Nanomedicine: Nanotechnology, Biology, and Medicine
xx (2017) xxx–xxx

nanomedicine
Nanotechnology, Biology, and Medicine

nanomedjournal.com

Special Topic: Two-Dimensional Biomaterials in Regenerative Medicine

Enhanced neural stem cell functions in conductive annealed carbon nanofibrous scaffolds with electrical stimulation

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Received 14 October 2016; accepted 6 March 2017

Abstract

Carbon-based nanomaterials have shown great promise in regenerative medicine because of their unique electrical, mechanical, and biological properties; however, it is still difficult to engineer 2D pure carbon nanomaterials into a 3D scaffold while maintaining its structural integrity. In the present study, we developed novel carbon nanofibrous scaffolds by annealing electrospun mats at elevated temperature. The resultant scaffold showed a cohesive structure and excellent mechanical flexibility. The graphitic structure generated by annealing renders superior electrical conductivity to the carbon nanofibrous scaffold. By integrating the conductive scaffold with biphasic electrical stimulation, neural stem cell proliferation was promoted associating with unregulated neuronal gene expression level and increased microtubule-associated protein 2 immunofluorescence, demonstrating an improved neuronal differentiation and maturation. The findings suggest that the integration of the conducting carbon nanofibrous scaffold and electrical stimulation may pave a new avenue for neural tissue regeneration.

Key words: Carbon nanofiber; Conductive nanomaterial; Electrical stimulation; Neural stem cell; Neural differentiation

Stem cell-based transplantation therapy has opened up new possibilities for repairing injured tissues or organs. As a multipotent cell population, neural stem cells (NSCs) are exhibiting promise for various neurodegenerative diseases and injuries. The therapeutic efficacy of NSCs is exclusive by a cell-replacement mechanism.[1] NSC line is capable of self-renewing and differentiating into neurons and other glial cells (astrocytes and oligodendrocytes) that can integrate with host tissues and repair nerve damages by improving neurogenesis and axonal growth.[2,3] The undifferentiated NSCs might also repair nerves by intrinsic neuroprotective ability in which NSCs release a series of bioactive molecules, *e.g.*, neurotrophic growth factors, immunomodulatory substances, for maintaining neural tissue homeostasis.[1] Despite great progression being achieved, there remains important issues that need to be solved regarding the NSC-based therapy. Direct transplantation of NSCs fails to offer the physiological stimulations that can promote proliferation and differentiation, and induce functional integration of NSC-derived neurons into healthy neural networks.[2,4] In order to support proper cell functions, NSCs are generally introduced to a target region by mixing with or being seeded on a functional scaffold. The functional scaffold provides mechanical support and physiochemical cues for guiding neural cell growth and differentiation as well as forming complex neural tissue patterns.[5,6]

Due to the intrinsic electroactivity of nerve cells, conductive scaffolds are of particular interest in neuroscience by offering means

Conflict of Interest and Disclosure: N/A.

All sources of support for research: This work was supported by the March of Dimes Foundation's Gene Discovery and Translational Research Grant, and NSF MME grant #1642186.

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http://dx.doi.org/10.1016/j.nano.2017.03.018
1549-9634/

to apply electrical stimulation. Carbon-based nanomaterials, including carbon nanotubes (CNTs), carbon nanofibers (CNFs), and graphene, hold potential for neural applications due to their unique electrical, mechanical, and biological properties.[7,8] In addition, neural tissue extracellular matrix (ECM) consists of various nanostructured components which directly interact with neural cells as well as stimulate cell growth and differentiation. In this regard, the nanoscale features of CNTs, CNFs, and graphene may provide a biomimetic nanostructured environment, which makes them superior to other conventional biomaterials in micro–macro dimension.[9] Actually, CNTs have been demonstrated to promote neuron proliferation, modulate neuronal behavior at either structural (neurite elongation) or functional (synaptic efficacy) level, as well as increase network activity of neuronal circuits.[10–12] Usmani et al found that conductive 3D CNT meshes were able to guide neural webs formation and facilitated signal transmission when cultured segregated spinal cord on them *in vitro*.[13] The *in vivo* implantation of the CNT meshes presented low tissue reaction as well. Graphene can also enhance neuronal differentiation, guide axons extension, and support functional neural circuit growth.[14–18]

In addition to the enhanced neural cell behaviors by conductive scaffolds themselves, external electrical stimulation is usually employed in an effort to restore injured neural functions. Despite exact mechanism of the interactions between electrical stimulation and neural cell/system has yet to be fully understood, it has been well-documented that electrical stimulation can increase neurite outgrowth *in vitro* and enhance functional recovery *in vivo*.[19,20] Furthermore, electrical stimulation delivered *via* carbon-based nanomaterials has been demonstrated to induce neuronal signaling.[21]

