

# Guided Neurite Growth on Patterned Carbon Nanotubes

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## ABSTRACT

A combination of microlithography and chemical vapor deposition is used to engineer patterned vertical multiwalled carbon nanotube substrates. They are used to demonstrate the formation of directed neuronal networks. Multiple substrate geometries and nanotube heights were fabricated to determine the most suitable combination for understanding the cell morphological changes. The interaction between the cell membrane and the nanotube substrate are visually characterized. The viability of the networks on the nanoscale substrates was observed.

**Keywords:** Nanoscale platforms, patterned carbon nanotube substrates, directed neuron growth

## INTRODUCTION

Nanostructured substrates have the capability for directing and guiding live biological cells. Not only do they provide support to the developing cells but also allow for in-situ monitoring. Neurons are electrically excitable cells that on network formation serve as conduits for information transfer. A vast amount of information is transferred through the cells in the spinal cord via synaptic and gap junctions in an electro-ionic fashion mediated by neurotransmitters. The failure of the mammalian spinal cord to regenerate following injury is not absolute, but appears to be amenable to therapeutic manipulation [1]. Promotion of axonal growth and support for long distance regeneration are the two requirements in the various experimental strategies for spinal cord repair [2]. The onus of achieving these goals in-vitro is on the substrate, which functions as the basis for the formation of neural bridges with high signal to noise ratio, for efficient signal transmission. Biomaterials play a major role in developing successful guiding strategies.

A novel technique is the use of patterned carbon nanotube (CNT) substrates for forming network hybrids and ensuring electrical signal transmission throughout the network. Here we show the potential synergy between MEMS fabrication and synthesis of nanostructured materials in developing platforms and devices amenable for point of care biological applications. We demonstrate the capability of functionalized patterned vertical CNT arrays as support platforms for guiding neurite growth and forming synaptically communicative networks.

## MATERIALS AND METHODS

For CNT array structures to function as effective platforms for scaffolding purposes, they need to demonstrate the ability to support and guide neuron growth over a combination of linear and circular geometries. To establish this capability, three patterns of CNT substrates were fabricated. The substrate fabrication comprised of two stages. The first stage is the development of a base substrate comprising of various optically formed patterns suitable for formation of vertical CNT arrays using standard photo lithography techniques. The second stage involves the formation of vertical multiwall carbon nanotube (MWNT) arrays of varying lengths using chemical vapor deposition process (Figure 1A). Functionalization of CNT substrates with growth adhesion factors was employed to improve the guided growth.

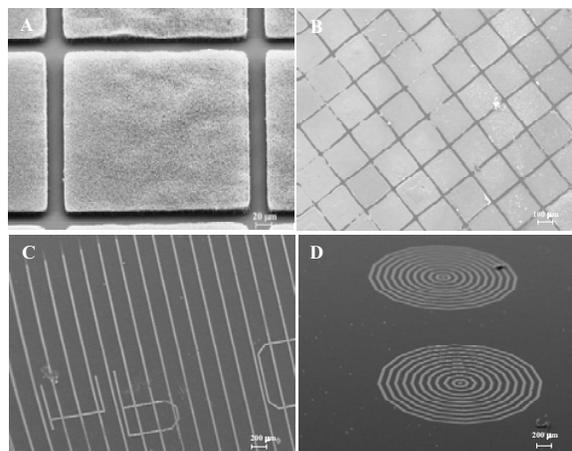


Figure 1: Scanning electron micrograph of the patterned CNT substrates. (A) 10  $\mu\text{m}$  long vertically grown CNT on Si substrate. (B) Square patterned CNT substrates suitable for understanding cell-cell interactions. (C) Parallel straight line substrates for understanding neurite extension. (D) Circular substrates of varying diameter for understanding guided growth.

A micro patterned substrate is first developed using conventional optical lithographic principles. This functions as the platform on which the vertically arrayed MWNT pattern 250  $\mu\text{m}$  x 250  $\mu\text{m}$  squares with 20  $\mu\text{m}$  spacing for determining the interaction between clusters of neurons on a MWNT substrate (Figure 1B). The second pattern consisted of 20  $\mu\text{m}$  thick lines with 200  $\mu\text{m}$  spacing, for determining the extension capability of a neuron process over a MWNT scaffold (Figure 1C). The third pattern

