Review

Recent Advances in Carbon Nanotubes for Nervous Tissue Regeneration

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Abstract: Nanomedicine has allowed for emerging advances in imaging, diagnostics and therapeutics. Regenerative Medicine has taken advantage of a number of nanomaterials for reparation of diseased or damaged tissues in the nervous system involved in memory, cognition and movement. Electrical, thermal, mechanical and biocompatibility aspects of carbon-based nanomaterials (nanotubes, graphene, fullerenes and their derivatives) make them suitable candidates to drive nerve tissue repair and stimulation. This review article focuses on recent advances on the use of carbon nanotube (CNT)-based technologies on nerve tissue engineering; outlining how neurons interact with the nanomaterials interface for promoting neuronal differentiation, growth and network reconstruction for their possible use in therapies of neurodegenerative pathologies and spinal cord injuries.

Keywords: carbon nanotubes; graphene; nanomaterials; nervous tissue; regeneration; neurons

1. Introduction

The emergent field of Nanomedicine proposes the application of precisely engineered nanomaterials for the prevention, diagnosis and therapy of certain diseases, including neurological pathologies [1]. These pathologies occur when basic units of the nervous system start to deteriorate. In these nerve cells, alterations cause them to function abnormally, which results in demise of cell functions. Initial symptoms of neuronal deterioration may include loss of coordination or the ability to remember names, which may worsen over time if a large number of neurons deteriorate [2]. Due to the complexity of the nervous system, recovering function of the injured nerves or repairing damages caused by mental diseases is still a major challenge in the biomedical field. Neurodegenerative diseases affect over 90,000 people every year, from which, spinal cord injuries alone affect 10,000 people yearly. Alzheimer's and Parkinson's disease are the most common neurological diseases and occur in 5 million and 1.2 million Americans, respectively [3]. Considering the high amount of nerve repair procedures being currently conducted, as well as an increasing and ageing world population, the numbers of patients in need of neural implants to improve the regeneration of damaged tissue will only substantially increase over the years.

Neuroregeneration is the regrowth, restoration or repair of degenerated nerves and nervous tissues, associated with the production of new axons, neurons, glia, myelin and synapses. The nervous system is divided into the peripheral nervous system (PNS), which has the innate capability for self-repair and regeneration, while the central nervous system (CNS) is unable to self-repair and regenerate. Silicon-based materials are the most common for peripheral nerve implants; studied since the 1960s they have been used as a model system giving fundamental insight on nerve tissue regeneration.

Silicone has been implemented in the diagnosis, monitoring and continuous treatment of nerve tissue damages [4]. These materials have been primarily used due to its biocompatibility, flexibility and the wide availability in different dimensions. However, their low impermeability and general

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inert properties do not actively prompt neural tissue regeneration and have led to research on substitute materials [5]. Today, the treatment for damages in the CNS (i.e. spinal cord), consist on physical therapy to help patients with limited mobility without a full regain and restoration of the tissue and motor function [6].

Although still narrow, the application of nanotechnology and nanomaterials to neuroscience has experienced an impressive growth over the past decades. With an increasing amount of studies proposing scaffolds based on nanomaterial as strategies to regenerate nerve cells and tissues [7]. An ideal scaffold for neural tissue application shall exhibit electrical activity to stimulate cell outgrowth, biodegradability and bioactivity for growth factor delivery, interestingly, a number of nanomaterials exhibit some of these properties and have proved relative long term success when implanted [8].

Carbon-based nanomaterials (CBNs) have shown great potential when interacting with neurons and nerve tissues [9–11]. The discovery and manipulation of innovative nanomaterials, like fullerenes and graphene, but especially carbon nanotubes (CNTs), are likely to have a major impact on neuroregenerative techniques and in the biomedical applications in general. CNTs have shown to interact with the nervous system promoting the neural development. Furthermore the outstanding mechanical, thermal and conductive properties make CNTs very promising for other technological fields as conductive composites and sensors [12]. This work intends to review some recent uses of CNTs in nerve regeneration. We start providing an overview of nerve architectures and recent progresses on carbon-based nanomaterials for regenerative therapies and neuron repair. We finish the review with some closing remarks on biocompatibility and toxicity challenges of CNTs when used as part of these therapies.

2. Central and Peripheral Nerve Regeneration

The human nervous system consists of the central nervous system (CNS) and the peripheral nervous system (PNS), which at the same time is compose of two cell types, neurons and neuroglia. Neurons are the brain's nerve cells that transmit information from electrical and chemical signals throughout the nervous system, while neuroglia are the most numerous cells and their purpose is to aid the function of neurons. Within these cells there are Schwann cells in the PNS and astrocytes and oligodendrocytes in the CNS. Researchers have shown that functional and structural recovery of the nerves depends on both, extrinsic and intrinsic factors [13].

2.1. Peripheral Nervous System Repair

The PNS consists of a complex collection of spinal nerves, brain nerves and neuron clusters called ganglia. These cells interact with other tissues transmitting sensory messages to and from the spinal cord [14]. The PNS has the ability of slowly regenerate on its own (axon growth 0.5– 1 mm/day), in case of small injuries nerve axons can regenerate by proliferating Schwann cells and macrophages to remove cellular debris from the injury side. Schwann cells infiltrate in the injury to stimulate and guide the new forming axon across the damaged nerve [15]. On the other hand, larger injuries need to be surgically treated, commonly using an autologous nerve implants. While extrinsic factors contemplate the environment at the injury site, intrinsic factors like the size of the injury and the ability of the neurons to regenerate by the synthesis of growth factors influence PNS regeneration [14–16]. In general, tissue engineering together with nanotechnology, aim to create innovative materials to help accelerate the PNS recovery since the delay in tissue regrowth may lead to muscle atrophy.

