

# Bombyx Mori Chitosan Nanoparticles: Synthesis and Properties

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**How to cite this paper:** Milusheva, R.Yu. and Rashidova, S.Sh. (2019) Bombyx Mori Chitosan Nanoparticles: Synthesis and Properties. *Open Journal of Organic Polymer Materials*, 9, 63-73.

<https://doi.org/10.4236/ojopm.2019.94004>

**Received:** September 12, 2019

**Accepted:** October 28, 2019

**Published:** October 31, 2019

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## Abstract

A synthesis of nanochitosan from Bombyx mori chitosan with particle sizes of 20 - 100 nm was carried out. The antibacterial and immunological properties of synthesized nanochitosan were first studied. It was revealed that preparations based on nanochitosan have pronounced antibacterial activity, and are also able to significantly increase the immune response of the living system.

## Keywords

Antibacterial Activity, Biodegradability, Chitosan, Nanoparticles

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## 1. Introduction

Chitosan and its derivatives are widely used in medicine due to a combination of valuable biochemical properties: bactericidal, biocompatible, and biodegradable. This allowed us to refer them to a group of parapharmaceuticals—natural substances with a pronounced pharmacological activity [1] [2] [3]. The chemical lability of this polymer allows, using relatively simple technological processes, to obtain homologues and analogues with different variants of physicochemical and biological properties. The compatibility of such compounds with biosystems, biodegradability in the body with the formation of harmless low molecular weight compounds, suggests of their practical use. So, for example, it has been established that pronounced anti-radiation activity is characteristic of chitosan, both in conditions of prophylaxis and in treatment. Intravenous administration of it 10 - 15 minutes before irradiation (385 - 365 rad), which causes a bone marrow form of radiation sickness, prevents the death of animals [4].

Chitosan is widely used to bind and excrete fats and cholesterol from the body, the excessive accumulation of which leads to the development of atherosclerosis, coronary heart disease, hypertension, diabetes mellitus and other dis-

eases. Chitosan binds fats 10 times more effectively compared to other glycans, lowering the level of high density lipoproteins, resulting in weight loss. In medical practice, aminoglucan preparations were used for alimentary obesity, hypercholesterolemia, biliary dyskinesia. A decrease in body weight was noted in patients who took chitosan by 8.7% for three weeks (dose 1.5 g) [5].

The main body of work on antibacterial activity is associated with chitosan, [6] [7] [8] [9] and there are almost no studies on the biological activity of nanochitosan (nChs) and the relationship between the chemical structure of the polymer and its biological effect on microorganism cells.

In addition, most studies of the biological activity of chitosan are carried out with chitosan isolated from crustaceans. In our study, we use *Bombyx mori* chitosan, which, unlike crab chitosan, is characterized by a low initial molecular weight, which makes its use in medicine promising.

In the literature, there are no data on the study of the bactericidal activity of chitosan *Bombyx mori*; the fact of using *Bombyx mori* chitosan is also important, which we emit from local raw materials, which are waste products silk, which annually accumulate in the amount of 10,000 - 15,000 tons [10] [11] [12].

The aim of this study was the synthesis of chitosan (Chs) *Bombyx mori*, the production of nanochitosan based on it, the study of their physico-chemical properties and the effect of polymer nanopreparations on the immune status, antibacterial activity, as well as the preparation of the bioactive nanopolymer coating for dentistry.

## 2. Data and Analyses

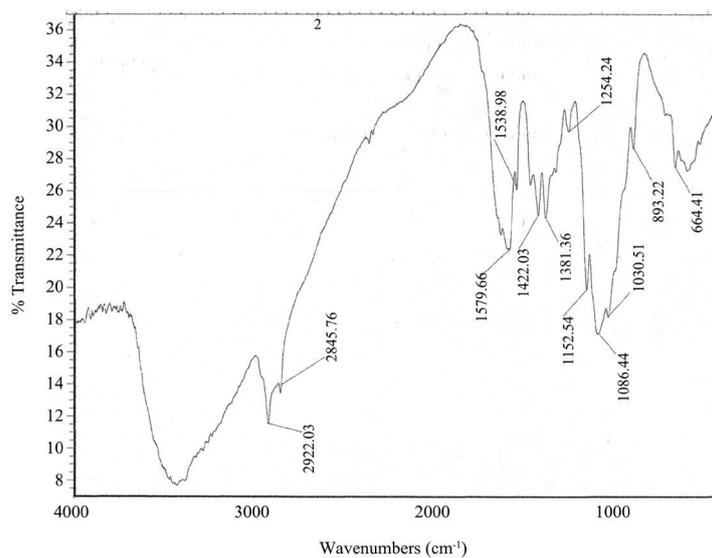
Chitosan is characterized by structural and chemical heterogeneity, since even after processing under narrow chemical conditions it contains a small amount of mineral and protein admixtures, and is also characterized by a wide molecular weight distribution. The latter causes the formation of insoluble gel particles when dissolving chitosan. These factors significantly limit its scope of using. Purification is needed to obtain chitosan nanoparticles.