consisted of circles with diameters ranged from 400  $\mu\text{m}$  to 2000  $\mu\text{m}$  and 20  $\mu\text{m}$  in width (Fig. 1D). To study the effects of curvature on neuronal guidance, circles with varying diameters were designed. Curved lines are crucial in determining the direction of axon growth into target region. It has been established that in-vitro process outgrowth associated with guided network formation from immobilized neuron soma and onto the substrate is curvature dependent [3]. The substrate comprised of silicon with a 0.5  $\mu\text{m}$  thick silicon dioxide, dielectric passivation layer. AZ 5214 was used as the photoresist (Microchem, MA). It was spin-coated over 1.5 cm x 1.5 cm silicon substrate at 3000 rpm for 30 seconds. As AZ 5214 is a positive photoresist suitable quartz masks were designed to ensure transfer of the appropriate patterns onto the substrates. The patterns were transferred using contact lithography. Exposure to UV light of intensity 300 mW/cm<sup>2</sup> for a period of 10 seconds was performed after masking. The chips were developed using AZ 5214 photo resist developer. The developer was mixed with DI water at a 1:5 ratio. The chips were immersed for a period of 60 seconds.

The second stage is the growth of the vertical arrays of carbon nanotubes using a catalyzed chemical vapor deposition technique. Typically, 10 nm thick of iron was deposited onto the patterned substrate using vacuum thermal evaporation. Iron acts as the catalyst in CNT growth. Lift-off was then performed, by immersing the substrate into acetone for a period of 60 seconds to leave the catalyst pattern on the bare silicon substrates. Following reported procedures, MWNTs were grown selectively on the catalyst patterns using a mixture of C<sub>2</sub>H<sub>2</sub> and H<sub>2</sub> [4]. MWNTs grown under these conditions were aligned vertically with two height steps.

The photolithographically patterned substrates were mounted on the surface of a stainless steel sample holder and the whole assembly was introduced into the thermal evaporation chamber. The chamber was pumped down to 1x10<sup>-6</sup> torr before the catalyst was deposited. During deposition the substrates were kept at room temperature. After depositing the catalyst layers the photoresist layer was lifted off the substrate in acetone, dried and, transferred into a quartz tube housed inside an electric furnace under argon. The temperature of the chamber was ramped up to the nanotube growth temperature of 850 °C under argon and hydrogen at flow rates of 2000 and 1000 sccm respectively. The nanotube growth was initiated by switching on a flow of C<sub>2</sub>H<sub>2</sub> at 200 sccm. The growth period varied between 1-10 min at the end of which, the chamber was cooled down to room temperature in argon. Varying heights were achieved by changing the duration of exposure of the substrate to the gaseous mixture.

The cells used were H19-7 cells (ATCC, VA), an SV40 Tts-immortalized rat hippocampal neuronal cell line. The cell density was adjusted to be approximately around 2000 cells/mL. The cells suspended in the growth buffer were flowed onto the growth permissive substrates and allowed

to settle for 20 minutes before incubation at 37 °C. Incubation was performed in a conventional cell incubator (Thermo Forma, Marietta, OH) for a period of 6 hours, to ensure cell viability and proliferation.

## RESULTS AND DISCUSSION

It was observed that the topography of the substrate played an important role in the scaffolding capability of the substrate. Neurons on the short MWNT substrates (500 nm) did not exhibit preferential directed growth over the MWNT array vis-à-vis the silicon substrate. In comparison guided growth of the neurites processes along the edges of the pattern of the long MWNT arrays (10  $\mu\text{m}$ ) was observed (Figure 2A, 2B). The neurite processes were restricted along the sidewalls of the vertical MWNT patterns for guided growth similar to trellis behavior.

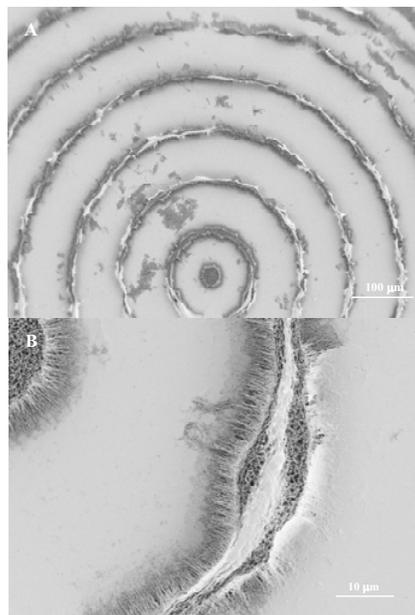


Figure 2: (A) SEM micrograph indicating guided neurite growth along long MWNT scaffolds. Neurons show preferential adhesion as well as proliferation along the MWNT pattern. Neurons do not show localization over the silicon substrate despite complete functionalization of the substrate. The substrate with the neuron networks is vacuum desiccated and sputtered with a thin film of gold (100Å) to facilitate imaging. (B) SEM micrograph of the selected area in figure 1B showing the positioning of the neurite on the MWNT scaffold. The extending neurite positions itself on the top surface of the pattern. Pattern deformation is observed at the areas of interaction between the neurite and the MWNT array.

