Materials Science and Engineering C 48 (2015) 620–631

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

The influence of electrospun fibre size on Schwann cell behaviour and axonal outgrowth

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ARTICLE INFO

Article history:
Received 7 February 2014
Received in revised form 17 September 2014
Accepted 17 December 2014
Available online 18 December 2014

Keywords:
Electrospun fibres
Extracellular matrix
Cell adhesion
Cell spreading
Axon outgrowth
Nerve tissue engineering

ABSTRACT

Fibrous substrates functioning as temporary extracellular matrices can be prepared easily by electrospinning, yielding fibrous matrices suitable as internal fillers for nerve guidance channels. In this study, gelatin micro- or nano-fibres were prepared by electrospinning by tuning the gelatin concentration and solution flow rate. The effect of gelatin fibre diameter on cell adhesion and proliferation was tested in vitro using explant cultures of Schwann cells (SC) and dorsal root ganglia (DRG). Cell adhesion was assessed by quantifying the cell spreading area, actin cytoskeleton organization and focal adhesion complex formation. Nano-fibres promoted cell spreading and actin cytoskeleton organization, increasing cellular adhesion and the proliferation rate. However, both migration rate and motility, quantified by transwell and time lapse assays respectively, were greater in cells cultured on micro-fibres. Finally, there was more DRG axon outgrowth on micro-fibres. These data suggest that the topography of electrospun gelatin fibres can be adjusted to modulate SC and axon organization and that both nano- and micro-fibres are promising fillers for the design of devices for peripheral nerve repair.

1. Introduction

In recent years, a considerable body of research has focused on the development of implantable polymeric scaffolds for repairing nerve gaps as alternatives to autografts [1–5]. The need for artificial scaffolds in nerve tissue engineering arises from the limited availability of autologous nerves and the requirement for secondary surgery that can result in donor sensory loss and/or pain from nerve autograft-based techniques, and from the immune-rejection problems common to allograft- and xenograft-based techniques [6–9].

Biomimetic materials for peripheral nerve reconstruction should: be biodegradable and biocompatible, display a large surface area and high porosity, and provide adequate mechanical support for re-growing axons. To achieve these goals, a scaffold for peripheral nerve repair can be functionalized by acting on the properties of the inner filler [1,6,10,11] in order to mimic the native structure of the nerve and to enhance nerve repair processes [12–14]. It is necessary to take account of (i) the three-dimensional architecture (topography) of the inner filler and (ii) its biochemical composition in developing innovative implantable nerve scaffolds, since these features profoundly influence cell behaviour and the organization of the regenerating tissue. In particular, the internal filler of the conduit (i.e. fibre composition, diameter, three-dimensional structure) can be diversified to modulate Schwann cell (SC) and axon behaviour (cell proliferation rate, number of migrating cells, number of re-growing axons, etc.).

Regarding the three-dimensional architecture of the inner filler, the basal lamina and extracellular matrix (ECM) structures have to be considered. The tissue basal lamina membrane has unique nano-fibrous characteristics, suggesting the importance of substrate topography [12–15]. A number of studies have demonstrated that micro-to-nano-scale topography is important for controlling the adhesion, proliferation and survival of different cell types such as human cord blood derived haemopoietic stem progenitor cells [16,17], bone marrow derived osteoprogenitors [18,19], mesenchymal stem cells [20], MC3T3-E1 preosteoblasts [17], hippocampal progenitor cells [21], human keratinocytes [22], neural stem cells [23], SC [24,25] and neurons [26,27].

Surface modification of the biomaterial, roughness, porosity, and topographical cues such as grooves and ridges or pits and pillars influence protein absorption, cell interaction and host response to the material [14,28]. Topographical modifications of implantable devices influence cell adhesion, growth and migration by affecting the size, shape, and distribution of focal adhesion plaques, actin cytoskeleton reorganization

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http://dx.doi.org/10.1016/j.msec.2014.12.055
0928-4931/

and/or *lamellipodium* and *filopodia* formation though integrin receptor signalling [13,14,28–30].

Ideally, a candidate biomimetic scaffold should mimic native ECM structure and function, defining the optimal three-dimensional tissue organization for maintaining normal cell organization, viability, and invasion, proliferation and differentiation behaviour [12–14].

Electrospinning is a fascinating and widely-used technique for producing fibres, mimicking the native ECM, from polymer solutions of both natural and synthetic origins. The resulting fibres are characterized by high surface to volume ratios, high porosity and enhanced physico-mechanical properties that can be adjusted by manipulating the polymer solution and process parameters to yield fibres with the desired morphology and mechanical strength [31]. Electrospun fibres mimicking ECM structure have been used in a variety of applications such as wound healing, drug delivery, enzyme immobilization, filtration and scaffold tissue engineering in nerve, dura mater, tendon/ligament and tendon-to-bone insertion repair [12,22,31–35].

Regarding biochemical composition, natural polymers are often used because of their high biocompatibility and presence of biofunctional cues, improving overall scaffold cytocompatibility [3,36,37]. Collagen, alginate, silk protein, hyaluronic acid, chitosan and other biomaterials have been used for preparing electrospun fibres [3,31,36,37]. However, ECM-based biomaterials have limitations such as poor mechanical properties and immunogenicity risk [28,38].

