

Carbon Nanotubes in Regenerative Medicine

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Abstract This chapter focuses on the latest developments in applications of carbonnanotubes (CNTs) for regenerative medicine. Regenerative Medicine focuses on technologies to create functional tissues to repair or replace tissues or organs lost due to trauma or disease. Carbon nonotubes (CNTs) have been under investigation in the past decade for an array of applications due to their unique and versatile properties. In the field of regenerative medicine, they have shown great promise to improve the properties of tissue engineering scaffolds. Drug delivery and imaging of engineering tissues. The chapter will review these latest advances

1 Introduction

The focus of regenerative medicine is on developing methods that can be applied to create functional tissues, to repair or replace tissues/organs lost due to trauma or disease [4]. Nanomaterials show potential and promise in regenerative medicine due to their interesting physico-chemical properties [47]. Carbon nanotubes (CNTs) are widely researched as multi-functional nanomaterials due to their unique electronic, mechanical, optical and chemical properties [5]. A CNT can either be single-walled (SWCNT) (Fig. 1a) or multi-walled (MWCNT) (Fig. 1b). In case of SWCNTs, a graphene sheet rolls up to form seamless one dimensional

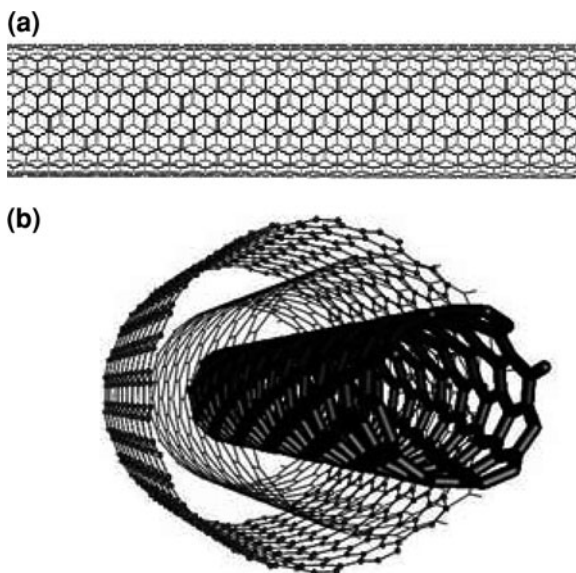
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Fig. 1 Depiction of **a** SWCNT with armchair structure, and **b** MWCNT with multiple concentric tubes of graphene



cylindrical tube, and for MWCNTs, the cylinder can be conceptualized as concentric layers of graphene sheets. The well-ordered arrangement of carbon atoms linked via sp^2 bonds provides superior mechanical stiffness, electrical and thermal conductivity. CNTs have a high aspect ratio. The diameter is between 0.8 and 2 nm for SWCNTs and 2–100 for MWCNTs. The length ranges between hundreds of nanometers to tens of micrometers.

In regenerative medicine, the structural and mechanical properties of CNTs make them applicable for use as composites for tissue engineering. CNTs can act as delivery vehicles for drugs and gene therapy and thus are suitable for therapeutics in regenerative medicine. Further, they also show promise as contrast agents for non-invasive in vivo molecular imaging. The recent developments in the aspects mentioned above are the focus of this chapter.

2 Nanocomposites and Nanoscaffolds

Biomaterials with excellent mechanical properties are of importance in the development of implants used for tissue regeneration. For instance, total joint arthroplasty requires the type of implant materials and design that can support large functional loads. To date, these implants have been designed with ceramic- or metal-based materials [31]. However, fatigue, corrosion, tissue infection, and poor implant-tissue interface create numerous problems in vivo. Polymers offer the flexibility to overcome these limitations. The mechanical properties of a polymer can be modified by composition, processing conditions and/or incorporating nanomaterials to create nanocomposites.

