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METHODS

Photocurable Hydrogels Containing Spidroin or Fibroin

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Abstract—Photocurable biocompatible hydrogels were fabricated from methacrylated gelatin and silk proteins (a recombinant analogue of spidroin from *Nephila clavipes* spider web and fibroin from the cocoons of the silkworm *Bombyx mori*). These polymers are widely applicable in tissue engineering due to their biocompatibility and biodegradability. Hydrogels were fabricated using two different methods that allowed to obtain either the macroscopic scaffolds or microstructures with a defined shape. Three-dimensional hydrogel samples were prepared via monomers solution photopolymerization for 10 min. As a result, the disk-shaped samples of hydrogels approximately 13 mm in diameter were fabricated. Their porous structure was confirmed by scanning electron microscopy. Microstructures were formed on coverslips using an Eclipse Ti-E microscope equipped with an A1 confocal module (Nikon, Japan) and 405 nm laser. This approach allows to control the topographic features of the obtained substrates, and it is viable for creating the micropattern surfaces to reveal for studying the interaction of cells with a substrate.

Keywords: hydrogels, photopolymerization, spidroin, fibroin, tissue engineering, methacrylated gelatin.
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The photocrosslinkable materials undergo covalent crosslinking upon exposure to light of a certain spectral region. Using them in both basic and applied biomedical research is of special interest. For example, they may be the basis of microstructured surfaces with controlled topography and distribution of ligands for cell receptors, which can simulate a variety of biological processes, i.e., axonal growth [1]. Photocuring is also the basis of a number of additive technologies that allow creating three-dimensional structures with submicron positioning accuracy [2].

Producing scaffolds for tissue engineering, which aims to find effective approaches to restore various damaged tissues and organs, is one of the main areas for applying these photocrosslinkable materials. Scaffolds are used as an artificial extracellular matrix on which the tissue is formed. Therefore, properties of the materials from which they are made are of great importance.

Methacrylated gelatin is a photocurable material that is widely used for various biomedical applications due to its nontoxicity, biodegradability, and the possibility to control its properties [3]. However, even the chemically crosslinked hydrogels consisting of this polymer do not possess the required mechanical properties nor do they have a high swelling ratio and biodegradation rate. One of the approaches allowing us to solve this problem has been proposed previously: an aqueous solution of methacrylated gelatin was supplemented with small amounts of silk fibroin (5–20%) prior to photopolymerization that made it possible to produce hydrogels possessing the structure of the interpenetrating networks [4]. These networks are composed of a C = C polymer based on a methacrylated gelatin crosslinked through double bonds and silk fibroin physically crosslinked through antiparallel beta-sheets. This composition ensures the optimal mechanical and technological characteristics of the biomaterial compared to the single-component counterparts.

Structural silk proteins, including fibroin, possess all the required properties to be used in tissue engineering and are characterized by strength and elasticity unique for natural polymers. The possibility to use spidroin, spider web protein, is of particular interest. It was previously shown that implantation of porous scaffolds made of recombinant spidroin in rat femur defect led to a significant acceleration of regeneration compared with the regeneration of the unfilled defect

femur or regeneration after the implantation of fibroin scaffold [5].

The goal of the present work was to obtain the photocrosslinkable hydrogels formed of methacrylated gelatin and structural silk proteins, the recombinant analog of spidroin of anchor thread of spider *Nephila clavipes* and silk fibroin of the cocoons of silkworm *Bombyx mori*.

MATERIALS AND METHODS

Materials. The following reagents were used: methacrylic anhydride (94%), diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (97%), dimethyl sulfoxide (99.9%), lithium bromide (99%) (Sigma-Aldrich, Germany), formic acid (99%) (Acros Organic, United States), ethanol (95%) (Medihimprom, Russia), crystalline gelatin (ultra-pure, Carl Roth, Germany), Dulbecco's modified Eagle Medium (DMEM, PanEco, Russia), fetal calf serum (HyClone, United States), paraformaldehyde (Sigma-Aldrich, Germany), SYTOX green nucleic acid stain (Invitrogen, United States), surgical silk thread (OOO Mosnitki, Russia), recombinant analogs of spider silk proteins 1F9 obtained according to [6], and 0.1 M potassium phosphate buffer solution (pH 7.2).

Synthesis of methacrylated gelatin. A portion of the crystalline gelatin (1 g) was dissolved in 20 mL of 0.1 M potassium phosphate buffer (pH 7.2) placed in a round-bottom flask fitted with a magnetic stirrer. Gelatin dissolution was performed in a water bath (50°C) under continuous magnetic stirring. The final concentration was 5 wt %. The resulting solution was supplemented with excess of methacrylic anhydride. The reaction was carried out for 3 h at 50°C under continuous stirring. Then, the reaction mixture was supplemented with 20 mL of 0.1 M potassium phosphate buffer (pH 7.2), after which the mixture was cooled to room temperature and purified by dialysis using the cellulose membrane against 20-fold volume of distilled water at constant stirring on a magnetic stirrer changing the solution every hour for 3 days before the disappearance of the methacrylic anhydride odor. The reaction product was placed into a Petri dish, frozen at –18°C, and lyophilized in an Alpha 1-2 LDplus freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) to a constant weight.