2.2. Central Nervous System Repair

The CNS includes the brain and the spinal cord, it is the responsible of interpreting and conducting signals as well as providing stimulation to the PNS and from there to other tissues [17]. In contrast to the PNS, the CNS does not support full tissue regeneration, this leads to permanent loss

of functions that can cause several physical and cognitive complications. Many factors such as the environment surrounding the CNS injuries and the neurons lack of regeneration capacity prevent cells from regenerating. Axonal regrowth is constrained by supporting cells, like myelinating oligodendrocytes, that create a growth inhibitor environment due to the formation of glial scar tissue and the lack of Schwann cells to promote axonal growth [18]. Therefore the overall regeneration strategies for CNS are to reactivate gliosis while promoting tissue regeneration, where nanomaterials incorporated as part of current implants may help with [6].

3. Current Materials for Nerve Tissue Regeneration and Stimulation

Neurological implants' success in enhancing survival of damaged neurons, axons growth, and neuronal synaptic signal transmission, is key to face the functional impairment that caused neuronal loss or degeneration. Basically, any strategy developed to fix an injury on the CNS should focus on regrowing injured axons, the plastic remodeling of neuronal circuitry and the construction of new neurons [7].

Upgrades in material synthesis have allowed to develop artificial nerve conduits build of absorbable synthetic materials. Materials like polyhydroxybutyrate (PHB), polylactic acid (PLA) or polyglycolic acid (PGA) are being investigated as biodegradable-absorbable synthetic polymers for neural cell growth and axon organization. In fact, absorbable synthetics (PLA and PGA), and nonabsorbable synthetics like poly lactic-co-glycolic acid (PLGA), are already used for nerve regeneration. These three polymers are mechanically fragile and lack regions suitable for further chemical modification [19], in any case, they have been FDA approved for use in several neuron repair devices.

3.1. Polycaprolactone (PCL)-based Materials

PCL has gained considerable interest in the field of nerve regeneration research. The main features of this biodegradable polyester are its ease of manipulation and low processing costs. Its high processability is given by the great solubility this substance has in many organic solvents and that its crystalline nature enables easy formability at low temperatures. Neurolac® (Polyganics Inc., The Netherlands) is a PCL nerve conduit approved by the FDA [20].

Other PCL co-polymers like the biodegradable polycaprolactone fumarate (PCLF) have recently allowed the fabrication of CNT composites [21]. These PCLF-CNT scaffolds not only exhibited excellent suitability to culture neuroblastic PC-12 cells (that can easily differentiate into neuron-like cells), but also allowed for good cell growth, differentiation, and electrical stimulation, which reflected in a neurite extension and promoted cellular migration and intracellular connections, which are all critical cellular behaviours for nerve regeneration [21].

3.2. Collagen-based Materials

Collagen comprises a large family of proteins with a wide range of biomedical uses including peripheral nerve repair. When adequately purified collagen becomes weakly antigenic, exhibits a smooth microgeometry and transmural permeability facilitate diffusion processes through collagen matrices [22]. Collagen type I constitutes an essential structural component of the extra cellular matrix and has been employed at fabricating nerve repair conduits. Of over a dozen nerve conduits currently FDA approved three are made of collagen type I: NeuraGen; NeuroMatrix and NeuroFlex [22].

The first semi-permeable type I collagen nerve guidance conduit approved by the FDA was NeuraGen® (Integra Life Sciences Corporation, Plainsboro, NJ, USA). A medical study on peripheral nerve reconstruction reported the clinical experience of using this implant, in which patients tolerated splinting and exercise without negative clinical repercussions. In another research, this conduit was compared with direct suture repair, in patients with complete traumatic nerve injuries. Results showed that patients who were treated with NeuraGen®, had less post-operative pain than those treated with direct suture repair. The main conclusion was that nerve repair using the NeuraGen® is a quite effective method of joining severed nerves [23]. As collagen-based nerve repair conduits still

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lack good mechanical stability, the possibility to reinforce them with CNTs seems appealing, in fact, this might improve their electrical conductivity, thus exhibiting good viability of neuronal cells as has been demonstrated in similar biomaterials [24].

4. Carbon Nanomaterials for Nerve Tissue Regeneration

Nanosized materials and nanoscale technologies seem to challenge many traditional paradigms in Materials Science. Since the discovery of CNTs by Sumio lijima in 1991 scientific literature on the physical and chemical properties of nanomaterials, especially carbon-based nanomaterials (CBNs), has grown significantly and so has the use of CBNs in nerve regeneration applications (Figure 1).

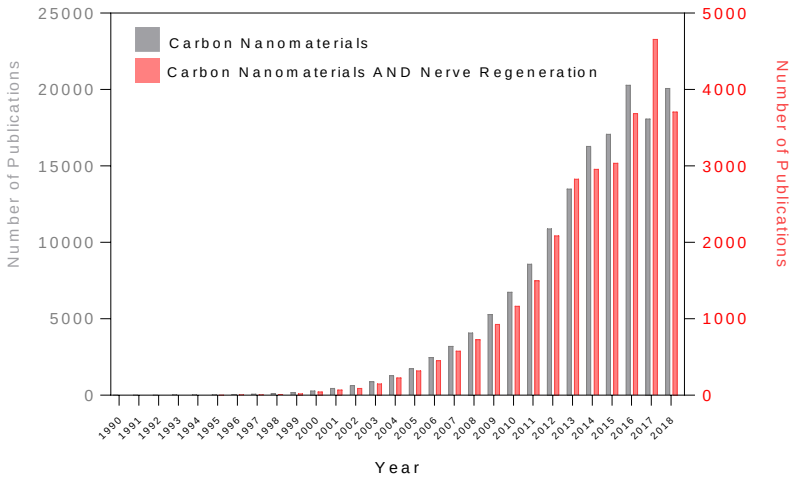

Figure 1. Increasing trend of reports on carbon nanomaterials (grey) and carbon nanomaterials and nerve regeneration (red). A Google Scholar search was performed on either "Carbon Nanomaterials" or "Carbon Nanomaterials AND Nerve Regeneration" on the specified time period.

