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Preparation of uniaxial multichannel silk fibroin scaffolds for guiding primary neurons

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ABSTRACT

Physical guidance cues have been exploited to stimulate neuron adhesion and neurite outgrowth. In the present study, three-dimensional (3-D) silk fibroin scaffolds with uniaxial multichannels (42–142 μm in diameter) were prepared by a directional temperature field freezing technique, followed by lyophilization. By varying the initial silk fibroin concentration, the chemical potential and quantity of free water around cylindrical ice crystals could be controlled to control the cross-section morphology of the scaffold channels. Aligned ridges also formed on the inner surface of the multichannels in parallel to the direction of the channels. In vitro, primary hippocampal neurons were seeded in these 3-D silk fibroin scaffolds with uniaxial multichannels of ~120 μm in diameter. The morphology of the neurons was multipolar and alignment along the scaffold channels was observed. Cell–cell networks and cell–matrix interactions established by newly formed axons were observed after 7 days in culture. These neurons expressed β-III-tubulin, nerve filament and microtubule-associated protein, while glial fibrillary acidic protein immunofluorescence was barely above background. The ridges on the inner surface of the channels played a critical role in the adhesion and extension of neurons by providing continuous contact guidance. These new 3-D silk scaffolds with uniaxial multichannels provided a favorable microenvironment for the development of hippocampal neurons by guiding axonal elongation and cell migration.

1. Introduction

In regeneration of the central nerve system (CNS), the microenvironment surrounding an injury site in the CNS is usually incompatible with axonal regeneration, as a result of the deficiency of extracellular matrix (ECM) and neurotrophic factors, the existence of inhibitive factors and glial scar, etc. [1,2]. This challenge motivates the continued development of novel materials and fabrication processes to produce scaffolds for the targeted guidance of regenerating axons. Oriented pores and topographical cues or micropatterns in the micron and nanoscale regime are effective for guiding adhesion, extension and migration of neurons and glial cells [3–5].

Numerous studies have indicated that artificial nerve grafts with special oriented channels provide physical cues to guide the linear growth of axons across a site of injury [6,7]. For example, the effect of chitosan biomaterials with different topologies (film, porous scaffold and multichannel conduits) on the differentiation and proliferation of neural stem cells was investigated [8]. After 5 days in culture, chitosan conduits prepared by lyophilization with multichannels in the inner part of the conduit assisted more neural stem cells than films or porous scaffolds, due to elongation along a preferred axis in the microchannels [8]. Fibronectin mats with oriented pores were prepared by lyophilization and implanted in the damaged spinal cord of adult rats, and robust, correctly oriented axonal growth was observed [7]. Freeze-dried agarose scaffolds with uniaxial channels also supported linear axonal growth in adult rats with spinal cord injuries [4]. Aligned polycaprolactone/gelatin (PCL/gelatin) nanofibrous scaffolds were fabricated by electrospinning. Schwann cells seeded on the PCL/gelatin nanofibrous scaffolds possess bipolar extensions with spindle-shaped morphology along with the aligned nanofibers [9]. Poly (ethylene glycol) hydrogel patterned surfaces formed by cross-linking permitted the adhesion and directed growth of neuronal cell, enabling regrowth of axons to bridge a spinal cord injury without interference from glial scars [10]. Many studies generally confirm that scaffolds with axially aligned pores support targeted axonal elongation and may also be an effective means for the local delivery of exogenous support cells and growth factors to the site of injury [11,12]. These studies suggested that

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nerve grafts with specially oriented channels are important for the extension of axons in spinal cord injury repair.

On the other hand, dimensions at the micron and nanoscale also influence cell differentiation and migration. Ridge/groove patterned arrays effectively induce the differentiation of human embryonic stem cells into a neuronal lineage, without the use of any differentiation-inducing agents, suggesting that special patterns may be useful in nanostructured scaffolds for nerve injury repair [13]. Cells exhibited morphological variations and contact guidance depending on the surface topography of the engineered scaffolds with different dimensions of the ridges [14]. Surface-patterned scaffolds with grooves, ridges, pores, steps and node topographies enhanced chondrocyte attachment and proliferation [15]. Rabbit bone marrow-derived mesenchymal stem cells preferred to adhere on the ridges of the scaffolds [16]. Human umbilical vein endothelial cells settled into micropatterned sheets with ridges and grooves. When the cells started to overgrow the ridges of the micro-patterns, the cells aligned themselves in the direction of the micro-pattern [3]. However, the continued lack of success of nerve regeneration strongly suggests that merely providing oriented channels or ridge/groove pattern through a conduit is insufficient. A scaffold with both special oriented multichannels and micron-nanoscale ridge/groove will be more competitive and has more potential for nerve regeneration.

Silk fibroin (SF) is a naturally occurring polymer that has been used for centuries in the production of textiles and clinical sutures [17]. SF materials can support the attachment, proliferation, and differentiation of primary cells and cell lines [18–20], and is easily prepared as films [21], porous scaffolds [22], gels [23], and mats [24]. The impressive cytocompatibility and malleability of SF materials make silk a popular starting material for tissue engineering scaffolds used in skin, bone, blood vessel, ligament, and nerve tissue regeneration [25–27]. Previous studies demonstrated that natural SF fiber was compatible with dorsal root ganglion neurons and Schwann cells in vitro [28] and SF tubes allowed for axonal regeneration across a 10 mm injury of the sciatic nerve [29]. Hippocampal neurons were also cultured on natural SF fibers and the results demonstrated good biocompatibility without any significant cytotoxic effect on any cell phenotype [30]. In the present study, silk guidance channels with micro-patterns were studied due to this combination of morphological features, to assess control of axon extensions. This feature, along with the robust material properties, future options to control degradation lifetime of the conduits from days to months or longer, as well as the ability to delivery bioactive compounds from these matrices, suggest novel control of nerve outgrowth would be an important combination of properties achievable with silk-based nerve conduits.

