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Successful Differentiation of Mouse Neural Stem Cells on Layer-by-Layer Assembled Single-Walled Carbon Nanotube Composite

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ABSTRACT

The same properties that made carbon nanotube (CNT) composites interesting for electronics, sensing, and ultrastrong structural materials also make them an asset for biomedical engineering. The combination of electron conductivity, corrosion resistance, and strength are essential for neuroprosthetic devices. All of the studies in this area demonstrating cellular adhesion and signal transduction activity on CNT matrixes were conducted, so far, with terminally differentiated primary cells and cancerous cell lines. Neural stem cells are very plastic neural precursors capable of adapting to environmental conditions and recreating signal transduction pathways. Their intrinsic biological functionality not only makes the transition to stem cell cultures a difficult-to-avoid step but also implies several fundamentally important challenges. Here we demonstrate that mouse embryonic neural stem cells (NSCs) from the cortex can be successfully differentiated to neurons, astrocytes, and oligodendrocytes with clear formation of neurites on layer-by-layer (LBL) assembled single-walled carbon nanotube (SWNT)–polyelectrolyte multilayer thin films. Biocompatibility, neurite outgrowth, and expression of neural markers were similar to those differentiated on poly-L-ornithine (PLO), one of the most widely used growth substratums for neural stem cells.

Introduction. The development of nanotechnology is penetrating biology and medicine at a remarkable speed.[1] Nanotechnology is poised to provide new tools to measure and understand biosystems,[2,3] bring insights to challenges in biotechnology and biomedicine,[4,5] and offer building components for advanced biomaterials.[6,7] One area that will significantly benefit from the development of nanoscale engineering is the technology for neural medicine in its many manifestations.[8] The reasons are multifold.

(1) Nanomaterials were originally developed and still have the most widespread applicability for electronics and information processing, which makes their functionalities quite similar to those of neural system.

(2) The neural tissue is highly complex in its anatomy, functional structuring, and information processing, which frustrates the applications of current technologies and requires the change of technological paradigm.

(3) The central nervous system is difficult to access and has heterogeneous cellular and molecular environment, which, in turn, means that size reduction of any devices to interact with neurons is required to create the least possible interference with and disruption to the central nervous system's functionality.

Indeed, recently we are beginning to see emerging applications of nanotechnology in neuroscience using primarily organic and polymeric materials as scaffolds. In this study, we focus on the development of a new platform biomaterial based on single-walled carbon nanotubes (SWNTs) that can find applications in the treatment of neurological disorders and injuries. Why carbon nanotubes? The unique physical, chemical, mechanical, and electronic properties of carbon nanotubes[9−14] can be, in part, transferred into SWNT composites to combine high electrical conductivity, chemical stability, and physical strength with structural flexibility.[15−19] Such a unique combination of characteristics and the possibility for surface functionalization[20,21] make these SWNT composites promising candidates for making neural prosthetic devices with capabilities for electrical field recording, sensing of neurotransmitters, and electrical stimulation. For example, several neurological disorders and injuries, such as Parkinson's disease, epilepsy, and stroke, require an implantable device to generate electrical activity in the damaged or diseased tissue. Some diseases, such as obesity, incontinence, heart problems, chemically resistant depression, and others find radically new solutions in application of implantable devices for neural stimulation, for instance Vagus nerve. One common problem of all chronically implanted

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electrodes lies in the tissue−electrode interface.[22] Tissue reactions typically lead to encapsulation (scarring) around the electrode, reducing its performance and lifetime. Current neural prosthetic technology is in active search of a biocompatible material that allows chronic implantation of miniaturized electrodes.[23,24] SWNT composites can satisfy the requirement for chronic implantation by providing a durable, electrically conductive, highly modifiable, and biocompatible interface. Various strategies can be similarly applied to SWNT composites to attract growing neurites[25,26] and reduce inflammatory response.[27] The first results on neuronal excitation through SWNT films made by the layer-by-layer (LBL) assembly had already been demonstrated.[28]

