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Electrical Stimulation of Neural Stem Cells Mediated by Humanized Carbon Nanotube Composite Made with Extracellular Matrix Protein

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ABSTRACT

One of the key challenges to engineering neural interfaces is to minimize their immune response toward implanted electrodes. One potential approach is to manufacture materials that bear greater structural resemblance to living tissues and by utilizing neural stem cells. The unique electrical and mechanical properties of carbon nanotubes make them excellent candidates for neural interfaces, but their adoption hinges on finding approaches for "humanizing" their composites. Here we demonstrated the fabrication of layer-by-layer assembled composites from single-walled carbon nanotubes (SWNTs) and laminin, which is an essential part of human extracellular matrix. Laminin-SWNT thin films were found to be conducive to neural stem cells (NSC) differentiation and suitable for their successful excitation. We observed extensive formation of functional neural network as indicated by the presence of synaptic connections. Calcium imaging of the NSCs revealed generation of action potentials upon the application of a lateral current through the SWNT substrate. These results indicate that the protein−SWNT composite can serve as materials foundation of neural electrodes with chemical structure better adapted with long-term integration with the neural tissue.

In recent years, carbon nanotubes have been widely explored for adoption in the biological realm in the form of biosensors and as agents of cellular transport and delivery.[1−4] Single-walled (SWNTs) and multiwalled carbon nanotubes (MWNTs) are also attractive candidates for developing interfaces with biological systems due to the promise of superior physical, electrical, mechanical, and optical properties in comparison with traditional materials.[5] This research direction is of particular relevance to the field of neural prosthetics field as strategies for the diagnosis, therapy, and treatment of neural disorders are increasingly relying on electrical stimulation techniques. However, these current techniques have several inherent problems that are associated with the traditional materials used for manufacturing of neural electrodes (NEs).[6,7]

The interactions between various cell lines and carbon nanotubes have been previously reported, including the adhesion, growth and differentiation of neuronal cells on carbon nanotube-based substrates.[1,3,4,8,9] Starting from 2006,[10,12] the ability of such substrates to electrically stimulate neuronal cells are being reported.[3,9−12] The strong interest in interfacing neuronal cells and carbon nanotubes is not accidental; the unique set of properties of carbon nanotubes make them an excellent candidate to address the acute need for better neural electrodes (NEs). The current problems facing neuroprosthetic devices include the following: (1) long-term inflammatory response of the neural tissues, which results in the neuron depletion around the electrodes and their replacement with reactive astrocytes preventing signal transduction, (2) delamination[6] and degradation[6,7] of thin metal electrodes off the plastic substrates that drastically decrease the stimulation abilities, (3) the need for significant miniaturization of the electrodes which is currently limited not by manufacturing abilities but by electrical properties of the cell-electrode interface, and (4) the need to achieve mechanical compliance with the neural tissues to attain adequate long-term performance, which cannot be addressed by semiconductor devices that can partially resolve some of the above-mentioned issues.[13−15]

In this work, we want to highlight three approaches to resolve these issues:

(1) Exploitation of the mechanical and electrical properties of nanostructured materials to develop new electrodes. Such materials, including carbon nanotubes composites would be able to trigger a drastic decrease of delamination, while meeting the necessary mechanical and miniaturization requirements for NEs.

(2) Incorporation of neural stem cells (NSCs) with the NE due to their ability to differentiate into functional neurons

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in damaged areas of the central nervous system.[16,17] This could result in significant reduction of inflammation of the neural tissue.

(3) Achieving better integration of tissues and NEs by "humanizing" the material, that is, making it structurally and chemically more similar to tissues surrounding neurons. To some degree, this approach can be compared to humanization of externally made antibodies used as drugs in order to reduce immune reaction of the body.

