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Microstructure and Mechanical Strength Properties of Chitosan Sponges Obtained from Polymer Solutions in Carbonic Acid

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Abstract—Polymer sponges based on chitosan are first obtained from chitosan solutions in carbonic acid and gels based on these solutions crosslinked by a noncytotoxic agent of natural origin, genipin. A comparative analysis of the structure and mechanical strength properties of sponges prepared from chitosan solutions in carbonic and acetic acids is carried out. It is shown that the addition of genipin in an amount of ~2 wt % to a chitosan solution in carbonic acid leads to a decrease in the average pore size by ~2.5 times and a significant increase in the strength characteristics of the material in comparison with the sponge prepared without genipin.

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INTRODUCTION

Currently, much attention of the scientific community is directed to materials obtained from renewable resources, in particular, from natural polymers and their derivatives, such as chitosan, collagen, and alginate. This is especially true for materials used in biomedicine where it is necessary to maintain biocompatibility and antimicrobiality, for example, in porous scaffolds for tissue engineering or matrices with encapsulated active substances [1, 2]. Thus, one of the promising areas is the creation of patches for transdermal drug delivery (TDL) which during the time the material is on the patient's skin are able to diffuse into the bloodstream [3].

The deacetylated chitin derivative, chitosan, is a polysaccharide widely used for the manufacture of biomedical materials, including TDL materials, in the form of porous sponges, microgranules, hydrogels, and films [4]. A polymer porous sponge made of chitosan is a particularly convenient material for contact with the skin. Mineral inorganic and some organic acids are used as solvents for processing this polymer into new forms of biomaterials. The “classic recipe” for producing porous chitosan sponges includes the stages of dissolving chitosan (1–2 wt %) in dilute acetic acid solution (1–2 vol %), freezing, and freeze-drying [5]. Despite a low content of acid in such materials, allergic reactions may develop on the skin upon contact with them. Therefore, it becomes extremely important to develop new methods for processing this polymer and to search for new media for its dissolution.

An alternative method for preparing chitosan solutions is the dissolution of the polysaccharide in water saturated with CO₂ under high pressure [6]. Carbonic acid is formed in water saturated with carbon dioxide, and under pressure, acidity as high as pH 2.8 can be reached at a temperature of 25°C and a pressure of 30 MPa [7]. An important feature of this method is that it allows obtaining of a biomaterial bypassing the stage of chemical neutralization. Indeed, the acid formed upon high-pressure contact of water with CO₂ is self-neutralized by simple pressure release to atmospheric. In addition, this biomaterial goes through the sterilization stage in parallel, since carbonic acid has an inactivating effect on microorganisms [8]. These features favorably distinguish this solvent as a medium for the preparation of chitosan sponges for use where high-purity materials are required.

It is important to note that porous sponges made exclusively of chitosan without any low or high molecular weight additives do not possess mechanical properties necessary for a number of medical tasks; in particular, they have low elasticity and strength [5, 6]. Meanwhile, the mechanical stability of TDL materials is especially important during their operation.

To improve the mechanical properties of polymer sponges based on chitosan, polymer chains are crosslinked using aldehydes (e.g., glyceraldehyde and glutaraldehyde), carbodiimides, and ionic crosslinkers [9–12]. To date, there are controversial data on the toxicity of widely used synthetic crosslinking agents. This is especially true for aldehydes. In any case, when

using them, it is necessary to control the residual content of unbound crosslinker and the appearance of possible byproducts resulting from side reactions occurring during crosslinking [13, 14].

Genipin is a chemical compound found in the gardenia fruit extract. It is obtained by the hydrolysis of geniposide, genipin β -D-glucopyranoside, extracted from gardenia fruits using β -glucosidase [15]. Genipin is a nontoxic bifunctional crosslinking agent with cytotoxicity many times lower than that of the common crosslinker, glutaraldehyde [16]. Genipin crosslinks primary amino groups. It is extensively studied for crosslinking both 2D gels and 3D scaffolds based on amino-containing polymers, such as chitosan, collagen, and gelatin [17]. The use of this crosslinking agent to obtain biocompatible mechanically strong TDL materials is extremely justified [18–20]. However, for the manufacture of patches or condensed polymer films with mechanical properties corresponding to a particular applied problem, there is need to choose the optimal ratio of genipin and polymer and the crosslinking time [21].

