

A unique and promising scaffolding structure for animal cell culture: Carbon nanotubes

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ABSTRACT

Recently the development of carbon nanotubes (CNTs) as a scaffold in animal cell culture has attracted a great deal of attention. The CNTs, which are macromolecular form of carbon of nanoscale dimensions, are viewed as a class of material with high potential for biological applications due to their unique mechanical, physical and chemical properties. CNTs may serve as an important component in imparting novel properties to the biomatrix necessary for cell growth and may provide the initial structural reinforcement needed for newly created tissue scaffolds. Supporting the use of CNTs as scaffold in tissue engineering many cell types like neuronal cells, fibroblasts and bone cells have been successfully grown on it. CNTs have also been used as in tracking of implanted cells, cancer therapy, biological sensors and as a bioactive delivery agent for DNA and drugs into the cells. Although significant promise has been shown by CNTs in emerging bio-technology, however the potential exposure of CNTs to environment and human has also shown great concern about their toxicological hazard and consequently more investigations are necessarily required.

Key words: Animal cell culture, Carbon nanotubes, Tissue engineering

The potential of using micro- and nanotechnology to recreate complex tissue architecture and *in vivo* conditions makes this an exceptionally exciting tool for tissue engineering. A particular area that is generating particular interest, illustrated by an increasing publication rate is the use of CNTs in tissue engineering (Edwards *et al.* 2009, Veetil and Ye 2009) as scaffolds for cell culture. With the expanding range of tissue types being considered for tissue engineering, the demand for novel scaffold is growing. The core of the tissue-engineered replacement is the biomaterial construct or scaffold, in which a given cell population is seeded. One of the challenges in tissue engineering is to find a more suitable method for the fabrication of scaffolds of defined architecture to guide cell growth and development. Ideally, a biomaterial scaffold should have well-controlled micro-architectures, including well controlled pore sizes and porosity, reproducibility, biocompatibility, thermal and biochemical stability, as well as no toxic solvents. A tissue engineered construct that has a well-controlled microstructure will better maintain cell morphology, differentiation, and functionality over long periods of time. A key feature of these constructs will be the replication of *in vivo* geometry and dimensional size scale that will aid in the maintenance of an

in vivo-like cell phenotype (Desai 2000). Other important fundamental requirements of scaffold are that it is fabricated from a biocompatible material that supports cell and tissue growth, which is able to be formed into a highly porous, 3D structure, with an interconnected network of suitably sized pores (Edwards *et al.* 2009). CNTs (Iijima 1991) are being increasingly studied due to their promising applications, not only in the electronics, optics, and mechanical materials, but also in biological applications, such as imaging, drug delivery and as a scaffold in tissue engineering. They have attracted this particular interest due to their unique morphology, nanosized scale, novel physico-chemical properties and, furthermore, their versatile applications. They are electrically conductive, chemically and thermally stable, and exceptionally strong. Given this unique combination of properties there has been much interest in CNTs, and finding applications for them. CNTs will impact tissue engineering for structural support. The matrix plays a critical role in tissue engineering. It is responsible for defining the space the engineered tissue occupies and aiding the process of tissue development. While popular synthetic polymers such as poly(lactic-co-glycolic acid) and poly(lactic-acid) have been used for tissue engineering, they lack the necessary mechanical strength. In addition, such polymers cannot easily be functionalized in contrast to CNTs which can be readily functionalized (Harrison and Atala 2007). In this review we

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have mainly focused on the biocompatibility and use of CNTs as a scaffold in cell culture with some other biological applications and toxicological issues. We foresee that CNTs will find numerous applications and take an important place in the development of emerging technologies in the near future.