Detailed evaluation of all of these approaches will probably require several years, detailed animal studies, and certainly cannot be done in one paper. Nevertheless, what we intend to do here is to demonstrate the technical feasibility of manufacturing electrodes that satisfy the requirements of these hypotheses so that their evaluation can be carried out. In particular, the focus will be placed on feasibility of developing CNT-based electrodes that incorporate extracellular matrix proteins, one of the key components of tissues surrounding neurons and stem cell compatible materials.

Results and Discussion. The development of these CNT-based electrodes for NEs hinges on the ability to process SWNTs or MWNTs in thin coatings or bulk composites. It is particularly important for the case of NSCs because some components of the composites and/or the nanoscale morphology of the interface can alter the differentiation pathway of the stem cells.[18,19] While there is a number of methods that can be used to yield carbon nanotubes film processing in substrates for neural growth, including simple solvent evaporation, polyelectrolyte layering, chemical vapor deposition, and electrochemical deposition,[20−29] not all of them may be suitable for NE purposes. In particular, many of these methods do not yield coatings that possess the mechanical stability and actual conductivity that are key for NE as discussed above. In many ways, the basic material of NE has to be engineered with great care to provide the required combination of properties. Besides electrical conductivity, mechanical characteristics are of great importance as well for NE because their optimization is critical to resolve the issues with long-term inflammation of neural tissues and formation of glial scar around implants. On the other hand, the technique of layer-by-layer assembly (LBL) provides a high level of control and tailoring with respect to the film composition, including the polymeric components used.[30] It also makes the coating exceptionally mechanically robust.[31] The ability of carbon nanotubes thin films, made by LBL to support growth, proliferation, and differentiation, to electrically stimulate adult neuronal cells of neuroblastoma NG108−15 and their ability to support the growth of NSCs in culture[32] was reported recently.[9,10] The LBL fabrication for the previous neuronal cell studies has however relied on the use of a synthetic nonbiological polymer,[9,10] which is one of the potential problems with this method. Along with other points addressing hypotheses 1 and 2, we demonstrate in this study that LBL films consisting of SWNTs and natural polymers such as laminin, serve as a biocompatible substrate for promoting adhesion and differentiation as well as for mediating electrical stimulation of neuronal cell lines. Overall, the interfacing of the biocompatible, electrically conductive and mechanically strong SWNT thin films with NSCs is likely to be of particular relevance for developing neural prostheses.

The favorable biological properties of carbon nanotubes that have been previously reported[9,23,24] provide an excellent foundation for exploitation of these properties in implantable medical devices. However, the critical issue of making tissue-friendly material still needs to be addressed for carbon nanotube-based NEs. One of the most logical ways to achieve this is to include molecules most familiar to the cells in the interfacial environment; such molecules are from extracellular matrix (ECM). While the incorporation of ECM proteins in carbon nanotubes composites made by many processing methods mentioned before, such as simple blending and solvent evaporation, might be faced with fast elution of the protein, the LBL technique can curtail this shortcoming. This technique, which is based on alternating the layers of nanotubes (or other nanoscale building blocks) and polymers, is versatile and can be easily adapted for the incorporation of biological components such as the ECM proteins. In this study, we explore and evaluate the performance of CNT-based NEs made by LBL method and that incorporate ECM proteins. The LBL films consisted from SWNTs (HIPCO, Tubes@Rice.com) wrapped with poly(styrene-4-sulfonate) (PSS, MW = 1 000 000, 1% in water, Sigma-Aldrich) and the glycoprotein laminin (MW ~ 900 kD, 100 μg/mL in PBS at pH 7.4). The latter is an important protein in the basement membrane that is widely used for coating substrate to promote cell adhesion and neurite outgrowth.[33] Dipping time of 30 min per single step of laminin and SWNT adsorption was used throughout the project.

