FABRICATION AND PHYSICO-CHEMICAL PROPERTIES OF PECTIN/CHITOSAN SCAFFOLDS

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Abstract

Scaffolds from chitosan and its combinations with other polymers are widely used for tissue engineering application. This is due to the favourable biological properties of chitosan such as antimicrobial activity, biocompatibility, biodegradability, cell adhesion and proliferation, etc. The aim of the study was the creation of 3D porous scaffolds based on pectin/chitosan polyelectrolyte complexes and study the influence of components ratio on their physico-chemical properties, as well as degradation behaviour in solutions modelling the media of the human body. Porous "sponge-like" polysaccharide films were produced using freeze-drying technique from gel-like pectin-chitosan polyelectrolyte complexes. The weight ratio of chitosan:pectin in complexes was varied in the range from 1:1 to 1:2. Obtained samples were characterized by infrared spectroscopy and scanning electron microscopy. It has been shown that pectin-chitosan films turned out to have sponge-like structure with highly interconnected pores with the size about 50-300 μm. It has been determined that all samples regardless of the chitosan:pectin ratio possess high swelling properties. The degradation profile of scaffolds in different media was studied. It has been determined that the largest weight loss is observed in water and reaches more than 80% after 1 day, while in NaCl and PBS solutions weight loss is approximately 50-60% after 25 days. For samples with different chitosan:pectin weight ratio, weight loss slightly rise with increasing amount of pectin. It has been shown that mesenchymal stem cells adhered to the surface of obtained pectin:chitosan porous scaffolds in viable state. Hence, it can be served as a potential material for tissue engineering applications.

Keywords: *chitosan, pectin, 3D scaffold, degradation*

[Engineering of Biomaterials 146 (2018) 2-7]

Introduction

The preparation of polyelectrolyte complexes (PECs) is an actual trend in modern material science due to their possible application in pharmaceutics, tissue engineering, food industry, for water purification, etc. [1-6]. Polyelectrolyte complexes possess unique properties, which are significantly different from those of the initial components. Polyelectrolyte complexes are products of intermolecular reactions of complementary polyelectrolytes and, depending on the preparation conditions, can be obtained in the form of nano- and microparticles, gels, films, membranes, porous structures and fibrous materials [2-7].

Currently, tissue engineering is an actively developing direction and important therapeutic strategy for present and future medicine. According to this strategy, tissue engineering scaffolds are used for the formation of new viable tissue for a medical purpose. Tissue engineering scaffolds have to include cells, scaffolds and growth factors. Hence, one of the main directions in tissue engineering is designing a suitable scaffold. The scaffold should have the following properties: biocompatibility, biodegradability, non-toxicity, non-immunogenicity, desired mechanical strength. The morphology of scaffolds should be suitable for cell attachment and allow providing the possibility of transporting nutrients, signal molecules, metabolites. For this reason, highly porous films are very attractive materials for use as scaffolds. It should be noted that the rate of scaffolds biodegradability has to be corresponding to the one of new tissue formation. There are a lot of biodegradable polymers that can be used for scaffolds creation, but one of the promising types among them are biopolymers (polysaccharides, proteins, etc.) due to their biological and chemical similarities to natural tissues.

In recent years, scaffolds based on polyelectrolyte complexes of chitosan (CS) with various anionic natural polyelectrolytes, such as alginates, carrageenans, gelatin, have attracted increasing attention for tissue engineering application due to their porous structure and beneficial biological properties [4,8-10]. Chitosan is a linear polycationic polysaccharide composed of randomly distributed β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It possesses such favorable biological properties as antimicrobial activity, biocompatibility, biodegradability, non-toxicity, cell adhesion and proliferation, as well as mucoadhesive behaviour. Combination of chitosan with other polymers allows to achieve improved physicochemical and permeability properties of material in use. For example, PECs on the basis of chitosan and pectin (Pect) have properties that overcome the limitations of individual polymers (stability, mechanical strength, sustained release of the entrapped substance, controlled degradation rate, etc.) [11,12]. Fabrication of highly porous scaffolds based on pectin/chitosan PECs are also gaining more attention due to anti-inflammatory activity of pectin, as well as simplicity of PECs formation from these two oppositely-charged polysaccharides. Pectin is a branched anionic heteropolysaccharide, which is rich in galacturonic acid. The residues of α-(1-4)-D-galacturonic acid partially etherified with methanol form the main chain of pectin.

