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MACROMOLECULAR COMPOUNDS AND POLYMERIC MATERIALS

Obtaining and Properties of L-Aspartic Acid Solutions of Chitosan

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Abstract—Conditions for obtaining L-aspartic acid solutions of chitosan were considered: dissolution of the polymer with the formation of chitosan aspartate occurs in pH range of 3.6–3.8 at a minimum stoichiometric ratio of [acid]/[chitosan ($–NH_2$)] ~ 0.43. The hydrodynamic, electrochemical, optical and biological properties of aqueous solutions of chitosan aspartic acid salt are investigated. It has been established that macromolecules of polymeric salt exhibit properties of a polyelectrolyte with a partially compensated charge in an aqueous medium. Huggins constants and temperature viscosity coefficients were calculated, which indicate an increased rigidity of the chitosan macrochain in the studied solutions and deterioration in the polymer–solvent interaction with increasing temperature. Chitosan aspartate powders in the form of lamellar microparticles with fractal ordering were isolated. Low biocompatibility and antibacterial activity of the polymeric salt are demonstrated, which allows recommending the production of biomedical products on its basis.

Keywords: chitosan; L-aspartic acid; degree of protonation; solutions; polyelectrolyte

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Chitosan belongs to the class of linear semi-rigid chain polysaccharides of copolymer structure. Due to its high melting point, significantly exceeding the temperature of thermal decomposition, the study of the properties and processing of this polymer into articles is carried out in solution.

Chitosan dissolves in aqueous acidic media at pH < 5.5–6.0 due to protonation of ionic amino groups with the formation of water-soluble salt complexes (polymeric salts). To dissolve chitosan, aqueous solutions of monobasic carboxylic acids: acetic, formic, are most often used, and mineral hydrochloric acid, less often [1, 2]. It is possible to dissolve chitosan even in the presence of unsaturated carboxylic acids: oleic, linoleic [3], of aromatic acids: benzoic [4], salicylic [5], as well as in the presence of biologically active acids promising in medical applications: ascorbic [6, 7], citric [8], azelaic, succinic, adipic [9], carbonic [10]. The latter also include one of the 20 proteinogenic acids of the body—aliphatic L-aspartic amino acid (Asp). In an aqueous solution, Asp exists in the form of an equilibrium mixture of a dipolar ion, cationic and anionic forms. The values of the dissociation constants are pK_a 2.1, 3.9, and 9.8 [11].

The nature of the acid has an important effect both on the dissolution of chitosan in an aqueous acidic medium, and on the physicochemical characteristics of polymeric salts and their solutions [12]. For example, in strong hydrochloric acid, the degree of protonation (α) of chitosan macromolecules is 90%. In the case of using a weak electrolyte, acetic acid, α = 50–60% [13].

Due to the presence of two types of functional groups, compositional heterogeneity, and the possibility of the formation of intra- and intermolecular hydrogen contacts that affect chain conformation, chitosan macromolecules are characterized by very complex behavior in solution [1, 14]. As a semi-rigid polymer, chitosan has high values of the limiting viscosity number, Huggins constant, and temperature viscosity coefficient [13, 15, 16]. Depending on the ionic strength of the medium, chitosan macromolecules can exhibit polyelectrolyte properties, which are more pronounced in solutions of

low *I*. Due to the low mobility of the polycation, chitosan polymeric salt solutions are characterized by reduced electrical conductivity compared to solutions of mineral electrolytes [17].

The solubility in water of air-dried samples of chitosan polymeric salts depends on the pK_a of the acid, the dielectric constant of the solvent mixture, and also the isolation method [18]. So, salt complexes of chitosan with carboxylic acids and hydrochloric acid, isolated from a solution by evaporation, are water-soluble, and by precipitation they are selectively soluble in water. For example, chitosan hydrochloride, isolated from an aqueous solution with an organic precipitant (methanol, isopropanol, acetone, dioxane), remains soluble in water, and acetic acid chitosan does not dissolve in water.

The nature of the acid also affects the structure and properties of salt form chitosan films obtained from solutions [19, 20]. Replacing the counterion, varying the physicochemical parameters and external conditions (temperature, solvent evaporation rate, etc.) make it possible to form both uniform transparent and composite films [4, 21]. In some cases, the formation of dendritic structures is observed [4, 22].