All of the latest studies of interactions of neural cells with carbon nanotubes were carried out with terminally differentiated primary cells or cell lines. The primary focus was on establishing biocompatibility of the proposed materials.[29,30] In addition, carbon nanofiber reinforced composites were shown to increase neuronal cell functions while providing a mechanically strong and electrically conductive substrate.[31] Patterned carbon nanotubes were utilized to guide neural cell growth and demonstrate preferential cell attachment on carbon nanotubes.[32,33] Neural signal transmission efficacy was also reported to increase on carbon nanotube-coated substrates.[34] We see strong possibilities, fundamental importance, and practical need to use neural stem cells (NSCs)[35] in the studies of SWNT materials for neural medicine. Terminally differentiated cells have limited capacities for extracellular matrix remodeling, axonal extension, and interfacing with implants.[36,37] Successful implantation and long-term performance of a neural prosthetic device may and most likely will require an interface that can recruit NSCs. Interfaces with pre-seeded NSC have a potential to greatly reduce scarring at the implant site. As well, in many cases NSCs, are necessary to regenerate the neural tissue,[38,39] while the implantable device will be needed to establish and train a particular signal transmission path. Despite the obvious importance of NSCs, the overall body of knowledge of their behavior on engineered materials remains very limited. Young and his colleagues have done work in this area with polymeric and biomimetic substrates.[40−42] Silva and Stupp have demonstrated the differentiation of NSCs in peptide fibrous matrices.[43] Interactions of quantum dots and nanoparticles have been investigated with neurons[44−46] and bone marrow stem cells[47,48] but not with NSCs. Likewise, no information is available about the interaction of any possible carbon nanotube materials and NSCs. Considering the potential importance of these composites for neural medicine, one has to ask the two fundamental questions that are necessary for further development of the field and acquisition of knowledge regarding the interaction of nanoscale materials and transformable cells. First, are SWNT composites biocompatible enough to support the differentiation of stem cells, which are well-known for their sensitivity to the environment? Second, if this is the case, do SWNT materials affect in any way the differentiation of NSCs? In this study, we demonstrate, for the first time, differentiation of mouse cortical NSCs on a SWNT-based composite. Our results provide us definite answers to the questions above regarding LBL-assembled SWNT−polyelectrolyte composite films. The choice of the particular SWNT composite was made on the basis of previous success with culturing NG108-15 murine neuroblastoma × glioma hybrid cells on SWNT LBL films[49] and their neural excitation through the LBL films.[28]

Results and Discussion. To investigate the behavior of NSCs on LBL-assembled SWNT composite films, mouse embryonic 14-day neurospheres (i.e., spherical clonal structures of NSCs) from the cortex were seeded and induced to differentiate on round glass coverslips (12 mm diameter) coated with six bilayers of SWNT−polyelectrolyte. SWNTs were dispersed in a 1 wt % poly(sodium 4-styrene-sulfonate) (PSS) solution and LBL assembled with the polyelectrolyte poly(ethyleneimine) (PEI). The resulting thin film of coating was designated as $(PEI/SWNT)_6$. As a control, neurospheres were also seeded and differentiated on coverslips coated with poly-L-ornithine (PLO), a standard substratum commonly used for NSC cultures and studies.

NSCs were first expanded in proliferation medium containing epidermal growth factor (EGF). To induce cell attachment and differentiation, they were seeded at a low density of 200 neurospheres per coated coverslip in 24-well plates and cultured with EGF-depleted differentiation medium. As suggested by Young et al.,[41] a high neurosphere seeding density encourages cell migration from neurospheres while a low seeding density does not support cell survival away from neurospheres and forces cells to extend long processes in search for other neurospheres. Therefore, a low neurospheres seeding density truly tests the interaction between cells and their substrate. In our study, neurospheres attached to both types of substrates and developed neural processes away from the edge of the neurospheres as early as 1 day in culture (Figure 1A). The lengths of processes from differentiated NSCs increased steadily over the 7-day culture period (Figure 1B). Throughout the culture period, neural processes developed on PLO-coated substrates remained longer than those developed on $(PEI/SWNT)_6$-coated substrates; however, the differences were not very significant. By day 5 in culture, NSCs on both types of substrates had extended long and complex processes into the area surrounding the neurospheres. It is highly likely that, if the neurospheres were seeded at higher densities, the extending processes from individual neurospheres could reach and form networks with their neighboring neurospheres.

