Biomaterials 34 (2013) 6402–6411

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Enhancement of electrical signaling in neural networks on graphene films

Mingliang Tang [1], Qin Song [1], Ning Li [1], Ziyun Jiang, Rong Huang, Guosheng Cheng*

Suzhou Key Laboratory of Nanobiomedicine & Division of Nanobiomedicine, Suzhou Institute of Nano-Tech and Nano-Bionics, Chinese Academy of Sciences, Suzhou Industrial Park, Jiangsu 215123, PR China

ARTICLE INFO

Article history:
Received 24 April 2013
Accepted 16 May 2013
Available online 4 June 2013

Keywords:
Graphene
Neural stem cells
Neural activity
Neural network

ABSTRACT

One of the key challenges for neural tissue engineering is to exploit supporting materials with robust functionalities not only to govern cell-specific behaviors, but also to form functional neural network. The unique electrical and mechanical properties of graphene imply it as a promising candidate for neural interfaces, but little is known about the details of neural network formation on graphene as a scaffold material for tissue engineering. Therapeutic regenerative strategies aim to guide and enhance the intrinsic capacity of the neurons to reorganize by promoting plasticity mechanisms in a controllable manner. Here, we investigated the impact of graphene on the formation and performance in the assembly of neural networks in neural stem cell (NSC) culture. Using calcium imaging and electrophysiological recordings, we demonstrate the capabilities of graphene to support the growth of functional neural circuits, and improve neural performance and electrical signaling in the network. These results offer a better understanding of interactions between graphene and NSCs, also they clearly present the great potentials of graphene as neural interface in tissue engineering.

1. Introduction

Reconstruction of neural circuit and tracts in the central nervous system (CNS) is especially difficult after large lesions or during chronic neurodegeneration, because of the intrinsic poor regenerative properties of this tissue [1,2]. Engineering neural interfaces are often introduced to promote nerve regeneration and to repair damage caused by injury. Recently, neural stem cells (NSCs), a self-renewing and multipotent cell population in the CNS, exhibit promising prospects in developing cell therapies for neural regeneration [3], while it usually requires grafts or scaffolds to provide additional treatments and appropriate regulation of NSCs. However, it still remains a big challenge to develop neural interfacing materials which can help rebuild lesioned CNS circuits or modulate the neural network activities after NSC differentiation with the scaffolds in the damaged area.

To date, neural tissue engineering, which aims at developing new strategies for neural repair and regeneration, has increasingly involved nanotechnology to improve material–neuron interfacing to sustain and promote neural regeneration following the injury [4]. Nanomaterials can be engineered and integrated into biological systems to provide supporting substrates to govern cell-specific behaviors. Graphene, in particular, has been at the forefront of nanotechnology due to their intriguing physical and chemical features [5], which allow the development of a variety of neural interfacing devices with remarkable properties. For example, it has recently been considered as a promising candidate for the fabrication of ultrafast nanoeletronic devices, quantum computers, transparent electrodes, nanocomposite materials and biomedical materials [5,6]. Interestingly, graphene and graphene oxide sheets show the potential as a non-cytotoxic, transferable, and implantable platform for stem cell culture [7–12], which can facilitate stem cell attachment and growth. Especially, graphene shows the remarkable ability to direct stem cell differentiation in a controllable manner. Currently, graphene was reported to be capable of directing stem cell fate by controlling and accelerating osteogenic differentiation of human mesenchymal stem cells (hMSCs) [10],

Abbreviations: AFM, atomic force microscope; CNS, central nervous system; CVD, chemical vapor deposition; hMSCs, human mesenchymal stem cells; mPSCs, miniature postsynaptic currents; NSC, neural stem cell; SEM, scanning electron microscopy; sPSC, spontaneous postsynaptic currents; TCPS, tissue-culture polystyrene; TEM, transmission electron microscope; XPS, X-ray photoelectron spectroscopy.

* Corresponding author.
E-mail addresses: gscheng2006@sinano.ac.cn, guosheng_cheng@hotmail.com (G. Cheng).

[1] These authors contributed equally to this work.

