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Cap.9: Carbon nanotubes in tissue engineering

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Abstract

For their peculiar features carbon nanotubes (CNTs) are emerging in many areas of nanotechnology applications. CNT-based technology has been increasingly proposed for biomedical applications, to develop biomolecule nanocarriers, bionanosensors and smart material for tissue engineering purposes. In the following chapter this latter application will be explored, describing why CNTs can be considered an ideal material able to support and boost the growth and the proliferation of many kind of tissues.

Keywords Carbon nanotubes, tissue engineering, bone replacement, neural regeneration, cardiac tissue engineering.

Table of Contents

1. Introduction
1.1 Biomedical applications of carbon nanotubes

2. CNTs for bone tissue engineering

3. CNTs for neural tissue engineering

4. Carbon nanotubes for cardiac tissue engineering

5. Other tissue engineering possibilities for CNTs

6. Toxicity of CNTs

7. Summary and conclusions

Abbreviations

BMP	bone morphogenetic protein
BP	buckypaper
CNF	carbon nanofibers
CNT	carbon nanotubes
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
ECM	extracellular matrix
Hap	hydroxyapatite
HIV	human immunodeficiency virus
MEA	multielectrode array
MWNT	multiwalled carbon nanotubes
NT-3	neurotrophin3
PLCL	poly (lactide-co-ε-caprolactone)
PLGA	poly (lactic-co-glycolic acid)
rhBMP-2	recombinant human bone morphogenetic protein-2
RNA	ribonucleic acid
SWNT	singlewalled carbon nanotubes

1. Introduction

At a time when the nanotechnologies are dominating the scene in almost all the branches of sciences, and even invading our daily life, the search for nanostructurable materials able to provide active support and effective interactions with biosystems at the molecular and submolecular level is very active. In this scenario, in recent years carbon nanotubes (CNT) are certainly numbered among the most interesting, fascinating and studied nanomaterials for a variety of applications. This particular allotropic form of carbon has in fact so peculiar and unique properties to be potentially exploitable in many application areas of nanosciences.

The need of materials able to interface with biological systems at the nanoscale is a very hot topic for the modern medicine. Carbon nanotubes have found many possible applications not only as promising materials for technological purposes and industrial applications but also in the biomedical field. There is a wide range of possible biological applications of CNTs reported in literature, therefore a complete review of this topic is a titanic job. We will only refer to representative examples, which can provide the reader with a flavour of the potential of CNTs in this exciting area.

1.1 Biomedical applications of carbon nanotubes

Motivated by the peculiar features of CNTs, research towards their biomedical applications has been progressing rapidly.

FIG 9.1: CNT features and their possible biomedical fields of application

Due to their ability to trespass biological membranes and the possibility to bear multiple functionalization on their backbone, CNTs have been studied as vectors for many different classes of therapeutic agents. Even though the specific mechanism of internalisation (endocytosis or needle like penetration) is still not fully elucidated, it is generally recognised that CNTs are able to enter cells, regardless of cell type and functional groups on their surface [1, 2]. In addition, their high surface area provides attachment sites for molecules, allowing for multiple derivatisation. Moreover, several in vitro and in vivo studies have shown, so far, that many types of chemically functionalised CNTs are biocompatible with the biological milieu, demonstrating how the in vivo behaviour of this material can be modulated by the degree and type of functionalisation, both critical aspects that need to be accurately tuned [3, 4, 5, 6]. For these reasons CNTs have been used as molecular carriers for a variety of therapeutic agents as antitumor drugs [7], antigens for an immunotherapeutic approach [8], targeting moieties (antibodies or peptides) [9] and also liposomes that can in turn act as vectors of molecules [10]. CNTs can be used as non-viral molecular transporters for the delivery of short interfering RNA (siRNA) into human T cells and primary cells. The delivery ability and RNA interference efficiency of nanotubes far exceed those of several existing non-viral transfection agents, including various formulations of liposomes. It was suggested that nanotubes could be used as generic molecular transporters for various types of biologically important cells, from cancer cells to T cells and primary cells, with superior silencing effects over conventional liposome-based non-viral agents [11, 12]. CNT-mediated nucleic acid transport has also been studied to deliver antisense oligonucleotide with proapoptotic activity [13, 14] to achieve gene transfer, or to combine a nucleic acid delivery system with photodynamic therapy [15].

The possibility of hosting small molecules inside the cavity of the nanotube has also been explored, allowing the depiction of CNTs as nanocapsules, role that may realize the “magic bullet” concept of a molecule capable of detecting and selectively destroying a cancer cell [16].

The transporter properties of CNTs can also be exploited for in vivo imaging application, for examples conjugating CNTs with traceable radionuclides or fluorescent probes [17, 18]. Nanotube-based optical biosensors may be used to detect specific targets inside the human body, e.g. tumor cells, wrapping the tubes by a protein that can link only to the targeted cells [19]. For example, a coordinated biosensor made of Au nanoparticles and SWNTs [20] has been studied for detecting the nanomolar scale of HIV-1 PR, an aspartic protease responsible for virion assembly and maturation [21]. The realization of high-sensitive detection of this protease was promising to expedite development of effective HIV-1 PR inhibitors.

