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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

# Three-Dimensional Scaffold Made from Recombinant Spider Silk Protein for Tissue Engineering[1]

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The natural polymers (biopolymers) and their derivatives—alginates, collagen, gelatin, chitosan, hyaluronic acid, and polyesters of bacterial origin—are now preferred as materials for reconstructive and substitutive medicine [1]. Biopolymers are highly biocompatible and also can act as biostimulants. After implantation they degrade to simpler compounds that are removed from the body or involved in cell metabolic processes [2–4].

Impressive mechanical properties of spider silk attracted attention of scientists and have been extensively discussing for ten years [5]. Viscoelastic fibers made of spider silk are highly elastic and resistant to rupture. Tensile strength of this material exceeds steel and is comparable with Kevlar. These characteristics of spider silk make this biopolymer a unique material among other natural and the majority of artificial materials [6]. Spider silk consists of two proteins, spidroin 1 and spidroin 2. These proteins are characterized by high molecular weight and a periodic structure and composed of a large number of direct repeats [7]. The repeats differ by deletions and insertions but their organization is similar. Each repeat contains hydrophobic poly-Ala segment (with a length of 4 to 8 amino acid residues) and a more hydrophilic sequence enriched with Gly residues. These proteins do not dissolve in water, weak acids, and weak alkali. Spider silk contain a considerable amount of crystals made of β-sheets (formed of poly-Ala blocks) and characterized by high level of structural organization, even in the Gly-rich regions [8].

Spidroins under the action of some factors undergo a rapid structural transition from a less organized to highly organized state and then to a water insoluble state [9]. Polymers can be modified by adding adhesion sites or, for example, cytokines, to side groups of amino acid residues.

Unique mechanical properties, environmental stability, biocompatibility and biodegradability make spider silk polymers a promising material for medical application.

The aim of this study was the production of a biocompatible three-dimensional scaffold made of recombinant spider silk proteins for cultivation of eukaryotic cells.

The structure of artificial analog of spidroin 1 gene was designed on the basis of known nucleotide sequence of the corresponding cDNA from *Nephila clavipes*. Four fragments that encode consensus primary repeats of natural spidroin 1 sequence were chosen for synthesis of the monomer of artificial gene. The monomer was produced by chemical-enzymatic gene synthesis, introduced into plasmid and amplified in *Escherichia coli* cells. The resulting gene that is composed of nine repeats of this monomer, was introduced into the genome of the yeast *Pichia pastoris*. The recombinant protein 1F9, an analog of spidroin 1, was separated from yeast cells, purified up to 98% [10], and used for scaffold synthesis.

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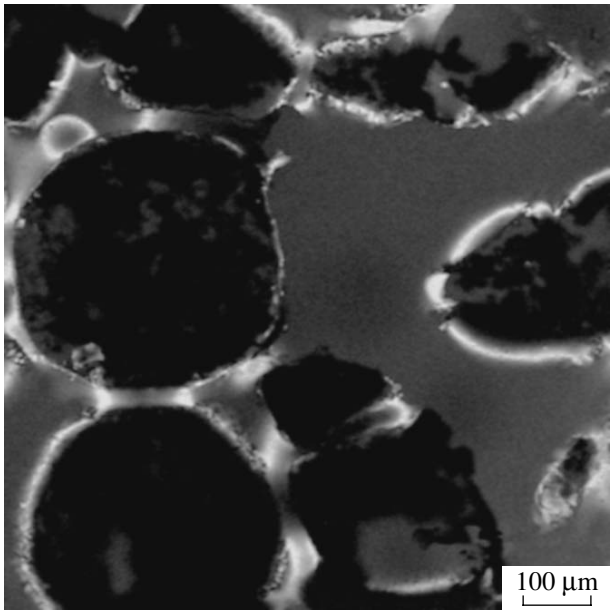


**Fig. 1.** A porous structure of the scaffold produced using 200–400 μm NaCl particles. Optical cross section was made is made at the level of 250 μm from the superficial level. The pore size corresponds to the size of NaCl particles. Some pore cavities are interconnected.

