

Carbon Nanostructures; The Current Potential Applications in Tissue Engineering

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Abstract

Tissue engineering aims to provide effective organs or substitute tissues for patients. One of the most important factors in this field is biomaterial so with the development of tissue engineering, new tools and materials for designing engineering scaffolds for cellular control and tissue growth are required. Carbon as a biocompatible and hemo-compatible material plays a significant role in the design of prosthetics. Carbon structures are used as implant coating, or as fibers in artificial organs as a strengthening agent. Characteristics of carbon with different morphologies in the body as a prosthetic have been demonstrated its high performance. One of the new structural forms of carbon is its nanostructure that can increase preformation of prostheses and be also used in different fields of medical sciences such as drug delivery and tissue engineering. Carbon nanostructures are new candidate that may meet the needs of various bio-medical applications and thus can be an important engineering material for tracking and detecting cells, delivering drugs and biological agents, and cellular scaffolds.

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1. Introduction

The concept of tissue engineering was formally presented by Langer and Vacanti in a historical article in *Science* in 1993, in which the properties and

applications of 3D biodegradable scaffolds were first described in detail [1]. Tissue engineering is a multidisciplinary fields for the reconstruction of tissues and organs, integrates various branches of



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science such as biology, biomedicine and biochemistry. An extremely important component defining the concept of engineering is the extracellular matrix (ECM) which links the design of scaffolds with biocompatible materials that support cell growth, differentiation and migration. Before attempting to reconstruct any tissue or organ, recognizing its anatomical structure and biography is important because it allows controlling the conditions that can affect tissue formation [1]. Several methods for the production of scaffolds as cell structure have been used in tissue engineering. The efforts of tissue engineers are aimed to design and construct scaffolds that support tissues during repair, healing and reconstruction, and to provide the suitable environment for cell growth and proliferation. Based on the nature of the biomaterial (natural or synthetic), its formability and manufacturability, the scaffold should be able to enhance and improve cell-matrix and cell-cell interactions [2-14]. Carbon as a biocompatible and blood compatible material plays a significant role in the design of prosthetics. Carbon is used as an implant coating, especially for heart valves, or as fibers in artificial ligaments and tendons as a strengthening agent [15]. Carbon nanostructure can increase preformation of prostheses and be also used in different fields such as drug delivery and tissue engineering [16-21].

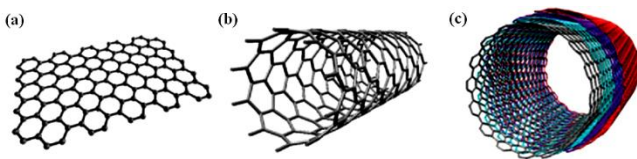


Figure 1. Schematic diagram of (a) graphene, (b) SWCNT and (c) MWCNT [15].

1.1. Carbon nanotubes (CNTs)

Carbon nanotubes (CNTs) are the first generation of nanoscale products discovered and launched in 1991. The nanotubes that are obtained from the complexity of graphite sheets are very long and thin and have stable, resilient, and flexible structures. If a carbon nanotube contains only one single graphite, is called single-wall nanotubes (SWCNTs), and if it contains a

number of concentric tubes, is called multiwall carbon nanotubes (MWCNTs). Carbon nanotubes in the scaffolds can enhance its structural properties, along with adding new properties such as electrical conductivity, which may also contribute to the direction of cell growth. One of the problems with the application of these nano-materials is the potential cytotoxic effects, which can be reduced by chemical agents [22]. The mechanical properties of carbon nanotubes, along with their unique structure and geometry, have attracted the interest of researchers in the preparation of high-strength composites and fracture toughness. In addition to the mechanical properties of these structures, the electrical, chemical and biochemical properties of carbon nanotubes can be mentioned. The use of carbon nanotubes, for trafficking of bio-molecules as well as medications, has been considered due to their unique surface properties; hence these nanostructures are subjected to surface modification, in order to link the bio molecules with ideal efficiency. Apart from the use of nanotubes in drug delivery or drug release, surface-modified carbon nanotubes interfere with the walls of molecules such as proteins, nucleic acids, carbohydrates, and other organic chemicals. CNTs have a large surface area that is exposed to biological tissues, but the asbestos-like appearance raises a serious concern about the toxicity to living organisms, and many studies have been performed to address these concerns. Recent studies have demonstrated that major CNT toxicities directly relate to specific properties of this material, such as their dimensions or the presence of impurities, such as metal particles or residues. In fact, accurate purification, and in particular the performance of CNTs, by choosing the appropriate size, results in a significant reduction in their toxic effects [23]. CNTs have certain physical and chemical properties, they are thermal and electrical conductive, but they are also very elastic. All of these properties theoretically make CNTs to become good candidates for the reconstruction and improvement of the function of tissues, particularly the nervous system or bone [24]. For example, the functional activity of carbon nanotubes has been proposed as a suitable scaffold for absorbing biological ions and forming bone mineralization. So

that the deposition of calcium and phosphate ions on this structure leads to the formation of a kind of calcium phosphate with a similar structure of the bone.

MacDonald *et al.* designed a collagen-SWCNT composite for the cell culture substrate. The SWCNTs were strongly entrapped by collagen and the composite showed high mechanical properties. And the cell showed good cell viability [25]. Also the cell adhesion on the MWCNT coated dish was much higher than that on the collagen-coated dish. Therefore, CNT-coating for dishes will be a useful new material for cell culture [26].

We will continue to explore the use of carbon nanotubes as well as graphene in the engineering of tissues like bone and nerve and cardiovascular tissues.

1.2. Graphene

Graphene is a thin two-dimensional structure of six-atomic carbon bonds, which form an atomic hexagonal network. The unique feature of graphene is hybridization SP^2 and its very thin atomic thickness [27]. The carbon atoms have six electrons, two electrons in the inner layer and four electrons in the outer layer. In graphene, each atom is connected to three other carbon atoms on a two-dimensional plate, so there's a free electron in the third dimension for electron conduction. Graphene sheets have special thermal, electrical, mechanical and optical characteristics, similar to carbon nanotubes [28]. Research has shown that the thermal stability of graphene is due to the strong carbon-carbon intermolecular bonding of this material, which prevents heat oscillations. The inherent strength of this material, due to the strength of carbon bonds, is another important characteristic of graphene. It is also elastic, which enables it to maintain its initial size after applying the pressure [29]. Graphene, like carbon nanotubes, has a potential for use in biological applications due to its structural characteristics. The chemical purity, the vast surface, and the low cost of producing large quantities of graphene films have increased the use of this material in biomedicine. Various studies have shown the bioavailability of graphene for cells. Based on cell culture experiments,

graphene sheets are biocompatible and have low toxicity for biomedical applications [30,31]. Graphene also possesses the features that have made it attractive for medicine regenerative, which will be further addressed in this review.