Despite its large size, laminin (isoelectric point is pH 7) appeared to interact strongly with the negatively charged, piranha-treated substrate upon deposition of the first layer. Atomic force microscopy (AFM) image of a single layer of laminin on a silicon oxide substrate indicated a uniform coating of the protein (Figure 1a). AFM was also utilized to assess the subsequent interactions between the deposited laminin and the SWNT dispersions and confirmed deposition of SWNT onto the laminin layer (Figure 1b). This observation is consistent with reports of strong electrostatic and nonspecific adsorption of proteins to carbon nanotubes.[34,35] The film progress of multilayer depositions was monitored by UV−vis absorption, which indicated linear increase in SWNT concentration on the thin films with increasing number of bilayers (Figure 1c) as expected for the traditional mode of LBL growth observed before.

As the first step in examination of suitability of SWNT−laminin composites suitability for the use as NEs, LBL films of up to 30 bilayers were made and the ability of cells to adhere on the SWNT/laminin substrate was assessed (Table 1). The architectures of the LBL films tested, that is, number of deposition cycles, their sequence, and the composition of the final layer, were chosen based on several factors: the fact that cells typically adhere better to positively charged surfaces and that heat treatment enhances cross-linking among the layers within SWNT LBL films and causes an increase in electrical conductivity.[36,37] In particular, the

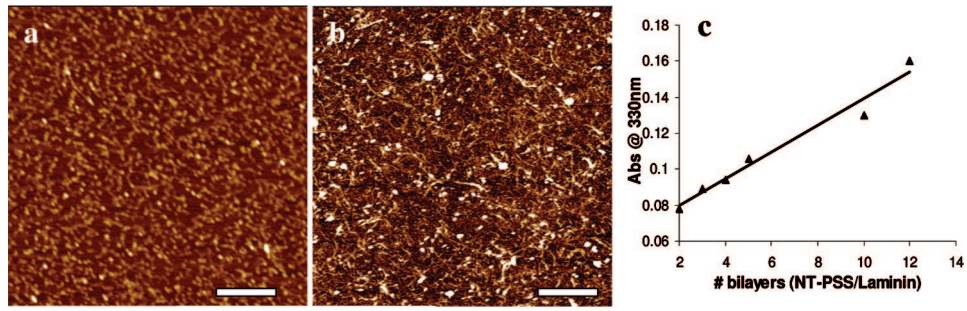

Figure 1. LBL fabrication. Atomic force microscopy image of (a) one monolayer of laminin on piranha-treated SiO_2 substrate and (b) six bilayers of SWNT/laminin on the same substrate. Scale bars are 1 μm. (c) Successful accumulation of laminin-SWNT layers on glass substrate monitored by UV−vis spectroscopy.