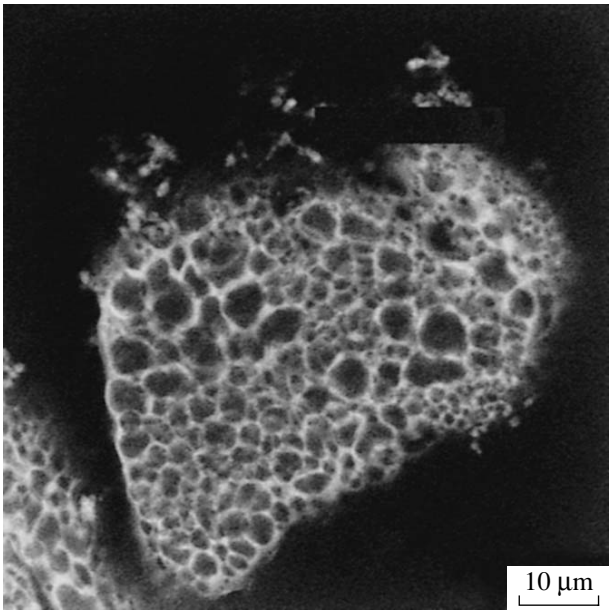


**Fig. 2.** Wall structure of scaffold pore produced using 50–100 μm NaCl particles. Optical cross section was made 100 at the level of 100 μm from the scaffold superficial layer. The pore size corresponds to diameter of used NaCl particles.

The three-dimensional scaffold was produced from lyophilized spidroin 1 by leaching. For this purpose, a sample of spidroin 1F9 (15 mg) was dissolved in 50 μl of 10% lithium chloride solution in 90% formic acid for 30 min at 40°C. The solution was centrifuged for 10 min at 11300 g. Then we mix 50 μl of the supernatant with 110 mg of sodium chloride particle (50–100 and 200–400 micrometers in diameter). Then y formed discs with a diameter of 10 mm and a thickness of about 1 mm, dried them at a room temperature, treated with 96% ethanol for 120 min and rinsed it in distilled water for 120 min. The specimens were degassed and stored in 96% ethanol. Scaffold pore size depends on used NaCl particle size and influences its mechanical properties, the rate of biodegradation, and its interaction with cells [11].

Scaffold specimens cells attached to the surface of material were studied with Zeiss Axiovert 200M LSM510 META, Leica TCS SP5 confocal microscope and Camscan S2 scanning electron microscope (Cambridge Instruments, United Kingdom). For studying the scaffold structure with the help of confocal microscopy we conjugated scaffold polymers with fluorochromes. The confocal images were processed and we calculated the cell quantity with the help of 3D for LSM Version 1.4.2 software.

The optical section of the scaffold (produced with NaCl particles of 200–400 μm in diameter) is represented in Fig. 1. The section was made at a distance of 250 μm from the scaffold surface. Figure 2 demonstrates the optical section through the scaffold pore (100 μm from the surface) produced with the NaCl particles of 50–100 μm in diameter. The pore surface is composed of units with a size of 1 to 10 μm formed by aggregated spidroin. The pose sizes of the specimens stored in aqueous medium correspond to sizes of NaCl particles used for their production (Figs. 1 and 2). Large pores are connected with one another via channels and orifices (Fig. 1), through which cells can migrate into deep layers. The interconnectivity of scaffold pores enhances homogeneity of cell cultivation environment in surface layers and in depth. The interconnectivity of pores was proven by a standard permeability test. For this purpose, scaffold specimens were brought in Indian ink for 2 h. The ink particles homogeneously stained the specimens, thus there are no closed pores in scaffolds.

To study the dynamic of cell growth, scaffold specimens (thickness of 700 μm) with pores produced with the NaCl particles of 400 μm in diameter were placed in wells of 24-well plate and supplemented with 2500 3T3 cells suspended in 300 μl of culture medium. After 2 h of incubation, the specimens were transferred into wells with 1 ml of culture medium. The specimens were studied on 1st, 4th, and 14th days of incubation.