Preparation of the aqueous solution of silk fibroin. Sample of surgical silk was dissolved in 9.3 M lithium bromide solution, and then dialyzed for 1 day against the distilled water which was refreshed 10 times during the process. Fibroin solution was frozen in 10-cm Petri dishes and lyophilized using an Alpha 1-2 LDplus freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The amount of protein required to obtain the solution with a desired concentration was dissolved in distilled water.

Hydrogel formation. To obtain the fibroin-based hydrogels, a sample of methacrylated gelatin was dissolved in dimethylsulfoxide by means of incubation in a thermostat at 50–60°C until complete dissolution and then supplemented with photoinitiator of polymerization, diphenyl (2,4,6-trimethylbenzoyl) phosphine oxide (97%, 3 wt % of methacrylated gelatin). Then, the aqueous fibroin solution was carefully added under continuous mixing. The concentration of methacrylated gelatin in the final solution was not less than 3 wt %, and the ratio between the monomers (methacrylated gelatin : fibroin) was 2 : 1.

To obtain spidroin-based hydrogels, a sample of the protein was dissolved in 90% formic acid and then the solution was accurately supplemented with methacrylated gelatin and mixed until complete dissolution. Then, a photoinitiator of polymerization diphenyl (2,4,6-trimethylbenzoyl) phosphine oxide (97%, 5 wt % of methacrylated gelatin) was added to the mixture. Methacrylated gelatin concentration in the final solution was not less than 10 wt %, and the ratio between the monomers (methacrylated gelatin : spidroin) was 2 : 1.

Photopolymerization. To obtain the macroscopic hydrogels, monomer solutions (300 μL) were placed into a polypropylene chamber 13 mm in diameter so that an even layer was formed. Then the mixture was photopolymerized under a 36 W UV lamp for 10 min. The chambers were filled with 95% ethanol, incubated for 1 h at an ambient temperature, and washed with excess of distilled water for 2–3 h changing the solution and under a continuous stirring. Hydrogel samples were transferred to 70% ethanol further storage.

Microscopic hydrogel structures were formed by photocuring using an Eclipse Ti-E microscope equipped with an A1 confocal module (Nikon, Japan). Templates with a required shape were obtained by means of NIS-Elements software (Nikon, Japan) and the corresponding regions of the mixture placed on the coverslip were irradiated using a 405 nm laser and a Plan Fluor 40x / 1.30 Oil DIC objective. The obtained microstructures were washed with distilled water and treated with 95% ethanol.

3T3 fibroblast cultivation on hydrogels. The 3T3 fibroblasts were suspended in DMEM medium containing 10% fetal calf serum. The concentration of fibroblasts was adjusted up to 24×10^3 cell/mL. Hydrogels were placed in 35 mm Petri dishes and then 2 mL of suspension were added. After 48 h of incubation, cells were fixed with 4% paraformaldehyde. The cell nuclei were stained with SYTOX green nucleic acid stain (Invitrogen, United States) and images were obtained using an Axiovert 200M LSM 510 Meta confocal microscope and 3D for LSM software (Zeiss, Germany).

