The Relation of Biological Properties of the Silk Fibroin/Gelatin Scaffolds with the Composition and Fabrication Technology

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The aim of the investigation was to research the effect of preparation method and composition of silk fibroin and gelatin scaffolds on biological properties.

Materials and Methods. Silk fibroin, gelatin and their blend with different mass ratio scaffolds were prepared by electrospinning. To research scaffold's structure light microscopy, scanning electron microscopy and confocal laser scanning microscopy were applied. Adhesion and proliferation of mice fibroblast 3T3 cell line were investigated to test biocompatibility of constructed scaffolds.

Results. Optimal parameters of device and fiber obtaining parameters were selected. Fibrous porous three-dimensional structure of investigated scaffolds was revealed. It was established that cell proliferative activity on electrospun scaffolds was significantly higher than on casting films. Addition of gelatin to scaffold composition increases cell proliferation.

Conclusions. Electrospun silk fibroin/gelatin scaffolds contain such polymers with mass ratio equal to 1:3 have significant greater ability to maintain cell and proliferation than fibroin and gelatin scaffolds.

Key words: biodegradable scaffolds; silk fibroin; gelatin; electrospinning.

Modern tissue engineering poses a set of problems, one of which is the development of structures (scaffolds) imitating the micro-architecture of native extracellular tissues' and organs' matrix and creating a favorable environment for cell culture. The correct choice of materials for scaffolds fabrication is very important. Currently, both synthetic, and natural polymers are used in tissue engineering and regenerative medicine. The advantages of natural polymers are the following: no toxic breakdown products and their inclusion in metabolic cell's pathways. Nature polymers such as gelatin, chitosan, alginates, etc. are successfully applied in tissue engineering; however, none of them is universal for scaffolds with desired properties, since natural characteristics of polymers (mechanical properties, solubility, immunogenicity level) impose some restrictions on their application.

Silkworm *Bombyx mori* silk fibroin is one of the most prospective nature polymers for tissue engineering. Different research teams have shown fibroin scaffolds to maintain intestine regeneration, nervous and bone tissue repair [1]. Fibroin is a fibrillar protein with a great number of repeats in primary structure. The secondary structure of fibroin contains anti-parallel β-layers united by hydrogen bonds. Amorphous areas of the protein form α-helices, the part of these helices increasing with fibroin

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hydration. Mechanical properties and biodegradation rate of fibroin products can be controlled by changing a proportion of β-layers in its structure [2]. Tertiary structure of fibroin has two chains: heavy and light, with molecular weight of 390 and 26 kDa, respectively, their ratio being 1:1, and they have a single disulphide bond [3]. Such structure of silk fibroin provides the presence of free chemical groups, which can be used for producing conjugates and composites with advanced properties [4–6].

Electrospinning is one of modern techniques used to fabricate scaffolds [7]. Principle of this technique is as follows: when exposed to electric field, thin fibers are spun from a polymer solution, and fall on cathode, where they ultimately dry. An installation for electrospinning consists of three main elements: a polymer solution supply device, high-voltage source, and a collector, which is a cathode. Depending on the task, various modifications of installations are applied using the altered position of polymer solution supply device, collector, and the distance between the installation parts, etc. Moreover, optimal parameters for fiber production are selected for a particular material depending on the experimental purposes.

Electrospinning allows to produce networks with both chaotic and oriented fiber distribution [8]. Oriented structure of constructions makes it possible to specify the cell growth direction that is of primary importance when working with muscular or nervous tissue. In addition, this technique enables to fabricate the constructions with the structure similar to that of bone tissue architecture indicating the prospects of the technique implementation in bone repair [9]. The advantages of the constructions fabricated by electrospinning include high surface-to-volume ratio, significant porosity, improved mechanical and physical properties, the capability to vary fiber thickness.

Electrospinning is widely used in modern researches to create artificial tendons [10], nervous tissue repair [11], vascular implants [12–14] and bone regeneration [15]. The construction's fabricated by electrospinning have been successfully tested both *in vitro*, and *in vivo* [16]. In our study for the first time we conducted comparative studies on biological properties of polymers and their composites using different manufacturing techniques.

