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The structural development of primary cultured hippocampal neurons on a graphene substrate

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ABSTRACT

The potential of graphene-based nanomaterials as a neural interfacing material for neural repair and regeneration remains poorly understood. In the present study, the response to the graphene substrate by neurons was determined in a hippocampal culture model. The results revealed the growth and maturation of hippocampal cultures on graphene substrates were significantly improved compared to the commercial control. In details, graphene promoted growth cone growth and microtubule formation inside filopodia 24 h after seeding as evidenced by a higher average number of filopodia emerging from growth cones, a longer average length of filopodia, and a larger growth cone area. Graphene also significantly boosted neurite sprouting and outgrowth. The dendritic length, the number of branch points, and the dendritic complex index were significantly improved on the graphene substrate during culture. Moreover, the spine density was enhanced and the maturation of dendritic spines from thin to stubby spines was significantly promoted on graphene at 21 days after seeding. Lastly, graphene significantly elevated the synapse density and synaptic activity in the hippocampal cultures. The present study highlights graphene's potential as a neural interfacing material for neural repair and regeneration and sheds light on the future biomedical applications of graphene-based nanomaterials.

1. Introduction

Despite numerous advances in the fields of neuroscience and regenerative medicine, effective nerve repair and regeneration and functional recovery after nerve injury remain a challenge in the clinic [1]. Tissue engineering with nanomaterials has gained more and more attention over the years, including the use of functionalized scaffolds, the use of guidance channels, and the controlled and sustained release of neurotrophic factors [1–3]. However, significant hurdles remain before full functional recovery of the nervous system can be accomplished, especially because of issues with delayed or incomplete neural tissue regeneration due to poor neural interfacing [4].

Graphene and its derivatives, including graphene oxide, reduced graphene oxide, and graphene composite, have emerged as promising nanomaterials with enormous potential for different biomedical applications and translational research since the initial report of their discovery in 2004 [5]. This potential is due to their excellent physical, chemical, and mechanical properties [6,7]. There are numerous studies and reports demonstrating that graphene and its derivatives can be widely used for molecular/cellular imaging [8,9], drug/gene delivery [8,10], bio-analysis [11], stem cell research [6,12–14], and photothermal therapy for tumors [15]. Graphene-based nanomaterials exhibit good biocompatibility and show little cytotoxicity for many different cell types [6]. Nevertheless, there is some discrepancy with regard to their cytotoxicity, which might

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be due to impurities in the materials during their preparation and surface functionalization [6,14,16].

For the repair and regeneration of neurons and the nervous system, the potential of graphene-based nanomaterials is largely uninvestigated. Neural cells are electroactive [17], and electrical stimulation plays an important role during neural repair and regeneration [18,19]. Graphene-based nanomaterials possess excellent electrical properties, and they can be tailored to match the charge transport requirements for electrical cellular interfacing. Also, the unique mechanical and chemical properties of graphene-based nanomaterials are beneficial for their integration with neural tissues as long-term implants.

The hippocampus plays a vital role in the consolidation of several forms of learning and memory, especially during the formation of declarative memories [20]. Hippocampus is one of the best characterized brain regions regarding both of the structure and functions. Besides, the structural development of the hippocampal neurons directly correlates with their functions and can be well characterized, and hippocampal neurons are well known for their plasticity and regenerative properties [21], which make them a suitable cell model for investigating the interactions between graphene-based nanomaterials and the nervous system. Thus, the present study aimed to measure the neuronal response to the graphene substrate in a hippocampal culture model as a way to determine graphene's potential as a neural interfacing material.

2. Materials and methods

2.1. Fabrication and characterization of graphene substrates

Graphene films were synthesized using the chemical vapor deposition (CVD) method as we previously described [22]. Briefly, a thin copper foil was heated to 1000 °C and annealed for 20 min under hydrogen and argon gases followed by exposure to hydrogen and methane for 5 min. Finally, the substrate was cooled down from 1000 °C to room temperature under hydrogen and argon gases. The copper foils were removed by etching in iron nitrate solution for at least 12 h. After rinsing sequentially with 1 M and 0.1 M HCl solutions and water for more than 72 h to remove the etching agents, the graphene films were carefully transferred to the FluoroDish (WPI, USA). The graphene films were sterilized with 75% alcohol and successively soaked into sterilized PBS buffer and coated with poly-lysine solution in PBS for at least 4 h at 37 °C. Just before cell seeding, the graphene films were soaked in the culture medium overnight.