The stability in solutions and the degradation rate of PECs strongly depends on pH, temperature, charge density and ionic strength, as well as other conditions [13]. Degradation of scaffolds is the key parameter for their application in tissue engineering, because scaffolds should degrade as new tissue formation takes place [14]. Therefore, it is important to establish the factors by which the biodegradation rate of scaffolds can be controlled. The key parameter for film based on PEC may be the ratio of components in it, especially if one of the component is water soluble. Thus, the aim of this work was the creation of 3D porous scaffolds based on pectin/chitosan polyelectrolyte complexes and study the influence of components ratio on their physico-chemical properties, as well as degradation behaviour in solutions modelling the media of the human body.

Materials and Methods

Low-molecular chitosan (M_v ~ $3.4 \cdot 10^5$, the content of NH_2 group = 74.8%) and citrus low methoxylated pectin (M_v ~ $1.4 \cdot 10^5$, the content of COOH group = 19.6%) used for this study were obtained commercially from Sigma-Aldrich.

Pectin/chitosan scaffolds in the form of porous "sponge-like" films were prepared by the freeze-drying of insoluble polyelectrolyte complexes formed by these two polysaccharides in an aqueous solution. For this, 5 mg/ml solution of chitosan in 1% acetic acid was added dropwise into 5 mg/ml water solution of pectin under vigorous shaking on the vortex to obtain a solution with a whole polysaccharide concentration 5 mg/ml (1.7-2.5 mg/ml chitosan, 2.5-3.3 mg/ml pectin). Mass proportions of the two polysaccharides (CS:Pect, w/w) was in the range from 1:1 to 1:2. The resulting gel-like complexes were left for 1 h to complete the formation of PECs, maintained at -20°C for 20 h and freeze-dried (Labconco FreeZone 1.0.) for 8 h.

The structure of pectin/chitosan PECs was studied by scanning electron microscopy (SEM, Jeol JCM-6000 Plus, Japan). Films were placed on a graphite tape, attached onto a metal support, and coated with platinum on Smart Coater (Neo Coater). SEM was also used for analysis the surface of pectin/chitosan films after culturing mesenchymal stem cells on them. The scaffolds, incubated with the cells (24 hours), were rinsed with PBS and fixed with glutaraldehyde (2.5% in PBS) for 10 min. Then films were dehydrated in graded ethanol of 70, 80 and 90% for 10 min each, frozen and freeze-dried. Finally, the obtained samples were coated with platinum (Smart Coater) and their morphology was investigated by SEM.

The infrared (IR) spectra of the initial polysaccharides and PECs were recorded on a Tensor 37 FTIR spectrometer ("Bruker", Germany) in the range of 4000-400 cm^{-1}. Samples for the study were prepared in tablets with KBr. To compare the intensity of the selected characteristic peaks of pectin and pectin/chitosan PEC, baseline correction of them was carried out before data analysis. Automatic baseline correction procedure was conducted using Bruker OPUS 6.5.97 Software.

The yield of pectin/chitosan films was calculated from the masses of chitosan and pectin used initially in preparation and the mass of the formed samples:

$$\text{Yield, \%} = \frac{m_{Pect} + m_{CS}}{m} \cdot 100\%$$

where m is the mass of pectin/chitosan film, m_{Pect} and m_{CS} are the respective initial masses of pectin and chitosan used for PEC film formation.