Carbon nanotubes (CNTs) have been characterized to possess excellent mechanical properties. Both SWCNTs and MWCNTs have high Young's modulus (~ 1 TPa) due to the flexible hexagonal network of carbon atoms [21]. Thus, CNTs have been incorporated as reinforcing agents into natural or synthetic polymer matrices. In these nanocomposites, CNTs have been recognized to improve substantially the mechanical and structural properties of polymer composites. Poly(propylene fumarate) (PPF), a linear biodegradable polyester, is a promising polymeric biomaterial for tissue regeneration applications. SWCNTs incorporated into PPF polymer have been shown to substantially improve the mechanical properties of PPF [37–39]. The presence of very low concentrations of SWCNTs (less than 0.5 weight percent) in the PPF polymer matrix substantially enhances (up to two- to threefold increase) the compressive and flexural properties of the nanocomposite as against PPF alone [41].

Carbon nanotubes (CNTs) have also been incorporated into polymeric scaffolds for tissue engineering. Tissue engineering can be considered as a sub-field of regenerative medicine. This emerging field seeks to combine materials and engineering principles to improve the biological properties of a tissue. Scaffolds are porous biomaterials and play a pivotal role in the tissue engineering paradigm by providing temporary structural support, guiding cells to grow, assisting the transport of essential nutrients and waste products, and facilitating the formation of functional tissues and organs. However, porous scaffolds do not possess mechanical properties necessary for *in vivo* applications [31].

The previously mentioned SWCNT–PPF nanocomposites have also been investigated to fabricate porous scaffolds with better mechanical properties for bone tissue engineering (Fig. 2) [38]. The investigations showed a general trend of enhancement in compressive mechanical properties of SWCNT–PPF scaffolds over pure PPF scaffolds. However, there were large variations in the mechanical properties depending on the porosity of the scaffold. Scaffolds porosities have been shown to play a major role in determining the mechanical properties of the scaffolds in accordance with power-law relationships. At 80 volume%, the porous SWCNT–PPF nanoscaffolds showed up to 40% increase in the compressive modulus compared to porous PPF scaffolds. At 90 volume %, there was no difference in the compressive modulus between porous SWCNT–PPF and PPF scaffolds (Fig. 3). The enhancement in the mechanical properties due to the presence of the SWCNTs in nanoscaffolds is not as significant as in nanocomposites.

An important consideration in the development of implants for tissue regeneration is the interaction of tissue and synthetic material used in the fabrication of the implant for artificial replacement of a body part damaged by disease or trauma. There is now a large body of work investigating the interactions of CNT-based nanocomposites and scaffolds with cells and tissues.

The ability of SWCNT and MWCNT composites to enhance cellular adhesion and proliferation has been investigated. These nanomaterials have been incorporated into synthetic and natural materials to create nanocomposites. Aoki et al. [3] cultured osteoblast-like cells on a porous SWCNT-polycarbonate

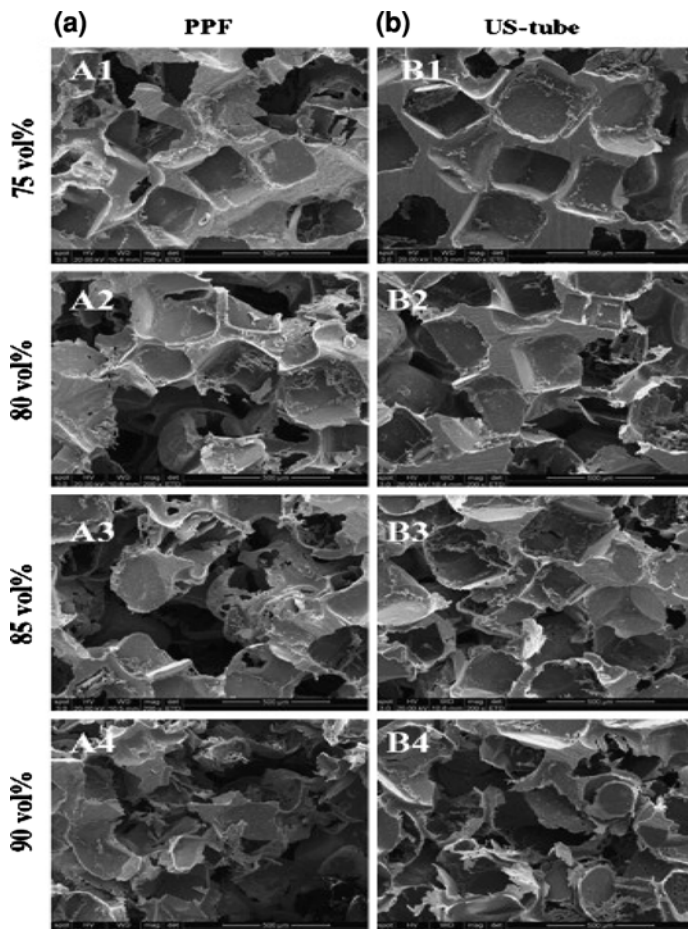
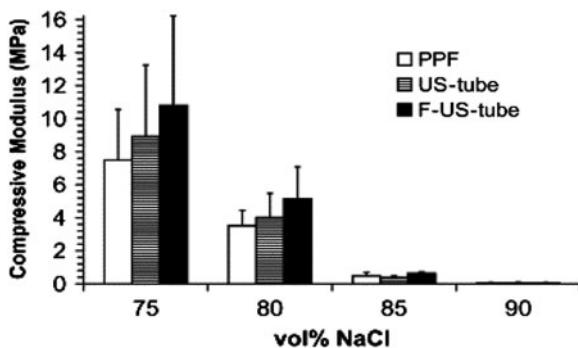