CNTs and their types

CNTs can be defined as rolled sheets of graphene with hollow core built from carbon atoms with sp² bond. They could be either single walled with diameters as small as 0.4 nm, or multi-walled consisting of nested tubes (e.g. 2–30 concentric tubes positioned one inside the other) with outer diameters ranging from 5 to 100 nm. CNTs are generally prepared via three methods: arc-discharge (Journet *et al.* 1997), laser ablation (Thess *et al.* 1996), and chemical vapor deposition (CVD) (Cassell *et al.* 1999). CVD is the most widely used commercial method of producing CNTs. The purification step is usually required before CNTs can be used for biological applications as metal catalysts (e.g., nickel) are being used for their growth.

Primarily there are two classes of CNT: single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNT). MWNT are larger and consist of many single-walled tubes stacked one inside the other. CNT are distinct from carbon fibers, which are not single molecules but strands of layered graphite sheets. CNT are reported to be physically strong and stiff and, e.g., SWNT can be as much as 10 times as strong as steel and 1.2 times as stiff as diamond (Walters *et al.* 1999, Yu *et al.* 2000). Ideally, it is enough to consider a perfect graphene sheet to roll it into a cylinder. There is no such restriction for nanotube length, which only depends on limitations brought by the preparation method and the specific conditions used for the synthesis. These features make SWNTs a unique example of single molecules with huge aspect ratios. The easiest MWNT to imagine is the concentric type (c-MWNT), in which SWNTs with regularly increasing diameters are coaxially displayed into a multiwall nanotube. The number of walls (or number of co-axial tubes) can be anything, starting from two, with no upper limit. The intertube distance is approximately that of the intergraphene distance i. e. 0.34 nm (as opposed to 0.335 nm in genuine graphite). As opposed to SWNTs, whose aspect ratio is so high aspect ratio for MWNTs are generally lower.

Biocompatibility of CNTs

It is essential that the materials used in scaffold fabrication are compatible with cells, the biological milieu in which cells are residing and surrounding tissue. A large number of CNT biocompatibility studies have been conducted that introduces CNTs to cells in a variety of ways. These include at different concentrations (Monteiro-Riviere *et al.* 2005), different degrees of purity (Lobo *et al.* 2008a), various chemical

modifications (Magrez *et al.* 2006), and both directly (Lobo *et al.* 2008b), or within composites (Abarrategi *et al.* 2008). Results have been found to be necessarily disparate, with interpretations difficult considering these many variables. In general biocompatibility studies can be grouped into either loose CNTs in suspension (Magrez *et al.* 2006), or CNTs physically contained within a structure (Firkowska *et al.* 2008, Jell *et al.* 2008, Lobo *et al.* 2008b). Studies of loose CNTs in cell culture generally found that nanotube concentration caused a dose (Magrez *et al.* 2006) and time dependent (Cui *et al.* 2005) decrease in cell viability. This has been accompanied by observations of nanotubes bound to the cell membranes (Garibaldi *et al.* 2006), within the cytoplasm (Yokoyama *et al.* 2005), and lying close to the nucleus (Monteiro-Riviere *et al.* 2005), impairing the cells ability to engulf (phagocytose) materials (Jia *et al.* 2005) after being exposed to CNTs. Conversely cells seeded directly onto the CNT containing structures exhibited good cell adhesion, spreading (Lobo *et al.* 2008b) and proliferation (Galvan-Garcia *et al.* 2007, Abarrategi *et al.* 2008), with cells extending projections onto (Lobo *et al.* 2008b), and intermingling with, the filamentary CNT surface. When considering these results it should be noted that these studies were performed on CNTs of different types (either SWNTs or MWNTs), of varying aspect ratios, functionalization (chemical modification) and levels of purity that may have influenced CNT biocompatibility. Functionalization of CNT is important when considering its biocompatibility issues. Recently (Liu *et al.* 2009) have reviewed that surface functionalization is critical to the behavior of CNTs in biological systems. As many *in vitro* and *in vivo* toxicity studies have revealed that highly water soluble and serum stable nanotubes are biocompatible, nontoxic, and potentially useful for biomedical applications.

