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Neurite Guidance on Laser-Scribed Reduced Graphene Oxide

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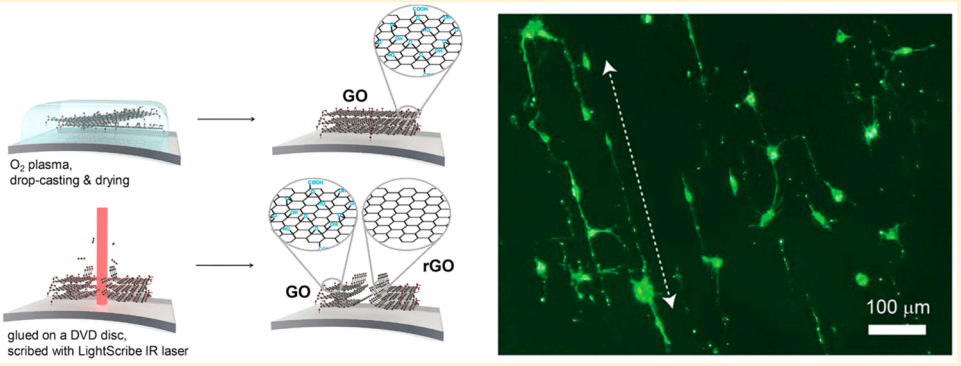
Supporting Information



ABSTRACT: This paper describes a one-step, chemical-free method to generate micropatterned in vitro neuronal networks on chemically unmodified reduced graphene oxide. The suggested method relies on infrared-based photothermal reduction of graphene oxide, which concurrently leads to the formation of submicrometer-scale surface roughness that promotes neuronal adhesion and guides neurite outgrowth. A commercially available laser source (LightScribe DVD drive) controlled by a computer software can be used to reduce graphene oxide (GO), and its repetitive scribing to a GO film brings about gradual increase and decrease in electrical conductivity and neurite guiding ability of the scribed regions, respectively. Our results also indicate that the observed adhesion-promoting and neurite guiding effect originate from the contrast in surface nanotopography, but not that in conductivity. This method is readily applicable to diverse graphene-based biomedical devices.

KEYWORDS: *reduced graphene oxide, neurite guidance, nanobio interfaces, conductive materials, neuron chips*

Graphene-derived materials (graphene, graphene oxide (GO), reduced graphene oxide (rGO), and their other chemical/physical derivatives) are attracting consistent interest from biomedical areas, as they have numerous properties adequate for being interfaced with biological systems.[1−3] Graphene—a molecular layer of sp^2-bonded carbons—exhibits exceptional electric conductivity along with thermal/mechanical stability and nonmetallic traits, such as flexibility and optical transparency, and it thus provides new ways to measure/provide signals to/from electrogenic biosystems. On the other hand, GO—an oxidized form of graphene—has different but yet more useful attributes for biomedical applications, including advanced water solubility, ease of chemical functionalization, massive synthesis, and many interesting biological properties including biocompatibility, antibacterial property, and influences on stem cell differentiation.[4,5] Since their discovery, graphene materials have been utilized as a carrier for delivering drugs,[6−8] a platform for biosensors,[9−12] tissue engineering,[13,14] and stem cell engineering.[15−18] The majority of these examples, however, have utilized graphene-derived materials as either a simple conductive matter (when used as graphene/rGO) or a biofunctionalizable substrate (when used as GO), as the advantageous properties of GO and graphene could not withstand simultaneously.

Graphene materials, considering the aforementioned advantages, are seemingly an attractive candidate to be applied for neurons. Many previous works showed the potential of graphene materials as neurofunctional substrates[15,19−21] or electrodes for being interfaced with nerve cells.[22−25] Bioelectric devices designed for interacting with living neurons often require sophisticated electric circuits with multiple bioactive surfaces assembled into delicate micro/nanopatterns.[26,27] This is particularly the case when dealing with neuronal networks, as microscale geometry of a neural network reflects its functions, and thus, it should be examined by measuring electrical signals from—or providing perturbations to—a selected set of cells.[28] However, there are critical issues that need to be addressed for graphene materials to be used for such purposes: (i) ways to directly functionalize pristine graphene, while keeping its electric conductivity within a reasonable range, are largely restricted.[29,30] Chemical functionalization of pristine graphene is inevitable for biological applications, since it is intrinsically inert for cell or tissues and is prone to aggregation in aqueous solutions,