Another example in viral disease diagnosis is the electrical detection of hepatitis C virus RNA [22]. A large surface-to-volume ratio and unique electronic properties made CNTs an optimal component for fabricating high-sensitive biodetectors, which were crucially needed in the diagnosis of viral diseases and the development of new anti-viral drugs. It was predictable, therefore, that CNTs might contribute considerably to the treatment of infectious diseases in the future.

Due to their ability to interact with the infrared radiation, CNTs can be used for hypertermal therapy of tumors. In fact, biological tissues are known to be transparent to 700- to 1,100-nm near-infrared light where CNTs show a strong optical absorbance. Appropriately functionalized CNTs with targeting moieties can reach the desired site (tumor) and release locally therapeutic molecules or cause an excessive local heating, leading in both cases to cell death [9].

Few studies have also described CNT antimicrobial activity: Kang et al. have demonstrated that highly purified pristine SWNTs with a narrow diameter distribution, coming in direct contact with cells can cause severe membrane damage and subsequent cell inactivation [23]. The same authors investigated the antimicrobial potential of SWNTs incorporated within the biomedical polymer poly(lactic-co-glycolic acid) (PLGA). They found that Escherichia coli and Staphylococcus epidermidis viability and metabolic activity were significantly diminished in the presence of SWNT–PLGA, and this effect was correlated with SWNT length and concentration [24].

Finally. and perhaps more importantly, the branch of biomedical sciences where CNTs are finding the widest variety of applications is represented by tissue engineering. This discipline studies the possibilities of replacing damaged, unfunctional or degenerated biological tissues by means of artificial (bio)materials able to mimic as much as possible the natural environment.

For their peculiar features of high mechanical strength, elasticity, good thermal and electrical conductivity CNTs are largely studied as key components for innovative materials in tissue engineering. They have shown in many cases to be biocompatible and to support the growth and the proliferation of many classes of cells. However as we will discuss more in detail in the following chapters, the toxicity of this carbon form is still an issue to be clarified.

2. CNTs for bone tissue engineering

Treatment of bone defects in humans, including those associated with the removal of tumors, trauma and abnormal bone development, faces important limitations. Current therapies such as autographs, allographs, and metal prostheses do not generally favour bone regeneration in itself. Instead, they replace the lost bone by an artificial material. One novel aspect of modern tissue engineering is the attempt to create tissue replacement by culturing bone cells on synthetic 3D scaffolds or live prosthesis. An ideal scaffold for bone tissue regeneration should possess mechanical properties similar to the bone tissue being replaced, good biocompatibility with surrounding tissue, large degree of porosity and high pore

size, high pore interconnectivity for bone tissue ingrowth. The synthetic scaffold material should either be biodegradable, disappearing as the new bone grows, or non-biodegradable. In the latter case, the non-biodegradable material behaves as an inert matrix on which cells proliferate and deposit new live matrix, which must become functional, normal bone. Despite extensive research, no existing man-made scaffold can meet all these requirements [reference here as above]. Hence the development of novel biomaterials and scaffold fabrication techniques is critical for the success of bone tissue engineering.

Bone structure and function depend intimately on the arrangement of cellular and noncellular components at the micro- and nanoscale level [25]. These include cell types such as osteoblasts, osteoclasts, and osteocytes embedded in a mineralized extracellular matrix consisting of collagen and a number of noncollagenous proteins [25].

The nanocomposite films or materials are expected to support the colonization with cells by some necessary requirements as nanoscale surface roughness, i.e., the presence of irregularities smaller than 100 nm, peculiar morphology or specific characteristics as hydrophilicity or conducibility.

The surface nano-roughness of the substrates to be colonized should mimic, as much as possible, the nanoarchitecture of the natural extracellular matrix (ECM) as well as of the cell membrane, such as the size of some ECM molecules, their folding and branching. The nanostructure of a material also improves the adsorption of cell adhesion-mediating ECM molecules, present in biological fluids or synthesized and deposited by cells contacting the material. On nanostructured materials, the cell adhesion-mediating molecules are adsorbed in advantageous geometrical conformations, allowing for good accessibility of their active sites by the cell adhesion receptors [26, 27].

In comparison with hydrophobic surfaces, wettable surfaces adsorb a lower amount of albumin, i.e., a non-adhesive protein for cells. However, the cell adhesion is optimal only on moderately wettable surfaces. Another property that can result to be very important is the electroactivity, as electrical charge, electrical potential and electrical conductivity, which could enable the electrical stimulation of cells [28]. Interestingly, the adhesion, growth, maturation and function of cells on electroactive surfaces are improved even without active stimulation of cells with an electrical current. The underlying mechanism probably includes enhanced adsorption of cell adhesion-mediating proteins, a more advantageous geometrical conformation of these proteins for their accessibility by cell adhesion receptors and facilitation of cellular processes, such as activation of ion channels in the cell membrane, movement of charged molecules inside and outside the cell, up-regulated mitochondrial activity and enhanced proteosynthesis (for a review, [29]). Furthermore electroactive substrates can significantly increase the mechanical and chemical resistance of the implant surface, preventing the release of ions and material particles from the bulk material.

