Modification of non-woven materials based on sodium alginate for tissue-engineering

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Abstract. This article describes the fabrication of non-woven fibrous materials based on sodium alginate by electrospinning and their modification with RGD peptides. The rheological properties of sodium alginate solutions are investigated. A defect-free fibrous material with an average fiber diameter of 180 nm was obtained. Ionic and covalent crosslinking of sodium alginate was carried out.

1. Introduction
Repair of damaged tissues and organs is one of the most acute problems in modern medicine. Various injuries, diseases, age-related changes can lead to a decrease in the regenerative properties of human body tissues and organs forcing them to be replaced [1].

At the moment, it is impossible to create artificial biocompatible materials, which are similar in their properties to natural human tissues. Numerous attempts to recreate organs using natural materials are far from complete. Therefore, the works devoted to modification of synthetic materials with biologically active compounds (growth factors, proteins) to increase their biocompatibility and the efficiency of the patient’s cells growth [2,3,4] are in progress.

Matrices can be made of synthetic or natural polymers, ceramics, composite materials. Recently, the interest in the preparation of matrices based on natural polymers has been growing due to their complex of “biosimilar” properties, e.g., biocompatibility, resorbability, hydrophilicity. The most widely used natural polymers are collagen, gelatin, hyaluronic acid, chitosan and alginate [5,6,7].

One of the methods for the manufacture of polymer matrices is electrospinning, which enables to fabricate ultrafine fibers with a diameter ranging from a few nanometers to several micrometers, and allows to create scaffolds with a high specific surface area, and with an architectural structure resembling the natural extracellular matrix.

This work focuses on development of fabrication method for non-woven fibrous materials based on sodium alginate and their modification with RGD peptides.

2. Objects and Methods

2.1. Preparation of spinning solutions
Polyethylene oxide (PEO) (Sigma-Aldrich, USA) with a molecular weight of 600 kDa and Pluronic F-127 (Sigma-Aldrich, USA) were used to prepare the spinning solutions of sodium alginate (W201502 Sigma-Aldrich, USA). The solutions were stirred for 10 hours at room temperature on a magnetic stirrer.
at 100 rpm. Thereafter, the solutions were stored in the refrigerator, and immediately before spinning, they were taken thereof and stirred for one hour at room temperature.

2.2. Optical microscopy
An optical microscope ZEISS Axio Imager 2 was used to study the process of fiber formation and the morphology of the obtained fibers.

2.3. Scanning Electron Microscopy
The surface morphology of the samples was investigated using a FEI Versa 3D DualBeam scanning electron microscope (SEM) in high vacuum mode. The images were obtained using a secondary electron detector at an ultra-low accelerating voltage of 1 kV.

2.4. Gamma irradiation of sodium alginate solutions
Sodium alginate solutions were irradiated with gamma radiation at a dose of 10 to 30 kGy based on the Cobalt-60 radionuclide.

2.5. Study of rheological properties of sodium alginate solutions
The rheological properties were studied with an Anton Paar Physica MCR 501 rheometer using a "cone-and-plate" measuring system, 50 mm (CP – 50).

2.6. Sodium Alginate Fibrous Material Crosslinking
For the ionic crosslinking, the fibrous material samples were placed in a 5% solution of CaCl₂ in 70% isopropyl alcohol (IA), then washed with distilled water to remove salt residues and dried at room temperature.

The covalent crosslinking of fibrous materials was carried out in a solution of epichlorohydrin in 60% ethanol for 20 hours. The concentration of epichlorohydrin was 4.3 mol / L, NaOH 0.14 mol / L, CaCl₂ 0.014 mol / L. After the crosslinking, the samples were washed with an IPA solution and dried at room temperature.

2.7. IR spectroscopy
The IR spectra of the samples were obtained on a Thermo Scientific Nicolet iS5 instrument using an iD5 ATR diamond attachment (spectral resolution of 4 cm⁻¹, 32 scans)

2.8. Immobilization of peptides with RGD sequence
The carbodiimide method was used for the peptides immobilization on the surface of the fibrous material [8]. The activation of carboxyl groups of sodium alginate was performed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma-Aldrich, USA) and N-hydroxysuccinimide (NHS) (Sigma-Aldrich, USA). With this objective, ion-crosslinked fibrous material was placed in distilled water, and then EDC was added in the amount equal to 50% of the total number of carboxyl groups in sodium alginate. NHS was added in a ratio of 1:2 to EDC, and then the solution was stirred for 5 minutes.

