

Matrices Based on Chitosan Nanofibers for Cell Technologies

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Received February 14, 2013; accepted for publication June 6, 2013

Abstract—A material constituting a nonwoven mat consisting of intersecting fibers 100 to 400 nm in diameter was fabricated by the electrospinning of a chitosan solution supplemented with a biocompatible polymer. Optimal compositions of solution and electrospinning conditions were selected. The material was tested for cultivating mesenchymal stem cells. The adhesion and proliferation rate of the stem cells applied on the surface of nanofiber matrix were investigated. The good compatibility of the obtained material with stem cells was shown.

DOI: 10.1134/S1995078013050054

INTRODUCTION

The application of nonwoven nanofiber materials based on biopolymers for medical purposes such as pads for treating wounds and burns, including ones with controlled drug release (matrices for tissue engineering that should be inert and provide required gas- and liquid exchange) are the most promising [1]. Polymers used for such matrices must be biocompatible, not exhibiting toxicity, and the material structure should have porosity with a pore size of up to tens of micrometers. It was reported recently that nanofiber materials on the basis of chitosan formed by electrospinning were used as matrices for cell-culture cultivation. These matrices demonstrate good adhesion and facilitate the preservation of cell morphology and viability [2, 3].

Electrospinning is a process of fabricating ultrathin fibers from a polymer solution or melt by spinning them in an electric field. Currently, the technology of electrospinning is experiencing a revival; the number of publications on this topic reached 1000 in 2011 and it continues to grow. Nonwoven materials can be produced by electrospinning from a large number of synthetic and natural polymers [4, 5]. Cellulose derivatives, chitin, chitosan, collagen, and polyethylene oxide, which have a long history of biomedical applications, are most often used biocompatible polymers. Chitosan is a polysaccharide with macromolecules consisting of β -(1-4)-D-glucosamine and N-acetyl-

D-glucosamine units. Chitosan is produced by the deacetylation of chitin, and the degree of deacetylation defines the difference between these two biopolymers. Commercially available chitosan has a degree of deacetylation of approximately 80–90%, and, hence, enough amino groups to be soluble in aqueous solutions of, e.g., acetic, fumaric, and lactic acids, in which chitin is not soluble [6].

The feasibility of electrospinning of any polymer, including chitosan, is determined by the selection of optimal conditions of spinning and primarily by the solvent selection. Results of the large number of experiments on the electrospinning of chitosan with a large range of molecular characteristics for different solvents and different spinning conditions are presented in the review [7]. The properties of the polymer itself, such as molar mass distribution, degree of deacetylation, and availability and nature of admixtures are crucial for the successful chitosan electrospinning. The large variety of raw material sources, methods of demineralization, deprotonation, deacetylation, and purification results in the production of chitosan samples with different sets of properties; hence, each chitosan sample requires the selection of unique optimal conditions for successful electrospinning. The selection of the solvent is very important. Trifluoroacetic acid was selected for the chitosan dissolution in [8, 9]. Its voluminous counter ions prevent the interaction of chitosan molecules in the solution, and high volatility is

favorable for the polymer fiber solidification in the process of formation. However, the high toxicity of this solvent limits the areas of its application. It was mentioned earlier that chitosan is soluble and capable of ionization in aqueous solutions of weak acids; however, their conductivity is significantly higher than of the polymers in organic solvents that are used most often for electrospinning [10, 11]. This significantly hinders the process of chitosan electrospinning. Repulsion forces between the ions of molecular chains increase upon spinning in an electric field, which makes it impossible to produce continuous fiber, especially upon the stretching and bending of the stream [12]. It is difficult to produce uniform material without defects when using concentrated aqueous solutions of acetic acid as a chitosan solvent due to the instability of the electrospinning process in these conditions [13, 14]. Small amounts of water-soluble polymers that demonstrate good suitability for electrospinning, such as polyethylene oxide or polyvinyl alcohol, are added to the solutions to improve their rheological and fiber-forming properties [15–17]. However, the increase in the relative fraction of water-soluble polymer results in the deterioration of properties of the obtained nanofiber material such as resistance to aqueous environment and air humidity.

