The control of neural cell-to-cell interactions through non-contact electrical field stimulation using graphene electrodes

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Electric field stimulation has become one of the most promising therapies for a variety of neurological diseases. However, the safety and effectiveness of the stimulator are critical in determining the outcome. Because there are few safe and effective in vivo and/or in vitro stimulator devices, we demonstrate a method that allows for non-contact electric field stimulation with a specific strength that is able to control cell-to-cell interaction in vitro. Graphene, a form of graphite, and polyethylene terephthalate (PET) was used to create a non-cytotoxic in vitro graphene/PET film stimulator. A transient non-contact electric field was produced by charge-balanced biphasic stimuli through the graphene/PET film electrodes and applied to cultured neural cells. We found that weak electric field stimulation (pulse duration of 10 s) as low as 4.5 mV/mm for 32 min was particularly effective in shaping cell-to-cell interaction. Under weak electric field stimulation, we observed a significant increase in the number of cells forming new cell-to-cell couplings and in the number of cells strengthening existing cell-to-cell couplings. The underlying mechanism of the altered cellular interactions may be related to an altered regulation of the endogenous cytoskeletal proteins fibronectin, actin, and vinculin. In conclusion, this technique may open a new therapeutic approach for augmenting cell-to-cell coupling in cell transplantation therapy in the central nervous system.

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1. Introduction

Neurons conduct incoming or outgoing signals through synaptic coupling and/or gap junctions (i.e., electrical coupling). Both synaptic coupling and electrical coupling play a critical role in shaping neuronal function but these connections can be transient [1–4]. To make the coupling stronger, various attempts have been made to find novel ways to facilitate coupling [5]. In particular, aiding contact between cells is a crucial beginning step of neural cell coupling.

It has been reported that various types of electrical stimulation can regulate cell physiological activities such as division, migration, differentiation and cell death [6–9]. Because of its noninvasiveness, electrical stimulation has been used in promoting healing for spinal cord repair and cancer therapy [10–13]. Moreover, direct current (DC) injection methods can dramatically induce a neural cell to align perpendicular to the direction of the applied electrical field. DC stimulation also induces axonal outgrowth toward the cathode with axons aligning with the direction of current flow [14]. Most of these cellular changes, however, occur by chronic exposure to DC electric field and may result in potential cellular damage to the underlying cells or tissue [15,16]. Therefore, in order to minimize cellular damage following electrical stimulation, it is necessary to develop an effective electrical stimulator that has a non-cytotoxic substrate that can serve as a stable interface between the stimulator and the cells [17,18].

Recently, nanocarbon materials such as carbon nanotubes and graphene have been considered to be new effective electrode materials with high conductivity. Graphene, a two-dimensional (2D) form of graphite, has high transmittance and excellent conductivity [19]. Recently developed large-area graphene has been applied to flexible thin film transistors [20] and touch panel electrodes [21–23]. In addition, polyethylene terephthalate (PET) is a well-known transparent polymer that is a non-cytotoxic material used in catheters for medical surgery [24].
Fig. 1. Schematic illustrating the morphological features of the graphene electric field stimulator and electric field stimulation protocol. A. (a) Side view and (b) top view of the PET/graphene film stimulator. The electrical field forms between two graphene electrodes. Neural cells located between two electrodes were observed by live optical microscopic imaging. B. (a) Optical microscopic images showing that the two graphene electrode edges were separated by a 2 mm gap. Neural cells were placed between graphene electrodes. (b) TEM images depicting cross-section view of 6 layers of the graphene stimulator. Total thickness of six layers is 2.3 nm. (c) AFM images of the graphene surface. C. Schematic illustrating the experimental stimulation and imaging protocol. A train of balanced biphasic stimulus charges of ±9 mV voltage was applied to the stimulator.
The purpose of this paper is twofold: i) to design a new flexible, transparent, and non-cytotoxic graphene/PET film stimulator in vitro and ii) to use this stimulator for non-contact electrical field stimulation and examine its effect on cell-to-cell coupling. This study will enable us to better observe cell-to-cell coupling in vitro in a weak electric field attributed to the high field enhancement factor of a thin graphene layer [25]. We will also focus on how the weak electric field stimulation changes endogenous cytoskeleton proteins, i.e., related with neuronal cell mobility.