The graphene films were transferred to coverslips for imaging. The crystallinity and number of the layers within the graphene were examined by Raman spectrometry (lamRAM HR800, HORIBA, France) and transmission electron microscopy (TEM) (Tecnai G2 F20 S-Twin, FEI, USA). The surface topological structures of the graphene films were determined by scanning electron microscopy (SEM) (Quanta 400 FEG, FEI, USA).

2.2. Isolation and primary culture of hippocampal neurons

All animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The protocol was approved by the Institutional Animal Care and Use Committee of Southeast University. All efforts were made to minimize the number of animal used and their suffering.

The isolation and culture of hippocampal neurons were conducted as previously described [23]. Briefly, postnatal day 0 Wistar rats were sacrificed and their bilateral hippocampi were dissected out and placed into 4 °C Neurobasal-A medium (Invitrogen, USA). The hippocampi were transferred to 1.4 ml papain solution (100 units/ml papain, 0.5 mM EDTA, 0.2 mg/ml cysteine; Sigma, USA) at 37 °C for 15 min for digestion. After digestion, 0.263 ml DNaseI (0.21%; Sigma) and 0.182 ml $MgCl_2$ (12.5 mM; Sigma) were added to the solution before incubation at 37 °C for 5 min. After incubation, 0.185 ml of horse serum (Sigma) was added and the cells were pelleted by centrifugation for 5 min at 4 °C. The cell pellet was resuspended in 4 ml of Neurobasal-A medium, and the solution/suspension was triturated with a fire-polished Pasteur pipette and passed through a 100 μm filter. Before cell seeding, both of the graphene substrates and commercial tissue culture polystyrene (TCPS, as control) substrate were coated with poly-lysine solution in PBS for at least 4 h at 37 °C, then were soaked in the culture medium overnight. The hippocampal neurons were seeded at a density of 1.0×10^5 cells/ml and cultured at 37 °C in 5% CO_2 for further experiments.

2.3. Immunostaining and fluorescence imaging of hippocampal neurons

The growing hippocampal neurons were immunostained and examined under a confocal microscope at different time points. Immunostaining followed a routine procedure. Briefly, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, then washed three times with PBS for 10 min. After permeabilization with 0.1% Triton X-100 for 15 min and blocking with 5% BSA/PBS for 20 min, cells were stained with the corresponding antibodies in 5% BSA/PBS at room temperature for 2 h. After washing with PBS for 2–3 times, cells were incubated with the secondary antibodies (Invitrogen) for 1 h. Cells were then imaged by Carl Zeiss scanning confocal microscope. To examine the growth of growth cones, hippocampal neurons were stained with antibodies against α-actin (Sigma) and β-tubulin (Sigma) 24 h after seeding onto the substrates. To visualize the morphology and dendritic complexity of hippocampal neurons at different time points, cells were immunostained with antibodies against β-tubulin (Sigma) and MAP-2 (Sigma), respectively. Axons were immunostained against Tau (CST, Danvers, MA). Synapses were immunostained with antibodies against the presynaptic marker synaptophysin (Millipore, USA) and the postsynaptic marker PSD-95 (Abcam, USA). Immunostaining and imaging with a confocal microscope (objective W Plan-Apochromat 20X/1.0 DIV M27 70 mm, pinhole 1AU, averaging num 2, line mode, 8 Bit depth) are routine experiments, and standard protocols in numerous previously published methods were followed in this study.

2.4. Imaging of dendritic spines in hippocampal neurons

Dendritic spines were imaged in hippocampal neurons that had been transduced with lentivirus encoding mCherry-actin (red). pLVX-mCherry-actin vector (Clontech, USA) was used for packaging the lentivirus. mCherry-actin fusion protein (red) can be incorporated into actin filaments, and this allows for visualization of actin-containing dendritic spines in live hippocampal neurons.

2.5. Analysis of dentritic spine types

Dendritic spines are identified as small projections extending ≤3 μm from the adjacent dendrite. According to the literature, they are usually classified as stubby, mushroom, and thin types based on the specific ratios of length of the spine/neck diameter and head diameter/neck diameter as previously described [24,25]. Stubby spines have a length similar to the diameter of the neck, which is similar to the diameter of the spine head. Mushroom spines are typically smaller than 1 μm in length with a neck diameter much smaller than the diameter of the head. Thin spines have lengths