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NATURAL SILK FIBER MICROCARRIERS FOR CELL CULTURE

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The development of effective and versatile microcarriers is a pressing issue in tissue engineering and regenerative medicine. The **objective** of this work is to create biocompatible fiber microparticles from the cocoons of the *Bombyx mori* silkworm, and to study their structure and biological properties. **Materials and methods.** In obtaining microparticles, the *Bombyx mori* cocoons washed from sericin were cryo-milled in liquid nitrogen. The structure of the resulting microparticles was analyzed via scanning electron microscopy. The cytotoxicity of the obtained fibers was assessed using MTT-cell culture assay of 3T3 mouse fibroblasts. Cell adhesion analysis was performed using the Hep-G_2 human hepatocarcinoma cell line. Cell visualization was performed by staining the nuclei with DAPI fluorescent dye. **Results.** Natural silk microparticles were obtained in the form of cylindrical fibers with 200–400 μm average length and 15 μm diameter. It was shown that the surface of the resulting microparticles has a rough relief; no pores were found. The microparticles are non-toxic for 3T3 mouse fibroblasts, they maintain a high level of adhesion by human hepatocellular carcinoma $HepG_2$ cells. **Conclusion.** The method developed by us for fabrication of biocompatible silk fibroin microparticles in the form of fibers without using toxic reagents and significant time costs is promising for cell cultivation and delivery to the damaged area for tissue and organ regeneration.

Keywords: microcarriers, silk fibroin, fibers.

INTRODUCTION

The traditionally used two-dimensional cell culturing distorts to some extent cell behavior and its biological functions in comparison to cells in native tissue [1, 2]. For example, 2D *in vitro* cultivation of tumor cells showed slower tumor growth and lower drug resistance compared to tumors in vivo, which leads to lower efficiency of screening and drug testing [3, 4]. Therapeutic use of cell culture is also ineffective because of the low level of cell viability and, as a consequence, minimal efficiency of regeneration of damaged tissue [5–7]. This is mainly due to the fact that in a 2D *in vitro* culture, cells are unable to recreate effective and multidirectional cell-cell and cell-extracellular matrix interactions present in the native microenvironment, which causes changes in cell morphology and gene expression. To obtain cells with normal morphology, metabolism, and functions, 3D culturing of cells on different microcarriers is used [8, 9].

In regenerative medicine, microcarriers are widely used both as a substrate for culturing various cell types and as a means of cell delivery to the damaged tissue or organ area [10]. When microcarriers are used as a delivery system, cells remain viable for a longer period of time [11], which allows them to secrete growth factors and actively participate in the formation of the intercellular matrix, promoting tissue regeneration [12]. Both natural and synthetic biopolymers are used to obtain microcarriers of various types and for regeneration of various tissues [13–16].

One of the promising materials for creating biocompatible microcarriers for tissue engineering and regenerative medicine is silk fibroin from the cocoons of the *Bombyx mori* silkworm [17]. The structure of silk fibroin provides unique properties that allow it to be used as a material for tissue engineering. The main advantage of silk over other biocompatible materials lies in its mechanical properties. The advantages of fibroin as a material used in regenerative medicine include the fact that fibroin-based constructs are biodegradable and biocompatible, they can be obtained under mild conditions and their production does not require special chemical treatment. Silk fibroin promotes cell adhesion, proliferation and differentiation, including with regard to mesenchymal stem cells [18–20]. For instance, silk fibroin-based microparticles are used for bone tissue [21] and nervous tissue [22] regeneration, as well as stem cell delivery [23]. Thus, fibroin has unique properties that makes it possible to form from it 2D and 3D constructs, including microcarriers, and widely use it as a biocompatible material in various areas of tissue engineering.

In this study, biocompatible microcarriers were developed from *Bombyx mori* silkworm cocoons in the form of fibers, their structure was studied, and the possibility of their application in tissue engineering was shown.

