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Carbon nanotubes: a promise for nerve tissue engineering?

Abstract: Owing to their peculiar physical and chemical properties, carbon nanotubes are intensively studied for many different applications, including those in the biomedical field. Carbon nanotubes are electrically conductive, elastic but mechanically resistant and these features, among others, have made them an ideal material for therapeutic applications at the neural tissue interface. The major recent advances in the study of carbon nanotube-based materials aimed at nerve tissue regeneration and functional recovery are reviewed here.

Keywords: carbon nanotubes; neural interface; neuronal tissue repair.

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1 Introduction

Carbon nanotubes (CNTs) are one of the more exciting and debated class of materials of the past decades. Since their discovery, more than 20 years ago [1], they have captured the attention of physicists, chemists and materials scientists, and they have been suggested as core materials for several applications in a variety of fields: electronics, aerospace technologies, catalysis, sensing and many others [2]. The study of CNTs in the biomedical field is one of the more popular in terms of research papers in the frame of the great development that nanotechnology for medical purposes has recently put forward [3, 4]. CNTs possess a very large exposed surface to interact with biological tissues, but their asbestos-like and biologically unfriendly appearance has raised serious concern about their possible toxicity for living organisms and several studies have supported this concept [5–7]. Recent studies suggest that CNT toxicity is directly related to specific features of this material, such as their dimensions [8] or the presence of impurities, for example, metallic particles or synthetic residues [9]. In fact, an accurate purification and especially the functionalization of CNTs, together with the selection of the appropriate CNT size and route of administration, lead to a dramatic reduction (or even elimination) of CNT toxic effects [10–12]. Indeed, it has been demonstrated that appropriately functionalized CNTs are uptaken by B and T lymphocytes as well as macrophages *in vitro*, without affecting cell viability [13]. Considering the toxicity at the neural tissue level, several studies report the biocompatibility of CNTs with neuronal cells *in vitro*, particularly when CNTs are functionalized with positively charged amino groups (see below). Moreover, the functionalization can make CNTs water soluble and easy to handle and the possibility of inserting different functional groups on the same CNT molecule is particularly promising to exploit them in the perspective of new strategies for improved therapeutic approaches [14–16].

CNTs possess peculiar physicochemical properties: they are highly thermally and electrically conductive, mechanically resistant but very elastic at the same time, they have large specific surface area and they have organized fractal-like nanostructure that mimics the extracellular matrix (ECM) [17]. All these features have made them theoretically perfect candidates for the development of central nervous system (CNS) interfaces. In general, neurons and glial cells prefer positively charged substrates for their proper adhesion, therefore a modification of glass and plastic culture substrates (i.e., with charged molecules such as poly-lysine or poly-ornithine, or with proteins such as laminin for optimum growth and neurite extension) is often required [18]. The possibility of functionalizing CNTs with this type of molecules makes them more neuro-friendly and improves their biocompatibility [19].

In this review, the evolutionary path followed by the development of CNT-based materials designed to be interfaced with neuronal cells will be described. The first part of the review will outline the *in vitro* studies that have demonstrated how CNTs can be an optimal substrate where neuronal cells are able to survive and grow. Different types of CNT-based materials are overviewed ranging from films to three-dimensional architectures. The last part is focused on the description of the impact that CNTs

have on the functional (electrical) activity of neurons (mainly studied by the authors).

2 CNTs sustain neuronal survival and promote neuronal outgrowth

The history of the study of CNTs as substrates for neuronal cells started from the initial observation that, when seeded on variously designed CNT-based substrates, neurons were able not only to survive but also to grow and extend their axonal processes. This first result was reported by Mattson and colleagues [20] who seeded dissociated embryonic rat hippocampal neurons on a mat of multi-walled CNTs (MWNTs) deposited onto polyethyleneimine (PEI) covered glass. In this condition, neurons were able to grow for and to elongate their neurites in all directions, but did not form branches on the CNT. CNTs were then noncovalently functionalized with 4-hydroxynonenal, a molecule that is known to induce increases of intracellular calcium levels and to modify cytoskeletal proteins regulating neurite outgrowth. When seeded on these modified CNTs, neurons increased the number and length of their neurites two/threefold more than when seeded on unmodified CNTs.

The same research group found that the surface charge of CNTs is an important feature to control the neurite outgrowth as demonstrated by the presence of increased growth cones, longer average neurite length and more elaborated neurite branching in neurons grown on positively charged MWNTs in contrast to neutral or negatively charged MWNTs [21]. The growth boosting effect of positively charged CNT surface was confirmed in a subsequent study where a copolymer of PEI was grafted on CNTs and the CNT-PEI copolymer was used as substrate for neuronal culture growth [22]. In this work, authors compare results obtained both on pristine MWNTs and on functionalized single-walled carbon nanotubes (SWNTs), without indicating any prevention in this comparison. In general, in the literature of this field experiments are often conducted both on single- and on multi-wall CNTs. There seems to be no indication at present that could highlight the advantages of one class vs. the other.

