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Transparent Conducting Films Based on Reduced Graphene Oxide Multilayers for Biocompatible Neuronal Interfaces

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Among the most critical components in neuronal interfaces is the implanted electrode which requires the long-term stability of its electrical performance and biocompatibility of electrode material in contact with live neuronal cells. Reduced graphene oxide (rGO) renowned for its high electrical conductivity and optical transparency has shown great potential for a variety of applications such as transparent conducting electrodes and biosensors, and might be a potential candidate material for the next-generation neuronal interfaces. However, there have been only few systematic studies on graphene-based neuronal interfaces in terms of electrical conductivity and biocompatibility. In this report, we maintained rat hippocampal neurons on top of the rGO multilayers and observed that the viability of neurons is minimally affected and comparable to those grown on a glass substrate up to 30 days *in vitro*. These results implicate that rGO multilayer can be utilized for excellent neuronal interfaces with its high electrical conductivity and biocompatibility.

KEYWORDS: *Graphene, Neuron, Transparent Electrode, Biocompatibility.*

INTRODUCTION

There is a growing interest in neuronal interfaces based on two-dimension electrode arrays. The planar-electrode-based interface is relatively easy to fabricate with conventional cleanroom techniques such as photolithography and metal/insulator film depositions and readily adaptable for the dissociated neuronal culture substrate with appropriate chemical coatings on top.[1] In conjunction with signal-amplifying circuits and data acquisition instruments, this system has been utilized to investigate *in vitro* neuronal network dynamics[2] and, recently, extended to neuronal recording/stimulation in live animal brains.[3] So far, most of the planar electrode arrays have been based on the micro-patterned metal electrodes which lack both prolonged biocompatibility for stable long-term electrical recording/stimulation and optical transparency necessary for simultaneous video recording during electrical communications at the bio-electronics interface.

Graphene, a monolayer of two-dimensional sp^2 carbon lattice, has emerged as a promising carbon nanomaterial in various fields with its remarkable electrical and thermal conductivities, mechanical properties, and large surface area.[4] Among various routes for preparation of graphene, a stable suspension of graphene oxide (GO) is the common choice over pristine graphene owing to its facile synthetic nature in a controlled, scalable, and reproducible manner.[5] The abundant oxygen-containing functional groups such as epoxide, alcohol, and carboxylic acids provide GO with

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excellent aqueous dispersity and also offer anchors for further chemical modifications.[6–8] Owing to these attributes, interfacing living cells with GO can be useful for the integration of dynamic cellular physiology with electrical readouts.[9] Recently, several attempts have been reported to interface GO with biomaterials; GO had been used for a membrane for DNA translocation,[10] quantitative measurement of the activity of helicase,[11] detection for the various biomolecules[12–14] and a supportive material for mammalian cell culture[15,16] and stem cell differentiation.[17] Although these recent studies suggested the high potential of GO for biomaterials and biological systems, the neuronal cytotoxicity of graphene and, particularly, reduced graphene oxide (rGO) has not been reported until now. In this paper, we report a simple yet versatile method for interfacing rGO multilayer film with neuronal cells. Moreover, we find that the rGO multilayer exhibits necessary biocompatibility with neurons which is prerequisite for the next-generation neural electrode.

EXPERIMENTAL DETAILS

Materials

Poly-D-lysine hydrobromide and fluorescein diacetate, propidium iodide (SigmaAldrich, Korea) were used without further purification. Hippocampal neurons were prepared from embryonic day (E18) Sprague Dawley rats via methods described in the previous literature,[18] and the chemicals and media for primary neuronal culture were purchased from Invitrogen (USA). Cover glasses (d = 12 mm) for primary culture substrates were supplied by Marienfeld-Superior (Germany).