The structural difference between the short and long MWNT arrays is believed to be the factor that determines

the scaffolding capability. Nanotubes have high tensile strength but their flexibility is a function of the length of the tube. Neurite outgrowth is elicited by the development of the growth cone. This structure located at the tip of the developing neural process determines the direction of extension of neurite. The growth cone shows preferential adhesion to flexible substrates in-vitro. A large percentage of the Short MWNTs during growth get pinned to the substrate. As a result nanoscale ordering between individual tubes is not maintained. This results in the formation of micron sized regions of similar surface topography. Neural cells adhere to any substrate via extracellular proteins most notably laminin. The dimension of this protein is approximately 70nm [5]. Surfaces that offer nanoscale roughness are hence most suitable for supporting and directing neurite growth. Thus short MWNT arrays despite the presence of the chemotropic guidance cues in the form of PLL do not offer the requisite roughness and hence there is no preferential neuron growth along the patterns. In the case of long MWNT arrays, the nanotubes during their growth remain upright. Hence nanoscale ordering of individual tubes in the array is observed. This results in increased surface roughness in the nanoscale. This offers a bio-mimetic topography similar to in-vivo conditions. Consequently guided neurite growth is observed along the patterns of long MWNT arrays (Figure 3).

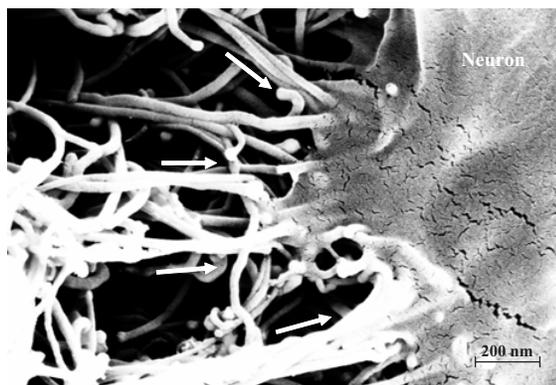


Figure 3: SEM micrograph shows the nanoscale interaction between the neurite and the nanotube bundles. The neurite extending from the motile growth cone grasps the long MWNT bundles and deforms them to assist in its elongation and morphological changes. The arrows show the direction of deformation of the nanotube bundles by the extending neurite.

We observe the formation of directed neural networks 24 hours after seeding the neurons onto the long MWNT substrates. The extending neurite process interacts with edges of the MWNT patterns (Figure 4). This occurs due to the deformation of the long MWNT's as a result of the extending process. The process continues extending along the nanotube surface due to the interaction of the growth

cone with PLL on the tubes that undergo deformation to accommodate neuron proliferation.

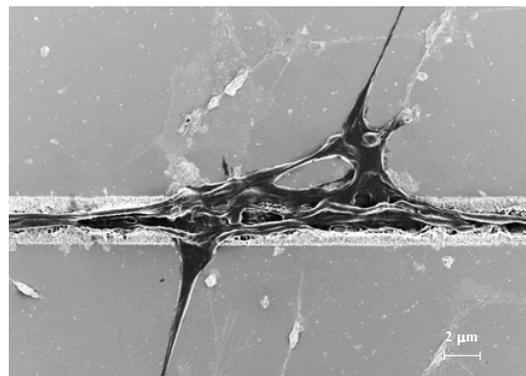


Figure 4: Scanning electron micrograph demonstrating guided neurite growth along the MWNT array pattern. The extending neurite is shown interacting with the edges of the pattern. This morphology is observed 24 hours after initial seeding of the cells.

To determine the effectiveness of a substrate as a two dimensional scaffold it is essential to establish the cell supporting capability of the vertical MWNT matrix over micron scale distances. The formation of neural bridges establishes the suitability of the substrate as a scaffold. Parallel straight lines comprising of MWNT arrays with separation widths of 20 μm were used. It was observed that the axons of the hippocampal neurons crossed over two pattern boundaries forming a synaptic bridge. The somas of the neurons forming the bridge were localized on the pattern edges. The neurite extended over a distance of 20 μm to form synaptic connections (Figure 5A). These observations indicate the potential of the substrate to support the formation of a communicative neural network in both two as well as three dimensions. Controlled surface coverage is a primary requirement for the eventual application of the substrate as a prosthetic scaffold implant. Optical visual characterization of neuron viability is achieved using fluorescent staining techniques. Viable in-vitro neural networks have high cytoplasmic calcium content with a high concentration of calcium ions at the pre and post synaptic terminal. The neurons on the patterned MWNT substrates were stained using fluo-3 calcium stain following standard immunohistochemical procedures. The stain binds to the free calcium ions within the cell. In viable cells the calcium concentration is approximately 0.2 M distributed evenly throughout the cytoplasm. Hence the entire cell bodies as well the neurite processes imbibe the stain in the case of viable. This is characterized on the guided networks using Nikon 600 upright fluorescent microscope (AG Heinze, CA) under FITC (Figure 5B).