Besides the surface charge, another important feature, able to positively affect cell growth, is the possibility of modulating the surface nanotopography of CNT substrates. Directionally oriented CNTs in the form of sheets and yarns were shown to serve as a viable substrate for the growth of a variety of cell types [23]. Fibroblasts grown on this material exhibited an increased diameter with numerous and longer cellular processes. In addition, fibroblasts migrated on the CNT support, presenting the characteristic migration morphology: frontal lamellopodial protrusions and posterior tail retractions. The average migratory velocity was doubled compared with that onto glass or plastics. Also neurons from murine cerebellum and cerebral cortex, which are normally more demanding in their requirements for survival and growth, and cortical neurons were observed to extend neurites onto the pristine CNTs similar to those grown on polyornithine treated glass, interacting seamlessly at the glass-CNT interfaces and showing directed growth along the orientation of the pulled CNT fibers. The precise mechanism by which pristine, hydrophobic CNTs provided a prime substrate for neural growth is not known. CNT roughness and the large exposed surface might play an important role in their ability to favor neuronal adhesion [24]. A further possibility is that CNTs could be selectively coated by serum proteins such as fibrinogen and apolipoproteins [25] thus favoring cell adhesion.

Scaffolds designed for neural tissue engineering are expected to favor and/or mimic the electrical properties of nerves and, possibly, to be able to electrically stimulate neurons; scaffolds electrical conductivity and its impact on neuronal behavior therefore appear important issues. To start exploring them, Malarkey and coworkers fabricated films of SWNTs covalently bound to polyethyleneglycol (PEG) chains with different thicknesses, obtaining films with different conductivity (but similar roughness), and observed that these differentially conductive materials affect the growth of neuronal cells when used as culture substrates. They used cells grown on PEI substrates as controls and observed that only CNT substrates of certain conductivity promoted the growth of longer neurites, without affecting the total number of processes. In addition, these authors observed that the mean area of the neuron cell body was also increased by conductive CNTs, whereas the number of growth cones at neurite tips was decreased. These observations should demonstrate, although the control with only PEG is not reported, that the positive CNT effects on neuronal morphology are promoted only in a narrow range of conductivity (Figure 1A) [26].

CNTs were demonstrated to positively affect the growth modalities of cultured neurons not only in the form of deposited substrates but also in solution as hydrosoluble compounds capable of efficiently delivering growth factors in the liquid culture medium. For example, MWNTs have been covalently coated with neurotrophins [nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF)] that are endogenous soluble proteins regulating

