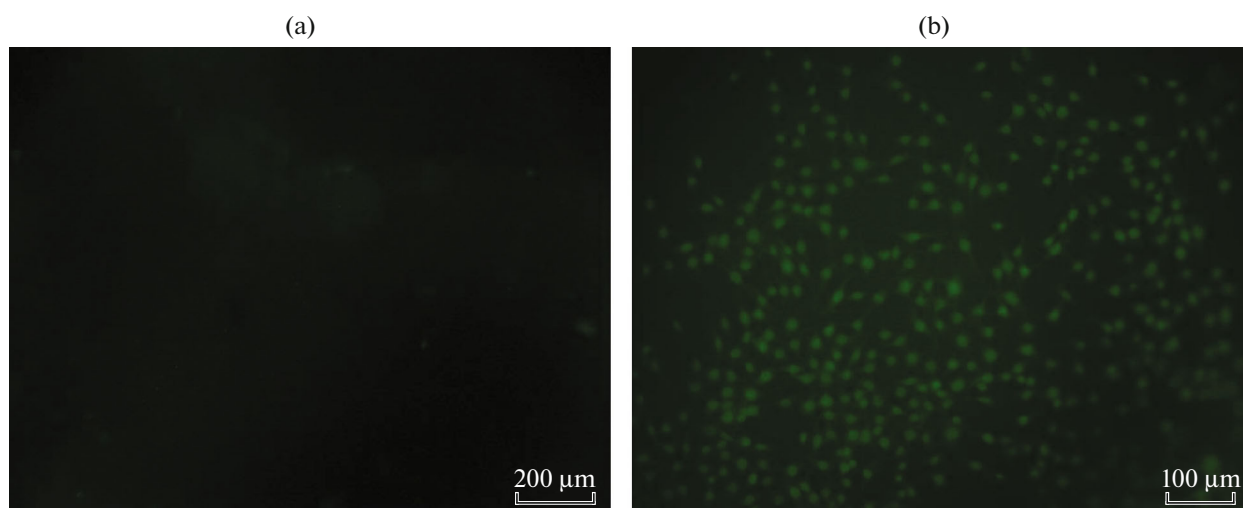


Fig. 4. Films of samples, after sowing of fibroblasts of the cell line hTERT BJ-5ta on their surface: (a) a composite based on chitosan containing 0.012 mol/L of ionic selenium [Se⁺⁴]; (b) a composite based on chitosan containing 0.012 mol/L of selenium NPs [Se⁰].

body cells, leading to a weakening of hydrophobic membrane bonds, an increase in permeability as a result of disruption of cell function or their death. In this work, we studied the effect of chitosan preparations containing Na₂SeO₃ at a concentration 3 times higher than the LD₅₀ for rats [13] and a nanocomposition of chitosan–selenium NPs at the same concentration on lipid peroxidation activity. The assessment was carried out according to the content of LOPs in the blood plasma of experimental animals DC, TC and SB—3 days after daily oral administration (Table 1).

It was found that the introduction of a chitosan solution does not lead to significant changes in the activity of LOPs processes. Chitosan may be viewed as a potential antioxidant and free radical scavenger. The non-enzymatic antioxidant defense system includes substances that mainly perform the role of traps (interceptors) of free radicals. They “extinguish” free radicals, take up excess energy, and inhibit the development of a chain reaction of the formation of new radicals.

In the 3rd experimental group (chitosan + Na₂SeO₃, in a concentration 3 times higher than the LD₅₀ for rats), an increase in the content of the products of POL-DC, TC, SB by 3–5 times is noted. This may be

due to the fact that the biological effects of selenium compounds are dose-dependent. That is, selenium can also act as a prooxidant. It can activate lipid peroxidation and reduce the activity of superoxide dismutase [5]. Selenium is indirectly involved in the formation of ROS during the oxidation of thiols since it is capable of forming an intramolecular S–Se bond with thiol groups [6]. Also, selenium compounds interact with glutathione to form intermediate metabolites (GS–SeH, GS–Se–SG), which can ultimately interact with oxygen to form free radicals [4].

In group 4 of experimental animals, which were injected with the nanocomplex “chitosan + selenium NPs”, the DC content corresponded to intact animals, and the content of DC and SB decreased by 2 and 4 times, respectively, which may be associated with the antioxidant effect of selenium NPs. Selenium acts as an activator of antioxidant defense enzymes, which, unlike the non-enzymatic link, can block free radical oxidation reactions much faster and more efficiently.

Thus, the results obtained indicate that the use of the nanocomplex “chitosan + selenium NPs” has a corrective effect on the oxidative processes of the body, reducing the activity of free radical oxidation in the blood of

Table 1. Content of LOPs in rat blood plasma 3 days after oral administration of solutions: chitosan, chitosan with Na₂SeO₃, and nanocomposite

LOPs	DC	TC	SB
Intact	0.171 ± 0.09	0.438 ± 0.35	35.39 ± 17.72
Chitosan	0.163 ± 0.051	0.634 ± 0.39	85.15 ± 36.31
Chitosan + Na ₂ SeO ₃	0.7054 ± 0.48**	2.73 ± 0.48	144.6 ± 20.8*
Chitosan + NP of Se	0.175 ± 0.021***	0.119 ± 0.05***	15.93 ± 9.47**

* $p < 0.05$ – in relation to the group “intact”; # $p < 0.05$ in relation to the group—“chitosan”; $p < 0.05$ – in relation to the group—“chitosan” + Na₂SeO₃.

animals. This makes it possible to recommend selenium nanocomplex for use in the manufacture of adaptogenic nanocomplexes to increase their efficiency.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on animal welfare. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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