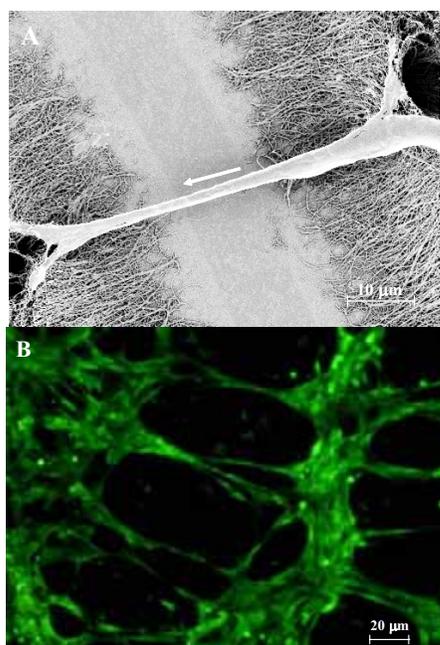


Figure 5: (A) SEM micrograph of a communicative synaptic bridge formed between two nanotube patterns with a distance of separation of 20  $\mu\text{m}$ . The soma of the neuron is positioned along the edge of the nanotube substrate due to the motile interactions between the cell body and the MWNT bundles. The axon extends over the pattern without guidance along the silicon substrate. The synapse is also formed at the pattern edge. The direction of process outgrowth is represented by the arrow. (B) Optical micrograph of a synaptically communicative neural network on MWNT patterned substrate. The viability of the network is determined by staining the neurons with Fluo-3. This is a calcium identifier and it binds to the free calcium ions in the cytoplasm of the viable neurons. At the synapses there is a burst of calcium ions that is represented by the bright fluorescent regions.

## DISCUSSION

We have demonstrated the capability to integrate micro and nano fabrication technology to develop substrates that function as scaffolds for forming guided two dimensional neural networks. The geometries of the micro scale patterns were so designed to address the three important factors in developing successful scaffolds namely cell process extension, guidance, and interaction. Neurite extension capability over artificially structured substrates is determined using parallel straight line features. Cell-cell interaction is analyzed using square patterns and finally neurite guidance is characterized over circular features.

Surface topography in terms of the length of the nanotubes was also observed to play an important role in process guidance. Neurite processes showed preferential

adhesion to the edges of long MWNT patterns whereas no selectivity was observed in the short MWNT patterns despite PLL functionalization of both types of substrates. This behavior is attributed to the adsorption of the PLL molecules onto the sidewalls of the long nanotubes and trapping of the PLL molecules in between the nanotubes at the pattern edges due to capillary action. In the case of short nanotubes during its growth a large percentage (>60%) of the tubes get pinned on to the substrate. These results in the absence of sidewalls for PLL functionalization also the short tubes do not promote capillary action. Also the rigidity of the short MWNTs do not offer the motile growth cone with a suitable surface for process development. The long MWNTs in comparison are flexible and undergo deformation to accommodate the proliferating neurite.

The substrate offers potential towards developing three dimensional scaffolds suitable for implants due to guided surface coverage as well as cell viability in two dimensions.

The major finding in the formation of the neuronal bridges was an understanding relating to the interaction between the neuron and the MWNT scaffold. As the neurite started to extend from the soma the growth cone extended to envelope the nanotubes in the areas where they were treated with PLL. The proliferation of the outgrowth resulted in enmeshing the tubes in the vicinity thus causing the nanotubes to cluster together resulting in disruptions in the patterns. We have established that guided neuronal networks can be formed on long vertical MWNT arrays by preferential adhesion to the pattern in comparison to short arrays. The neurite extends itself by contracting bundles of nanotubes due to the deformation it causes as a result of its extension. Thus probing the bundles of MWNTs in contact with the neurite process with electrical back contacts will allow for a better understanding of the signal transduction pathways. Future applications could include a new diagnostic tool and signal analysis after breakdown of the neuron pathway.

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