Viability of neurospheres was evaluated using the MTT assay (Figure 1C). There was no noticeable difference between the viability of neurospheres on $(PEI/SWNT)_6$- and PLO-coated substrates. Cell viability was maintained at a steady level after day 3. The initial decline in cell activity between day 1 and day 3 is likely to be a consequence of cell damage during the EGF depletion process (repeated centrifugation and dispersion) and lost of weakly attached neurospheres during medium change. In any case, our viability assessment indicates that $(PEI/SWNT)_6$-coated substrates are as biocompatible as PLO-coated substrates in supporting neurosphere differentiation. The differentiated morphology of neurospheres was confirmed by scanning

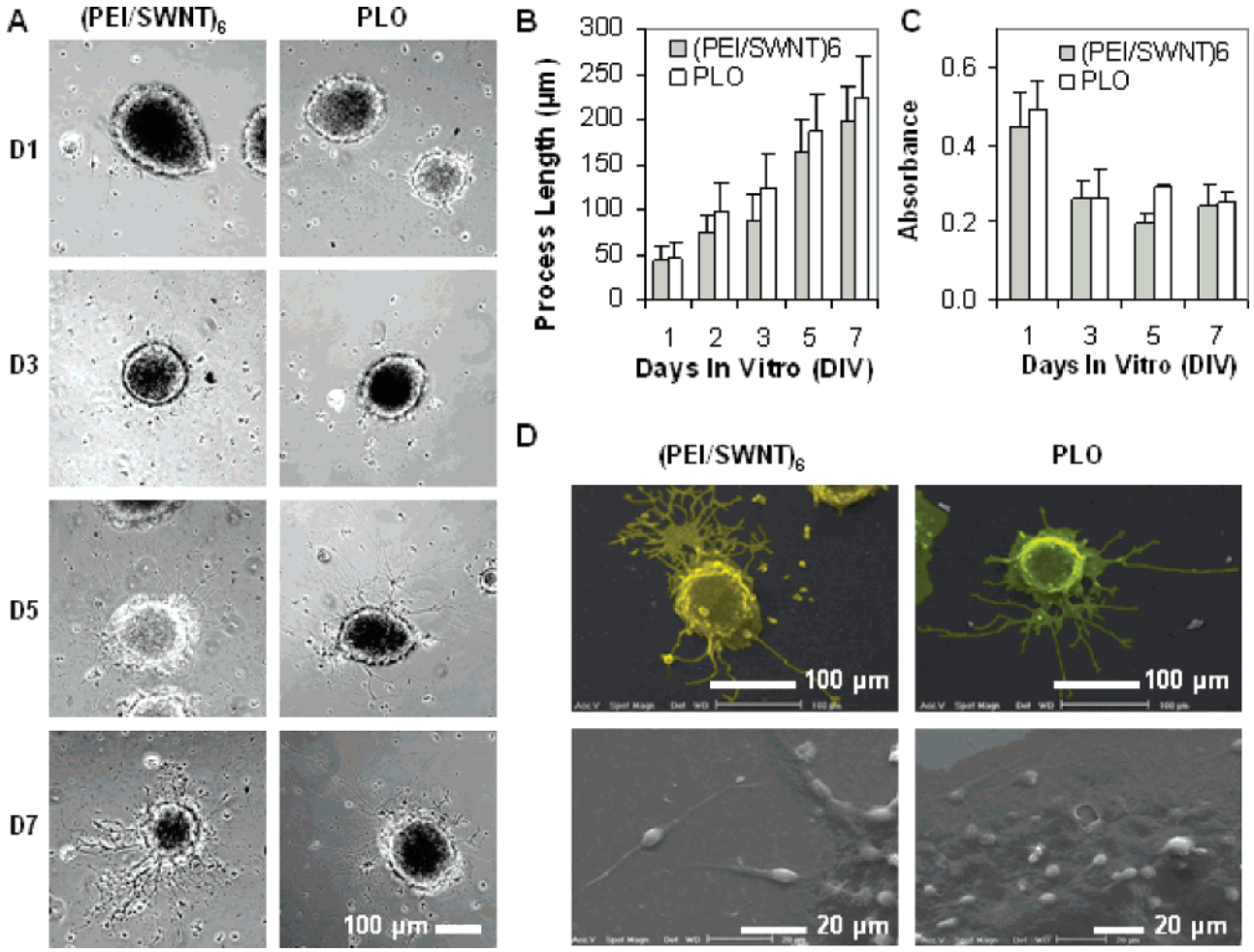

Figure 1. Mouse embryonic 14-day cortical neurospheres differentiated on and $(PEI/SWNT)_6$- and PLO-coated coverslips in 24-well plate at a low seeding density of 200 neurospheres per well. (A) Light microscopy images of differentiated neurospheres. (B) Evaluation of the lengths of processes extending from the differentiated neurospheres for the 