2. Application of carbon nanostructures in bone tissue engineering

Bone tissue is a natural composite mainly comprised of collagen fibers and hydroxyapatite crystals. Osteoblastic cells secrete the protein matrix and then form the inorganic phase with hydroxyapatite. Polymers and polypeptides, as well as bio ceramics such as hydroxyapatite and tri-calcium phosphate, are used to make bone scaffolds. These materials have relatively low mechanical strength and of them are very sensitive to immune response. Carbon nanotubes can be considered to be a very good option in the design of composite scaffolds for bone tissue engineering, due to their flexibility, elasticity and low density properties.

Zhang *et al.* studied effect of SWCNT, double walled carbon nanotubes (DWNTs) and MWCNT on the proliferation, differentiation, trans-differentiation and mineralization of primary osteoblasts. They treatment of CNTs could reduce the viability of primary osteoblasts and inhibit the mineralization of osteoblasts in a dose dependent manner. Also CNT reduced the adipocytic trans-differentiations. However, the inhibition was not strong enough to reverse the cytotoxicity and suppression on viability and mineralization of primary osteoblasts [32].

Pristine SWCNTs do not show any inherent properties which support new bone growth. Specifically, pristine SWCNTs do not contain functional groups that can attract calcium cations that initiate the crystallization of HA [33]. Therefore, in order to use CNTs as a scaffold for bone regeneration, one can modify CNTs with some functional groups that attract calcium cations [33]. For example, SWCNTs were functionalized with poly(amino benzene sulfonic acid) (PABS)[33]. Thin films of either SWCNT-COOH or SWCNT functionalized with PABS (SWCNT-PABS) were deposited on glass slides. After soaking the films in a solution of $CaCl_2$ and Na_2HPO_4 , showed a large amount of

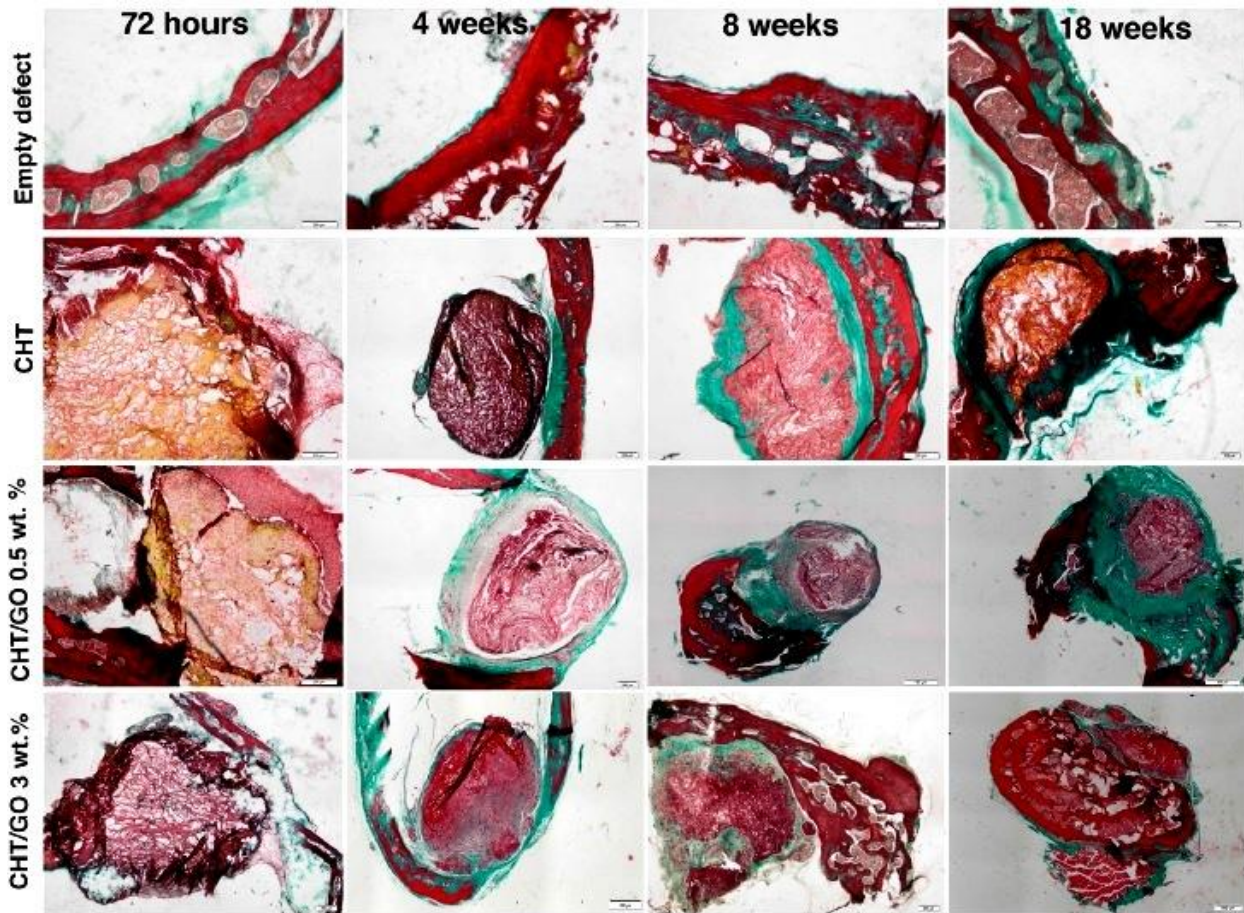


Figure 2. Histology of the repaired calvaria after 72 hours, 4, 8 and 18 weeks of CHT-GO scaffold's implantation [37].

“plate-shaped HA crystals” throughout the whole surface of SWCNT–PABS thin films and the thickness of the HA layer was found to be 2.4 μm . Price *et al.* also reported that greater weight percentages of carbon nanofibers in the PCU (polycarbonate urethane)/CNF (carbon nanofibers) composite increased osteoblast adhesion while at the same time decreased fibroblast adhesion. A material that can promote osteoblast adhesion can lead to faster integration of the bone to the implant surface *in vivo* [34].

Graphene as a coating can cause pro-osteodifferentiation on implants and scaffolds [35]. The ability of graphene to improve biological properties

of scaffolds and their ability to increase adhesion, proliferation and osteogenic differentiation of MSCs or osteoblasts has been studied in many studies. Kalbacova and colleagues [35] were able to cultivate osteoblasts on silica (SiO_2) and SiO_2 coated with graphene. After 48 hours, the cells covered homogeneous graphene substrate. The imaging data demonstrated that the number of osteoblasts doubled in the graphene bed. In another study, Lee *et al.* [36] observed a positive relationship between graphene bed and osteogenic differentiation. This study revealed the ability of graphene substrate to serve as a stimulant for osteogenic differentiation factors, such as dexamethasone and β -glycerophosphate [36].