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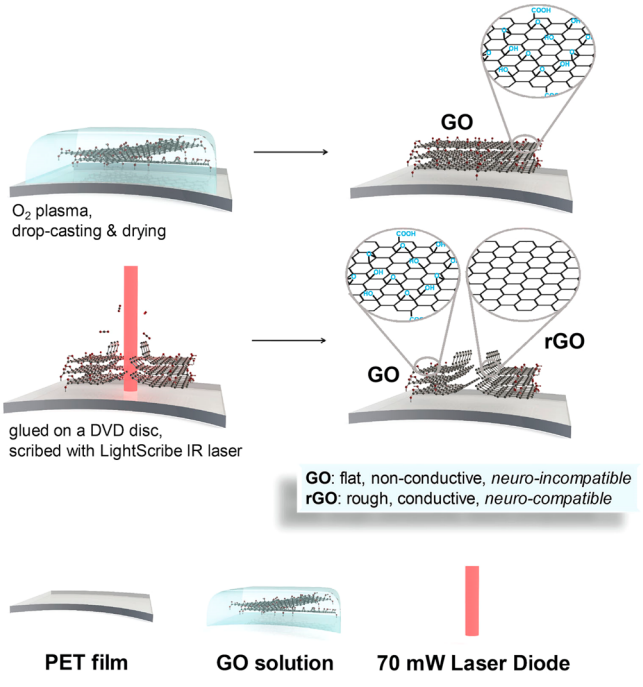
Nano Lett. XXXX, XXX, XXX−XXX

complicating its handling in many biologically relevant conditions. (ii) GO, on the other hand, has functional groups more susceptible to chemical derivatization than a simple C═C bond, but at the same time is lack of conductivity. These intrinsic limitations have hampered widespread applications of graphene materials to neuron-based chips or neural prosthetics, and their usage has been limited to supportive roles in composites with other materials. One way to surmount such limitations—and to expand the applicability of graphene materials to electrogenic biosystems—is to arrange GO and graphene in a single, carefully designed device so that one can benefit from the materials properties of both.

Here we describe a facile method to generate micro- or macropatterns of reduced graphene oxide (rGO) on a GO film, which can promote the adhesion and survival of rat primary neurons and, at the same time, guide the subsequent elongation of neurites without any chemical functionalizations. The suggested method uses IR laser-scribing that leads to the partial reduction of GO in the irradiated area, leaving rGO micropatterns having enhanced conductivity and microscale roughness, which simultaneously play a role as a physical guidance cue for neurons to attach/develop faster. It uses a commercially available IR laser source (LightScribe DVD drive),[31−33] which ensures ease of process and large flexibility in design. We show that the electrical and morphological properties of laser-scribed rGO (LGO) are controllable by varying the number of repetitive scribing procedures. O_2-plasma treatment of the LGO substrates allowed us to conclude that the major factor that contributed to the observed physical guidance of neurite outgrowth is the surface topography, rather than the electrical conductivity, of the LGO surface.

Scheme 1 depicts the procedure for the generation of LGO patterns. Reduction of GO by laser-scribing, first developed by