7-day culture period. (C) MTT reduction activity of differentiated neurospheres. (D) Color-enhanced SEM images highlighting neurite outgrowth of differentiated neurospheres on day 7 (first row). SEM images at higher magnification showing migrating NSCs around neurospheres (second row).

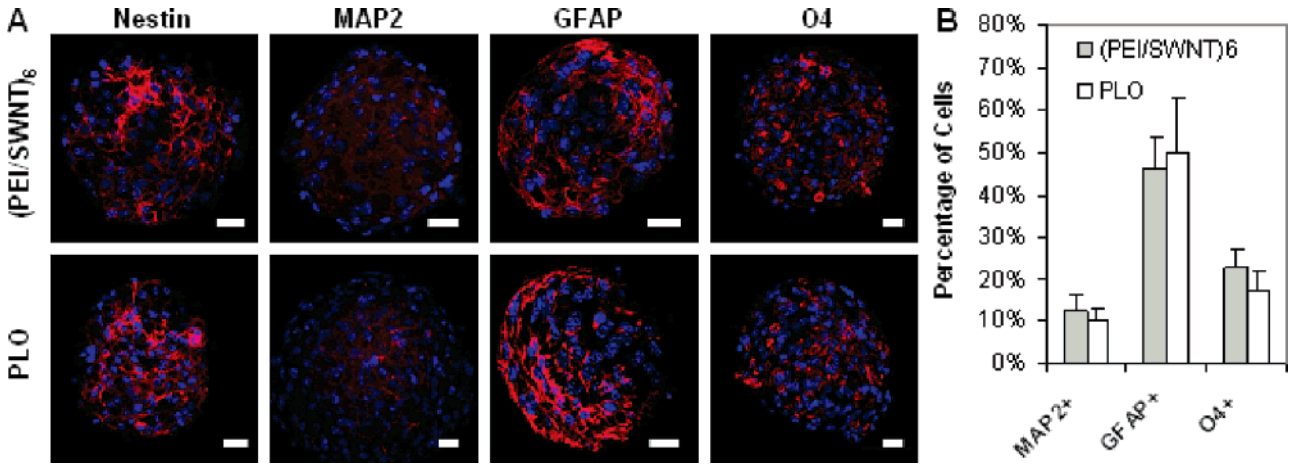

Figure 2. (A) Confocal microscopy images differentiated neurospheres on day 7. Neurospheres were stained for markers of NSCs (nestin), neurons (MAP2), astrocytes (GFAP), and oligodendrocytes (O4). Neural markers are shown in red, while the cell nuclei, counterstained with DAPI, are shown in blue. Images represent scans near the center of the neurospheres. Scale bars: 20 μm. (B) Average percentages of differentiated cell phenotypes after 7 days in culture.

electron microscopy (SEM) (1D first row). Differentiated neurospheres developed abundant and highly branched neural processes on both types of substrates. SEM also revealed migration of individual cells away from the original neurospheres (1D second row). In addition, we observed more pronounced spreading of extracellular matrix (ECM) on