The process of purified chitosan obtaining from pupae of the silkworm *Bombyx mori* includes successive stages of dissolution of technical chitosan in aqueous acetic acid, precipitation at pH 8 - 9, coagulation, centrifugation for 10 min at 7000 rpm. After centrifugation, the precipitate was redispersed and again sedimented via centrifugation twice and freeze drying using an ALPHA 1 - 2 LD plus dryer at  $T = -50^{\circ}\text{C} - 55^{\circ}\text{C}$ ,  $P = 0.3 - 0.5$  mbar [13].

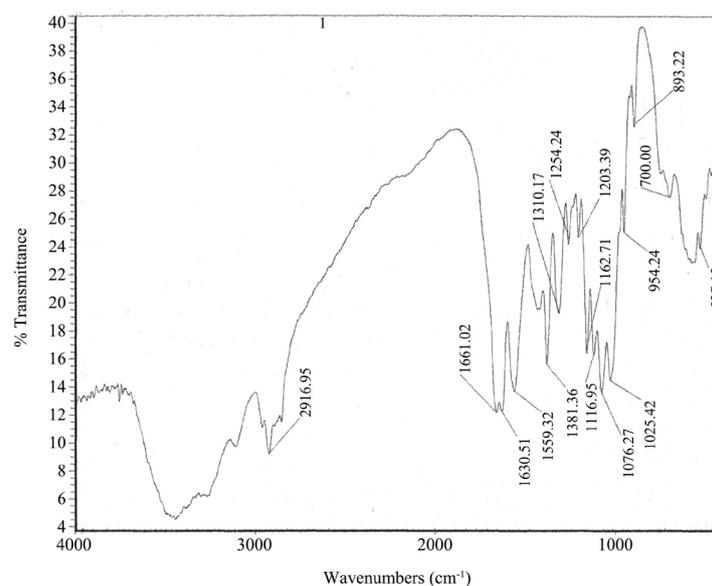
The physico-chemical properties of the initial chitosan were studied by elemental analysis, IR -spectroscopy, X-ray diffraction analysis, degree of deacetylation (DDA), and molecular weight (MM). Samples were characterized by the nitrogen content of 7.42%, ash content-3.37%, solubility-86.05%, and degree of crystallinity-41%. Intrinsic viscosity and viscosimetric average molecular weight: before purification  $[\eta] = 0.314$  L/g,  $M_v = 170 \times 10^3$  g/mol, DDA, determined conductometrically-71.5%.

On the IR-spectra of the native chitosan (Figure 1(a)) it could be seen an in-

tense broad absorption band with a peak at  $3460\text{ cm}^{-1}$  (valent vibrations of OH-groups included in the hydrogen bonds and the absorption bands at  $2900 - 3120\text{ cm}^{-1}$ , which correspond to valent vibrations of CH- and  $\text{CH}_2$  - groups). On the right side of the peak of OH - groups is visible the superposition of the absorption band of NH - groups at  $3220\text{ cm}^{-1}$ . IR-spectrum of purified chitosan (**Figure 1(b)**) is also characterized by absorption bands of Amide - I ( $1665\text{ cm}^{-1}$ ) and Amide - II ( $1600\text{ cm}^{-1}$ ), due to the valent vibrations of C = O and NH - groups, as well as several bands associated mainly with the deformation vibrations of  $\text{CH}_3$ -methyl, =CO-carbonyl, -COC-ether group, and -C=C-double bonds in the range of  $1300 - 1450\text{ cm}^{-1}$  and  $1000 - 1260\text{ cm}^{-1}$ .