Preparation of Negatively and Positively Charged GO

Graphite oxide was prepared as modified Hummers method[19,20] and exfoliated under horn-type ultrasonication. The resulting graphene oxide (GO) has negative charges due to chemical functional groups such as carboxyl acid groups at the edges. Positively charged GO was synthesized by using 1.25 g of *N*-ethyl-*N*′-(3-dimethyl aminopropyl) carbodiimide methiodide (EDC, SigmaAldrich), 10 mL of ethylenediamine (99%, SigmaAldrich) with 50 mL of GO suspension of at a concentration of 0.5 mg/mL with vigorous stirring for 12 hr. The resultant suspension was dialyzed (MWCO 12000–14000, Spectra/Por) for 3 days to remove any unreacted reagents and by-products.

rGO Film Preparation

Silicon or quartz substrate was cleaned by piranha solution and oxygen plasma to remove any contamination and produce hydrophilic surface. Positively charged GO suspension (0.5 mg/mL) adjusted to pH 3.5 was dropped on the silicon or quartz substrate which was loaded on a spin-coater (ACE-200, Dong Ah Tech), maintained for 2 min as a deposition time, and spun at 3000 rpm for 30 s. As a rinsing step, pH-adjusted deionized water was dropped on the substrate coated with positively charged GO, maintained for 10 sec to remove unbound GO^+, and spun at 3000 rpm for 30 s. Next, negatively charged GO solution (0.5 mg/mL) adjusted to pH 10 was spin-coated in the same manner, followed by the rinsing step, which afford one-bilayer film of $(GO^+/GO^-)_1$ multilayer. The above processes were typically repeated to 10 bilayers. Reduced GO (rGO) multilayer films were prepared by thermal annealing in home-made furnace up to 300, 600, 1000 °C (heating ramp: 15 °C/min) under argon atmosphere for 30 min.

rGO Surface Modification

The rGO substrates were immersed in 0.10 mg/ml poly-D-lysine (PDL) aqueous solution and cured in a CO_2 incubator (Thermo, 37 °C, 5% CO_2) for 3 hr. Afterward, the substrates were rinsed with sterilized DI water several times to remove unbound PDL coatings and maintained in a water-filled jar before use.

Primary Culture and Cell Viability Test

Hippocampal neurons were prepared from embryonic day (E18) Sprague Dawley rats following the previously-reported methods with slight modifications.[18] Briefly, the cortex is removed from the embryonic (E18) rat brains. Hippocampi are separated from the cortex with dissection tools and collected in a sterilized petri dish. Subsequently, hippocampal tissues are immersed in HBSS (Hank's buffer salt solution) solution containing 20 unit/mL papain and the whole dish is placed in a CO_2 incubator. After 15 min, the tissues are mechanically dissociated with a sterile pasture pipette. Finally, neuronal cells are plated on to the pretreated substrates with a cell density ~100 cells/mm.[2] The viability of neuronal culture is examined by selectively staining live cells with fluorescein diacetate (FDA, SigmaAldrich) and dead cells with propidium iodide (PI, SigmaAldrich). The fluorescene images were taken by inverted fluorescent microscope (LEICA DM IRB, Leica) and analyzed with Image J software. All animal procedures were approved by the GIST Institutional Animal Care and Use Committee (GIST-2012-11).

Exfoliation Test of rGO Multilayer Film

We prepared two identical rGO films on quartz substrates after thermal annealing at 300, 600, and 1000 °C, respectively. Each rGO film was dipped in a phosphate buffered saline (PBS) solution and neurobasal media for 3 and 7 days and the film delamination was monitored by tracking the optical absorbance change before and after dipping in each solution using a Perkin Elmer Lambda 750 UV-Vis-NIR spectrometer.