The swelling behavior of scaffolds was investigated at 37ºC by exposing them for 1 h to solutions with different pH and ionic strength: distilled water, 0.9% NaCl (pH 5.5, ionic strength 0.17 M), Dulbecco's phosphate buffered saline (PBS, 8 g/L NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 Na_2HPO_4 g/l, pH 7.0-7.4, ionic strength 0.15 M). The wet weight of the scaffold was determined by first blotting the scaffold surface with filter paper, to remove the excess surface water, and then weighed immediately. The percentage of water uptake (W) by the scaffold was calculated from the equation [12]:

$$W, \% = \frac{m_t + m_0}{m_0} \cdot 100\%$$

where m_0 is the mass of the initial dry sample (mg) and m_t is the weight of the hydrated sample (mg).

Degradation behavior of scaffolds was investigated by immersion them into solutions with different pH and ionic strength (distilled water, 0.9% NaCl, PBS) with weight loss determination during 25 days. For this, samples of porous pectin/chitosan films were dipped into 10 ml of a model solution and incubated at 37°C (LOIP LB-140 water bath, Russia) for 25 days. The samples were removed within 1, 7, 14 and 25 days, carefully washed with distilled water to remove low molecular weight electrolytes, frozen, freeze-dried for 8 h and finally weighed. For each kind of scaffolds, ten samples were tested. Data were plotted as mean ± SD. Stability of scaffolds was estimated as weight loss (WL, %):

$$WL, \% = \frac{m_0 - m_1}{m_0} \cdot 100\%$$

where m_0 is the mass of initial dry sample (mg) and m_1 is the mass of sample after aging in the test medium and dried at time t (mg).

Rat mesenchymal stem cells (3rd passage) obtained from adipose tissue were used to assess the possibility of application the obtained porous pectin/chitosan films as scaffolds. The viability of cells taken off from the surface of scaffolds was evaluated using 0.4% trypan blue solution.

Results and Discussions

Chitosan (polycation) and pectin (polyanion) are electrostatically complementary to each other and form PECs by mixing their solutions due to electrostatic interactions between their ionized amino (NH_3^+) and carboxy groups (COO^-), respectively. The resulting PEC can be also stabilized by hydrophobic and van der Waals interactions, as well as hydrogen bonds between the different groups in polymer-polymer complexes. Chitosan and pectin are weak polybase and polyacid respectively, so the optimal pH range in the reaction mixture for the formation of PECs is between the values of their pKa when the macromolecules are charged. For pectin the value of pKa is ~ 2.9 [15], therefore, at a lower pH its carboxyl groups are unionized and the charge density of macromolecules is insufficient to form a complex. The amino groups of chitosan will be ionized at pH <6.5 (pKa = 6.5 [16]) and are able to interact electrostatically with COO- groups of pectin. Therefore, in this work the formation of complexes was carried out in acetic acid medium at pH ~ 4-5.

The formation of pectin/chitosan PECs was confirmed by IR spectroscopy. The IR spectra of pectin (FIG. 1) contain characteristic bands: the band identified at 1750 cm^{-1} corresponds to the stretching vibrations of the carbonyl group (vC=O) of the methyl ester ($COOCH_3$) or in the undissociated carboxyl group, and the one at 1627 cm^{-1} deals with the asymmetric stretching vibrations of the dissociated carboxyl (COO-) group [17-19]. In the spectrum of chitosan, there are overlapping bands at 1670 cm^{-1} and 1660 cm^{-1}, corresponding to stretching vibrations of the vC=O of the amid group of the acetylated units of chitosan (Amid I band) and deformation vibrations δN-H of the primary amine (Amid II band) [17,20]. The main changes in the IR spectra of pectin/chitosan PEC are in the region between 1800 and 1400 cm^{-1} and associated with the formation of intermolecular ionic salt bonds between chitosan and pectin (FIG. 1). Thus, a decrease in the intensity of the band at 1750 cm^{-1}, associated with the vC=O vibration of the non-ionized carboxyl group, is observed. Also, a hypsochromic shift to the short-wave region and intensification of the band at 1620 cm^{-1}, assigned to the stretching vibrations of the vC=O of the dissociated carboxyl (COO-) group, were determined.