Scheme 1. Schematic Illustration of the Formation of LGO Micropatterns



Kaner et al., has proven powerful in the design of all-carbon electric circuits composed of GO (insulative) and rGO (partially conductive) surfaces. It relies on properties of carbon materials that convert IR absorption into heat, resulting in photothermal reduction of GO, and has been applied to supercapacitors, electrocatalysts, electronic devices and sensors, but not to biomedical devices.[31,34−37] A single laser-scribing process does not completely reduce GO, and thus repetitive scribing alters the electrical properties of the target GO substrate incrementally. In consistent with the previous reports, our repeated laser-scribing processes lead to visible color-change accompanied by formation of microscale roughness (Figure 1a), and a scanning electron microscopy (SEM) analysis ensures that the topography is uniform and reproducible throughout the entire scribed area. Figure 1b shows magnified views of scribed areas, which exhibit heterogeneous roughness at scales of 1−10 μm. Many types of surface topography at this scale have been reported to influence cellular behaviors in various ways. The C 1s X-ray photoelectron spectra of GO and LGO show clear differences in the ratio between C−C/C═C and O−C═O/C═O/C−O components, confirming that the laser-scribing effectively reduces GO to rGO (Figure 1c). Figure S1 shows that repetitive scribing does improve the electrical conductivity of the LGO: ten scribing steps can increase the linear conductivity of 100-μm line up to 350 μS cm^{-1}. The measured conductance of LGO, lower than what was reported by Kaner et al., lies within a range of semiconductor, but it is applicable for measuring bioelectric signals. Possible reasons of such difference would include the properties of the initial GO films used and the performance of the IR-laser diodes in the DVD drive.

With LGOs fabricated and characterized, we first set out to examine the adhesion and survival of neurons on GO and LGO substrates. Despite many examples that demonstrated biocompatibility of GO-coated films for mammalian cells,[38] primary hippocampal neurons rarely adhere and survive on GO substrates (Figure 2a). This is somewhat predictable, given that primary neurons are known to be more sensitive to changes in surface chemical properties (charge density, in particular),[39] and the negative surface charge of GO may affect adversely the adhesion and development of neurons. Laser-scribing processes, on the other hand, dramatically enhances the neurocompatibility of the substrate. Figure 2b shows neurons grown on a linearly scribed (>10 times) LGO substrate (with line patterns roughly 15 μm in width with 8 μm-spacing). Neurons on LGO adhere and develop normally, and have multiple neurites which can be visually categorized into putative axons and dendrites. The viability of neurons at 8 days in vitro (DIV) cultured on LGO is 60 ± 10% in respect to a control, whereas only 11 ± 12% of those are alive on GO (Figure 2c). The relative viability on LGO may seem low compared to other neurocompatible substrates, but it should be noted that a significant part of the LGO substrate is not laser-scribed, and thus still remained as GO. More remarkable is that the topographical patterns generated by anisotropic laser-scribing can effectively guide neurite elongation, as shown in Figure 2b (fluorescence image) and d (SEM image). SEM analyses show that somas tend to attach on scribed regions, making discontinuous and irregular contact with surface (Figure 2d, inset). We also observed that majority of neurites are confined within grooves generated by the laser scribing (Figure 2e). We and others previously shown that surface roughness of submicrometer-scale (>200 nm) elicits accelerated[40−42]—in one case, completely distinct[43]—development of primary hippocampal neurons, by mechanotransductive mechanisms (one or many) that rely strongly on cytoskeletal

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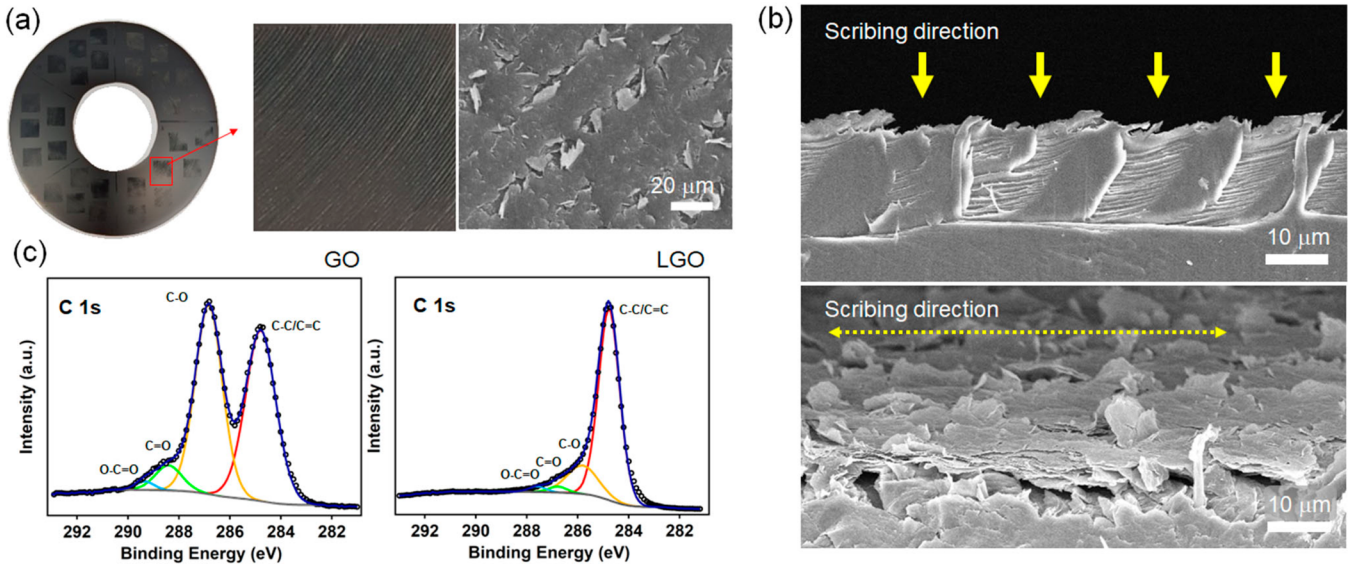

