

Interactions Between Cultured Neurons and Carbon Nanotubes: a Nanoneuroscience Vignette

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Carbon nanotubes, owing to their electrical, chemical, mechanical and thermal properties, are one of the most promising nanomaterials for the electronics, computer and aerospace industries. More recently, these unique materials are finding their niche in neuroscience. Here, we discuss the use of carbon nanotubes as scaffolds for neuronal growth. The chemical properties of carbon nanotubes can be systematically varied by attaching different functional groups. Such functionalized carbon nanotubes can be used to control the outgrowth and branching pattern of neuronal processes. We also discuss electrical interactions between neurons and carbon nanotubes. The electrical properties of nanotubes can provide a mechanism to monitor or stimulate neurons through the scaffold itself. The ease of which carbon nanotubes can be patterned makes them attractive for studying the organization of neural networks and has the potential to develop new devices for neural prosthesis. We note that additional toxicity studies of carbon nanotubes are necessary so that exposure guidelines and safety regulations can be set.

Keywords: carbon nanotubes, modification, substrates/scaffolds for neuronal growth, electrical recordings, interface

1. Introduction

Nanoneuroscience is a recently coined term describing the convergence of the existing but different worlds of nanotechnology, chemistry, engineering and neurobiology. A large body of research is emerging that hints at the potential applications of nanotechnology in neuroscience, although the basic scientific and clinical progress is limited by the intrinsic complexities of the mammalian central nervous system (CNS; Silva, 2006). Nevertheless, recent studies interfacing nanomaterials with neural systems have provided a foundation for generating a new class of multifunctional devices and hybrid systems that could help in the repair of damaged CNS tissue and that have paved the road to nanoneuroscience as a new discipline which can aid in unveiling functional properties of brain.

Carbon nanotubes (CNTs) have been at the forefront of nanotechnology due to their unique electrical, mechanical and chemical characteristics, which allow the development of variety of miniaturized devices with outstanding properties (Krishnan et al., 1998). CNTs are cylindrically shaped carbon nanostructures, discovered in the 1990s (Iijima, 1991; Iijima and Ichihashi, 1993; Bethune et al., 1993). The simplest geometry of CNT is that of a single-walled nanotube (SWNT). Here, a single graphene sheet is rolled up and closed at each end by a hemispherical fullerene cap; SWNT diameters typically range between 0.8 and 2 nm. Multi-walled nanotubes (MWNT), on the other hand, are composed of numerous concentric graphite cylinders and can reach diameters of up to 100 nm. Interestingly, depending on hexagonal lattice structure, the resulting electronic conduction within SWNTs approaches the properties of an insulating, conducting or semiconductive material (Krishnan et al., 1998). CNTs can be systematically manipulated by attaching different chemical groups and can be functionalized to display, for example, variety of surface charges, such as positive, neutral or negative. The ease of such chemical modifications combined with their innate characteristics make CNTs an excellent candidate for interfacing them with neural systems. Indeed, there have been developments of biocompatible, durable and robust substrates/scaffolds and devices to affect neuronal growth and potentially provide therapies for CNS disorders (reviewed in Malarkey and Parpura, 2007). Approaches toward the characterization of the interactions between CNTs and brain cells and their circuitry offer great opportunities for nanotechnological applications for the nervous system. This review article briefly summarizes recent advances in the study of the effects that CNTs exert on neural cells when used as substrates for cellular growth. We also discuss the inherent capacity of such substrate for interfacing with neuronal electrical activity.

2. CNTs as substrates/scaffolds for neuronal growth

Recently, CNTs have attracted tremendous attention due to their use as scaffolds for neuronal growth; the extension of this is their potential use for re-establishing intricate connections between neurons after injury. Such possible future applications in tissue engineering require an understanding of mechanisms and chemical modifications that may alter the interactions of biological cells and tissues with the nanomaterials (Fortina et al., 2005). During the last decade, several research groups have assessed CNTs as substrates for neuronal growth, demonstrating the biocompatibility of these materials. The application of CNTs in neuroscience research has been oriented towards the use of both MWNTs and SWNTs.