CBNs offer unequal advantages, like high electrical conductivity, high surface-volume ratio, powerful mechanical strength and chemical stability [25]. CBNs are held in high steem in the biomedical materials community, and constant efforts have been made to integrate them into existing materials and devices like cellular sensors, tissue scaffold reinforcements, and drug delivery systems [26]. Fullerenes, graphene and carbon nanotubes (CNTs) are the most studied CBNs; they have attracted significant attention regarding their unique optical, electronic, mechanical, thermal, and chemical properties [27].

4.1. Fullerenes

Fullerenes are CBNs of great importance in biomedical research. Since their discovery in 1985 it was evident that this polyaromatic, symmetrical, and hollow spherical cage C_{60} molecule was meant to find a number of versatile applications in antiviral therapies [28], energy production, flat panel displays, semiconductors, environmental technologies, cosmetics [29] and food industry [30]. C_{60} has been recently used as an in vitro vehicle for therapeutic astrocyte delivery to neural lesions [31]. PCL and C_{60} were electrospun into 200 nm diameter nanofibers that showed good cell attachment and promising potential as drug delivery devices (Figure 2) [31].

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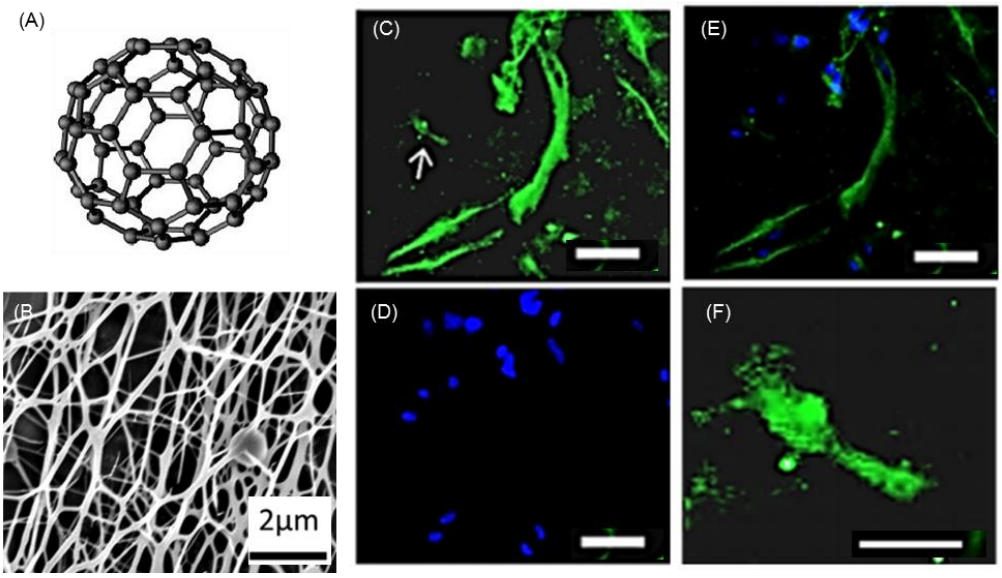

Figure 2. (A) Representation of C_{60} fullerene [32]. (B) SEM images of PCL-fullerene nanoscaffolds fabricated by electrospinning. (C-E) Immunocytochemical micrographs of astrocytes cultures on the scaffolds and labeled with anti-glial fibrillary acidic protein antibody (anti-GFAP, green) and Hoechst dyes (blue, scale bar: 50 μm). (F) Magnification images of cells pointed with an arrow in panel (C) (scale bar: 25 μm) [31].

4.2 Graphene

Graphene is a polycyclic aromatic molecule that is composed of a two-dimensional sheet of sp^2 bonded carbon atoms. This dimensional feature grants graphene and its derivative graphene oxide (GO) with high elasticity, conductivity, remarkable mechanical strength, rapid heterogeneous electron transfer and high surface area [33]. Graphene is considered a versatile building block for functional nanoelectronics, energy storage and production [34] as well as antibacterial [35], biosensing [36] and anticancer therapies [37].

Different amounts of layers grants the graphene with different properties, going from one-layered graphene to multilayered graphene structures [38], and interaction between these graphene-based nanomaterials and neurons have been recently explored [36]. In a recent study by Pampaloni et al it is shown that single-layer graphene (SGL) is able to tune astrocytes excitability and increases neuronal firing by altering membrane-associated functions in vitro. The authors hypothesise that graphene restricts the mobility of K+ ions in close proximity to the SGL surface, but only when SLG is deposited on electrically insulating substrates. In this fashion, graphene properties might affect neuronal information processing (Figure 3) [36].