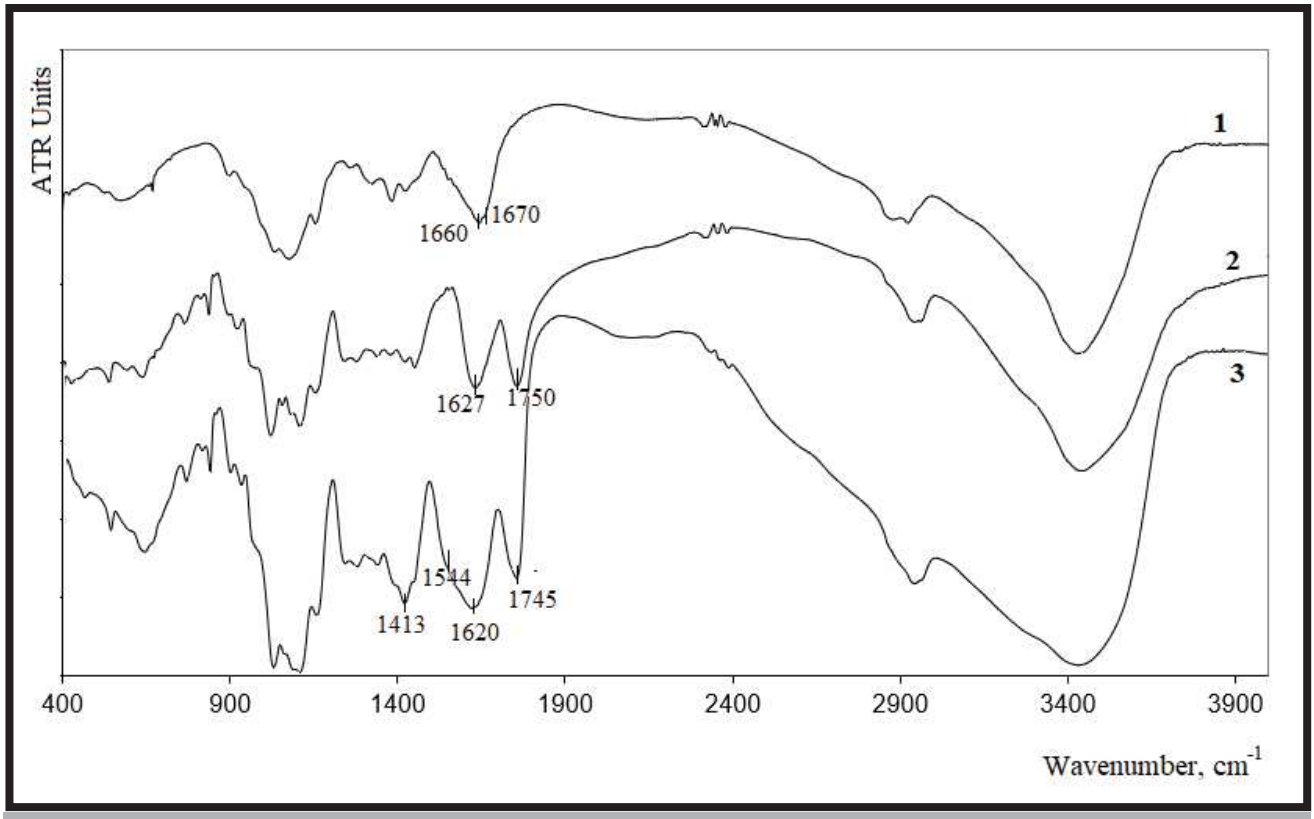

FIG. 1. IR spectra of chitosan (1), pectin (2) and polyelectrolyte complex pectin-chitosan (3).

TABLE 1. Yield of PECs and their swelling behaviour in dependence of chitosan:pectin weight ratio in scaffolds.