Mattson et al. (2000) applied MWNTs dispersed in ethanol onto polyethyleneimine (PEI) coated glass coverslips and then allowed evaporation of ethanol to generate an MWNT layer on top of PEI. They demonstrated that rat hippocampal neurons plated on such a substrate survived and continued to grow for at least 8 days in culture. These results indicated for the first time that CNTs support relatively long term cell survival in culture (Mattson et al., 2000). Other researchers confirmed these findings, by characterizing the presence of growth cones, neurite

outgrowth and branching in rat hippocampal neurons grown on MWNTs (Hu et al., 2004). Since then MWNTs have been considered as biocompatible substrates, although they did not support as much neurite outgrowth and branching as other known permissive substrates for neuronal growth, such as PEI (Mattson et al., 2000; Hu et al., 2004). SWNTs were also tested for their biocompatibility (Liopo et al., 2006). This material allowed long term survival of NG108 neuroblastoma cells in culture. NG108 cells grown on SWNT films displayed attachment and growth albeit with a reduced rate when compared to their growth on more permissive substrates, such as tissue-treated polystyrene (Liopo et al., 2006). When combined, these reports support the notion that CNTs, MWNTs and SWNTs, could be used as biocompatible materials permitting cell viability. However, they also indicated some limits in respect to CNTs' ability to support neurite elongation and branching. Such parameters were quantified in neurons and/or NG108 cells grown on CNTs and appeared reduced when compared to those gathered from neurons grown on widely accepted permissive substrates (Hu et al., 2004; Liopo et al., 2006). This issue was addressed by Galvan-Garcia and co-workers (Galvan-Garcia et al., 2007). They reported that directionally oriented CNTs in the form of sheets or yarns offer an alternative presentation of CNTs to cells. These CNT forms promoted cell attachment, differentiation and cell growth. Additionally, when highly purified, these CNTs also allowed neurons to extend processes of which the number and length were comparable to those of neurons grown on a permissive substrate, represented by polyornithine pre-treated glass (Galvan-Garcia et al., 2007). Thus, the interaction between neurons and CNTs may be affected by the purity of CNTs as well as by the 3-dimensional organization of the CNT substrate/scaffold.

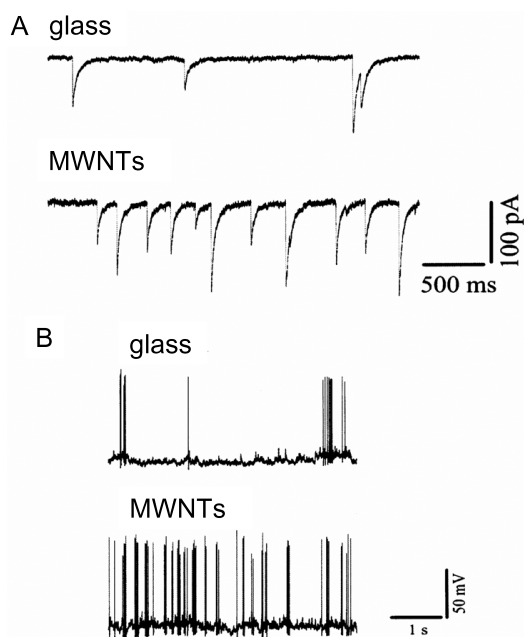


Figure 1 Carbon nanotube substrate affects synaptic activity and neuronal excitability. Neurons grown on MWNT based substrate display increase in the frequency of A) spontaneous post-synaptic currents and B) “firing rate” when compared to neurons grown on glass coverslips. Modified from Lovat et al, 2005.

Although highly informative, the above studies, that assessed the viability of neurons grown on CNTs and their morphology, have not investigated the presence of functional synaptic transmission. Indeed, the electrophysiological properties of neurons grown on CNTs and the functional status of their synaptic connections were addressed by Lovat et al. (2005). These authors reported for the first time the effects of CNT substrates on the electrical behavior of

neurons and neuronal networks in culture. The CNTs used for neuronal growth were first functionalized allowing uniform solvation and then deposited onto a glass substrate. Following evaporation of the solvent, CNTs were defunctionalized by thermal treatment generating glass coverslips covered by a film (i.e., a nano-meshwork) of native/non-functionalized CNTs. This strategy allowed a long term and stable retention of CNT films on glass and, moreover, long term neuronal cell survival in culture. When compared to control plain glass surfaces, MWNT substrates boosted neuronal network activity under culturing conditions (Lovat et al., 2005). Similar effects were found when using SWNTs (Mazzatenta et al., 2007). CNTs increased the frequency of spontaneous postsynaptic currents in hippocampal neurons; the recording of these currents provide clear evidence of the existence of functional synapses. Additionally, neurons grown on CNTs displayed increased excitability as evidenced by the increased frequency of action potential discharges, or so called “firing rate” (Figure 1). Thus, the growth of neurons on a CNT meshwork was accompanied by a significant increase in the network activity. Since there is a lack of evidence for specific chemical interactions between neurons and non-functionalized CNT, it is likely that the electrical conductivity of the CNT substrate might be underlying this specific electrophysiological effect (Lovat et al., 2005; also see below).

