Acta Biomaterialia 35 (2016) 42–56

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Glial progenitor cell migration promotes CNS axon growth on functionalized electroconducting microfibers

Jorge E. Collazos-Castro *, Concepción García-Rama, Alexandra Alves-Sampaio

Neural Repair and Biomaterials Laboratory, Hospital Nacional de Parapléjicos (SESCAM), Finca La Peraleda s/n, 45071 Toledo, Spain

ARTICLE INFO

Article history:
Received 6 August 2015
Received in revised form 25 January 2016
Accepted 12 February 2016
Available online 13 February 2016

Keywords:
Conducting polymers
Microfibers
Axonal
Glial
bFGF
Fibronectin

ABSTRACT

Electroactive systems that promote directional axonal growth and migration of glial progenitor cells (GPC) are needed for the treatment of neurological injuries. We report the functionalization of electroconducting microfibers with multiple biomolecules that synergistically stimulate the proliferation and migration of GPC, which in turn induce axonal elongation from embryonic cerebral cortex neurons. PEDOT doped with poly[(4-styrenesulfonic acid)-*co*-(maleic acid)] was synthesized on carbon microfibers and used for covalent attachment of molecules to the electroactive surface. The molecular complexes that promoted GPC proliferation and migration, followed by axonal extension, were composed of polylysine, heparin, basic fibroblast growth factor (bFGF), and matricellular proteins; the combination of bFGF with vitronectin or fibronectin being indispensable for sustained glial and axonal growth. The rate of glial-induced axonal elongation was about threefold that of axons growing directly on microfibers functionalized with polylysine alone. Electrical stimuli applied through the microfibers released bFGF and fibronectin from the polymer surface, consequently reducing GPC proliferation and promoting their differentiation into astrocytes, without causing cell detachment or toxicity. These results suggest that functionalized electroactive microfibers may provide a multifunctional tool for controlling neuron-glia interactions and enhancing neural repair.

Statement of Significance

We report a multiple surface functionalization strategy for electroconducting microfibers (MFs), in order to promote proliferation and guided migration of glial precursor cells (GPC) and consequently create a permissive substrate for elongation of central nervous system (CNS) axons. GPC divided and migrated extensively on the functionalized MFs, leading to fast elongation of embryonic cerebral cortex axons. The application of electric pulses thorough the MFs controlled glial cell division and differentiation. The functionalized MFs provide an advanced tool for neural tissue engineering and for controlling neuron-glia interactions. CNS axonal growth associated to migratory glial precursors, together with the possibility of directing glial differentiation by electrical stimuli applied through the MFs, open a new research avenue to explore for CNS repair.

1. Introduction

Lesions of the human brain and spinal cord frequently extend for several centimeters [1] and are accompanied by scars and cavities that lack ordered guidance cues for regenerating cells [2,3]. Hence, central nervous system (CNS) repair necessitates providing molecular cues for promoting long-distance, guided axonal elongation and glial cell migration across the damaged tissue. Because both neurons and glia are strongly influenced by substrate-anchored molecular complexes, organized neural regeneration might be promoted by implantable scaffolds modified with multiple, spatially arranged biomolecules. Using electroconducting materials to fabricate those scaffolds will enhance the probability of succeeding in CNS repair, because they will enable the application of electrical stimuli to steer axonal growth [4] and myelination [5].

From the group of electroactive materials, conducting polymers (CP), and particularly those containing poly(3,4-ethylenedioxythiophene) (PEDOT), are good candidates for the fabrication of

* Corresponding author.
E-mail address: jcollazos@sescam.org (J.E. Collazos-Castro).

http://dx.doi.org/10.1016/j.actbio.2016.02.023
1742-7061/

neurological devices because of their superior electronic and ionic conductivity, stability and electric charge injection capacity [6–9]. Moreover, multiple biomolecules can be anchored to these polymers for controlling neural cell development on electroactive surfaces. For instance, extensive growth of embryonic CNS neurons was achieved by electroadsorption or covalent bonding of polylysine (PLL) to the polymer surface [9,10]. Axonal elongation was inhibited when heparin was added on the PLL layer, and the inhibition was overcome by electrical stimuli that released the heparin molecules from the polymer without causing cytotoxicity [9]. Additionally, when bFGF was linked to the PLL/Heparin complex, massive proliferation of glial precursors took place [9]. A further achievement was the incorporation of L1 and N-Cadherin to the CP functionalization-complex [10], given that these cell adhesion molecules play a critical role in axonal growth and fasciculation in the developing nervous system [11,12]. Carbon microfibers (MFs) coated with poly[(4-styrenesulfonic acid)-*co*-(maleic acid)]-doped PEDOT (PEDOT:PSS-*co*-MA), and functionalized with L1 or N-Cadherin, promoted long (up to 7 mm in 10 days) axonal elongation from cerebral cortex neurons [10]. Remarkably, the pioneering axons were followed by numerous axons that extended aligned on the former, resulting in the formation of thick axonal fascicles on the MFs. However, glial progenitors showed little proliferation and migration on microfibers functionalized with L1 or N-Cadherin, being always far behind the front of axonal growth.

For CNS repair applications, it is desirable that the implanted biomaterials also support glial cell growth. This is a very important issue, not only because astrocytes and oligodendrocytes are essential for the appropriate function of the nervous system, but also because astrocytes may provide a permissive substrate for axonal regeneration [13,14]. Hence, the present work investigated additional functionalization schemes for PEDOT:PSS-*co*-MA-coated MFs, in order to promote proliferation and migration of CNS glial progenitor cells (GPC). It likewise addressed the possibility that migrating GPC induce axonal elongation from cortical neurons. Finally, it developed electrostimulation protocols to control GPC proliferation and differentiation on the MFs.

