On the cytotoxicity of carbon nanotubes

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The cytotoxicity of carbon nanotubes (CNTs) is a major concern today well before its unusual physico-chemical, mechanical, and electrical properties are fully exploited for commercial interests and subsequent mass production leading to greater possibilities for its exposure to humans and the environment. Contradictory reports on cytotoxicity of CNTs often appear in the literature and a mechanistic explanation of the reported toxicity remains obscure. We review here the conflicting results to focus categorically on an array of issues in CNT cytotoxicity. They include dispersion, aggregation status, coating or functionalization and immobilization, cellular uptake or internalization, purity in terms of metal catalyst contaminants, size and size distribution, surface area, surface chemistry and surface reactivity, cell types selected for experimentation as well as bioassay of nanotoxicity itself attesting as an issue in cytotoxicity. Recently a general agreement has emerged towards the potential toxicity of CNTs, although various paradigms explaining the mechanisms of CNT cytotoxicity continue to be elusive in the literature. A lack of synergy among various issues while studying cytotoxicity and most developed paradigms for the mechanism of CNT toxicity is highlighted.

Keywords: Carbon nanotubes, cytotoxicity, nanocomposite, tissue engineering.

THE urgent need for toxicological studies on carbon nanotubes (CNTs) has arisen from the rapidly emerging applications of CNTs well beyond materials science and engineering. Especially the potential medical and environmental problems, including the associated toxicity and biocompatibility issues have attracted a great concern among scientists[1–6]. Therefore, determining the cytotoxicity of CNTs has been one of the most pressing questions in nanotechnology. Before reviewing the details on toxicity, we briefly describe CNTs in the next section.

CNTs are well-ordered, high aspect ratio allotropes of carbon. The two main variants, single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) both possess a high tensile strength, are ultra-light weight, and have excellent chemical and thermal stability. They also possess semi- and metallic-conductive properties. The SWCNT is a one-atom thick sheet of graphite (called graphene) rolled up into a seamless cylinder with diameter of the order of a nanometre. This results in a nanostructure where the length-to-diameter ratio exceeds 1,000,000. Such cylindrical carbon 'molecules' have novel properties that make them potentially useful in many applications in nanotechnology, electronics, optics and other fields of materials science. They exhibit extraordinary strength and unique electrical properties, and are efficient conductors of heat as well. All these unique physico-chemical properties of CNTs and the unusual one-dimensional hollow nanostructure have also rendered them useful in biological applications, particularly as a novel drug delivery tools and imaging agents. Scientists also eye upon a great promise of CNTs to impart mechanical strength to relatively weak but biologically important biomaterial scaffolds in the area of regenerative medicine or tissue engineering. However, such biomedical applications will not be realized if there is no proper assessment of the potential hazards of CNTs to humans and other biological systems. Several issues relevant to cytotoxicity have been discussed and results from various scientific tests on cells have so far proven confusing[7–15], with some results indicating it to be highly toxic[16–21], and others showing low toxicity or no signs of toxicity at all[22–24].

In this review we compile a range of scattered issues in CNT nanotoxicology as published in the literature so far and also critically review major reports concerning CNT biocompatibility in following sections.

Issues in CNT nanotoxicology

As seen in the literature, CNT cytotoxicity can be attributed to a range of issues such as metal impurities, length and size distribution, surface area, dispersion and aggregation status, coating or functionalization, immobilization, cellular uptake or internalization and cytotoxic response of different cell types to CNTs as well, among others. In this section we review and organize the published results from the literature into sub-sections to focus on these different issues in CNT cytotoxicity.

Cell types and CNT cytotoxicity

Relatively more challenged and easily accessible organs in a given CNT-polluted environment are the skin, lungs and blood-borne-cells. Therefore, these organs and organ-

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specific cell lines have been studied substantially[7,16–18,25–35]. Nevertheless, nanotoxicity data of CNTs have been made available on several other types of cell lines as well, namely kidney cells[9], stem cells[29] and cancer cells[7,34].