It was previously reported that chitosan can be dissolved in an aqueous solution of L-aspartic acid with the formation of a salt complex of chitosan aspartate [23]. However, the process of dissolution of chitosan in an aqueous medium in the presence of Asp and the properties of the resulting solutions are not described in the literature. Research in this direction seems to be very relevant, since the biological activity of Asp allows considering its aqueous solutions as a biocompatible medium for the dissolution of chitosan.

The aim of this work was to obtain a solution of aspartic acid chitosan and the study of its hydrodynamic, electrochemical, optical, and biological properties.

EXPERIMENTAL

As starting reagents, powdered chitosan with a molecular weight of 200 kDa, a degree of deacetylation of 82 mol %, manufactured by Bioprogress CJSC (Schelkovo) was used; Asp produced by Bioamid CJSC (Saratov) of analytical grade; distilled water, degassed from CO_2 and O_2 by boiling at 373 K for 1 h; NaCl (chemically pure). Aqueous suspensions of chitosan, freshly prepared aqueous solutions of chitosan in Asp, and air-dried samples of chitosan aspartate isolated from the corresponding solutions were selected as the objects of the study.

Chitosan suspensions with a concentration of c'_{CS} = 0.05–0.3 g dL^{-1} were prepared according to the standard method with preliminary swelling of the polymer powder in distilled water for 20 min at 293 K. The suspension hydromodule was varied in the range of 83–500 mL g^{-1}. Aqueous Asp solutions with c_{Asp} = 0.02–0.80 g dL^{-1} were prepared according to procedure in [23]. The initial chitosan aspartic acid solution with a concentration of c_{CS} = 1.2 g dL^{-1} was obtained by dissolving the polymer powders and Asp in distilled water with stirring on a magnetic stirrer for 2 h at 293 K. Working solutions with c_{CS} = 0.04–1.20 g dL^{-1} were obtained by diluting the initial chitosan solution with Asp. Air-dried samples of chitosan aspartate were isolated from solutions by drying on a glass substrate at 293 K.

Hydrogen index (pH) was measured on a pH-meter pH-150 MI, optical density (*A*) was measured on KFK-3-ZOMZ, wavelength range λ = 315–590 nm. The viscosity properties of solutions (viscosity number η_{sp}/c_{CS}, dL g^{-1}; limiting viscosity number [η], dL g^{-1}) were evaluated in a Ubbelode capillary viscometer with a capillary diameter of 0.56 mm at 298–328 K, electrical conductivity (æ, S m^{-1}) were determined on an Anion 4120 conductivity meter, and the refractive index (n_D^{25}) was determined on an RM-40 refractometer. Ionic strength (*I*, M) was calculated by the formula $I = 0.5\sum c_i Z_i^2$, where c_i is the molar concentration of individual ions, Z_i is the ion charge; turbidity (τ, cm^{-1}) was calculated according to the formula $\tau = 2.3A/l$, where *l* is the optical path length (cm); Huggins constant (K_H) and temperature coefficient of viscosity ($-\delta_T$, K^{-1}) was calculated according to [15]; equivalent electrical conductivity (λ, S mol^{-1} m^2), degree of dissociation (α′, %) and conditional dissociation constant (K_d) were obtained in accordance with [24] and degree of protonation (α,%), with [1]. The morphology of air-dry powders was evaluated using a MIRA II LMU SEM. A gold layer of 5–10 nm thick was preliminarily sprayed onto the sample using an Emitech K 4 device.

Biocompatibility was investigated in vitro on a model of the cell line of human fibroblasts. In vials with DMEM nutrient medium, an aspartic acid solution of chitosan and a suspension of fibroblasts (300 thousand cells per cm^2) were added. Cells were cultured for 3–4 days in a CO_2 incubator at constant temperature (310 K), humidity (90%), and CO_2 content (5%). The spreading and proliferation of cells were observed using an inverted