Weight ratio of CS:Pect in PEC	Yield, %	Water uptake, %		
		NaCl	H_2O	PBS
1:1	108.2 ± 5.4	3000 ± 450	2900 ± 300	2200 ± 400
1:1.5	117.6 ± 4.3	3530 ± 290	2240 ± 100	2470 ± 220
1:2	119.3 ± 3.6	3230 ± 230	2310 ± 210	2250 ± 300

In the IR spectrum of PEC also appears a band at 1413 cm^{-1}, corresponded to the symmetric stretching vibrations vC=O of the dissociated carboxyl (COO-) group [19], and a shoulder at 1544 cm^{-1}, which is responsible for the symmetric bending vibrations of the protonated NH_3^+ group [20].

In addition, IR spectra of pectin/chitosan PEC in comparison with the spectrum of individual polysaccharides shows an increase in the intensity of the absorption band in the region between 3500 and 2900 cm^{-1}, which is assigned to the superposition of stretching vibrations of NH groups of chitosan and free (3550-3500 cm^{-1}) hydroxyl groups. It should be noted that a wide intense band at the 3500-3200 cm^{-1} region also indicates the formation of intra- and intermolecular hydrogen bonds. The bands in the region of 950-1150 cm^{-1}, related to the pulsation vibrations of the pyranose ring skeleton, remain unchanged [20].

The yield of PEC films based on chitosan and pectin is more than 100% (TABLE 1). It increases with the raise of pectin content in the complexes. This can be caused by the retention of small amounts of water by the sample due to the hydrophilic nature of pectin. The amount of retained water, indeed, raises with increasing weight fraction of pectin in PEC.

Highly porous structure is favorable for tissue engineering as interconnected porous and cavity structure have to ensure a biological environment conducive to cell attachment and proliferation as well as tissue growth. Based on the observations of SEM micrographs, obtained pectin/chitosan polyelectrolyte complexes turned out to have sponge-like structure regardless of the polysaccharides ratio in the complex (FIG. 2). The scaffolds present an irregular, highly porous structural form (from sheet-like to fibrous-like structures). Moreover, samples possess highly interconnected pores with the size about 50-300 μm (FIG. 2). Increases in pectin content in the films leads to smaller pores formation (FIG. 2). The porous structure of obtained films is due to the formation of ice crystals during freezing process. Their removing by lyophilization causes pores formation. Apparently, an increase in the content of pectin during the PECs preparing leads to the formation of a denser network of intermolecular bonds between polysaccharide macromolecules resulting in smaller voids which are filled with the dispersion medium (water).