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.05.024

accelerating bone marrow derived MSCs to grow toward osteogenic lineage [8] and enhancing differentiation of human NSCs into neurons [11]. The strong interest in interfacing graphene with neural cells is mainly based on the unique electrical properties of graphene, which can be tailored to match the charge transport required for electrical cellular interfacing [13]. To the best of our knowledge, there is no report yet about the neural network formation developed by NSC differentiation and their activities on graphene films. The potential impact of interfacing graphene with stem cells prompted us to investigate the formation of functional neural network and their performances after stem cell differentiation on the graphene substrates.

To better utilization of graphene as a neural interface for stem-cell based therapy, it is critical to make sure the cells after stem cell differentiation could form functional connections from each other and preserve normal or even enhanced activities. In order to address whether and how neural circuit could be structurally and functionally formed on graphene substrates and whether synaptic activity could be affected by graphene coupling, we attempted to investigate the growth, functional formation and neural activities in the networks on graphene substrates by combining calcium imaging, single-cell electrophysiology, immunofluorescence and scanning electron microscopy (SEM).

2. Materials and methods

2.1. Graphene substrate preparation

Graphene samples were synthesized according to previously published chemical vapor deposition (CVD) method [14]. Briefly, a thin copper foil was heated to 1000 °C and annealed for 20 min under H_2 and Ar gases, followed by exposure to H_2 and CH_4 for 5 min. Finally, the substrate was cooled down from 1000 °C to room temperature under H_2 and Ar gases. Graphene films were removed from the Cu foils by etching in an aqueous solution of iron nitrate. After the copper film was dissolved, a tissue-culture polystyrene (TCPS, taken as control in the following studies) substrate was brought into contact with the graphene film and it was pulled from the solution. Finally, a graphene substrate was acquired using this method. Then, the graphene substrates were mounted with a chamber and immersed in milli-Q water overnight to remove residual soluble toxic components. After sterilization by 75% alcohol, graphene substrates were successively soaked into sterilized PBS buffer and coated with laminin (5 μg/mL, Sigma, USA) solution in PBS for at least 4 h at 37 °C. Just before cell seeding, graphene films were soaked in the culture medium overnight.

2.2. Characterization of graphene substrates

After graphene was transferred to TCPS dish, the surface morphologies of graphene substrate and bare TCPS were determined by atomic force microscope (AFM) (Dimension 3100, Veeco, USA) using tapping mode operated at the room temperature. The crystallinity and number of the layer presented within graphene were examined by Raman spectrometer (lamRAM HR800, HORIBA, France) and transmission electron microscope (TEM) (Tecnai G2 F20 S-Twin, FEI, USA). The surface chemistry of the graphene film was examined using X-ray photoelectron spectroscopy (XPS) (Axis Ultra DLD, Kratos, UK) utilizing an Al Ka X-ray source operated at 40 eV.

2.3. NSCs culture

NSCs were derived from both hemispheres hippocampus of postnatal day 1 ICR rat (Animal Center in SooChow University). Hippocampus was removed from blood vessels and meninges to be collected in falcon tubes in hank's balanced salt solution (HBSS) at 4 °C, then rinsed with HBSS solutions for two times. After centrifugation (1000 r/min for 5 min), tissues were digested in TryplE (Life Technologies, USA) for 15 min at 37 °C, then gently triturated mechanically by using pipette tips to NSC suspension in DMEM-F12 medium containing 2% B-27. NSCs were cultured at 37 °C in humidified atmosphere with 5% CO_2. Passage of NSCs was carried out every 7 days. NSCs were seeded in the DMEM-F12 medium containing 2% B-27, 20 ng/mL EGF and 20 ng/mL FGF-2 (R&D Systems, USA) by supplementation of fetal bovine serum (FBS, Gibco, USA) and 1 μM RA (Sigma). The care and use of animals in these experiments followed the guidelines and protocol approved by the Care and Use of Animals Committee of Suzhou Institute of Nano-Tech and Nano-Bionics. All efforts were made to minimize the number of animals used and their suffering.

2.4. Immunofluorescence staining of NSCs

Cells were washed with PBS, fixed in 4% paraformaldehyde for 45 min, blocked and permeabilized for 90 min. Primary antibodies were incubated for 90 min, and secondary antibodies were incubated for 60 min, followed by DAPI staining. Antibody panel used includes primary antibodies against β-tubulin (Sigma), GAPDH (Sigma), MAP-2 (Abcam, USA).