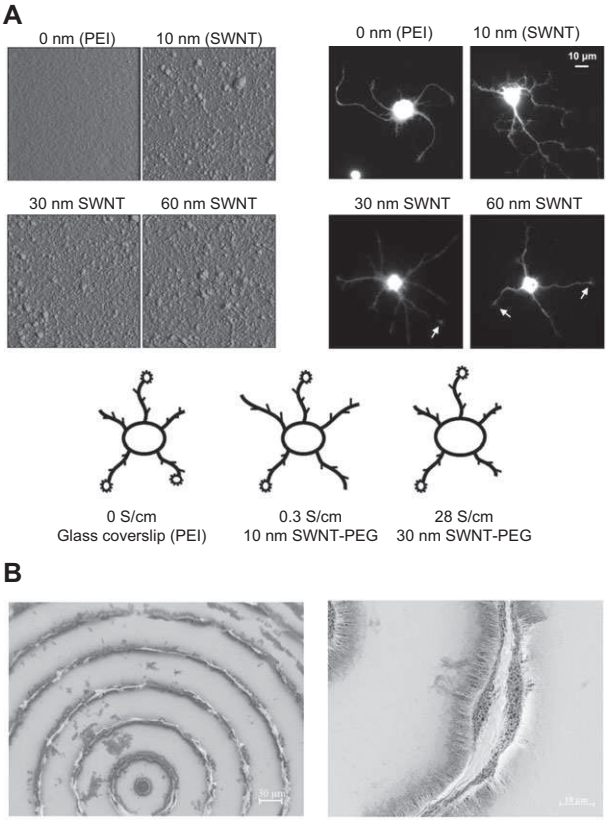


Figure 1 CNTs differentially modulate neuronal growth and morphology.
(A) left, atomic force microscopy (AFM) images showing SWNT-PEG films of different conductivity, used as substrates for the growth of hippocampal neurons (PEI was used as control substrate). Right, fluorescent images of live neuronal cells (stained with calcein) showing different growth and morphology depending on substrate conductivity. Bottom, summary of the impact of differentially conductive substrates on neuronal growth and neurite outgrowth [26]. Reprinted (adapted) with permission from Malarkey et al. [26] Copyright (2009) American Chemical Society. (B) left, SEM micrograph showing a patterned vertical MWNT substrate used for neuronal cell culturing. Cells preferentially adhered and grew on patterned MWNTs, with neurites specifically growing along MWNT arrays (right) [27]. Reprinted from Zhang et al. [27] with permission from Elsevier B.V.

the survival, growth, morphological plasticity and protein synthesis, in functionally differentiated neurons. When the neurotrophin-coated CNTs were added to the cell culture medium, they promoted neurite outgrowth. This demonstrated that the neurotrophins were not altered in their functionalities when linked to CNTs [28]. In another study, water soluble PEG-functionalized CNT derivatives (without neurotrophins) directly applied to the culture medium resulted in an altered growth and morphology of neurons, notably an extended neurite length and a reduced number of neurites [29]. The explanation of these phenomena could be ascribed to the fact that, when cells are exposed to soluble CNTs, the endocytic processes are inhibited but not the exocytotic ones [30]. This could be due to the nanotubes mechanically affecting the endocytotic vesicle: the relatively long length of the nanotubes could prevent the vesicle from closing and pinching off from the membrane, trapping it and preventing endocytosis. Also, this configuration of nanotubes physically sticking in vesicles would not prevent exocytosis, as the nanotube would simply be expelled as the vesicle collapses into the membrane during full fusions. The observed increase in total membrane area during neurite outgrowth would occur by incorporating the new membrane by vesicle fusion or exocytosis [30].

3 CNT/polymer-based composites

A step forward in the study of CNT-based materials for nerve tissue engineering was the development of polymeric composites incorporating CNTs into biocompatible networks to produce conductive neuron-comfortable scaffolds. This strategy should improve the biocompatibility of unconstrained CNTs, in particular using biopolymers such as chitosan, agarose or collagen. Furthermore, these composites should be supple materials that can be adapted to directly interface complex neuronal tissues (e.g., to create a nerve guidance or conduit at a spinal lesion site).

CNTs have been incorporated into chitosan films deposited onto poly(methyl methacrylate) (PMMA) supports and into chitosan fibers obtained by coagulation and hydrodynamic focusing methods [31]. To render these materials more “cell friendly” they were coated with laminin (LN). PC12 cell growth (a model system for neuronal differentiation) was evaluated to verify the biocompatibility of the substrates. Experimental results showed that PC12 cells grew and extended their neurites on both films and fibers. The neurites of PC12 cells extended in the same direction as LN-coated CNT/chitosan fibers, regardless of fiber width (i.e., LN-coated CNT/chitosan fibers induced directional neurite growth). Conversely, neurites on LN-coated CNT/chitosan films had no discernible pattern.

In another study, PC12 cells were grown on electrically conductive CNT/collagen composites: collagen is an important component of basal membranes and promotes cell adhesion, differentiation and survival. With the right ratio between CNTs and collagen, it was possible to achieve a very good substrate for neuronal growth and differentiation while, upon electrical stimulation, cells

were also able to increase their neurite spreading on this surface [32].

CNT fibers with agarose were produced by wet spinning and by molding the fiber in a hollow tube [33]. Fibers were then functionalized with laminin. Cytotoxicity and cell attachment were evaluated showing that these conductive substrates are biocompatible and allow the growth of astrocytic cell cultures. CNT/agarose fibers were also implanted *in vivo* in rat brain for a preliminary evaluation of the foreign body reaction to the fiber insertion: interestingly, the authors observed only a moderate glial activation, similar to that reported for other biocompatible materials such as silicon, thus confirming the biocompatibility of the CNT composite [34].

Other examples of hybridization of CNTs with biopolymers with the aim of obtaining a biocompatible scaffold able to accommodate neuronal cells include electrospun fibers of poly(D,L-lactic-co-glycolic acid (PLGA) [35] and of poly (L-lactic acid-co-caprolactone) (PLCL) [36]: in both cases, functionalized CNT-based substrates were shown to be able to promote neurite outgrowth in *in vitro* neural cells.