CNTs as a scaffold in animal cell culture

There are various reports which suggest use of CNTs as a scaffold for cell culture (Table 1). One of the first studies using microfabricated substrates to study cellular behaviour was performed by Brunette and colleagues (Brunette *et al.* 1983). Further Mattson and colleagues reported for the first time the application of CNTs as substrate for neuronal growth. They reported that neurons can attach and grow across the surfaces of CNTs and when nanotubes were coated with bioactive molecule 4-hydroxynonenal, neurite branching and total neurite outgrowth were greatly enhanced. Because MWNTs can have diameters approximately 100 nm, they can possibly be used to mimic neural fibers for neuronal growth (Mattson *et al.* 2000). It has been shown that hippocampal neurons from 0- to 2-day-old Sprague-Dawley rats were able to grow on CNTs coated with 4-hydroxynonenal (Hu *et al.* 2004). Lovat and colleagues demonstrate the possibility of using CNTs as potential device to improve neural signal transfer as well as helps in dendrite

Table 1. Various cell types grown successfully on carbon nanotubes

Cell type	Observations	References
Neuronal cells	Proper neuron attachment and growth across surfaces of CNTs Enhanced osteoblast proliferation on carbon nanofibres Hippocampal neurons grows on CNT coated with 4-hydroxynonenal CNTs as potential device to improve neural signal transfer and helps cell adhesion Osteoblast-like cells showed excellent proliferation with extension of cell morphology on CNT scaffold CNTs as the best scaffold material for cell culture in comparison with other isomorphs of carbon.	Mattson <i>et al.</i> 2000 Elias <i>et al.</i> 2002 Hu <i>et al.</i> 2004 Lovat <i>et al.</i> 2005 Aoki <i>et al.</i> 2005 Aoki <i>et al.</i> 2006
Bone cells	CNTs carrying neutral electric charge sustained the highest growth of osteosarcoma ROS 17/2.8 cells Cell response to a wider range of patterns of micro and nano scale lengths supporting the use of carbon nanotubes as scaffold Number of attached cells on the MWNT sheets seemed to be greater than on SWNT sheets and the cell adhesion to CNT sheets were influenced by serum proteins Proliferated cell numbers largest on SWCNTs, followed by MWCNTs, and are very low on graphite	Zanello <i>et al.</i> 2006 Eliason <i>et al.</i> 2008 Akasaka <i>et al.</i> 2009 Watari <i>et al.</i> 2009
Fibroblast cells	L929 mouse fibroblasts successfully grown on carbon nanotube scaffolds Long term growth of skin fibroblast and other cell types on highly oriented sheets and yarns reported Growth of L929 mouse fibroblast cells on vertically aligned CNTs shows very high cell viability and proper cell adhesion	Correa-Duarte <i>et al.</i> 2004 Galvan-Garcia <i>et al.</i> 2007 Lobo <i>et al.</i> 2008b
Muscle cells	Blends of SWNT with collagen support smooth muscle cell growth	MacDonald <i>et al.</i> 2005
Myoblastic cells	C2 C12 multipotent cell line from mouse shows good proliferation, adhesion and viability onto MWNT/Chitosin scaffolds	Abarrategi <i>et al.</i> 2008

elongation and cell adhesion (Lovat *et al.* 2005). The first application of carbon nanofibres in osteoblast culture has been reported by Elias and colleagues. They showed the enhanced long-term (days to weeks) functions of osteoblasts as well as the evidence of enhanced osteoblast proliferation on carbon nanofibres and predicted carbon nanofibres as a promising class of orthopedic as well as dental implant formulations (Elias *et al.* 2002). Aoki and colleagues formed the CNT scaffolds on porous polycarbonate membrane to study the osteoblast-like cells (Saos2) interaction with CNTs. They reported that the osteoblast-like cells showed excellent proliferation with extension of cell morphology in all directions as well as their tight interaction with CNT scaffold (Aoki *et al.* 2005). The functionalization of CNTs have demonstrated well growth of certain cell types e.g., osteosarcoma ROS 17/2.8 cells were cultured on chemically modified single walled and multiwalled CNTs. CNTs carrying neutral electric charge sustained the highest cell growth (Zanello *et al.* 2006). Supporting the use of CNTs as scaffold. Eliason and colleagues reported the interaction between osteoblast cells and micropatterned CNTs on a polymer cell substrate and cell response to a wider range of patterns of micro and nano scale lengths (Eliason *et al.* 2008).

