Perspective

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Carbon Nanotubes in Biomedical Applications: Factors, Mechanisms and Remedies of Toxicity

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1
Abstract

Carbon nanotubes (CNTs) represent one of the most studied allotropes of carbon. The unique physicochemical properties of CNTs make them among prime candidates for numerous applications in biomedical fields including drug delivery, gene therapy, biosensors and tissue engineering applications. However, toxicity of CNTs has been a major concern for their use in biomedical applications. In this review, we present an overview of carbon nanotubes in biomedical applications; we particularly focus on various factors and mechanisms affecting their toxicity. We have discussed various parameters including the size, length, agglomeration, and impurities of CNTs that may cause oxidative stress, which is often the main mechanism of CNTs’ toxicity. Other toxic pathways are also examined and possible ways to overcome these challenges have been discussed.

Keywords: carbon nanotubes, SWCNT, MWCNT, toxicity, mechanism, inflammation, nanotoxicity, cytotoxicity
1. Introduction

Merging of nanotechnology with medicine presents an unprecedented opportunity for developing novel materials that can significantly improve treatment and diagnosis of diseases. It is also expected that the ability to make and use nanomaterials at industrial scales will be the driving force for the emerging new economies and industries. Carbon nanotubes (CNTs) are novel nanomaterials that have unique properties and potential to be developed into useful products at industrial scales, as they are attractive for a variety of applications in various fields. More specifically, CNTs have the potential to revolutionize biomedical research due to their important electrical, chemical, thermal, mechanical and structural properties which have made them an area of great research interest. They have the ability to display metallic, semi-conducting and superconducting electron transport properties. They are also known for their high elastic modulus compared to all other materials. In the last decade, numerous studies have been conducted on the application of CNTs in biomedical field. It has been shown that CNTs can be used in numerous applications including diagnostic tools and devices in radiation oncology, biosensors, probes and quantum dots, nanofluidic systems, biopharmaceutical applications such as drug delivery and drug discovery, implantable biomedical devices, such as nanosensors and nanorobots, and tissue engineering applications.

However, in order for the CNTs to be more broadly used and eventually end up in a clinical settings, their biological properties, behavior and performance needs to be thoroughly understood. Furthermore, when produced at large scales, CNTs should also have well characterized biological, environmental and safety profiles. However, CNTs unlike many of their chemical agent counterparts do not always come with well-defined structure and purity. CNTs can significantly vary in size, morphology, structure and purity based on the
preparation, purification and functionalization method used to synthesize them\textsuperscript{5,6}. Therefore, the interaction between CNTs and the biological environment is very complex and sometimes unpredictable. Similarly, researchers have found that CNTs can show different levels of toxicity depending on their manufacturing method, surface-to-volume ratio, shape, concentration, aspect ratio, extent of oxidation, composition, functional group(s), and the applied dosage\textsuperscript{7-10}. In addition, CNTs are highly hydrophobic, which plays a major role in reducing their biocompatibility\textsuperscript{11}. They have the ability to damage the DNA and the cell membrane as well. They can also cause toxicity through oxidative stress, mitochondrial activities modification, protein synthesis and altered intracellular metabolic routes\textsuperscript{10}. The most common CNT mechanisms that lead to cytotoxicity also include necrosis and apoptosis\textsuperscript{10}.

In this review, we discuss the critical roles of CNTs in biomedical applications. We state the synthesis methods of CNTs, and we summarize the recent advances in application of CNTs in drug delivery, bioimaging, biosensing and tissue engineering applications. The paper also summarizes how physicochemical properties of CNTs such as size, shape, surface chemistry and the route of synthesis can affect their toxicity. Finally, the mechanisms responsible for CNTs’ toxicity and the potential remedies to overcome their pitfalls have also been presented.