Fig. 2 SEM image of porous scaffolds made using varying percentages of porogen (NaCl); made of Poly propylene fumarate (*PPF*) (A1–A4), Ultra short SWCNT reinforced PPF (*US-tube*) (B1–B4). Adapted from [38]

Fig. 3 Compressive moduli of porous scaffolds made of polypropylene fumarate (*PPF*), ultra short SWCNT (*US-tube*) and functionalized ultra short SWCNT (*F-US-tube*) as a function of the porogen (*NaCl*) fraction used. Adapted from [38]



membrane to study cellular adhesion. When the cells were cultured on polycarbonate membranes without SWCNTs, fewer lamellipodia (cytoskeletal) extensions, and shorter lamellipodia extensions were apparent in comparison to the lamellipodia extensions of cells cultured on the polycarbonate membranes containing SWCNTs [3]. The filipodia were bound to the SWCNTs preventing the cells from lifting off of the polycarbonate CNT composite membrane [3]. Abarrategi et al. [1] created SWCNT-incorporated Chitosan scaffolds. They demonstrated the ability of these nanocomposites to support cell growth in vitro using C2C12 cells which are derived from the C2 myogenic cell line. Hirata et al. [19] studied cellular proliferation and adherence on a collagen sponge honeycomb scaffold, and compared the cellular proliferation and adhesion to a similar collagen sponge honeycomb containing a MWCNT film. They cultured MC3T3-E1 cells, a mouse osteoblast-like cell line, onto the honeycomb structures. Their results showed that the addition of the MWCNT onto the scaffold significantly increased cellular adhesion and proliferation [19]. Meng et al. [30] created polyurethane MWCNT nanocomposites, and cultured fibroblast cells onto these nanofibrous scaffolds. Their results show favorable interactions between the cells and the polyurethane surface. These fibroblasts were capable of proliferating and secreting proteins. Yildirim et al. [45] have created an alginate SWCNT composite scaffold which also showed greater cellular adhesion in comparison to the non-SWCNT alginate scaffold. In this case, the alginate scaffold was composed of 1% SWCNTs. The 1% addition of SWCNTs increased the mechanical strength and integrity of the scaffold. The SWCNTs also increased endothelial adhesion, and proliferation onto the alginate scaffold. The authors hypothesized that the increase in cellular adhesion and proliferation to the nanocomposite is due to factors that include the large aspect ratio of the SWCNTs, the increase in surface roughness of the scaffold, and the flexibility of the CNTs [45].

Carbon nanotubes can also effect the differentiation of cells. Chao et al. [8] cultured human embryonic stem cells onto poly(acrylic acid) grafted MWCNT substrates and their results indicate that this substrate increased cellular differentiation towards neurons in comparison to the embryonic stem cells cultured on poly(acrylic acid) acids without MWCNTs. The incorporation of MWCNTs into the substrate not only increased cellular differentiation to neurons, but also increased the level of neural adhesion [8].