RESULTS

Properties of rGO Multilayer Films

High-temperature thermal reduction of GO was previously reported by Ruoff group as a means to enhance the conductivity of the GO films.[21] In this study, GO multilayer films treated under different temperatures are carefully characterized in terms of conductivity, surface morphology, and chemical functional groups. As shown in Figure 1, after thermal treatments under different temperatures, sheet resistance values were dramatically dropped, for example, to 10 kohm/sq after 1000 °C annealing. As expected, XPS analysis demonstrated that the high-temperature induced to remove both plain and edge functional groups such as epoxy, hydroxyl (286.6 eV), carbonyl (287.8 eV)[22] and carboxylic acid groups (288.9 eV).[23] Moreover, as functional groups of GO multilayer films disappear after the heat treatment, surface morphology of rGO films becomes smooth with a significantly reduced roughness as shown in the AFM images.

Stability of rGO in PBS

The rGO substrates used in this work were prepared by layer-by-layer (LbL) deposition of oppositely charged GO based on the electrostatic interaction, followed by the thermal annealing process according to our previous reports.[24–27] It is known that the intermolecular interactions between rGO sheets are highly dependent on the surface functional groups as well as the interlayer distance. As such, the rGO nanosheets can be disassembled from the multilayer, which might cause the cellular uptake or increase the cytotoxicity. Therefore, to ensure biocompatibility of thermally-annealed rGO substrates, we first tested the stability of rGO film in a phosphate-buffered saline (PBS). Based on the characteristic UV/Vis absorbance of rGO at 268 nm, we could monitor the stability of the LbL-assembled rGO films. As shown in Figure 2(a), rGO substrates annealed at 300 °C were delaminated in PBS solution. It implies that rGO annealed at 300 °C are not sufficiently reduced and adhered to the substrate. Therefore, their interlayer spacing is still large and readily delaminated. However, we found that rGO film annealed at above 600 °C does not yield any measurable changes even after 7 days of incubation, suggesting significant integrity of rGO film and potentially minimal cytotoxicity caused by delaminated rGO flakes in a culture media.

Evaluation of Neuron Viability on rGO

With its high conductivity as well as excellent optical transparency, graphene is an ideal candidate for biointerface electrode materials for neuronal recording and stimulation. In order to take full advantage of such benefits, the neuronal culture viability is a most important issue. We employed a cell-viability test method based

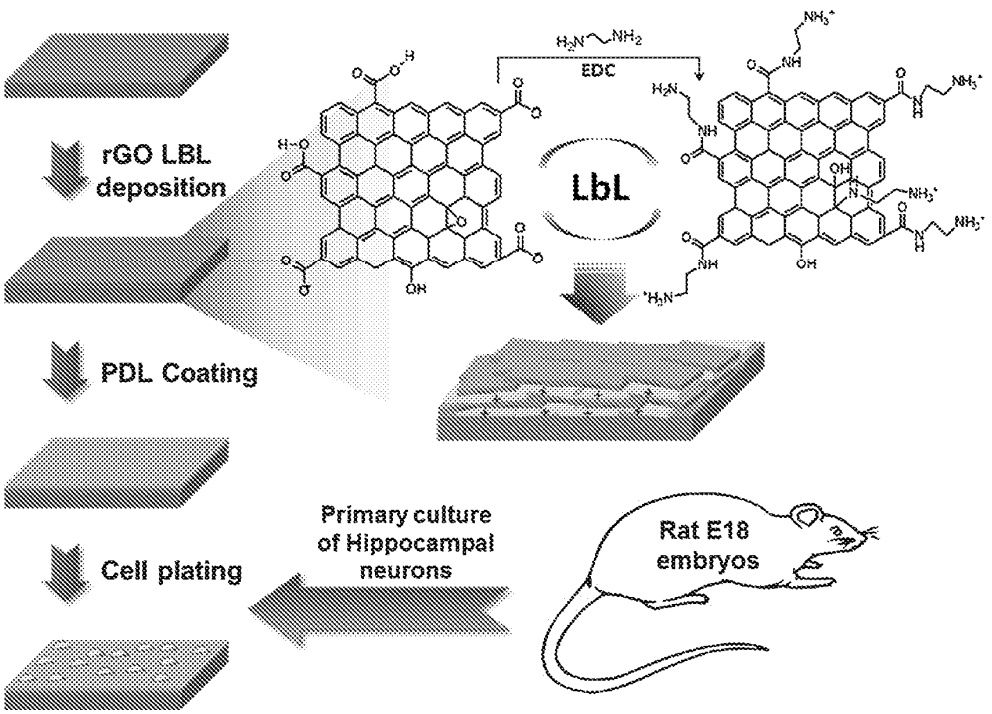

Figure 1. **Schematic diagram of substrates preparation and cell plating on rGO multilayer and glass substrates.**