The purpose of this work was to demonstrate the fundamental possibility of obtaining chitosan sponges from polymer solutions in carbonic acid, water saturated with carbon dioxide under high pressure, to study their internal microstructure, porosity, and mechanical strength properties and to compare these data with the parameters of polymer sponges obtained from a traditional solvent, acetic acid.

EXPERIMENTAL

We used chitosan powder (catalog number 448877, Sigma-Aldrich) and genipin (catalog number 4796, Sigma-Aldrich), high-purity CO_2 (99.995%, Moscow Gas Processing Plant), water purified before each experiment on a Millipore Milli-Q installation, and chemically pure glacial acetic acid and sodium hydroxide.

Purification of Chitosan

Chitosan was purified by reprecipitation of the polymer in its base form. Chitosan (1 wt %) was dissolved in a 0.5 mol/L hydrochloric acid solution. The acid-insoluble precipitate was separated by centrifugation at 10 000 rpm. At the next stage, the pH of the solution was brought to 5.4 by adding dropwise a 0.5 mol/L sodium hydroxide solution. The prepared chitosan solution was precipitated with 0.05 mol/L sodium hydroxide solution under vigorous stirring. The precipitate was centrifuged, repeatedly washed with distilled and deionized water (final washing), and freeze-dried at a residual pressure of 12 Pa.

The molecular weight of chitosan after reprecipitation was determined by the viscometric method. The polymer was dissolved in an acetate buffer consisting

of 0.3 mol/L acetic acid and 0.2 mol/L sodium acetate. The efflux times of the buffer and polymer solutions of different concentrations were measured on an Ubbelohde viscometer with a capillary diameter of 0.54 mm. The viscosity average molecular weight was calculated using the Mark–Houwink equation at $K = 0.074$ and $a = 0.76$ [22]. The degree of deacetylation of the reprecipitated chitosan was determined by linear potentiometric titration according to the procedure described in detail in [23].

Obtaining Polymer Sponges by Casting Chitosan Solutions in Acetic Acid

The reprecipitated chitosan (100 mg) was dissolved in an aqueous solution of 0.2 mol/L acetic acid (10 mL) under stirring for a day. The resulting chitosan solution was poured into Petri dishes 5.6 cm in diameter and frozen at -25°C for two days. The resulting samples were freeze-dried in an Alpha 1-2 LD unit (Christ, Germany) at -50°C and a residual pressure of 12 Pa.

Obtaining Polymer Sponges by Casting Chitosan Solutions in Carbonic Acid

Ten milliliters of deionized water and 100 mg of reprecipitated chitosan were loaded in a 30-mL autoclave. At a temperature of 25°C , the reactor was filled with CO_2 to create a pressure of 30 MPa. The contents of the reactor were stirred on a magnetic stirrer for 7 days at room temperature. Thereafter, the autoclave was slowly decompressed at a rate of 0.5 MPa/min. As a result, a chitosan solution in carbonic acid was obtained. Polymer sponges were prepared by casting the solution in Petri dishes, freezing, and freeze-drying.

Obtaining Chitosan Sponges Crosslinked by Genipin

To prepare a chitosan sponge crosslinked by genipin, 500 μL of 0.32 wt % genipin solution (1.6% in terms of chitosan weight) was added dropwise to a chitosan solution in carbonic acid. According to [21], such a ratio of genipin and polymer should provide the maximum strength of the sponges. The solution was left stirring on a magnetic stirrer for 10 min. The solution was then poured into Petri dishes, frozen, and freeze-dried.

Methods for Studying the Structure of Chitosan Sponges

The IR spectra of chitosan sponges were measured on a Thermo Nicolet Nexus IS5 FT-IR spectrometer (United States). The spectra were taken on a multiple attenuated total internal reflection accessory using a zinc selenide crystal.

The morphology of transverse cleavages of freeze-dried chitosan sponges was studied by low-voltage scanning electron microscopy on a Scios microscope

(FEI, United States) at an accelerating voltage of 1 kV in the secondary electron mode. Samples were cleaved after freezing in liquid nitrogen immediately before imaging. The surface of the cleavage perpendicular to the plane of the sample was investigated. The fraction of pores on the cleavage surface and the porosity were estimated from electron microscopic images using the Image J program.