2. Carbon Nanotubes Synthesis

CNTs have been extensively studied in many disciplines such as engineering, medicine, biology and chemistry due to their unique properties and promising applications\textsuperscript{12,13}. Their diameter is approximately 1 nm and their length varies from 1 to 100 µm. The carbon atoms in CNTs are arranged to form a cylinder of graphite layers\textsuperscript{14}. There are mainly two types of CNTs: single-walled nanotubes (SWNTs) which are formed of one layer of graphene and multi-walled nanotubes (MWNTs), which are formed of multiple layers of graphene, as
shown in Figure 1. Other types of carbon based nanomaterials include carbon nanohorns, fullerenes, carbon nanotubulars, carbon nanopeapods and carbon nanobuds. CNTs are produced using three different techniques: the carbon arc-discharge technique; the laser-ablation technique; and the chemical vapor deposition (CVD) technique, shown in Figure 2. Arc-discharge technique was first used by Ijima in 1991 to develop the MWNTs. Shortly after that, Ijima and Ichihashi and Bethune et al. used metal catalyst in the same technique to produce the first SWNTs. Later, Thess et al. used the laser-ablation technique to synthesize bundles of aligned SWNTs. Yacaman et al. was the first team to propose catalytic growth of MWNTs by CVD. One synthesis method used carbon fly ash as a catalyst for the CNTs growth. Yasui et al. adopted the use of coal fly ash which contains relatively small amount of carbon. This method is very promising as it is very cost effective and can extend the applications of CNTs. In addition, cobalt (Co) catalyzed MWCNTs films have been produced using low pressure chemical vapor deposition, on silicon oxide grown silicon substrate. These Co MWCNTs can be used as sensors for detecting carbon mono-oxide (CO) gas.

Pristine CNTs often lack the necessary solubility that impose great limitations for their biomedical applications. As-produced CNTs are almost completely insoluble in any aqueous solution or organic solvent except by sonication that usually only leads to CNT dispersions. Therefore, it is critical to functionalize CNTs to not only make them more soluble but to allow their integration into many organic, inorganic, and biological systems and applications. There are three main approaches for CNT modification, namely (a) the covalent functionalization of the π-conjugated skeleton of CNT using various chemical groups and reactions; (b) the non-covalent adsorption of numerous functional bio/molecules; and (c) the endohedral filling of their inner empty cavity (Figure 1B and 2D). For example, 1,3-
dipolar cycloaddition of azomethine ylides to CNTs is one of the simple, powerful and highly utilized functionalization approaches that leads to soluble CNTs that can be used for further reactions and an array of functional group and biomedical applications\(^4,24\) (Fig. 2B).

3. CNTs in Biomedical Applications

CNTs possess numerous unique features that make them a promising material for an array of biomedical applications. Some of their unique features include their unique structure giving rise to an extraordinary combination of mechanical, electrical, and optical properties\(^25\). Although CNTs are hydrophobic in nature, they can be functionalized to suit particular applications. Several of these functionalization techniques have useful biomedical applications. Functionalization moieties can be introduced to CNTs such that they specifically interact with cell surface receptors that can guide their internalization. These receptor-mediated targeting strategies can facilitate specific cell loading, thereby lowering the quantity of drugs needed in disease treatment. In addition, these strategies can help in minimizing systemic toxicity and inflammation\(^26\).

3.1 Drug and gene delivery

Many drug delivery systems have been designed using CNTs for treatment of a variety of diseases. In fact, CNT-based anticancer drugs have attracted much attention, and many depends on two strategies: selective targeting, which is accomplished through functionalization with specific tumor receptors, and secondly, the controlled release of the drugs usually present in tumor environment such as lower pH\(^26-30\). In fact, this enables CNTs to deliver small amounts of drugs to the specific tumor site by which minimization of systemic toxicity and reduction of the undesirable side effects of normal anti-cancer drugs is achieved. Cheng et al., 2011 developed a CNT-based anticancer drug that overcame the problem of multidrug resistant cancer cells, and at the same time, was effective on sensitive
cancer cells without affecting cell proliferation and cell cycle. In addition, using a non-covalent approach, they developed peptide-modified SWCNTs that could be loaded with the anticancer drug, tamoxifen. This system when tested in vitro and in vivo showed high anti-tumor effect and efficient tumor targeting. There are many other important types of CNT-based drugs, for example using CNTs as carrier for immunization against some antigens. In fact, induction of immune response against tumors can be achieved through CNT based vaccination as in Villa et al., 2011 work, which used SWCNTs as a tool for successfully delivering tumor antigens. Other types of substances such as acetylcholine (Ach) that cannot be transported to the brain by traditional ways in which normal drugs are transported, can be successfully transported using SWCNTs. The lack of Ach is associated with Alzheimer’s disease where neurons may be unable to synthesize it. Yang et al. showed the capability of SWCNTs to deliver Ach into the brain of mice. Moreover, cell-selective nuclear targeting of anticancer drugs has been achieved using functionalized MWCNTs.