Despite the large number of publications, there is no universal approach for fabricating nonwoven material on the basis of chitosan; the vast majority of studies are phenomenological. The goal of this work is to investigate the properties of chitosan solutions and prepare nonwoven materials from them for biomedical applications.

EXPERIMENTAL

MATERIALS AND PREPARATION OF ELECTROSPINNING SOLUTIONS

Chitosan with viscosity-average $MM = 200$ kDa, deacetylation degree 82 mol % (Bioprogress, Russia), was used to prepare nonwoven materials. Polyethylene oxide $MM = 600$ kDa (Sigma-Aldrich, United States) was added to the solution to improve fiber-forming properties. Aqueous solutions of acetic acid of different concentrations were used as a solvent. Glacial acetic acid was diluted with deionized water to the required concentration and added to a chitosan aliquot. Dissolution was performed by stirring with a magnetic stirrer at room temperature for one day. An aliquot of a PEO powder (10 wt % relative to chitosan) was added to the prepared solution and stirred for one more day to prepare the visually homogenous solution. In some cases the prepared solution was subjected to a vacuum at room temperature to remove air.

INSTRUMENTATION AND METHODS

The electrical conductivity of acetic acid of different concentrations was measured with an Ekspert-002

electrical conductivity meter (Russia). Dynamic viscosity was determined with a Heppler viscometer.

Electrospinning was performed with a setup consisting of a device for the polymer solution supply, collecting electrode, and Spellman SL130PN10 (Spellman Inc., United States) high voltage supply. High negative voltage from 25 to 40 kV was applied to a vessel with a polymer solution. Electrospinning was conducted through thin-walled metal capillaries with hydrodynamic resistance 900 Pa. A metal disk with 35 cm diameter or a metal drum with 22 cm diameter performing rotational and forward–backward movements were used as collecting electrodes. The inter electrode distance between the capillary and collecting electrode was from 20 to 23 cm. The morphology of the obtained materials was investigated with a method of optical (Olympus CX41, Olympus, Japan) and scanning electron microscopy (Zeiss SUPRA55VP, Carl Zeiss, Germany). Fiber diameter, pore sizes, and their distribution were estimated with the Image Pro 4.5 program.

The pH of the water extract was measured to determine the residual content of acetic acid in the nonwoven material. An aliquot of nonwoven material (0.01 g) was placed into 10 ml of deionized water followed by stirring with a magnetic stirrer for 2 h at 50°C and 200 rpm; the obtained extract was filtered through a paper filter and cooled to room temperature. pH was measured with a SEVEN MULTI S40-K (Mettler Toledo, Switzerland) instrument at 25°C. Measurements were conducted with 3 samples taken from three different areas.

Cultures of mesenchymal adipose stem cells (ASCs) were used for a cytological investigation of materials on the basis of chitosan. White adipose tissues obtained with the lipoectomy procedure was homogenized, treated with 0.2% collagenase solution (Sigma, United States), and centrifuged. Cells were cultivated in a MEM Alpha Modification nutrient medium (Gibco, United States) supplemented with 15% fetal calf serum (Gibco, United States) and antibiotics (100 units/ml penicillin and 100 g/ml streptomycin, Gibco, United States). The cultivation of stem cells was conducted in a CO₂ incubator in atmosphere of 70% humidity at $T = 37^\circ\text{C}$ and the CO₂ content was 5–7%; subculturing of the cells into fresh flasks was conducted when 80% confluence was reached.

The stem cells were deposited onto a chitosan matrix from the liquid nutrient medium containing the cells; the time of contact of the tested sample with the matrix was from 1 h to 2 weeks. The cells were fixed on the surface of the matrix after cultivation using 0.25% glutaraldehyde solution (Sigma, United States) following dehydration with ethanol. Microscopic investigations were performed with a Zeiss SUPRA55VP scanning electron microscope (Carl Zeiss, Germany). The samples were coated with a thin layer of gold.