Hermenean *et al.*[37] investigated osteogenic differentiation; along with bone repair capacity of 3D chitosan (CHT) scaffolds enriched with graphene oxide (GO) in critical-sized mouse calvarial defect. Results showed that CHT/GO scaffolds could represent a promising tool for the reconstruction of large bone defects, without using exogenous living cells or growth factors. The CHT/GO 0.5 wt.% group showed more fibrous tissue infiltration at weeks 4 and 8 compared with CHT group. New bone formation observed outlying from the surgical margins, while osteoid tissue and calcified bone spicules appeared neighboring connective tissue at week 18. The CHT/GO 0.5 wt.% material most resorbed than CHT alone after 18 weeks post-implantation, but large fibrous capsule observed.

La *et al.* [38] examined the potential of titanium coated with graphene oxide (GO) (graphene oxide) in loading and release of type 2 morphogenic protein (BMP-2). Osteogenic differentiation of human bone marrow MSCs (hBMMSCs) was tested on Ti and Ti / GO substrates. Finally, the high osteogenic potential of hBMMSCs showed Ti / GO substrates compared to graphene-free substrates. The Ti, Ti / GO, and BMP-2 substrate group was evaluated for rat calvaria defects. After 8 weeks, no new bone tissue was formed without BMP-2, but the Ti / GO / BMP-2 substrate showed better bone formation than Ti / BMP-2 [38]. Crowder and colleagues have proven the potential for osteogenic differentiation of MSCs on graphene 3D (three-dimension) substrates. The authors stated that a 3D foam structure provides a suitable basis for differentiating bone cells [39]. The effect of graphene and carbon nanotubes on the osteogenic differentiation of MSCs was also studied by Duan *et al.* Nano-fiber composite scaffolds of poly-L-lactide (PLLA) and graphene were designed with carbon nanotubes and were biologically evaluated *in vitro* and *in vivo*. Nanofibrous structures significantly increased cell adhesion, osteogenic proliferation and differentiation of hBMMSCs, although graphene substrate showed a greater effect on osteogenic differentiation of hBMMSCs than CNT. The results of *in vivo* test also showed that both nanocomposite scaffolds had good biocompatibility and excellent ability to trigger osteogenesis [40].

Tavarty and colleagues synthesized a new biocompatible nano-composite combination of graphene oxide-calcium phosphate (GO-CaP) and studied its ability to induce osteogenic differentiation in MSCs. All three GO, CaP and GO-CaP substances induced calcium in the osteogenic medium significantly higher than that of in control, while negative controls did not show calcification. Also, in comparison to GO-CaP nanocomposites, they had higher osteo-inductivity than CaP or GO alone [41]. Xie *et al.* investigated various ratios of graphene to improve the load-bearing implant surface. The results obtained from the study of surface properties of graphene-calcium silicate composite coatings showed that the graphene coatings are homogeneous in the calcium silicate matrix. Coverage of surfaces has been shown to be beneficial for cellular behavior and early fixation of bones. CNTs can cause stem cell differentiation among bone cells, mostly due to high protein uptake by CNT substrates. Because CNTs are less compact than ceramics and metals, they produce lighter, more flexible, but more stable scaffolds for bone tissue engineering [42]. In a study by Lin *et al.*, Poly (lactic co-glycolic acid) (PLGA) films modified with MWCNT with carboxylic group were used. Rectal Mesenchymal Stem Cells (MSCs) on the film showed good adhesion and significantly increased levels of alkaline phosphate compared to the control group [43]. Gupta and his colleagues synthesized SWCNT / PLGA composites with varying amounts of SWCNTs and studied the behavior of human bone marrow MSCs on this scaffold. They observed that cell proliferation and osteoblastic differentiation of stem cells in composites with SWCNTs content increased compared to the PLGA substrate [44]. Zhang *et al.* synthesized three-dimensional PLGA / MWCNT nanofibers for bone tissue engineering. Rat MSCs showed a higher reproductive rate on PLGA / MWCNT nanofibers compared to PLGA nanofibers alone [45]. Mackie *et al.* designed nanofibers of poly (lactic acid) (PLA) along with CNT by electrolysis. They showed that PLA / CNT scaffolds are more stable than PLA nanofibers in buffered saline [46]. Baik and colleagues reported the osteogenic differentiation of human MSCs on SWCNT substrates. They used oxygen plasma to functionalize

the CNT surface and showed that the adhesion, proliferation and differentiation of stem cells increased compared to CNT substrates without surface modification [47]. Nayak *et al.* used MWCNTs modified with PEG (MWCNTs PEG-modified) (MWCNT-PEG) to differentiate human MSCs in the absence of biochemical induction factors. They measured the amount of osteopontin expression (OPN) as a biomarker for osteogenesis of stem cells, CD44 (for human MSC) and osteocalcin (for osteoblasts) on MWCNT-PEG, PEG and substrates coated with and without the addition of bone morphogenetic protein (BMP-2). The stem cells cultured on MWCNT-PEG and show that CD44 stem cell surface marker did not express, but differentiated human MSCs into osteoblasts compared to other substrates. Evaluating the differentiation of human MSCs into osteoblast showed that differentiation was possible even in the absence of osteogenic inducer [48]. Namgung and colleagues used oriented and random networks on the gold substrate to be able to control the orientation and differentiation of human MSCs. They observed that stem cells were able to detect CNTs and were directed along CNTs. In addition, when CNTs were oriented, the osteogenic differentiation of human MSCs increased compared to random and irregular CNTs even without the use of differentiation factors [49]. Facca *et al.* used CNTs reinforced with hydroxyapatite (HA) on titanium to regenerate bone. They used three different scaffolds (titanium, HA on titanium and HA / CNT on titanium) in an intranasal study in a mouse model. After a month of cultivation, cortical bones were completely restored, and all specimens showed good compatibility [50]. Li and colleagues examined the ability MWCNT to induce osteogenic differentiation of human adipose tissue derived stem cells (ASCs). They observed that MWCNTs caused bone formation after implantation in the body. The nano porous structure of MWCNT deemed to be useful in stimulating stem cells to produce bone tissue with the accumulation of bone inducing proteins in the body [51]. Shao *et al.* successfully developed the nanofibrous oriented and random mesh of PLA / MWCNTs. The presence of CNTs has greatly improved the mechanical and electrical properties.