Also, synthetic polymers have been used as partners in the production of CNT composites. CNTs have been used as dopants for the production of a very stable poly(3,4-ethylenedioxythiophene) (PEDOT)-based coating for neural stimulation electrodes [37]. Positive charge-modified SWNTs have been assembled using a layer-by-layer technique together with a negatively charged polymer (polyacrylic acid, PAA) achieving a freestanding film that has been used as substrate for a neuroblastoma/glioma hybrid cell culture: the film resulted to be biocompatible and to induce strong cell differentiation towards neuronal lineage, as differentiated cells extended several neurites on the surface [38]. In a later study, the same group investigated the stimulation of cells grown on this substrate through the substrate itself, demonstrating that this material possesses a sufficiently high electrical conductivity to electrically stimulate excitable neuronal cells [39].

4 Patterned CNT substrates for cell culture growth

When deposited or grown on a patterned array, CNTs were shown to allow the growth of differentiated neuronal cells whose extended neuronal processes followed the CNT tracks. CNTs can, in fact, be deposited or grown directly from patterned catalysts to create aligned patterns with a variety of geometries. This could be a very important issue in order to give a preferential direction to the (re)growth of neuronal processes. Correa-Duarte et al. [40] fabricated a three-dimensional (3D) CNT structure starting from vertically aligned thin films and obtaining pyramid-like structures and cavities by means of capillary forces. These structures were able to support the growth, spreading and proliferation of fibroblast cell lines. CNT patterning on silicon oxide surfaces by microlithography can achieve the proper topography suitable to control neural cell adhesion, spreading and growth [40].

Zhang et al. [27] engineered patterned vertical MWNTs of different lengths using a combination of microlithography and chemical vapor deposition. MWNTs were then coated with poly-L-lysine (PLL), that is known to enhance neuron adhesion and protein absorption by altering surface charges on the culture substrate. These authors observed that neuronal cells exhibited neurite growth along the edges of the patterned substrate (Figure 1B) only onto longer MWNTs. It was suggested that long CNTs are more flexible and can deform their shape and this would result in an increased surface roughness at the nanoscale, which offers a biomimetic topography similar to *in vivo* conditions. Furthermore, the authors observed the formation of neural bridges among patterned boundaries and the neurites extended over a longer distance (around 20 μm) to form synaptic connections. These results confirm the importance of morphology and topography of substrates for proper neuronal adhesion and growth. Similar results have been obtained by Gabay et al. [41]: islands of CNTs were patterned onto quartz surfaces and dissociated cortical neurons were seeded on them. After several days of incubation, cells aggregated and accumulated on the CNT covered regions from where they spread processes that tended to bridge gaps between neighboring islands. In general, the processes consisted of single axons or bundles of axons and dendrites. The formed networks have been tested for their electrical activity by the patch-clamp technique and their functionality was found to be well preserved, giving a further proof of the full neurocompatibility of these materials.

Regarding the mechanism of attachment of cell processes to CNT substrates, Sorkin et al. [24] observed that this type of cells tend to intertwine, bend and curl when cultured on a substrate that possesses a roughness comparable with the length scale of its processes (as CNTs are). High resolution scanning electron microscopy (SEM) images revealed that the curly appearance of the processes results from their curling and looping around the CNT surfaces.

In 2007, Galvan-Garcia et al. have obtained a guiding substrate for neurite outgrowth patterning CNT in the form

of parallel or crosslinked yarns [23]: seeded hippocampal neurons have shown to extend neurites only along CNT yarns and these neurites have little or even no branching. It should be noted that a reduced axonal branching means a better axonal pathfinding: indeed, in another study this type of axonal behavior has been shown to be at the basis of the excellent and precise recovery of function observed after neuronal lesion [42].

5 CNTs improve *in vitro* neuronal electrical performance

Once established that CNT-based substrates are not only biocompatible but can also stimulate and improve neuronal cell growth and ability to extend processes, the study of CNTs for tissue regenerating purposes provided further outstanding surprises when CNTs effects on the electrical activity of neuronal networks were investigated.

In the past decade, it has been shown that CNT-based substrates are indeed able to profoundly impact on neuronal physiology from the functional (electrical) point of view [43–46]. CNTs were used as substrates for neuronal growth in the form of a mat of pure, non-functionalized MWNTs, obtained by CNT functionalization (by 1,3-dipolar cycloaddition of azomethine ylides) (Figure 2A), deposition and thermal defunctionalization, yielding a stable and homogeneous CNT meshwork (Figure 2B) [47, 48]. The pioneering study of 2005 [43] compared the spontaneous synaptic activity of hippocampal neuronal networks directly grown on this MWNT mat with that of control networks grown on pure glass by means of the patch-clamp technique. The frequency of spontaneous events (postsynaptic currents, PSCs) in networks cultured on CNTs showed a very strong increase (approx. sixfold) compared with controls (Figure 3A). CNT substrates effect on the excitatory and inhibitory components of network activity was comparable, with no alteration of the balance between inhibition and excitation in the neuronal network. Accordingly, CNT substrates strongly increase the spontaneous firing frequency, further confirming that neuronal network operations are promoted by the CNT meshwork [43], with no impact of CNTs on neuronal survival or neurite extension [43]. Interestingly, SWNTs have been shown to be as efficient as MWNTs in boosting neuronal network activity in hippocampal cultures [43, 49]. The contact between neuronal membranes and CNTs, as revealed by SEM and

