Biomaterials 35 (2014) 664–674

Contents lists available at ScienceDirect

Biomaterials

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

Differentiation of neuronal stem cells into motor neurons using electrospun poly-L-lactic acid/gelatin scaffold

Loïc Binan [a,b], Charlène Tendey [a], Gregory De Crescenzo [a,b], Rouwayda El Ayoubi [c], Abdellah Ajji [a], Mario Jolicoeur [a,b,*]

[a] Department of Chemical Engineering, École Polytechnique de Montréal, P.O. Box 6079, Centre-Ville Station, H3C 3A7 Montréal, Québec, Canada
[b] Groupe de Recherche en Science et Technologie Biomédicale (GRSTB), École Polytechnique de Montréal, P.O. Box 6079, Centre-Ville Station, H3C 3A7 Montréal, Québec, Canada
[c] New World Laboratories, 500 Boulevard Cartier Ouest, H7V5B7 Laval, Quebec, Canada

ARTICLE INFO

Article history:
Received 20 August 2013
Accepted 24 September 2013
Available online 22 October 2013

Keywords:
Electrospinning
Drug delivery
Motor neuron
Nerve
Regeneration
Differentiation

ABSTRACT

Neural stem cells (NSCs) provide promising therapeutic potential for cell replacement therapy in spinal cord injury (SCI). However, high increases of cell viability and poor control of cell differentiation remain major obstacles. In this study, we have developed a non-woven material made of co-electrospun fibers of poly L-lactic acid and gelatin with a degradation rate and mechanical properties similar to peripheral nerve tissue and investigated their effect on cell survival and differentiation into motor neuronal lineages through the controlled release of retinoic acid (RA) and purmorphamine. Engineered Neural Stem-Like Cells (NSLCs) seeded on these fibers, with and without the instructive cues, differentiated into β-III-tubulin, HB-9, Islet-1, and choactase-positive motor neurons by immunostaining, in response to the release of the biomolecules. In addition, the bioactive material not only enhanced the differentiation into motor neuronal lineages but also promoted neurite outgrowth. This study elucidated that a combination of electrospun fiber scaffolds, neural stem cells, and controlled delivery of instructive cues could lead to the development of a better strategy for peripheral nerve injury repair.

1. Introduction

Traumatic injury to the spinal cord causes cell death and axonal degeneration resulting in functional motor and sensory loss [1]. With 12,000 new cases every year in the US only [2], spinal cord injury results in a dramatic loss in life quality. The process of spinal cord repair is often complex due to cell death during the first two weeks, the fragile nature of the cells, and the appearance of scar tissue that is chemically and mechanically hostile to the re-growth of functional tissue [3]. The lack of treatment is directly related to the lack of grafts. In this context, cell transplantation therapy using neural stem cells (NSC) provides a promising therapeutic potential to replace the damaged cells after SCI [4]. Studies by several groups, have demonstrated the potential of restoring functional motor activity after transplanting motor neurons derived from neural stem/progenitors cells in paralyzed animals [1,5,6]. Unfortunately, neural stem cells transplanted into the spinal cord have much tendency to differentiate into either astrocytes or oligodendrocytes and rarely undergo differentiation into neuronal lineages [7,8]. Therefore expandable sources of motor cells and optimal scaffolds that can promote neural differentiation to specific lineage are needed for effective spinal cord repair.

Various attempts have been made to develop a scaffold to support neural tissue growth, but a remaining limitation is related to the need of a continuous delivery of the appropriate biomolecule(s). Their periodic injection is not a viable option as it requires several heavy surgical procedures, and their concentration should not significantly vary with time [9–11]. Therefore, neural tissue regeneration requires a scaffold permitting the controlled release of specific instructive cues in situ, in addition to harboring adequate mechanical properties. In that context, electrospun fibers have attracted interest for nerve tissue regeneration. The versatility of electrospinning allows for the fabrication of non-woven meshes of fibers of diameters and mechanical properties that can be adjusted by properly choosing the polymer to be processed and its concentration [12]. Furthermore, it offers a possibility to incorporate instructive cues within the nanofibers, which will serve as a controlled delivery system [13]. The high surface-to-volume ratio of the electrospun fiber mats as well as their structure that mimics

* Corresponding author. Department of Chemical Engineering, École Polytechnique de Montréal, P.O. Box 6079, Centre-Ville Station, H3C 3A7 Montréal, Québec, Canada. Tel.: +1 514 340 4711x4525; fax: +1 514 340 2990.
E-mail address: mario.jolicoeur@polymtl.ca (M. Jolicoeur).