Other advantages with functionalization of CNTs have been reported in many studies using covalent conjugation strategies. For example, Khazaeei et al., found that covalent functionalization of SWCNT with amide and ester bonds facilitates the slow administration of drug for longer periods and improves the solubility in aqueous and organic media. In another study, covalently attached liposomes were found to enable the delivery of large doses of a drug. Baligelli et al., 2013 found that targeting intracellular organelles like mitochondria with covalently functionalized CNTs for the delivery of therapeutics could be one effective strategy to better understand different genetic disorders. Also, different types of chemically functionalized MWNTs have been used to study their interaction with neural tissue cells and such studies could help in developing effective gene and drug delivery systems and future use of CNTs in CNS applications. On the other hand, some molecules that cannot form covalent linkages (such as aromatic compounds), can be easily attached by
strong non-covalent bonds. Non-covalent functionalizations of CNTs have been used in many medical applications including many successful models for CNT-based drugs. Many successful drug models were developed using non-covalent interactions allowing for selective targeting and proper release of the drug in tumor environments at reduced pH and the effective killing of cancer cells. Further non-covalent functionalization of SWCNT with modified polyethylenemines has been shown to be a successful gene delivery vector system in vivo.

Another great advantage of CNTs is that it can be loaded with a variety of biomolecules like siRNAs, genes and DNA. This feature can make them an effective tool in gene silencing and gene delivery. Efforts have been made for developing efficient and specific non-viral gene delivery systems using CNTs. For instance, functionalizing CNTs with poly (lactic-co-glycolic) (PLFA) was shown to deliver pro-apoptotic protein caspase-3 (CP3) into osteocarcinoma cells. CNTs have also been used to deliver the GFP gene into cultured cell lines. On the other hand, gene silencing was also successfully performed on many cell lines. In vivo, gene silencing was achieved in mice model without any signs of toxicity or induction of immune response. Huang et al. showed that functionalizing MWCNTs with polyethylenimine was able to deliver siRNA successfully into the HeLa-S3 cells. In addition, DNA molecules were delivered using SWCNTs that not only transported them but also protected them from digestion by nucleases present in the cytoplasm.

3.2 Biomedical Imaging

Biomedical imaging is a field that is being heavily studied and that incorporates different approaches from different fields of science. It is an emerging tool that can offer high resolution imaging of the behavior of cells, tissues, organs or complete body. CNTs, having unique physical properties, can be manipulated to be subjected to different methods of imaging.
biomedical imaging in order to analyze and improve their functionalities and response to their environment\textsuperscript{53}.

One method of biomedical imaging is fluorescence emission in the near infrared region, being a label-free technique that can be done at high speed, giving access to monitor the nanotubes in live cells\textsuperscript{54}. Photoacoustic imaging (which allows deeper tissues to be imaged, using different contrast agents to target specific sites\textsuperscript{55}) and magnetic resonance imaging are two additional methods that can be performed on CNTs, due to their high absorbance and the impurities in the form of metallic nanoparticles they contain. In fact, results of in vivo experiments done by Vittorio et al. have shown that CNTs can be guided by an external magnetic source towards a specific organ\textsuperscript{56}. In addition, a method used is scanning gate imaging of two coupled quantum dots, done by electron transport through the wall can be performed\textsuperscript{57}.