Figure 1. (a) Low- and high-magnification photos (left) and a SEM image (right) of LGO attached onto a PET film. (b) Magnified SEM side-view images of scribed regions of an LGO substrate. (c) X-ray photoelectron spectra (C 1S) of a GO film (left) and a LGO film (right). The peaks were deconvoluted with respect to the characteristic peaks of carbon-containing functional groups.

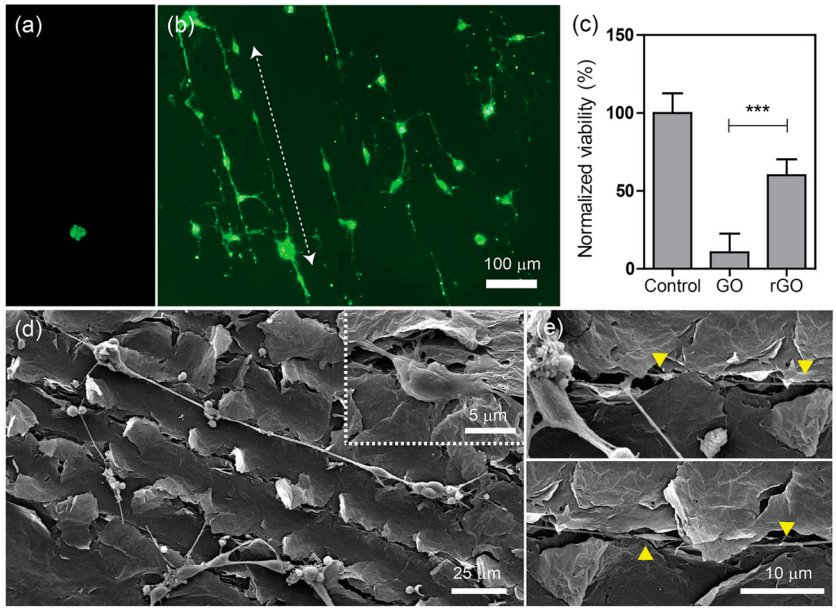

Figure 2. Survival of neurons on GO and LGO and axon guidance on LGO micropatterns. Fluorescence micrographs of hippocampal neurons (stained with FDA) cultured on (a) GO and (b) LGO at 8 DIV. The double-headed arrow indicates the direction of laser-scribing. (c) Relative viability of hippocampal neurons cultured on control (PDL-coated coverslip), GO, and LGO. The values are normalized to those of the control, and compared with control by one-way ANOVA. (d) A representative SEM image of a neuron at 5 DIV cultured on LGO. The inset image shows a soma attached to a scribed region. (e) SEM images showing that neurites passing in between grooves of LGO. The arrowheads indicate neurites.

dynamics (F-actin, in particular). The present results indicate that the roughness generated during by the laser scribing falls into the "active" topographical range previously shown to promote the adhesion and development of primary neurons.[40,41] This provides a waiver of using a substrate patterned with different molecules, which would generally require lithographic techniques.[44−47]