The aim of the investigation was to carry out a comparative study of biological properties of silk fibroin, fibroin composite and gelatin scaffolds, and assess the effect of their preparation method on their ability to support cell adhesion and proliferation.

Materials and Methods

Silk fibroin preparation. Silk fibroin was obtained from silkworm (*B. mori)* cocoon provided by Republic Scientific Research Silk Spinning Station, Russian Academy of Agricultural Sciences. At the first stage the cocoons were cleared of sericin. A weighed sample of silk cocoons of 1 g was heated in water bath with 500 ml of bidistilled water containing 1,260 mg of sodium hydrocarbonate (PanReac Applichem, Germany) for 40 min. Than washed with 3.6 L of distilled water. After that boiling in 500 ml of distilled water for 30 min and then washed by 3.6 L of distilled water. The last procedure was repeated 3 times. Purified silk fibroin was air-dried at room temperature.

Preparation of aqueous solution of silk fibroin. To prepare aqueous solution of fibroin, the weighed sample of silk was put into lithium bromide solution (Sigma-Aldrich, USA) with concentration 9.3 M, at the rate of 150 mg/ml, and dissolved in water bath for 5 h. The solution was centrifuged for 7 min at 12,100 g. The supernatant was dialyzed against 500 ml of bidistilled water. Totally, there were carried out 10 dialysis changes by 30 min. Fibroin solution was centrifuged for 7 min at 12,100 g, fibroin concentration being measured spectrophotometrically, at wavelength of 280 nm, molar extinction coefficient being taken equal to 473,480 $M^{-1}cm^{-1}$ that corresponds to an extinction coefficient theoretically calculated by amino acid sequence of heavy chain of silk fibroin (UniProt No.P05790).

Preparation of fibroin solution in 1,1,1,3,3,3-hexofluoroisopropanol-2. Preliminarily, dry fibroin films were prepared. To prepare a film, 1 cm in diameter, 100 μl of silk fibroin aqueous solution was applied on the surface of polished teflon, and dried for 2 days at room temperature. Total protein concentration in the solution for film preparation was 20 mg/ml. The films were removed from teflon surface by scalpel, the films had been preliminarily incubated in ethanol for 15 min, and store in 96% ethanol at 4°C. The polymer solution was prepared by dissolving a dried silk fibroin film in 1,1,1,3,3,3-hexofluoroisopropanol-2 (PiM-invest, Russia) at 37°C till the concentration of 75 mg/ml was reached.

Preparation of gelatin solution in 1,1,1,3,3,3-hexofluoroisopropanol-2. To prepare gelatin solution, the weighed sample of type A dry gelatin (Sigma-Aldrich, USA) was dissolved in 1,1,1,3,3,3-hexofluoroisopropanol-2 at 37°C till the concentration of 75 mg/ml was reached.

Fabrication of silk fibroin and gelatin scaffolds. Scaffolds were fabricated by electrospinning from prepared polymer solutions in 1,1,1,3,3,3-hexofluoroisopropanol-2, concentration being 75 mg/ml. Were prepared 5 groups of specimens, by 5 in each group. Each group had different weight distribution of the proteins used: group 1 — fibroin; group 2 — fibroin and gelatin (1:3); group 3 — fibroin and gelatin (1:1); group 4 — fibroin and gelatin (3:1); group 5 — gelatin. The obtained polymer solutions immediately before electrospinning were centrifuged within 10 min at 12,100 g. The scaffolds were fabricated on the surface of round glasses, 2 cm in diameter, and processed by 96% ethanol. For solution supplement was used a needle

22G, its inside diameter being 0.41 mm, outside diameter: 0.70 mm (Messe Düsseldorf Japan Ltd.).

To prepare fibers from the s specimens of groups 1 and 5 we used the following parameters: source voltage: 5.8 kV (Scientific Electronics, Russia), solution supplement speed: 0.2 ml/h. For specimens of other groups, we used the following parameters: source voltage: 7 kV, solution supplement speed: 0.3 ml/h. Spraying time of a polymer was 20 min. The glasses with scaffolds were dried at room temperature within 2 h. Scaffolds of 100% gelatin were additionally treated with 0.3% glutaric dialdehyde solution (Serva, Germany) for covalent crosslinking in ethanol for 12 h under darkroom conditions at room temperature. All scaffolds were finally treated with 96% ethanol within 1 h followed by drying and store at room temperature.