Modification of CNTs and addition of elements to their structure can bring new perspectives of analysis of their behavior. One technology is assembling gold nanostructures, which has proven to enhance fluorescence intensity and has achieved ferritin receptor-mediated targeting and biomedical imaging both in vitro and in vivo, proving itself as an important biomedical imaging agent. Effective multifunctional platforms can be made out of the nanotubes, thus enhancing their properties and the width of their application, as is for the effectiveness of the imaging. This can be done by adding the different types of nanoparticles to CNTs that include among others: gold nanoparticles, quantum dots, iron oxide nanoparticles, upconversion nanoparticles, PET imaging nanoprobes\textsuperscript{58}. Some of these nanoparticles may contribute, in some cases, to the toxicity of the CNTs\textsuperscript{58}, which makes the platform unsuitable for cellular life. However, this can be avoided by not exceeding the toxic amount of the element to be used. In fact, two published papers so far have used ligands to
target tumors in vivo, and it has been proven efficient by modifying the ligands used and their concentration. This technology is proving itself as a new, powerful technique to treat cancer.

Biomedical imaging is proving itself as a watershed in nanotechnology and biomedical engineering, providing easy-to-control platforms in living cells and allowing the emergence of new solutions and perspectives of analysis in different fields.

### 3.3 Carbon Nanotubes in Biosensors

Biosensors are one of the most important application of CNTs, where they combine their biological recognition with a chemical/physical transduction in order to detect biomolecules. CNTs have excellent mechanical, electrical and electrochemical properties, high surface area, and high exposure sensitivity to various biomolecules; this has made CNTs highly effective sensing elements for biosensors. The high surface area-to-volume ratio of CNTs has made them a powerful tool to obtain fast biological species detection at low concentration, which made CNT-based biosensors important in the field of ultra-sensitive biosensing applications (Figure 3).

CNT-based biosensors have many advantages over commercially available, silicon based or other material-based sensors. These advantages include: i) high sensitivity, due to their high surface-to-volume ratio and hollow tubular structure. CNTs can offer high biological activity because of their ability to be used to immobilize enzymes, ii) fast response time; CNTs can promote electron-transfer reactions due to their high ability to mediate fast electron-transfer kinetics, i.e. NADH and hydrogen peroxide reaction, iii) less surface fouling effects and low potential of redox reaction, and iv) long life span and high stability.

As mentioned earlier, CNTs are known for their high surface area to volume ratio which can be a contribution to the biomolecular conjugation, and it is being taken into consideration in...
constructing enzyme biosensors. For this reason, enzyme biosensors are one of the most commonly used type of biosensors. As an example, Vicentini et al. was able to develop a tyrosinase biosensor which is glassy carbon electrode based, adjusted with functionalized MWCNT, 1-butyl-3-methylimidazolium chloride (IL) and tyrosinase (Tyr) within a dihexadecylphosphate (DHP) film. This combination of the electrocatalytic activity of MWCNTs and the biocompatibility and the conductivity of IL has enhanced the biosensors’ response signal; this biosensor has proven its durability and stability. Another enzyme biosensor was developed to detect androsterone by using 3α-hydroxysteroid dehydrogenase immobilized onto a CNTs/IL/NAD+ composite electrode. The most unique characteristic of CNTs taken into consideration while developing an enzyme biosensor is their large surface area, which contributes to the biomolecular conjugation. Enzyme biosensors can act as glucose detectors in the blood. Glucose detection is one of the most important application of currently used enzyme biosensors.