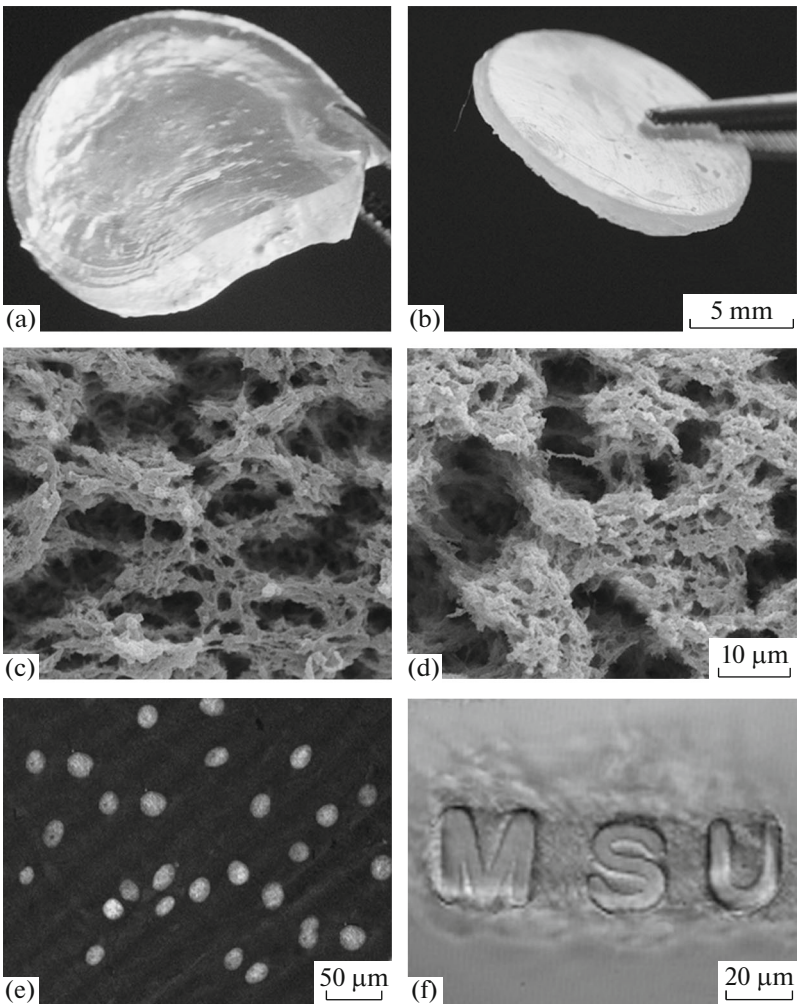

Fig. 1. Photocurable hydrogels containing (a, c, e) spidroin or (b, d, f) fibroin: (a) discs formed from hydrogels based on methacrylated gelatin and spidroin, (b) discs formed from methacrylated gelatin and fibroin, (c, d) SEM images of the structure of the disks based on methacrylated gelatin in combination with (c) spidroin or (d) fibroin, (e) nuclei of 3T3 fibroblast cultured for 48 h in the surface of the hydrogel based on methacrylated gelatin in combination with spidroin, (f) microstructure formed from the hydrogel based on methacrylated gelatin and fibroin.

Scanning electron microscopy. Hydrogels were dehydrated by passing through a series of increasing ethanol and acetone concentrations, dried using a Critical point HCP-2 dryer (Hitachi Ltd., Japan), and coated with a 20 nm platinum layer using the IB-3 Ion Coater (Eiko Engineering Co., Ltd, Japan). The specimens were examined using a CamScan S2 microscope (Cambridge Instruments, United Kingdom).

RESULTS AND DISCUSSION

Hydrogels are three-dimensional polymeric networks capable of absorbing and retaining large amounts of water. Numerous studies have focused on applying them in tissue engineering, targeted delivery of drugs, and other biomedical applications [7].

The development of wound dressings based on hydrogels is an area of their active application, since they have the ability to retain exudate in the wound providing for the accelerated healing. Another advantage of hydrogels is the possibility to introduce antibiotics and other pharmaceuticals into their structure [8]. It was previously shown that fibroin and spidroin microcarriers introduced in full-thickness skin wounds accelerated healing of a skin wound and restorating all the structural skin components [9, 10]. The wound dressings formed from hydrogels based on these structural silk proteins may also contribute to regeneration. In the present work, the prototypes of such products consisting of methacrylated gelatin combined with spidroin or fibroin have been produced. Disk-shaped hydrogel samples 13 mm in diameter were obtained (Figs. 1a, 1b).

Diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (97%), which is a highly reactive water-insoluble compound applied for rapid photocuring, for exam-

ple, in laser stereolithography, it was used as the photoinitiator of polymerization. In the previously described system based on methacrylated gelatin and fibroin, 2-hydroxy-1-[4-(hydroxyethoxy)-phenyl]-2-methylpropanone was used [4]. Application of diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide made it possible to reduce the duration of UV radiation by 5–10 times and performed photopolymerization in the solution layer up to 5 mm in thickness.

The internal space of the dried disks was characterized by a porous structure (Figs. 1c, 1d). To evaluate the biocompatibility of photocrosslinkable hydrogels in model systems in vitro, 3T3 mouse fibroblasts were used. A representative image of cell nuclei 48 h after placing the fibroblast suspension on the surface of spidroin-containing hydrogel is shown in Fig. 1e. The cell density indicated their active proliferation. Similar results were also obtained for the fibroin-containing hydrogels. Thus, the photocrosslinkable fibroin or spidroin derivatives in model systems in vitro retained biocompatibility after their modification with methacrylated gelatin and photoinitiator.

The possibility to obtain the microscopic structures was accessed using an optical system of a confocal microscope. The relief elements consisting of the silk fibroin hydrogel, with one of the linear dimensions not exceeding 5 μm, were formed on the surface of the coverslip (Fig. 1f). The microstructured surfaces can be used in model systems to study the effect of the microenvironment on cell function. For example, the algorithms for the automated study of single cells can be developed [11]. Furthermore, application of methacrylated gelatin derivatives makes it possible to adapt structural silk proteins to additive and prototyping technologies.

Thus, photocurable hydrogels based on methacrylated gelatin and recombinant spidroin, as well as methacrylated gelatin and silk fibroin, which can be used in the formation of scaffolds for tissue engineering and wound dressings, including microstructured, were obtained. Moreover, the biocompatible photocrosslinkable spidroin hydrogels were obtained for the first time.

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