Adhesion of osteoblast-like cells to a multi-walled and single-walled CNT sheets has been recently reported and compared with that of cell culture polystyrene dishes. The number of attached cells on the MWNT sheets seemed to be greater than on SWNT sheets and cell culture polystyrene and the cell adhesion to CNT sheets were influenced by serum proteins (Akasaka *et al.* 2009). Watari and colleagues performed cell proliferation on the scaffolds of single-walled CNTs, multiwalled CNTs, and on graphite and their scanning electron microscopy observation of osteoblast-like cells (Saos2) showed the morphology fully developed for the whole direction. Proliferated cell numbers were reported largest on SWNTs, followed by MWNTs, and are very low on graphite. In addition, the nanosize meshwork structure with large porosity was found responsible for the excellent cell adhesion and growth on CNTs (Watari *et al.* 2009). Many cellular tests have been conducted during cell culturing on CNTs scaffold. The high level of viability of fibroblast and osteoblast in contact with the, the slight increase of collagen formation, the lack of pro-inflammatory IL-6 cytokine as well as the induction of free radicals, confirm a good biocompatibility of nanotubes (Choplek *et al.* 2006). Aoki and colleagues reported that the CNTs as the best scaffold

material for cell culture in comparison with other isomorphs of carbon. The morphology of cells cultured on CNTs scaffolds investigated using confocal laser scanning microscope and scanning electron microscope revealed that the cells extended strikingly in all directions (Aoki *et al.* 2006). Aoki and colleagues studied the response of osteoblast cells on SWNT, MWNT and graphite (all forms of carbon) scaffold. Their results have shown the good biocompatibility of CNT scaffold in terms of cell morphology, cell proliferation (Aoki *et al.* 2007). Apart from neuronal and osteoblasts, other cell types have been successfully grown on CNTs or nanocomposites. For example L929 mouse fibroblasts have been successfully grown on CNT scaffolds (Correa-Duarte *et al.* 2004), blends of SWNT with collagen support smooth muscle cell growth (MacDonald *et al.* 2005). Further Lobo and colleagues reported growth of fibroblast L929 mouse cells on vertically aligned CNTs used. Their results show high cell viability (close to 100%) after 96 h of incubation, proper cell adhesion and spreading on the surface formed by nanotube tips (Lobo *et al.* 2008b). Supporting the use of CNTs in tissue engineering Galvan-Garcia and colleagues demonstrated that highly oriented CNT sheets and yarns, produced with a minimal residual content of catalytic transition materials, support the long-term growth of a variety of cell types ranging from skin fibroblasts and Schwann cells, to postnatal cortical and cerebellar neurons (Galvan-Garcia *et al.* 2007). Cell adhesion, viability and proliferation onto the MWCNT/Chitosin scaffolds with C2C12 cell line (myoblastic mouse cell), which is a multipotent cell line has been evaluated *in vitro* by Abarrategi and colleagues recently. Their results have also supported the use of MWNTs as scaffold for cell culture (Abarrategi *et al.* 2008).