In vitro studies with the SWCNT-PPF scaffold have shown it to support cell attachment, and proliferation [39]. In vivo studies of SWNT-PPF scaffolds implanted into rabbit tibia showed that after 12 weeks there was a change in the hard tissue response with increased levels of collagen in the extracellular matrix of these scaffolds [41]. Inflammatory cells appeared within the scaffold after 4 weeks, but this level decreased after 12 weeks. The results also suggested that these SWNT-PPF scaffolds were potentially bioactive and could promote osteogenesis.

3 Therapeutics

Controlled production and/or delivery of tissue-inducing macromolecules such as cytokines and growth factors is a widely used strategy in regenerative medicine. The physical and chemical properties of CNTs make them useful for a variety of therapeutic and drug-delivery applications in regenerative medicine. The external carbon sheath of the CNTs can be covalently or non-covalently functionalized with biological moieties that target specific cell or tissues types and/or pharmaceutical agents. Here, the CNTs target a specific cell or tissue type, and act as biological cargo vehicles to transport and deliver therapeutic agents via a biochemical or biophysical stimulus. Furthermore, the CNTs themselves can be used as a therapeutic agent by exploiting their unique physical properties. For example, the strong optical absorption properties of SWCNTs render them capable of generating acoustic waves upon irradiation. These waves have shown to induce differentiation of cells [17]. Other advantages of using SWCNTs for therapeutic purposes, and as delivery vehicles include their nanoscale dimensions, which enhance their retention and permeability in diseased tissues (e.g. tumors) [13]. Additionally, their large aspect ratio allows attachment of multiple functional groups for the targeted delivery of multiple therapeutic entities.

Carbon nanotubes can be functionalized using numerous methods of covalent interactions [12]. Carboxylic acid groups can be formed via oxidation by creating defects using acid treatments. The CNTs can undergo sidewall functionalization which includes fluorination, radical addition, nucleophilic addition, electrophilic addition, and cycloaddition. CNTs can also be functionalized using non-covalent interactions [20]. These include weak van der Waals interactions between the CNTs and the functional group; polymer (e.g. DNA) wrapping around the CNTs.

Post functionalization, the CNT conjugates can be used to translocate biological moieties into cells, and as transfection agents. SWCNT conjugates have been combined with proteins or oligonucleotides to transport these biological moieties into the cells via energy-dependent endocytosis [23]. SWCNT-based transfection agents have been reported to be superior to preexisting transfection agents due to their hydrophilic interaction with the cellular surface that allows them to bind to the cell surface, and subsequently translocate into cells. For example, Kam et al. [23] demonstrated that functionalized SWCNTs could deliver small interfering RNA (siRNA) against CXCR4 to T cells which could inhibit HIV entry into T cells. They compared this SWCNT–siRNA complex to multiple liposomes such as Lipofectamine2000TM–siRNA, and this liposome showed no effect on the T cells. A study by Zhang et al. [48] demonstrated that functionalized SWCNTs could carry siRNA and suppress the growth of tumor (HeLa) cells. Another method of cell transfection is “nanotube spearing,” a process used to penetrate cellular membranes and the nucleus thereby delivering plasmid DNA. In this study, transfection was achieved by creating a magnetic field by placing a magnet below the cellular surfaces and rotating the magnet [6]. Due to the presence of

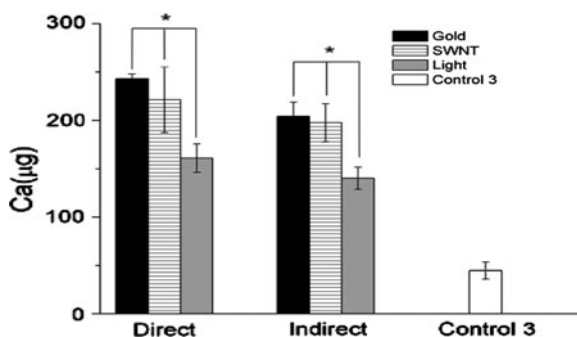


Fig. 4 Calcium content measured in the cells after 16 days in groups subjected to photoacoustic (PA) treatment and control. *Direct* samples indicate groups that were present in the path of the laser light during PA stimulation. *Indirect* samples include groups that were in wells adjacent to the direct laser light path. Mean $\pm$ SD, $n = 4$, $P < 0.05$ between PA groups and *control*. Adapted from [17]