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All these peculiar requirements for an optimal bone-compatible scaffolds can be met by carbon based materials and composites as demonstrated by an increasing number of scientific publications. Indeed, the tensile strength of SWNT is about one hundred times higher than that of the steel, while their specific weight is about six times lower [30, 31, 32]. Thus, CNTs could find ideal applications in hard tissue surgery, e.g., to reinforce artificial bone implants, particularly scaffolds for bone tissue engineering made of relatively soft synthetic or natural polymers.

CNTs have shown to be fully biocompatible with osteocytes and bone cells [33]. MWNTs adjoining bone induce little local inflammatory reaction, show high bone-tissue compatibility, permit bone repair, become integrated into new bone, and accelerate bone formation stimulated by recombinant human bone morphogenetic protein-2 (rhBMP-2) [34]. CNTs have been shown to support nucleation of hydroxyapatite (Hap) in correspondence of their defect sites [35]. Moreover CNTs can inhibit osteoclastic bone resorption also in vivo as reported by Narita and coworkers [36].

Osteosarcoma cells were cultured on chemically modified single-walled and MWNTs [33]. CNTs carrying neutral electrical charge (PEG functionalized) sustained the highest cell growth and production of plate-shaped crystals of mineralized bone matrix. There was a dramatic change in cell morphology in osteoblasts cultured on MWNTs, which correlated with changes in plasma membrane functions

As a consequence of these encouraging preliminary results, the number of studies on CNTs or CNT composite-based scaffolds for the replacement of defecting bone tissue has increased dramatically.

Many naturally occurring biopolymers have been studies in hard tissue engineering to replace bone tissue. Their major problem consists essentially in the low mechanical strength and CNTs have been considered the perfect material to reinforce three-dimensional structures formed by natural polymers. Many bionanocomposites containing SWNT or MWNT have been developed with biopolymers like chitosan [37], alginate [38], hyaluronate [39, 40], collagen [41], polylactic acid (PLA) [42]. All these composites have demonstrated lack of cytotoxicity, to be more stable and more mechanically resistant with respect to their homologs without CNTs.

Fig. 9.2 The whole shape of the uncoated collagen sponge honeycomb (a) and MWNT-coated sponge (b). (c) SEM image of an MWNT-coated sponge. (d) SEM image of the inside surface of an uncoated sponge. (e) SEM image of the inside surface of the MWNT-coated sponge.(reprinted with the permission of [40].

Also synthetic polymers have been utilized together in an attempt to reduce biodegradation rates, although maintaining the tissue requirements, as poly(lactide-co-glycolide) (PLGA) [42], polymethyl methacrylate [43], polypropyl fumarate [44, 45], polyurethanes [46], polycarbosilane [47].

Hydroxyapatite, the inorganic calcium-containing constituent of bone matrix and teeth, has been integrated in CNT based structures to create a CNT reinforced brittle HAp bioceramic [48]. In order to make them more "bone-friendly" unfunctionalized CNTs have been simply mixed in HAp matrices but they have shown better results on the spontaneous mineralization of HAp crystals when functionalized. Functionalized CNTs have been also further derivatized by in situ deposition of HAp providing a good biocompatibility with osteoblasts [49].

Another strategy to improve the performances of CNT-based implants as replacement of bone tissue provides for a scaffold designed to deliver useful molecules such as bone trophic factors, immobilized and with reproducible gradients, or that can be further structured to incorporate cell transplants. In a recent study neurotrophin-3 (NT-3) was incorporated in a chitosan-SWCNT hydrogel and, under electrically simulated conditions, a steady release of the agent (NT-3) was observed, suggesting an electrically controlled factor delivery. The presence of CNTs into the biohydrogel composite facilitated the electron transfer more efficiently [50]. A similar strategy can be adopted for releasing bone specific factors from functionalized CNT dispersed polymer scaffolds for effective bone tissue engineering as already done in the case of bone morphogenetic protein (BMP) adsorbed on MWNT-chitosan scaffolds [51].

Electrical stimulation of osteoblast cells may not seem intuitive for any practical advantage, but exposure to alternating currents increased bone cell proliferation and extracellular calcium production of osteoblasts grown on CNT-polylactic acid composites, demonstrating application for accelerated bone repair [28].

Some in vivo studies have been conducted in animals, implanting CNT composites in defecting bones: COOH-functionalized MWNTs reinforced with polymethyl methacrylate/HAp have been implanted in some holes of a sheep tibia and the cellular response has been examined [43]. The authors have found that this novel composite accelerates cell maturation by providing a mechanically competent bone matrix; this likely facilitates osteointegration in vivo. In another study hyaluronic acid functionalized SWNT were injected in rat tooth sockets under conditions in which bone formation is compromised (diabetic rats) [38]: results indicate that restore the bone repair process in the tooth sockets in diabetic rats was significantly restored 14 days after first molar extraction, suggesting that these materials can be potentially useful in therapies for bone tissue reconstruction in normal and adverse metabolic states.

