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Rolled graphene oxide foams as three-dimensional scaffolds for growth of neural fibers using electrical stimulation of stem cells

Omid Akhavan [a,b,*], Elham Ghaderi [c], Soheil A. Shirazian [a], Reza Rahighi [a]

[a] Department of Physics, Sharif University of Technology, P.O. Box 11155-9161, Tehran, Iran
[b] Institute for Nanoscience and Nanotechnology, Sharif University of Technology, P.O. Box 14588-89694, Tehran, Iran
[c] Nanobiotechnology Research Lab., Division of Advanced Materials, Azadi Ave., Tehran, Iran

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ABSTRACT

Graphene oxide foam (GOF) layers with thicknesses of ~15–50 μm and density of ~10 graphene oxide (GO) sheets/μm were fabricated by precipitation of chemically exfoliated GO sheets in an aqueous suspension at ~80 °C under UV irradiation. Then, rolled GOFs with desirable scales were developed as electrically conductive 3D-scaffolds and applied in directional growth of neural fibers, through differentiation of human neural stem cells (hNSCs) into neurons under an electrical stimulation. X-ray photoelectron spectroscopy indicated that the UV irradiation resulted in partial deoxygenation of the layers. Scanning electron microscopy and Raman spectroscopy confirmed the presence of multilayer GO sheets in the foam structure. The electrical sheet resistance of the GOFs was found low enough to produce the electrical stimulation currents used in differentiation of the neural cells, under low voltages. Rolling the GOFs (with hydrophilic surfaces) resulted in formation of cross-sections with superhydrophilic characteristics, inducing effective proliferation and differentiation of the hNSCs throughout the pores and interfaces of the scaffold. The electrical stimulation induced more proliferation of the cells and acceleration of the differentiation into neurons (rather than glia). These results suggest the GOFs as flexible and conductive scaffolds for regeneration of nervous systems and tissue engineering.

1. Introduction

Graphene (as a realization of two-dimensional (2D) nanomaterials with graphitized structure) has presented fascinating characteristics such as exceptional electrical [1] and mechanical [2] properties, the largest surface area [3], easy functionalization [4] and low-cost production [5]. Hence, graphene has promisingly been used in a broad range of nanotechnology-based bio-applications including DNA/RNA extractions [6,7], extremely sensitive biosensing [8], disease diagnosis [9,10], drug delivery [11,12], anti-bacterial [13–15], parasitical [16] and viral [17] intentions, effective cancer cell targeting, imaging and therapy [18–23], and altering the genetic characteristics of spermatozoa [24] and stem cells [25]. Recently, graphene layers have successfully been utilized in upcoming stem cell-based tissue engineering as 2D-scaffolds [26–28].

Due to the superior electrical properties of graphene (e.g., the ballistic electron transport property) which can effectively contribute in the electrical stimulations of neurons, its promising bio-applications in regeneration of nervous systems have highly attracted the attentions of researchers. Meanwhile, graphene can generally supply biocompatible surfaces with suitable chemical properties for effective attachment, proliferation and differentiation of neural cells. The effective attachments of cells to graphene templates can provide effective transports of electrical charges required in neuronal differentiation and/or stimulation [29]. For instance, Park et al. [30] applied graphene sheets as 2D-conductive templates in differentiation of human neural stem cells (hNSCs) into neurons (rather than glia) using electrical stimulation. Graphene/polyethylene terephthalate film was also utilized for managing the neural cell-to-cell interactions by non-contact electrical field stimulation [31]. Recently, injection of the photoexcited electrons from semiconductors (such as TiO_2 [32] and graphene nanomeshes [33]) into hNSCs attached on surface of graphene layer was proposed to realize flash photo stimulation of the cells in neural differentiation. The flash photo stimulation was also utilized for generation of a 2D-neural network on graphene nanogrids [34]. The injection of electrons from graphene oxide (GO) sheets stimulated by pulsed laser irradiation was also resulted in self-organized differentiation of hNSCs into neurons [35].

* Corresponding author at: Department of Physics, Sharif University of Technology, P.O. Box 11155-9161, Tehran, Iran.
E-mail address: oakhavan@sharif.edu (O. Akhavan).

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In recent years, graphene foams have successfully been developed [36] and suggested as three-dimensional (3D) scaffolds providing differentiation of cells into the desired topographies (i.e., similar to their in vivo counterparts) [37,38]. Although the chemical exfoliation of graphite is known as one of the most comfortable and popular methods for production of GO and reduced graphene oxide (rGO) (especially in low-cost and mass production of graphene [39]), all the graphene foams used for cell proliferation and differentiation have been synthesized by chemical vapor deposition (CVD) method. Of course, synthesis of graphene foams by using Hummers' method was already proposed by Dikin et al. [40] and called "graphene oxide papers". However, there has been no report on application of such GO and/or rGO layers as 3D-scaffolds in cell proliferation and differentiation.

In this work, at first, graphene oxide foam (GOF) layers were fabricated by precipitation of chemically exfoliated GO sheets in an aqueous suspension under UV irradiation. Then, the GOF layers (with desirable scales) were rolled to obtain 3D-scaffolds with capability of directional proliferation and differentiation of neurons in the direction of the main axis of the rolls. X-ray photoelectron spectroscopy (XPS) was utilized to study the chemical state of the UV-irradiated carbons in the foam. Scanning electron microscopy (SEM) and Raman spectroscopy were applied to confirm the presence of single- and/or multi-layer GO sheets in the foam structure. The electrical sheet resistance of the GOFs was measured to examine the capability of the foams in electrical stimulation of the neural cells under low voltages. Hydrophilicity of surface of the layers and cross-section of the rolled foams were also investigated. Finally, the rolled GOFs were applied as 3D-scaffolds in directional growth of neural fibers by differentiation of hNSCs into neurons under electrical stimulation.