2. Materials and methods

2.1. Design of the in vitro graphene electrical stimulator

The graphene/PET film electrical stimulator was fabricated in order to stimulate neural cells in a cultured dish. Large-area graphene was synthesized by chemical vapor deposition (CVD). A few layers of graphene (thickness: 0.5–5 nm) electrodes were grown on Ni film (Sigma-Aldrich, St. Louis, MO, USA) with CH4 as a carbon source at 880 °C. The graphene/Ni layer was coated by polymethyl-methacrylate (PMMA, MicroChem Corporation, Newton, MA, USA) as a supporting layer and dipped in Ni etchant (type CE-100, Transene Company Inc., Danvers, MA, USA) in order to remove the Ni from the graphene-PMMA layer. After the removal of Ni, deionized water (Sigma-Aldrich, St. Louis, MO, USA) was flushed onto the graphene until the residual etchant was cleaned off.

Two graphene/PMMA pieces were transferred onto PET film and separated by 2 mm. This graphene/PET film was air-dried and the PMMA was removed by acetone (Fig. 1A). To connect the graphene and the external Au electric wire, a Cr/Au layer was deposited at the edge of the graphene. For this process, an electron beam evaporator was used to deposit 5 nm of Cr as an adhesion layer followed by the deposition of a 100 nm Au layer. Finally, the graphene/PET film was assembled in a 35 mm culture dish. The 2 electrodes were separated by a gap of 2 mm and connected to an Au wire which connects to a power generator for stimulation (Fig. 1A(a, b)). The cells were placed onto the PET film surface directly over the middle of the 2 mm gap space between the graphene electrodes. Therefore, the neuronal cells remained intact during the experimental stimulation session. The neural cells were maintained in a CO2 incubator.

2.2. Cell culture

SHSY5Y human neuroblastoma cells (ATCC, Manassas VA, USA) were adhesive neural cell line and grown in DMEM (Gibco BRL, NY, USA) with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis MO, USA) in a T75 Flask (Techno Plastic Products AG, Switzerland). Cells were subcultured every 3 days by...
resuspending in 1X cell dissociation solution (Sigma-Aldrich, St. Louis MO, USA) and washing with 1X phosphate-buffered saline (PBS; Gibco BRL, NY, USA) for the experimental session. A density of $3 \times 10^5$ cells was plated onto the graphene/PET substrate in a 30 mm stimulating culture dish and maintained in a 5% CO2 incubator (Sanyo, Japan) for 16 h prior to stimulation in order to allow cell attachment and growth.

2.3. Electric field stimulation and optical microscopic imaging

The neural cells and the graphene stimulator were placed on the optical microscope stage. Charge-balanced biphasic pulse trains with a frequency of 1 Hz frequency were repeatedly delivered onto the bipolar graphene/PET stimulator in order to generate electric fields. The electrical pulse was generated using an eight-channel programmable stimulator (Master-8, A.M.P.I., Israel) and two stimulus isolators (ISO-Flex, A.M.P.I., Israel). The pulse train duration of 10 s (1 Hz) with an interval of 10 s was delivered for 32 min (Fig. 1C). The electric field strength varied from ±4.5 mV/mm to ±450 mV/mm. In order to study the effect of stimulation on the cells’ morphological features, the live cell images were obtained before and after each electrical pulse train, or every 50 s, by a CCD camera (Leica, Germany) attached to a fluorescent microscope (DMI6000B, Leica, Germany). A total of 40 frames of live images was obtained and analyzed. The stimulated cells were then processed for immunocytochemistry or RT-PCR analysis.

2.4. Data analysis and statistics

The images from the live video file were examined and the area and length of each neuron were measured using AF6000 imaging software (Leica, Germany). After careful visual inspection, we categorized each cell’s behavior pattern of cell-to-cell interaction into four groups (Fig. 4A): cell wavering (CW), cell-to-cell decoupling (CD), cell-to-cell coupling (CC), or cell shrinking (CS). The CC group was further divided into two subcategories: newly formed cell-to-cell coupling (NCC) and strengthened cell-to-cell coupling (SCC). The NCC subcategory represents cell-to-cell coupling that is newly formed between two cells that were originally separated from each other while the SCC subcategory represents existing cell-to-cell couplings that became a more strengthened interactive coupling.

For data quantification, the number of cells in each category were counted and compared with a control group, the cells of which were placed on the graphene/PET film stimulator without electric field stimulation applied. In order to quantify the immunocytochemical data, the fluorescence intensity of each region of interest (ROI) was measured and normalized to the DAPI intensity of the same ROI. All numerical data are expressed as mean ± SEM (standard error of the mean). Statistical evaluation was performed using ANOVA (InStat, GraphPad Software Inc., La Jolla, CA, USA). Differences in intensity were considered to be statistically significant, when *p < 0.05, **p < 0.01, or ***p < 0.001.