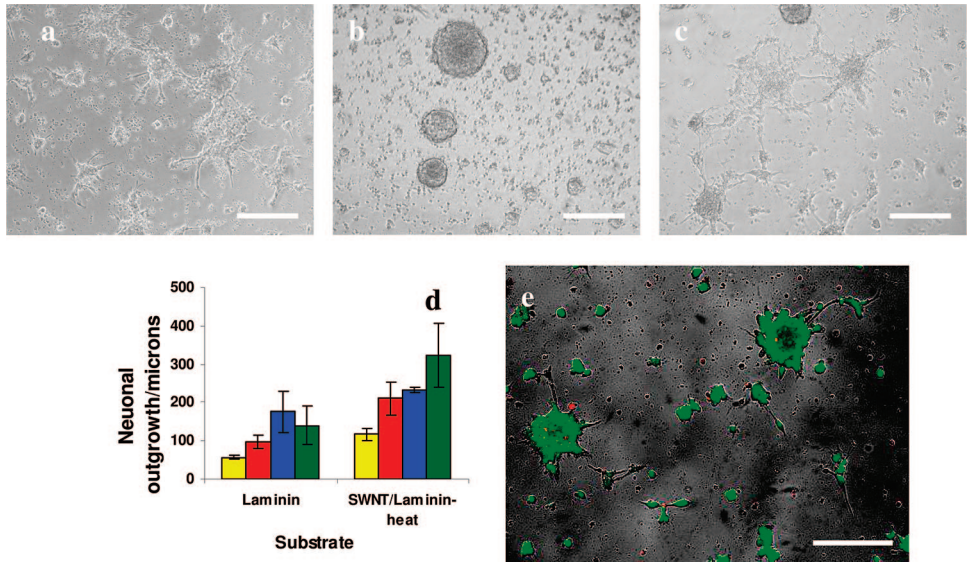

Figure 2. Micrograph assessing NSC cell adhesion and differentiation 72 h after initial seeding on (a) laminin-coated glass slides and on 10 bilayers SWNT/laminin thin films that were (b) used as is or (c) heated at 300 °C for 10 min. (d) Distance of outgrowth from neurospheres after 24 h (yellow), 48 h (red), 72 h (blue), and 120 h (green) on laminin-coated slides and heat-treated SWNT/laminin film on slide. (e) Live-dead viability assay on seeded cells where live cells are stained green and dead cells are red. Scale bars are 200 μm.

Table 1. Summary of Various LBL Configurations Investigated in Cell Seeding and Cell Adhesion Experiments

LBL film configuration (15 bilayers)	cell adhesion (day 1)	cell adhesion (day 7)
laminin as final layer	yes	no
PSS-wrapped SWNT as final layer	yes	no
laminin as final layer, annealed at 300 °C (10 min)	no	no
PSS-wrapped SWNT as final layer, annealed at 300 °C (10 min)	yes	yes

influence of the structure of the polymeric component(s) of the LBL film and the annealing step were investigated. We found that cell adhesion markedly depended on the composition of the final layer. The initial ability of cells to adhere to the substrate was assessed 24 h following seeding and at various time points leading to 7 days postseeding (Table 1). The substrate that was most conducive to cell adhesion and attachment was the LBL film that contained SWNT as the topmost layer and that was heat treated. Control experiments with laminin-coated slides (no SWNT) that were either used as made or following heat treatment were also carried out in parallel. Interestingly enough, not all "humanized" SWNT-protein composites revealed sufficient adhesion of the cells. As-made films did not reveal any cell adhesion after 7 days (Table 1). This is likely to be related with gel-like behavior of laminin. Swelling of its composites in water apparently reduced stiffness of the substrates and prevented cell adhesion. On the contrary, SWNT−laminin films obtained after annealing, that is, after cross-linking, exhibited sufficient stiffness[38] and served as excellent adhesion support for cells (Table 1). Note that SWNTs and other inorganic materials substantially increase thermal stability of proteins and other macromolecules and drastically reduce the decomposition of these materials at high temperature.[39,40] So, while altering the structure of the proteins, thermal annealing for a fairly short time used here does not certainly destroy laminin structure completely as one might expect from pure laminin coatings.

The interfacing between neuronal cells and the heat-treated SWNT/laminin LBL film was monitored more closely by measuring neuronal outgrowth from the cells. When compared to neuronal behavior on laminin-coated glass slides, longer outgrowths were noted at every time point (24−120 h) on the heat-treated SWNT/laminin substrate. The bio-

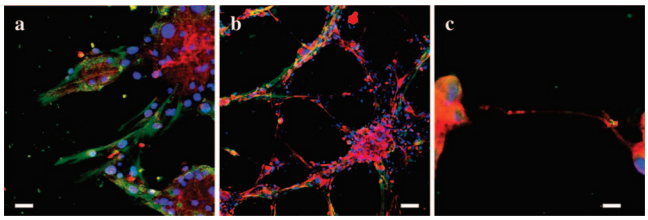

Figure 3. Immunostaining of spontaneously differentiated NSCs on heat-treated SWNT/laminin thin films. The blue color in all images indicates DAPI staining of cell nuclei. (a) GFAP staining is red and MAP-2 staining is green. (b) GFAP staining is red and nestin staining is green. (c) Cells were stained red for the presence of synapsin. Scale bar is 2 μm.

compatibility of the annealed SWNT/laminin substrate was also verified via a commercial cell viability assay that confirmed previous reports on the biocompatibility of SWNT substrates (Figure 1e). They indicated that 98% of cells on the surface of SWNT−laminin composite were viable.