In this work, gelatin, a natural polymer obtained from the thermal denaturation of collagen, was chosen to produce random electrospun fibres owing to its advantageous features. In comparison to collagen, an animal protein, gelatin is biocompatible, biodegradable and does not induce immune-rejection problems, maintaining molecular cues that are key regulators of cell behaviour [38]. Despite these properties, the difficulty of dissolving gelatin in water at mild temperatures (e.g. 37 °C) limits its use in biomedical applications [38–41]. In a previous study, the authors successfully established a protocol for producing electrospun gelatin fibres. Gelatin was dissolved in distilled water, avoiding the use of potentially cytotoxic solvents and acidic solutions [39]. Furthermore, to increase gelatin stability in a physiological environment, γ-glycidoxypropyltrimethoxysilane (GPTMS) was used as cross-linker. GPTMS cross-linking has been reported not to alter the morphology of fibre matrices but it decreases the gelatin degradation rate in physiological solution (from few hours to more than seven days for gelatin and cross-linked gelatin nano-fibres, respectively). In that study, the authors identified the process and solution parameters to be applied in fabricating homogenous nano-fibres. In detail, by tuning the gelatin concentration over the range 8 to 15% w v^{-1}, the solution flow rate over the range 8 to 15 ml min^{-1} and the nozzle collector distance over the range 7 to 19 cm, electrospun fibres with diameters ranging from 200 to 700 nm were obtained. The biocompatibility of the nano-fibrous gelatin substrates was tested by culturing Neonatal Olfactory Bulb Ensheathing Cells (NOBEC) on 300 nm fibres [39].

On the basis of these previous results [39], we developed nanofibrous matrices characterized by different diameters in order to analyse the influence of fibre size on nerve tissue behaviour. Gelatin-GPTMS cross-linked electrospun fibres with diameters ranging from 300 nm to 1300 nm were prepared and in vitro and ex vivo primary SC culture and dorsal root ganglia (DRG) explants were established to investigate whether the topography of the micro-to-nano-scale fibres can modulate SC adhesion, proliferation and migration and axonal outgrowth.

2. Materials and methods

2.1. Fibre preparation

Gelatin solutions were prepared as previously described [39]. Briefly, gelatin (type A from porcine skin) and GPTMS were supplied by Sigma Aldrich. Gelatin was dissolved in demineralised water at 50 °C to obtain the desired concentration (15 or 20% w v^{-1}) and 137 μl of GPTMS was added to the solution and mixed for 1 h before spinning.

The electrospinning system used for fibre preparation consists of an isothermal chamber equipped with: a high voltage generator (PS/EL30R01.5-22 Glassman High Voltage) providing a voltage of 0 to 30 kV and a current of 0 to 1.5 mA with reversible polarity; a volumetric pump (KDS210, KD Scientific); an electrode; a mobile syringe support; a syringe and a 1.5 mm thick flat aluminium collector.

The gelatin solutions (containing 15% or 20% gelatin) were spun at 50 °C, 30 kV, flow rate 10 μl min^{-1} or 15 μl min^{-1} and a nozzle–collector distance of 15 cm, as previously reported [39]. Briefly, 15% of the gelatin solution was spun at 10 or 15 μl/min flow rate to yield fibres of 300 or 600 nm in diameter, respectively. Twenty percent of the gelatin solution was spun at 12.5 or 15 μl/min to yield fibres of 1000 or 1300 nm in diameter, respectively (Fig. 1A).

Electrospun scaffolds were prepared using a vertical electrospinning set-up to generate randomly-orientated nano- or micro-fibres on glass coverslips.

2.2. Scanning electron microscopy

The morphology of the electrospun matrices was examined using a scanning electron microscope (STANDARD DEVIATION Philips 525 M) at an acceleration voltage of 15 kV. The fibre samples were coated with gold using a sputter coater. The STANDARD DEVIATION magnification (6000×) was selected for 50 μm square fields, allowing fibre distribution to be described.

Pore and fibre diameters were quantified by analysing SEM micrographs with *ImageJ* software as previously reported [23,39]. For each fibre type, three images from three different samples were examined and the diameters were recorded as mean value ± standard deviation.

2.3. Cell culture

The RT4-D6P2T SC line and primary SC were grown on monolayers at 37 °C in a humidified atmosphere of 5% CO_2. The RT4-D6P2T were purchased from ATCC (American Type Culture Collection, 10801 University Blvd, Manassas, VA 20110-2209) and grown in complete high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) according to the designated ATCC protocol.

SC for primary culture were isolated from sciatic nerves harvested from adult female Wistar rats (Charles River Laboratories, Milan, Italy) weighing approximately 190–220 g. All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Adequate measures were taken to minimize pain and discomfort taking human endpoints for animal suffering and distress into account. The sciatic nerves were isolated, cut into 3 mm section and incubated at 37 °C in 5% CO_2 in complete medium consisting of low glucose DMEM (Gibco) supplemented with 100 units ml^{-1} of penicillin, 0.1 mg ml^{-1} of streptomycin, 1 mM of sodium pyruvate, 2 mM of L-glutamine, 10% of heat-inactivated foetal bovine serum (FBS, Invitrogen) 63 ng/ml of glial growth factor (GGF, R&D Systems) and 10 μM of forskolin (Sigma). The medium was changed every three days. After two weeks, 2 ml of digestion solution consisting of 0.6 mg/ml of collagenase type IV (Sigma) and 0.5 mg/ml of dispase (Invitrogen) diluted in low glucose complete medium was added. After 24 h the incubated nerve segments were transferred to a 50 ml tube and suspended in 5 ml low glucose complete medium using a glass Pasteur pipette. The cell suspension was filtered through a 70 μm strainer (Falcon), centrifuged at 900 rpm for 5 min, re-suspended in 10 ml of complete SC medium and seeded on a poly-L-lysine (PLL, Sigma) coated plate. In order to remove contaminating fibroblasts, the confluent SCs were immunodepleted. Briefly, the confluent SC were harvested by trypsinization, re-suspended in 500 μl low glucose complete medium containing mouse anti-rat Thy1.1 antibody diluted 1:500 (Serotec, MCA04G) and incubated for 10 min at