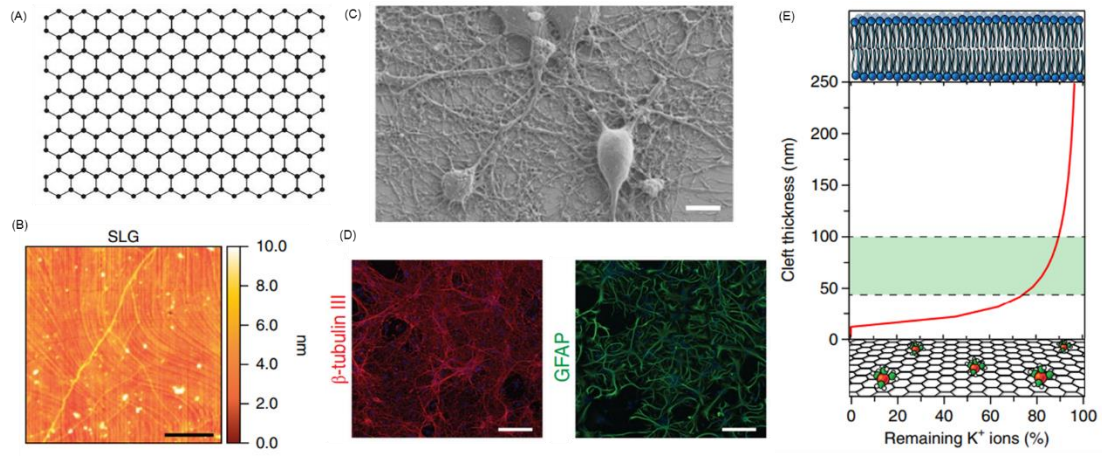

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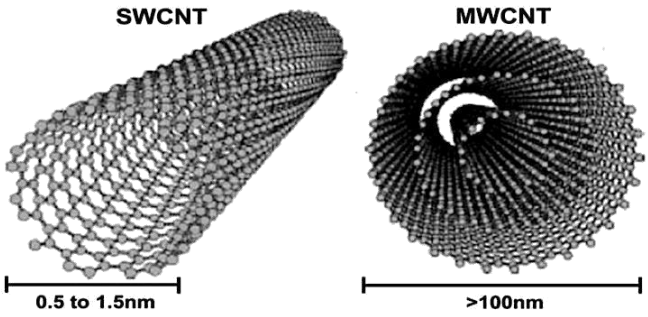
Figure 3. (A) Molecular representation of graphene [39]. (B) AFM topography of single layer graphene (SGL, scale bar: 5 μm). (C) Scanning electron micrograph depicting hippocampal neuron morphology cultred onto SLG (scale bar: 10 μm). (D) Fluorescent microscopy images showing dissociated hippocampal networks labelled with class III β-tubulin (for neurons) in red and GFAP (for astrocytes) in green (scale bar: 100 μm). (E) Sketch of the local amount of K+ depletion in the space between the cell membrane and the SLG surface (membrane/surface cleft) due to graphene trapping as function of cleft thickness. The light green region shows the extrapolated K+ depletion values (red line) within the range of the estimated cleft dimensions (40–100 nm). See Reference [36] for more details.

4.3 Carbon Nanotubes (CNTs)

Carbon nanotubes (CNTs) are the most widely used in nerve regeneration CBN to date [22]. Different from other CBNs, CNTs exhibit tunable physical (length, diameter, single-walled SWCNTs vs multiwalled MWCNTs, chirality) [40] and chemical properties (surface functionalization, high electrochemical surface area) [26]. CNTs can be envisioned as cylinders made of rolled-up graphene layers with diameters in nanometer scale (Figure 4). CNTs possess an extended conjugated sp^2 carbon network that renders a π-electron system which extend over the nanostructure originates either highly conducting CNTs or semiconducting ones, this also provide tunable band gaps compatible with neural activity [10]. The following section addresses the specific use of CNTs in neural regeneration and stimulation in deeper detail.

Figure 4. Representation of a single-walled carbon nanotube (SWCNT) and a multi-walled carbon nanotube (MWCNT) [41].



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5. CNTs for Neural Regeneration and Stimulation

CNTs are generating an attractive approach in the treatment of neural pathologies and nerve tissue damaged. On top of their aforementioned capabilities, CNTs show morphological similarity to neurites, small CNT bundles have dimensions similar to those of dendrites (the branched extensions of neuron cells), enhancing possibilities for not only probing, repairing, stimulating or reconfiguring neural networks [10] but gaining insights into basic mechanisms of neuronal functions [11]. Success on the application of CTNs is strongly connected to the ability to control the interaction between them and the neurons' changes in ionic conductance and synaptic transmission, this being a perk when incorporated into electrodes and conductive probes [1,42]. In the following sections we provide a comprehensive view of the use of CNTs in neural regeneration and stimulation.

5.1. Improving CNTs Neurocompatibility

CNTs are synthesized through a number of treatments that renders them positively or negatively charged, and be further modified to incorporate various functional groups via covalent [43] and non-covalent pathways [44].

Incorporation of hydrophilic polymers such as polyethylene glycol (PEG) increases CNT solubility in aqueous solution, increasing their biocompatibility and facilitating the fabrication of CNT-based medical materials and common use polymers such as poly-ethyleneimine (PEI) and poly-L-ornithine (PLO) have been reported to promote neural attachment and subsequent neurite outgrowth, these non-covalent functionalization examples represent a valuable resource when manufacturing the coating for neural interface devices [45,46]. Use of CNTs decorated with these poly-cations generally promotes neuron growth, this is most likely due to an enhanced electrostatic interaction between the CNTs and the plasma membrane of neural cells that has a negative charge [47]. Recent biofunctionalisation approaches [48] appear as promising alternatives for increasing CNTs water solubility and further neuron growth stimulation and cell membrane incorporation.

5.2. CNTs Application Strategies

Literature reports on two possible strategies to control neural cell functions through biofunctionalized CNTs. One is through the addition of soluble CNTs directly to neuronal cell culture medium, and the other is by utilizing them for surface modification of supporting substrates such as scaffolds. The first strategy requires a direct application of the CNTs to the nerve tissue allowing the carbon structures to interact directly with nerve culture and to expand or disperse within the cells.

The second strategy employs CNTs as modifiers (either surface or bulk) of other materials to enhance their neuro-functionality either as part of a multi composite scaffolds, implantable devices cell guiding matrices, enhancing activity of other components such as the therapeutic drugs (small molecules), proteins (neurotrophic factors or extracellular matrix (ECM) components), and nucleic acids (siRNA, miRNA, pDNA, etc.) [45].

Properties like roughness, charge, polarity, and chemistry of CNT scaffolds, can alter the affinity of neurons linked to CNT-containing surfaces. Direct interactions between neurites and CNTs act as an exoskeleton, with more membrane/material tight junction formation. Greater surface area of CNT significantly results in stronger charge injection capacity and lesser interfacial impedance as they help in electrochemical coupling via electron transfer between CNTs and neurites [5,46,49,50].

5.3. Neural Response Mechanisms to CNTs

CNTs have demonstrated to play an important role mediating interactions between neurons and their environment. When used as a scaffold CNTs act not only as a reservoir of adsorbed proteins, but also play a dynamic role in boosting neuronal electrical performance. Observed discontinuous and tight contacts between MWCNT or SWNT bundles and neuronal membranes favor the hypothesis of a direct electrical coupling (Figure 5). The work of Cellot et al demonstrate that meshwork of MWCNTs outside neurons is in intimate contact with a small area of the neuritic

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membranes, this report constitutes the very first attempt at linking electrical phenomena in nanomaterials to neuron excitability [11].