The lungs as a whole have been subjected to testing for the potential hazards of inhalation exposure to carbon nanotubes *in vivo* in rat models. The pulmonary toxicity in such experiments was found due to mechanical blockage of the large airways[16,25]. The physiological relevance of these findings remains to be determined, since the SWCNTs have a strong tendency to agglomerate following intratracheal exposures[16]. Lam *et al.*[17] also reported similar work on the pulmonary toxicity of SWCNTs in mice, where SWCNTs were found to induce dose-dependent interstitial granulomas and pulmonary injuries. In addition to mechanical blockage and distinct granulomas, the pharyngeal aspiration of CNTs in animal models resulted in a pronounced cellular response and increase in various cytotoxicity/inflammatory markers in the lungs[26]. These included a significant increase in total bronchoalveolar lavage (BAL) cells and polymorphonuclear leukocytes and also protein, lactate dehydrogenase (LDH), tumour necrosis factor (TNF)-alpha, interleukin (IL)-1beta, and mucin levels[26]. Not only have the lungs as a whole at the organ level and *in vivo*, but the cytotoxicity effect of CNTs has also been evaluated *in vitro* on cultured lung cells. Davoren *et al.*[27] reported SWCNTs to have low acute toxicity to the A549 cells cultured *in vitro*. However, the presence of increased number of surfactant storing lamellar bodies as seen in TEM ultrastructural studies of SWCNT-exposed lung cells indicated a defensive response of these cells that might be the cause of the low cue cytotoxicity observed[7]. Casey *et al.*[28] demonstrated the cause of such apparent cytotoxicity to the A549 cells to be due to media constituent depletion and referred to it as a case of a false positive result. In their experiment Casey *et al.*[28] suspended SWCNTs in the culture medium and then removed all the tubes through ultracentrifugation and filtration. Media constituent depletion caused due to nutrient biomolecule deposition on SWCNTs may immediately appear as a convincing explanation given by the authors, since it makes the cells starve for essential nutrients in the medium. Also, any hydrophobic surface is a preferred site for biomolecule deposition and hence being inherently hydrophobic, well-dispersed CNTs will adsorb most of the nutrient biomolecules from the medium proportional to the concentration and surface area of CNTs, thereby depleting nutrient biomolecules in the culture medium. Nevertheless, if media depletion is the cause for the loss in cell viability, this needs further exploration as there are reports at the molecular level showing genotoxicity being induced due to SWCNTs. Kisin *et al.*[29] have reported loss of viability in lung fibroblast (V79) cell line in a concentration and time-dependent manner after the exposure of cells to SWCNT, demonstrating the genotoxic effect of SWCNTs at the molecular level in terms of DNA damage.

Since alveolar macrophage constitutes the first line of immunological defence against the invading particles in the lung, researchers have conducted a cytotoxicity study of carobon nanotubes with macrophages as well. Jia *et al.*[18] observed profound dose-dependent cytotoxicity of SWCNTs in alveolar macrophage isolated from guinea pigs *in vitro* for 6 h. The macrophages exposed to SWCNTs or MWCNTs showed characteristic features of apoptotic cell death at different dosages, toxic response being more with SWCNTs compared to MWCNT, quartz and fullerene used in this study[18]. There are reports showing contradictory results of CNT cytotoxicity to macrophages. Kalbacova *et al.*[30] found SWCNTs to be toxic to monocytes/macrophage (THP-1) cells, while Fiorito *et al.*[31] reported insignificant toxicity of SWCNTs to human macrophage cells.