Thereafter, RGD peptide (Sigma-Aldrich, USA) was added to the solution (1 mg peptide per 1 g of the fibrous material). The modification was performed at room temperature for 15 hours, and then the modified material was washed thoroughly with distilled water.

2.9. Nuclear magnetic resonance
To record nuclear magnetic resonance spectra (NMR), Bruker 600 MHz instrument was used. For this purpose, the studied samples were dissolved in deuterated water.

3. Sodium Alginate Solutions Electrospinning
Since sodium alginate solutions do not have fiber-forming properties, polyethylene oxide with a molecular weight of 600 kDa and the surfactant Pluronic F-127 were introduced into the solution. Table 1 presents the compositions of the prepared solutions and the parameters of their electrospinning.
Table 1. Composition of the spinning solutions and parameters of electrospinning

| Alg, % | MW of PEO, kDa | PEO, % | F-127, % | Voltage, kV | Flow rate, ml/h |
|--------|----------------|--------|----------|-------------|----------------|
|  3     |  600           |  2.2   |  0.5     |  1.5        |  2             |  25            |  0.2            |
|  600   |  1.6           |        |          |             |                |                |                |

When 2.2% PEO was added to the sodium alginate solution, fibers on the receiving electrode were not formed. However, after the introduction of Pluronic F-127 into the spinning solution, defect-free fibers were detected on the precipitation electrode (Figure 1).

Figure 1. Micrographs of the fibers obtained from a 3% solution of sodium alginate with the addition of 2.2% PEO (600 kDa) and Pluronic F-127: a) 0.5% b) 1% c) 1.5% d) 2%

To obtain fibers with a higher content of sodium alginate, the concentration of PEO in the spinning solution was reduced. It was found that the optimal concentration of PEO in the solution to obtain defect-free fibers is 1.6%. At lower concentrations of PEO, either the electrospray of the solution (in the absence of PEO), or the formation of defective fibers were observed.

However, despite the optimization of PEO content in the solution of sodium alginate, the process of electrospinning was unstable, which led to the traces of drops on the surface of the fibrous material (Figure 2).

Figure 2. Photographs of the fibrous materials: (a) with defects from digging up the solution before irradiating the solution; and (b) containing no defects after gamma irradiation of the solution.

High molecular weight of sodium alginate is the reason for high viscosity of its solutions. To reduce it, gamma radiation was used for destruction of glycosidic bonds in the polymer chain [9]. As shown in Figure 3, when a 4% solution of sodium alginate was irradiated with a dose of 10 kGy, a significant
decrease in the viscosity of the solution was observed. Higher radiation doses did not lead to a notable change in the viscosity. This allowed the use of more concentrated solutions of sodium alginate.

![Graph showing the dependence of viscosity on absorbed dose.](image)

**Figure 3.** The dependence of the viscosity of the sodium alginate solution on the radiation dose.

Figure 2b shows micrographs of the fibrous materials fabricated from a 6% solution of irradiated sodium alginate with the addition of 1.6% PEO and 1.5% F-127.

4. Ionic crosslinking of fibrous materials based on sodium alginate

To maintain the fibrous structure of materials based on sodium alginate in aqueous media, they must be crosslinked. The main method for crosslinking of sodium alginate is the ion crosslinking with calcium chloride (CaCl₂).

Figure 4 shows SEM-images of the fibrous materials before and after ion crosslinking. As can be seen, after ionic crosslinking, the fibrous structure of the material is preserved, however, the fibers partially change their morphology (spread) due to swelling in water. In this case, adhesion of the fibers between themselves and a decrease in pore size are observed. The material retained a fibrous structure when it was kept in water for a week. However, apparently, a rather slow dissolution of sodium alginate in water takes place, since after 7 days a decrease in the average fiber diameter from 180 to 140 nm was detected. Another reason for the reduction in the fiber diameter can be a leaching of poly(ethylene oxide) and Pluronic F-127 from the fibers.

Figure 5 shows the IR spectra of the ion-crosslinked and original fibrous materials based on sodium alginate. In the spectra of non-crosslinked material, there are peaks related to poly(ethylene oxide) and pluronic, while for the crosslinked materials these peaks were not observed.

![SEM images of fibrous materials.](image)

**Figure 4.** SEM images of the fibrous materials: a) before crosslinking, b) after crosslinking and storage for 1 hour in water, c) after 3 days in water, d) after 7 days in water.