(a)



(b)

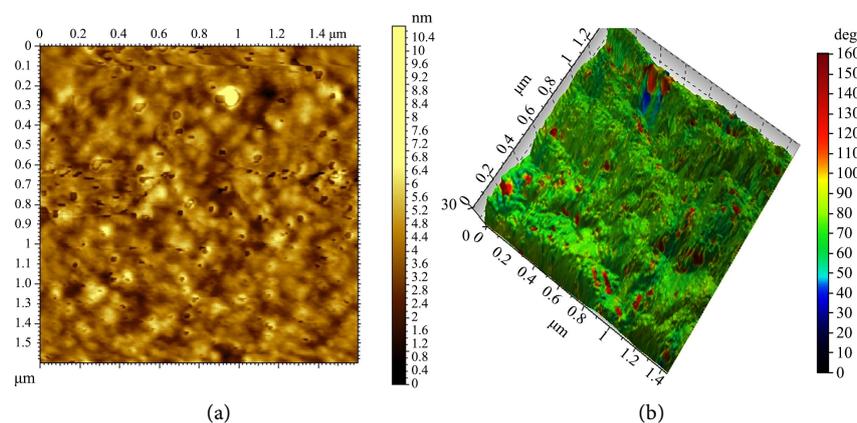
**Figure 1.** IR-spectra: (a)-native; (b)-purified chitosan,  $N_6$ -8.48%, ash-1.58, solubility-98.8%,  $I_k$ -36%.

On the diffractogram of purified chitosan, the degree of crystallinity of the samples decreased from 41% to 36% - 39%, which indicates the increased amorphization of the obtained chitosan samples. Nitrogen content and solubility of the obtained samples significantly increased and reached 8.48% and 98.8%, respectively. A decrease in the ash content of the samples was almost twofold, indicating an increasing of the purity of sample. The degree of deacetylation of the purified samples was 75%, the molecular weight of the purified chitosan samples decreased and waved from  $120 \times 10^3$  g/mol to  $130 \times 10^3$  g/mol.

The formation and use of polymer nanomaterials is widely demanded in science, industry, biotechnology and medicine in connection with the ability to achieve a significant improvement in the physicochemical, mechanical, barrier properties of polymers and use them as carriers of drug substances, which is especially promising for biodegradable polymers such as chitosan and their derivatives. Extremely important and relevant is the development of experimental methods for creating nanoparticles (NPs) and nanostructures in such polymers, determining the possibilities of their chemical and physical modification, the characteristics of molecular and supramolecular organization and obtaining polymer materials with unique properties on their basis. Owing to their unique features, such as a large specific surface of nanoparticles, they exhibit properties different from ordinary polymers. This opens up great opportunities for using them in various fields of science and technology, in particular when creating medicinal substances. As a result, we synthesized NPs based on chitosan *Bombyx mori*.

Synthesis of nanochitosan from purified chitosan was carried out by the method of fractional precipitation in the presence of a surface modifier [14]. The proposed method for the preparation of nanoparticles of *Bombyx mori* chitosan includes the preparation of a solution of pre-purified chitosan in 2% acetic acid, the addition of solutions of alkali metal hydroxides (Na or K) to pH 9 - 10 at the temperature of 20°C - 25°C in the presence of a surface modifier preventing aggregation of nanoparticles of chitosan, fractionation of the dispersion obtained by settling in a separatory funnel into fractions with different nanoparticle sizes. To stabilize chitosan nanoparticles the surface modifier polyoxyethylene sorbitol monooleate (TWEEN-80) has been selected from the group of non-ionic surfactants. The dispersion is then centrifuged at 6000 - 7000 rpm. The resulting precipitate was washed with distilled water to neutral pH. Then, the precipitate was freeze-dried using an ALPHA 1 - 2 LD *plus* instrument at - (50 - 55)°C and P = 0.3 - 0.5 mbar. Chitosan nanoparticles were obtained as a light-creamy friable powder with a particle size of 20 to 800 nm.

When nanochitosan is produced from the initial purified chitosan, the molecular weight decreases more than twice, and the solubility of nano-Chs reaches 99%. The production of nano-Chs was confirmed by the AFM method. The particle size of nano-Chs without ultrasonic dispersion was in the range of 90 - 200 nm. The method of ultrasound provided nano-Chs-P with sizes from 20 to 100 nm (Figure 2).