Apart from the concern for inhalational entry of carbon nanotubes into the biological system, a direct contact with the skin is of equal concern. Carbon fibre dermatitis, hyperkeratosis, and naevi for example, have been linked with exposure to carbon nanomaterials and graphite. Hence several investigators have been carried out to determine cytoxicity of carbon nanotubes on skin cells. Shvedova *et al.*[7] reported ultrastructural and morphological changes, formation of free radicals, accumulation of peroxidative products, antioxidant depletion, and loss of cell viability in a culture of immortalized human epidermal keratinocytes (HaCaT) exposed to unrefined SWCNTs, indicating dermal toxicity of SWCNTs. Surprisingly, Tian *et al.*[33] used highly refined SWCNTs and found that they induced more cellular apoptosis/necrosis in human fibroblast cells and proved to be more toxic than their unrefined counterpart. However, a serum supplementation to the cell culture medium probably made it non-toxic, as demonstrated recently by Yehia *et al.*[34], when they dispersed the purified or refined and characterized carbon nanotubes termed as DM-SWCNTs in the medium with cultured human epithelial-like HeLa cells. Even MWCNTs and MWCNOs (multiwalled carbon nano onions) have been shown to be toxic to human skin fibroblast. The toxicity was demonstrated by cell-cycle arrest and increased apoptosis/necrosis at cytotoxic doses, with MWCNOs showing ten times less cytotoxicity than MWCNTs[35]. MWCNT cytotoxicity was also demonstrated by Monteiro-Riviere *et al.*[36] on human epidermal keratinocytes, where TEM examination of cells confirmed the presence of chemically unmodified MWCNTs within the cytoplasmic vacuoles. The study further showed that MWCNTs induced the release of the proinflammatory cytokine interleukin-8 from human epidermal keratinocytes in a time-dependent manner[36]. Compared to oral and nasal ingestion, dermal penetration route of nanotubes into the living tissue does not warrant much attention. More so for nanoparticles with size exceeding

100 nm, with no evidence in the literature showing penetration through the skin barrier. Nevertheless, a size less than 100 nm should be thoroughly investigated[37].

Among other cell types tested for toxicity include mouse embryonic stem cells[29] and human embryo kidney cells[9], popularly known as HEK293. These cells were evaluated for toxicity at the molecular level and CNTs were found to inhibit the proliferation of these cells by inducing cell apoptosis and decreasing cellular adhesive ability. By and large, all the above-mentioned studies report an assorted degree of CNT cytotoxicity to the cells. These discrepancies may find bases in various other issues mentioned below, rather than the cell types alone.

Bioassay of nanotoxicity

One of the most recent topics that has been added to the list of issues in nanotoxicology of carbon nanomaterials is the commonly used colorimetric and fluoresecence-based assay method itself[38–40]. Bioassay of cell viability normally involves assessment of metabolic activity (using alamar blue or AB), lysosomal activity (using neutral red dye or NR), mitochondrial activity (using MTT assay), total protein content of the cells (using coomassie brilliant blue or CB assay), loss of cell membrane integrity (using adenylate kinase or AK release assay) and inflammation response (using interleukin-8 or IL-8 assay). Carbon nanotubes have been found to interact with these colorimetric and fluorescent dyes used to determine toxicity and interfere with absorption/fluorescence data. For instance, NR for cell lysosomal activity was found to adsorb onto carbon nanomaterials to yield false reading of absorption spectra[39]. Similarly, cytokine assay was postulated to be objectionable as being the probable case of IL-8 adsorption to carbon black by the same group[39]. Wörle-Knirsch *et al.*[40] demonstrated interference of SWCNTs with the MTT assay. Due to such interactions between organic indicator dyes and SWCNTs, many investigators are not in favour of employing such dyes for cytotoxicity screening of carbon nanoparticles[5,39–42]. An alternative way to use these dyes ensuring complete absence of SWCNTs in the test solution was attempted[28] using the highly sensitive alamar blue assay that is nontoxic, water-soluble and stable in the culture medium. However, complete elimination of nanotubes from the test medium needs great care, including centrifugation, filtration and spectroscopic characterization[28]. Nevertheless, the removal of nanotubes from the test solution or medium so that they do not interfere with the absorption spectrum of the dye, does not completely solve the problem of cytotoxicity determination; rather it creates another concern, i.e. of media nutrient depletion. A notion of an indirect cytotoxicity to the cells has developed due to media depletion of essential nutrients not being available to the cells simply because the organic nutrients get eliminated through adsorption on hydrophobic and inherently adsorptive surfaces of carbon nanomaterials[28]. Indeed, SWCNTs have been observed to bind various organic molecules such as sugars, proteins and culture medium components[41,43–45] as well as lung surfactant proteins[46]. Therefore, another best alternative to address the problems with colorimetric or fluorescence assay remains the old clonogenic assay that does not involve any absorbance or fluorescence measurements of indicator dyes[47–50]. The clonogenic assay can further distinguish between the effect, for example, of carbon nanotubes on cell viability and cell proliferation[28,50]. Media depletion due to cabon nanomaterials and an indirect cytotoxicity to cells influencing cell proliferation[28,50] also find support from the well-known effect of nutrient-deficient environments on cells that respond by reducing cell proliferation leading to reduced colony size[51]. Such indirect effect reflecting on the cytotoxicity of carbon nanomaterials may possibly be a wrong information or a false positive result in many cytotoxicity studies rather than the toxicity being an inherent property of the carbon nanomaterials themsevles specially SWCNTs.