Study of Mechanical Strength Properties of Chitosan Sponges

The mechanical strength properties of polymer sponges were studied on a TA.XT Plus texture analyzer (Texture Technologies, United States). A sponge sample 40 mm long, 10 mm wide, and 0.3 mm thick was used for testing. The stress–strain curves were measured for three samples, and using the data obtained, the average strength characteristics were calculated. The tensile strength of the sponge σ , i.e., the maximum stress that the material can withstand under tension, was estimated from the ratio

$$\sigma = \frac{F}{S}, \quad (1)$$

where F is the load at failure and S is the cross-sectional area of the sponge.

Elongation at break of the sponge ε was estimated through the equation

$$\varepsilon = \frac{\Delta l}{l}, \quad (2)$$

where Δl is the absolute elongation of the sample before rupture and l is the original length of the sample.

The Young modulus E was determined from the ratio

$$E = \frac{\sigma}{\varepsilon}. \quad (3)$$

Porosity of Chitosan Sponges

The porosity of the sponges was determined by filling the void space of the sponge with 95% ethyl alcohol. The polymer sponge sample with the known geometric parameters and weight was immersed in a vial with alcohol. After 5 min, the sample was removed from the vial, excess ethanol was removed using filter paper, and the sample was weighed. Porosity ϕ was calculated by the formula

$$\phi = \left(\frac{m_f - m_i}{\rho V} \right) \times 100\%, \quad (4)$$

where m_f is the weight of the sponge after exposure in alcohol, m_i is the weight of the initial sponge, V is the volume of the sponge calculated from the geometric parameters of the sample, and ρ is the density of 95% ethyl alcohol.

Table 1. Samples of chitosan sponges obtained in the work

Sample code	Source system
CTS_AA	Chitosan solution in 0.2 mol/L acetic acid
CTS + Gen_AA	Chitosan solution of in 0.2 mol/L acetic acid with the addition of genipin (1.6% in terms of the chitosan weight)
CTS_CA	Chitosan solution in carbonic acid
CTS + Gen_CA	Chitosan solution in carbonic acid with the addition of genipin (1.6% in terms of the chitosan weight)

RESULTS AND DISCUSSION

At the first stage, the commercial chitosan preparation was purified by reprecipitation and characterized. The molecular weight of chitosan determined by viscometry was 283×10^3 . According to the potentiometric titration data, the degree of deacetylation, that is, the molar fraction of *D*-glucosamine units, was 0.73 ± 0.04 .

As a result of chitosan dissolution, subsequent casting, solution freezing, and freeze-drying, polymer sponges were obtained (Fig. 1; Table 1). Acetic and carbonic acids were used as solvents for chitosan.

For a medical object (e.g., for a patch used as a bio-compatible matrix for the introduction of bioactive substances through the skin or for a material for tissue engineering), structural stability, that is, the ability to withstand various mechanical impacts that inevitably arise during the life of the patient, is very important. To investigate the mechanical properties of polymer sponges stress–strain curves were taken for them (Fig. 2).

It was found that the tensile strength for the sponge obtained from 1 wt % chitosan solution in acetic acid (0.05 ± 0.01 MPa) coincides with that for the sponge prepared from a carbonic acid solution of the same concentration (0.05 ± 0.02 MPa).

The obtained σ values agree with the data on the tensile strength of chitosan sponges, 0.01–0.07 MPa [24–26]. To increase the strength of chitosan sponges, the polymer was crosslinked in solution using a natural crosslinking agent, genipin.

A small addition of genipin (1.6% in terms of the chitosan weight) to a chitosan solution in carbonic acid makes it possible to obtain a much stronger and harder material (increase in the ultimate stress for $\Delta\sigma = 40 \pm 15\%$ and decrease in rupture strain for $\Delta\varepsilon = 150 \pm 20\%$) with the stress–strain curve characteristic of elastic materials (Fig. 2, Table 3). On the contrary, the crosslinking of chitosan by genipin in an acetic acid solution under the same conditions leads to a twofold decrease in the sponge strength.

The ATR IR method, which makes it possible to probe the composition of the near-surface layer of the sample, proved the presence of genipin in the chitosan matrix. It was also used to study the nature of interac-

Fig. 1. Schematic of obtainment of chitosan sponges from polymer solution in carbonic acid. Color drawings can be viewed in the electronic version.

Fig. 2. Stress–strain curves for chitosan sponges obtained from polymer solutions in carbonic and acetic acids: (1) CTS_CA, (2) CTS + Gen_CA, (3) CTS_AA, and (4) CTS + Gen_AA.