**Figure 2.** Nano-Chs BM with stabilizer + UZD: Dimensions of nano-Chs-P: length—120 nm, width—7 nm.

The obtained polymers of chitosan and its nanostructured form of various concentrations were tested for the sensitivity of various strains of microorganisms *in vitro*. The sensitivity test of microorganism strains was carried out with 3 groups of microorganisms: gram-positive, gram-negative and fungi. It was shown that depending on the concentration of Chs it has an antibacterial effect on the microorganisms *Klebsiella* and *Actinomyces* at 0.1%, on *Proteus vulgaris* at 0.5% concentration. It should be noted that with an increasing of chitosan concentration to 1%, an antibacterial effect is also observed on the anaerobic bacteria *Pseudomonas aeruginosa*.

Tests for antibacterial activity of nano-Chs found that the drug had a pronounced effect on almost all groups of microorganisms: *Staphylococcus saprophyticus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Escherichia coli LP*, *E.coli LN*, *Proteus vulgaris*, *Klebsiella*, and *Actinomyces* regardless of concentration.

Based on these studies, it can be concluded that Nano-Chs is a broad-based drug. One of the positive actions of nano-Chs is its anti-staphylococcal effect. In a concentration of 1.0% nano-chitosan has a pronounced effect on the entire staphylococcal group: *S. aureus*, *S. epidermidis*, *S. saprophyticus*, and *St. pyogenes*, which, of course, will be important in the treatment of purulent-inflammatory diseases [15].

The influence of chitosan and nanochitosan on immunological parameters was determined: the immune response to the erythrocyte antibody titre of the sheep, in the blood serum of mice, the number of cells in the central and peripheral organs of immunity, the number of erythrocytes and leukocytes in peripheral blood of mice.

The effect of Nano-Chs-P on the state of peripheral immune organs shown that with the introduction of Nano-Chs-P the total number of thymocytes significantly increased for 1.29 times  $(45.9 \pm 1.8) \times 10^6$ , in control  $-(35.7 \pm 1.4) \times 10^6$  cells. Thus, Nano-Chs-P is able to increase the number of cells in the central body of immunity - thymus (Table 1).

**Table 1.** Effect of nanopreparations on the number of cells in the central and peripheral immune organs in mice ( $M \pm m$ ,  $n = 6$ ).

№	Group	Dose, mg/kg	Thymus cells $\times 10^6$	IP	Bone marrow cells $\times 10^6$	IP	Lymphoidal cells $\times 10^6$	IP
1	Control	-	$42.2 \pm 2.1$	-	$9.7 \pm 0.4$	-	$26.7 \pm 1.5$	-
2	Chitosan	100.0	$39.3 \pm 2.1$	-1.07	$18.8 \pm 0.5^a$	+1.94	$26.3 \pm 1.4$	-1.02
3	Control	-	$35.7 \pm 1.4$	-	$10.6 \pm 0.3$	-	$19.5 \pm 0.3$	-
4	Nano-Chs	100.0	$45.9 \pm 1.8^a$	+1.29	$13.3 \pm 0.3^a$	+1.25	$25.7 \pm 0.4$	+1.32

<sup>a</sup>significantly to 1 g.

Similar data were obtained in the study of the effect of Nano-Chs on another central immune body—the bone marrow. In the control group, the number of cells in the bone marrow was  $(10.6 \pm 0.3) \times 10^6$ . Under the influence of Nano-Chs, the number of cells in the bone marrow increased significantly by a factor of 1.25  $(13.3 \pm 0.3) \times 10^6$ . Consequently, the bone marrow, like the thymus, was sensitive to the stimulating effect of Nano-Chs [16].

The influence of Chs and Nano-Chs on some hematological parameters of peripheral blood of mice (erythrocytes, leukocytes) was studied (Table 2).

In the control group, the number of blood leukocytes was  $(6.8 \pm 0.2) \times 10^6/\text{ml}$ . Under the influence of chitosan, the number of leukocytes significantly increased for 1.38 times  $(9.4 \pm 0.3) \times 10^6/\text{ml}$ , the number of erythrocytes significantly increased for 1.45 times  $(7.1 \pm 0.2) \times 10^9/\text{ml}$ , *i.e.* chitosan increased the proliferation of hematopoietic cells in the body of experimental mice. Nano-Chs increases the number of erythrocytes for 1.08 times, and the number of leukocytes for 1.09 times.