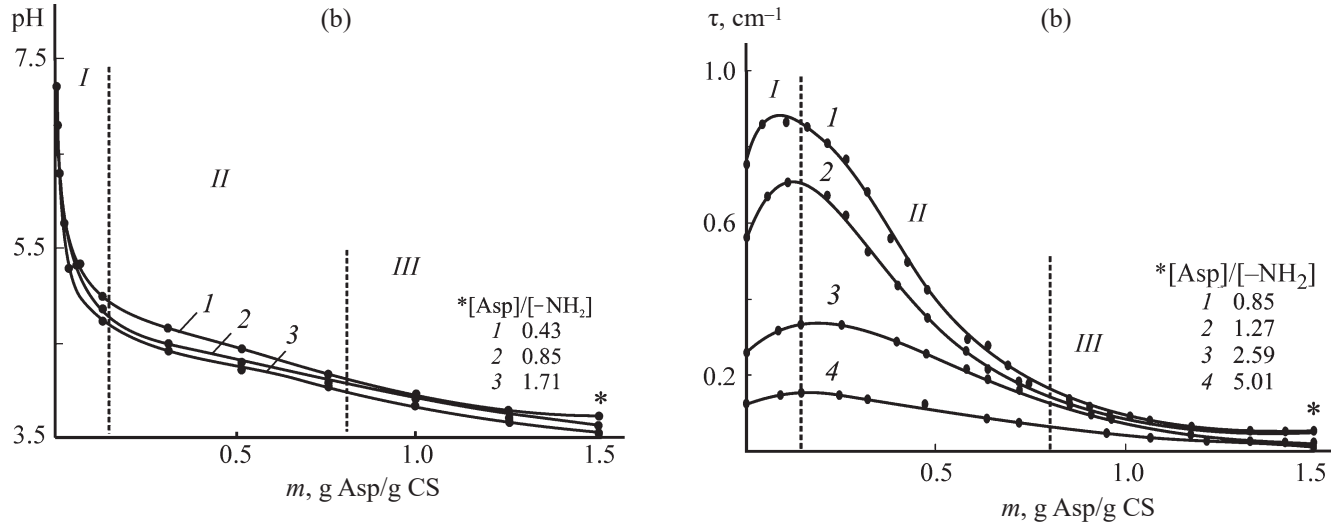

Fig. 1. (a) pH and (b) turbidity of the chitosan + Asp + H_2O system vs. the acid/chitosan mass ratio upon fractional introduction of the Asp solution into the chitosan suspension. (a) c'_{CS} = 0.3 g dL^{-1}, c_{Asp} = (*1*) 0.2, (*2*) 0.4, (*3*) 0.8 g dL^{-1}; (b) c_{Asp} = 0.4 g dL^{-1}, c'_{CS} = (*1*) 0.3, (*2*) 0.2, (*3*) 0.1, and (*4*) 0.05 g dL^{-1}. (*) The molar ratio [Asp]/[–NH_2] (mol/base mol) was calculated taking into account the change in the volume of the system upon addition of the Asp solution.

Biolam P-3 microscope (LOMO, Russia). The nutrient medium in the vials was not changed until the end of the observation period. Antibacterial activity was determined by agar diffusion test with a daily culture of the reference strain of *Staphylococcus aureus* 209 P. Agar medium for culturing microorganisms and solutions with c_{CS} = 0.04–1.20 g dL^{-1} were used. Equal aliquots of a solution of different concentration of aspartic chitosan solution were introduced into the wells of the agar medium, kept for 1 h at room temperature for diffusion of the polymeric salt into agar, incubated at 310 K for 18–20 h, and the diameter of inhibition growth zones of the test strain were measured.

RESULTS AND DISCUSSION

L-aspartic acid, unlike traditional solvent media of chitosan, aqueous solutions of mineral and monobasic carboxylic acids, belongs to 310the class of amino acids, which may also affect the process of its salt formation with chitosan. Therefore, at the first stage, it is advisable to study the polymer dissolution processes.

The dissolution of chitosan in an aqueous medium in the presence of Asp was studied by fractional injection of an acid solution into a polymer suspension. In one series of experiments, the concentration of the Asp solution was varied with a constant hydromodule of [water]/[chitosan] suspensions, in another, the hydromodule was changed, and the Asp concentration was set constant. Therewith the change in the acidity of the medium and the turbidity of the system were controlled (Fig. 1). A quantitative change in the composition of the system was expressed by the acid/chitosan mass ratio.