In the present study, conductive carbon nanofibrous scaffolds have been fabricated by annealing polymeric precursor electrospun fiber mats. Unlike the CNTs or CNFs synthesized by other methods, such as laser ablation, and chemical vapor deposition, this annealing approach can directly generate an integrated network structure in the absence of substrate for deposition and any catalyst. It was demonstrated that the electrospun carbon nanofibers (ECNFs) fabricated by annealing electrospun polymeric nanofibers can support human endometrial stem cells to give rise to neuron-like cells,[22] showing great promise in neural regeneration. Considering the advantages of conducting ECNFs and electrical stimulation on neural tissue engineering, we postulate that the combination of them will generate a robust strategy for neural regeneration. Therefore, we investigated here the electrical properties of annealed ECNFs and bioactivity by culturing NSCs. Additionally, we applied electrical stimulation on the NSC seeded ECNF scaffold; the cell proliferation was studied associating with the detection of neural differentiation by quantitative real-time polymerase chain reaction (RT-PCR) and immunocytochemistry.

Methods

Fabrication of conductive ECNF scaffolds

The ECNF scaffolds were fabricated *via* a single-spinneret electrospinning and post-thermal treatment similar to our previous work.[23] In a typical procedure, 0.5 g of terephthalic acid (PTA) was first dissolved in 10 g of *N*, *N*-dimethylformamide (DMF) by mixing at room temperature for 10 min, and next 1 g of polyacrylonitrile (PAN) was added and further mixed at 80 °C for 3 h. The prepared solution was then electrospun using a 12 mL syringe with a 25 gauge blunt needle (NNC-PN-25GA, Nano NC) at a flow rate of 1 $mL.h^{-1}$ (NE-300, New Era Pump Systems Inc.). The voltage of 10 kV was applied, and the syringe needle-to-collector distance was maintained at 10 cm. A rotating drum wrapped with an aluminum foil (1000 rpm) was used to collect the fibers for 4 h. The as-spun fibers were first dried in an oven (VWR Force Air Oven) at 60 °C for 1 day, then heated in a tube furnace (OTF-1200 × −80, MTI Corporation) from room temperature to 280 °C for 2 h in air for stabilization (heating rate of 2 °C min^{-1}), followed by further heated up to 1000 °C for 1 h in N_2 for carbonization (heating rate of 5 °C min^{-1}). The annealing time and temperature were optimized based on our previous study.[23]

Scaffold characterization

The morphology of fibers before and after annealing was examined by scanning electron microscope (SEM, FEI Teneo LV SEM). All samples were coated with iridium for 30 s prior to imaging. Element compositions of samples were examined by energy-dispersive X-ray spectrometer (EDS). 200 fibers from at least 10 different images were analyzed for fiber diameter with ImageJ software (National Institutes of Health, USA). The Raman spectra of the samples were characterized by a Raman spectrometer (Horiba Scientific) with a 532 nm laser excitation. For the electrochemical measurement, the cyclic voltammetry was performed using potentiostat (Digi-IVY DY 2013) with a standard three-electrode electrochemical cell. The cell assembly composed of a commercial Ag/AgCl reference electrode (Sigma–Aldrich), a counter electrode of platinum, and a working electrode with phosphate buffered saline (PBS) solution at 25 °C. The voltammogram curves were observed within the potential window from −0.8 V to 0.8 V at a scan rate of 100 mV/s, starting from zero current.

NSC culture

Mouse NSCs were obtained from ATCC (NE-4C). The cells were cultured in Eagle's Minimum Essential Medium (ATCC) supplemented with 2 mM L-Glutamine (Thermo Fisher) and 10% fetal bovine serum (FBS, Gemini) and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Cell expansion was conducted on 15 μg/mL poly-L-lysine coated flasks. Passages 4 to 6 cells were used for all experiments. For NSC differentiation, cells were cultured in a differentiation medium composed of complete culture medium with 10^{-6} M retinoic acid (Sigma–Aldrich).

NSC viability study

To evaluate the biocompatibility of ECNFs, NSCs were cultured on ECNF scaffolds. PAN scaffolds and glass were selected as controls. Prior to cell seeding, 12 mm diameter samples were fixed on the bottom and rinsed with 70% ethanol for 20 min. After washing three times with PBS, samples were pre-wetted with complete medium overnight. NSCs were then