On the basis of the obtained data, it can be concluded that the studied systems based on Chs and Nano-Chs possess of immunostimulating properties: they increase the number of AFC (antibody-forming cells) in the spleen, the total number of cells in the thymus, bone marrow, lymph nodes, and the level of erythrocytes and white blood cells of mice.

Investigations on the creation of bioactive nanocoatings based on *Bombyx mori* chitosan on titanium implants for dentistry have been conducted.

Calcium-phosphate coatings on dental implants are known to accelerate bone growth and improve bone fixation [17] [18]. In addition, their low surface tension, as well as their high degradability, can help prevent delamination of the coating under natural conditions [19] [20].

An increase in biocompatibility can be achieved by electrolytic coating due to the introduction of certain biologically active agents, such as chitosan.

It was found that chitosan enhances the differentiation of oste (base) cells, supports the expression of extracellular matrix proteins using human osteoblasts and chondrocytes [21]. The inclusion of chitosan in electrolytically deposited TCF will improve the biocompatibility of the coating, while retaining its original mechanical properties.

**Table 2.** Effect of chitosan and nanochitosan on leukocytes and red blood cells number of the mice peripheral blood.

Group	Dose, mg/kg	Red blood cells $\times 10^9/\text{ml}$	IC	White blood cells $\times 10^6/\text{ml}$	IC
Control	-	$4.9 \pm 0.1$	-	$6.8 \pm 0.2$	-
Chitosan	100.0	$7.1 \pm 0.2^*$	+1.45	$9.4 \pm 0.3^*$	+1.38
Control	-	$6.6 \pm 0.2$	-	$7.4 \pm 0.3$	-
Nano Chitosan	100.0	$7.1 \pm 0.2$	+1.08	$8.1 \pm 0.2$	+1.09

Studies on the creation of bioactive nanocoats on the basis of chitosan *Bombyx mori* on titanium implants for dentistry have been carried out. An electrolytically deposited coating of tricalcium phosphate/chitosan on titanium plates was prepared. A solution of chitosan was prepared for electrolytic deposition of chitosan on a titanium plate, which is added to a supersaturated 0.2 M solution of tricalcium phosphate (TCF)  $-\text{Ca}_3(\text{PO}_4)_2$ . Precipitation was made at  $52^\circ\text{C}$  for 15 hours in a supersaturated buffer solution with  $\text{pH} = 6.6$  and a current of  $2.0 \text{ mA}/\text{cm}^2$ .

The coating was examined by scanning electron microscopy (SEM). As can be seen from **Figure 3** the coating deposited on the surface of the titanium plate consists of TCF beads with a diameter of  $10 - 30 \mu\text{m}$ . The chitosan film is aggregated around the TCF beads. These aggregations of chitosan are integrated with both calcium phosphate balls and with each other, forming cross-linked structures (**Figure 3**).

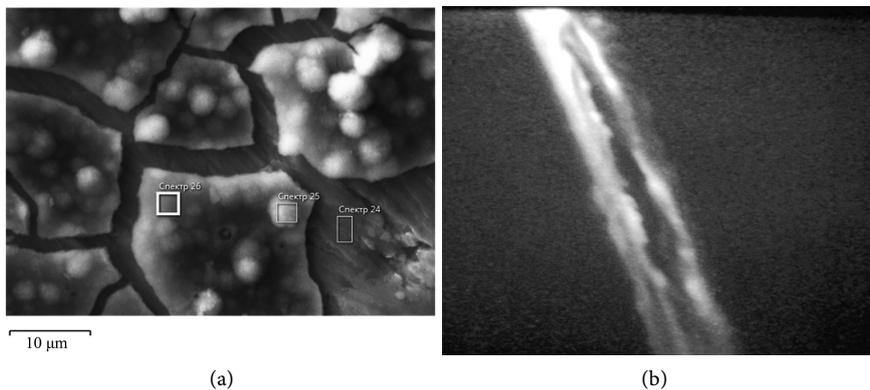
The quantitative spectrum of the content of elements in the titanium plate showed its homogeneity, with a predominant content of elements of Ti-96.2% and an insignificant content of other inorganic impurities in the form of Al, Mn and the residual content of Ca elements (**Figure 4(a)**).