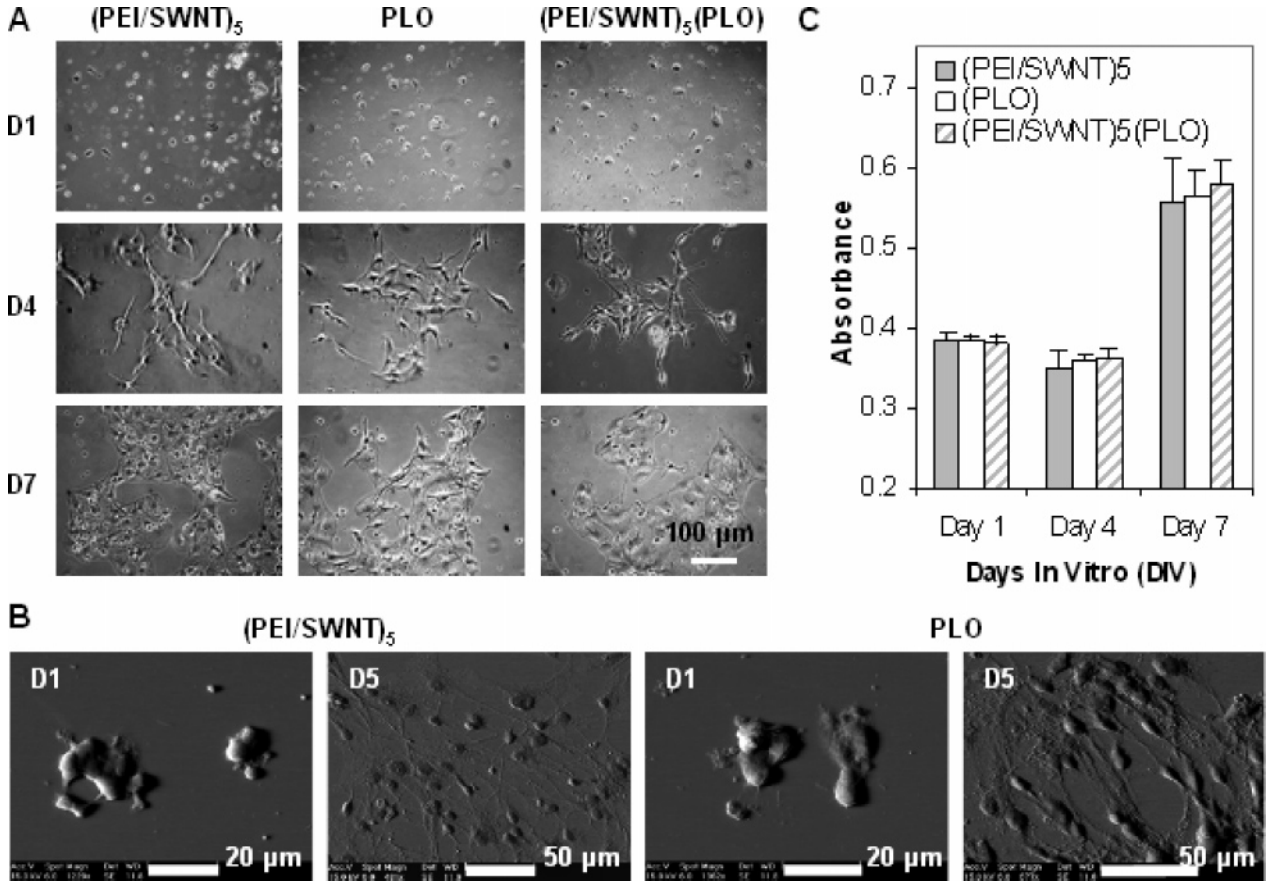

Figure 3. Mouse embryonic 14-day cortical neurospheres differentiated as dissociated single cells and small clusters on and $(PEI/SWNT)_6$-, PLO-, and $(PEI/SWNT)_6$(PLO)-coated coverslips in 24-well plate. (A) Light microscopy images of differentiated NSCs. (B) SEM images of differentiated NSCs. (C) WST-8 reduction activity of differentiated NSCs.

PLO-coated substrates, which we believe is a consequence of PLO's adhesive nature. The SEM images are a straightforward indication, together with Figure 1A,B, that LBL-assembled SWNT films can support the differentiation of neurospheres and their neurite outgrowth.

It has been known since the discovery of NSCs that NSCs differentiate into a heterogeneous population of cells consisted of neurons, astrocytes, and oligodendrocytes.[35] To analyze the differentiated phenotypes, neurospheres cultured for 7 days were immunostained with antinestin-, antimicrotubule-associated protein 2 (anti-MAP2), antiglial fibrillary acidic protein (anti-GFAP), and anti-oligodendrocyte marker O4 (anti-O4) and counter stained with DAPI nuclei stain (Figure 2A). Nestin expression has been adopted as a marker for neural stem and precursor cells.[50] MAP2 is a cytoskeletal protein component in mature neurons and dendrites,[51] while GFAP and O4 are established markers for intermediate filament proteins in astrocytes[52,53] and surface antigens of developing oligodendrocytes,[54,55] respectively.