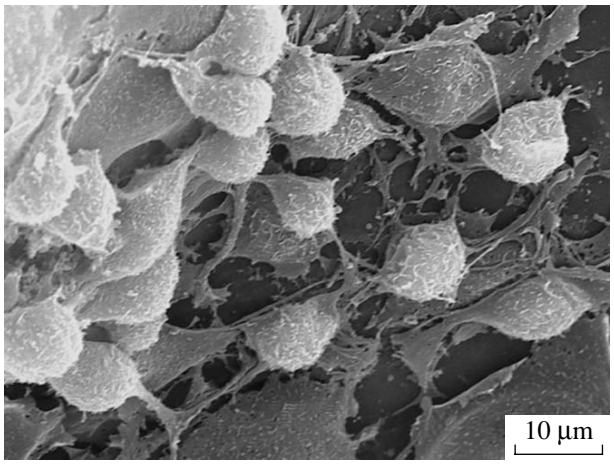


**Fig. 3.** 3T3 cells on the surface of the scaffold made of recombinant spidroin 1 after 24-h incubation under the conditions described in the text. Scanning electron microscopy.

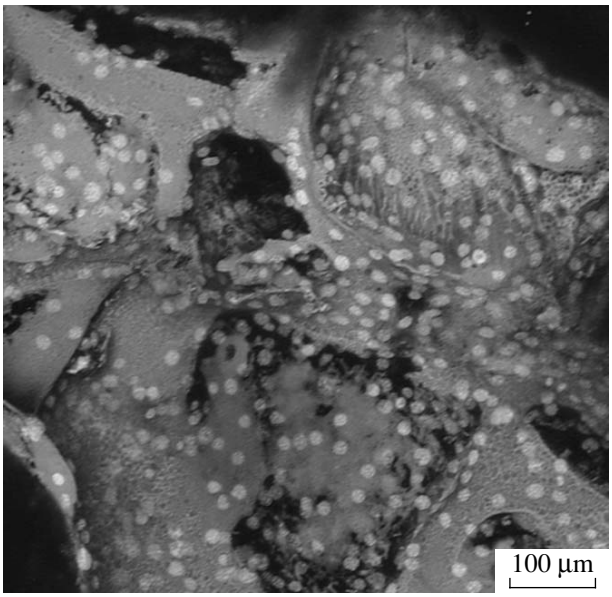


**Fig. 4.** Plane projection of serial optical cross sections of 1 μm from the surface to a depth of 200 μm. The scaffold structure and DAPI-stained 3T3 fibroblast nuclei are seen. The situation of nuclei suggests that cells formed a monolayer and are attached to pore walls.

For scanning electron microscopy the specimens were fixed with glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated in solutions with increasing ethanol concentrations. Then specimens were transferred to acetone, and critical point dried in a special device. Specimens were sputter coated with gold (thickness of 20 nm) at 6 mA and under of 0.1 mm Hg in an argon atmosphere using a sputter coater Ion Coater IB-3 ("Eico", Japan). We used Camscan S2 under Secondary Electron Image mode (SEI) (optical resolution of 10 nm, operating voltage of 20 kV).

For confocal microscopy, the specimens were fixed for 30 min with 4% formalin, treated for 10 min with 0.1% Triton X-100 in PBS, and stained by DAPI or CYTOX green solution to detect cell nuclei.

Scanning electron microscopy data demonstrate that the cells efficiently attached to the scaffold in the first 24 h of cultivation (Fig. 3). Confocal microscopy allowed us to study cells in deep layers of scaffold and observe the change in quantify of cells during cultivation. After 24 h of cultivation, the cells were predominantly localized in surface layers of the scaffold and only some cells were detected at depth over 200 μm from the scaffold surface. Cell density averaged for all layers was $355 \pm 64$ cells per 1 $mm^3$ of scaffold. After 4 days of cultivation fibroblast nuclei were detected in deep layers of the scaffold. The cells were situated on the surface of pore walls (Fig. 4). The cell density increased to $861 \pm 118$ cells per 1 $mm^3$. The ratio of cell density in superficial layer and 200 μm deep was approximately 3:1. After 14 days of cultivation cell density in deep layers of the matrix did not differ from that in superficial layers of scaffold and was around $1924 \pm 212$ cells per 1 $mm^3$.

Thus, we have demonstrated that the three-dimensional scaffolds made of the recombinant spidroin 1 display a good compatibility with the cell culture, providing efficient cell adhesion and proliferation over a long time period. It has been earlier found that recombinant spidroin 1 displays a weak immunogenic activity [12], which also confirms its biocompatibility and increases its potential as an implanted material.

Our results suggest that the recombinant spidroin 1 is promising for reconstructive and substitutive medicine, including designing of hybrid organs and tissues.

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