We also tried to generate large-area micropatterns of rGO based on light flash, which was previously reported to reduce GO in a similar manner to laser. Exposure of a GO film to a light flash results in microscale surface topography of similar roughness to that generated by laser, but it is isotropic, as expected (Figure S2a). Such a difference, however, brings about a huge difference in long-term viability and neuritogenesis of neurons. Initially, neurons adhere normally on flash-exposed GO film (Figure S2b), although no observable neurites exist. The absence of neurites resultantly causes neruonal death and leaves no viable neurons at 8 DIV (Figure S2c). The result implies that, at least in our case, anisotropicity of the surface topography is crucial not only for the guidance but also for the formation of neurites, which is indispensible for survival of neurons.

Although multiple laser scribing can improve the electric conductivity of the resultant LGO, it also generates microscale variances in surface topography, and, thus, may affect behaviors

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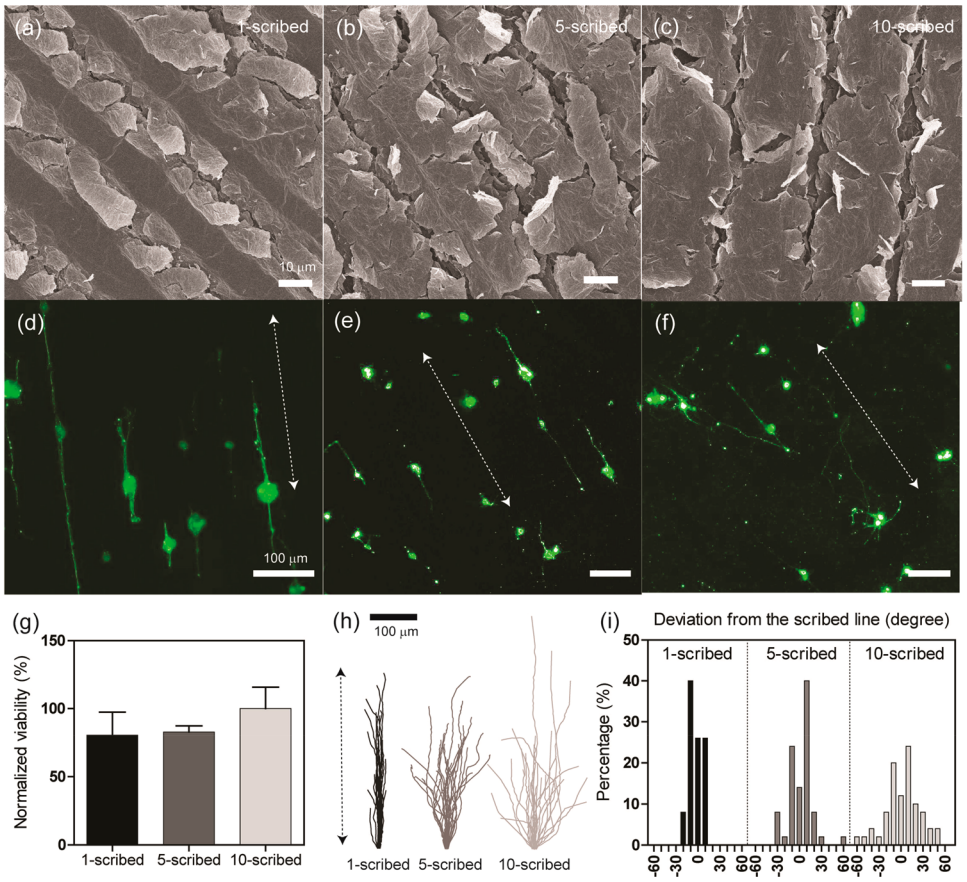

Figure 3. Influences of repeated scribing on axon guidance and neuronal viability. SEM images of (a) 1-scribed, (b) 5-scribed, and (c)10-scribed LGO. (d–f) Hippocampal neurons at 7 DIV, cultured on each substrate, respectively. The double-headed arrows indicate the direction of laser-scribing. (g) Viability of hippocampal neurons cultured on the substrates. The values are normalized to those of 10-scribed samples, and compared with control by one-way ANOVA (n.s.). (h) Axons or major neurites tracings on the substrates, aligned in respect to the scribing direction and the location of the somas. (i) Angle of deviation of tips of neurite tracings on the three substrates with respect to the scribed line.