ferromagnetic catalyst nickel particles present in their tips, the CNTs responded to the magnet, allowing transfection of DNA plasmids across the cellular membrane. This study also suggested that the effect of spearing on cellular viability was minimal due to the small size of CNTs. This though may be dependent on the concentration of functionalized CNTs because increased concentration of CNTs implies greater levels of spearing. Adeli et al. [2] functionalized MWCNTs with polyglycerol to examine their potential as a drug delivery agent. They showed that MWCNT-polyglycerol conjugates are biocompatible, with promise as a versatile platform for drug delivery applications. Riggio et al. [36] have created an alginate-polymeric film embedded with SWCNT, and have studied the release of bovine serum albumin from this SWCNT-incorporated polymer. Their results indicate that the proteins released from the MWCNT polymer construct maintained their biological function, and that it shows potential for drug delivery applications.

Sitharaman et al. have recently reported that SWCNT-enhanced photoacoustic stimulation differentiates stem cells towards osteoblasts [17]. Photoacoustic stimulation refers to the generation of acoustic waves by the absorption of electromagnetic energy, such as optical or radiofrequency wave [44]. An electromagnetic source with a low fluence (number of particles intersecting a unit area) irradiates non-ionizing electromagnetic energy onto an absorbing surface giving rise to transient acoustic waves through a thermoelastic mechanism induced by a slight temperature rise (milli-Kelvin range). SWCNTs, which show strong intrinsic electromagnetic absorption at visible, near infrared wavelengths and in the radiofrequency domain, have been used as contrast agents for photoacoustic imaging. In their study, the authors demonstrated that a brief (10 min) daily exposure of stem cells to pulse laser-induced PA stimulation promotes differentiation of mesenchymal stem cells towards osteoblasts. This osteodifferentiation is substantially enhanced by SWCNTs present in the beam path, showing increased calcium deposition (Fig. 4) and osteopontin levels compared to the controls [17].

Their results suggest that PA stimulation of SWCNT-incorporated bone tissue engineering polymer scaffolds should assist the process of osteoinduction (differentiate osteoprogenitor cells towards osteoblasts). This approach is novel since it introduces a nanoparticle-based biophysical rather than biochemical cue to affect the osteodifferentiation of mesenchymal stem cells with potential implications for other bone tissue engineering strategies. For instance, mesenchymal stem cells could be labeled *ex vivo* with nanoparticles that enhance the PA effect, seeded onto a carrier scaffold, implanted into a bone defect, and stimulated towards osteoblasts. Further, this non-pharmacological strategy based on bone's sensitivity to mechanical/acoustic signals does not possess the limitations of pharmacological growth factor-based approaches for bone regeneration such as unstable biological activity, short half-life, minimal tissue penetration.

4 Bio-Imaging

The limitations of standard diagnostic tools and techniques for detecting, and monitoring the process of tissue regeneration in small animals are well known [16]. The most robust technique for the evaluation of *de novo* tissue formation, neo-vascularization or monitoring the fate of transplanted cells is histological analysis. Because histology is an endpoint evaluation, and large variation is observed, it is difficult to assess temporal results in a statistically significant manner. In search of alternatives, much progress has been made on new approaches for non invasive *in vivo* imaging using photoacoustic imaging, positron emission tomography (PET), magnetic resonance imaging (MRI) and X-ray computed tomography (CT). These imaging modalities offer scientists the spatial and temporal information in a faster and more convenient manner. For each imaging modality, substantial attention has been devoted to the development of contrast agents. Novel agents, such as CNT-based contrast agents, may enhance molecular imaging by improving detection sensitivity and selectivity. The strategies developed for design of CNT-based contrast agents for biomedical imaging include encapsulation of medically relevant metal ions within their carbon sheath, the functionalization of the carbon sheath with a variety of imaging agents, and exploiting the intrinsic physical properties of the CNTs.