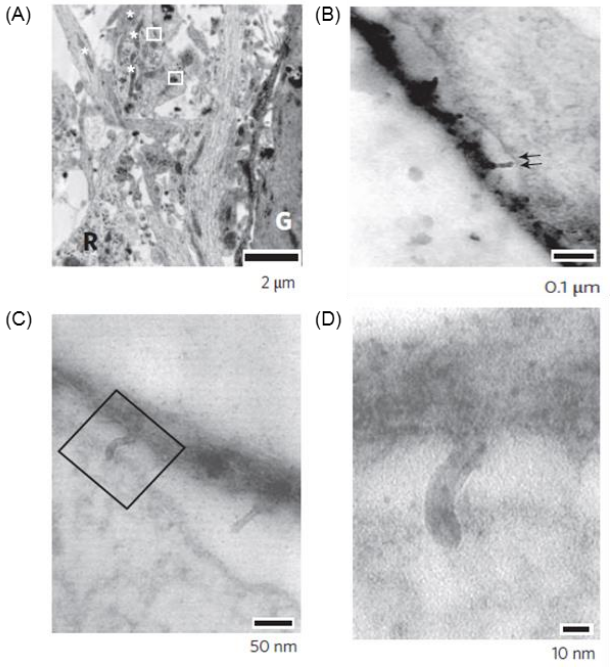

Figure 5. Interaction between MWCNTs and neurons. (A) Transmission electron micrographs (TEM) sections of neurons grown on MWCNTs showing functional synaptic contacts (rectangular box). (B) Arrows indicate MWCNT–membrane contacts on this TEM sections from Panel (A). (C, D) High-magnification micrographs from a section consecutive to those of (B) illustrating how MWCNTs 'pinch' neuronal membranes [11].

Hippocampal neurons cultured on MWCNTs were studied by Fabbro et al [51] demonstrating an increase in expression of paxillin, this membrane protein is involved in focal adhesions-mediated intracellular signaling pathways, demonstrating that electrophysiological cues provided by CNTs can be translated into specific neuronal signals. However, more detailed mechanistic studies between the neuronal tissue and CNTs interface are still required to engineer further applications of CBNs [5,50,52]. Some recent applications are presented in the following section.

5.4. Aplications

CNTs have been incorporated in the design and manufacturing of a number of biomedical technologies. A number of comprehensive reviews on the use of CNTs for neuron regeneration can be found in literature [7,15,22,53]. This section aims to present recent examples of CNT-based materials used as scaffolds (hard printed or in the form of hydrogels) for neuron culture and conduits for nerve reconstruction.

5.4.1. 3D printing nanoconductive MWCNT scaffolds for nerve regeneration

Aminated MWCNTs have been recently been incorporated in a poly(ethylene glycol) diacrylate (PEGDA) matrix [54]. This report shows how CNTs can be easily incorporated in emerging 3D printing technologies to render scaffolds that supports differentiation and growth of neural cells while having microelectroporous characteristics, the manufacturing process is depicted in Figure 6.

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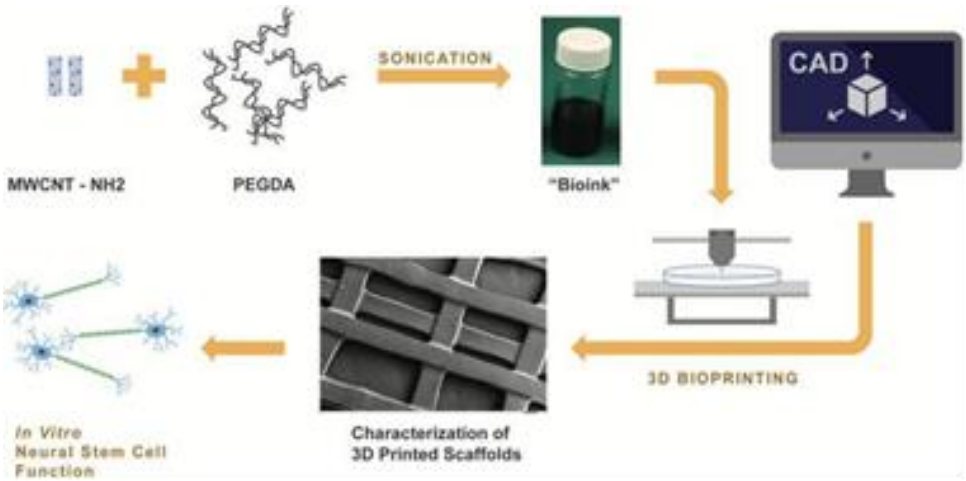

Figure 6. Representation of the manufacturing process of 3D-printing nanoconductive MWCNT-PEGDA scaffolds [54].

In this study on MWCNT-PEGDA scaffolds concentration of CNTs was evaluated, finding that proliferation at 0.02% of amine-containing MWCNT was the highest after four days of culture, whereas the highest proliferation of neural stem cells (NSCs) in all the concentration groups occurred in the 0.1% amine-containing MWCNT scaffolds. This proliferation was obtained until day seven, and the researchers believe that this delay was caused by the adaptation process of the NSCs to the substrate. Higher concentrations of MWCNTs in the printed scaffolds showed a higher positive charge which could promote a better development and greater nerve cell growth [54].

5.4.2. CNT-interfaced glass fiber scaffold for regeneration of transected sciatic nerve

Peripheral nerve injuries are common in clinical settings yet the possibility of the nerve to regenerate spontaneously will vary according to the severity of the injury, which will be limited if the injury is too severe. The study by Ahn et al addresses this issue by fabricating a phosphate glass microfibers (PGF) scaffold that incorporates aminated-CNT [55].