Fig. 3. IR spectra of chitosan sponges obtained from (a) carbonic acid and (b) acetic acid: (1) sponges without crosslinker and (2) sponges with the addition of 1.6 wt % genipin.

tion between macromolecules and the crosslinker. The penetration depth of the incident radiation is on the order of several microns and enables analysis of the composition of the substance in the volume of sponges.

The IR spectra of chitosan sponges obtained from carbonic acid solutions without crosslinker (CTS_CA) and with the addition of genipin (CTS + Gen_CA) are shown in Fig. 3a. The spectra of the sponges show absorption bands characteristic of the base form of chitosan; namely, bands at 3350 cm^{-1} are due to N–H and O–H stretching vibrations, bands at 1645 cm^{-1} are due to C=O bending vibrations (amide I), bands at 1551 cm^{-1} are due to N–H bending vibrations (amide II), and bands at 1159 cm^{-1} are due to C–O–C stretching vibrations in the glycosidic bond [27]. A decrease in the intensity of the band with a wavenumber of 1551 cm^{-1} which corresponds to N–H vibrations in the amino group and the shift of the amide I band to large wavenumbers indicate the appearance of a covalent bond between the amino group of chitosan and the olefinic carbon atom of the genipin molecule and the subsequent formation of a heterocyclic amine [28]. The spectra of sponges obtained from acetic acid solutions (CTS + GEN_AA) lack peaks and shifts indicating formation of the covalent bond between the amino group of chitosan and the olefinic carbon atom of the genipin molecule (Fig. 3b).

Such a difference in the spectra of genipin sponges prepared from chitosan solutions in carbonic and acetic acids is associated with the difference in the degree of crosslinking of polymer chains by genipin molecules. Indeed, it is known that the rate and efficiency of crosslinking of chitosan gels by genipin depends on the pH of solution [29]. The pH of the chitosan solution in acetic acid is 3.86 ± 0.05 . In contrast, the pH of the chitosan solution in carbonic acid rapidly increases after pressure release, and under the used experimental conditions, the pH is 5.70 ± 0.05 . With a decrease in the acidity of the medium, the proportion of free nonprotonated amino groups that are able to go into the crosslinking reaction with genipin molecules increases.

A significant difference in the strength of the sponges obtained from solvents of two types, in particular, an abnormal twofold decrease in strength when a crosslinker is added in an acetic acid solution, can be explained by influence of the conformational features of polymer chains in solution and the difference in the density of crosslinking of chains by genipin. Indeed, without a crosslinker, the strength of the sponges is approximately the same and is determined by physical crosslinks owing to the mutual interlacement of macromolecules in solution (Fig. 4a). When genipin is added, chemical crosslinks, that is, covalent intermolecular and intramolecular bonds between the amino groups of chitosan and genipin, are formed. Owing to intramolecular crosslinking, the conformation of macromolecules changes and domains develop.

In a carbonic acid solution where a high density of crosslinking of polymer chains is realized, interdomain binding is much stronger than that in an acetic acid solution (Figs. 4b, 4c). In the case of formation of numerous crosslinks, as in CA solution (Fig. 4c), the integral (total) binding of all macromolecules in the system is higher than that in solution without crosslinker. On the contrary, adding genipin to chitosan solution in acetic acid decrease the integral binding of macromolecules in the system (Fig. 4b) owing to a decrease in the total number of crosslinks. Actually, in

Table 2. Average pore size and fraction of pore surface area of transverse cleavages of polymer sponges

Sample	Average pore size, μm	The proportion of pores on a cleavage surface area of 3 mm^2 , %
CTS_AA	25 ± 1	4.5 ± 0.3
CTS + Gen_AA	20 ± 1	4.1 ± 0.3
CTS_CA	67 ± 3	9.4 ± 0.3
CTS + Gen_CA	25 ± 1	5.0 ± 0.3

Fig. 4. Conformations of chitosan macromolecules in aqueous solution (a) without crosslinker, (b) with genipin at a low crosslinking density (the case of chitosan crosslinking in acetic acid), and (c) with genipin at a high crosslinking density (the case of chitosan crosslinking in carbonic acid).

the absence of the crosslinker, chitosan macromolecules in solution and upon further freezing adopt an equilibrium conformation (Fig. 4a), and the number of physical crosslinks is much higher than that in the case of a weak genipin crosslinking where domains with chains in the nonequilibrium (compressed) conformation are weakly bound between themselves and with free macromolecules (Fig. 4b).