The addition of the first portions of the Asp solution (m ~ 0.08–0.13 g Asp/ g CS) to the chitosan suspension was accompanied by a sharp decrease in pH from 7.3 to 4.7–5.0 and the increase in turbidity of the system to a greater extent, the higher c_{CS} (Fig. 1, region *I*). The first is a consequence of a change in the concentration of hydrogen ions when acid is added, the second is an increase in the particle size of the dispersed phase due to the swelling of the polymer powder. It is quite possible that partial protonation of the amino groups of chitosan occurs at this stage, but the realized values of α are still insufficient for its dissolution. With further addition of Asp and, correspondingly, an increase in the acid/chitosan ratio, not only the pH decreases, but also the τ of the system (region *II*). Together, these results indicate the formation of a protonated form of chitosan and, accordingly, its dissolution in an aqueous medium. With such a shift in the acid-base equilibrium, the dispersion medium is enriched in salt complexes of chitosan with the corresponding counterions ~–NH_3^+ + –OOC–CH_2–CH(NH_2)–COOH. At m ~ 0.80–1.25 g Asp/g CS, the pH and τ values continue to decrease, but not so intensively (region *III*). The introduction of more than

1.25 g Asp/g CS leads to complete dissolution of the polymer. The system becomes optically transparent, the turbidity does not exceed 0.03 cm^{-1}, and the pH reaches values of 3.5–3.8. Along with this, in the interval with c_{Asp} = 0.2–0.4 and c_{CS} = 0.05–0.30 g dL^{-1} (marked with *), the molar ratio of [Asp]/[–NH_2] varies in the range of 0.43–5.01. It is logical that the dissolution of chitosan is intensified by an increase in the concentration of Asp and the hydromodule of the suspensions, since the molar ratio of [Asp]/[–NH_2] and, accordingly, the fraction of protonated amino groups of the polymer increase (Table 1).

Thus, chitosan in the aqueous Asp solution dissolves at pH 3.5–3.8, the minimum [Asp]/[–NH_2] stoichiometric ratio ~ 0.43 (Fig. 1), and the minimum degree of protonation ~6.1% (Table 1). It is believed that chitosan is soluble in an aqueous acidic medium with the degree of protonation ≥50%, however, the data presented indicate the possibility of the formation of a polymer solution even with a lower value of α. Apparently, this is due to the nature of L-aspartic acid, which is prone to bipolar ionization in an aqueous medium depending on pH of the solution.

The data of IR spectroscopy confirm the electrostatic nature of the interaction of chitosan with L-aspartic acid, leading to the formation of a salt complex in the form of chitosan aspartic acid [23].

A study of the hydrodynamic properties of aqueous solutions of chitosan in Asp showed that the chitosan aspartate macromolecules exhibit the properties of a polyelectrolyte with a partially compensated charge: the viscosity number η_{sp}/c_{CS} increases with decreasing c_{CS}, while the dependence $\eta_{sp}/c_{CS} = f(c_{CS})$ passes through a maximum and has a dropping branch (Fig. 2a, curve *1*). It is likely that the presence of free Asp ions [$K_d(I) = 1.29 \times 10^{-2}$] causes some screening of the polycation charge. In addition, polymeric salt macromolecules exhibit mixed polyelectrolyte/ionomer behavior when only part of the low molecular weight counterions are in a "bound" state with the macroion. This phenomenon has much in common with the effect of the formation of "charged microgels" of polysaccharides [25].

Table 1. The degree of protonation of chitosan macromolecules in an aqueous solution of L-aspartic acid

c_{CS}, g dL^{-1}	α, %, at c_{Asp}, g dL^{-1}	
	0.40	0.80
0.04	46.8	94.8
0.08	23.1	47.4
0.15	12.2	25.1
0.30	6.10	12.2

The addition of a low molecular weight NaCl salt to a solution of chitosan aspatate suppresses the polyelectrolyte effect and compacts (compresses) macromolecular tangles up to the transition to the ionomeric state. At a