The observation of neuronal outgrowths from the adhered cells is a promising indication of successful cell differentiation. However, in the context of this study more concrete evidence is required to indicate that NSCs are capable of transforming into neuronal cells following adhesion to the SWNT substrate. NSCs have been shown to transform into functioning neurons as well as glial cells such as astrocytes and oligodendrocytes.[17,41] Following cell seeding on the SWNT substrates, the NSCs were immunostained with neuronal markers and observed by confocal fluorescence microscopy. The protein markers studied included MAP-2 (for neurons), glial fibrillary acidic protein, GFAP (for astrocytes), and nestin (for NSCs). The results indicate that even though neural stem cells precursors still exist 7 days postseeding on the heat-treated SWNT/laminin substrate, differentiated neurons and glial cells are also present in large amounts due to spontaneous differentiation caused probably by the physical properties of the SWNT−laminin composites. Interestingly, most of the adhered cells showed some signs of differentiation, which is clearly important from NE perspective. The strong presence of neurons (MAP-2, Figure 3a, green staining) provides indication that successful neuronal communication could possibly be established between cells grown on the CNT substrate and the surrounding tissue.

It is interesting to verify the presence of synaptic connection between differentiated cells, which would also show the possibility of connectivity between the cells grown on the SWNT-composite and fairly efficient signal transmission. Synapses or synaptic nodes are crucial elements in the propagation of electrochemical signals from cells to cells that ensure communication within a neuronal population.[42] The cells seeded on the CNT surface were thus immunostained for the presence of synapsin protein with the appropriate markers. Synapsin is present between the differentiated cells, thus further confirming that neuronal cell growth and differentiation on the SWNT/laminin thin films result in functional neuronal networks (Figure 3c).

Once the biocompatibility and cellular composition on the laminin-SWNT substrates is established, it is necessary to demonstrate the possibility to excite the neuronal cells residing on the nanotubes−protein films. The conductivity of a 10-bilayer SWNT/laminin thin film was estimated to be 430 and 2140 S/m before and after heat treatment, respectively. Drastic increase of conductivity after heat treatment is not surprising. In large part, it is related to reduced swelling behavior. It is certainly beneficial for the design of NEs based on these composites. An electrical signal was applied to the cells via application of a lateral current through the nanotube substrate. The electrical stimulus was applied in a similar fashion as in the previous study,[43] via electrical leads that induce a lateral electric field within the entire SWNT/laminin substrate. The device set up used is shown in Figure 4a; two cloning rings were mounted on the LBL film to serve as chambers for the cell and aqueous media. The electrical leads were attached directly the dry area of the SWNT film by using silver paste and copper tape. The input stimulation was applied with the aid of a function generator (Instek Instruments) that gave flexibility in the stimulus signal to be applied. Constant DC signals as well as pulsed signals were utilized in this study and were typically verified by the use of an oscilloscope prior to cell stimulation/recording.

Cell response to chemical and electrical stimuli typically results in a change in the membrane voltage of the cells, commonly referred to as an action potential. Detection of action potentials can be achieved via the invasive patch clamp method[44] used before or via noninvasive optical detection techniques. Transparency of SWNT LBL films previously reported by our group[45] affords the utilization of optical methods for monitoring that can be a great advantage for fundamental studies. The fluorescence detection method was chosen for this work due to its convenience, clarity, and high information content. Ion-sensitive dyes undergo a change in fluorescence intensity as a result of change in ion concentrations upon application of an action potential. A Ca^{2+} dependent dye (Fluo-4 a.m., Molecular Probes) was used for our study that is membrane-permeable, thus allowing facile imaging of a large population of live cells.[46] The versatile nature of the LBL fabrication allowed for simultaneous detection of fluorescence changes in cells preincubated with Fluo-4 a.m. during electrical stimulation. The experimental setup for the cells thus underwent minor adjustments to ensure that (1) the cells were kept in their physiological media during experiment, (2) electrical leads were kept isolated from any aqueous media, and (3) that a no. 1 coverglass slide was used as the substrate for the LBL film to allow cell recording with an inverted confocal fluorescence microscope.