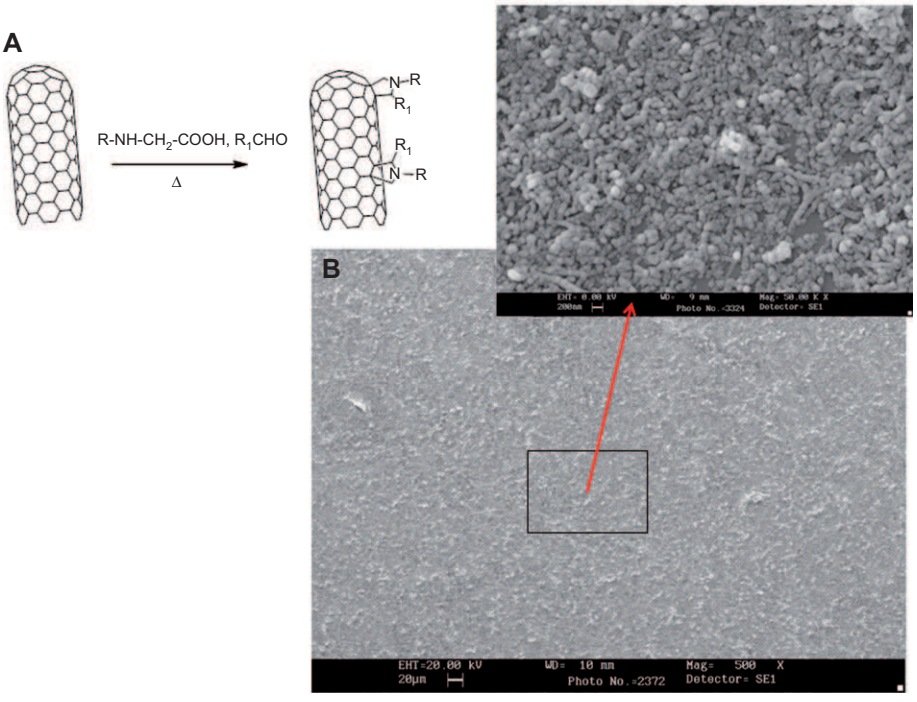
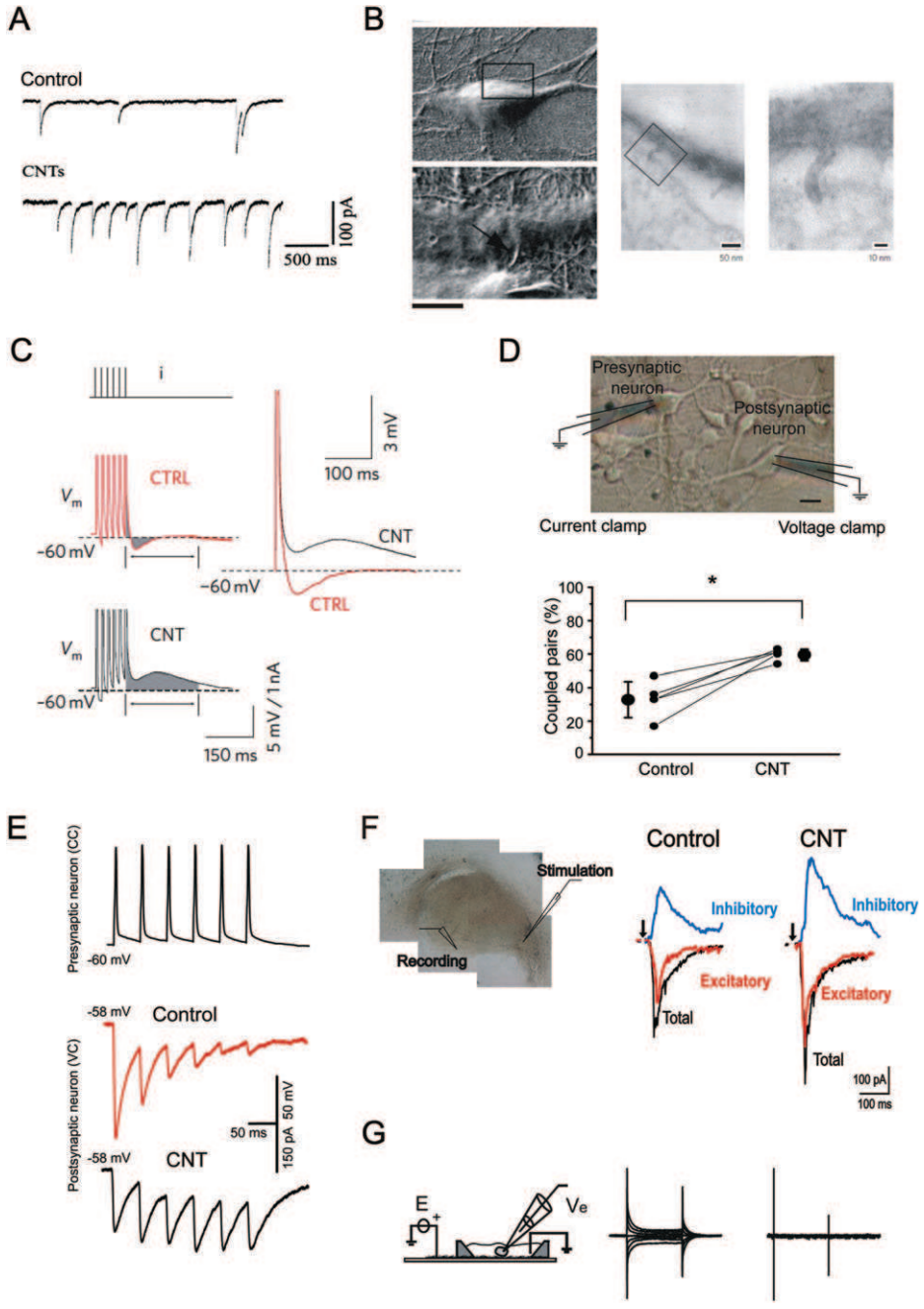