Purity vs catalyst metal contaminants in CNTs

Among the issues that complicate the matter are the catalyst metal contaminants in CNTs, which have so far been impossible to remove entirely without destroying the structural entity of CNTs. A recent report on the genetic response to CNTs indicates that risk-assessment studies of CNTs to date may be viewed as a sum of the effects of CNTs and the transition metals[35], some of which are known to be toxic by themselves[1]. But whether impurity of CNTs is the concern that induces cytotoxicity to cells remains a dilemma when investigators find both positive and negative toxicity while using highly refined preparations. Kalbacova *et al.*[30] claimed using pure SWCNT free of metal catalyst contaminant as confirmed by Raman and UV–VIS–NIR spectroscopy to investigate cytotoxicity on macrophage and showed it to be toxic to monocytes/macrophage (THP-1) cells used in this study through metabolic activity assessment. On the other side of the spectrum, Fiorito *et al.*[31] also claimed using highly purified fullerenes and SWCNT on murine and human macrophages to investigate the cytotoxic effects, but found that these materials did not stimulate the release of the inflammatory marker nitric oxide by murine macrophage cells in culture and that each possessed a low toxicity against human macrophage cells. Another such study claims focusing on the transition-metal impurity-free preparation of well-characterized homogenous solution of SWCNTs of approximately 130 nm size showing uptake into the cytoplasm and causing only low level of cytotoxicity[38]. SWCNT with varying metal content has been evaluated for toxicity. A 30 wt%, iron-

rich SWCNT was reported to cause oxidative stress and loss of cell viability, including ultrastructural and morphological changes in human epidermal keratinocytes (HaCaT)[7] and a 26 wt%, iron-rich SWCNT resulted in a significant loss of intracellular low molecular weight thiols (GSH) and accumulation of lipid hydroperoxides in murine macrophages[32]. Tian *et al.*[33] compared the refined nanomaterial that introduced the strongest toxic effect to its unrefined version and found that refined SWCNTs are more toxic than their unrefined counterparts. Yehia *et al.*[34] characterized and confirmed the purity of the SWCNT sample using scanning electron microscopy, thermal gravimetric analysis, atomic force microscopy, inductively coupled plasma–mass spectrometry, and absorption and Raman spectroscopies. They have concluded that dispersions of purified SWCNTs are not inherently cytotoxic to HeLa cells used. However, unlike others, serum was used in this study[34].