2.5. Calcium imaging

The cultures were washed with the standard external solution containing (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 D-glucose, and buffered to pH 7.3. The standard external solution was continuously bubbled with 95% O_2 and 5% CO_2. Cells were then loaded with 2.5 μmol Fluo-4-AM (Dojindo Laboratories, Japan) and pluronic F-127 [Sigma, 0.004% (*w*/*v*) final] at 37 °C for 45 min. After Fluo-4-AM was removed, the cells were incubated for an additional 25 min in the external solutions, to allow for hydrolysis of the ester bond in fluorescent Fluo-4. Subsequently, cultures were imaged with a Carl Zeiss scanning confocal microscope (Carl Zeiss Company). Epifluorescent excitation for Fluo-3 was at 488 nm, and emission was collected at 510 nm. Camera gain was adjusted to give baseline maximal fluorescence levels of 40–100 (arbitrary units) of a maximal eight-bit signal output of 256. In the experiments of external stimulations, cell fluorescence during the 5 min baseline period was F. Fluorescence measurements for each cell were normalized to the average fluorescence intensity. Region of indexes (ROIs) were defined in the first image, and the normalized fluorescence changes $\Delta F/F$ were measured throughout the image sequence. All settings of the canning system and the complete data acquisition were controlled and collected by the LSM 510 software (Carl Zeiss Company). For electrical stimulation, an electrical lead was attached directly the dry area of the graphene substrate by using silver paste and copper tape. The input stimulation was applied with the aid of a function generator (S3K, Grass Technologies, USA) that gave flexibility in the stimulus signal to be applied. A series of 10–100 ms monophasic cathodic pulses were applied with intervals of 5 s, the stimulation threshold was 0.5–1 μA. For high K^+ stimulation, 50 mM KCl solutions were acutely exposed to the cultures.

2.6. Electrophysiological recordings

Whole-cell voltage clamp recordings were made from visually identified neurons in the cultures, using a CCD camera, connected to an infrared video microscopy (E600-FN, Nikon, Japan). The healthy neurons were identified with following criteria: the cell was bright, kept its shape (no swollen), had a clear edge, and its content could not be seen. Patch pipettes were pulled from borosilicate glass capillary and had resistance of 3–4 MΩ, when filled with the pipette solution. The pipette solution contained (in mM): potassium gluconate 70, KCl 60, ethylene glycol-bis-(aminoethyl ethane)-N,N,N′,N′-tetraacetic acid (EGTA) 5.0, N-2 hydroxyethyl piperazine-n-2 ethanesulphonic acid (HEPES) 10, $MgCl_2$ 2, $CaCl_2$ 0.5, adenosine 5′-triphosphate (ATP) disodium 2.54, pH 7.25 (adjusted with KOH). Currents were recorded using an EPC-9 amplifier (HEKA, Germany) in voltage clamp mode. Only cells whose series resistance ranged from 10 to 30 MΩ and changed less than 20% throughout the experiment were taken into consideration. Fast and slow capacitances were neutralized, and series resistance was always compensated by 70–80%. All signals were digitized at 10 kHz, filtered at 2 kHz, stored on a computer hard disk, and analyzed offline with Igor Pro 4.05 software (Wavemetrics, USA).

2.7. Data analysis

The electrophysiological data files were imported into Igor Pro 4.05 for events detection and analysis. Only those spontaneous and miniature events, whose tails did not contain subsequent contaminating events, were identified as good peak events, else were identified as bad peak events. The frequency of spontaneous and miniature events was represented as the total number of events occurring in 1 s (Hz). Cumulative probability plots of intervals between successive IPSCs and amplitudes were generated from more than 100 good peak events for each condition. The significance of the effect was analyzed by Kolmogorov–Smirnov (K–S) test. All data are expressed as mean ± SEM. *p* value less than 0.05 is considered significant.

3. Results

3.1. Preparation of graphene substrates for NSC culture

We crafted conductive substrates of purified graphene to sustain NSC growth. Graphene sheets exhibit unusual mechanical strength coupled with remarkable flexibility, also feature large electrical conductivity, allowing graphene substrates to conduit electrical current between electrochemical interfaces. The surface properties of graphene films were firstly investigated by AFM. Compared with TCPS, graphene substrate was consisted of many ripples and