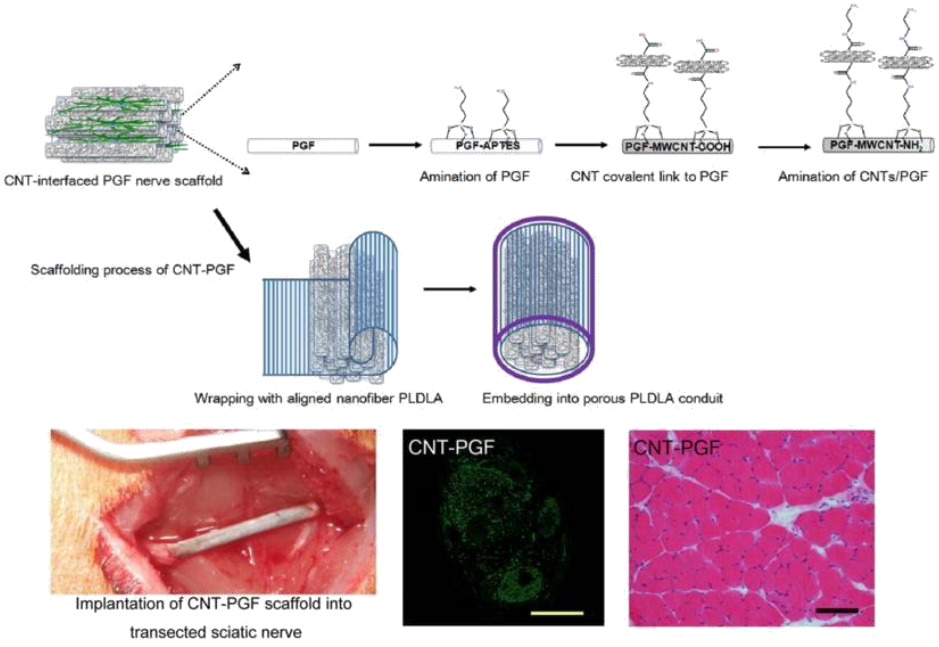

Figure 7. Functionality of a CNT-interfaced PGF scaffold used as nerve conduit. The aligned PGF bundle interfaced with CNTs for neurite outgrowth [55].

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The researchers performed a surface coupling of aminated CNTs to aligned PGF bundles and used the resulting fibers as interfacing material for neuron physical guidance. In vivo cell guidance studies were performed after wrapping the CNT–PGF substrate around poly(l/d-lactic acid) (PLDLA) electrospun nanofibers. The nerve guidance device was made in the shape of a cylindrical tube (as shown in Figure 7) and tested in a rat sciatic injury model [55], exhibiting good neural interaction, cell viability and physicochemical integrity. Finally, the implant displays effectiveness in restoring motor functions, indicating that the muscle in the animal was functionally improved as a result of the CNT interfacing, as the scaffold-crossing axons reinnervated into the gastrocnemius muscles [55].

5.4.3. Polysaccharide/CNT hydrogel hybrid as neuronal growth substrates

Hydrogels are attracting much attention in biomedical applications given their molecular-scale control over mechanical and bioresponsive properties [56]. Most hydrogels still lack good mechanical strength and electrical conductivity, thus limiting their biomedical applications, but CNT hydrogels hybrids have emerged as candidates to overcome this. These composite hydrogels have rapidly gained attention in developing regenerative therapies for skeletal muscles, cardiac and neural cells [57].

A recent study by Wu et al explores the potential of chitin-based composite hydrogels incorporating MWCNT [58]. These hybrid hydrogels originated from a chitin/NaOH/urea aqueous solution blended with modified MWCNTs. Hydrogels bulk consisted on bundles of chitin nanofibers and carbon nanostructures that were stabilized through intermolecular forces like hydrogen bonding, electrostatic and hydrophobic interactions (Figure 8). The resulting hydrogels showed improvements in thermal stability, a better hemocompatibility, good mechanical properties, while slowing down biodegradability rates and the swelling ratio compared to control chitin hydrogels [58]. On top of these improvements, in vitro evaluation of Schwann cells was performed, resulting in a successful proliferation of the neuronal cells with little cytotoxicity and neurotoxicity, displaying a promising potential as neuronal growth substrates for peripheral nerve regeneration [58].

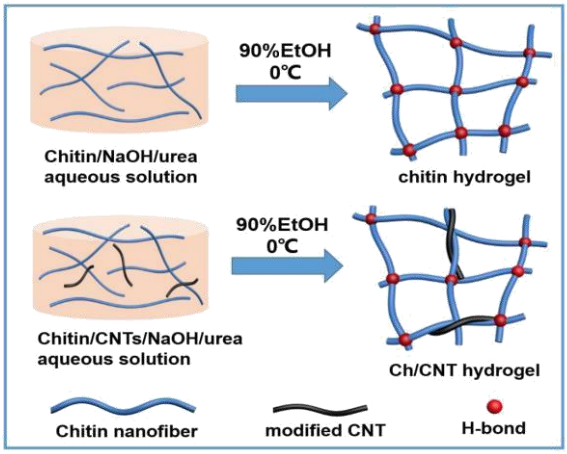

Figure 8. Schematic representation of the synthesis process of Chitin-MWCNT hydrogel hybrids [58].

5.4.4. Nerve Guide Conduits Based on Protein/CNT Composites

The study presented by Mottaghitalab et al introduced a clever nerve guide conduit (NGC) design that merged the mechanical advantages of the naturally occurring proteins silk fibroin (SF) and SWCNTs for use in nerve grafts. The resulting conduit showed stable chemical, physical and electroconductivity properties due to his uniformity, plus the addition of fibronectin contain nanofibers (FN) through a electrospinning process rendered an addition extracellular matrix guidance for neuron growth and migration (Figure 9). FN conferred the SF/SWNTs NGCs conduits bioactivity allowing the growth and adhesion of U373 cell lines. NGCs were studied in vivo,

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implanted to 10 mm left sciatic nerve defects in rats, resulting in nerve regeneration in the proximal regions of the implants after five weeks. In both SF/SWNT and SF/SWNT/FN NGCs more myelinated axons were present as well as higher nerve conduction velocities, indicating a functional recovery for the injured nerves [59].