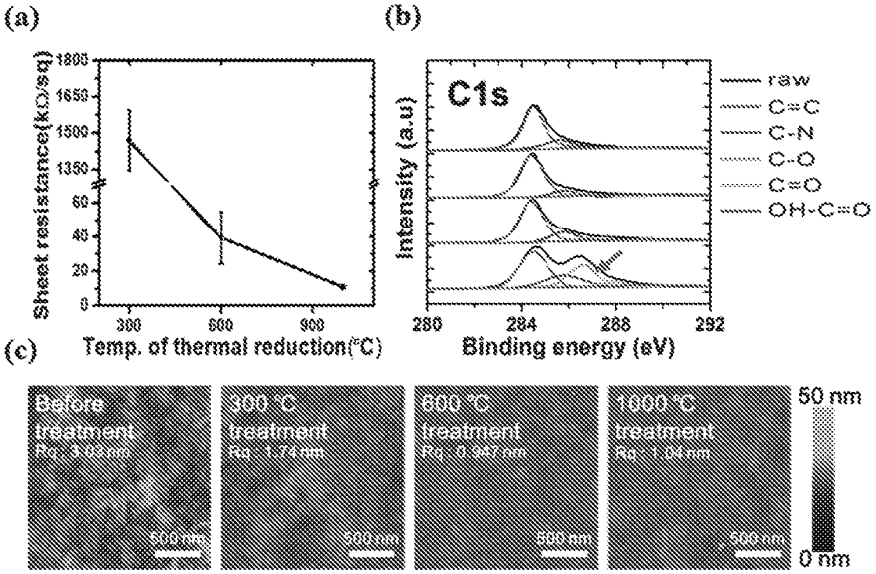

Figure 2. **rGO multilayer film characterizations. (a) sheet resistance, (b) XPS analysis, (c) AFM images of 10-bilayer rGO substrates with different thermal treatment temperatures.**

on fluorescein diacetate (FDA) and propidium iodide (PI) which selectively stain live and dead cells, respectively. The same density of rat hippocampal neurons were plated onto a glass and rGO substrate with PDL modification for cell attachment and stained with FDA and PI. As shown in Figure 3(a), the density of live cells with green fluorescence (FDA) in rGO substrate is almost the same as that in control plain glass substrate. This indicates that the viability of dissociated neuronal culture is minimally affected by rGO coatings. Furthermore,