Size, size distribution and surface area of CNTs

A structure–activity relationship for asbestos-like pathogenicity linked with carbon nanotubes based on length-conforming asbestos and other pathogenic fibres has been demonstrated by Poland *et al.*[52]. The long exposure of MWCNTs to the mesothelial lining of the body cavity of mice resulted in asbestos-like, length-dependent, pathogenic behaviour, including inflammation and granulomas formation[52]. Sato *et al.*[53] conducted a detailed study on the effect of length on CNT cytotoxicity using the human acute monocytic leukaemia cell line THP-1 *in vitro* and could not see any significant effect. However, the degree of inflammatory response in subcutaneous tissue in rats showed length-dependent inflammation. Carbon nanofibres being lengthier compared to SWCNTs, have been shown to be comparatively more cytotoxic in a study[30] involving mesenchymal stem cells and monocyte/macrophage cell line THP-1. Magrez *et al.*[54] studied the cellular toxicity of MWCNTs and other carbon-based nanomaterials as a function of their aspect ratio and surface chemistry using lung tumour cells *in vitro* and found the hazardous effect to be size-dependent. Kang *et al.*[55] conducted experiments to show the antibacterial effect of the size of SWCNTs and MWCNTs. They found that SWCNTs were much more toxic to bacteria than MWCNTs, attributing size to the degree of cytotoxicity. Similarly, ultrafine/nanoparticles were found to produce enhanced toxicity responses compared with larger-sized particles of similar chemical composition[56,57]. These investigators have also indicated transmigration of ultrafine/nanoparticles to the pulmonary interstitium escaping alveolar macrophage watch[56,57]. Tian *et al.*[33] found surface area as an important variable that best predicts the potential toxicity of these refined carbon nanomaterials. SWCNTs produced by high pressure carbon monoxide (HiPCO) chemical vapour deposition process[58,59] having greater surface area showed higher toxicity compared to SWCNTs produced by arc discharge method, in media depletion experiments[28]. However, while considering various carbonaceous materials, Raja *et al.*[60] reported an inversely proportional relationship between carbon nanomaterial size regimes and cell growth inhibition.

Surface chemistry and surface reactivity at the CNT interface

Particle surface and interfaces have significance as important nanoscale material components. With a reduction in particle size, surface atoms are proportionately enhanced compared to the proportion inside its volume, resulting in more reactive nanoscale particles. Reactive surfaces may act as an effective catalyst, but in terms of biological use reactive groups present on surfaces may find health implications, thereby making surface chemistry at the interface or of the shell, an important parameter to look at from the toxicity point of view. Pertaining to lung cytotoxicity, Warheit *et al.*[61,62] have indicated the importance of particle surface reactivity in playing a definite role in terms of eliciting inflammatory response rather than the core particle or simply the particle size and surface area. Saxena *et al.*[63] demonstrated the role of surface charge present on acid-functionalized SWCNT preparation unlike pristine SWCNTs in eliciting strong cytotoxicity *in vitro* and *in vivo*, that could be reversed by neutralizing their surface charge with poly (L)-lysin.

Dispersion/suspension, aggregation status and sedimentation of CNTs

Another issue is the lack of methods to prepare water-soluble CNT particles that are homogeneous enough to ensure validity of the studies on the alleged nanosize effects. The uncontrollable aggregation behaviour of CNTs, for instance, bundle formation, poses a primary problem that hampers risk assessment studies[4,5]. Therefore, an effort to achieve a stable suspension of CNTs in water has been a familiar concern[64–76]. While most specific ways to solubilize CNTs are mentioned in the next section dedicated to functionalization of nanotubes, we summarize the commonly used surfactant-based methods here. Even though the use of surfactant is straightforward and simple, it may be noted that the surfactants used widely for solubilization of CNTs[77,78] are toxic by themselves and must therefore be avoided. Monteiro-Riviere *et al.*[79] used nontoxic surfactants, namely pluronic F127 and Tween, to see the effect on reducing aggregation of MWCNTs and achieving dispersion to confirm whether large aggregates of MWCNTs had any contribution to cytotoxicity. This study[79] found surfactants to disperse and