Fabrication of films from polymers by casting. To fabricate films by casting, gelatin or silk fibroin solution in 1,1,1,3,3,3-hexofluoroisopropanol-2 with concentration of 75 mg/ml was applied on glasses, 2 cm in diameter, and dried within 12 h at room temperature. Gelatin films were additionally treated with 0.3% glutaric dialdehyde solution in ethanol for 12 h under darkroom conditions at room temperature.

Analysis of scaffold structure by scanning electron microscopy. Scaffold samples were fixed in 2.5% glutaric dialdehyde solution in phosphate buffer saline followed by dehydration by ethanol of increasing concentrations: 10, 30, 50, 70, 95%, and placing in acetone (Chemmed, Russia). Then the samples were dried in a critical point dryer HCP-2 (Hitachi Ltd., Japan). Dried samples were covered by a chrome layer, 20 nm thick, in argon atmosphere, at ion current 6 mA and pressure 0.1 mm Hg using IonCoater IB-3 (Eiko Engineering, Japan) followed by the analysis using a scanning electron microscope Camscan S2 (Cambridge Instruments, Great Britain), resolution 10 nm, working voltage 20 kV. Images were captured using MicroCapture software.

Analysis of adhesion and proliferation activity of 3T3 line mice fibroblasts on scaffolds. To study adhesion and proliferative activity, the glasses with scaffolds were placed in culture plates, 35 mm in diameter. As controls was used a glass with no scaffold. Before the test, the scaffolds in plates were sterilized by 70% ethanol treatment within 12 h. Then, the plates were added a sterile solution of phosphate buffer saline for 30 min, the change of phosphate buffer saline was repeated thrice. The plates were added 1.5 ml of incubation medium consisting of Dulbecco's Modified Eagle's Medium — low glucose (Sigma-Aldrich, USA) and Ham's F12 (Flow laboratories, Great Britain) in the ratio 1:1 containing 10% fetal bovine serum (HyClone, USA), gentamicin, 40 μg/ml (Ferein, Russia), glutamine, 4 mM (PanEco, Russia), and incubated for 15 min.

Mice 3T3 fibroblast suspension in incubation medium, 2 ml, was added in culture plates, at the rate of 9,000 cells per 1 cm^2 of the surface. The cells were incubated in 5% CO_2 medium at 37°C. Adhesion was observed 12 h after culture, and proliferative activity of the cells was estimated on days 3 and 5 of culture.

Cell proliferative activity was assessed visually using a microscope Carl Zeiss Axiovert 25 (Carl Zeiss, Germany). The images of cells on scaffolds in transmitted light and in phase contrast regime were captured using AxioCam HRC (Carl Zeiss, Germany). The images were processed in a program AxioVision 3.1 (Carl Zeiss, Germany). According to the images we calculated the cells per microscope field of vision. Mean number of cells at this magnification was converted per scaffold area.

Analysis of scaffold structure by laser confocal scanning microscopy. Scaffold specimens were fixed by paraformaldehyde (Sigma-Aldrich, USA) followed by washing from the fixator by phosphate buffer saline for 10 min, the buffer being changed 3 times. The solution of TRITC (Sigma-Aldrich, USA), with concentration of 10 μg/ml was prepared in phosphate buffer saline, and added in culture plates with scaffolds in phosphate buffer saline, incubated for an hour in phosphate buffer saline at room temperature. The reaction was stopped by putting a scaffold in 0.1 M solution of tris(hydroxymethyl) aminomethane (Helicon, Russia) for 30 min. The scaffolds were washed in phosphate buffer saline 3 times for 10 min, followed by the incubation in 70% ethanol for 30 min. Then the scaffolds again were washed by phosphate buffer saline three times. The obtained specimens were studied using a laser scanning confocal microscope, Axiovert 200M LSM510 META (Carl Zeiss Jena, Germany). Fluorescence in the specimens was induced by a helium-neon laser, wavelength 543 nm. The images were analyzed using the program LSM Image Browser (Carl Zeiss Jena, Germany).