To analyze the features of the deformation behavior of the obtained sponges, the internal microstructure of the samples was investigated. Electron microscopic images of the surface of the transverse cleavage of freeze-dried sponges obtained from polymer solutions in acetic and carbonic acids are shown in Fig. 5. The microstructure of the chitosan sponge prepared from acetic acid solution contains both large pores up to 100 μm in size and pores with sizes on the order of a few microns, which significantly differs from the microstructure of the sponge obtained from carbonic acid solution (Figs. 5b, 5d), which contains pores of a much larger size (while maintaining small pores with a diameter of several microns). The average pore size in the sponge prepared from chitosan solution in carbonic acid is $67 \pm 3 \mu\text{m}$ which is almost 3 times higher than the average pore size in the sponge obtained from acetic acid solution (Table 2). In this case, the pore surface area of the CTS_CA sponge is 2 times higher than that of the CTS_AA sponge. This macroporous

architecture of the polymer sponge can be especially useful for tissue engineering applications [30]. The pores in the volume of the material after crosslinking the sponge with genipin somewhat decrease, which indirectly indicates the loss of mobility of macromolecules owing to chemical crosslinking, which also manifests itself as a slight decrease in the ultimate elongation of the sponges ($\Delta = 10 \pm 3\%$; Table 3).

Figure 5 shows that the microstructure of the chitosan sponge obtained from carbonic acid solution and crosslinked by genipin is close to the structure of the chitosan sponge crosslinked by genipin but prepared from acetic acid solution under normal atmospheric conditions. Thus, the genipin crosslinking of the sponge obtained from chitosan solution in carbonic acid makes it possible to achieve the standard pore size distribution for which the effectiveness of loading and prolonged delivery of drugs is proven (it is known that chitosan sponges obtained from acetic acid solutions are successfully used in drug delivery owing to their developed porous microstructure [31]). Note that sponges obtained from solutions in water saturated with CO_2 under high pressure have undeniable advantages: the solvent used is absolutely biocompatible and environmentally friendly and does not require postprocessing, for example, neutralizing the acid used to create the material.

Table 3. Mechanical strength characteristics and porosity of chitosan sponges obtained by freeze drying of polymer solutions in carbonic and acetic acids

Sample	σ , MPa	Strain at the beginning of rupture, %	Strain at the end of rupture, %	ϕ , %	E , MPa
CTS_AA	0.05 ± 0.02	4.5 ± 0.5	15 ± 1	78 ± 5	12.2 ± 0.4
CTS + Gen_AA	0.021 ± 0.001	3.7 ± 0.4	19 ± 2	80 ± 5	0.6 ± 0.1
CTS_CA	0.05 ± 0.01	5.0 ± 1.0	15 ± 5	85 ± 5	1.0 ± 0.1
CTS + Gen_CA	0.07 ± 0.01	4.5 ± 1.0	8 ± 4	85 ± 5	2.5 ± 0.2

Fig. 5. Electron microscopic images of the surface of cleavages of chitosan sponges obtained from polymer solutions in carbonic and acetic acids: (a) CTS_AA, (b) CTS + Gen_AA, (c) CTS_CA, and (d) CTS + Gen_CA.

CONCLUSIONS

It was first shown that chitosan sponges with good mechanical strength properties can be obtained from polymer solutions in a biocompatible, self-neutralizing solvent, carbonic acid. The comparative study of the morphology and mechanical strength characteristics of the sponges obtained from carbonic acid solution and in the solvent traditional for chitosan, acetic acid, was carried

out. It is shown that chitosan sponges prepared from a polymer solution in carbonic acid are characterized by the presence of large pores with sizes up to 300 μm , which is twice as large as that for sponges obtained from acetic acid. It was found that the crosslinking of the sponges by genipin leads to a decrease in the average pore size for both sponges, while for the sponge obtained from carbonic acid the average pore size decreases by a factor of 2.5. It is

demonstrated that the mechanical strength characteristics of the polymer sponges obtained from carbonic acid solutions and additionally crosslinked by genipin are significantly better than those of traditional composites prepared from acetic acid solutions at normal atmospheric pressure. In particular, their Young modulus is 2–4 times higher. Such mechanical characteristics will make it possible to use new porous chitosan materials even under difficult operating conditions, for example, as a component of a patch with bioactive substances on the human skin.

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