The cell response appeared to be independent of the type of stimulus applied. The results reported here were obtained from cells that were excited with a pulsed signal. The latter was generally the preferred stimulus in order to minimize any heating effect in the SWNT thin films and to ensure that SWNT electrodes as well as cells are not constantly under stress during stimulation. The typical stimulation signal employed consisted of a series (∼10−15) of 1 ms pulses spaced in 1−10 s intervals. The pulses were applied at these intervals rather than continuously in order to allow both the

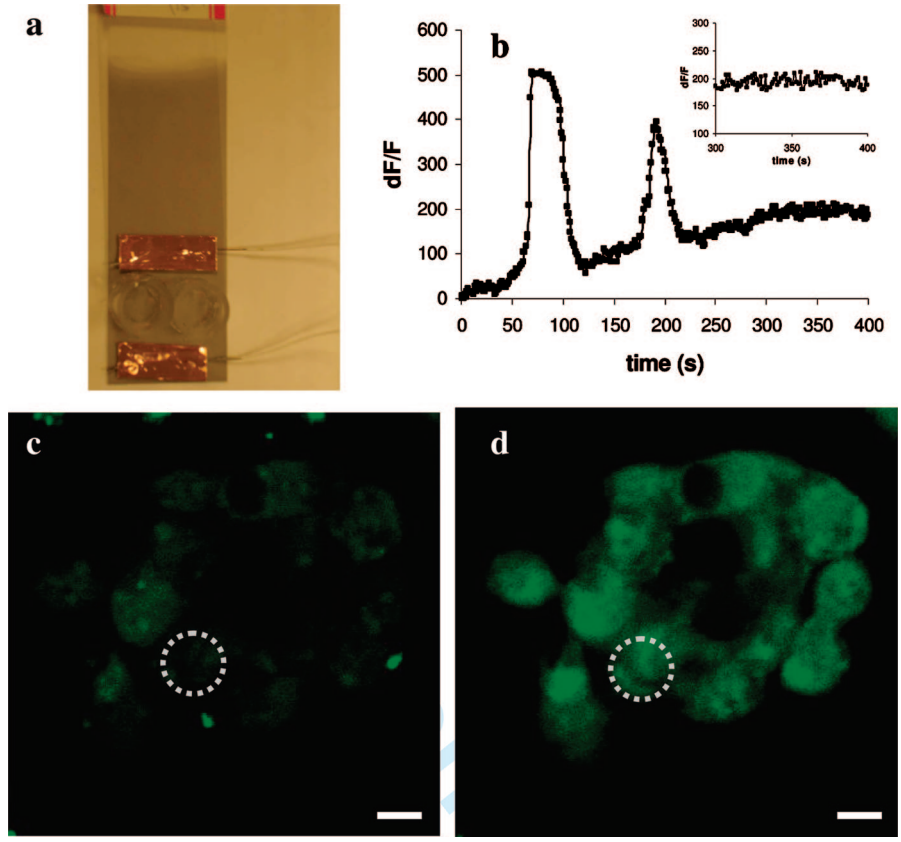

Figure 4. (a) Experimental setup for electrical stimulation studies indicating the cell chambers and electrical leads. (b) Change in fluorescence intensity of Fluo-4 a.m. dye ($\Delta F/F$) in one cell during electrical stimulation by CNT substrate. Change in fluorescence once stimulation is turned off at time $t = 300$ s is shown in inset. (c) Confocal image of a cell cluster before start of stimulation and (d) confocal image of same cluster during stimulation. Data shown in panel b is from the circled cell in panel c and panel d. Scale bar is 10 μm.

electrodes and the cells to discharge. Cellular stimulations were carried out over areas of the films of about 4 cm^2, and output currents from the film range from the order of 1−10 μA/cm^2.