Data statistic processing. The data were processed by ANOVA. Statistical significance of the results was assessed by Mann–Whitley test. The level of statistical significance, p was taken equal to 0.05.

Results and Discussion. Electrospinning is one of the techniques used to fabricate artificial structures for tissue engineering, its advantage is the possibility to produce micro- and nanofibrous structures imitating structure of organs' and tissues' extracellular matrix. Figure 1 presents the scheme of a standard electrospinning installation for scaffold fabrication.

The installation consists of a pump, a syringe with a blunt-pointed needle, coupled to a high-voltage-source, and a negatively charged collector. A series of experiments allows to select optimal parameters for the installation to work with silk fibroin and gelatin solutions: a pump was placed at an angle of 45° to the supporting plane, the distance between the collector and the needle tip was equal to 3 cm. The variation of the installation parameters enables to modify the diameter and distribution density of fiber that allows to fabricate structures with required porosity.

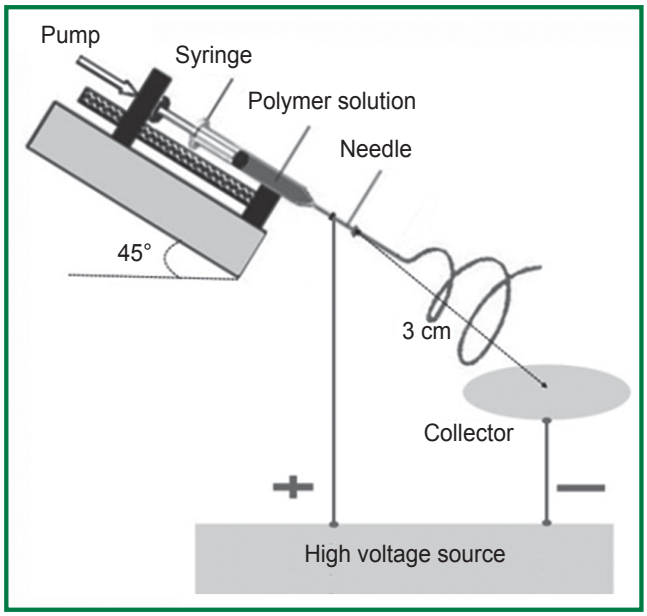

Figure 1. Diagram of the unit for fabricating silk fibrin and gelatin scaffolds

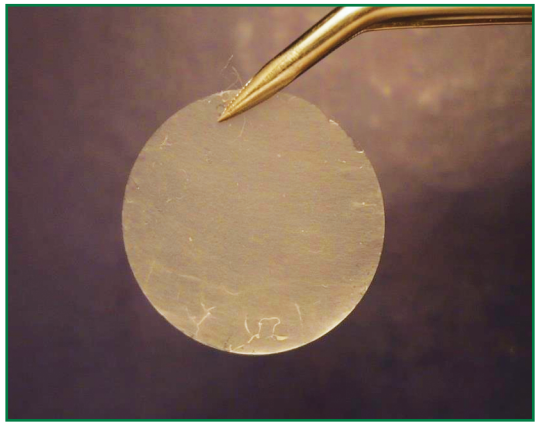

Figure 2. The appearance of silk fibroin on a glass, the fibroin produced by electrospinning; glass diameter is 2 cm

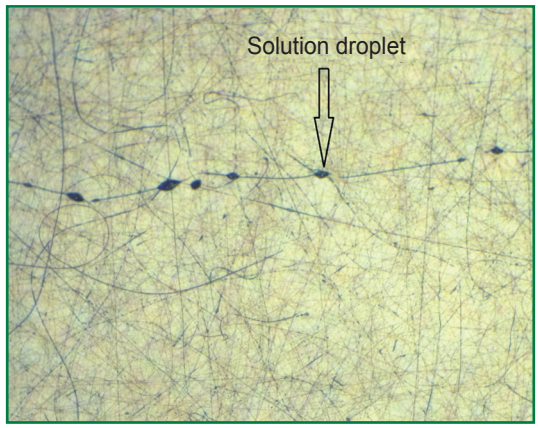

Figure 3. Fiber defect caused by spraying parameter mismatch (silk fibroin; ×100; light microscopy; uncolored fibers)

1,1,1,3,3,3-hexofluoroisopropanol-2 was chosen as a dissolving agent, widely applicable in electrospinning due to its high volatility that enables to provide rapid drying of collecting fiber.