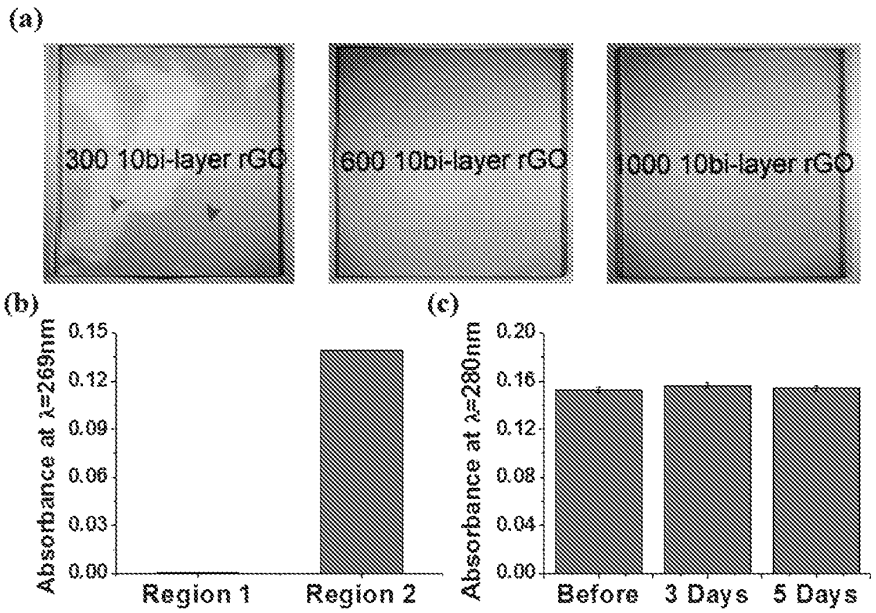

Figure 3. **Evaluation of rGO exfoliation ratio with varying dipping time. (a) Images of graphene substrates after dipping in PBS solution for 3 days. Red and blue arrows represent exfoliated and non-exfoliated regions, respectively. (b), (c) absorbance peak intensity graphs referring to rGO substrate annealed at (b) 300 °C at 3 days in PBS and (c) 1000 °C with varying dipping time in PBS. Region 1 and 2 are the parts indicated by red and blue arrows, respectively.**

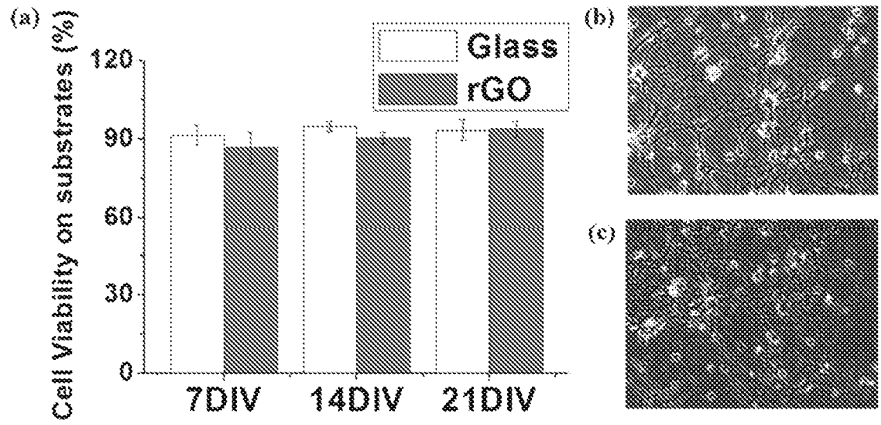

Figure 4. **(a) Viability of neurons on rGO substrates at 7 DIV, 14 DIV and 21 DIV. (b, c) phase contrast microscopy images of neurons on (b) rGO substrate and (c) glass slide at 30 DIV.**

the viability test implies that thermal annealing at sufficiently high temperature induces strong adhesion among rGO flakes and thereby minimized film delamination (Fig. 3). Indeed, dissociated neuronal culture is maintained over 30 days *in vitro* with marginal difference in cell morphology between control glass and rGO substrates (Figs. 4(b and c)).

CONCLUSION

In this report, we observed that solution-processed rGO substrates with enhanced conductivity and optical transparency support *in vitro* dissociative culture of rat hippocampal neurons over 30 days. First, we confirmed the reduction of graphene oxide films by XPS spectra and the improved electrical conductivity and optical transparency after thermal treatment. In addition, the integrity of rGO films is maintained in a neuronal culture environment after thermal annealing at high temperature and neuronal cells survive on rGO substrates over 30 days *in vitro*, which proves the potentials for further sophisticated applications such as *in vitro* neuronal interfaces and long-term implanted stimulation electrodes. We anticipate that micro-patterned rGO-based electrodes will serve as excellent multi-functional platforms for the fundamental neuroscience studies and biomedical applications when this is combined with appropriate biochemical/neuropharmacological modifications.

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