2. Experimental section

2.1. Fabrication of rolled GOFs

The GO aqueous suspensions were obtained by sonication of the graphite oxide suspensions which synthesized using an improved Hummers' method. The details of the procedure were previously reported elsewhere [41]. The graphene oxide suspensions with concentrations of ~5 mg/mL were loaded in flexible PET glasses (with volume of 50 mL) and dried at ~80 °C for 24 h under UV irradiation of a florescent UV lamp (Philips, 10 W). Then, the films achieved from the sedimentation of the GO sheets at the bottom of the glasses were separated and cut as squares. The thickness of the GOF layers was measured ~15–50 μm, using a micrometer. The GOF layers were uniform and dark brown under transmitted environmental light and almost black in reflection, especially for thicker ones. The GOF layers were incubated in laminin solution (10 μg/mL in the culture medium) for 2 h for better attachment of the cells on the layers, as previously shown by Park et al. [30]. The incubated layers were rinsed three times with phosphate buffer saline (PBS). The rolled GOFs were obtained by soft rolling the laminin-functionalized GOF layers around one of the edges. This method resulted in formation of a cylindrical-like scaffold with an outer diameter corresponding to stacking ~30 GOF layers.

2.2. Material characterizations

Morphology of the cross section of the GOFs was studied by using a SEM (TESCAN) at 25 keV. XPS was utilized to examine the chemical state of carbon atoms contributed in the structure of GOFs. The XPS were obtained by using a hemispherical analyzer supplied by an Al Kα X-ray source operating at energy of 1486.6 eV in a vacuum better than 10^{-7} Pa. The XPS data were calibrated by fixing the C(1s) core level at 285.0 eV. To confirm this, a thin Au layer was coated on some masked samples using a desktop sputtering system (Nanostructured Coating Co., Iran) and then the calibration was checked by fixing the Au($4f_{7/2}$) peak at 83.7 eV. The XPS peaks were deconvoluted using Gaussian components after a Shirley background subtraction. The carbon structure of the GOFs was investigated using Raman spectroscopy (HR-800 Jobin–Yvon) supplied by a 532 nm Nd-YAG excitation source. The hydrophilicity of the surface of the GOF layers and cross section of the rolled GOFs were studied using water contact angle measurements at room temperature. The electrical conductivity of the GOF layers was evaluated by measuring the sheet resistance of the layers using four-point probe technique.

2.3. Cell culture

The hNSCs (obtained from Gibco) were cultured in a proper medium (Dulbecco's modified Eagle medium (DMEM) including 2% bovine serum albumin (BSA), 1% penicillin/streptomycin, 2 mM L-glutamine, 10 ng/mL epidermal growth factor (EGF), and 10 ng/mL basic fibroblast growth factor (bFGF)) under 5% CO_2 ambient at 37 °C. The culture medium was refreshed every 3 days. The hNSC suspensions obtained at the third passage (based on the Gibco user manual with catalog Nos. N7800-100 & N7800-200) were seeded among the unrolled GOF layers with cell density of ~6×10^4 cells/cm^2, and subsequently, the unrolled GOFs were rolled again, according to the characteristics of the initial rolling. The proliferation of the cells was performed in 5% CO_2 atmosphere at 37 °C for 72 h.

2.4. Differentiation and electrical stimulation of stem cells

Immediately after the cell proliferation, the neural differentiation of the stem cells was initiated by removing the growth factors (i.e., EGF and bFGF) from the culture medium [42]. The period of differentiation was considered 2 weeks. For electrical stimulation, at first, the culture medium was replaced by an extracellular medium including 140 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 15.0 mM ascorbic acid, 10 mM glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH ~6.5. Then, a series of 100 ms cathodic voltage pulses were applied on the two ends of the graphene rolls (electrically connected to a function generator through gold wires and silver paste) in intervals of 10 s. The stimulation threshold was found at the current of ~20 mA.

2.5. Fluorescence imaging of cells

The cells grown on the rolled GOFs were fixed by PBS solution containing 4 vol% paraformaldehyde for 20 min. Then, the samples were rinsed 3 times with PBS and incubated in PBS solution containing 2% BSA, 0.1% Triton X-100, and anti-nestin (Millipore, with dilution of ~1:500) for 60 min. After that, the cells were incubated with goat anti-rabbit TRITC (with dilution of ~1:500) for 60 min. Once again, the cells were rinsed three times by using PBS. Neuron-specific class III β-tubulin (TUJ1, as a neural cell marker with green color), glial fibrillary acidic protein (GFAP, as a glial cell marker with red color) (both of them with dilution of ~1:500), and ~1:100 diluted 4′,6-diamidino-2-phenylindole (DAPI, as a cell nucleus marker with blue color) were added into the PBS solution and allowed to incubate with the differentiated cells for 30 min. The stained cells on cross section (obtained by cutting the rolls) and interior surfaces (obtained by slowly unrolling) of the rolled GOFs were rinsed by PBS and were monitored by a confocal fluorescence microscope (Zeiss LSM 510). The average numbers of cells per unit area were obtained by counting the stained cells over