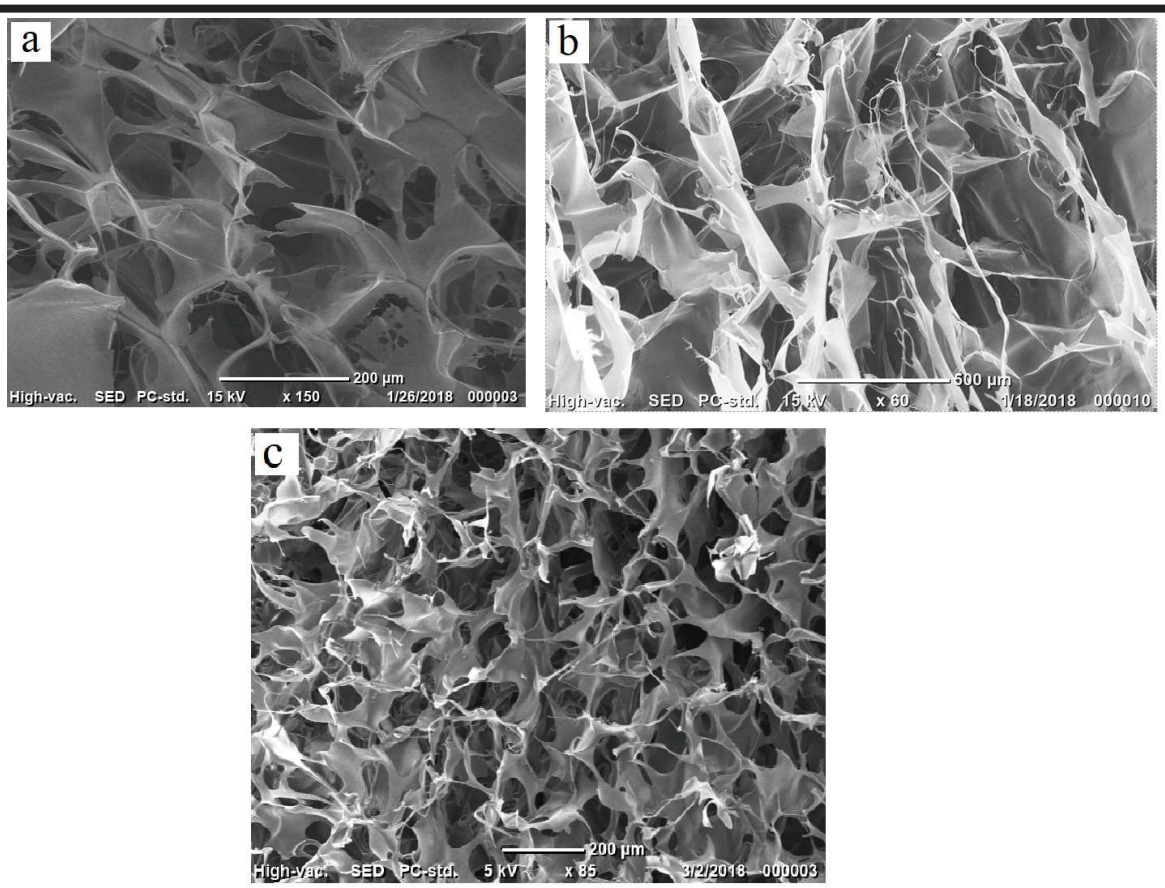

FIG. 2. SEM image of pectin/chitosan PECs. The weight ratio of CS:Pect are 1:1 (a), 1:1.5 (b) and 1:2 (c).