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.09.097

extracellular matrix, make them well-adapted scaffolds for neuronal tissue engineering [14]. The high number of polymers that can be electrospun also makes it possible to obtain a wide range of material properties [15–17]. However, most of these materials need to be coated to improve cell adhesion [12,18]. To reduce this limitation, more and more scientific reports explore the use of coaxial electrospun materials where two immiscible polymer solutions are electrospun using concentric needles [19,20]. Two important uses of this technique can be as follows: one is the use of extracellular matrix as an outer shell to favor cell contact and the other is the use of the inner shell as a reservoir of instructive cues for progressive delivery [21–23]. Another characteristic reported in literature is that fiber diameter itself plays an important role in cell adhesion and differentiation [24]. In fact, according to this study, an ideal fiber diameter for nerve tissue engineering would be between 400 and 600 nm to allow for a maximum neurite outgrowth.

Fibroblast and epidermal growth factors (FGF and EGF) are biomolecules commonly used to favor neuronal stem cell (NSC) proliferation, and a cocktail of retinoic acid and purmorphamine has been widely shown to promote NSC differentiation into motor neurons [25]. These are issued from stem cells by the successive expression of Islet-1 and HB-9 homeogenes. Therefore, since choactase is the enzyme that catalyzes the biosynthesis of acetylcholine, the neurotransmitter used by motor neurons, it is commonly admitted that cells positive for Islet-1, HB-9 and choactase genes are motor neurons [26].

In this work, we have combined the co-electrospinning technology to generate fibers with concentric layers of two different polymers, i.e. poly-L-lactic acid (PLLA) as a core and gelatin as a shell, with a drug delivery strategy since the gelatin layer was loaded with RA and purmorphamine.

We thus report the fabrication of co-electrospun fibers with tunable mechanical properties, degradation and drug release rates. The nanofibers matrices were characterized morphologically, physically, chemically, and biologically to evaluate their efficacy to promote cell survival and differentiation towards motor neuronal lineages, which could lead to better strategy for peripheral nervous system repair.

2. Materials and methods

2.1. Fabrication and characterization of scaffolds of nanofibers

2.1.1. Fabrication of the fibers

PLLA (Purac, USA) and gelatin (Sigma, Oakville (ON), Canada) were electrospun using a co-axial needle (Linari, Pisa, Italy). Briefly, PLLA and gelatin were each dissolved in trifluoroethanol (Sigma, Oakville (ON), Canada) to final concentrations of 10% and 7% w/v, respectively. Solutions were agitated overnight and the gelatin solution was then heated at 70 °C to allow gelatin dissolution. Fibers were collected on a rotating drum set to 1400 rpm with an electrospinning distance of 15 cm and an applied voltage of 24 kV. The flow rate of both solutions was set to 1 mL/h in order to form fibers with an average diameter of 1.6 μm. For the fabrication of loaded fibers, retinoic acid (Sigma, Oakville (ON), Canada, R2625) and purmorphamine (Cayman Chemical Company, Ann Arbor, MI, USA, 10009634) were dissolved in the gelatin solution prior to electrospinning to reach 0.2% w/w purmorphamine and 0.08% w/w retinoic acid in the dry gelatin.

2.1.2. Crosslinking of the fibers

In order to control the degradation rate of the gelatin outer shell, a crosslinking step was performed following fiber fabrication. Co-electrospun fiber mats were cut to fit in a 24 well plate containing 0.5% or 0.7% w/w glyceraldehyde in 70% ethanol. Samples were left 19 h in these solutions at room temperature according to the protocol by Sisson et al. [27].

2.1.3. Scanning electron microscopy (SEM)

Samples were imaged after with a JSM-7600TFE microscope equipped with a field emission gun from JEOL, after their fabrication and after cell culture. For sample preparation, fibers were dried using successive dilutions of ethanol (50%, 75%, 90%, 100%) to avoid shrinkage, and stored under vacuum before imaging. All samples were coated with gold (2 times for 15 s). Observations were performed with a LEI (low secondary electron image) detector and an acceleration voltage of 2 kV.

2.1.4. Transmission electron microscopy (TEM)

TEM was performed with a JEM-2100F microscope equipped with a field emission gun from JEOL, to further confirm the core/shell structure of the fibers. Fibers were co-electrospun directly on a copper grid and observed at 200 kV for bright field imaging.

2.1.5. Diameter measurement

Mat samples were prepared as described for cell culture, and placed in culture medium (50% DMEM; 50% F12 during 7days) in an incubator (37 °C) to assay for their degradation in culture conditions. Fiber diameter was measured with Photoshop software on SEM images at 70 different locations for each mat sample for statistical analysis.