In this investigation two types of scaffolds were fabricated on glass surface by electrospinning method. The fabricated scaffolds were based on two polymers: silk fibroin and gelatin and fibroin-gelatin blend in different ratios. Every scaffold took the form of several layers of evenly distributed fibers (Figure 2).

Quantitative characteristics of a scaffold depend on the parameters of fiber preparation. Among these parameters are: solution pumping speed, voltage at a needle tip, as well as the time of fiber application [7]. Besides, the work polymer solution concentration and the needle diameter have an effect on scaffold properties. Parameter mismatch results in polymer fiber breakout and polymer solution droplet formation into the scaffold structure (Figure 3).

These defects impair the characteristics of the fabricated structure followed by a negative effect on cell adhesion and proliferation. The parameters selected in our study cause no above mentioned processes and result in no defects.

The structure of fabricated scaffolds was studied by microscopy. Light microscopy analysis showed scaffolds consist of uniformly distributed fibers assembled in several layers on the glass surface (Figure 4 (a)). The obtained structure was proved by scanning electron microscopy (Figure 4 (b)). The analysis of a scaffold showed that silk fibroin fibers form multi-layer porous structure which can contribute to cell proliferation and migration within a construction, and by that improving the properties of a construction as an implant [17]. The similar structure was observed for other scaffold groups.

To analyze the structure of silk fibroin scaffolds in aqueous surrounding, laser scanning confocal microscopy was applied also the thickness of fibers composing scaffolds was measured. Silk fibroin fibers were visualized by laser scanning confocal microscopy. 3T3 fibroblasts (Figure 5) having been preliminarily cultured on fabricated scaffolds. Mean fiber thickness was equal to 0.5±0.2 μm.

To prove the effect of the scaffold structure, the nature and composition of the applied polymers on cell adhesion and proliferation, we compared the activity of adhesion and proliferation of 3T3 fibroblasts on silk fibroin and gelatin scaffolds fabricated by two techniques: electrospinning and casting (Figure 6). In contrast to scaffolds fabricated by electrospinning, the scaffolds obtained by casting exhibit no porosity.

Adhesion was evaluated 12 h after the experiment had started. The number of 3T3 fibroblasts adhered to different scaffolds was not significantly different. Cell

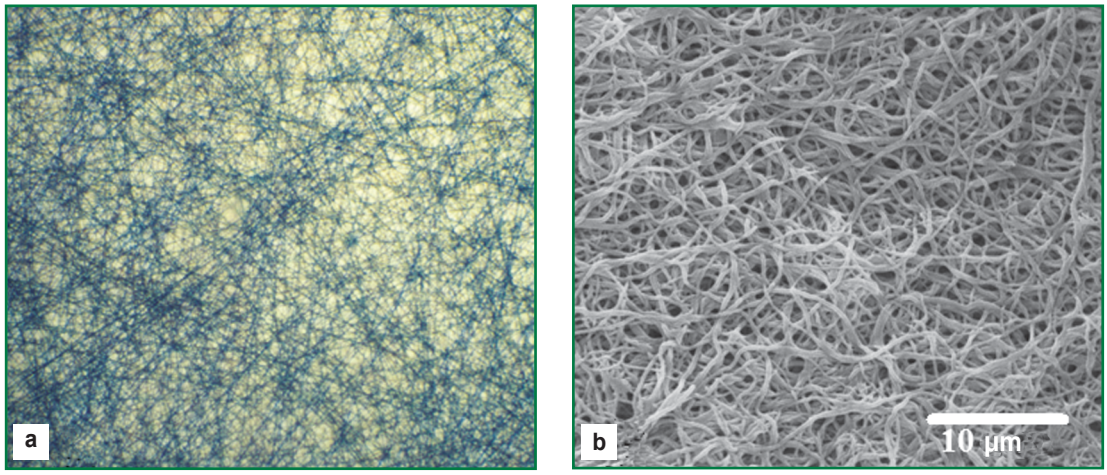

Figure 4. Silk fibroin scaffold structure; the images of silk fibroin fibers obtained by: (a) light microscopy, ×100, unstained sample; (b) scanning electron microscopy