The results showed that the oriented nanofibers were more effective in signaling and conducting osteoblasts compared with random samples [52]. Mei *et al.* developed an electrospun mat composed of PLLA, MWCNTs, and (HA). They found that the presence of CNTs increased the adhesion and proliferation of periodontal biosynthesis cells (PDLs). The PLLA / MWCNTs / HA scaffold grown with PDLs was implanted into the mouse muscle. PDLs that are attached to the scaffold demonstrated good performance *in vivo* and with no-inflammation detected around the scaffold [53]. Rodrigues *et al.* used butylene adipate-co-terephthalate-based fibers with low amount of super-hydrophilic MWCNTs for bone regeneration. Fibers showed good biocompatibility with osteoblast MG63 cells. Also, osteogenic differentiation of MG63 cells and the formation of nodules in (minerals formation) PBAT / 0.5% MWCNTs showed a significant increase compared to the control group and PBAT alone [54]. Liu *et al.* designed PLA, HA, and GO-based electrospun mats, which demonstrated that adding a small amount of GO (from 1 to 3 wt.%) would strengthen PLA / HA mat. In addition, GO along with calcium phosphate increased alkaline phosphate activity and calcium deposition of osteoblasts [55]. Shao *et al.* added electrophoretic GO to PLGA and fibroin silk (SF) for the purpose of bone tissue engineering. That GO results in simultaneous increase of mechanical and biological properties of the materials. Compared to PLGA or PLA / SF, the elastic modulus and tensile strength of GO-containing materials were significantly increased [56]. An and colleagues designed PLA / PU composites with 3 and 5 wt.% GO. The results showed that these materials have excellent biocompatibility and antimicrobial activity. Therefore, scaffolds with to bone and cartilage regeneration, they can simultaneously reduce the risk of chronic infection of the surrounding tissue [57]. Pereira *et al.* designed PLA and nano diamond-based electrospun mat for bone tissue engineering. The combination of nanoscale diamonds (Nano-diamonds) in the biopolymer increased the hydrophilicity and show positive effects on cell adhesion and proliferation. The combination of MWCNTs and GO with HA in many ways represents

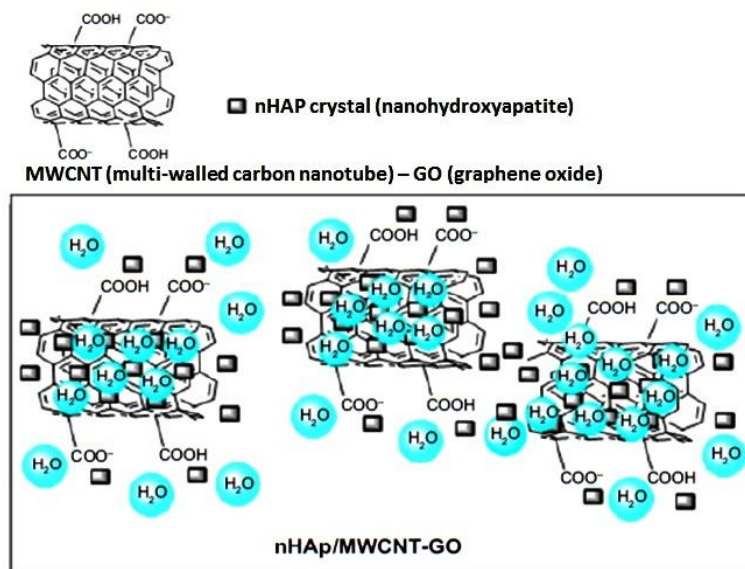


Figure 3. Scheme illustrating the growth of nHAp on MWCNT-GO surfaces and their interaction with water molecules [63].

an appropriate replacement for the chemical, physical and biological properties of polymer composites, as well as the production of materials that can simulate production of bone tissue [58]. Balani *et al.* reported an improvement in the fracture toughness and crystallinity of Hydroxyapatite (HAP) / MWCNT coatings prepared by plasma spray [59]. Lahari *et al.* showed that MWCNTs increased the adhesion of osteoblasts in HAP / MWCNT nanocomposites [60]. In a study, aGO-functionalized Ti porous scaffold (GO/Ti scaffold) designed by depositing GO onto polydopamine (PDA) modified Ti scaffolds. The mussel inspired PDA modification facilitated the interaction between GO and Ti surfaces, leading to a uniform coverage of GO on Ti scaffolds. BMP2 and vancomycin separately encapsulated into gelatin microspheres (GelMS). The mussel-inspired GO/Ti hybrid scaffold combined the good mechanical properties of Ti scaffolds and the advantages of GO nanosheets. GO nano sheets with their unique nanostructure and functional groups, together with GelMS on Ti scaffolds, can be used as suitable carriers for drug delivery and provided adhesive sites for cell adhesion and created nanostructured environments for bone regeneration [61].

Nunez *et al.* reported nHAP growth on CNTs through the wet-chemical *in situ* precipitation route [62]. Rodrigues *et al.* synthesized nHAp / MWCNT-GO nano composites for producing materials with bone properties for orthopedic applications. That nHAp / MWCNT-GO scaffolds were very porous (~60-70%) and were favorable for cell proliferation and penetration for bone regeneration purposes. Moreover, increasing MWCNT-GO concentration resulted in the absorption of nHAP and consequently an increase in hydrophilicity property. The synthesized nano composites were biologically active and had a good antibacterial effect against *S. aureus* and *E. coli*, and no osteoblastic toxicity was observed [63].

Elkhenany *et al.* evaluated the effect of graphene on the growth and differentiation of mesenchymal stem cells under *in vitro* conditions. Proliferation and differentiation between TCPS (Tissue culture polystyrene) control and graphene-coated control were compared. The cells cultured on a sample with oxidized graphene exhibited osteogenic differentiation in a culture medium containing a bovine serum without adding glucocorticoid or specific growth factor. These findings showed that graphene could act as an osteo-inducer, and that a

combination of graphene and mesenchymal stem cells could provide a suitable structure for bone tissue engineering [64]. Zanello *et al.* investigated the proliferation and function of osteoblastic cells implanted on various functionalized nanotubes and demonstrated that bone marrow cells preferred electrically neutral CNTs that increase osteoblasts and bone formation [65]. Domke *et al.* showed that cell adhesion increased with roughness of the surface. In this study, the cells were adhered to cavity structures and exhibited a higher adhesion strength ($E = 5.43 \pm 2.05$ kPa) compared to osteoblasts distributed on random CNTs ($E = 4.14 \pm 1.69$ kPa). The regular tomography compared to irregular surface could have a greater effect on the adhesion of osteoblast cells [66]. MacDonald and colleagues also found that modified-carbon nanotubes could be used as an appropriate scaffold for the growth of osteoblasts [67]. Nayak *et al.* examined the effect of graphene on the growth of stem cells. Four substrates with different hardness and roughness were used: polyethylsiloxane (PDMS), polyethylene terephthalate (PET), glass slide and silicon wafer with 300 nm SiO₂ (Si / SiO₂), graphene-free surface coating as control. Survival and differentiation of stem cells were investigated on samples. The results of MTT analysis for cell biocompatibility showed that there was no significant difference in the survival of cells between substrates with and without graphene coating, indicating that cell growth was really affected by the presence of graphene. In addition, the presence of graphene did not affect the shape and growth of cells in normal stem cell media. In the presence of osteogenic environments, graphene coating clearly accelerates the differentiation of hMSCs at a similar rate to substrates with the BMP-2 [68].