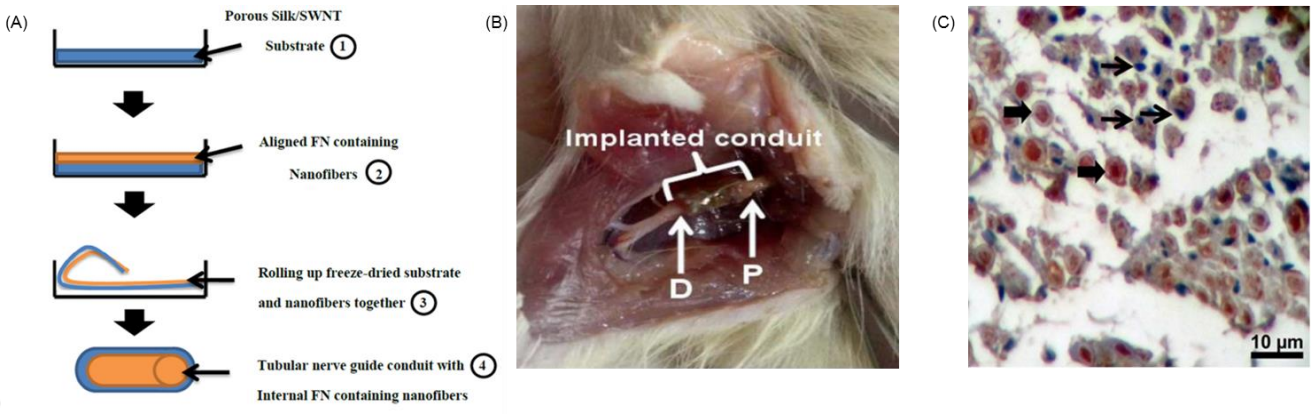

Figure 9. (A) Schematic representation of rolling up protocol for nerve guide conduit (NGC) containing SF/SWNT/FN. (B) Implanted conduit in left sciatic nerve defects in a rat model after 5 weeks implantation. (C) Cross sections of regenerated nerves taken from nerve conduits implanted in rats for 5 weeks [59].

6. Biocompatibility and Neurotoxicity of CNTs

CNTs show in general good compatibility in vivo with neuronal tissues. One of the main concerns when CNTs are applied in biomedicine is the possibility of generating accumulation-related adverse reactions in the tissues [60]. Collateral diseases have been reported in different studies due to the application of mostly non functionalised CNTs, such as cardiopulmonary diseases, inflammation and fibrosis, among others [61,62]. Among this reasons are the superficial load, the distribution of the functional groups of the nanotubes and the difference of rugosity, all these reasons can be enclosed within the methods of manufacture of these structures [62,63], which we describe in the following section.

6.1. CNTs Neurotoxicity Related to Manufacturing and Functionalisation

As previously mentioned, CNT can be manufactured by different methods (arc discharge, chemical vapor deposition, laser ablation of graphite among others) which generate a very wide spectrum of shape, length, chirality and impurities. Despite the fact that a number of studies highlight toxic effects in cells upon CNTs exposure, these adverse effects are largely due to heavy metal nanoparticles (Fe, Co, Ni, Y) produced during their synthesis [64].

Further functionalization steps tend to remove these metals and also to reduce CNTs' tendency to bundle, thus improving further biodistribution and lowering inflammatory responses [65–68]. Table I shows some toxicity related effects as consequence of different CNTs solubilizing and functionalisation treatments in biological tissues with special emphasis on neuron related reports [64,69].

Additionally, the length and shape of CNTs also influence their toxicity, depending on these physical characteristics, their interaction mechanism can be altered and generate different immunological responses [70,71]. Several studies have shown a lower immune response as CNT decreases, since short CNT can cross cell membranes more easily, whereas longer CNTs remain in the extracellular space [72].

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411 **Table 1.** Toxicity study of various carbon nanotubes solubilisation and functionalisation
412 schemes of biological interest.

Small molecules as solubilizing agents	**Toxicity**	**Reference**
Tetrahydrofuran	Tumorigen, mutagen	[73]
Dichlorocarbene	Harmful	[73]
Anthracene	Possible tumor promoter	[74]
Pyrene	Carcinogenic, mutagenic	[75]
Zn-porphyrin	Unknown, likely safe	[76]
Phenylethyl alcohol	Topical irritant	[77]
n-octyl-*β*-d-glucoside	Unknown	[78]
n-decanoyl-*N*-methylglucamide	Unknown	[78]
Triaminopyrimidine	Unknown	[79]
Lysophosphatidylcholine	Unknown	[79]
Barbituric acid	Not pharmacologically active	[80]
Sodium cholate	Unknown	[78]
Taurine	Safe up to ~28.57 mg/mL	[81]
Thiolated organosilane	Unknown	[82]
Macromolecules as solubilizing agents	**Toxicity**	**Reference**
Chitosan	Mostly safe	[83]
Helical amylase	Unknown	[84]
Poly(phenyleneethynylene)	Possible antimicrobial properties	[85]
Poly(aminobenzene sulfonic acid)	Hazardous to blood, nervous system, liver	[86]
PAA	Severely irritating and corrosive	[87]
PEG	Acute oral and dermal	[86]
Sulfonated polyaniline	Unknown	[88]
Functionalisation approach	**Toxicity**	**Reference**
MWCNT-NH_3^+	Weak transient inflammatory response on glial cells	[89]
^{13}C enriched SWCNTs + Tween-80 1%	Moderate (mouse lungs and liver), biodistribution study	[90]
PEG-modified SWCNTs	Mostly safe (spinal chord injury)	[91]
[^{111}In]DTPA-MWCNTs	Not determined (blood-brain barrier in vitro model)	[92]
PEG-Oligodeoxynucleotide (CpG)	Mostly safe (glioma tumor model)	[93]

413 Abbreviations:
414 PEG: Polyethylene glycol
415 PAA: Poly(acrylic acid)
416 DTPA: diethylenetriaminepentaacetic acid.
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6.2. Neuron Interaction Mechanisms

So far, it is understood that there are two possible mechanisms that CNTs can present to enter neurons or potential host cells: a. Active transport through endocytosis/phagocytosis and passive transport or simple diffusion (also known as nanopenetration) [69].

