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Impact of crystalline quality on neuronal affinity of pristine graphene

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ABSTRACT

Due to its outstanding mechanical and electrical properties as well as chemical inertness, graphene has attracted a growing interest in the field of bioelectric interfacing. Herein, we investigate the suitability of pristine, i.e. without a cell adhesive coating, chemical vapor deposition (CVD) grown monolayer graphene to act as a platform for neuronal growth. We study the development of primary hippocampal neurons grown on bare graphene (transferred on glass coverslip) for up to 5 days and show that pristine graphene significantly improves the neurons adhesion and outgrowth at the early stage of culture (1–2 days in vitro). At the later development stage, neurons grown on coating free graphene (untreated with poly-L-lysine) show remarkably well developed neuritic architecture similar to those cultured on conventional poly-L-lysine coated glass coverslips. This exceptional possibility to bypass the adhesive coating allows a direct electrical contact of graphene to the cells and reveals its great potential for chronic medical implants and tissue engineering. Moreover, regarding the controversial results obtained on the neuronal affinity of pristine graphene and its ability to support neuronal growth without the need of polymer or protein coating, we found that the crystallinity of CVD grown graphene plays an important role in neuronal attachment, outgrowth and axonal specification. In particular, we show that the decreasing crystalline quality of graphene tunes the neuronal affinity from highly adhesive to fully repellent.

1. Introduction

In the field of bioelectronics, a growing interest concerns the materials at the interface with living tissue. Besides the basic requirement of biocompatibility, which relies on the chemically inert nature of the sensor material, intimate coupling with the cells is one of the key features for sensitive and stable neural interfacing. Therefore, materials promoting a direct neural adhesion and (regenerative) outgrowth are of primary interest as they provide a direct electrical coupling between the neurons and the sensor.

A variety of materials are currently used to stimulate and to detect signals from electrogenic cells such as neurons [1]. However, cells can barely attach and grow (directly) on those materials. Typically neuronal attachment to any substrate is mediated by a cell adhesive coating such as poly-lysine, polyornithin, laminin or matrigel, which mimics the extracellular matrix. A disadvantage of this method is that these coatings affect the electrical coupling of the cell to the sensor material. They increase the background noise and the distance to the cell, reducing the sensitivity and the reliability of the recording [2–4]. Further surface topography modifications improving cellular adhesion also improve the electrical coupling and allow the detection of very small signals [5]. Thus, besides the excellent electrical properties, the sensor material should also offer a tight contact to the cells.

Neuroprostheses can barely rely on the chemical and topographical (substrate) modifications used in vitro and still suffer from a poor coupling between the tissue and the implanted electrode. The cell–device interface degrades in time until the signal is finally lost, the reject of the implant being partially due to the mechanical mismatch and the inflammatory response of the surrounding tissues (elasticity of neurons being around 200Pa against 1 MPa–10 GPa for elastomers or silicon). In this context soft electronics are promising tools but they are still limited by the electrodes material exposed to the cell which may cause cell apoptosis and subsequent glial scarring [6]. Highly biocompatible carbon based materials like diamond have increased the acceptance of the implants [7], opening a way for long lasting recordings.

For the purpose of neural interfacing, graphene emerges as one

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of most promising candidates [8–10]. Besides its outstanding chemical stability [11], stretchability [12,13] and exceptional electrical properties [14], which have already made possible the detection of action potentials of electrogenic cells [15,16] and first generation of flexible and transparent neural interfaces [17], graphene seems to provide a unique level of bioacceptance. Indeed, pristine graphene (uncoated) increases the stem cell differentiation into neurons [18] and combining graphene with an adhesive coating (poly-L-lysine, laminin) improves neurite sprouting [19] and enhances the electrical activity of neural networks in culture [20]. However the demonstration of a superior cytocompatibility of pristine graphene is unclear. Previous reports on bare graphene showed either a similar neural attachment on the uncoated sapphire substrate, thus raising the possibility of an indirect effect of the substrate [21], and/or neurite and soma conglomerations which are first indications of poor attachment or cell detachment from the substrate [22]. Moreover, differences in the cell types, culture protocols or substrates (under the graphene layer) may contribute to some variability in the reported results.

Herein, we show remarkable neurons adhesion and healthy growth on bare graphene (Fig. 1) and reveal that high quality of single layer graphene is an unyielding requirement for neurons attachment at otherwise constant experimental conditions (cell, culturing protocol, medium, and substrates). In particular, we compare bare monolayer graphene and glass coverslip coated (and not coated) with poly-L-lysine (PLL), in terms of viability and growth over a period of 5 days in culture. The study assesses the main stages of neuronal development, from adhesion kinetics (DIV1-2) and neuritogenesis (DIV4) towards the formation of the first synaptic contacts (DIV5). Moreover, we show that poor -crystalline graphene monolayers fully repel the neurons. In combination with adhesive patterns (PLL Patterns) this repellent nature could be used for effective in-vitro designing of neural networks.