Figure 2 Functionalization of CNTs by 1,3-dipolar cycloaddition of azomethine ylides and deposition on glass coverslip.
(A) Scheme of the reaction of 1,3-dipolar cycloaddition of azomethine ylides used for the functionalization of CNTs in order to obtain derivatives that are soluble in many organic solvents [43]. (B) SEM images of a typical sample of CNTs deposited onto a glass coverslip (A. Turco and M. Prato, unpublished).



transmission electron microscopy (TEM) investigations, is very tight, but discontinuous (Figure 3B; [44, 49]), an observation which suggested the hypothesis that nanotubes could provide a shortcut for the direct electrotonic current transfer between different neuronal compartments, causing a redistribution of charge along the surface of the membrane and reinforcing direct coupling among neurons. This hypothesis has been tested in a subsequent study by single cell electrophysiology experiments, where neurons were forced to fire train action potentials and the presence of an extra membrane afterdepolarization potential (ADP; Figure 3C) was monitored [44]. ADP is an indirect effect of Ca^{2+}-mediated electrogenesis and represents backpropagating action potentials, a neuronal electrical regenerative property implicated in many important cellular processes such as the fine-tuning of synaptic activity, the expression of spike timing-dependent plasticity, the release of modulatory messengers and the modulation of synaptic plasticity [50–54]. The study showed that the occurrence of backpropagating action potentials is more frequent in neurons interfaced to CNTs, indicating that CNTs modulate single cell electrical regenerative