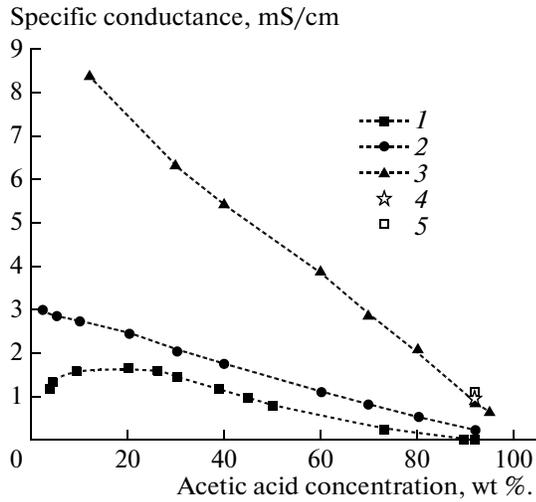


Fig. 1. (1) Dependence of the electrical conductivity of acetic acid solution on concentration. (2) Electrical conductivity of 1.5 wt % chitosan solution in acetic acid solutions of different concentrations; (3) electrical conductivity of 7.5 wt % chitosan solution in acetic acid solutions of different concentrations. (4) Electrical conductivity of 7.5% chitosan solution in 92% acetic acid with the addition of 10 wt % PVP; (5) the same, but with the addition of 10 wt % PEO.

**RESULTS AND DISCUSSION
ELECTRICAL CONDUCTIVITY
AND VISCOSITY OF SOLUTIONS,
ELECTROSPINNING**

The electrospinning of fibers and fabrication of nonwoven materials on their basis was conducted from chitosan solutions in acetic acid. The conducted experiments on electrospinning from aqueous solutions of lactic and fumaric acids produced negative results, the formation process was unstable, the final material had a large number of defects in the form of droplets and spindles of different shapes. As was mentioned above, aqueous solutions of weak acids have a higher electric conductivity than water or organic solvents, which are successfully used as solvents on the electrospinning of fibers. This can explain the difficulties of fiber formation from the chitosan solutions in weak acids. That is why special attention was given in this work to an investigation into the electric conduc-

tivity of acetic acid solutions in a wide range of its concentrations, as well as chitosan solutions in it.

As is evident from Fig. 1, the specific conductance (SC) of acetic acid solutions has a maximum at an acid concentration of around 20 wt % and decreases both upon a decrease and increase in its amount. The decrease in electrical conductivity of highly concentrated solutions is related to the significant reduction of the dissociation degree of acid due to the shortage of available water molecules; the SC adheres to Ostwald’s law of dilution at low concentrations of the solution. However, the pattern of the dependence changes to monotonously decreasing with an increasing concentration of acetic acid upon the addition of chitosan to the solution. We suggest that the lack of a bend on curves 2 and 3 in Fig. 1 in the range of acid concentrations 0–20% is related to the increase in the ion mobility against the background of a decrease in dynamic viscosity of the solutions.

The low electrical conductivity of the polymer solution at high concentrations is required for the stable electrospinning process: breaking of the polymer solution into microjets in an electric field and the formation of fibers. It can be seen from the data that the 7.5 wt % chitosan solution in 92% acetic acid suits these requirements; the value of its specific conductance is below 1 mS/cm.

The electrospinning of pure chitosan solutions did not allow conducting the process in a stable manner; the obtained fiber material contained defects in the form of droplets and fibers have a large spread in diameters. Microphotographs of such samples are presented in Fig. 2.