The cells were seeded on the preheated SWNT/laminin substrate for at least 72 h, incubated with Fluo-4 a.m. dye, and washed prior to electrical stimulation studies. The cells were observed by confocal microscopy during the application of the stimulus. A representative confocal microscope image of a cell cluster is shown after Fluo-4 a.m. incubation/washing step but prior to stimulation is given in Figure 4c. The fluorescence level was adjusted such that minimal background fluorescence from the cells was present. A clear increase in fluorescence was observed in the cell cluster following electrical stimulation (Figure 4d). In order to obtain a quantitative measurement of the fluorescence fluctuation in the cells, the image processing software ImageJ was used to measure the fluorescence intensity in the cells during stimulation. The change in fluorescence over stimulation time is plotted as $\Delta F/F$ (Figure 4b) and is shown for one cell from the cluster (circled in Figure 4c,d). In addition to the increase in fluorescence observed, the presence of two peaks on the fluorescence curve suggests that an individual cell can be stimulated more than once upon application of the pulsed stimulus in the form of the sequence of two pulses, which is expected from NEs. The fluorescence level is also monitored after the electrical stimulation is stopped at time $\sim$ 300 s. Upon turning off of the electrical pulses, a leveling off is noticed in the cell fluorescence (Figure 4b). This indicates that the observed cell signal is indeed due to the stimulation through the electrode and not due to spontaneous firing. Recording of the cell excitation after the excitation process also indicated that generation of action potential in individual cell is dependent on the status of the surrounding cells. Once one cell is excited, the train of the excitation is spreading from one cell to another in a nearly sequential manner, which can be identified in the video recording of excitation (Supporting Information). Although the main source of stimulation comes from the electrode underneath the cells, excited-state of the neighboring cells causes the next cell to fire with lower threshold. This is another fact that confirms communication between the NSCs differentiated probably as early neurons.

In conclusion, this study establishes the ability of composite SWNT/laminin thin films to mediate the differentiation and electrical stimulation of NSCs. The protein−SWNT composite can serve as a foundation of a new type of NEs which (1) incorporate SWNTs as superior conductor and strengthening agent; (2) are conducive to NSCs growth and proliferation; (3) and are suitable for excitation of NSCs and the products of their differentiation. Additional studies to further understand the effect of electrical stimulus on NSC during the differentiation cycle and in a longer time frame are still needed.

Materials and Methods. ***Thin Film Fabrication.*** CNT thin films were fabricated by an LBL method with alternating layers of SWNT and the protein laminin. SWNT made by the Hipco process were obtained from Carbon Nanotechnologies Inc. and dispersed by bath sonication in a 1% aqueous solution of polystyrene-4-sulfonate (PSS, MW 1 000 000). The laminin was obtained from Sigma-Aldrich and used as a 100 μg/mL solution in PBS. Thin films were fabricated on a no. 1 glass coverslide by dipping the substrate in the SWNT suspension and protein solution alternatively with thorough rinsing in DI water at each step. The dipping time for each step of laminin and SWNT adsorption was 30 min.

Cell Lines. NSC were purchased from Stemcell Technologies and were grown and differentiated according to the company's protocols.

Cell Interfacing with Thin Films. To enable cell seeding onto the SWNT substrates, cloning rings are mounted on the substrate with high-vacuum grease serving as the adhesive. The attached cloning rings provide a chamber (with volume capacity of ~400 μL) for holding culture media required for cell growth as shown in Figure 6. After the sterilization process, the SWNT samples are passivated with the appropriate cell media for at least 12 h at 37 °C and in a humidified, 5% CO_2 environment.

NSC cell suspensions are then seeded within the designed cell chambers in the appropriate differentiation medium. The cells are allowed to incubate for at least 12 h prior to subsequent studies.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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