Figure 3 CNT scaffolds strongly improve neuronal electrical performance.
(A) Patch-clamp recordings of spontaneous network activity (PSCs) from cultured hippocampal neurons grown on control substrate (glass) or on a CNT substrate. Neurons grown on CNTs show a marked increase in the frequency of spontaneous PSCs with respect to controls [43]. (B) Top left, SEM micrograph of a hippocampal neuron grown on a SWNT scaffold. Bottom left, higher magnification of the region highlighted in the box. Note the close and intimate contact between CNTs and neuronal membrane (arrow). Scale bar: top image 10 μm, bottom image 450 nm [49]. Right, TEM micrographs from planar sections of hippocampal cultures grown on CNTs, showing the neuronal membrane (in the bottom-left portion of the image) "pinched" by a single CNT [44]. (C) Hippocampal neurons grown on a control substrate [glass; control (CTRL); red] or on a CNT scaffold (CNT; gray) were forced to fire a train of action potentials by the application of high frequency current pulses (top), to assess the presence of an additional afterhyperpolarization or afterdepolarization (ADP) at the end of the train (gray shadows). The presence of an ADP is significantly increased in CNT neurons with respect to control [44]. (D) Top: bright-field image of the experimental setup used for paired patch-clamp recordings from hippocampal neurons grown on glass or on CNT substrates. The presynaptic neuron is forced to fire one or more action potentials and the evoked PSCs are recorded from the postsynaptic neuron. Scale bar: 15 μm. Bottom: the probability of finding synaptically connected cell pairs was doubled in CNT cultures with respect to control [45]. (E) In paired recordings a train of action potentials, elicited in the presynaptic neuron, usually evoked depressing PSCs in the postsynaptic neuron in control conditions (red), whereas in neurons interfaced to CNTs (black) PSCs were usually not depressing [45]. (F) Left: bright-field image of a cultured spinal explant, with the indication of the experimental setting employed for recording the response of spinal interneurons to the stimulation of afferent pathways. Dorsal root ganglia were electrically stimulated, while the response was recorded via patch-clamp from neurons in the premotor area. Middle and right: superimposed evoked PSCs recorded from interneurons from explants interfaced to glass (control) or to CNTs (black: total PSCs recorded at the resting membrane potential, -56 mV; orange: excitatory component of the response, recorded at -40 mV; blue, inhibitory component of the response, recorded at 0 mV). Interfacing spinal explants with CNTs strongly increased the amplitude of both the excitatory and the inhibitory components of the response evoked by dorsal root ganglia stimulation [46]. (G) Left: sketch of the experimental setup used to stimulate hippocampal neurons via SWNTs. Neurons were cultured on a SWNT substrate; in a modified chamber an Ag electrode was positioned in electrical contact with the SWNT layer in a small dry area, isolated from the recording chamber, and voltage controlled stimulations (E) were delivered to SWNT via the Ag electrode. SWNT stimulation elicited current steps in a patch-clamped neuron on SWNTs (middle), whereas no currents were recorded if stimulation was performed on a control (glass) preparation [49].

properties (i.e., excitability). This observation, together with the theoretical simulations reported in the same study [44], strongly supports the so-called "electrotonic hypothesis": by the discontinuous but tight proximity between CNTs and cellular processes, nanotubes could create an electrotonic shortcut from somatic to dendritic compartments, accounting for the generation of extra ADP at the level of dendritic membranes [44].

The impact of CNTs directly at the synaptic level has been explored in the same type of preparation (hippocampal cultures on MWNTs or on control glass) [45]. Simultaneous whole-cell recordings from pairs of neurons (Figure 3D, top) showed that the probability of finding synaptically connected pairs is almost doubled by the presence of the CNT scaffold (Figure 3D, bottom). Accordingly, an increased number of synapses was observed in the presence of CNTs by immunostaining experiments. By paired recordings, the same showed that, in addition to synaptic connectivity, CNT scaffolds strongly impact also on synaptic plasticity. Indeed, the induction of a presynaptic spike train causes short-term synaptic "depression" at the postsynaptic level in control conditions, converted into short-term synaptic "potentiation" by the presence of the CNT scaffold (Figure 3E). Interestingly, the CNT-driven changes in synaptic dynamics depend on network activity and spike propagation, as the CNT-induced effects were removed by a chronic pharmacological block of synaptic transmission. There is no shortage of CNT features that can be put forward to explain their strong impact on neuronal functions: CNT high conductivity, topographical features or physical chemical properties could all play an important role. Notably, the boosting in the ability to generate backpropagating potentials brought about by CNTs does not underlie the observed increased connectivity, although improved dendritic regenerative properties in neurons grown on CNTs may play a role in the fine-tuning of synaptic dynamics [45].

Recently, the ability of CNT substrates to impact on CNS 3D tissue has been tested by co-culturing embryonic spinal cord and dorsal root ganglia (DRG) explants chronically interfaced to a film of purified MWNTs (Figure 3F, left) [46]. In this study, CNTs promoted the development of neuronal processes emerging from DRGs and growing in direct contact with the substrate, as neurites from explants grown on CNTs were more numerous, longer and with a higher number of growth cones at their tips with respect to neurons grown on control substrate. Interestingly, a different morphological adaptation of neuronal fibers to the CNT substrate was detected, i.e., a tendency of neuronal processes to slack above the CNT substrate, with an increase in their adhesion area. Furthermore, also the fiber intrinsic elastic properties were remarkably affected by the growing substrate, as fibers grown on CNTs were less stiff than the control ones. Tight contacts between CNTs