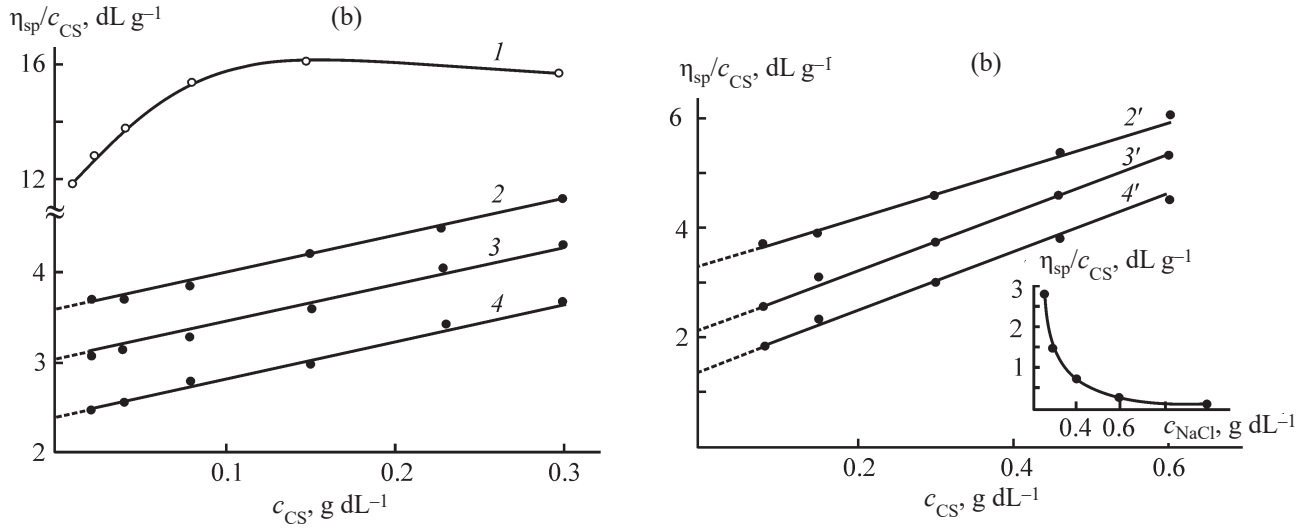

Fig. 2. Concentration dependence of the viscosity number of aqueous chitosan solutions in Asp with c_{Asp} = (a) 0.4 and (b) 0.8 g dL^{-1} without (*1*) and with addition of 0.17 M NaCl (*2–4*, *2′–4′*) at 298 (*1, 2, 2′*), 308 (*3, 3′*), and 328 K (*4, 4′*). The inset shows η_{sp}/c_{CS} of chitosan solutions in Asp vs. concentration of NaCl, 298 K.

Table 2. Hydrodynamic parameters of chitosan macromolecules in an aqueous solution of L-aspartic acid, $I = 0.17$ M

c_{Asp}, g dL^{-1}	Hydrodynamic parameter	Temperature, K		
		298	308	328
0.4	[η], dL dL^{-1}	3.56	3.10	2.40
	K_H	0.32	0.80	1.06
	$\delta_T \times 10^{-2}$, K^{-1}		–2.1	
0.8	[η], dL dL^{-1}	3.35	2.25	1.40
	K_H	0.48	1.13	1.19
	$\delta_T \times 10^{-2}$, K^{-1}		–2.0	

background electrolyte concentration of $c_{NaCl} \geq 0.8$ g dL^{-1}, complete suppression of polyelectrolyte swelling is observed (Fig. 2b, see the inset). The dependences of the viscosity number of the "neutralized" chitosan macromolecules are linear (Figs. 2a, 2b, curves *2* and *2′*), which does not change with increasing temperature (curves *3*, *3′*, *4*, *4′*).

The value of the limiting viscosity number of a water-salt solution of chitosan aspartate naturally decreases with increasing Asp concentration and temperature (Table 2), which is typical for chitosan solutions with high ionic strength [15], as well as nonionic semi-rigid natural polysaccharides and their derivatives. The values [η] of chitosan solutions in Asp over the entire range of studied temperatures are quite high, which is due to the increased rigidity of chitosan macrochains. An increase in temperature from 298 to 328 K leads to an increase in the Huggins constant.

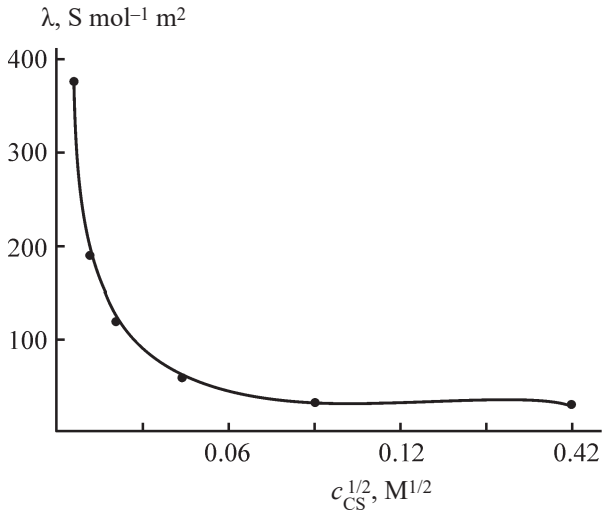

Fig. 3. Equivalent electrical conductivity of aspartic acid solutions of chitosan vs. the polymer concentration, c_{Asp} = 0.8 g dL^{-1}.