In conclusion, CNTs could be a very good choice as structural and functional constituents of 3D scaffolds for bone tissue engineering. Probably the best solution consists in a complex composite of nanocarbon materials, biopolymers and biominerals enriched with bone growth factors, to take advantage of all the positive features typical of each class of materials. An important issue is that so far no sufficient pre-clinical or clinical studies with nanomaterials for bone repair have been conducted while exhaustive toxicological studies on these materials have to be performed.

3. CNTs for neural tissue engineering

Due to the complexity of the nervous system anatomy and function, repairing damaged nerves as well as recovering full function of injured nerves have been particularly challenging when compared to other tissue repairs (such as bone repair). Traditional neural implantation and surgery (such as using autografts, allografts, xenografts, and silicon probes for the continuous diagnosis and treatment of neural tissue or other biomaterial nerve graft devices) have posed a variety of problems as rejection, immune response, incomplete functional recovery, instability of the materials. For these reasons the demand for new biocompatible and long–term stable materials for neural regeneration and total functional recovery is very urgent. Current strategies to approach neuronal regeneration use nerve conduits and synthetic guidance devices, made of degradable or non-degradable compounds, that can guide and facilitate peripheral nerve regeneration. Various conduits have been fabricated for bridging nerve gaps after injury, and both natural and synthetic materials have been used [53]. The main characteristic of these materials is a longitudinal organization mimicking the natural structure of the nerve pathway within the brain and spinal cord. They are designed to serve as conduits for axonal elongation and to constrain the direction of regenerative outgrowth. Moreover, they should be able to direct regenerating axons to reconnect with their target neurons and enhance functional restoration of the nerve [54]. Many experiments have been performed to study functional recovery after injury in animal models. A promising strategy for treatment of neuronal injuries is to support and promote axonal growth by the use of nanometer-scale materials, especially nanotubes and nanofibres. They mimic tubular structures that appear in nature, such as microtubules, ion channels and axons. Nanotubes can be produced from various materials, such as carbon, synthetic polymers, DNA, proteins, lipids, silicon and glass. With their exceptional properties of small size, flexibility, strength, inertness, electrical conductivity and ease of combination with various biological compounds are the perfect candidates for interfacing successfully with damaged neuronal tissues.

Since when, in 2000, Mattson and colleagues have found that CNTs deposited on functionalized CNTs were not only surviving but also elongating their neurites in all directions [55], the study of these materials as functional components of composites for the support of the regeneration of neural tissue has been set up by many research groups.

CNTs seem to be particularly appealing in these applications for all their physical features but above all for their relatively high conductivity, useful to sustain the electrical communication among neuronal cells. Moreover, as in the case of bone regeneration, they can be functionalized with chemical groups or molecules able to improve the growth and the survival of cells. It has also been demonstrated that the charge surface on the CNT wall is crucial for the cell wellness as indicated by the presence of increased growth cones, longer average neurite length and more

elaborated neurite branching. These boosting effects are mainly achieved when positive charges are exposed on CNT surface [56].

Neurons and several other cell types appear to adhere and grow extremely well on surfaces with topography on the nanoscale [57]. Just varying its degree of roughness, a substrate can be cell adhesive or non-adhesive depending exclusively on the surface roughness (as observed for rough or smooth SiO2). CNTs can be deposited to form bidimensional films or can give the formation of 3D structures in such a way to control their surface roughness. It was shown that neuronal cells are able to grow and elongate their neurites onto CNT-based substrates with a precise nanotopography [58]. Conductive CNTs have demonstrated to modulate the growth and the morphology of neuronal cells in a narrow range of conductivity promoting the outgrowth of neurites with a decrease in the number of growth cones as well as an increase in cell body area [59]. Furthermore, the orientation of CNTs can be controlled and is able to affect the direction of neurites outgrowth [60] and accordingly it should be possible that they drive the direction of the electric signal propagation. CNTs deposited via a combination of microlithography and chemical vapor deposition supported the growth of neurons, affecting their capability of extending neurites and guiding these cell processes along their length. Surface topography in terms of length of nanotubes was observed to play an important role in process guidance [61]. Neurite processes showed preferential adhesion to the edges of long CNT patterns whereas no selectivity was observed in the short CNT patterns. This behaviour could also be due to the rigidity of CNTs: short CNTs do not offer the motile growth cone with a suitable surface for process development. The long CNTs in comparison are flexible and undergo deformation to accommodate the proliferating neurite.

Fig. 9.3: Scanning electron micrograph demonstrating guided neurite growth along a MWNT array pattern. The extending neurite is shown interacting with the edges of the pattern. This morphology is observed 24 h after initial seeding of the cells (reprinted with the permission of Zhang X et al)

In order to further improve the biocompatibility of unconstrained CNTs and to produce 3D-structures able to be colonized by neuronal cells and to foster the communication among them, many research groups have tried to incorporate CNTs in polymeric scaffolds where they can play a strengthening and electrically functional role. CNTs have been integrated into various biopolymer-based hydrogels as collagen [62], chitosan [63], agarose [64]. In general, all these substrates are very good supports for neuronal cells, able to sustain their growth and their ability to extend neurites and growth cones without a remarkable toxicity. None of these scaffolds has however been tested in vivo yet.