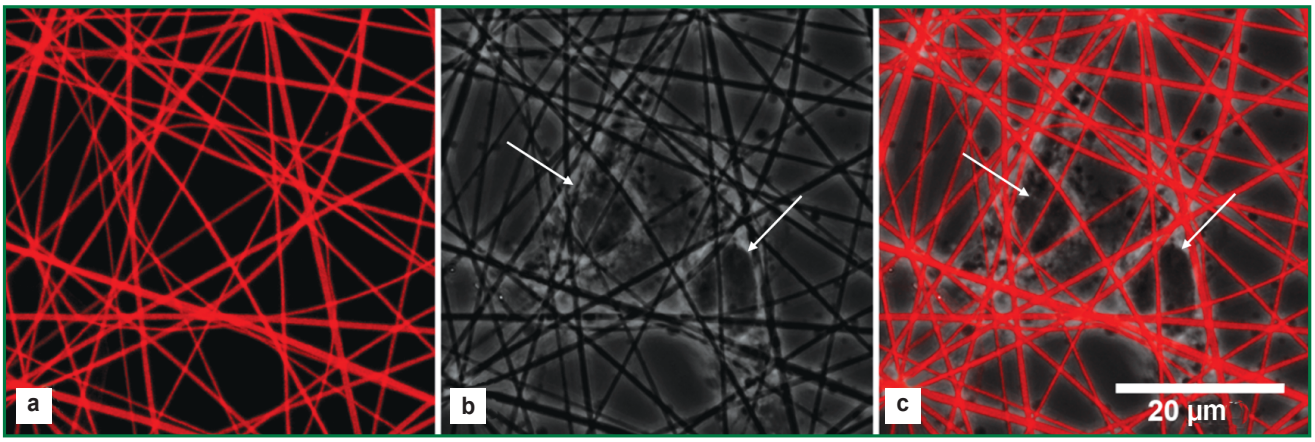

Figure 5. The image of 3T3 line fibroblasts on silk fibroin scaffolds obtained by confocal scanning microscopy: (a) silk fibroin scaffold stained by TRITC, ×200; (b) an image in transmitted light; (c) the overlapping of (a) and (b) images; white arrows indicate cells

proliferation was estimated on days 3 and 5 of the experiment. The number of cells on gelatin scaffolds fabricated by electrospinning, on day 3 of the experiment was insignificantly larger than on those fabricated by casting. However, on day 5 of the experiment the examined scaffolds showed no difference between cell proliferative activity. It can be explained by the fact that gelatin is collagen denaturation product, and its structure contains RGD sequence, which binds to integrin receptors on fibroblast membranes and by that triggering their proliferation [18]. Thus, in this case the differences in construction porosity have no effect on cell proliferative activity. Gelatin is capable to maintain cell proliferative activity [19].

By contrast, cell proliferative activity on silk fibroin scaffolds fabricated by two different techniques significantly differed, and was higher on the scaffolds fabricated by electrospinning. It is elucidated by the fact that the construction obtained by electrospinning is characterized by high porosity and fibrous structure, which provides conducive environment for fibroblast proliferation, since it imitates the architecture of native extracellular matrix. Decreased cell proliferative activity on scaffolds fabricated by electrospinning on day 5 of the experiment can be explained by contact inhibition [20].