Other biological applications of CNTs

Apart from being use as scaffold in cell culture, a lot of investigations into CNTs are being made for biomedical application which has been reviewed recently by Li and colleagues (Li *et al.* 2010). Endo and colleagues in different studies have fabricated highly small-sized CNT-filled nanocomposite-derived microcatheter which exhibit good biological properties that make them applicable in various fields from hemodialysis cardiopulmonary bypass, intra-vascular catheters, left ventricular support to vascular dilating devices (Endo *et al.* 2001, Endo *et al.* 2005a, Endo *et al.* 2005b). As the ability to track implanted cells and to monitor the progress of tissue formation *in vivo* is important in tissue engineered-constructs of clinically relevant sizes. In this relevance, the CNTs display good photostability and are feasible as imaging contrast agents for optical, magnetic resonance, and radiotracer modalities. Raman scattering and fluorescence spectroscopy may be promising methods for tracking CNTs in cells over long durations of time (Cherukuri *et al.* 2004, Heller *et al.* 2005). Singh and colleagues have shown the biodistribution of functionalized nanotubes in

BALB/c mice. These functionalized SWNTs were shown not to accumulate in any specific organ and were rapidly cleared from blood system without any toxic side effects and could be visually observed in the urine. Their work suggests that nanotubes have good biocompatibility and can be functionalized to serve as a vehicle for carrying imaging agents (Singh *et al.* 2006). CNTs provide several attractive features that make them ideal components for nanosensors including their electrical properties, large surface area, and the capacity to immobilize DNA or other proteins. For example, MWNTs have also been modified with putrescine oxidase for detection of putrescine (Rochette *et al.* 2005), free cholesterol in blood can be measured using a MWNT electrode placed on a biocompatible substrate (Tan *et al.* 2005). Polyaniline and nanotube composite sensors have been shown to form flexible pH sensors (Kaempgen *et al.* 2006). CNTs have been also used for many other cell altering applications like localized drug delivery (Martin *et al.* 2003), delivery of genetic material (Singh *et al.* 2005) and proposed as ion channel blockers (Joseph *et al.* 2003). The transporting capabilities of CNTs combined with suitable functionalization chemistry and their intrinsic optical properties had lead to new classes of novel nanomaterials for drug delivery and cancer therapy. As selective cancer cell destruction has been achieved by functionalization of SWNT with a folate moiety, selective internalization of SWNTs inside cells labeled with folate receptor tumor markers, and NIR-triggered cell death, without harming receptor-free normal cells (Kam *et al.* 2005a). The ease of functionalization of CNTs gives them overwhelming advantage over spherical nanoparticles in many biological applications. For example, Kam and colleagues reported that biotin functionalized CNTs bound to fluorescent dyes were capable of intercellular transport of fluorescent streptavidin (Kam *et al.* 2004), Singh and colleagues reported that the cationic functionalization of nanotubes facilitates their penetration in human and murine cell types and such nanotubes were complexed with plasmid DNA for facilitative transfection of cells (Singh *et al.* 2005). Similarly, DNA or siRNA can be bound to nanotubes with cleavable disulfide bonds and delivered to mammalian cells (Kam *et al.* 2005b). A novel SWNT-based tumor-targeted drug delivery system (DDS) has been reported, which consists of a functionalized SWNT linked to tumor-targeting modules as well as prodrug modules and shows high potency toward specific cancer cell lines (Chen *et al.* 2008). Further it has been demonstrated that use of MWCNTs to generate heat in response to near-infrared radiation (NIR) results in thermal destruction of kidney cancer *in vitro* and *in vivo*. The use of MWCNTs enables ablation of tumors with low laser powers and very short treatment times with minimal local toxicity and no evident systemic toxicity. Thus making use of MWCNTs with NIR is effective in anticancer therapy (Burke *et al.* 2009).