The temperature coefficient of the limiting viscosity number of solutions is characterized by negative and large in magnitude δ_T values (Table 2). The negative sign of the temperature coefficient [η] is due to a decrease in the size of macromolecular tangles with increasing temperature. Together, the dependences [η] = $f(T)$, $K_H = f(T)$, and the negative δ_T sign of water-salt solutions of chitosan aspartate indicate the deterioration in the polymer–solvent interaction with increasing temperature.

The polyelectrolyte nature of the salt form of chitosan determines the electrical conductivity of its solutions. The concentration dependence of the equivalent electrical conductivity of chitosan aspartic acid solutions in the coordinates of the Kohlrausch equation is typical for weak polyelectrolytes (Fig. 3).

The calculated degree of dissociation and conditional dissociation constant also confirm that the studied polymer salt belongs to low-dissociating polyelectrolytes. With increasing concentration of chitosan, the values of α′ and K_d decrease, and the refractive index, pH, and optical density of solutions increase (Table 3).

The morphology of the powders of the initial chitosan and that isolated from aspartic acid solution differs significantly (Fig. 4). The polymeric salt is characterized by tightly packed microparticles in the form of plates ranging in size from 45 to 80 μm. It should also be noted that the particles are uniform in thickness, their relative orientation, and fractal packing.

In a separate series of experiments, the biocompatibility and antibacterial activity of aqueous solutions of chitosan aspartate were studied. It was found that the addition of a polymeric salt solution to the nutrient medium positively affects the rate of spreading and proliferation of human fibroblasts (Fig. 5). So, after 1 h of cultivation

Table 3. Physicochemical characteristics of aqueous solutions of chitosan in L-aspartic acid with concentration 0.8 g dL^{-1}

c_{CS}, g dL^{-1}	Degree of dissociation α′, %	Constant of dissociation, $K_d \times 10^3$	Refractive index n_D^{25}	pH	Optical density	
					λ = 364 nm	λ = 400 nm
0.04	60.5	3.29	1.3337	3.17	0.05	0.04
0.08	29.9	0.74	1.3337	3.25	0.08	0.05
0.15	19.3	0.45	1.3339	3.24	0.12	0.06
0.30	9.10	0.19	1.3341	3.30	0.21	0.09
0.60	5.70	0.14	1.3346	3.52	0.42	0.19
1.20	3.40	0.09	1.3357	4.82	0.99	0.45