Similar results have been achieved by CNT composites based on synthetic polymers, mostly polyesters polymers such as electrospun fibers of poly(d , l -lactic-co-glycolic acid (PLGA) [65] and of poly (l-lactic acid-co-caprolactone) (PLCL) [66].

The most outstanding results concerning the interface between CNTs and neurons are related to the effects on the electrical activity of neuronal networks. In a study performed in 2005, we compared the electrical 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 [67]. The frequency of spontaneous events (postsynaptic currents, PSCs) in networks cultured on CNTs was strongly boosted and increased (approximately six fold) compared with controls. Moreover the balance between inhibitory and excitatory components in the neuronal network was not affected. By means of single-cell electrophysiology techniques, electron microscopy analysis and theoretical modelling, it has been hypothesized that CNTs can provide a kind of shortcut between the proximal and distal compartments of the neuron [68]. This theory, supported by the observation that neuronal membranes establish a tight but discontinuous contact with the CNT substrate, was further corroborated by other experiments where, when cells were forced to fire trains of action potentials, the presence of extra-membrane after depolarization potentials was detected, and this was much more frequent on CNT deposited cells with respect of those grown on an inert glassy support. This kind of backpropagating action potentials represents a regenerative ability that neurons exhibit in cellular processes as the tuning of synaptic activity, the expression of spike timing-dependent plasticity, the release of modulatory messengers and the modulation of synaptic plasticity [69]. Another interesting observation concerns the impact of CNTs on the synaptic activity of neuronal networks: the probability of finding synaptically connected pairs of neurons is almost doubled in presence of the CNT substrate. Moreover the synaptic plasticity was also affected because cells grown on CNTs demonstrate potentiated short-term synaptic condition instead of a normal depression after a presynaptic spike train. All these impressive effects are entirely attributable to the peculiar features of conductivity and physical chemical properties of CNTs that impact on the network activity and spike propagation.

Not only cells but also more complex neuronal systems have been tested on CNTs: embryonic spinal cord and dorsal root ganglia (DRG) explants have been interfaced to a film of purified MWNTs [70]. With respect to the controls, DRG cultured on CNTs displayed a higher number of longer neuronal processes growing in tight contact with the substrate bearing a higher number of growth cones at their tips. These neuronal processes seemed to slack on the CNT carpet increasing their contact surface and were less stiff than in the control. The overall interaction of the DRG with the substrate appears to be very intimate and similar to that reported for cell cultures. DRGs were stimulated and the response to an afferent stimulation was registered by single neurons located in a portion of the slice that was not in contact with the CNTs layer. We found that the amplitude of the response to DRG stimulation was strongly increased in both its excitatory and inhibitory components but the ability to integrate repetitive stimulations was preserved. In addition, the spontaneous activity was also preserved.

CNT coated surfaces can potentially be used for a wide variety of applications such as retinal implants, network repair, and neuro-welding.

Recently many research groups have dedicated their attention to the production and the study of neuronal performances. One of the first contributions in this field was that of Khraiche et al. (2009). The authors cultured rat hippocampal neurons on multi-arrayed electrodes (MEAs), whose tips were covered with CNT (SWNT). They observed that the electrical activity of the neuronal networks was detectable four days after seeding and continued to grow until day 7, while neurons developed on control (bare gold) electrodes showed no electrical activity until day 7. The hypothesis is that the rough SWNTs surface provides cells with a larger surface area to adhere leading to an increased activation of adhesion molecules (such as integrins), which might in turn promote a faster neuronal differentiation [71]. In this direction Shein and coworkers coated MEA electrodes with CNTs, obtaining islands with a conductive, three-dimensional, exceptionally high surface area [72]. Dissociated cortical neurons cultured on these electrodes adhered only and directly to these islands, and self-assembled in neuronal networks patterned on the CNT neurochip. Once the neurons had adhered and self-organized, the CNT–MEA allowed very high fidelity, direct recording of neuronal activity, and an effective electrical stimulation of neurons at the electrode sites. An interesting application of this kind of devices was explored by Shoval et al.: they investigated the use of MWNT coated microelectrodes as an interface for retinal recording and stimulation applications [73]. Whole-mount retinas isolated from neonatal mice were placed on the electrodes allowing electrical recordings of the spontaneous, typical, propagating retinal waves. With respect to commercially available electrodes, recordings from MWNT–MEAs showed a consistently higher signal-to-noise ratio and a relevant increase in the amplitude of the recorded spikes over a period of minutes to hours was observed. The proposed hypothesis is that a dynamic interaction between MWNTs and neurons produces an improvement in cell–electrode coupling, resulting in the phenomena detected. Finally the authors validated the suitability of their MWNT electrodes for sustained neuronal stimulation.

In an additional paper where SWNTs were deposited directly on standard platinum electrodes to fabricate MEAs for electrophysiological recordings, the advantages of CNT–MEAs over metal electrodes in neuronal recordings were further confirmed [74]. In this report, the application of SWNT-modified MEAs to record electrical activity from whole-mount rabbit to standard, platinum electrode-based MEAs.