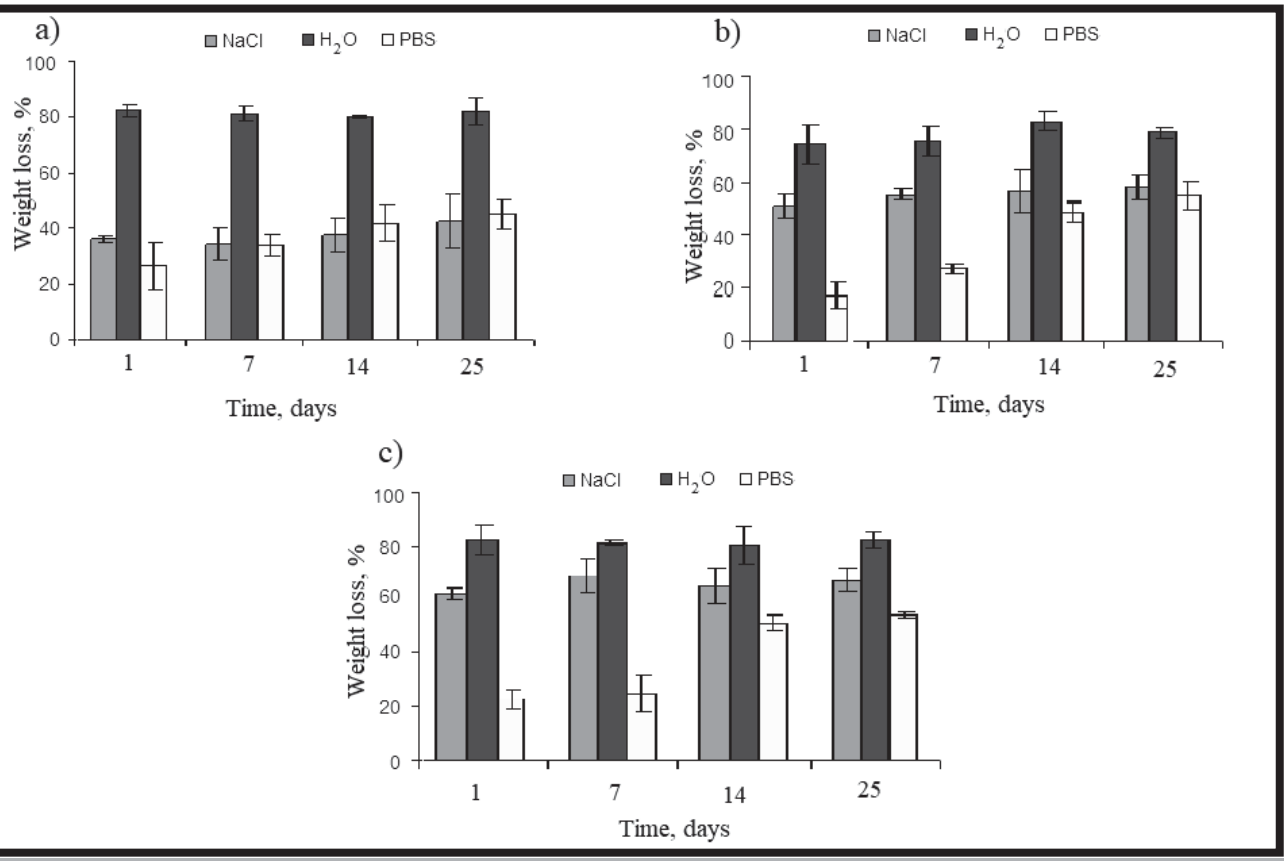

FIG. 3. Weight loss behaviour of the obtained scaffolds after submersion in NaCl, PBS and distilled water. The weight ratio of CS:Pect was 1:1 (a), 1:1.5 (b) and 1:2 (c).

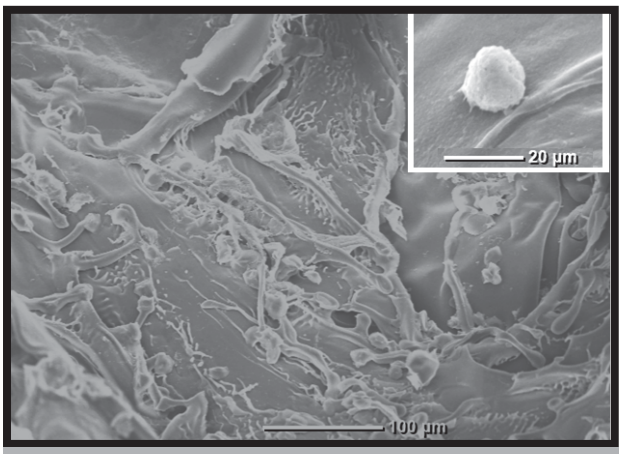

FIG. 4. SEM image of pectin/chitosan PEC with mesenchymal stem cells. The weight ratio of CS:Pect was 1:1.

Swelling behaviour and structural stability of scaffolds are critical for their practical using in tissue engineering. It is well known that natural polymers swell readily in biological fluids. Pectin/chitosan complexes are stabilized due to ionic interactions between positively charged chitosan and negatively charged pectin and exhibited ionic strength and pH-sensitive swelling (TABLE 1). So, the swelling behaviour of the scaffolds differed distinctly in used solutions (TABLE 1). The maximum swelling of films appeared for physiological solution (0.9% NaCl): water uptake is 3000-3530%. Decrease in solution ionic strength while preserving the pH value results in slightly reduction in water uptake (up to 2240-2900%). As swelling medium pH changes from 5.5 (NaCl) to 7.4 (PBS), the water uptake decreases in approximately 1.4 times (TABLE 1). From the data obtained by swelling tests for different scaffolds, it can be concluded that the addition of pectin does not influence on scaffolds' swellable and all samples characterized by high swelling behaviour.