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MATERIALS AND METHODS

Obtaining microcarriers

Cocoons from mulberry silkworm *Bombyx mori*, provided by Bogoslovsky V.V. (Zheleznovodsk, Stavropol Krai), director of the Republican Research Station for Silkworm Breeding, were used as a source of silk fibroin fibers. At the first stage, the cocoons were purified of sericin according to the following procedure. 1 g of silk from cocoons was boiled in 500 mL of double-distilled water water with 1260 mg of soda for 40 min in a water bath, then washed with 3.6 L of distilled water. Next, it was boiled in 500 mL of double-distilled water water for 30 minutes and washed with 3.6 L of distilled water. The last procedure was repeated 3 times. The purified silk fibroin was air-dried at room temperature. Then silk fibroin weighing 80 mg was placed in a 3% aqueous solution of glycerin, incubated for 120 minutes and frozen in liquid nitrogen, after which the fibroin was crushed for 5 minutes using a mortar and pestle to an average fiber size of 200–400 μm. The resulting fibers were transferred into 5 mL of 70% ethanol for 30 minutes, and then the ethanol was changed [24].

Analysis of microcarrier structure by scanning electron microscopy

The fiber samples under study were fixed in a 2.5% glutaraldehyde solution in phosphate-buffered saline with pH = 7.4 for 2 hours in the dark at 4 °C, the samples were washed of the fixing solution with phosphate-buffered saline (pH = 7.4) 5 times for 5 minutes each. Then the samples were dehydrated with increasing ethanol concentrations. Ethanol concentrations used were 10%, 20%, 50%, 70% and 96%, the samples were incubated for 1 hour in ethanol of each concentration. Samples were then transferred to acetone for 30 minutes, and then the acetone was changed.

The samples were dried by critical point transition ($T_{cr.CO_2}$ = 31 °C, $p_{cr.CO_2}$ = 72.8 kg/cm^2)) using a K850 unit (Quorum Technologies, UK). The dried samples were coated with a 10 nm thick gold layer in argon atmosphere at 20 mA ion current and 1 mbar pressure using the Q150R ES vacuum sputtering machine (Quorum Technologies, Great Britain). The obtained samples were analyzed using a Tescan Vega3 SBU scanning electron microscope (Tescan, Czech Republic) at 15 kV operating voltage. Images were obtained and analyzed using the VegaTC software (Tescan, Czech Republic).

Analysis of microcarrier cytotoxicity

The mouse 3T3 fibroblast cell line was used in the experiments. Cells were cultured in plastic vials in DMEM low glucose medium containing 10% fetal bovine serum, 0.324 mg/mL glutamine and 10 mg/mL gentamicin at 37 °C, 5% CO_2. The culture medium was changed every 48 hours. Cell monolayer was disaggregated using a trypsin-Versene solution. Cells were counted in the Goryaev chamber and seeded in a 1 : 3 ratio.

Cytotoxicity of all obtained samples was analyzed in accordance with the GOST ISO 10993-5-2011 standard [25] using the MTT test [26] in the mouse 3T3 fibroblast cell line model.

For this purpose, mouse 3T3 fibroblasts were cultured in 300 μL of culture medium in a 96-well plate in a thermostat at 37 °C and 5% CO_2 for 3 days. Then, the culture medium was changed and 50 μL of fiber suspension was added to the wells of the plate. The culture plate was used as a control. The plates were incubated in an incubator at 37 °C, 5% CO_2. Cytotoxicity was assessed on days 3, 5 and 7 of the experiment. For this purpose, 60 μL of MTT solution with 5 mg/mL concentration was added to each well of the plate. It was incubated in a thermostat at 37 °C and 5% CO_2 for 4 hours until dark blue crystals of formazan precipitated out. Fibers were then removed from the wells, and the plate was centrifuged for 5 minutes at 885 g. The supernatant was removed, the formazan precipitate was dissolved in 300 μL dimethyl sulfoxide for 20 minutes, and the optical density of the solution was measured at 540 nm wavelength on a Picon device (Picon incorporated company, Uniplan, Russia).

Analysis of cell adhesion on microcarriers

Human liver cancer cell line Hep-G2 was used in the experiments. The cells were incubated in plastic culture vials in a 1 : 1 mixture in DMEM high glucose medium and in Ham's F-12 medium containing 10% fetal bovine serum, 0.324 mg/mL glutamine and 10 mg/mL gentamicin at 37 °C, 5% CO_2.

The experiment was performed in a 96-well plate. For the experiment, a 150 μL fiber suspension was transferred to the wells of the plate. The culture plate was used as a control. Sterile phosphate-salt buffer solution (pH = 7.4) was then added to the wells of the plate and incubated for 15 minutes, after which the phosphate-buffered saline was changed. This procedure was repeated three times. Next, 300 μL of incubation medium was added to the plate wells for 30 minutes.