2. Materials and methods

2.1. Preparation and biofunctionalization of electroconducting MFs

Poly[(4-styrenesulfonic acid)-*co*-(maleic acid)] (20 kDa) and 3,4-ethylenedioxythiophene were purchased from Sigma–Aldrich. PEDOT:PSS-*co*-MA was electrosynthesized on carbon MFs (7-μm diameter, Goodfellow, UK) applying a constant anodic current of 1 μA/mm^2 and a polymerization charge (Q_{POL}) of 96 mC/cm^2 (3.8 mC per 10 MFs). The preparation and the structural and electrochemical properties of the CP-coated MFs have been reported elsewhere [10]. The first molecule (PLL, Sigma–Aldrich, 30–70 kDa) was covalently conjugated to the carboxylic groups of the dopant [10]. Subsequently, 10 mM heparin (Sigma–Aldrich H5515, 11-12.5 kDa) dissolved in PBS was applied for 4 min. Then, recombinant human bFGF and/or platelet-derived growth factor AA (PDGF-AA) (PeproTech 100-18B and 100-13A, respectively) were applied at 1 μg/ml in PBS for 1 h. Finally, the MFs were incubated at 37 °C for 1, 2 or 3 days in PBS or DMEM containing rat vitronectin (VN) (Sigma–Aldrich, V0132), bovine fibronectin (FN) (Invitrogen, 33010-018), fetal bovine serum (FBS) (Gibco, 10108) or normal rat serum (NRS) (Invitrogen, 10710C). We tested different concentrations of matricellular proteins (2, 10 or 20 μg/ml) and serum (10–20%). Consistent effects on cell growth were obtained when the MFs were incubated for three days in solutions with either 20 μg/ml of the purified proteins or 10% serum. Therefore, these concentrations were used for all the studies. For control experiments, we functionalized the MFs with PLL but omitted some of the other molecules as indicated in the text. Additionally, we used MFs functionalized with N-Cadherin [10] as control in tests with AraC.

2.2. Surface characterization

For an initial evaluation of the functionalization procedures, PEDOT:PSS-*co*-MA was electrosynthesized on gold-coated glass slides (Phasis, Switzerland), processed for molecule attachment, and then immunostained with antibodies anti-bFGF (Millipore 05-118, 1:250), anti-PDGF-AA (PeproTech, 500-P46, 1:500), anti-fibronectin (BD Biosciences 610078, 1:200) or anti-vitronectin (Santa Cruz Biotechnology, SC30979, 1:500). The same antibodies were used to confirm growth factor and fibronectin binding to PEDOT:PSS-*co*-MA–coated MFs, and for assessing the proteins at the surface after electrostimulation. The immunostaining procedure has been described elsewhere [9,10].

Polymer functionalization was also investigated by X-ray photoelectron spectroscopy (XPS). XPS data were collected on a Thermo Scientific electron spectrometer equipped with a monochromatic Al Kα (1486.6 eV) X-ray source and a flood gun charge compensation device. The base pressure in the analysis chamber was maintained below 10^{-7} mbar during data acquisition. Survey (pass energy 200 eV, step size 1 eV) and high-resolution (pass energy 40 eV, step size 0.1 V) scans were acquired from surface spots of 400 μm at a takeoff angle of 30°. 40 scans were collected for survey analyses, whereas 75 (C 1s, O 1s), 150 (N 1s), or 300 (S 2p) scans were obtained for detailed atomic investigations. Spectra were analyzed using the XPS Advantage software of Thermo Scientific. The binding energies were referenced to the binding energy of the C 1s core-level spectrum at 285 eV. Atomic fractions were calculated from the peak areas of high-resolution spectra.

2.3. Cell culture and immunocytochemistry

Cell culture wells had a borosilicate glass (100 μm thick) as bottom for allowing cell visualization by confocal microscopy. The ends of the MFs rested on a support (700 μm high) to keep them elevated from the bottom glass. A polystyrene chamber (11 × 11 × 20 mm width, height and depth, respectively) was bonded to the glass and the support of the MFs with medical grade silicone (MED-4210; NuSil, USA). The MFs ended in graphite electric contacts outside the chamber.

The use of animals for cell culture was in accordance with the European and Spanish regulations for the protection of experimental animals (86/609/CEE, 32/2007 and 223/1988) and the protocols were approved by the Ethical Committee for Animal Research of the Hospital Nacional de Parapléjicos. In brief, Wistar rats were anesthetized to extract E18-embryos under sterile conditions. The meninges of the embryonic brain were carefully removed and pieces of cerebral cortex (0.5 × 1.5 mm) were cut and directly placed on the MFs. The ventral (subventricular) region of the cerebral cortex contacted the MFs. At E18, the subventricular region contains mostly glial stem cells, whereas the upper layers contain neurons. The explants maintained that orientation and the cells at the subventricular region proliferated (Suppl. Fig. 1). The cortical explants were kept at 37 °C in Neurobasal™ cell culture medium supplemented with L-glutamine, B27, Penicillin-Streptomycin and Gentamicin. Half of this medium was replaced every other day after the fifth day of cell culture. At 5, 10 or 15 days *in vitro* (DIV), the cells were fixed in Neurobasal™ with 2% paraformaldehyde.

Indirect double-immunofluorescent labeling combined with Hoechst 33342 nuclear staining (Molecular Probes, 2 μl/ml in PBS