The degradation profiles of the obtained pectin/chitosan scaffolds in different media are presented in FIG. 3. For all samples, the largest weight loss is observed in distilled water, reaches more than 80% after 1 day, and does not change after 25 days regardless of the type of used films. Obtained scaffolds are less degradable in 0.9% NaCl. For films with weight ratio of CS:Pect = 1:1, weight loss in NaCl solution was about 40% after 1 day and does not vary significantly after 25 days. Increase in pectin content in the PEC leads to decrease in sample stability in NaCl solution (FIG. 3). For example, weight loss of scaffold with weight ratio of CS:Pect = 1:2 reaches approximately 60% within 1 day storage in physiological solution. Another situation was observed for PBS: in this media the scaffolds decreases gradually with time. In PBS scaffolds showed reduction in weight 17-26% and 45-55% after 1 and 25 days, respectively (FIG. 3). Weight loss occurs mainly due to the break of ionic interactions, established between the chains of two polymers. The observed slowest destruction of scaffolds in PBS may be due to the interaction of chitosan chains with presented in the buffer hydrogen phosphate anions acting as additional physical crosslinkers that can lead to strengthening of the polymer matrix. So, it is known [21], that the addition of hydrogen phosphate anions to chitosan resulted in the gel formation at 37°C. The author [22] also shown, that chitosan fibers and films treatment with solutions containing phosphate ions resulted in samples' mechanical properties enhance. The obtained experimental data on the degradation of pectin/chitosan scaffolds are in good agreement with the literature: the authors [13] also showed that percentage of weight loss in the pectin/chitosan polyelectrolyte complex scaffolds with higher pectin content was slightly superior compared to the one with less it amounts. They suggested that the weight loss is mainly due to the loss of pectin chains rather than to an equal loss of both polysaccharides and with time the scaffolds become rich in chitosan [13].

The possibility of application the obtained porous pectin/chitosan complex films as scaffolds in tissue engineering constructions for cell culturing was investigated using mesenchymal stem cells as model culture. It has been shown that mesenchymal stem cells effectively adhered on the surface of films after 1 day of incubation. The data of scanning electron microscopy after 1 days of stem cells seeding on the surface of pectin/chitosan films indicated the presence both individual and clusters or agglomerates of cells (FIG. 4). It has also been determined that adhered stem cells are in predominantly viable state: the viability of cells was >90%. According to the obtained data, the adhesion of mesenchymal stem cells to the surface of pectin/chitosan porous films doesn't depend on the content of pectin and chitosan in the scaffold.

Conclusions

Highly porous "sponge-like" scaffolds were obtained based on pectin/chitosan interpolyelectrolyte complexes using freeze-drying technique. Formed pectin/chitosan films turned out to have sponge-like structure with highly interconnected pores with the size about 50-300 µm and theirs morphology depends on the weight ratio of CS:Pect in complexes. Increase in pectin content leads to the formation of a denser network of intermolecular bonds and results in smaller pores size. All obtained samples regardless of the chitosan:pectin ratio possess high swelling behaviour. The investigation of degradation profiles of pectin/chitosan scaffolds showed that the largest weight loss is observed in water and reaches more than 80% after 1 day, while in NaCl and PBS solutions weight loss is around 50-60% after 25 days. For samples with different CS:Pect weight ratio, weight loss slightly increase with improving amount of pectin. It has been shown that mesenchymal stem cells adhered to the surface of obtained pectin-chitosan porous scaffolds in viable state. Hence, it has been demonstrated that obtained pectin-chitosan scaffold have structure and possess properties which are favourable for their application in tissue engineering.

Acknowledgments

This research has been financial supported from the State Program of Scientific Research "Convergence-2020" (task 3.05).

The author sincerely wishes to express their gratitude to the staff of the Laboratory of Biochemical Methods of the Belarusian State Medical University, headed by Dr. Zh.A. Ibragimova, as well as to Dr. A. Zhura for their help in carrying out experiments on cell seeding.

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