3. Application of carbon nanostructures in nervous system engineering

The reconstruction of post-traumatic neural tissue as a complex and difficult process which led researchers to attempt regenerate damaged tissues with the help of cellular technology and tissue engineering. Due to the fact that carbon nanotubes are electrically

conductive and also have a diameter close to that of nerve fibers, they are an ideal material to grow and repair neurons.

CNFs/CNTs have exceptional electrical, mechanical and biocompatible properties, are excellent candidates for neural tissue repair [69–72]. CNFs have excellent properties comparable to CNTs but at a lower cost and are fabricated through an easier scale-up process [72,73], thus, CNFs have generated much interest in regenerative neural tissue engineering applications.

Nguyen-Vu *et al.* fabricated a vertically aligned carbon nanofiber (VACNF) electrode coated with a thin film of electronic conductive polypyrrole polymers for neural implants [74-76].

The study showed that the vertical CNF arrays helped to form an intimate neural–electrical interface between cells and nanofibers for neural prosthesis. Many researchers have fabricated various patterned and random CNF nanocomposites for potential tissue regenerating applications [77,78]. McKenzie *et al.* investigated astrocyte (one of the glial scar tissue forming cells) function on CNFs/polycarbonate urethane (PCU) composites [77]. They demonstrated that astrocyte adhesion can be effectively inhibited when incorporating and increasing the surface energy of CNFs in the polymer composites.

Similar to CNFs, CNTs also potentially serve as substrates to impregnate progenitor cells (such as stem cells) and selectively differentiate them into favorable neuronal cells at injury sites.

Because MWNTs can have diameters approximately 100 nm, they can possibly be used to mimic neural fibers. MWNTs have been shown that hippocampal neurons from Sprague-Dawley rats were able to grow on carbon nanotubes coated with 4-hydroxynonenal [79].

Jan *et al.* [80] investigated the efficacy of SWCNT / PEI (Poly ethylene-imine) composite as a nerve stem cell culture substrate. An increase in the growth of neuronal cells and increased expression of protein 2 associated with microtubules (MAP-2) were observed. Therefore, CNT was proposed to be as a suitable culture substrate for nerve stem cells. The results indicated that composites were not only cytocompatible for stem cell growth, but also

contributed to differentiating stem cells to neuronal cells. Studies have shown that CNTs functionalized with bioactive molecules can improve neural regeneration and attachment of growth cones [81]. SWCNT with polyethyleneimine (PEI) copolymer has been synthesized to effectively lengthen neurites and increase neurite branches approximately comparable to those on polyethyleneimine [81]. Matsumoto *et al.* demonstrated that MWCNTs with neurotrophin can regulate and promote neurite outgrowth [82].

The use of graphene oxide and carbon nanotubes combined with other biomaterials can increase the responsiveness of the neurons [83-90]. Liu *et al.* graphene oxide and carbon nanotubes were covalently functionalized to obtain cross linkable grapheme oxide acrylate (GOa) sheets and carbon nanotube poly(ethylene glycol) acrylate (CNTpega). An electrically conductive reduced GOa–CNT pega–oligo (polyethylene glycol fumarate) (OPF) hydrogel (rGOa–CNTpega–OPF) was successfully fabricated by chemically crosslinking GOa sheets and CNTpega with OPF chains followed by in situ chemical reduction in L-ascorbic acid solution. Results were demonstrated robustly stimulated neurite development in PC12 cell on a conductive rGOa–CNTpega–OPF composite compared with that on neutral OPF hydrogels. The material illustrated a promising potential as conduits for neural tissue engineering [91].

Park and colleagues showed that by controlling the shape of the CNT substrates, the growth, polarization and differentiation of NSCs can be controlled. In controlled substrates with CNT, NSCs showed a better differentiation into astroglial and neural like cells [92]. Chao *et al.* cultivated the hESCs (human embryonic stem cells) on a CNT-poly (acrylic acid) composite. The results showed that the differentiation of hESCs into neurons on composite content CNTs was significant compared to hESCs cultured with poly (L- ornithine) (PLO) [93]. Sridharan *et al.* used a CNT / collagen composite for nerve differentiation. They modified type I collagen by CNT. CNT not only improved collagen biocompatibility, but also increased interactions between hESCs [94]. Tay *et al.*, designed the fibrocetin-coated SWCNT substrate to enhance the adhesion of hMSCs compared to conventional culture media, they illustrated an increase in the expression of a neuron gene such as nestin and MAP-2, which is a cytoskeletal protein in neurons and dendrites. Coating CNT with biocompatible proteins is a solution to reduce the immune response [95]. Kam *et al.* designed SWCNTs / laminin layer structures. As a result, SWCNT-laminin films minimized immune responses without affecting neuronal differentiation; this study was significant because it demonstrated that CNT linked with such proteins could be used as a potentially biocompatible material for neural tissue engineering [96]. Park *et al.* used graphene substrates to differentiate human NSCs to neurons. Human NSCs were grown on graphene and glass substrates. Adhesion and cell differentiation into neurons were observed on graphene substrates, while more glial cells than neurons were found on the glass substrate [97]. Hong and colleagues cultivated PC-12 cells on glass substrates coated with and without graphene. Cell adhesion was observed with higher cell proliferation and nerve differentiation on graphene-coated substrates [98]. Wang *et al.* cultivated MSCs on fluorinated graphene plates and observed that plates showed a significant increase in the differentiation to neurons [99]. Li and colleagues used a graphene based foam scaffold that controlled the

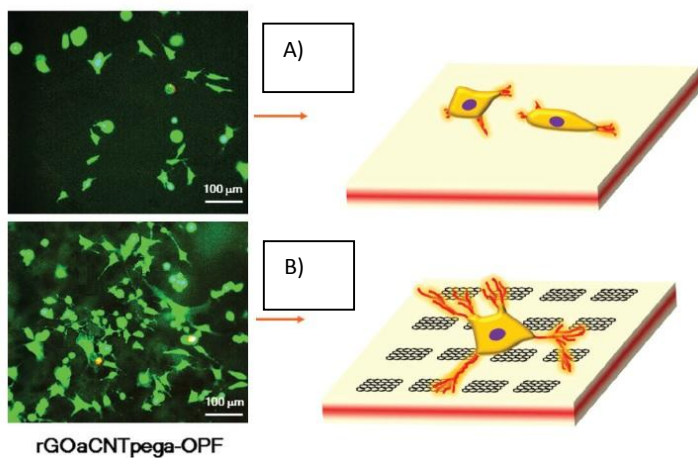


Figure 4. Live (green) and dead (red) staining of PC12 cells on (a) OPF and (b) rGOaCNTpega–OPF hydrogels [91].