Although the way to a functionally efficient neural prosthesis is still very long to go, there are some promising nanomaterials that seem to be very useful for this purpose and CNTs are definitely among them. Their good conductivity and efficient supporting ability together with a confirmed biocompatibility with neuronal cells make CNTs particularly interesting as constituents in biomimetic scaffolds to guide axon regeneration and improve neural activities.

4. Carbon nanotubes for cardiac tissue engineering

Another tissue in which electrical signals are propagated that can potentially be successfully interfaced with an electroconductive material like CNTs is the cardiac tissue. As for the neuronal system, the possibility to have a bi- or three-dimensional substrate able to reinforce and regenerate the cardiac functionality could be an incredible progress in many heart pathologies, including heart failure (myocardial infarction) and congenital cardiovascular defects.

Cardiac tissue engineering aims for the development of a bioengineered construct that can provide physical support to the damaged cardiac tissue by replacing certain functions of the damaged extracellular matrix and prevent adverse cardiac remodelling and dysfunction after myocardial infarction. Cardiovascular biomaterials can be based on either biodegradable or on non-biodegradable materials. Within this matrix of conductive vs. non-conductive and biodegradable vs. non-biodegradable materials lie the most commonly studied materials and techniques used to promote heart health. Synthetic polymers offer advantages in their ability to tailor the mechanical properties, and natural polymers offer cell recognition sites necessary for cell, adhesion and proliferation. The most of the injectable scaffolds developed for myocardial applications are however non-conductive, lack nanofibrous architectures at submicrometer scale (10-100 nm in diameter) and are typically mechanically weaker than the native heart tissues. For these reasons CNTs seem to be theoretically the ideal material for a successful biomaterial for cardiac applications.

The first study of biocompatibility of CNTs with cardiac cells has been performed with rat cardiac cells cultured onto a suspension of SWNTs [75]. Within short term (3 days) CNTs did not display a significant toxicity while for longer time the toxic effect have been ascribed to physical interactions. These long-term negative effects have been evidenced after reseeding the cardiac cells: non-viable cells coming from SWNT-treated samples increased by 25%, when compared to reseeded cells not treated with SWNT.

We discovered outstanding effects on cardiac cells cultured on CNTs substrates. Neonatal rat ventricular myocytes (NRVM) were able to interact with non-functionalized CNTs (MWNT) deposited glass coverslips by forming tight contacts with the material (fig 4.1) [76]. Cardiac myocytes modify their viability, proliferation, growth, maturation and electrophysiological properties when interacting with CNT scaffolds. CNTs appear to have two opposing effects on the development: they prolong the proliferative state, which maintains some cells in an undifferentiated state, and they accelerate the maturation of the differentiated cardiac myocytes, in terms of a more negative NRVM resting potential compared to control, indication of the fact that the cells become more adult-like. The mechanism that regulates these effects is not clear yet but we observed by TEM microscopy that CNTs develop irregular tight contacts with the membranes, contacts that are morphologically similar to those seen in neurons cultured on MWNTs [67]. Moreover it is not excluded that other modifications, indirectly brought about by MWNTs, such as the deposition of the extracellular matrix or the cell contact

driven cytoskeletal dynamics, are ultimately responsible for the detected positive effects.

Fig. 9.4: Characterization of MWNT substrates and ultrastructural interaction between MWNTs and cultured cardiac myocytes. TEM planar section (c) of NRVM grown on carbon nanotube layer reveals a healthy organization of cardiac myocyte networks, accompanied by the presence of desmosome-like contacts (arrows). TEM sagittal sections (d–f) illustrate nanotube–membrane contacts. In panel d, it is possible to appreciate the continuous layer of MWNTs interacting with cells (arrowheads); panels e and f are a series of further high-magnification micrographs from the same section. Note how nanotubes are "pinching" cell membranes (arrows). Reprinted with the permission of [67].

The development of three-dimensional architectures of cardiac cells at the nanoscale able to improve next generation transplantable cell-enriched devices for tissue implants is the main requirement for the progress towards a practical application of these materials for the heart regenerative medicine [77]. Carbon nanomaterials in the form of carbon nanofibers (CNFs) have been integrated in composites in order to achieve conductive matrices able to accommodate myocardial cells. Carbon nanofibers have been added to biodegradable PLGA to increase the conductivity and cytocompatibility of pure PLGA [78]. Human cardiomiocytes proliferated on the different PLGA: CNF ratios, an increase in proliferation density from 530% on day 1 to 700% on day 5 resulted between the 100:0 and 25:75 (PLGA:CNF wt.%) ratio. CNF are characterized by a structure called stacked-cup carbon nanotubes (the overall structure appears like concentric cylinders) hence CNFs possess nanoscale geometries which imitate the extracellular matrix of various tissues (such as the heart), potentially leading to improved cytocompatibility of these materials [79]. Although requiring further study, CNF can play a similar important role in promoting cardiomyocyte by increasing victronectin and laminin adsorption, two adhesion glycoproteins of the extracellular matrix that in turn will induce cell adhesion and proliferation. While the mechanism of enhanced cardiomyocyte density is not clearly detailed at this time, it could have to do with the topography of PLGA-CNF composites and/or the increased presence of CNF on PLGA surfaces, which can control initial protein adsorption through altered surface energetics. Pedrotty et al. showed that numerous cardiac cell functions (including adhesion, proliferation, and migration) might be modulated by electrical stimulation [80], hence requiring the use of a conductive material in cardiac applications. Also, Mihardjo et al. demonstrated that enhanced myocardial repair following ischemic injury could be achieved using conductive polymers, such as polypyrrole [81]. The conductivity values measured for PLGA-CNF substrates were lower than those of heart tissue (ranging from 0.16 longitudinally to 0.005 S m^{-1} transversely) [82], but future techniques (such as CNF or CNT alignment) may increase the anisotropic conductivity to match that of heart tissue [83]. It is

also important not to exceed the stimulatory conductivity of the cells, avoiding a possible decreased cell function.