2.5. Immunocytochemistry and fluorescence microscopy

After electric field stimulation, the neural cells on the culture dish were fixed for 20 min with 4% paraformaldehyde in PBS, pH 7.4 and rinsed three times with PBS. Cells were then incubated in PBS containing 4% normal goat serum (NGS; Vector laboratories, CA, USA), 0.2% Triton X-100, 2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis MO, USA), 2% PBS (Sigma-Aldrich, St. Louis MO, USA), and the appropriate primary antibodies for 60 min at room temperature (RT) or overnight at 4 °C. After washing with PBS, secondary Alexa fluor 568 goat anti-mouse IgG (H + L) and Alexa fluor 488 goat anti-rabbit IgG (H + L) were applied to the cells for 60 min at RT in the dark. Cells were then rinsed three times with PBS. For nuclear staining, DAPI (1:100 dilution) was added to the last PBS buffer wash and cells were incubated for 30 min at RT and then washed one more time before mounting. TMR Red solution (Roche, Germany) was used for in situ TUNEL staining in order to label apoptotic cells. Cells were then incubated in the CO2 incubator at 37 °C for 40 min. After final washing with PBS, cells were mounted with fluorescent mounting medium (DakoCytomation, CA, USA) and then covered with cover glass.
The primary antibodies used and their dilutions were as follows: Rabbit anti-Fibronectin (1:500, Millipore, USA); mouse monoclonal anti-F-actin (1:1000, Millipore, USA); Rabbit anti-vinculin (1:500, Millipore, USA). After immunostaining, dishes were examined with fluorescence microscopy (DM16000B, Leica, Germany).

2.6. RT-PCR analysis

Total RNA was purified from SHSY5Y human neuroblastoma (Rneasy Micro Kit, QiAGEN, AMBION, Inc., Texas, USA). For RT-PCR, RNA of SHSY5Y was reverse-transcribed with specific primers: F-Actin primers, ACC CAG AAG AAG CAG ATG GA (forward) and ATC TTA ACC AGG CCA ACC AC (reverse); these primers should produce a 256 base pair (bp) specific product; fibronectin primers, ACC CGC CAC GTG CCA GGA TTA C (forward) and CTG ATG GGG GTG GCC GTT GTG G (reverse), which should produce a 439 bp specific product; vinculin primers, ACC TGC AGA CAA AAA CCA AC (forward) and TIG CAG CTC AGA TCC TG (reverse), produced a 317 bp product and GAPDH used as a standard. One microliter of each reverse transcription reaction product was used in the PCR reactions (RT-Premix kit, Cosmo Genetech, S. Korea). The amplifications were performed with the following protocol: 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 65 °C for 30 s. The PCR products were subjected to electrophoresis on an agarose gel and obtained a band data.

2.7. TEM and AFM image

High-resolution transmission electron microscopy (HRTEM; JEM 2100F, JEOL, Japan) was used to investigate the number of layers and the morphology of the graphene sheets. The transmission electron microscopy (TEM) image was scanned at a magnification of 3830,000X with an exposure time of 1.0 s at 200 kV. For the TEM sample preparation, the synthesized graphene on Ni film was submerged in diluted nitric acid. The graphene layer was suspended in Etchant solution diluted by deionized water, which was used to etch away the Ni sub-layer completely. The graphene layer was suspended in Etchant solution diluted with a drive amplitude and microscopy (DFM; Japan) mode in order to study the surface morphology. Image data was acquired at scan rates between 0.5 and 1 Hz with a drive amplitude and contact force maintained around 7 V. The image seen in Fig. 1B(c) was analyzed (AFM) imaging under ambient conditions. Graphene was characterized using the biocompatibility of our developed graphene/PET film substrate was compared to a conventional culture dish substrate without any coating with substances. The stimulator developed herein did not affect cell viability (Fig. 2A) and therefore produced no differences in cell proliferation between the graphene/PET film substrate and the conventional culture dish substrate (Fig. 2B). These results showed that the PET/graphene film stimulator had no cytotoxicity to the SH-SY5Y cells. Also, because the hydrophilic nature of the PET film substrate enables extracellular matrix proteins such as laminin, fibronectin or gelatin to be easily coated onto the substrate [26], our PET/graphene film stimulator becomes a more useful experimental device for biological electric stimulation in vitro cellular research.