of the neurons on top. To this end, we sought to investigate the influences of multiple scribing on the observed neurite-guiding ability of the LGO substrates. One, five, or ten repetitive scribing steps, while keeping scribed regions unchanged, were conducted on GO substrates, which caused gradual changes in surface morphology. Figure 3a–c shows broadening of the rough areas and increase in their roughness according to additional scribing steps, all of which can accommodate neurons (Figure 3d–f). Trends in such morphological changes correlate with those in the electrical conductivity of the substrates. In terms of neuro-compatibility, additional scribing steps do not alter cellular adhesion and survival significantly (Figure 3g), but do show negative influences on the neurite guiding ability. Neurites are clearly better aligned to the scribing direction on 1-scribed LGO substrates, compared to those on 5- or 10-scribed LGO, as shown by manually aligned tracings of neurites (Figure 3h). Such a tendency was more apparent when the tracings were quantified by measuring angles of deviation from the scribed line for each tip of neurites. More than 90% of neurites on 1-scribed LGO stay within ±10 degrees from the scribed line, but 78 and 56% of those do so on 5-scribed, and 10-scribed LGO, respectively (Figure 3i). We also proved that more complicated geometries than linear patterns can be used to guide neurites, by conducting two single scribing steps perpendicular to each other, as shown by neurites guided on grid-patterned LGO (Figure S3). We note: controlling neurite outgrowth with a nonlinear geometry is not as effective as is doing so linearly, because the LightScribe device used in this study can only work linearly, thus one needs to conduct sequential linear scribing steps in order to obtain more complex patterns. Here, the latter scribing step may partially override the preexisting surface topography, attenuating influences of the former scribing step. Such a limitation, however, can be overcome easily by using a different way of laser scribing, depending on the desired geometry.

Ballerini et al. have performed a series of studies on altered electrophysiological properties of neurons on carbon nanotube-coated substrates.[48−50] In their cases, the observed changes were attributed not to the topography of the substrates, but to their conductivity. In order to better understand the origin of its adhesion-promoting and neurite-guiding ability, we sought to ask whether the surface topography is the most deterministic factor among others (e.g., conductivity and chemical structure) in the neurocompatibility and neurite guidance ability of LGO substrates. To regulate substrate conductivity, we treated line-patterned LGO substrates with O_2 plasma. This process oxidized the entire surface, and thus converted the entire region back to GO, while retaining the surface topography (Figure 4a). Neurons cultured on 10-scribed, O_2 plasma-treated LGO substrates show similar growth with those without O_2 plasma (Figure 4b). Quantitative analyses of viability (Figure 4c) and neurite guidance (Figure 4d) also indicate that O_2 plasma do not impede neurite guiding, and adhesion/survival of neurons significantly, implying that surface nanotopography, but not contrasts in conductivity or surface charge, is the major factor that influences

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Nano Lett. XXXX, XXX, XXX−XXX