Great attention has been directed towards generating CNT scaffolds that may guide nerve tissue regeneration after injury. Conceptually, this aim could be achieved by designing substrates able to promote controlled neurite outgrowth and branching. In this respect, several research groups modified CNT substrates/scaffolds by conjugating them with biologically active compounds or with molecules yielding various charges at the surface of modified CNTs. Mattson et al. (2000) reported that rat hippocampal neurons elaborated multiple neurites, with extensive branching, when they were grown on pre-coated MWNTs. MWNTs were pre-coated via physisorption, with the bioactive molecule 4-hydroxynonenal, a lipid peroxidation product that in a biological environment controls neurite outgrowth (Mattson et al., 2000). In addition, it has been observed that neuron-like differentiated PC12 cells display improved attachment to the substrate when grown on MWNTs pre-coated with a thin layer of type IV collagen, an extracellular matrix protein (Nguyen-Vu et al., 2006). These results indicate that modifications of CNTs by physisorption of functional groups can be used to affect the interaction between neurons and nanomaterials. It should be noted, however, that molecules attached by physisorption do not exhibit a stable and long lasting retention to the CNTs.

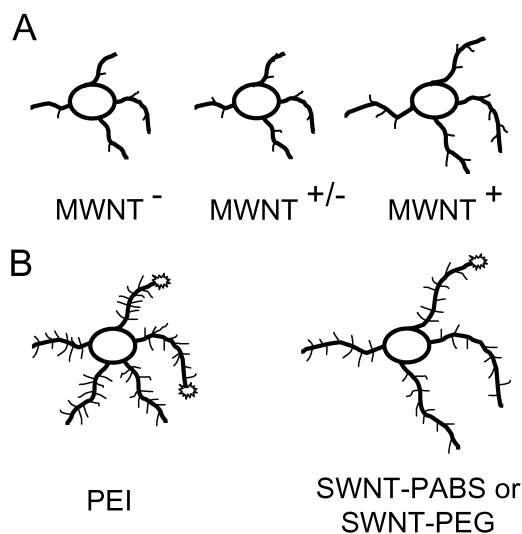


Figure 2. The effects of carbon nanotubes on growth cones, neurite outgrowth and branching. A) Manipulating the charge carried by functionalized MWNTs can be used to control the outgrowth and branching pattern of neuronal processes. B) Water soluble SWNT-PABS and SWNT-PEG copolymers

when added to the culturing medium can increase the length of selected neuronal processes while reducing the number of growth cones. a) Modified from Hu et al, 2004 and b) Modified Ni et al, 2005.

This issue can be resolved by chemical modification of CNTs using covalent attachments of functional groups to CNTs (Hu et al., 2004). A downside of covalent modification using biologically active compounds is the possibility of losing their specific activity. However, Matsumoto et al. (2007) demonstrated that neurotrophin, a key protein in differentiation, maintains its biological activity, promoting the neurite outgrowth of chick dorsal root ganglion neurons (Matsumoto et al., 2007), despite its covalent attachment to CNTs. Covalent modifications of CNTs have also been used to manipulate the charge carried by functionalized CNTs. Namely, it is accepted that neuronal growth and development on glass or plastic is enhanced by treating their surfaces with positively charged polymer molecules such as polylysine or polyornithine. Hence, Hu et al. (2004) chemically functionalized MWNT with carboxyl group, poly-m-aminobenzene sulfonic acid (PABS) or ethylenediamine to obtain CNTs that exhibited negative, neutral or positive surface charges, respectively, at the physiological pH of extracellular medium. They observed that rat hippocampal neurons grown on positively charged MWNTs showed longer neurites with more extensive branching and a larger number of growth cones when compared with neurons grown on neutral or negatively charged CNTs (Hu et al., 2004; Figure 2). Similar results have been reported for neuronal growth on chemically modified SWNT, where their functionalization with the positively charged PEI promoted neurite branching and outgrowth in comparison with that of neurites extending from neurons grown on native CNTs (Hu et al., 2005). On the contrary, the functionalization of SWNT substrates with 4-benzoic acid or 4-tert-butylphenyl to obtain negatively charged or neutral SWNTs, respectively, reduced both attachment and survival of cells grown on these modified substrates (Liopo et al., 2006). Chemical modification of CNTs is therefore emerging as a powerful tool to design substrates which can control the growth and morphology of neuronal cells.