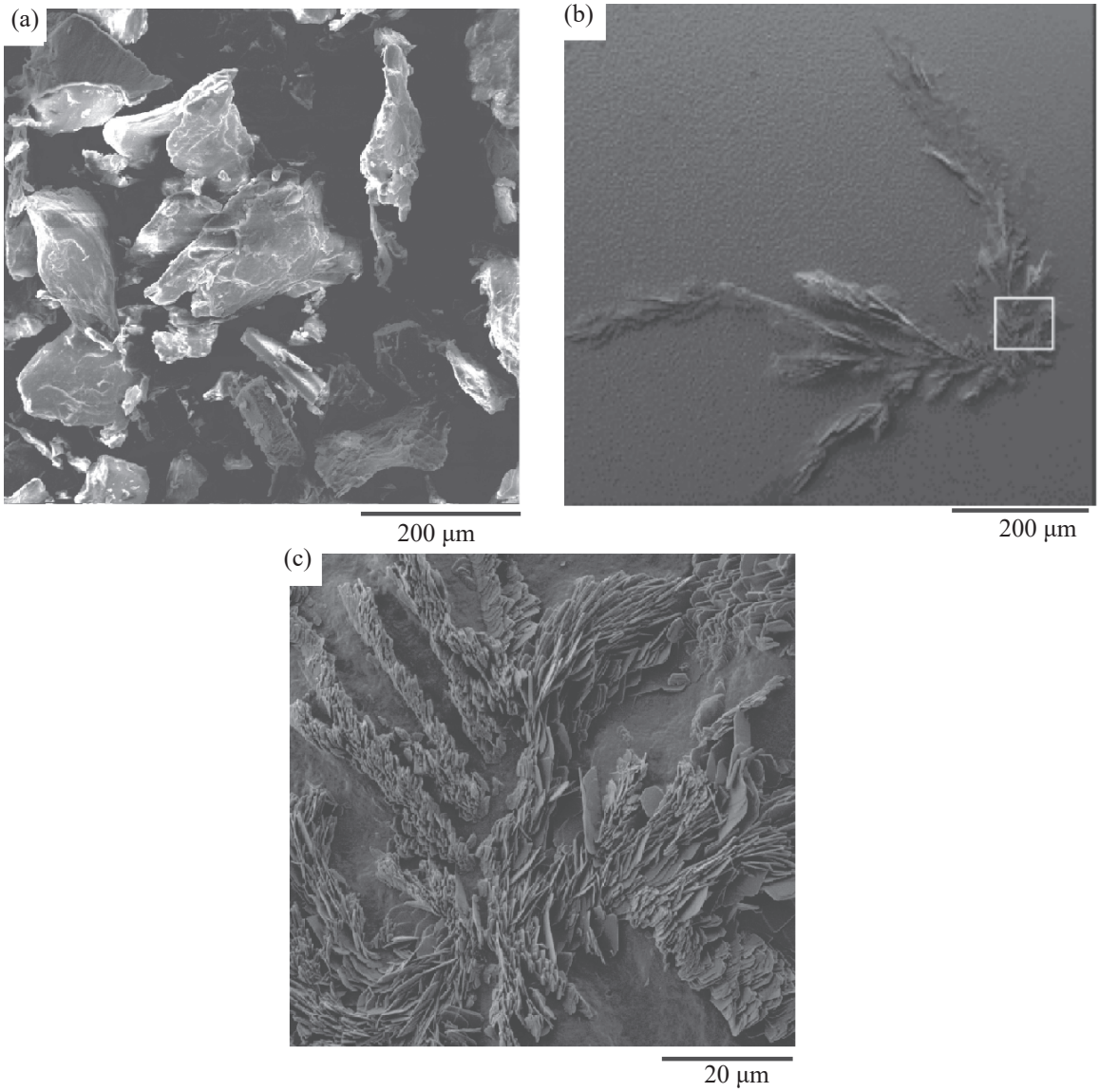

Fig. 4. Images of air-dried powders obtained using scanning electron microscopy: (a) original chitosan; (b, c) chitosan aspartate isolated from a solution of the composition c_{CS} = 0.6 g dL^{-1}, c_{Asp} = 0.8 g dL^{-1}. (a, b) Overview view; (c) magnified fragment of the site marked in Fig. 4b.

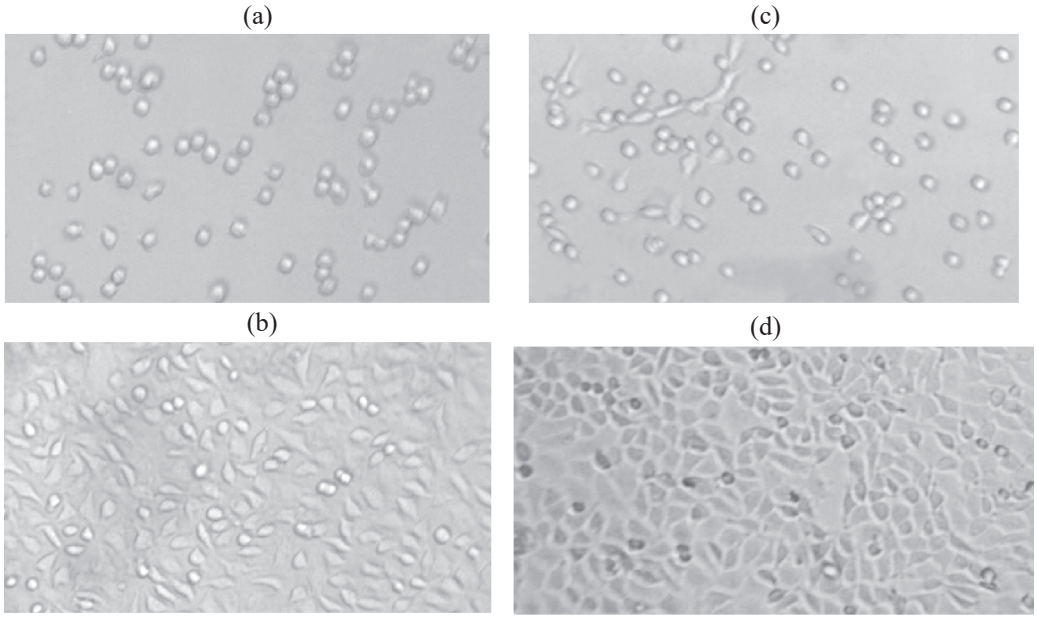

Fig. 5. Fibroblasts after (a, c) 1 and (b, d) 72 h cultivation without (control) and with chitosan aspartic acid solution, magnification 100×.