Single-walled CNTs (SWCNTs) can be used in optical imaging as they exhibit a near-infrared photoluminescence in the 'biological spectral window' between 700 and 1,100 nm [32]. In this range, interference such as absorption, scattering, photobleaching and the autofluorescence effects in water, tissue and cells are minimized [18]. Cherukuri et al. [9] have utilized near infrared (NIR) fluorescence microscopy to image SWCNTs in phagocytic cells. They used SWCNTs at varying concentrations to track their *ex vivo* uptake in mouse peritoneal macrophage cells. NIR fluorescence imaging revealed that there was no difference in population growth, adhesion, morphology and confluence between the control and the cultures containing SWCNTs. Detectable emission was seen only in cells incubated with

SWCNTs. Based on the image taken for these cells, it was interpreted that the uptake of nanotubes was through phagocytosis. Weisman et al. [25] have used in vivo NIR imaging to assess the biocompatibility of SWCNTs in an intact organism, *Drosophila melanogaster*. This study suggests the effectiveness of SWCNTs as NIR probes for studying individual nanotubes in tissue specimens or inside living organisms during the course of tissue regeneration. Thus, fluorescence imaging may be a promising method for tracking SWCNTs in cells and small animals over long durations of time.

Raman spectroscopy is a technique that studies the vibrational and low frequency modes of CNTs based on inelastic scattering called Raman scattering. In both SWCNTs and MWCNTs, Raman modes also appear at $1,590\text{--}1,600\text{ cm}^{-1}$, which is known as the G band, due to stretching along the C–C bond of graphene. Further, resonance-enhanced Raman bands for SWCNTs appear at $150\text{--}300\text{ cm}^{-1}$ called the radial breathing modes, and can be related to the diameter of the SWCNTs [24]. Various features can be understood about SWCNTs by Raman spectroscopy, such as diameter and semiconducting or metallic properties [11]. Thus, Raman microscopy and imaging allows for the spatial and chemical imaging of SWCNTs in cells and small animals. Raman microscopy has been applied to monitor cells labeled with CNTs [18]. Raman scattering and fluorescence measurements of SWCNTs encapsulated by DNA oligonucleotide (DNA–SWNTs) in the stained, and live cells showed continuous emission, and spectral changes on uptake, respectively [18]. These nanotube aggregates remained in the cell, and displayed scattering even after several cycles of cell division. Thus, they show potential as markers in assessing the proliferation, and differentiation of cells in culture, long-term labeling of cell populations, and continuous monitoring of the cellular environments. In another study by Liu et al. [27] Raman microscopy was used to study biodistribution, and blood circulation of PEGylated SWCNTs in a mouse model. The results showed SWCNT accumulation in the intestine, feces, kidney, and bladder of mice, and suggested excretion and clearance of SWCNTs from mice via the biliary and renal pathways. Another study carried out by Zavaleta et al. [46] indicates the use of Raman imaging for real-time monitoring of SWCNTs for disease (tumor) targeting and localization. Specifically disease targeting SWCNTs (RGD–SWCNT, arginine–glycine–aspartic acid) were examined for localization in diseased (tumor) mice as against plain SWCNT. The images showed a higher accumulation of RGD–SWCNTs in the tumor compared to the non-functionalized SWCNT mouse group, and there was significantly less accumulation in the liver and spleen.

Carbon nanotubes have also been assessed for increasing contrast in photoacoustic (PA) imaging in vivo [10]. PA imaging is based on the photoacoustic effect (see last section), and occurs when non-ionizing laser pulses are absorbed by the tissue to generate acoustic/ultrasonic emissions that can be used to generate images. PA imaging allows for high spatial resolution and deep tissue imaging up to $700\text{ }\mu\text{m}$ resolution and 50 mm depth using 3.5 MHz ultrasound detectors [43, 44]. Pramanik et al. in recent in vivo studies in mice have shown that SWCNT-enhanced PA imaging of the lymph nodes and vasculature with a high contrast to