In **Figure 4(b)**-The spectrum of the  $\text{Ca}_3(\text{PO}_4)_2$  beads is shown. As can be seen from the spectrum, the Ti content sharply decreased to 7.1% (almost 13.5 times), which indicates an almost complete coverage of the titanium plate by the TCF layer. The content of Ca was  $-25.5\%$ , phosphorus- $12.7\%$  and O- $53.7\%$ , which agrees well with the chemical formula of TCF.

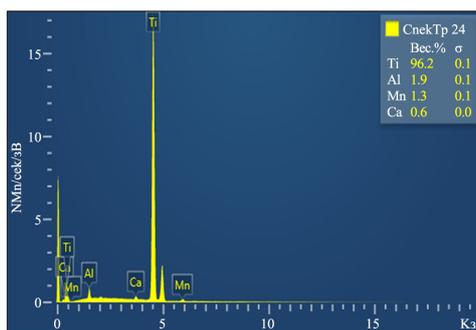
In **Figure 4(c)**, the spectrum of a chitosan film in which the appearance of carbon in the amount of  $-9.9\%$ , which indicates the presence of a polymer of chitosan on this coating, and the presence of calcium-Ca, phosphorus-P and oxygen, which characterize the presence of a combined coatings Chs-TCF

Results of histo-morphological studies of bone tissue are shown in **Figure 5**. **Figure 5(a)**-Formation of bone tissue after application of osteoplastic material 1 month after implantation of dental implants with deposition of bioactive layer: in the bone tissue of rabbits, a significant 40% - 50% filling of the implant surface outside with a loose, sometimes dense connective tissue was noted. There are fibroblasts with collagen fibers leaving from them.

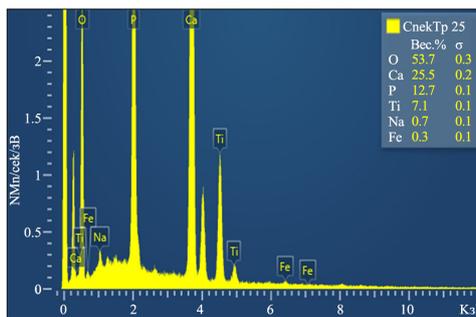
**Figure 5(b)**-2 months after implantation: in the regeneration zone, a formed lamellar bone tissue is found. On the periphery, active osteoblasts are determined. In the regeneration zone, active macrophages and osteoclasts are found. In the interosseous cells, the elements of the bone marrow are determined.



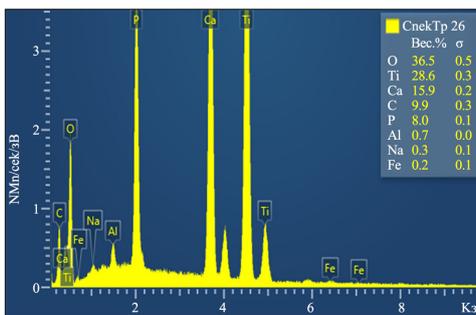
**Figure 3.** Chs-TCF coating image on a titanium plate, scale-10 microns. (a) CHS-Ca/P on a titanium plate, scale-10 μm; (b) SEM-image coating of the end of the plate: the outer layer of Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, the inner (dark) layer of chitosan.



(a)-titanium plate

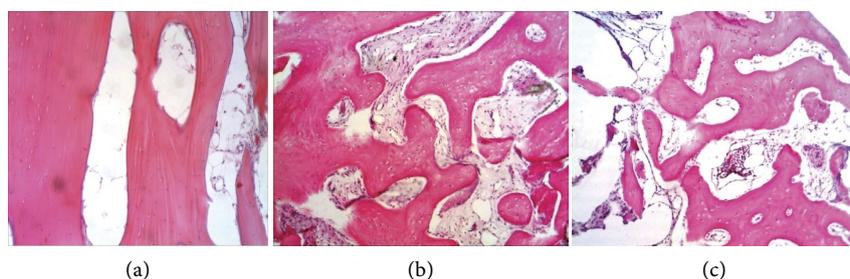


(b)-Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>



(c)-Spectrum of chitosan

**Figure 4.** The quantitative spectrum of the content of elements in a titanium plate (a); TCF coated titanium plate (b); Chs + TCF coated titanium plate (c).