3.2. Neural cells’ morphological changes in relation to varying electric field strengths

Overall, the majority of neural cells underwent cellular morphological changes when electric field stimulation ranging from ±4.5 mV/mm to ±450 mV/mm was applied through the graphene/PET film stimulator (Fig. 3A). There was no effect of electric field strength on general cellular morphology (Supplementary Fig. 1). There was, however, a significant effect of electric field when we quantified the percentage of shrunk cells compared to the total number of morphologically changed cells (Fig. 3B, C, D). The largest percentage of shrunk cells was observed at the 450 mV/mm electric field while the smallest percentage of shrunk cells was observed at the 4.5 mV/mm electric field. Fig. 3A illustrates the pattern of neural cell behavioral changes at 4.5 mV/mm and 450 mV/mm electric fields, respectively. The majority of cells shrunk significantly when the electric field strength was raised up to 450 mV/mm (Fig. 3B). Thus, the strength of the electric field is critical in determining cell-to-cell interaction patterns in vitro.
3.3. The effect of transient electric field on cell-to-cell interactive behavioral changes

As we stated in the Materials and Methods section, the cell-to-cell interactive changes following electric field stimulation were visually inspected and categorized into 4 major categories: cell waviering (CW), cell-to-cell coupling (CC), cell-to-cell decoupling (CD), and cell shrinking (CS). In particular, CW, CC, and CD were used to categorize responsive cells which exhibited behavioral cell-to-cell interaction (Fig. 4A). The CC group was further classified into two groups: NCC (newly formed cell-cell coupling) and SCC (strengthening cell-cell coupling) group (Fig. 4A). The NCC group represented a group of cells that responded to the electric field stimulation by forming new contacts between cells. The SCC group represents a group of cells strengthening existing contact between cells after electric field stimulation.

The varying electric field strengths significantly affect the number of cells in each category (Fig. 4B). The lowest level of stimulation, 4.5 mV/mm, resulted in the highest percentage of cells categorized as CC compared to other levels of electric field stimulation and this result was highly significant compared to the control group (p < 0.001, **). This low voltage stimulation with high CC is a consequence of implementing thin graphene electrodes which provide high local field by the large field enhancement factor. To the contrary, the percentage of cells categorized as CD out of the total altered cells was the lowest percentage at the 4.5 mV/mm electric field and this result was also significantly different when compared to the control group (p < 0.001, **). While stimulation at 22.5 mV/mm induced a significant increase (p < 0.001, ***) in the percentage of CC cells, no other electric field levels resulted in a significant stimulation effect on the CC and CD groups.

When we further analyzed the pattern of cell-to-cell coupling behavior, there was a significant effect of electric field on the percentages of cells’ showing NCC and SCC (Fig. 4C). In particular, the rates of NCC and SCC were the highest at 4.5 mV/mm, which was highly significantly different compared to the control group (p < 0.001, ***). No other electric field levels induced significant changes on the percentages of NCC and SCC.

Fig. 4D illustrates the cellular morphological changes following low and high stimulation and shows examples of CC, CD and CW cells. The pattern of cellular changes in the control group with no electric field stimulation is also presented in Fig. 4D as a comparison. With no stimulation, the majority of cells are categorized into the CW group. Few NCC and SCC are noted with no stimulation. With weak biphasic electric field stimulation (4.5 mV/mm), the largest percentage of cells was categorized as CC with abundant observations of NCC and SCC cells. With high biphasic electric field stimulation (450 mV/mm), the majority of cells reacted and were categorized into the CD and CW groups.

Most previous in vitro studies have applied long-term unidirectional DC electrical stimulation in order to induce morphological changes within cells [6–8,10,11,14]. Also, cells under a DC-generated electric field become perpendicularly aligned in relation to the direct of current flow [27]. On the contrary, in this study, we generated a non-contact electric field stimulation with biphasic pulses at low frequency (1Hz). In addition, we also limited the stimulation period to only 32 min whereas previous studies sustained stimulation for several hours and up to several days. As a result, our stimulation induced changes in the cells’ responsive behavior which eventually leads to the increases in cell-to-cell interactions as opposed to changes in the cell’s alignment or migration. Therefore, a transient non-contact electric field stimulation is a particularly effective means of stimulation for shaping a cell’s protrusion. Protrusion plays an important role in guiding the directional position of a cell’s movement for cell-to-cell contact. Therefore, in our results, non-contact weak electric field stimulation facilitates cell-to-cell coupling without cellular death whereas high stimulation enhances cell-to-cell decoupling.