reduce MWCNT aggregation in the medium. However, MWCNTs were found to be cytotoxic to cells independent of surfactant exposure. Polyoxyethylene sorbitan monooleate, yet another surfactant, was found to well-disperse CNTs and is also considered to be nontoxic[80]. While comparing the cytotoxic effects of well-dispersed CNTs using polyoxyethylene sorbitan monooleate with that of conventionally purified, rope-like agglomerated CNTs and asbestos as a reference, suspended CNT bundles were found less cytotoxic than asbestos[80]. Further, the rope-like agglomerates induced more pronounced cytotoxic effects than asbestos fibres at the same concentrations[80]. Murr *et al.*[19] used well-characterized, SWCNTs (ropes) and two different MWCNT aggregates with 1–2 μm mean diameter as shown by TEM and found strong concentration and toxicity relationship for all the carbon nanotube materials relative to the chrysotile asbestos nanotubes and black carbon nanoaggregates being used as toxicity standards. Raja *et al.*[60] tested the isolated effect of SWCNT aggregates by subjecting rat aortic smooth muscle cells (SMC) to grow in two types of media, both exposed to SWCNTs but later, one type was made free from SWCNT through filtering and the other type was used as such with suspended SWCNT aggregates in it. At low dosage of SWCNTs (below 0.1 mg/ml), removal of nanotube aggregates ensured better growth of SMC in the filtered medium compared to the unfiltered medium. But at 0.1 mg/ml cell growth was affected equally in both filtered and unfiltered media relative to the control. Soto *et al.*[81] demonstrated varying degrees of the cytotoxic effect of a series of nanomaterials, including MWCNT aggregates on murine alveolar macrophage cell line and human macrophage and epithelial lung cell lines.

Coating or functionalization and immobilization of CNTs

Functionalization of the CNTs seems important as solubilization and stable suspension has been reported to be influenced by functionalization[76]. Most importantly, solubilization through functionalization rules out a toxic effect by avoiding the use of surfactants. Several investigators have attempted specific physico-chemical ways to functionalize the nanotubes to achieve solubilization[82–100]. Recent cytotoxicity studies on carbon nanotubes have shown that the biocompatibility of nanomaterials might be determined mainly by surface functionalization, rather than by size, shape and material[101,102]. Hu *et al.*[12] reported the use of chemically modified carbon nanotubes as a substrate for cultured neurons, where they systematically varied the chemical properties of carbon nanotubes by attaching different functional groups that conferred known characteristics to the substrate. By manipulating the charge carried by functionalized carbon nanotubes, they could control the outgrowth and branching pattern of neuronal processes. Magrez *et al.*[54] studied the cellular toxicity of MWCNTs and other carbon-based nanomaterials as a function of their aspect ratio and surface chemistry using lung tumour cells *in vitro*. They found that the carbon nanomaterials were toxic and cytotoxicity was enhanced when the surface of the particles was functionalized after an acid treatment. Contradictory to this, Sayes *et al.*[102] showed that the cytotoxic response of cells in culture was dependent on the degree of functionalization of the SWCNT, but with an inverse relation; as the degree of sidewall functionalization increased; the SWCNT sample became less cytotoxic. However, functional groups attached to the carbon nanotubes were different in these two studies[54,102]. Zhang *et al.*[103] showed that the lower concentration of 5 ng/ml of 6-amino-hexanoic acid-derivatized SWCNTs (AHA-SWCNTs) maintains cell viability and induces a mild cytotoxicity, but 50,000 ng/ml of AHA-SWCNTs demonstrated an irritation response by an increase in IL-8. While proper functionalization of CNTs facilitates their solubilization and makes them suitable for a given application, there is an apprehension that this may also accelerate their uptake in the systemic circulation, thereby sourcing their translocation and distribution to different organs of the body[4,104].