Polymer additives that showed good electrospinning properties were used to improve the fiber-forming properties of the polymers. Biocompatible polyvinylpyrrolidone (PVP) and polyethylene oxide (PEO) were among them. It was found that the addition 10 wt % of PVP to chitosan resulted in the formation of separated fibers on electrospinning (Fig. 2c). However, the addition of the same amount of PEO demonstrated the best results with the electrospinning process proceeding stably. The role of a water-soluble polymer addition probably lies in the redistribution of hydrogen bonds, which results in a decrease in the interchain interaction between the chitosan molecules

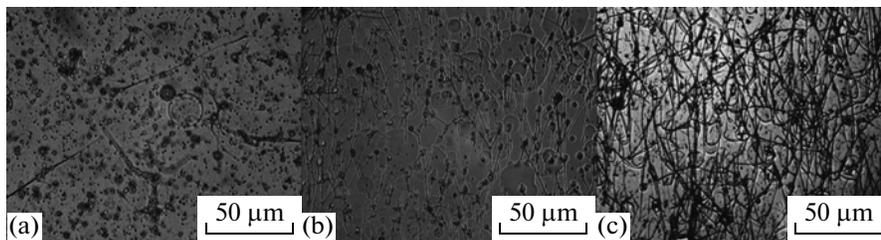


Fig. 2. Microphotographs of samples fabricated at nonoptimal electrospinning conditions. (a) Episodic fibers, (b) beginning of formation (spindles), and (c) material with large number of defects.

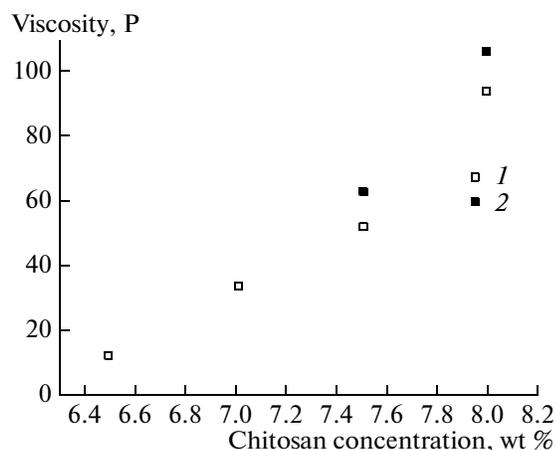


Fig. 3. (1) Dependence of spinning solution viscosity on chitosan concentration; (2) the same with the addition of PEO.

in the solution due to the formation of hydrogen bonds between the molecules of chitosan and the added polymer. The solution becomes more homogenous, which facilitates the successful electrospinning and fabrication of material with the required properties. It should be noted that the addition of both PEO and PVP did not affect the specific conductance because both additives are nonionic and do not affect the viscosity at lower amounts (Fig. 1).

The increase in the polymer concentration in the solution is important for increasing electrospinning

productivity, but it is necessary to prevent the formation of the gel in the case of chitosan. The maximal possible polymer concentration of 8.5 wt % was determined using 92% acetic acid as a solvent, but this solution did not demonstrate stable electrospinning. The 7.5 wt % chitosan solution in 92% acetic acid with the addition of 10 wt % of PEO relative to chitosan yielded repeatable results and required productivity.

The dependence of viscosity of the spinning solutions presented in Fig. 3 demonstrates its significant increase with the increase of chitosan concentration; the reduction of the chitosan concentration below 5% does not make sense due to the low productivity of the process. The difficulties in determining the rheological characteristics of chitosan solutions should be mentioned; the decrease in the viscosity by 10% or more occurs even in solutions with low acid concentration within several days after preparation. Moreover, the rate of the viscosity decrease, which we relate to the decrease in chitosan MM due to its destruction, depends on the acid concentration in the solution. For example, the viscosity decrease of the 1.5 wt % chitosan solutions in 2.5 and 92% acetic acid after 6 days was 13 and 26%, respectively, and the fiber-forming properties of the solutions deteriorated.

An insignificant viscosity increase is observed upon the addition of the PEO in the solution as compared with the pure chitosan solution, which is related to the increase in the total polymer mass in the solution.