3.4. Effect of electric field stimulation on cytoskeleton proteins

We investigated the effects of the electrical field-induced cell-to-cell coupling behavior on gene and protein synthesis mechanisms by RT-PCR and immunocytochemistry. The human neuroblastoma cell line SHSY5Y, which has abundant adhesion proteins distributed through the cytoplasm, was used since it is an ideal model for studying cellular movement. Various molecules play a significant role in cell motility and cell-to-cell contact [28,29]. Actin is a primary cytoskeleton protein related to cellular mobility. Focal adhesions (FAs) and fibrillar adhesions (FBs) contribute to morphological adhesive changes in cell [28] and can be labeled by vinculin and fibronectin, respectively. The expression of the cytoskeleton gene filamentous actin (F-actin) was increased by the weak field stimulation (Fig. 5A). This implies that cell mobility was increased along with weak electric field influence. A slight decrease in vinculin gene expression indicates that FAs formed between cells and PET substrate were weakened due to the protrusion edge of contacting cell with adjacent cells. On the other hand, an increase in gene expression of fibronectin indicates an upregulation of FBs under the weak electric field influence. Fibronec tin’s alterations of intracellular distribution were confirmed by immunocytochemistry (Fig. 4B). The distribution of intracellular fibronectin proteins was changed from broader sites in the cytoplasm to perinuclear locations (c,d), resulting high concentration of fibronectin around the perinuclear site (e). The central localization of FBs (c,d) indicates that the cellular edge is more actively modified under the weak electric field influence than the central cellular part. Therefore, the weak electric field in this study affects the levels of the intracellular genes and proteins related to cell mobility and cell adhesion and

Fig. 4. Cell-to-cell interactive reactions to electric field stimulation. A. Schematic illustrations of cell-to-cell interactive reactions between two separated cells under electric field stimulation. (a) Cell-to-cell decoupling (CD). Cells belonging to the CD group separated from each other after stimulation. (b) Cell-to-cell coupling (CC). The CC group was further classified into two groups: The newly formed cell-cell coupling (NCC) group and the strengthened cell-cell coupling (SCC) group. The NCC group represents a group of cells that respond to electric field stimulation by forming new contacts between cells. The SCC group represents a group of cells strengthening existing contact between cells after electric field stimulation. C. Heo et al. / Biomaterials 32 (2011) 19–27 25

C. Heo et al. / Biomaterials 32 (2011) 19–27 25
The stimulated neuronal cell shows the higher contrast optical images of cells on the stimulator. (c & d) The corresponding RNA normalization. B. Immunocytochemical analysis of and vinculin levels decreased slightly. GAPDH was used as a volumetric standard for membrane protrusion without cytotoxicity. These results may yield effective applications in neural cell transplantation therapy. Cell therapy is being utilized to cure neuronal cell loss or neuronal functional loss [30]. However, a question of whether the newly injected neural cells are able to thereby enhance cell-to-cell contact beyond the level of normal cell membrane protrusion without cytotoxicity.

These results may yield effective applications in neural cell transplantation therapy. Cell therapy is being utilized to cure neuronal cell loss or neuronal functional loss [30]. However, a question of whether the newly injected neural cells are able to couple with existing host cells still remains [31]. This issue is particularly important in the central nervous system (CNS) consisting of the fine neural networks. The formation of new neural cell-to-cell coupling between injected cells and host cells is crucial in forming functional neuronal circuits and therefore, in the success of the transplantation.

4. Conclusion

We have developed a graphene/PET stimulator and non-contact electric field stimulation protocol that can enhance neural cell-to-cell interaction in vitro. A weak non-contact electric field promotes new cell-to-cell coupling and strengthening existing cell-to-cell coupling. The weak field operation with high field enhancement factor is another benefit of using graphene/PET stimulator in addition to high transmittance and flexibility. These cell-to-cell interactive changes were obtained via alterations in the regulation of protein synthesis involved in cell mobility in relation to the cytoskeleton. The findings from this study may aid in the development of a new therapeutic stimulator for neurological diseases and cell transplantation therapy in CNS.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2010.08.095.

Appendix

Figure with essential color discrimination. Figs. 1–5 in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2010.08.095.

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