Both mechanisms are presented in Figure 8, where a. represents the absorption of CNTs using the deformation of the plasma membrane to form a vesicle that internalizes into the cytoplasm [94,95]. Phagocytosis is a process similar to endocytosis but is characterized by its specialized exogenous material of greater size as bacteria or microorganisms. Both mechanisms are dependent on energy (ATP) and temperature [96,97].

Nanopenetration is a passive mechanism that allows the CNT to cross the membrane without the need to generate a vesicle, it can be compared with the simple diffusion that some substances of interest for the membrane present [98]. The results of several studies of both mechanisms suggest that they can generate different immune reactions, as they activate a number of transport routes at the same time and also DNA damage can be involved at some point [98].

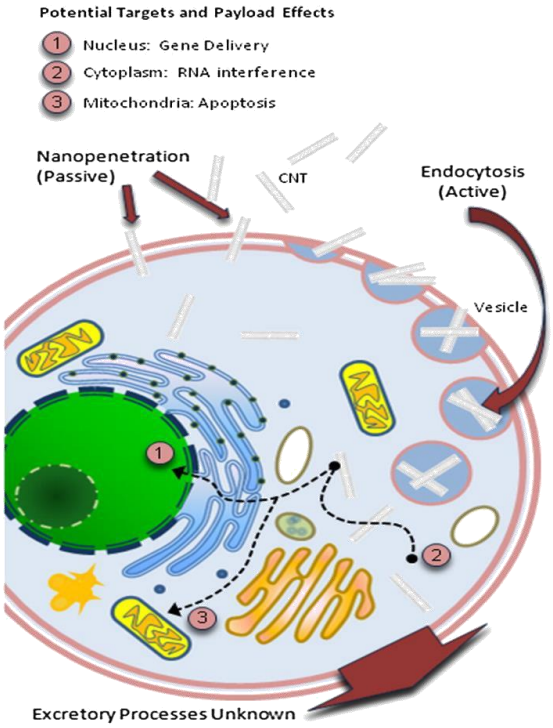

Figure 8. Graphic demonstration of endocytosis and nanopenetration [69].

A number of studies have tested how a number of small and macromolecules can travel across the blood-brain barrier (BBB) [99], in fact CNTs are no exception to this. When ^{13}C-enriched SWCNTs were administered to mice it was found they accumulate in the animal's brain, but showing little no none acute toxicity while also accumulating in liver, lung and spleen, organs where CNTs persistence may lead to long-term toxicity effects [90].

MWCNTs are also able to cross the BBB as the work of Kafa et al demonstrated. In this study radiolabelled MWCNTs were intravenously administered to a murine model in order to study the molecular mechanism mediating CNTs crossing the BBB, finding that micropinocytosis is the prevalent internalization mechanism and therefore transcellular uptake is hypothesized as the primary mechanism behind the BBB crossing [92].

Gastrointestinal administration of SWCNTs can lead to accumulation across the BBB, it also known that SWCNTs tend to accumulate in neurons' lysosomes, Yang et al took advantage of these

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observations to treat Alzheimer's disease model mice by delivering acetylcholine using the CNTs [100]. This study based the release of cargo based on a pH change in neuron lysosomes, but it has been demonstrated that CNTs can be enzymatically degraded by peroxidases in immune cells, glia cells and the extracellular space as well [64], therefore, lessening concerns about their use in neuron therapies.

7. Concluding Remarks and Future Perspectives

Several areas of biomedical engineering have also benefited greatly from CBNs in recent years because incorporating CBNs is effective not only as injectable nanoscale devices but also as components to enhance the function of existing biomaterials significantly.

Despite safety concerns over CBNs, many studies have reported the successful use of CBNs in biological applications. In addition, several chemical modification strategies have been developed to circumvent toxicity issues and to increase the biocompatibility and functionality of CBNs.

More studies should be carried out that relate both the shape, size and functional group with the toxicity of the CNT, because the various studies present varied results related to their manufacturing methods.

The characterization of the mechanisms of interaction of the CNT remains uncertain as it varies depending on the physicochemical characteristics of the same, likewise these mechanisms can generate a variety of immune responses of the cell causing high toxicity, which is why continue studying these mechanisms and relating them to the appropriate physicochemical factors for each of the cells of interest.

Although all kinds of carbon nanoparticles have a potential use in different areas of science, CTN are the most important ones used in the subject of nervous system repair.

While these results are of great interest, there is a significant theoretical gap in terms of exactly how neurons and CNTs interact which requires further in-depth experimentation, knowledge that will be vital if these technologies are to be fully realized. Moving forward, an important goal is the interface of CNTs with other electrically active cells such as other forms of cardiac, muscle, and sensory receptor cells. It can be hoped that such advances will help muscular regeneration and amelioration of impaired sensory input. [10]

Nevertheless, it should be noted that more systematic toxicology studies are needed to determine the toxicity and pharmacokinetics of CBNs.

This paper has introduced several successful applications of CBNs in drug delivery, tissue imaging, and scaffold reinforcement. With the popularity of CBNs as highly versatile and useful nanomaterials, we expect to see continued use of CBNs in many facets of biomedical engineering.

In particular, there is great promise in applying the biocompatible and multifunctional nature of CBNs to the areas that interconnect mechatronics and biology, such as microelectromechanical systems ("MEMS") for biological sensors and actuators.48

Furthermore, more recent studies suggest that CBNs may also be used to regulate cellular behavior.49,50 Although research efforts have largely been focused on utilizing CNTs, other types of CBNs; especially graphene, which has gained wide recognition in recent years; are expected to be investigated extensively in the near future.

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