behavior of NSCs. The scaffold showed an excellent biocompatibility. In addition, the 3D foam structure had greater electrical stimulation compared to the 2D graphene structure [100]. Yang *et al.* used three substrates; GO, graphene and CNT to induce dopamine neuronal differentiation of rat embryonic stem cells (ESCs). ESCs were cultured in all substrates. Graphene and CNT did not show any significant progress, but the GO substrate resulted in a significant increase in dopamine neural differentiation [101]. In a study by Tang *et al.*, neurospheres were grown on graphene substrates, and after cultivation, the formation of neural networks was observed. The formed neuritis begins to form synapses, so graphene can be considered as a good substrate for increasing neuronal activity [102]. Shah and colleagues designed nano-graphene-based materials for making nanofibrous scaffolds to guide the differentiation of NSCs into oligodendrocytes. The use of GO in combination with electrospun nanofibers is effective in differentiating NSCs into oligodendrocytes. Also, the amount of GO in the scaffolds was directly proportional to the expression of the key neural markers [103]. Song *et al.* studied the anti-inflammatory effect of graphene foam cultured with microglia cells [104]. Li and colleagues showed good potential of graphene for neural tissue. Designed graphene foam greatly differentiated NSCs into neurons and could be used as nerve scaffolds [105]. Meng *et al.* showed that electrical stimulation increased the growth of neurite. The positive effect of graphene on neurite growth and propagation was studied when graphene was coated with fetal bovine serum (FBS) [106]. Convertino *et al.* examined the potential of graphene as an interface and conductor of peripheral nerves [107]. Mattson *et al.* studied the effect of MWCNTs on increasing adhesion and growth of neurons. They cultivated hippocampal neurons on glass substrate coated to polyethylene amine (PEI) and multi-walled CNTs (MWNTs) mats. Neurons were able to grow and strengthen their neurites in all directions. Also CNTs were functionalized using 4-hydroxynonenal. Neurons increased the length and number of their neurites (2-3 times) compared to that of when they grow on unmodified CNTs [108]. Hu *et al.* showed that

functionalized CNTs with active biochemical molecules can increase and improve neural regeneration [109]. Hung *et al.* designed the CNT rope substrate for the growth and differentiation of NSCs. They observed that the electrical stimulation of cells cultured on the CNT rope increased the growth and direction of neurite and increase the early differentiation of NSCs into adult neurons [110]. Chen *et al.* also observed that carboxylated MWCNTs can increase the MSCs neuronal differentiation without using an external differentiation factor [111]. Kim and colleagues designed and synthesized MWCNT-oriented planes to control the proliferation and differentiation of human MSCs into neurons, and observed that human MSCs expanded and proliferated after a day of cultivation. Stem cells were cultured on a glass slide as controls, and it was reported that neuronal markers were observed for cells cultured on CNT oriented plates compared to controls [112]. Kabiri *et al.* designed a PLLA-CNT nanofibrous scaffold for cultivating and differentiating mice ESCs. They showed that PLLA-CNT nanofibers produced good electrical conductivity due to the presence of CNTs, as well as increased the adhesion, amplification and differentiation of mice ESCs compared with controls [113]. Zang *et al.* designed poly (ethylene terephthalate) (PET) fiber matrix coated with MWCNTs. They observed that the biocompatibility and adhesion of the mouse ESCs increased on MWCNT scaffolds compared to PET due to increased scaffold roughness with CNTs. Also, CNTs facilitated the differentiation of mouse ESC to neurons and the formation of a neural network [114]. Chen and colleagues designed silk-MWCNT scaffolds to improve human ESCs' neural differentiation. They cultivated stem cells on silk-MWCNT, silk, and poly (L- ornithine) PLO substrates. The cells cultured on silk-MWCNT scaffolds exhibited more neuronal differentiation, as well as complex 3D axon connections, while this preformation was not observed on other scaffolds [115]. Roman *et al.* showed axonal growth, and functional recovery of spinal cord injury by chemically modified SWCNTs with PEG [116]. In another study, Lee *et al.* showed that amine

functionalized SWCNTs protect the neurons and increase the behavioral performance of induced

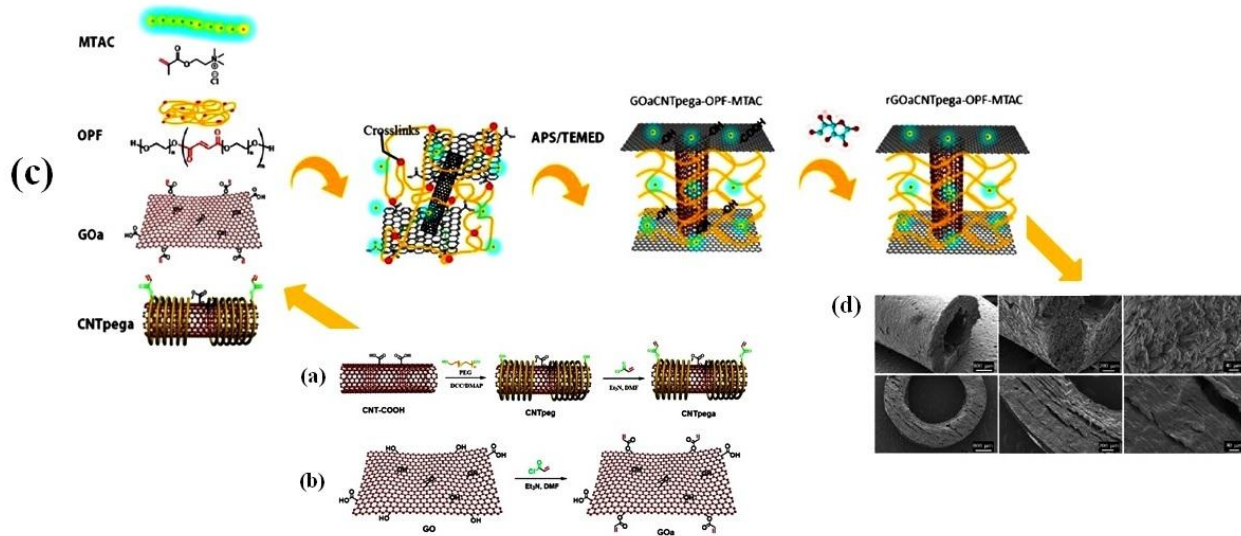


Figure 5. Synthesis procedures for obtaining CNTpega carbon material and Goa (a and b). Fabrication of conductive GOaCNTpega-OPF-MTAC hydrogel and in situ reduction of GO sheets in L-ascorbic acid solution (c), and SEM images of the fabricated tubular conduit (d) [126].