The number of cells on gelatin scaffolds increased within the experiment, while the cell distribution in the constructions was uniform that also proves gelatin capability to maintain cell proliferation. It is consistent with a great number of reports in literature demonstrating that the application of gelatin as the material for cell culture and scaffold fabrication for tissue engineering significantly raises the level of cell adhesion [21–23], triggers proliferation and differentiation of osteogenic cells, since gelatin functions as a natural component of extracellular matrix [24]. On the other hand, there are researches, which have investigated insufficient values of mechanical characteristics of gelatin scaffolds for surgical

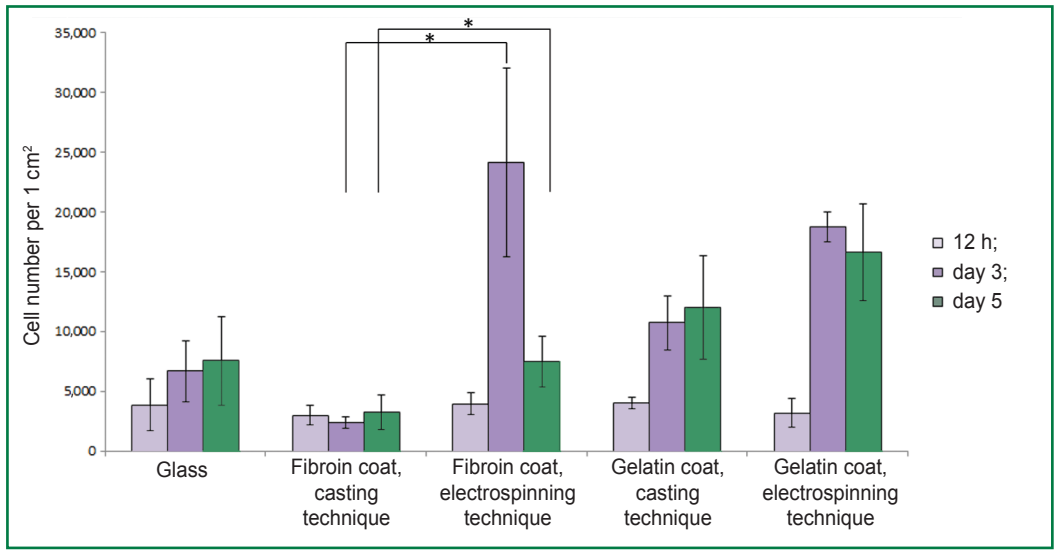

Figure 6. The comparison of silk fibroin and gelatin scaffolds fabricated by casting and electrospinning. The data on adhesion and proliferative activity of mice 3T3 fibroblasts on different products 12 h after the experiment start, and on days 3 and 5 of the experiment. Standard error values are given for 5 independent experiments. The values with $p<0.05$ are marked with an asterisk

procedures; it constrains their potential application. In addition, gelatin application is limited by its high water solubility and quick biodegradability [25].

At the same time, silk fibroin is characterized by unique mechanical properties; however, cell proliferative activity on silk fibroin scaffolds is lower than that on gelatin constructions [26, 27]. One of possible problem solution is the fabrication of composite materials with improved adhesion properties and optimal values of mechanical properties.

Therefore, the second experiment aimed at searching an optimal ratio of gelatin and fibroin in the blend, which could provide cell adhesion and proliferation, but at the same time enable to avoid rapid fiber resorption.

Three groups of scaffolds with different mass ratio of silk fibroin and gelatin: 3:1, 1:1 and 1:3, were fabricated. There was successful and nearly equal fibroblast adhesion to all scaffolds 12 h after the experiment had started. Cell proliferative activity on different constructions was estimated on days 3 and 5 of the experiment (Figure 7).

On day 3 of the experiment there was observed the increase in cell proliferative activity on scaffolds containing gelatin, on day 5 the number of cells on all composite coatings. Moreover, there was direct dependency of cell proliferative activity on gelatin content in a blend. However, on gelatin scaffolds the number of cells on day 5 of the experiment suffered no changes compared to that on day 3. This can be explained by the fact that the gelatin scaffolds without fibroin addition were fixed by 0.3% glutaric dialdehyde solution to avoid their destruction due to gelatin dissolution during the experiment. Glutaric dialdehyde is known to be toxic to cells and undermine cell proliferation in a product [28].