**Figure 5.** Results of histo-morphological studies of bone tissue: (a)-1 month after implantation; (b)-2 months after implantation; (c)-3 months after implantation.

The osteoplastic material is gradually replaced by a connective tissue and forming bone beams. In the interosseous cells, the elements of the bone marrow are determined.

**Figure 5(c)**-mature bone, remote from the implantation zone after 3 months of experiments: formation of bone beads of the lamellar bone is observed. Between the bone beams there are channels with loose unformed connective tissue containing fibroblasts.

### 3. Conclusions

The synthesis of nano chitosan *Bombyx mori*, a particle size of 50 -200 nm was carried out. It was revealed that preparations based on Chs and nano chitosan have a pronounced antibacterial activity, which mainly refers to nano chitosan. The nano chitosan had a pronounced effect on almost all groups of microorganisms: *St. saprofiticus*, *Str. pyogens*, *Ent. faecalis*, *Esch. Coli LP*, *Esch. Coli LN*, *Prot. vulgaris*, *Klebsiella*, *Aktinomitset*, regardless of its concentration. Nano chitosan has the ability to significantly increase the immune response of living systems: it increases the number of cells in the central (thymus, bone marrow) and in peripheral (lymph nodes) immune organs in mice.

Bioactive nanopolymer coating tricalcium phosphate + chitosan on dental titanium implants was created. Morphological analysis of bone tissue after implantation of implants with a deposition of bioactive layer allowed to evaluate the effect of coating on the process of reparative regeneration. Restoration of defects in bone tissue occurs as complete healing.

### Acknowledgements

The authors want to thank Dr. F.K. Usmonov (Tashkent State Dental Institute, Uzbekistan) for the histo-morphological investigations.

### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

### References

- [1] Muzzarelli, R.A.A. and Muzzarelli, C. (2002) Chitosan in Pharmacy and Chemistry,

- Atec, Italy.
- [2] Schuetz, Y.B., Gurny, R. and Jordan, O. (2008) A Novel Thermoresponsive Hydrogel Based on Chitosan. *European Journal of Pharmaceutics and Biopharmaceutics*, **68**, 19-25. <https://doi.org/10.1016/j.ejpb.2007.06.020>
  - [3] Fischer, S., Foerg, C., Merkle, H.P. and Gander, B. (2004) Chitosan Coated Plga-Microspheres—A Modular System for Targeted Drug Delivery. *European Cells and Materials*, **7**, 11-12.
  - [4] Andrianova, I.E. (2001) New Advances in the Study of Chitin and Chitosan: Mater. *VI International Conference*, Moscow-Schelkovo, VNIRO, Moscow, 22-24 October 2001, S.126-S.127.
  - [5] Bygchkov, A.V., Byghkova, V.M. and Krivosheina, L.I. (2003) New Advances in the Study of Chitin and Chitosan: Mater. *VII International Conference*, St. Petersburg-Repino, VNIRO, Moscow, 15-18 September 2003, S156-S157.
  - [6] Jeon, Y.J., Park, P.J. and Kim, S.K. (2001) Antimicrobial Effect of Chitoooligosaccharides Produced by Bioreactor. *Carbohydrate Polymers*, **44**, 71-76. [https://doi.org/10.1016/S0144-8617\(00\)00200-9](https://doi.org/10.1016/S0144-8617(00)00200-9)
  - [7] Qi, L.L., Xu, Z.R., Jiang, X., Hu, C. and Zou, X.F. (2004) Preparation and Antibacterial Activity of Chitosan Nanoparticles. *Carbohydrate Research*, **339**, 2693-2700. <https://doi.org/10.1016/j.carres.2004.09.007>
  - [8] Rabea, E.I., *et al.* (2003) Chitosan as Antimicrobial Agent: Applications and Mode of Action. *Biomacromolecules*, **4**, 1457-1465. <https://doi.org/10.1021/bm034130m>
  - [9] Ravi Kumar, M.N.V. (2000) A Review of Chitin and Chitosan Applications. *Reactive & Functional Polymers*, **46**, 1-27. [https://doi.org/10.1016/S1381-5148\(00\)00038-9](https://doi.org/10.1016/S1381-5148(00)00038-9)
  - [10] Milusheva, R.Yu., Rakhmanova, V.N., Inoyatova, F.K. and Rashidova, S.Sh. (2012) Synthesis of Chitosan Derivatives of *Bombyx mori* and Their Use in Diseases of Various Etiologies. *XI International Conference of Modern Prospects in the Study of Chitin and Chitosan*, Murmansk, June 25-30 2012, 69-72.
  - [11] Milusheva, R.Yu., Ibragimov, K.S. and Rashidova, S.Sh. (2014) Bioactive Polymers from Waste from Silk Production. XII International Conference of Current Prospects in the Study of Chitin and Chitosan, (RosHit, Perm, Tentorium Hall.), 230-232.
  - [12] Vasquez, D., Milusheva, R., Baumann, P., Constantin, D., Chami, M. and Palivan, C. (2014) The Amine Content of PEGylated Chitosan *Bombyx mori* Nanoparticles Acts as a Trigger for Protein Delivery. *Langmuir*, **30**, 965-975. <https://doi.org/10.1021/la404558g>
  - [13] Millusheva, R.Yu., Pirniyazov, K.K. and Rashidova, S.Sh. (2016) Purification of Chitosan *Bombyx mori*. *Journal of Bulletin of Tver State University, Series: Chemistry*, No. 2, 119-124.
  - [14] Millusheva, R.Yu. and Rashidova, S.Sh. (2017) Bioactive Properties of Nanochitosan *Bombyx mori*. *Polymer Science, Series C*, **59**, 29-34. <https://doi.org/10.1134/S1811238217010088>
  - [15] Rashidova, S.Sh. and Millusheva, R.Yu. (2009) Chitin and Chitosan *Bombyx mori* Synthesis, Properties and Application. Tashkent, FAN, 246.
  - [16] Millusheva, R.Yu., Muhamedov, I.M., Batirbekov, A.A. and Rashidova, S.Sh. (2016) Nanochitosan *Bombyx mori* Synthesis and Aspects of Biomedical Application. Oral Report at the XIII International Conference of Modern Prospects in the Study of Chitin and Chitosan, (RosHit-2016).