In terms of carbon nanomaterial-based devices for functional regenerative purposes, an interesting cardiac construct has been produced by Shin and colleagues [84]: CNTs were embedded into photo-crosslinkable gelatin methacrylate (GelMA) hydrogels, resulting in ultra-thin 2D patches where neonatal rat cardiomyocytes were seeded. These cells showed strong spontaneous and stimulated synchronous beating. In addition, a protective effect against doxorubicin (cardiotoxic) and heptanol (cardio-inhibitor) was observed. When released from glass substrates, the 2D cardiac patches (centimeter size) formed 3D soft actuators with controllable linear contractile, pumping, and swimming actuation behaviours. CNT concentration of 3 mg/mL led to tissues with optimal electrophysiological functions, while 5 mg/mL showed the maximal protective effect. CNTs formed electrically conductive and collagen fibril-like nanofibers bridging pores, which mechanically strengthened the gel, promoted cardiac cell adhesion and maturation, and improved cell-cell electrical coupling. Compared to existing scaffold materials, CNT-GelMA seems to be a very promising multifunctional cardiac scaffold.

5. Other tissue engineering possibilities for CNTs

Some recent papers describe attempts to explore CNTs as substrates for a variety of different tissues.

Rat hepatocytes have been seeded onto CNTs-coated surfaces and their morphological and their functional behaviour has been studied [85]. Primary hepatocytes exhibit different morphological and functional characteristics depending on the surface properties on which they are deposited. Hepatocytes in a serum-containing medium adhered on the CNT surface and formed monolayer configuration in form of spheroids. This peculiar shape seems to be due to the hydrophobic features of the CNT substrate. Furthermore the expression levels of connexin-32 (a molecule that forms gap junctions for cell–cell communication) were higher on the CNT-coated than on the collagen- and CNT/collagen-coated surfaces used as controls, indicating the development of intracellular communication between cells under those conditions. This study is a very preliminary exploration of CNT-based substrates for hepatocyte cultures that needs further data to verify the real efficacy.

Another interesting practical application of CNT-based composite materials involves the regeneration of dermal tissue in wound healing. From the combination of SWNT and polyvinylpirrolydone in aqueous media, Simmons and coworkers have produced a highly pure microporous film that, due to the iodine non-covalently linked to SWNT, have antiseptic properties and can be used as an antiseptic bandage. Electrical pulses sent through the composite may allow for enhanced cell growth and faster reconstitution of the damaged tissue [86].

Lima and coauthors described a faster and longer-stroke artificial muscle based on yarns made from sheets of CNTs with a solid guest or filler material such as wax; melting and solidifying the wax twists or untwists the yarn and generates

motion [87]. Other guest materials are activated by chemical absorption or illumination by light. The new artificial muscle outperforms existing artificial muscles, allowing possible applications such as linear and rotary motors, and might replace biological muscle tissue if biocompatibility can be established.

The so-called buckypaper (BP), a macroscopic assembly of entangled carbon nanotubes, is a relatively new material that can be formed by single, double, multiwalled CNTs with different lengths, diameters or aspect ratios. BP has been proposed for the encapsulation of islet cells for diabete treatment, as an artificial membrane for retinal and iris pigment epithelial transplantation, as a flexible antiseptic bandage, as immune shielding for cells and tissues, as a carrier for gene or drug delivery, and as a scaffold for tissue engineering. BP was recently shown to be not toxic and not to affect the in vitro proliferation and viability of both normal human arterial smooth muscle cells and human dermal fibroblasts. Martinelli et al. have studied the adhesive properties of BP on a wet compliant substrate [88]. By means of shear and peeling adhesion tests they have showed that BP readily and strongly adhere to a trimmed muscular fascia of a rabbit abdominal wall, chosen as the model substrate. The material has been compared to commercially available prosthetic materials and it was demonstrated to possess superior properties of adhesion and stability. BP could find applications in abdominal prosthetic surgery or for wound closure, thus allowing not only easier surgery procedures but also reduction in the use of conventional perforating fixation, to which serious postoperative complications are usually associated.