Cellular uptake or internalization of CNTs

There have been difficulties in spotting the CNTs entering the cells and differentiating them from other organic carbon-based cell structures, such as membranes. This has warranted the need for alternative ways to study their uptake and cytotoxic effects in cells. A recent study by Alexandra *et al.*[105] shows that once the CNTs are inside the cell, they accumulate in the cytoplasm and cause cell death in a dose-dependent manner. Chemically unmodified MWCNT uptake was demonstrated by Monteiro-Riviere *et al.*[36] with human epidermal keratinocytes under TEM examination. They confirmed the presence of MWCNTs within the cytoplasmic vacuoles. Yehia *et al.*[34] used confocal micro Raman spectroscopy to demonstrate that SWCNTs were taken up by HeLa cells in a time- and temperature-dependent fashion. They also used TEM that spotted SWCNT-like materials in intracellular vacuoles. Earlier, Kam *et al.*[23] also observed internalization of functionalized carbon nanotubes by adherent as well as nonadherent human cancer cells showing no toxicity to the cells. However, when a fluorescenated protein, streptavidin that cannot enter the cells by itself was allowed to bind biotin-functionalized nanotubes, it could be internalized through adsorption-mediated endocytosis of the so-called CNT–cargo complex and caused dose-dependent cytotoxicity. Similarly, internalization of MWCNTs by phagocytic cells and brain cells was demonstrated and was not found toxic

to the cells tested, suggesting the potential use of MWCNTs as a novel, non-toxic, and biodegradable nano-vehicle for targetted immunotherapy in brain cancers[106]. Recently, Saxena *et al.*[107] have patented a new technique to isolate deposited carbon particles from lung epithelial cells and alveolar macrophages.

CNT nanocomposites and biocompatibility

CNTs can form several categories of nanobiocomposites, including biometals, bioceramics such as hydroxyapatite, tricalcium phosphate, wollastonite, bioactive glasses and polymer (natural and synthetic) e.g. collagen, polyhydroxyalkanoates, polylactides and polyglycolides. Other general-purpose, hydrogel-forming polymers are polyethylene oxide, polyvinyl alcohol, polyvinyl pyrrolidone and polyhydroxyethyl methacrylate.

Several investigators who worked with nanocomposites of CNT with biomaterials have reported non-cytotoxicity of CNTs[108–110]. The application of 'fixed' or embedded CNTs in nondegradable nanocomposite scaffolds has been felt advantageous over 'loose' or unattached CNTs from a toxicological point of view[108]. Purified SWCNTs, SWCNTs functionalized with 4-*tert*-butylphenylene, and ultra-short SWCNTs at 1–100 μg/ml concentrations were mixed with biodegradable poly(propylene fumarate) and the crosslinking agent propylene fumarate-diacrylate to prepare injectable nanocomposites, which when tested for cytotoxicity to fibroblast cell line *in vitro* were not found to be toxic. Nearly 100% cell viability was observed on all crosslinked nanocomposites, except the degradation products of the nanocomposites that displayed a dose-dependent adverse effect on the cells[109]. Kawaguchi *et al.*[110] reported non-cytotoxicity and usefulness of CNT–alginate hydrogel nanocomposite as a scaffold material in tissue engineering. Studies of bone cell interactions with the 3D polyurethane and CNT nanocomposite foams were not found cytotoxic and revealed no detrimental effects on osteoblast differentiation or mineralization[108].