CNTs toxicology

In spite of many attractive features, the toxicity of CNTs is a prime concern, with several groups pointing to their similarity to asbestos fibers (Poland *et al.* 2008). CNTs were reported to be harmful to living organisms (Hoet *et al.* 2004, Colvin 2003) and consequently extensive nanotoxicological investigations are necessary to determine their biocompatibility and cytotoxicity. The identification of possible risks to human health and environment has been reviewed by many groups (Lam *et al.* 2006, Helland *et al.* 2007, Firme and Bandaru 2010) and is a prerequisite for a successful introduction of CNTs in future applications. The properties of CNTs suggest a possible accumulation along the food chain and high persistence. In organisms the absorption, distribution, metabolism, excretion, and toxicity of CNTs depend on the inherent physical and chemical characteristics including agglomeration state that are influenced by the external environmental conditions during CNT production, use, and disposal stages (Helland *et al.* 2007). SWNTs and MWNTs elicited pathological changes in the lungs, and SWNTs further produced respiratory function impairments, retarded bacterial clearance after bacterial inoculation, damaged the mitochondrial DNA in aorta, increased the percent of aortic plaque, and induced atherosclerotic lesions in the brachiocephalic artery of the heart, it is speculated that exposure to combustion-generated MWNTs in fine particulate matter may play a significant role in air pollution-related cardiopulmonary diseases (Lam *et al.* 2006). Further SWNTs have been known to cause several cytotoxic effects, induce oxidative stress upon pharyngeal aspiration or inhalation and have ability to cause an inflammatory response to humans (Shvedova and Kagan 2010). CNT toxicity in both *in vivo* and *in vitro* studies has been attributed to various factors, for instance, length, type of functionalization, concentration, duration of exposure, method of exposure, and even the dispersant used to solubilize the nanotubes. Yet many studies also seem to suggest that such attributes for CNT toxicity are unfounded. These inconsistencies seem to arise largely due to differences in experimental protocol, and whereas some points of view have been reconciled, most aspects of CNT toxicity remain uncertain (Firme and Bandaru 2010). In cases where CNTs have a toxic interaction with cells, the mechanisms of toxicity are coming into focus. Results suggest CNTs may cause harm to cells by activating many pathways at once, mostly involving DNA damage (Pacurari *et al.* 2008). In one study, mesothelial cells exposed to SWNTs at concentrations ~25 $\mu\text{g}/\text{cm}^2$ activated DNA recovery along with changes in the cell cycle and generation of apoptotic signals. Another approach showed that most cells incubated with CNTs halt at the G1 phase of the cell cycle (Jacobsen *et al.* 2008). It was also observed that CNT/DNA interaction was the preferred route of toxicity in a 3 h incubation study with 96 μg SWNT/ cm^2 , which induced DNA damage (through

micronucleus generation) in lung fibroblasts (Ulrich *et al.* 1999). Jia and colleagues reported that when SWNTs and MWNTs were incubated with alveolar macrophages (AM), significant (35% increase) in cytotoxicity was observed after 6 h of exposure (Jia 2005). It should be possible, through the observation of specific toxic events that result from incubations with different types of functionalized-CNTs, to test for functional groups that reduce the severity of such events. Evaluating the adverse effects of MWNT Tabet and colleagues recently demonstrates that MWNT produced for industrial purposes exert adverse effects without being internalized by human epithelial and mesothelial pulmonary cell lines (Tabet *et al.* 2009). There is need of more studies on CNT toxicity, with emphasis on issues such as cell specific tolerance, rates of toxic events, mechanism of cell injury, and organ-specific biodistribution.

Conclusion and future prospects

CNTs are considered one of the most promising materials in nanobiotechnology with applications ranging from electronics to biology. CNTs appear well suited as a biomaterial and may serve as an important scaffold in tissue engineering for directing cell growth. CNTs have shown as a promising material as a scaffold in cell culturing for many types of cells e.g. fibroblast, osteoblast and neuronal cells. In addition CNTs, have the capacity to be used in tracking of implanted cells, cancer therapy, biological sensing and as a bioactive delivery agent for DNA and drugs into the cells. Although the use of CNTs in tissue engineering represent a challenging task but they will provide key contribution for development of engineered biomaterials and further research into the fundamental aspects relevant for nanotube application, such as intrinsic defects, methods of synthesis, nature of the functional group, and so forth, all of which underlie toxic and biocompatibility issues and eventual widespread application.

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