6. Toxicity of CNTs

The main prevention in the use of CNTs based materials in biological environment is the controversial question of their potential toxicity. In the literature there are a number of conflicting reports concerning this issue: some investigations have reported toxic effects following the exposure of several cell types to both SWNTs and MWNTs, while others demonstrate very low or insignificant cellular responses. This debate is mainly due to the fact that toxicity depends by factors like purity (metal content), surface modification (charge), dimensions (aspect ratio <3), layer number, degree of dispersion (aggregate formation) [89]. (See table 9.1).

	cell types/animals	**type of CNTs**	**CNT toxicity**
metal impurities	H460	SWCNTs containing 19.4%Ni/5.49%Y; 14.3%Ni/2.09%Y; 3.15%Ni/9.21%Co; 22.8%Ni/4.79%Y; 24.1%Ni/4.17%Y ; 3.3%Co/1.27%Mo	nickel is bioavailable at toxicologically significant concentrations
	NR8383; A549	purified SWCNTs; SWCNTs con-	dose- and time-

		taining 0.009%Fe/2.8%Co/4.2%Mo; purified MWNTs; MWNTs containing Ni	dependent increase of intracellular ROS; decrease of mitochondrial membrane potential
	RAW264.7	SWCNTs containing 26% Fe or 0.23% Fe	hydroxyl radical generation: loss of intracellular low molecular weight thiols; accumulation of lipid hydroperoxides
	HaCaT	SWCNTs containing 30% Fe	formation of free radicals; accumulation of peroxidative product; antioxidant depletion; loss of cell viability
surface charge and modification	HMMs	acid-treated, water-soluble SWCNTs	acid-treated SWCNTs are less aggregated within lysosomes and cytoplasm and cause no significant changes in cell viability or structure
shape	HUVEC	pristine SWCNTs; oxidized SWCNTs	functionalized and pristine SWCNTs have limited cytotoxicity
	normal mice	MWNTs 1520 μm or longer	length-dependent inflammation and formation of granulomas
	human primary macrophages	short CNTs; long, tangled CNTs; long, needle-like CNTs	tangled CNTs are swallowed into cells; long, needle-like CNTs activate secretion of IL-1α and IL-1β
lenghth	normal mice	MWNTs 15–20 μm or longer	long MWNTs cause inflammation and granulomas
	human primary macrophages	short CNTs; long, tangled CNTs; long, needle-like CNTs	long, needle-like CNTs activate secretion of IL-1α and IL-1β
	THP-1; rat	MWNTs 500 nm to 5 μm	MWNT with an average length of 825 nm induce higher inflammation than those with an average length of 220 nm
	A549; THP-1; normal mice	MWNTs: length 5–15 μm, diameter 20–60 nm; length 1–2 μm, diameter 60–100 nm; length 1–2 μm, diameter <10 nm	long and thick MWNTs induce the strongest DNA damageand increase the total cell number in abdominal lavage fluid while similar SWCNTs caused little effect
	P53+/-mice	long MWNTs 1–20 μm	Long MWNTs (short not included) can form

			fibrous or rod-shaped particles of length around 10-20 micrometer (μm) and induce mesothelioma
agglomeration	SPC; DRG	agglomerated SWCNTs; better dispersed SWCNT bundles	highly agglomerated SWCNTs significantly decrease the overall DNA content
	MSTO-211H	CNT agglomerates; CNT bundles	suspended CNT-bundles are less cytotoxic than asbestos, rope-like agglomerates
layer number	RAW 264.7	pristine graphene	depletion of the mitochondrial membrane potential and increase of intracellular ROS and apoptosis
	alveolar macrophage	SWCNTs; MWNTs (diameters 10–20 nm)	SWCNTs > MWNTs

Table 9.1: Basis of CNT toxicity. Reprinted with permission of [89]

Metal impurities, especially catalyst metal contaminants, such as Fe, Y, Ni, Mo, and Co coming from production methods are the most important factor in CNT cytotoxicity. Even if it is almost impossible to remove all the impurities completely because they are entrapped into graphitic shells, they can be released in the biological medium causing negative effects [90]. However acid treated SWNT and MWNT with a very small metal content are commercially available. The functionalization of CNT surface is determinant to reduce the toxicity: 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 [91]. Furthermore the functionalization and the surface charge affect the binding of blood proteins and this could greatly alter their cellular interaction pathways and their metabolic fate and can reduce the cytotoxicity [92]. We have demonstrated that chemical functionalization reactions and appended functionalities that lead to shortening or untangling/debundling of aqueous dispersions of f-MWNTs will help to resolve toxicological risks associated with long-fibre exposure [93]. Another important aspect to take into consideration is the administration route used combined with the dose. In general it is very difficult to evaluate the toxicity of CNTs because of the extreme heterogeneity of samples described through the literature. To make experimental results comparable, it is needed to establish recognized standard CNT samples in toxicity testing and also to establish standardized and reliable methods for evaluating CNT toxicity.

3 Summary and conclusions

Among the different possibilities of application of CNTs for biomedical purposes, tissue engineering can be acknowledged as one of the more interesting. Thanks to their physical and chemical features CNTs can provide the final composite with those biomimetic requirements that are fundamental for a full biocompatibility and a functional efficiancy. In general the research in this field is still at an early stage and there is still much to do in order to improve the interaction between tissues and materials at cellular and sub-cellular level and to clarify all the doubts regarding toxicity issues but, as reported in this overview, preliminary results are very promising, indicating CNTs as ideal support for tissue growth and recovery.

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