Paradigms for the mechanism of CNT cytotoxicity

Among the current hypothesized toxicity mechanisms – disruption of intracellular metabolic pathways, oxidative stress, and physical membrane damage causing ruptures – the generation of reactive oxygen species (ROS) and oxidative stress is the most developed paradigm for the mechanism of CNT toxicity[55]. All stress-related reports on toxicity are mostly investigated at the molecular level exploring the genotoxicity of CNTs in mammalian cells. Cytotoxic doses of MWCNTs induce cell-cycle arrest, increase apoptosis/necrosis, perturb multiple cellular pathways, activate genes involved in cellular transport, metabolism, cell-cycle regulation, stress response and show that interferon and p38/ERK-MAPK cascades are critical pathway components in the induced signal transduction[35]. Cui *et al.*[9] carried out extensive molecular characterization of HEK293 responses to SWCNTs showing secretion of some 20–30 kDa proteins, aggregation of cells attached by SWCNTs, G1 arrest and cell apoptosis, up-regulation expression of cell cycle-associated genes such as *p16*, *bax*, *p57*, *hrk*, *cdc42* and *cdc37*, down-regulation expression of cell-cycle genes such as *cdk2*, *cdk4*, *cdk6* and *cyclin D3*, and down-regulation expression of signal transduction-associated genes such as *mad2*, *jak1*, *ttk*, *pcdha9* and *erk* and of adhesion-associated proteins such as laminin, fibronectin, cadherin, FAK and collagen IV, suggesting that down-regulation of G1-associated *cdks* and *cyclins* and upregulation of apoptosis-associated genes may contribute to SWCNT-induced G1 phase arrest and cell apoptosis. Zhu *et al.*[111] assessed the DNA damage response to MWCNTs in mouse embryonic stem (ES) cells and found that MWCNTs can accumulate and induce apoptosis in mouse ES cells and activate the tumour suppressor protein p53 within 2 h of exposure. They have warned for a careful scrutiny of the genotoxicity of nanomaterials like MWCNTs reportedly to be of limited or no toxicity. Garza *et al.*[112] have investigated the cytotoxicity and ROS generation for various carbonaceous materials, including MWCNT aggregates. The data demonstrate that cytotoxicity is related to ROS generation.

Conclusion and future considerations

As mentioned in the preceding sections, there exist discrepancies among the reports on cytotoxicity of CNTs. A close look at the published literature on this issue reveals that the resulting positive or negative reports on cytotoxicity may be due to the way CNTs have been used in the experiments by different researchers. By and large, two ways could be identified. First, CNTs are used as suspension in the media and secondly, they are immobilized as a layer, on a culture dish using a polymer or through funtionalization to the scaffold in tissue-engineering pursuits. Invariably CNTs are shown to be toxic to cells when used as a suspension in cell culture media in any given experiment, while they appear as non-toxic if immobilized to a matrix or to a culture dish. A summary of such trends reported in various studies is given in Table 1.

Further, nanocombinatorial library approach may be a good futuristic consideration in nanomedicine and nanotoxicity research, as recently demonstrated by Zhou *et al.*[113]. Finally, adequate material characterization remains a challenging and exhaustive exercise to be practised by all prudent investigators in order to make the cytotoxicity results meaningful in future[114].

Table 1. Cytotoxicity trend as dictated by different modes of carbon nanotube (CNTs) usage irrespective of various other issues mentioned in this review

Experimentation	Cell type	Toxicity result
CNTs in suspension		
Suspended in cell culture media[9,27,33,35,54,60,80,111,115]	Human HEK293 cells[9], human fibroblast[33], 3T3 fibroblast cell[115], mesothelioma cell line (MSTO-211H)[80], lung tumour cell lines (H596, H446, Calu-1)[54], human skin fibroblast (HSF42) and human embryonic lung fibroblast (IMR-90)[35], human A549 lung cell[27], smooth muscle cells[60], stem cells[111]	+
CNTs on substratum, adhered/static		
Nonwoven SWCNTs with nanotopographic structure and macroscopic volume[116]; chemically modified CNT to have neutral, negative and zwitterionic charge[117]; SWCNT films on poly styrene[30]; CNTs as substrate for neuronal growth[12]	3T3-L1 mouse fibroblasts[116], osteoblast[117], mesenchymal cells, monocytes/macrophage[30], neurons[12]	–
CNTs composite		
CNF and PCU[118]; MWCNT and hydroxyapatite[119]; MWCNT and hydroxyapatite[120]; CNT and collagen composite[14]; porous ultra-short SWCNT nanocomposite[121]; PLA-CNT conducting polymer nanocomposite[122]; polystyrene–SWCNT nanocomposite[123]	Osteoblast[118,119,122], rat aortic smooth muscle cells[14], MSC[121], mouse fibroblast (L-929)[123]	–

+, Toxic; –, Non-toxic.

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