The highest cell proliferation was obtained on scaffolds with mass ratio of fibroin and gelatin 1:3. The number of cells on these scaffolds was larger by 2.5 times compared to that on gelatin scaffolds, and fivefold compared to silk fibroin scaffolds. The reached effect could be connected with the following: such combination of polymers provides optimal distribution of amino acid sequence RGD as a part of gelatin on the fiber surface, as well as the formation of insoluble high-porous scaffold due to the presence of silk fibroin in the composite. Moreover, the charge of substrate surface also has an effect on cell adhesion efficiency, viability, proliferation and migration rate. Negatively-charged gelatin (as fibroin pH is neutral) added in films composition could improve the efficiency of cell adhesion to such substrate. So, porous scaffolds and microgels from recombinant analogue, spidroin: a structural protein of spider's silk with positive charge at physiological pH values, are perfect substrates for *in vitro* cell adhesion and proliferation, as well as they also induce *in vivo* neuro- and angiogenesis that is of primary importance for different types of tissue regeneration including gastrointestinal organs, bone tissue, skin, etc. [29, 30].

Conclusion. The comparative analysis of biological properties of silk fibroin and gelatin scaffolds fabricated by different techniques showed electrospinning to enable to fabricate products with improved physicochemical properties, which have a positive effect on their biocompatibility. Optimum compromise of structure and biocompatibility can be achieved by composite scaffolds application with the mass ratio of fibroin and gelatin equal to 1:3, fabricated by electrospinning.

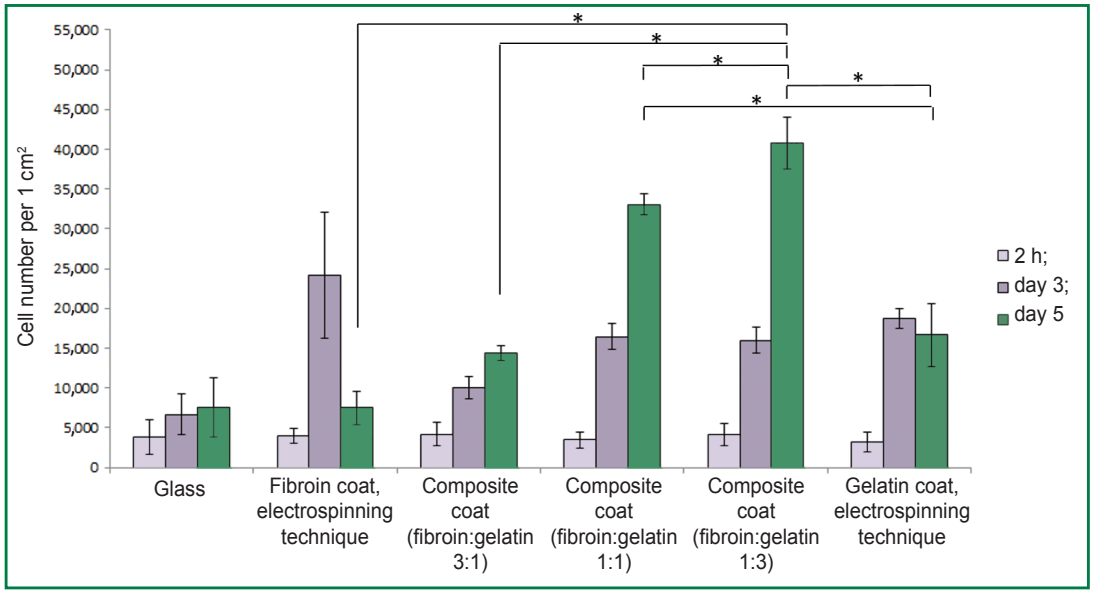

Figure 7. The comparison of scaffolds from pure polymers with composite scaffolds with different mass ratio of fibroin and gelatin. The data on adhesion and proliferative activity of mice 3T3 fibroblasts on different scaffolds 12 h after the experiment start, and on days 3 and 5 of the experiment. Standard error values are given for 5 independent experiments. The values with $p<0.05$ are marked with an asterisk

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Conflicts of Interest. The authors have no conflicts of interest related to the present study.

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