The hypothesis in the present study is that bioactive SF scaffolds with both oriented multichannels and micro-patterns on the inner surface of the channels will guide neuron extensions. To confirm this hypothesis, three-dimensional (3-D) SF scaffolds were prepared by a directional temperature field freezing technique. The direction, quantity, morphology and size of the channels were controlled by adjusting the growth of ice crystals, as were the ridges on the inner surface of the channels. The adhesion, spreading, differentiation and migration of hippocampal neurons in the scaffolds were investigated.

2. Materials and methods

2.1. Materials

Bombyx mori (*B. mori*) raw silk fibers were purchased from Zhejiang the Second Silk Co. Ltd. (Huzhou, China). Bone marrow derived stromal cells (BMSCs) were isolated from the femoral bone marrow of Sprague–Dawley (SD) rats (120–150 g, Si Laike Animal Centre, Shanghai, China). Hippocampal neurons were isolated from E18 SD rats (Si Laike Animal Centre). Dulbecco's modified eagles medium (DMEM), neurobasal® medium, and fetal bovine serum (FBS) were purchased from Shanghai Pufei Bio-Technology Co. Ltd., China. *N*-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-morpholinoethanesulfonic acid (MES), methyl thiazolyl tetrazolium (MTT), mouse monoclonal anti-β-III-tubulin antibody, Hoechst 33258, FITC-labeled goat anti-mouse IgG, and phycoerythrin (PE)-labeled goat anti-mouse IgG were purchased from Sigma–Aldrich (Shanghai) Trading Co. Ltd., China. Mouse monoclonal anti-glial fibrillary acidic protein antibody (anti-GFAP), microtubule-associated protein (MAP2) and mouse monoclonal anti-neurofilament 200 antibody (anti-NF-200) were purchased from Chemicon International Inc., USA.

2.2. Preparation of the SF porous scaffolds

SF solution was prepared following the procedure described previously [22]. Briefly, 150 g raw silk fibers were degummed three times in 5 l Na_2CO_3 solution with a concentration of 0.05% (w/w) at 100 °C for 30 min, rinsed thoroughly, and dried in an oven. The extracted SF was dissolved in a ternary solvent of $CaCl_2$:CH_3-CH_2OH:H_2O (1:2:8 M ratio) with 1:10 g ml^{-1} bath ratio at 70 ± 2 °C for 1 h. A 3.2 wt.% SF solution was obtained after dialysis of 4 days in deionized water followed by filtration. SF scaffolds were prepared by referring to the method of preparation of aligned porous PVA scaffolds [31,32]. In our study, SF solution was concentrated by controlling the very slow drying at 37 ± 2 °C for 48 h to obtain 18.7 wt.% of SF solution. The SF solution was then diluted to 3, 5, 7, 9 wt.% with deionized water, respectively. NHS, EDC, and MES were added to the SF solution at 10%, 20%, and 20% of the SF weight, respectively. The mixture was injected into a mould (35 mm in length, 3 mm inner diameter, Fig. 1) surrounded with thermal insulation. The mould was then fixed vertically on a metal plate sitting in a pool of liquid nitrogen (LN_2). After freezing for 1 h, the mould was lyophilized for 3 days. Scaffolds were removed from the moulds, and cut with stainless steel blades to a length of 3 mm and a radius of 1 mm for further characterization. To prepare control samples, the diluted 3 wt.% SF solutions mixed with NHS, EDC, and MES were injected into 3 mm inner diameter silicone tube

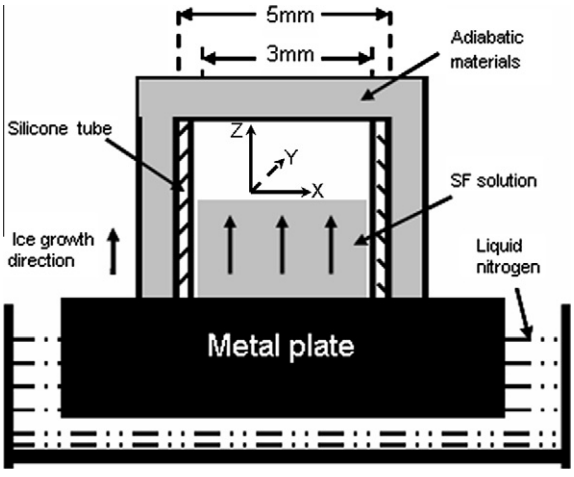

Fig. 1. Schematic of the freezing apparatus for SF scaffolds with oriented channels. A metal box is placed into the chamber filled with LN_2 and kept at the bottom of a metal container in contact with the LN_2. A silicone tube with inner diameter of 3 mm and outer diameter of 5 mm is fixed on the bottom of the metal box. The silicone tube is surrounded with adiabatic materials to ensure a uniaxial temperature gradient field. SF solution was injected into the tube, frozen for 1 h and lyophilized for 3 days. The bold arrows show the direction of the temperature gradient field and ice crystal growth.