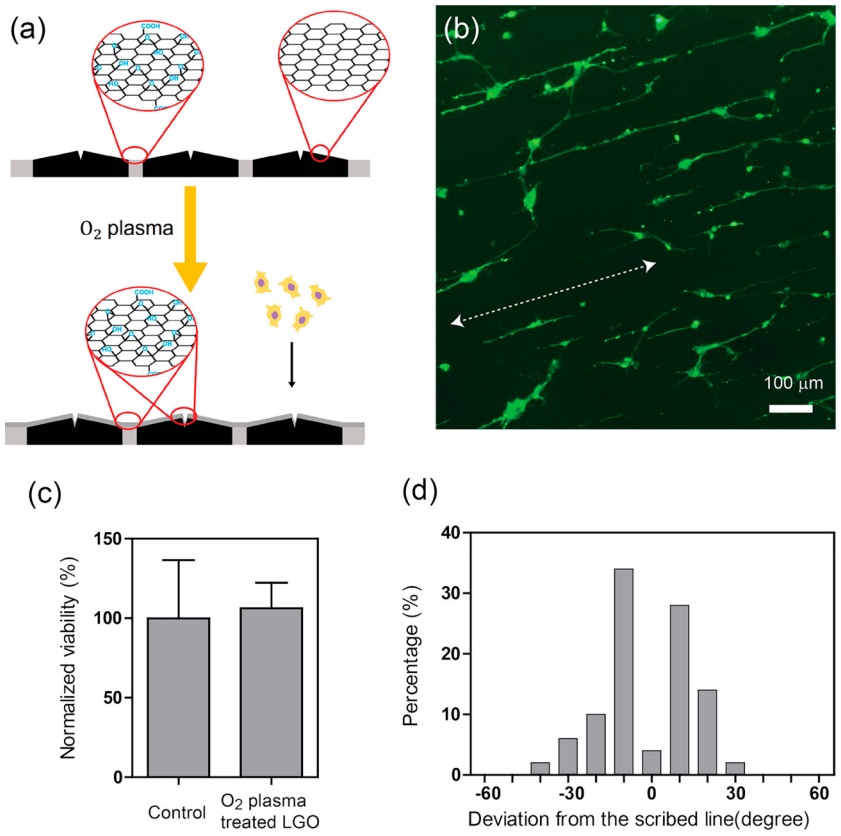

Figure 4. (a) Schematic illustration of O_2 plasma-treatment of LGO. (b) Hippocampal neurons cultured on O_2 plasma-treated LGO at 7 DIV. The double-headed arrow indicates the direction of laser-scribing. (c) Relative viability of hippocampal neurons cultured on control (PDL-coated coverslip) and O_2 plasma-treated LGO. The values are normalized to those of the control, and compared by one-way ANOVA (n.s.). (d) Angle of deviation of tips of neurite tracings on 10-scribed substrates with respect to the scribed line.

neuronal adhesion and neurite guidance. This result—neurite guidance is determined dominantly by surface topography over the chemical properties of the surface—provides flexibility of chemical functionalization when functions other than guidance is additionally required.

Numerous recent evidence suggests that nanotopography alone (i.e., without chemical treatment) can make the surface functional for adhesion and survival of neurons and neuronal differentiation of various stem cell lines.[51−55] There have been, however, relatively few examples that directly utilized carefully designed nano- or microrough surfaces for regulating cellular behaviors in neuro-electric devices. This is presumably because (i) the relationship between surface topography and neuronal behaviors and functions is not fully understood yet, and (ii) the method for fabricating nanostructured surfaces used in the previous studies were not compatible with materials used for current bioelectronics devices.

This paper, on the other hand, demonstrates a practical example of projecting recent findings regarding neuronal behaviors regulated by surface topography to designing bioelectric devices for neuron. The laser-based reducing of GO film is emerging as a simple and versatile tool for graphene-based electronics, but its extension to biological devices—for bioelectrogenic systems, in particular—has not been pursued yet. In addition, similar topographical derivatizations can readily be conducted to other types of reducible materials, because the observed cellular behaviors are irrespective of material.

The suggested method have many advantages to be readily applied in graphene-based biomedical devices: (i) the substrate contains both conductive rGO regions and biocompatible and functionalizable GO region, taking advantage of well-established useful material properties of the both. (ii) It does not require usage of any chemical modification, so it can be combined with conventional methods of chemical functionalization orthogonally. (iii) It ensures an extremely large degree of freedom in the design of a device at microscale, since the fabrication does not require to use lithographic techniques but simply a computer software. The ease of design is particularly advantageous when applied to neuroelectric devices, in which conventional cell-patterning methods suffer from high spatial complexity required to investigate in vivo neural networks.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.8b01651.

Experimental procedure and electrical characterization of LGO (PDF)

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Nano Lett. XXXX, XXX, XXX−XXX

Author Contributions

K.K. and M.H.L. conceived and designed the experiments. H.B.L. and Y.K. performed the biological experiments. S.H.L. and J.R.J. fabricated and characterized the substrates. K.K. and M.H.L. cowrote the paper.

Author Contributions

†S.H.L., H.B.L., and Y.K. contributed equally.

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Notes

The authors declare no competing financial interest.

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