in a medium with additive (chitosan + Asp), there were 4 times more spread cells were observed than in the control (Figs. 5a, 5c). Over the next 72 h, in the presence of chitosan aspartate, the formation of a monolayer of cells also proceeded at a faster pace (Figs. 5b, 5d). On the fourth day, the shape and size of the cells of the mature monolayer corresponded to the norm, which indicates a high compatibility of aspartic acid chitosan with human tissues and the absence of a cytotoxic effect.

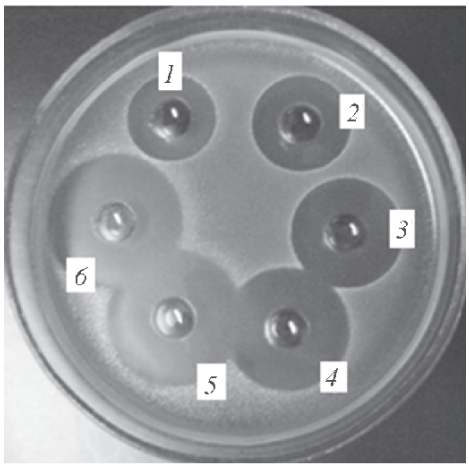

Fig. 6. Zones of growth inhibition of colonies of *Staphylococcus aureus* 209 P reference strain with chitosan asparatic acid solutions of concentrations (*1*) 0.04,(*2*) 0.08, (*3*) 0.16, (*4*) 0.32, (*5*) 0.64, and (*6*) 1.20 g dL^{-1}.

It was revealed that chitosan aspartic acid salt exhibits high antibacterial activity against the opportunistic culture of *Staphylococcus aureus* (Fig. 6). All studied solutions of this polymeric salt successfully inhibited the growth of colonies of *Staphylococcus aureus* 209 P. At the same time, clear zones on bacterial lawn were formed, the diameter of which decreases as the concentration of chitosan aspartic acid decreased.

In conclusion, it can be stated that as a result of the interaction of chitosan with Asp in an aqueous medium, a visually homogeneous molecular solution of the corresponding salt is formed. The values of the degree of protonation of macromolecules depend both on the concentration of chitosan in the system and on the concentration of acid. Chitosan aspartate in an aqueous medium exhibits the properties of a semi-rigid chain polymer with high biological activity. The specificity of morphology of air-dried polymeric salt powders during their separation from the solution manifests itself in the locally directed formation of lamellar oriented particles. The desired biological properties of chitosan aspartic acid salt established in experiments suggest that preparations from this polymeric salt will be widely used in medicine, in particular, in replacement therapy to stimulate tissue regeneration and osteogenesis, as well as to prevent the development of wound infection.

CONCLUSIONS

Chitosan is dissolved in an aqueous solution of L-aspartic acid at a pH of 3.5–3.8 and the minimum [Asp]/[$–NH_2$] stoichiometric ratio ~ 0.43. An increase in the concentration of Asp and the hydromodule of the [water]/[chitosan] suspensions intensifies its dissolution. Chitosan aspartic acid salt in an aqueous medium exhibits the properties of a polyelectrolyte with a partially compensated charge. An increase in Asp concentration and temperature leads to a decrease in the hydrodynamic size of macromolecules and a deterioration in the polymer–solvent interaction. With an increase in the concentration of aqueous chitosan solutions in Asp, the degree of protonation, the degree and constant of dissociation decrease, and the refractive index, pH, and optical density of the solutions increase. The morphology of chitosan aspartic acid salt isolated from the solution is characterized by densely packed homogeneous microparticles (45–80 μm) in the form of plates with fractal packing. Aqueous solutions of chitosan aspartate exhibit antibacterial activity and can be used to produce promising biologically active preparations and chitosan-containing materials.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest requiring disclosure in this article.

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