stroke rats [117]. Malarkey *et al.* designed SWNT films that were covalently linked to PEGs. They observed that these substances affected growth of neurons [118]. In another study, Cho *et al.* cultivated PC12 cells on an electrically conductive CNT / collagen composite. Collagen caused adhesion, differentiation and survival of the neurons, and also provided electrical stimulation due to the presence of carbon nanotubes, so the cells were able to expand their neurite on this surface [119]. Zhang and his colleagues synthesized patterned vertical MWNTs with different lengths. These MWNTs were then coated with poly-l-lysine (PLL). The neural cells increased neurite growth along the edges of the patterned bed. Additionally, authors observed the formation of neural bridges across patterned borders and neurites were drawn at long distances of about 20 microns to form synaptic connections [120]. Gabay *et al.* cultivated neurons on CNT islands on quartz surfaces. Several days after cultivation, the cells were accumulated in the areas covered by CNTs. Generally, the processes included single axons or axons and dendrites. The networks formed using the

patch-clamp techniques were evaluated for electrical activity. Overall, good performance was shown [121]. Galvan-Garcia *et al.* designed a CNT substrate for neurite growth in the form of oriented, aligned or cross-linked strings. Hippocampal neurons cultivated on substrates expanded only along CNT strands and these neurites were non-branching or had very small branches [122]. Jin *et al.* designed PLCL nanofibers coated to ad hoc functionalized-MWCNTs to increase neurite adhesion and growth. The scaffold coated with MWCNT showed increased adhesion, proliferation and growth of PC-12 cells compared to non-coated PLCL scaffolds [123]. Massoumi *et al.* designed gelatin and GO-based nanofibrous scaffolds. They covalently linked GO to (poly(2-hydroxyethyl methacrylate)-graft-poly caprolactone). The electrical conductivity of the obtained electrospun nanofibers indicated the proper performance of such scaffolds for damaged nerve tissue regeneration [124]. Aznar-Cervantes *et al.* designed fibrin nanofibers with GO for biomedical applications. Tensile tests on matrix and nanocomposites containing GO or RGO (Reduced

Graphene Oxide) showed that coatings with graphene compounds reduced the elasticity of SF fibers, while increasing elastic modulus and ultimate strength. Among the designed specimens, those containing GO

graphene oxide acrylate (GOa) and carbon nanotube poly-(ethylene glycol) acrylate (CNTpega) for nerve regeneration applications. The conductive hydrogel fabricated by covalently embedding GOa and CNTpega within oligo(poly(ethylene glycol) fumarate) (OPF)hydrogel through chemical cross-linking followed by in situ reduction of GOa in L-ascorbic acid solution. In previous study of this group [127], OPF cross-linked with [2-(methacryloyloxy)ethyl]trimethylammonium chloride(MTAC) to form a positively charged OPF-MTAC hydrogel and showed enhanced effects on neuronal cell adhesion, proliferation, and differentiation during *in vitro* studies. The obtained rGOa-CNTpega-OPF-MTAC composite hydrogel showed good biocompatibility and excellent enhancement for PC12 cell proliferation and spreading. These results demonstrated promising potential for the rGOa-CNTpega-OPF-MTAC hydrogel to use as conduits for neural tissue engineering. In addition graphene and carbon nanotubes-based materials have been used as biocompatible substrates for the growth and differentiation of cells, including nerve cells.

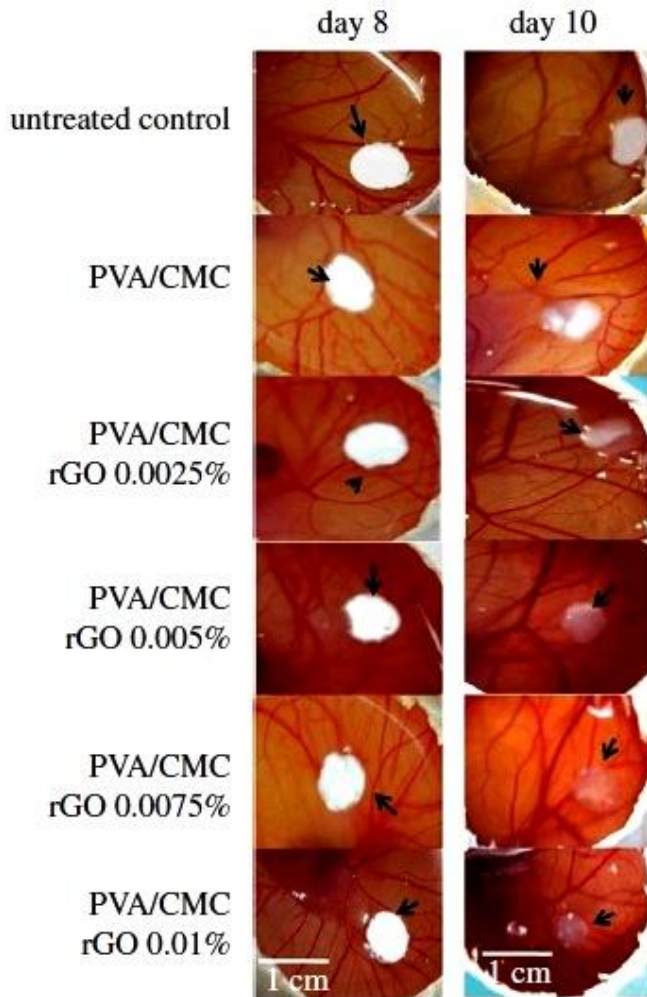


Figure 6. Digital images of the untreated and treated chick chorioallantoic membrane (CAM) assay. A significant increase in the number of blood vessels was observed in the PVA/CMC scaffolds incorporated with 0.005, 0.0075 and 0.01% rGO compared with the control (sterile filter paper discs)[133].

showed the best results in terms of fibroblast proliferation, while the RGO-coated mesh exhibited the highest electrical conductivity, in general, such graphene design and their derivatives are very suitable for nerve tissue engineering purposes [125]. In other study, Liu *et al.*[126] designed an electrically conductive hydrogel composed of functionalized

4. Application of carbon nanostructures in cardiovascular tissue engineering

Being a cell-based substrate, CNTs can cause electrical stimulation of neural tissue cells as well as, heart tissue. Mooney *et al.* designed a carboxylated PLA-SWCNT nanoclay scaffold in their study to enhance the differentiation of MSCs into cardiomyocytes. The differentiation of MSCs in the presence of CNTs increases after electrical stimulation [128]. Lee *et al.* showed that vitronectin-coated graphene increases the differentiation of human ESCs to cardiomyocytes. Human ESCs were also cultured on Matrigel-coated glass. The results showed that human ESCs cultivated on graphene enhanced the expression of genes involved in specific differentiation to mesodermal and endodermal cell lines and differentiated cardiomyogenic cells [129]. Hosseinpour *et al.* studied the effects of MWCNTs on mouse heart tissue. The effects of MWCNTs on the ECG signal were investigated in a group of mice before and after nanotube injection. The MWCNTs

did not significantly alter the sympathovagal balance. It was also observed that CNTs injections increase heart beating rate. In addition, even though nanotubes did not cause serious problems in normal autonomic nervous system (ANS) activity; however, they were not fully compatible with cardiomyocytes [130]. Liu *et al.* also designed poly (ethylene glycol) -poly (D, L-lactide) copolymers (PELA) containing 6% CNTs to create synthetic micro-environment to improve the function of cardiomyocytes. Loading large amounts of CNTs into fibers increased cellular contraction and the production of contractile proteins, as well as the synchronous beating behavior of cardiomyocytes [131]. Zhou *et al.* used nano-conducting materials for the repair of damaged heart tissue. They designed gelatin hydrogels contain single-wall carbon nanotubes (SWNTs). They found that SWNTs can provide micro-cellular environment in suitable laboratory conditions for contraction of the heart and expression of relevant proteins. In this study, the heart tissue based on composite gelatin / SWNTs demonstrated stronger contractile and electrical properties in laboratory conditions [132].