One corollary of the ability to specifically affect neurite outgrowth and branching by modification of CNTs is to apply them in the future for neurite regeneration after injury. To achieve such an ambitious goal there are at least several additional requirements to be fulfilled: (i) the substrate would have to be a freestanding structure without support by glass or plastic, (ii) CNTs would need to be patterned, (iii) water dispersible CNTs should be available, and (iv) these materials should be tolerated by cells. There have been advances in all of the above categories. (i) Freestanding SWNT composite cylinders formed by rolling up poly(N-cetyl-4-vinylpyridinium bromide-co-N-ethyl-4-vinylpyridinium bromide-co-4-vinylpyridine)-SWNT copolymers were made and used to support the growth of NG108 cells; neurite outgrowth was prominently displayed (Gheith et al., 2005). (ii) Micropatterned arrays of CNT islands were generated and used to study self-organization of neural networks (Gabay et al., 2005; Sorkin et al., 2006). This advance makes it possible to grow cells in defined regions. More generally they can be used for biocomputational purposes and/or for studying neural networks. (iii) Chemically functionalized water dispersible soluble SWNTs, SWNT-PABS or SWNT-polyethylene glycol copolymers, were designed and applied to neurons which displayed enhanced outgrowth of selected neurites (Ni et al., 2005; Figure 2). Such SWNTs could be injected at the local site of nerve injury to increase the chance of bridging the damaged site. (iv) CNTs turned out to be substantially less toxic than carbon black when applied to cultured cells (Magrez et al., 2006).

Therefore, CNTs have the potential to be the next generation of materials for use in implantable neuroprosthetic devices and in injectable local treatment to promote nerve regeneration after injury.

2. Electrical interactions between neurons and CNTs

One of the exciting developments at the juncture between neuroscience and (nano)technology is that of designing neural interfaces. For example, devices for CNS implantation were developed to

control motor disorders (Benabid et al., 2005) and drug-resistant psychiatric conditions (Nuttin et al., 2003; Mayberg et al., 2005) and to translate willful brain processes into specific actions through the control of external devices (Llinas et al. 1973; Mussa-Ivaldi and Miller, 2003; Lebedev et al., 2006). A prerequisite for exploiting CNTs in such biomedical devices is to understand their actions on neurons, especially in respect to neuronal excitability, changes in ionic conductances, and in intercellular signaling via synaptic transmission. Such knowledge would help designing CNT-based neuronal interfaces, while minimizing unwanted interactions.

The first evidence that CNTs can affect ionic conductance came from the ability of SWNTs to block ion fluxes through potassium ion channels expressed in a cell line (Park et al., 2003). The authors speculated that the CNTs blocked the channel pore and interrupted ion permeability. Similarly, SWNTs caused a significant impairment in cytoplasmic Ca^{2+} elevation when neurons were depolarized; this may be due to CNTs interfering with the functioning of Ca^{2+} channels (Ni et al., 2005). Thus, these results caution that CNTs used as a structural component in biological applications may inadvertently affect the activity of cells with which they come into contact.

As mentioned earlier Lovat et al. (2005) and Mazzatenta et al. (2007) studied the electrical properties of neurons grown on CNTs. These studies strongly suggested that growing neurons on conductive CNTs platforms promoted a significant increase in network operation. Such an effect was not related to an increased number of surviving neurons in the presence of CNTs. These authors determined that the cellular composition of 8-day-old hippocampal cultures, using immunocytochemical markers for astrocytes¹² and neurons (Lovat et al., 2005; Mazzatenta et al., 2007), was similar for cells grown either on CNT-coated or plain glass coverslips. The authors speculated that CNTs might provide a bidirectional electrotonic current transfer, causing a redistribution of charge along the surface of the membrane, ultimately increasing neuronal excitability (Lovat et al., 2005). It is interesting to note that several electrophysiological membrane properties measured on the cultured hippocampal neurons did not indicate the occurrence in these cells of changes in ionic conductance brought about by the CNTs layers directly (Lovat et al., 2005; Mazzatenta et al., 2007).