Cell suspension in the incubation medium was transferred to 96-well plates at 1000 cells per well. The plates were incubated in a thermostat at 37 °C and 5% CO_2 for 7 days.

Cell adhesion was assessed visually using a Carl Zeiss Axio Vert.A1 microscope (Zeiss, Germany). For this purpose, samples were stained with DAPI fluorescent dye, which binds to cell DNA. Before staining, samples were washed twice of the incubation medium and non-adherent cells with phosphate-buffered saline (pH = 7.4). Afterwards, an aqueous dye solution with 3 μg/mL concentration was added at a rate of 300 μL per well and incubated in the incubation chamber at 37 °C and 5% CO_2 for 5 minutes. Samples were then washed twice with phosphate-buffered saline (pH = 7.4) to remove unbound dye. The obtained samples were analyzed on a

Fig. 1. Fabrication of fiber microcarriers. a) *Bombyx mori* silkworm cocoons; b) sericin-free silk fibroin; c) fiber microcarriers. Scale bar 100 μm

fluorescence microscope using a filter with 360–370 nm excitation range, and 420–470 nm emission range.

Cell images were obtained using an Axiocam 305 color camera (Carl Zeiss, Germany). The images were processed using the Zen 2.3 Blue Edition software (Carl Zeiss, Germany).

Statistical processing of results

The data were processed by analysis of variance. Statistical significance of the results was assessed using the Mann–Whitney U test. The statistical significance level α was taken to be 0.05.

RESULTS AND DISCUSSION

In this study, a suspension of microparticles from the cocoons of the *Bombyx mori* silkworm was obtained using cryogenic grinding (Fig. 1). The microparticles are cylindrical fragments of silkworm cocoon fibers, 200–400 μm in average length and 15 μm in diameter. This microparticle shape has a high surface area to volume ratio and, therefore, a larger surface area available for cell adhesion and for formation of more cell contacts compared to spherical particles. Silk fibroin is one of the promising materials for tissue engineering due to a unique combination of mechanical properties and a high level of biocompatibility and can be used in many areas of tissue engineering, both as an independent material for creating constructs and as a carrier for cell delivery or targeted drug delivery [27]. The advantage of the proposed technology for the manufacture of microcarriers in the form of fibers is to obtain biocompatible carriers for cells of controlled size and shape without the use of toxic reagents and significant time costs. The size and shape of the microcarriers can be regulated by varying the cryogenic grinding time and the volume of the washed silk fibroin. Also, the shape of microcarriers in the form of fiber allows for easier positioning of the cells during targeted delivery and facilitates cell orientation.

The structure of the resulting microcarriers was studied by scanning electron microscopy. The surface of the fibers has micro- and nanorelief in the form of roughness (Fig. 2). This method did not reveal any pores in the microcarrier structure. The surface structure of the construct is known to influence cell adhesion [28, 29]. This is because the presence of roughness on the substrate surface increases the surface area available for cell adhesion. At the same time, there is an optimal level of substrate roughness for each cell culture [30].

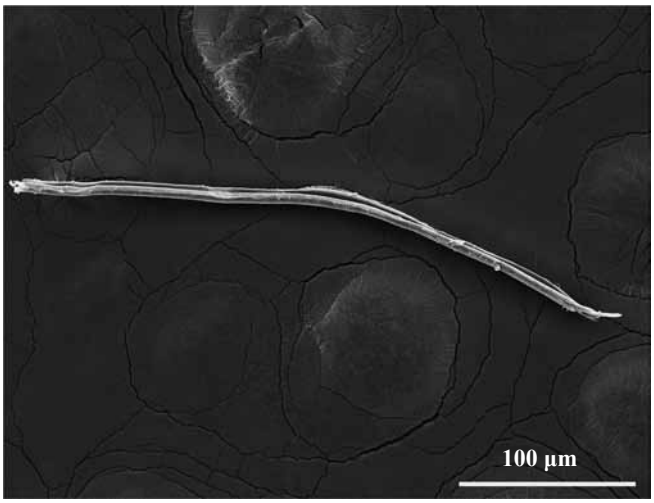

Fig. 2. Image of a microcarrier obtained using scanning electron microscopy. Scale bar 100 μm

The resulting microcarriers were tested for cytotoxicity by MTT. During the study, the effect of samples on the number of cells cultured on the plate was evaluated. The culture plate was used as a control. No cytotoxic effect of the obtained fibers was detected during the experiment; the number of cells did not differ in the experimental and control samples.