noise ratio (CNR) of ~ 79 and good resolution of $\sim 500 \mu\text{m}$ [17, 34]. In most tissues, light-absorbing components such as hemoglobin of blood are the strongest endogenous PA generators. Under illumination by NIR laser light at a wavelength of 793 nm, the optical absorption of SWCNTs is much stronger than biological tissues. The absorption coefficient of SWCNTs at this wavelength is nearly 200 cm^{-1} which is almost 10 times higher than that of epidermis and nearly 500 times higher than that of dermis. Therefore, SWCNTs generate strong PA signals, and manifest high image contrast, causing the vasculature in organs to stand out prominently in PA images. Functionalization of the SWCNTs with targeting groups has also been shown to allow molecular imaging of diseased (tumor) tissues. Zerda et al. [10] used RGD–SWCNTs to target tumors in mice, and demonstrated that an eight fold greater PA signal was seen in these compared to non-targeted SWCNTs. The results suggest that SWCNT-enhanced non-invasive deep tissue PA imaging at high spatial resolution can be obtained for imaging nanotherapeutics in the body. The PA techniques can also help understand several intrinsic factors of tissues and cells, for example vascularization and oxygen saturation in diseased tissues and tissue engineering constructs.

Carbon nanotubes have also been shown to amplify the contrast enhancement efficacy of metal ions such as Gadolinium; widely used in the development of clinical MRI contrast agents. MRI is a non-invasive imaging modality that provides anatomical details for diagnosis of various diseases, and physio-chemical states of tissue and blood flow [28, 33]. With an applied external magnetic field, and radio-frequency pulses, proton spin states are excited. The time required for the excited state spin to return to equilibrium is the relaxation time, which can be either longitudinal T_1 or transverse T_2 . This relaxation causes a change in the flux of the receiving coil of the MRI scanner, inducing a magnetic resonance signal. Therefore, the relaxation time affects the quality of the image, especially the contrast. Contrast agents can modulate the relaxation time to improve contrast. An important measure of an MRI contrast agent efficacy is a term called “relaxivity”. Relaxivity is the change in the relaxation rate of the water protons per unit concentration of the contrast agent and has a unit of $\text{mM}^{-1} \text{ s}^{-1}$.

Recently, Sitharaman et al. [40] reported a novel method of using SWCNTs as carriers of gadolinium (Gd). Until now, Gd has been used in a chelated form in order to sequester its toxic effects in vivo [7]. According to this study, the SWCNTs not only encapsulate the Gd^{3+} ions to sequester the toxic effect of Gd^{3+} ions, but also, amplify the contrast enhancing efficacy of Gd^{3+} ions. The SWCNT-based MRI contrast agent increases the proton relaxivities (T_1 weighted) by 40 at the standard MRI field strengths used for clinical imaging (20–60 MHz). With such a large contrast enhancing efficacy, SWCNT-based MRI contrast agents could be dispersed throughout an engineered tissue to monitor the process of the tissue regeneration [42].

The examples of CNT-based contrast agents discussed in this section have focused on studies that harness the intrinsic properties of SWCNTs. However, a number of studies have shown that “passive” functionalization of the external carbon sheath of CNTs (SWCNTs and MWCNTs) with suitable imaging agents

allows molecular imaging with MRI, PET, and nuclear imaging modalities [26, 29, 35]. Therefore, CNTs in a true sense show promise and potential as multi-modal contrast agents for molecular imaging.

5 Summary

Current research suggests that CNTs as biomaterials have immense potential for applications in regenerative medicine. CNTs have shown to improve the mechanical properties of implants. Implants containing scaffolds and composites that are incorporated with CNTs as reinforcing agents have superior mechanical properties and they have been tested for cytocompatibility (in vitro) and biocompatibility (in vivo). The external carbon sheath of the CNTs can be functionalized for targeting, drug delivery, and imaging agents. Further their intrinsic physical properties can be harnessed for therapeutics and imaging. However, significant challenges exist towards their eventual translation for clinical applications. A challenge is obtaining pure CNTs, free of metal catalysts (iron, nickel, cobalt, yttrium) or amorphous carbon [22]. Dispersion of CNTs in the polymer matrix remains a major challenge in spite of CNT-reinforced nanocomposites exhibiting enhanced mechanical properties compared to the polymer alone. CNTs usually exist as bundled ropes of many individual nanotubes, and also show a propensity to aggregate into micrometer-sized agglomerates. Further, while the CNT-based nanocomposites offer promising properties for tissue engineering scaffolds, little is known about their long-term biocompatibility, and biodistribution upon their release from the biodegradable polymers in vivo [14, 15]. In conclusion, even though the above challenges need to be overcome, CNTs show great promise as a single platform with multi-functional capabilities for regenerative medicine.

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