The absorption band at 1100 cm⁻¹, characteristic of C–O–C bonds, decreases in intensity after crosslinking, whereas the bands in the region of 1500–1300 cm⁻¹, which can be assigned to asymmetric and symmetric deformation vibrations in –CH₃ groups, disappear. The remaining bands are
characteristic of glycosidic bonds in alginate. The absorption band at 2890 cm\(^{-1}\), which corresponds to the –CH stretching vibrations in –CH\(_3\) groups, also decreases in intensity. Thus, one can conclude that incubation of the fibrous material for one hour in the crosslinking solution (2% CaCl\(_2\) in 70% IA) results in almost complete washing out of PEO and Pluronic from the fibers. However, an increase in incubation time does not lead to notable change in the IR spectra.

![IR spectra of the fibrous materials before (1) and after their crosslinking, and after incubation in water for 1 h (2), 24 h (3), 3 days (4) and 7 days (5).](image)

**Figure 5.** IR spectra of the fibrous materials before (1) and after their crosslinking, and after incubation in water for 1 h (2), 24 h (3), 3 days (4) and 7 days (5).

5. **Covalent crosslinking of fibrous material based on sodium alginate**

Ion-crosslinked alginate fibrous materials are stable in distilled water, but they dissolve in a medium with sodium ions. This is due to the fact that the calcium ions that “crosslink” the alginate are replaced by sodium ions. The chelate complex that holds the alginate molecules destroys, and the material begins to dissolve.

In order to increase the stability of the fibrous materials in buffer solutions, additional covalent crosslinking is necessary. This crosslinking can be carried out through two functional groups, i.e. –OH or –COOH. But, since peptides are grafted through carboxyl groups, the hydroxyl groups were used for crosslinking.

Epichlorohydrin was selected as a crosslinking agent. Since its solubility in water is relatively low, the crosslinking reaction was carried out in a 60% ethanol solution. To catalyze the reaction, the alkaline pH of the solution was maintained with NaOH. Figure 6 shows the proposed reaction scheme:

![Scheme of covalent crosslinking of sodium alginate with epichlorohydrin.](image)

**Figure 6.** Scheme of covalent crosslinking of sodium alginate with epichlorohydrin.

After the covalent crosslinking, the samples were washed in water and dried at room temperature. Figure 7 shows the IR spectra of ionically and covalently crosslinked fibrous material.
Figure 7. IR spectra of covalently (1) and ionically (2) cross-linked sodium alginate.

As can be seen, the band at 3,300 cm\(^{-1}\) increases in intensity, indicating the growth of hydroxyl groups. Also, for the covalently cross-linked material, the intensity of the bands at 1418 cm\(^{-1}\) and 1600 cm\(^{-1}\) increases. In addition, a new band at 872 cm\(^{-1}\) appears. These observations can be explained by incomplete washing of the material from the inorganic salts.

Samples of the covalently and ionically cross-linked material were placed in a sodium phosphate buffer. The ionically crosslinked material dissolved for one hour. However, the covalently crosslinked material not only kept its shape, but as shown in Figure 8, retained the fibrous structure. The SEM images also show salt crystals, indicating insufficient washing of the material.

Figure 8. SEM image of covalently crosslinked material after storage in PBS for one hour.

6. Modification of the fibrous material with a RGD sequence

The immobilization of the RGD sequence on the surface of the fibrous material was carried out by the carbodiimide method. NMR spectroscopy was used to confirm the modification. The fibrous materials were placed in deuterated water with sodium chloride, then the solution was actively stirred until the material was completely dissolved.

Figure 9 shows 1H NMR spectra of the original and modified sodium alginate. A comparison of the NMR spectra shows that after carbodiimide cross-linking of the peptide, the signals corresponding to the protons of the methylene groups of arginine and aspartic acid (2.2–2.45 ppm) appear in the spectra. Due to the low integral intensity of these signals, it is difficult to quantify the modification. Based on the literature data [89], one can conclude that the degree of peptide grafting is less than 1-5%. The spectra also contain signals corresponding to low-molecular-weight by-products (n-acylurea) at 1.15 and 3.45 ppm.
7. Conclusion
The process of electrospinning of a fibrous material based on sodium alginate is studied. It is shown that the use of preliminary gamma irradiated spinning solutions is advisable to form the defect-less fibers. In addition, it is necessary to use fiber-forming additives, i.e. PEO with MW of 600 kDa at concentration of 1.6% and Pluronic F-127 at concentration of 1.5%. The fibrous material was covalently crosslinked with epichlorohydrin and its stability in buffer solutions was established. The RGD peptide was immobilized on the surface of the fibrous material using the carbodiimide method, which was confirmed by 1H NMR spectroscopy.

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**Figure 9.** 1H NMR spectra of sodium alginate (A) and sodium alginate modified with RGD (B).