- [17] Clemens, J.A., Klein, C.P., Vriesde, R.C., Rozing, P.M. and de Groot, K. (1998) Healing of Large (2mm) Gaps around Calcium Phosphate-Coated Bone Implants: A Study in Goats with a Follow-up of 6 Months. *Journal of Biomedical Materials Research*, **40**, 341-349. [https://doi.org/10.1002/\(SICI\)1097-4636\(19980605\)40:3<341::AID-JBM1>3.0.CO;2-F](https://doi.org/10.1002/(SICI)1097-4636(19980605)40:3<341::AID-JBM1>3.0.CO;2-F)
- [18] Moroni, A., Aspenberg, P., Toksvig-Larsen, S., Falzarano, G. and Giannini, S. (1998) Enhanced Fixation with Hydroxyapatite Coated Pins. *Clinical Orthopaedics and Related Research*, **346**, 171-177. <https://doi.org/10.1097/00003086-199801000-00024>
- [19] Ban, S. and Maruno, S. (1993) Deposition of Calcium Phosphate on Titanium by Electrochemical Process in Simulated Body Fluid. *Japanese Journal of Applied Physics*, **32**, 1577-1580. <https://doi.org/10.1143/JJAP.32.L1577>
- [20] Habibovic, P., Barrère, F., van Blitterswijk, C.A., de Groot, K. and Layrolle, P. (2002) Biomimetic Hydroxyapatite Coating on Metal Implants. *Journal of the American Ceramic Society*, **85**, 517-522. <https://doi.org/10.1111/j.1151-2916.2002.tb00126.x>
- [21] Lahiji, A., Sohrabi, A., Hungerford, D.S. and Frondoza, C.G. (2000) Chitosan Supports the Expression of Extracellular Matrix Proteins in Human Osteoblasts and Chondrocytes. *Journal of Biomedical Materials Research*, **51**, 586-595. [https://doi.org/10.1002/1097-4636\(20000915\)51:4<586::AID-JBM6>3.0.CO;2-S](https://doi.org/10.1002/1097-4636(20000915)51:4<586::AID-JBM6>3.0.CO;2-S)