and cell membranes were observed at the bottom of the explant core, thus suggesting that CNT growth interfaces could directly affect the bottom layer of the tissue explant in a way similar to what have previously been reported for dissociated hippocampal cultures, that is, by driving the building up of a potentiated hybrid network (see above). The possibility of a remote effect of CNTs was tested in the same study by patch-clamp experiments in which the DRGs (the physiological input pathway of afferent sensory signals) were stimulated and the response to afferent stimulation was recorded in the premotor area of the explant, from interneurons located approximately five cell layers far from the CNT scaffold, that is, not in direct contact with it (Figure 3F, left). CNTs improved the neuronal ability to respond to afferent stimulations, as the amplitude of the response to DRG stimulation was strongly increased (in both its excitatory and inhibitory components; Figure 3F, middle and right), whereas the ability to integrate repetitive stimulations was preserved. CNTs also impacted on spontaneous spinal network activity, increasing the amplitudes of spontaneous PSCs, whereas the number of synaptic sites was unaffected by the CNT scaffold [46]. The hypothesis put forward to explain the reported observations is that a boosting of neuronal activity took place at the bottom layer of the spinal explant in direct contact with CNTs and that this boosting is remotely transferred to neuronal layers not in contact with CNTs, probably by activity-dependent forms of plasticity, or unknown second messenger pathways. This would lead to a synchronization of the neuronal network and/or the synapses, leading to the observed increase in currents amplitude [46]. Unfortunately, the ultimate, precise mechanisms accounting for the observed impact of CNTs on neuronal performance are not easy to elucidate and still remain unknown.

An interesting feature of CNT-based substrates is that they can be used not only to improve neuronal performance but also to directly, electrically stimulate neurons, an issue explored in 2007 by Mazzatenta and colleagues [49]. In this study, a layer of SWNTs deposited on a glass coverslip has been used as growth substrate for hippocampal neurons; the neuronal responses to the direct stimulation of the SWNT layer was investigated by administering square-pulse voltage steps of distinct positive and negative amplitudes via SWNTs (Figure 3G, left) and observing the appearance of neuronal current responses induced by this stimulation by means of single cell patch-clamp recordings (Figure 3G, middle). When the same type of voltage steps were applied to control glass substrates, no current responses were detected (Figure 3G, right). Importantly, the delivery of brief voltage steps of sufficiently large amplitude to SWNTs was able to induce the appearance of Na^+ fast inward current in the recorded neuron (i.e., action potential). This finding represents convincing evidence that CNTs are effective tools to transfer exogenous electrical signals to excitable neurons [49], thus strongly encouraging CNT exploitation in the design of efficient neural interfaces and stimulating devices.

6 CNTs in complex tissue engineering

Several strategies for engineering complex tissues have been approached, exploiting 3D polymeric scaffolds able to mimic *in vivo* morphological and physiological features [55] and leading in some cases to the production of innovative and effective devices. For example, cardiac patches have been developed in which neonatal cardiac cells with a mixture of prosurvival and angiogenic factors were seeded into an alginate scaffold capable of factor binding and sustained release [56]. Very often, however, polymeric scaffolds present some limitations such as weak mechanical properties, lack of electrical conductivity, absence of adhesive and microenvironment-defining moieties, and the inability of cells to self-assemble to 3D tissues. Carbon nanostructures have been incorporated into such scaffolds to compensate for these deficiencies. One of the most promising fields of application of CNTs as components of nanocomposite materials is bone tissue engineering. Some recently developed materials have been tested *in vivo* and have demonstrated a good biocompatibility, mechanophysical reinforcement of the whole structure and fostered osteointegration with surrounding bone tissues [57].

Concerning neural interfaces, promising *in vivo* results have been achieved by implantable multielectrode array (MEA) electrodes coated by CNTs: these devices have shown to improve electrochemical and functional properties not only in cultured neurons but also *in vivo* [58]. Moreover, the application of these types of electrodes for retinal recording and stimulation [59] seems very interesting. However, despite all reported promising evidence, no implantable CNT-based device able to act as a prosthesis or a nerve conduit has been produced yet to re-engineer *in vivo* neuronal performance.

7 Conclusions

More experimental studies are necessary to outline the preclinical issues of CNT-based devices, such as their

biodegradability or biostability *in vivo*, their functional performance *in vivo* and their potential long-term toxicity. Moreover, the design and shape of the device would vary depending on its final application: it could consist of a film that could wrap the nerve bundles, or a sponge-like scaffold in which neurites could be extended, or fiber bundles that can physically and functionally replace the injured tissue. Nevertheless, at present, CNTs are certainly one of the most promising classes of materials that has the potential for successful engineering of nervous tissue and, in particular, for its functional recovery after injury.

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