Chakraborty *et al.* [133] assessed the potential of reduced graphene oxide (rGO) for enhancing angiogenesis in tissue engineering applications. Polyvinylalcohol/carboxymethyl cellulose (PVA/CMC) scaffolds loaded with different concentrations of rGO nanoparticles were synthesized via lyophilization process. The scaffolds containing 0.005 and 0.0075% rGO enhanced the proliferation of endothelial cells *in vitro*. *In vivo* studies showed that the scaffolds containing rGO significantly enhanced angiogenesis and arteriogenesis.

5. Cytotoxicity

It seems that the development of CNTs with almost no hazard to human health is possible, because it has been reported that the physical properties (length, thickness, rigidity) of the CNTs greatly contribute to their toxicity [134,135]. Several studies showed that carbon nanotubes are cytotoxic. Cytotoxicity was observed after 6 h of exposure (AM) of SWNTs and MWNTs with alveolar macrophages [136]. *In vitro* studies demonstrated that highly purified single walled carbon nanotubes (SWCNTs) induced the

release of inflammatory makers (nitric oxide and Interleukin-8 (IL-8)) [137,138-145]. Casey *et al.* showed the possible effect of media components on SWCNT-induced cellular responses [146]. Most cases of *in vivo* and *in vitro* studies involved high concentrations (50~ 800µg/ml) and exposure times (>24 hours) [146,147]. Mooney *et al.* showed that functionalized CNTs were easier to disperse in human mesenchymal stem cell media. The COOH-functionalized SWCNT were least toxic to the cells [148]. Kalbacova *et al.* indicated that differently prepared SWCNT films are not toxic for osteoblasts and could be used for biomedical applications [149]. In a phagolysosomal assay; carboxylated SWCNTs showed both longitudinal splitting and de-bundling as well as oxidative degradation of the side walls producing ultrafine carbonaceous particles. The oxidatively-degraded carbon nanotubes may be more readily cleared from the lungs and induce less toxicity than native or other types of surface-functionalized single-walled carbon nanotubes [150].

Graphene has low toxicity and a large dosage loading capacity, making it a potential efficient carrier for therapeutic proteins or tissue regeneration [151]. With the increasing interest in the use of graphene in biomedical applications, a number of studies have attempted to investigate the toxicity of graphene. Studies have shown that suspended hydrophobic graphene particles show more toxic than hydrophilic GO or functionalized graphene [152]. Graphene particles tend to agglomerate in cell culture medium with increasing concentration. In contrast, GO and chemically functionalized graphene (functionalized with carboxyl, hydroxyl, tween, dextran, chitosan, polyethylene glycol, proteins, etc.) tend to adsorb proteins on their surface, limiting direct interaction with cells, thereby minimizing cytotoxicity [153]. In addition, studies showed that the cytotoxic effects of suspended graphene-based materials are highly dependent on surface chemistry, particle size, shape, and concentration [154-156]. Non-functionalized graphene tends also to form strong multilayered aggregates while GO and rGO are generally present in single or few-layers. The use of polymers with a low content of graphene minimizes potential toxicity as it is slowly released from the degradable polymeric

matrix. In a study, chitosan matrix with GO enhanced cell proliferation, and importantly, the release of GO during degradation of chitosan did not elicit a toxic effect on the cells [157]. The collagen matrix with GO supported mesenchymal stem cell attachment and proliferation with no observable toxicity [158]. The studies demonstrated that there is strong evidence that biological response to graphene-based substrates is markedly different than that to suspended graphene-based particles. These substrates include small molecules and polymers to functionalize graphene and the use of metallic and ceramic decorated hybrid graphene substrates to modulate cell adhesion, assembly, proliferation, and differentiation. Morphological characteristics of the materials play also a fundamental role in influencing the toxic effects. For example, comparing GO to carbon nanotubes, it was shown that they display different toxicity for neurons [159]. Among the structural characteristics of graphenes, it was demonstrated that size is relevant on the internalization mechanism into the cells. Indeed, studies on macrophages pointed out that the intracellular localization of GO was dictated by size, thus leading to different compartmentalization's [160]. Also, bigger GO flakes induced a much stronger inflammatory response with high release of pro-inflammatory cytokines [161].

A Study was shown that GO biodegradation can be modulated by dispersibility [162]. The GO can be digested by peroxidases naturally present in cells. The biodegradation of graphene materials can avoid bio accumulation thus limiting its long-term toxicity. Moreover, studies from different groups incontrovertibly demonstrated that most of the already mentioned clinical side effects may be sensibly reduced or avoided by surface functionalization [163,164]. If substrate degrades, graphene may elute out over time, and the risk, if any, that it may pose needs to be thoroughly characterized in long-term animal experiments. Graphene released from the scaffolds as the polymer degrades may be taken up in cells via various endocytosis pathways. Thus, there is a need for better understanding of the uptake of the eluted graphene from the scaffold. The cellular uptake of protein-coated graphene depends on the size of the graphene sheets. Small nano sheets

of graphene enter cells mainly through clathrin-mediated endocytosis, whereas large graphene sheets enter by phagocytotic uptake. Nevertheless, the findings by different groups suggest that graphene-based biomaterials hold exciting promise in tissue regeneration, underscoring the need for continued investigations [165].

6. Conclusion

Nanotechnology has been shown to be effective in various medical areas. In this review, we have covered tissue engineering applications for the carbon nanostructures. Due to the unique properties of carbon nanostructures, such as desirable mechanical and electrical properties, these structures can be effective for bone, nerve and cardiovascular tissue regeneration especially in the form of coatings or in combination with other materials. Electrical stimulation can stimulate the cells in the tissues and ultimately accelerate cellular processes leading to repair of damaged tissues. Biocompatibility reports have demonstrated that carbon based matrix's have excellent potential for tissue regeneration and device integration. However, the performance of carbon-based substrates *in vivo* is not well understood, especially to address potential concerns of toxicity.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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