Immunostaining results on PLO- and $(PEI/SWNT)_6$-coated substrates are qualitatively comparable (Figure 2A). Both types of substrates supported differentiation of neurospheres into the three primary neural cell types: neurons, astrocytes, and oligodendrocytes. In general, nestin expression is intense and abundant near the center of neurospheres. MAP2-positive cells are faintly stained and located near the center, while O4-positive cells are sparsely distributed throughout the neurospheres. GFAP is heavily expressed at the periphery of neurospheres, indicating that most migrating cells might be astrocytes. Quantitative assessment of cell phenotypes shows that there were slightly more neurons and oligodendrocytes on $(PEI/SWNT)_6$-coated substrates and more astrocytes on PLO-coated substrates (Figure 2B), although the differences between PLO and SWNT results are close to the margin of error. In general, the cell phenotype analysis is consistent with our observations from Figures 1 and 2A. Longer neural process formation (Figure 1A,B) and increased spreading of ECM (Figure 1D, second row) on PLO-coated substrate correlate with increased astrocyte population (Figure 2). Fibrous astrocyes are known to have long and slender processes, and ECM formation is characteristic for these cells as well. Interestingly, Young and his colleagues[41] also observed that, under serum-free condition, a different substrate that promotes longer processes generated primarily fibrous astrocytes and fewer neurons.

Having successfully demonstrated the differentiation of mouse neurospheres on LBL-assembled SWNT composite films, we sought to elucidate the differences between the LBL and PLO substrates by increasing cell−substrate contact. To accomplish this, we chemically dissociated neurospheres into single cells and differentiated them on $(PEI/SWNT)_5$-, PLO-, and $(PEI/SWNT)_5$(PLO)-coated glass