2. Methods

2.1. Graphene substrates

High-quality monolayer graphene was grown on copper foil (25 μm thick, 99.8% purity, Alfa-Aesar) using thermal chemical vapor deposition (CVD) as reported earlier [23]. We used monolayer graphene obtained with pulsed CVD growth. While in the conventional CVD growth of graphene a continuous CH_4 flow is added to diluted H_2 atmosphere (1000 sccm, 25 mbar pressure), here pulses of CH_4 (2 sccm 10 s, then 60 s off) are used. Continuous CH_4 flow usually results in an increasing amount of carbon atoms dissolved in Cu foil defects. The following segregation of carbon atoms to the surface of the Cu foil leads to an uncontrolled formation of graphene multilayers. In contrast, using pulsed CH_4 flow the copper foil is periodically exposed to pure hydrogen, which binds the segregated/dissolved carbon atoms and carries them out from the growth chamber, preventing the development of multilayer patches. Before the growth, Cu foil is cleaned in acetone and annealed in diluted H_2 atmosphere (dilution in Ar at 10%) at 1000 °C for 2 h. Pieces of about 4 × 4 mm^2 (taken from same graphene monolayer) are then transferred on glass coverslips (Marienfeld) by polymer assisted wet transfer technique. Cu foil is first covered by PMMA on the graphene side, and then wet etched in ammonium persulfate solution (0.1 g/ml, 2 h at room temperature). Once Cu foil is completely dissolved, graphene-PMMA bilayer remains floating on the surface of the etchant solution. Since both, copper and ammonium persulfate, are toxic for biological applications, great care was taken to ensure a complete Cu etching and removal of etchant residuals (6 subsequent washings in DI water). Then a cleaned and hydrophilic glass coverslip is brought into contact with the transparent graphene-PMMA film and pulled from the solution. Directly after fishing, the glass coated with graphene-

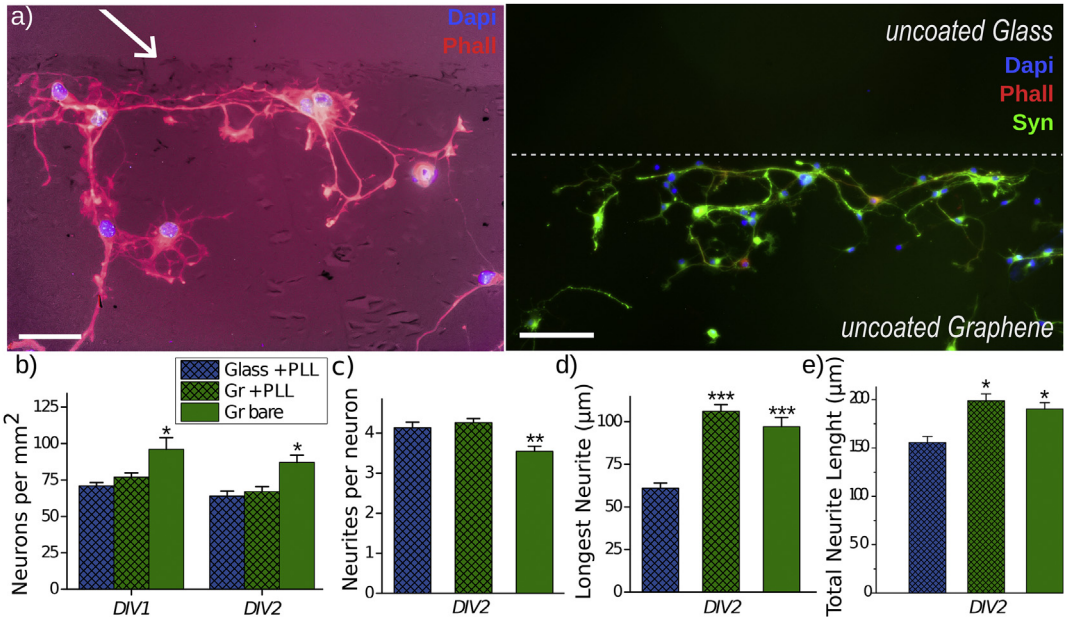

Fig. 1. Adhesion of hippocampal neurons on pristine graphene. a) Representative immunofluorescence images of primary mouse embryos hippocampal neurons grown on bare monolayer graphene (B1 transferred on glass) after 5 (left) and 4 days (right) in culture (without poly-L-lysine coating). The arrow and dashed line indicate the border between glass (top) and graphene (bottom). Neurons are stained with Dapi (blue), Synapsin (green) and Phalloïdin (red), labeling the nucleus, synaptic vesicles and actin filaments respectively. Scale bar 50 μm b) Density of attached neurons on bare graphene (Gr bare) in comparison with the coated glass (Glass + PLL) and graphene (Gr + PLL) samples at first and second day in-vitro. Scale bar 100 μm c) Number of neurites per neuron, (d) longest neurite per neuron and (d) total neurite length per neuron. The total length is the summation of all neurites length of a neuron, averaged on all the counted cells per sample. Means and standard error of the mean are presented. *: $p < 0.05$, **: $p < 0.005$ and ***: $p < 0.001$, compared with PLL-coated glass control. (counted cells n > 140 per condition and time point.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)