The analysis of cell adhesion on the obtained microcarriers in the form of fibers was performed visually using a fluorescent microscope; the cells were pre-stained with the DAPI fluorescent dye to visualize the nuclei. Fibers were positioned in the wells of culture plates; the culture plate was used as a control. The studies were carried out using human liver cancer cell line Hep-G2. The experimental results are shown in Fig. 3.

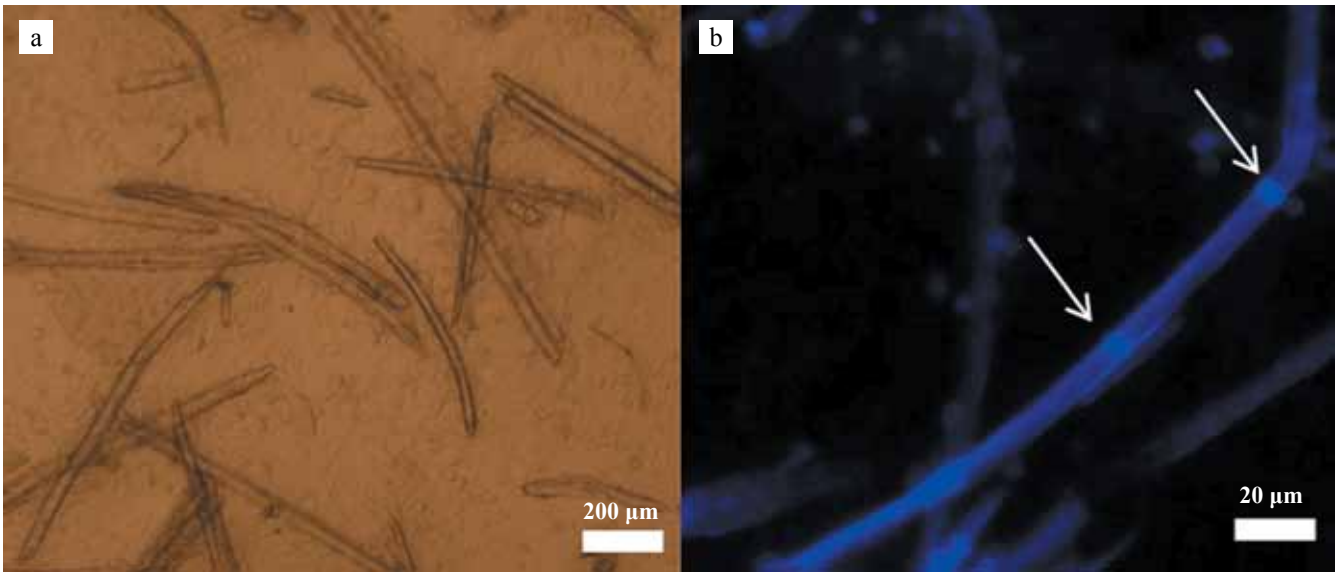

Fig. 3. Adhesion of Hep-G_2 cells on the obtained microcarriers. a) optical image, scale bar 200 μm; b) fluorescent image, nuclei of dapi-stained adherent cells are indicated by arrows, scale bar 20 μm

Cell morphology was assessed using a light microscope; cells formed filopodia and adhered tightly to the substrate, while cell adhesion occurring irregularly. It was found that the obtained microcarriers were biocompatible and maintained a high level of cell adhesion, which can be attributed to the optimal level of fiber roughness and biocompatible properties of silk fibroin [31, 32]. Thus, the resulting silk fibroin microcarriers in the form of fibers are biocompatible and can be further used in regenerative medicine as universal carriers for cells obtained without the use of toxic reagents and significant time costs.

CONCLUSION

As part of the presented work, microparticles were obtained from the cocoons of the *Bombyx mori* silkworm in the form of fibers with 200–400 μm average length and 15 μm diameter. It was shown that the surface of the obtained microparticles has a rough relief; no pores were detected. The microparticles are non-toxic for the culture of mouse 3T3 fibroblast cells, they maintain a high level of cell adhesion of human liver cancer cell line Hep-G2. The technique developed by us for creating biocompatible microcarriers from *Bombyx mori* silkworm cocoons in the form of fibers is promising for cell culture and cell delivery to the damaged area for tissue and organ regeneration.

The authors declare no conflict of interest.

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