The samples of nonwoven material from chitosan with the addition of 10% PEO were investigated for the

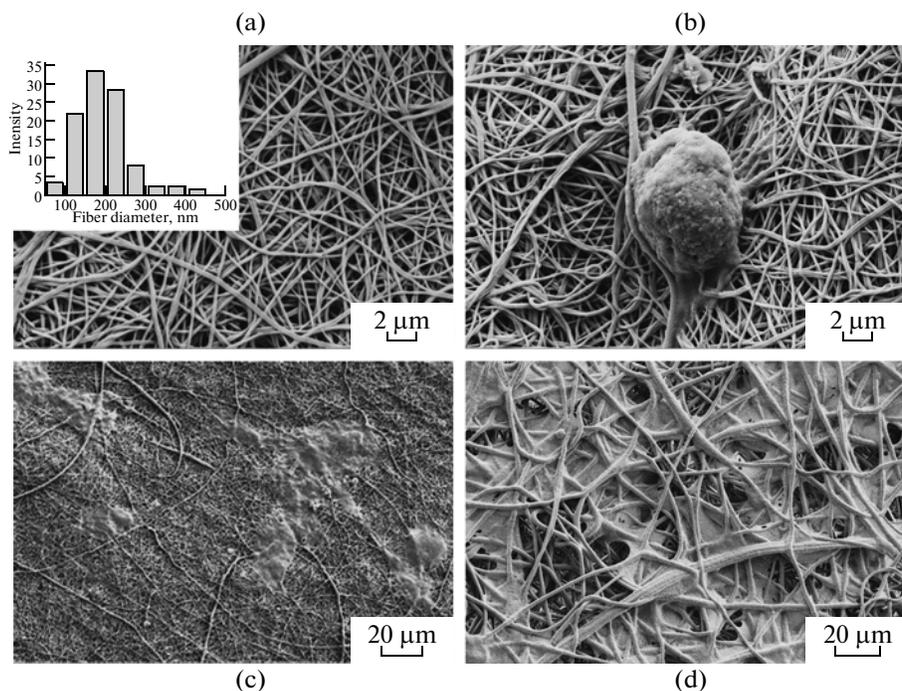


Fig. 4. Microphotographs of chitosan nanofibers (a) and matrices after the cultivation of stem cells on them for 1 h and 7 and 14 days (b, c, d).

fiber distribution by the diameter and uniformity of the material by the area. Microphotographs of the nonwoven material are presented in Fig. 4a. The material consists of intersecting fibers with diameters from 100 to 400 nm and an average diameter of approximately 180 nm (fiber distribution by diameter is presented in the inset in Fig. 4a); the number of fibers with diameters of over 300 nm is insignificant. The surface of the fiber is uniform without pronounced microstructure. The structure of the material is dense; significant surface defects such as droplets and holes are absent.

It is important to control the amount of residual acid in the nonwoven material, because the pH of the medium is an important parameter for the application of matrices for cell technology. The nonwoven material exhibits a distinct smell of acetic acid immediately after fabrication. The hydrogen indicator of the water extract of the material after prolonged (more than 30 days) storage was 5.22 ± 0.04 , with the control value for the deionized water being 5.65.

CELL ADHESION

The samples were used as matrices for the cultivation of mesenchymal stem cells. The cells were inoculated onto the samples using the protocol described above. A typical microphotograph of the matrix with the cell 1 h after inoculation is presented in Fig. 4b. It can be seen that the stem cell has a globular shape characteristic of the cells in the liquid matrix. The change in the shape and number of the cells after incubation for 7 days in the cell matrix can be observed in Fig. 4c. The cells are well spread and their number on the surface of the matrix increased significantly. This indicates good compatibility of the material with stem cells and a lack of fiber toxicity. The good compatibility of nanofibers facilitating intensive cell proliferation is confirmed by the results of prolonged incubation of the material in the cell matrix. It can be seen in Fig. 4d that the entire surface of the investigated matrix is covered with stem cells after 14 days, which confirms the high proliferation rate of stem cells on the chitosan nanofiber matrix.

CONCLUSIONS

Hence, optimal conditions were selected for electrospinning. Nonwoven material on the basis of chitosan with the addition of polyethylene oxide was prepared, which was tested as a support in experiments on stem-cell cultivation and showed good cytocompatibility. The good adhesion of cells on chitosan nanofi-

bers, the conservation of their viability, and the proliferation up to the degree at which the entire matrix surface is filled was observed.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 11-03-01062a).

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Translated by L. Brovko