Liopo et al. (2006) suggested that the conductive nature of CNTs could be used to stimulate neurons. They created a stimulation chamber containing dorsal root ganglion neurons grown on top of a SWNT film. Application of a current through the CNTs resulted in an inward transmembrane current, measured in neurons by a whole-cell patch clamp. Such current was indistinguishable from those induced by direct patch-clamp electrode-mediated depolarizing voltage steps (Liopo et al., 2006). Similarly, the electrical properties of SWNT films made by the layer-by-layer method have been utilized for external stimulation of NG108 cells in culture (Gheith et al., 2006). Again, such external stimulation via SWNTs evoked inward currents in NG108 cells that were indistinguishable from those evoked by intracellular stimulation via whole-cell voltage clamp. These rapid inward currents were reminiscent of those usually associated with the ion flux through voltage-gated Na^+ channels. Taken together, these reports indicate the possibility of stimulating neurons in culture via SWNTs.

Mazzatenta et al. (2007) employed scanning electron microscopy, electrophysiology and computational modeling in order to understand the nature of electrical coupling between neurons and CNTs. Scanning electron microscopy showed intimate contacts between hippocampal neurons and the substrate, composed of purified SWNTs, used to support cellular growth. Such contacts might represent a physical conduit for electrical coupling between SWNTs and neurons, since stimulation via SWNTs reliably evoked postsynaptic responses in neurons. The recordings suggested that coupling between neurons and SWNTs is in part resistive; further studying involved mathematical modeling. This combined approach implicated that any resistive coupling between bio-membranes and SWNTs is qualitatively indistinguishable from a coupling between SWNT and the patch-pipette through the patch-seal path to ground (Mazzatenta et al., 2007). Thus, whole-cell patch-clamp recordings from neurons stimulated by SWNTs (see also Liopo et

al., 2006; Gheith et al., 2006) may yield deceiving results. Hence, due to the non-idealities of the single electrode voltage clamp, eliciting Na⁺-currents in neurons through SWNT stimulation does not conclusively prove a resistive coupling between SWNTs and neurons. Rather, this can be accomplished by detecting synaptic responses, evoked by action potentials elicited in not-clamped neurons using the electrical stimulus delivered via SWNTs (Mazzatenta et al., 2007).

The use of a combination of electrophysiology and computational/mathematical modeling perhaps represents an entry into the realm of computational (nano)neuroscience. Owing to the recent availability of powerful computer simulation software (Carnevale and Hines, 2006; Markram, 2006) this emerging field has promising future.

The studies discussed above demonstrated the ability to stimulate neurons via CNTs. In many cases, electrical stimulation of neurons employed in neural prosthesis and neurological therapies requires microelectrode arrays. Indeed, CNT based micro arrays have been constructed (Gabay et al., 2005; McKnight et al., 2006; Nguyen-Vu et al., 2006; Nguyen-Vu et al., 2007; Yu et al., 2007; Wang et al., 2006) by growing vertically-aligned carbon nanofibers (VACNFs). Such electrodes offer higher limits for charge injection than presently used metal based devices. Thus, not only can VACNFs arrays be used for electrochemical applications (Wang et al., 2006; Gabay et al., 2005; McKnight et al., 2006), but they can also be utilized for repetitive stimulation of, for example, rat hippocampal neurons grown on them (Wang et al., 2006). Recently, these VACNF devices were also employed for extracellular recordings and stimulations in cultured hippocampal organotypic slices (Yu et al., 2007). Taken together, it appears that CNT based arrays offer an improved platform for interfacing with neurons and neural tissue and hold great promise for use in studying networks and medical applications.

Concluding remarks

The intent of this review was to put forward recent advances in the generation of CNT scaffolds for neuronal growth. We also discussed the effects that CNTs exert on neural cells when used as substrates for cellular growth and their potential for an electrical interface with neural cells. It is apparent from the presented body of work in this review that CNTs have great potential in biotechnology and medicine. Some of the initial concerns regarding their toxicity (Service, 2003; Service, 2004) have been somewhat alleviated by the fact that they seem to be less hazardous than carbon black, a different form of carbon which has been widely used and has defined exposure guidelines. As it is inevitable that widespread commercialization of CNTs will occur, we still need to stay alert in respect to the potential harm that these new nanomaterials could exert on human health and must employ additional toxicity testing. For now, it appears, however, that the potential benefit warrants further investigations in research laboratories from a biological standpoint.

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