2.1.6. Degradation rate of the gelatin outer shell

The degradation of the gelatin shell was assayed at different time points by placing fiber mat samples in medium in an incubator at 37 °C. Gelatin amount on fiber mats was determined by measuring the amount of free amine groups of the gelatin by adapting the Orange II dye protocol already published [28]. Fiber mat samples (approx. 3 mg) were incubated 30 min in 1 mL of a solution of 40 mM Orange II dye (pH 3). Unbound Orange II dye was removed by three 1 mL washes (pH 3). Remaining dye was then desorbed by immersing the samples in a NaOH solution (pH 12, 15 min). Supernatants were acidified with 1% v/v of pure HCl. Eluted Orange II dye concentration was then measured by spectrophotometry (480 nm). The amount of eluted Orange II dye was normalized relative to the sample weight in order to derive the amount of remaining gelatin per g of fiber.

2.1.7. Mechanical testing

Mechanical testing was performed using an INSTRON instrument (Norwood, MA, USA). Fiber mat samples (2 cm × 5 mm) were crosslinked as they were for cell culture. Samples were then placed between pneumatic grips so that 5 mm of sample remained free. The Young's modulus was calculated as the slope of the initial linear part of the curve of the tensile stress (in MPa) plotted against tensile extension.

2.1.8. Enzyme-linked immunosorbent assay (ELISA)

To determine the release profile of retinoic acid from the fibers, the meshes were cut into round patches (16 mm of diameter) and a time dependent release of RA was performed by ELISA. The concentration of RA in medium was determined by ELISA assay (MyBioSource, San Diego, USA, MBS705877) following the manufacturer's instructions. Briefly, the samples were placed in medium (50% DMEM, 50% F12 with antibiotics) and samples of 25 μl of liquid medium were taken periodically and placed at −80 °C. Taken medium was replaced by fresh, and the suspension medium was changed every 3 days. Samples and standards were added to the 96-well plate and incubated for 1 h at 37 °C, which was followed by the addition of TMB substrate (20 min at 37 °C). Optical density was determined using a plate reader set to 450 nm with a correction at 540 nm.

2.2. NSLC culture and differentiation

2.2.1. Cell culture

Engineered Neural Stem-Like Cells (NSLCs) provided by New World Laboratories Inc. (Laval (QC), Canada) were cultured in neural progenitor medium (Lonza, Waskerville, USA, CC-4461) supplemented with laminin (Sigma, Oakville (ON), Canada, L2020-1 MG) (5 mg/L). For proliferation studies, cells were cultured on tissue culture plate or fiber mats, with EGF and FGF (Lonza, Waskerville, USA) in the medium. For the characterization of NSLC differentiation, cells were cultured on fibers loaded with purmorphamine (0.2% $w/w_{gelatin}$) and retinoic acid (0.08% $w/w_{gelatin}$) without any growth factor in the medium.

2.2.2. Fiber mat sample preparation and cell seeding

Fiber mat samples (10% PLLA, 7% gelatin) were cut to the size of a well from a 24 well plate (16 mm) using a punch. Samples were considered sterile after the crosslinking step during which they stayed overnight in 70% ethanol. Samples were then rinsed 3 times with PBS (1 mL) and cells were seeded at 45,000 cells/well in 2 mL of neural progenitor maintenance medium (Lonza, Waskerville, MD, USA, CC-4461). Cells were trypsinized and spun down at 1000 rpm for 5 min and the resultant pellet was re-suspended and dissociated in fresh growth medium. The cell suspension (45×10^3 cells/well) was seeded on nanofibers with and without the presence of purmorphamine (0.2% $w/w_{gelatin}$) and RA (0.08% $w/w_{gelatin}$). Cells were incubated at 37 °C, 5% CO_2 for 3 weeks and maintenance medium was changed every three days.

2.2.3. Immunofluorescent staining and microscopy

To assess cell phenotype, samples were rinsed three times with 1 mL PBS and fixed in 1 mL formaldehyde (3.7%, Sigma) during 30 min. Samples were permeabilized in Triton (0.5%, 20 min), blocked with donkey serum 5% (Sigma, D9663-10 ML, 1 h) and immunostained at 4 °C overnight with different primary antibodies: goat anti-actin, -nestin, -choactase, -HB-9, -Islet-1 (sc-1616, sc-21248, sc-19057, sc-22542, sc-23590, all from Santa Cruz Biotech, Dallas, TX, USA) or mouse anti-TUJ-1 (Neuromics, Edina, MN, MO15013). Primary antibodies were dissolved in 0.1% Triton,