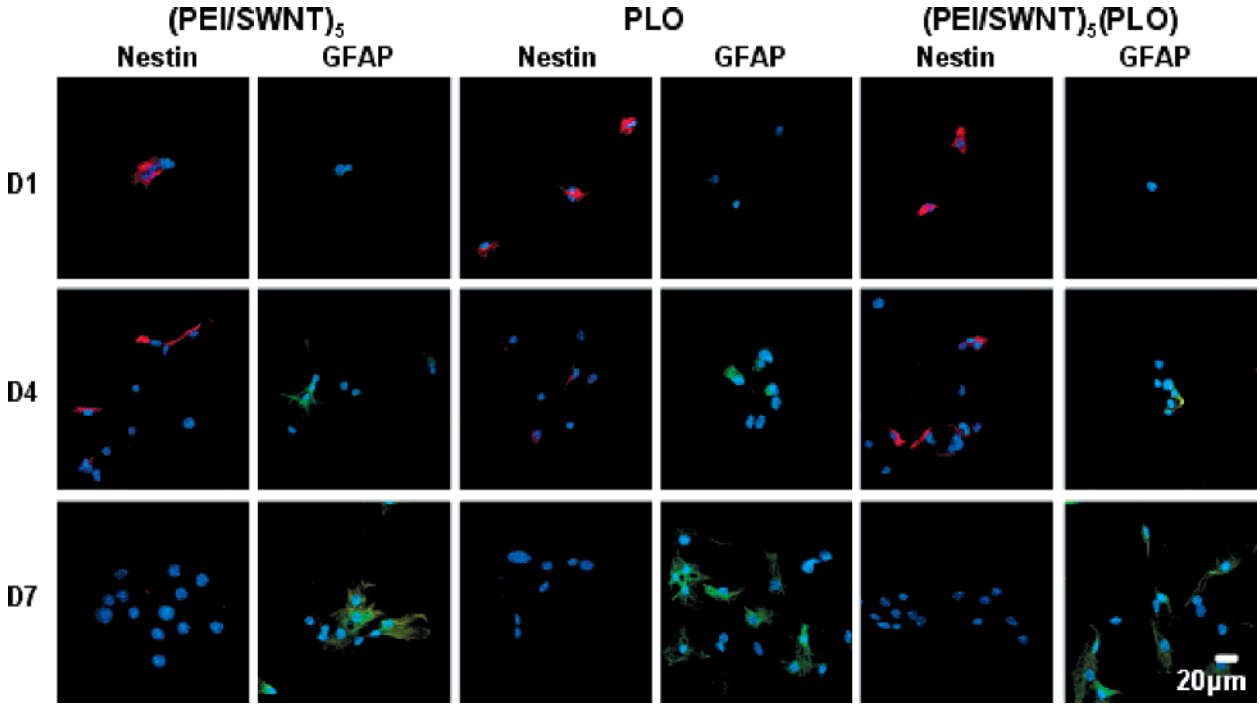

Figure 4. Confocal microscopy images of differentiated NSCs. Cells were stained for markers of NSCs (nestin, shown in red), astrocytes (GFAP, shown in green), and neurons (MAP2, not shown due to low level of expression). Cell nuclei were counterstained with DAPI and shown in blue.

coverslips. We added $(PEI/SWNT)_5(PLO)$-coated substrates to help us determine whether the combination of PLO's adhesiveness and SWNT's nanotopology (see Supporting Information) would produce any additive cell behavior. As time progressed, NSCs transformed from a spherical morphology to a more elongated and spread-out morphology representative of differentiated neural cells (Figure 3A). Differentiated NSCs also developed neural processes that intertwine with one another. The change in cell morphology was confirmed by scanning electron microscopy (SEM) images acquired on day 1 and day 5 after seeding (Figure 3B).

Viability of differentiated single cells was assessed using the WST-8 assay. We switched to using WST-8 because a previous study reported MTT's tendency to associate with carbon nanotubes to give false and cytotoxic results and recommended the use of water-soluble tetrazolium salts.[56] WST-8 is a tetrazolium salt that produces a highly water-soluble formazan upon cellular reduction and has been recently adopted as an indicator for use in viability assays.[57] Overall, NSCs on the three different substrates demonstrated similar viability throughout the 7-day culture period (Figure 3C). Cell activity appeared to be constant between day 1 and day 4 but increased significantly from day 4 and day 7. We suspect this increase in cell activity to be the result of increased metabolic activity of the extensively differentiating cell populations.

The progression of NSC differentiation was monitored by immunostaining for nestin, MAP2, and GFAP expressions (Figure 4). Identical trends were observed on all three types of substrates, and differentiation was not affected by the SWNT-based substrates in comparison to each other. While about 10% of differentiated neurospheres expressed MAP2, we observed an insignificant amount of MAP2 expression in differentiated *single cells* throughout the culture period consistent with preferential migration of astrocytes on the films. Our finding also indicates that the three-dimensional structure of neurospheres and the junctions betweens individual cells in neurospheres may provide an environment similar to the neural stem cell niche and therefore may be essential for neuronal differentiation. Despite the lack of MAP2 expression, NSC differentiation was nevertheless confirmed through changes in nestin and GFAP expressions. The entire cell population was nestin-positive and GFAP-negative on day 1, indicating an undifferentiated state. As time progressed, NSCs began to lose their nestin expression and acquire GFAP expression. By day 7, none of the cells stained positive for nestin and approximately 80−90% of all cells were GFAP-positive. As NSCs proliferate and differentiate into neurons and glia, nestin is down-regulated and distinct intermediate markers are expressed.[58]

Conclusion. In conclusion, we have demonstrated for the first time that the differentiation of environment-sensitive NSCs, both as neurospheres and single cells, on a CNT-based material. Our study is essential for further development of nanomaterials for neural interfaces. The nanostructure of the LBL-assembled SWNT−polyelectrolyte composite, which gives rise to the composite's structural flexibility, chemical stability, and physical properties, showed no adverse affect on the differentiation of NSCs. NSCs behaved similarly to those cultured on the standard and widely used PLO substratum in terms of cell viability, the development of neural processes, and the appearance and progression of neural markers. We also see indications that SWNT sub-

strates might induce preferential differentiation into the specific lineage; however, this aspect of the study as well as the potential mechanism(s) of the influence requires further investigation.

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Supporting Information Available: Methods: preparation of substrates, culture of mouse cortex embryonic 14-day neurospheres, quantification of process growth, assessment of cell viability, scanning electron microscopy, immunocytochemistry, SEM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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