The other well-known CNT based biosensors are the DNA biosensors. They have been heavily used for medical diagnostics, forensic science and many other applications. DNA, whether it is single stranded (ssDNA) or double stranded (dsDNA), is the main sensing element in the DNA biosensors. Researchers have found ssDNA to be highly adsorptive to CNTs, unlike dsDNA which cannot bind to CNTs. Researchers have taken advantage of this unique property of DNAs and used it to build CNT-DNA biocomplexes for biosensing technologies to target various molecules. Tang et al. were able to fabricate a SWCNT-FET-based electronic DNA biosensor; these biosensors can be used in systems-on-chip applications. DNA biosensors are simpler in their set up and chemistry, compared to other commercial biosensors such as electrochemical and optical DNA biosensors. In addition, DNA methyltransferase (DNA MTase) control and inhibition is now possible due to the ultra-sensitive DNA biosensor that was developed using the fluorescence polarization.
detection and MWCNT signal amplification. Furthermore, a glassy carbon based sensitive DNA biosensor was recently designed by Zhang et al.; it is modified with MWCNTs, polydopamine (PDA) and gold nanoparticles, and used as a DNA sequencing detector. This innovative sensing approach has proven its sensitivity and selectivity; it is being used as human serum samples in experiments for targeting DNA complements and have shown promising results so far. Several types of the CNT biosensors are illustrated in Fig 3. The unique characteristics of CNTs have made them extremely important for biosensing applications.

3.4 Tissue engineering and Regenerative Medicine

Tissue engineering and regenerative medicine are new approaches in medicine intended for developing engineered artificial tissues for applications in replacement grafts, and tissue models for in vitro disease studies and drug discovery. Usually, in these approaches, cells are seeded or encapsulated in a suitable biomaterial for growing engineered tissues. Ensuring proper mechanical, electrical and biological properties of biomaterials is a challenge in the field of tissue engineering where CNTs can play important roles. Recently, CNTs have been used in tissue engineering in varieties of applications including improving the mechanical and electrical properties of scaffolds, sensing the cell microenvironments, tracking of cells and delivery of suitable chemical and biological agents. The success of some studies in using CNTs as scaffolds for nerve generation in vitro has opened a new horizon for neural tissue engineering. Use of CNT-based scaffolds has been reported to improve neural growth, bone growth, cardiac tissue growth. While in neural and cardiac tissue growth the CNTs are generally used as enhancer of electrical properties of the scaffolds, the bone growth can be achieved by functionalization of CNTs with groups attracting calcium cations. Recently, MWCNTs functionalized with fibroblast
growth factors also displayed ability to be used as scaffolds for bone formation. Another potential use of CNTs in tissue engineering is to engineer biohybrid tissue actuators. Inspired by muscle tissues, biological machines, sensing and adapting to the environment, biohybrid tissue actuators can be built; these devices and actuators can be used to handle numerous challenges in biorobotics and drug screening. Furthermore, electrospun gelatin MWCNTs fibers were used as scaffolds for myoblasts (C2C12) growth; MWCNTs method has enhanced the mechanical properties of the resulting fibers. Under electrical stimulation, the fibers have enhanced the myotube contraction amplitude, as well as myotube’s maturation. Thus CNTs are also emerging as a potential useful component for various biomaterials in tissue engineering applications.

4. Toxicity of CNT

Numerous toxicological studies of CNTs have been reported in literature, both in vitro (Table 1) and in vivo (Table 2), some of which are contradictory to each other due to the variabilities in parameters including the type of functionalization, method of preparation and the doses of CNTs. In addition to these variables, other simple laboratory variables such as different types of cell-viable indicator dyes can contribute to different cytotoxicity profiles. The most common indicator dyes include commassie blue, alamar blue, neutral red, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide), and WST-1 a water-soluble tetrazolium salt). Hence, it is important to mention this simple observation before assessing parameters that can lead to CNT cytotoxicity results and their variability.

4.1 What makes CNTs cytotoxic?

Factors that contribute to cytotoxicity of CNTs include the amount and kind of metal impurities; length and type of carbon nanotubes; the presence of surface functionalization and its type, and the existence of dispersant or surfactant in dispersant solution (Tables 1 and...
2. Below, we discuss the factors contributing to toxicity and the plausible mechanisms by which CNTs exert their toxicological effects (Figure 4).

4.2 Effect of metal catalyst impurities

Several studies have demonstrated that metal impurities introduced during the synthesis of CNTs could have a significant impact on toxicity. Pulskamp et al, in one of their studies, have shown the toxicological effect of different purified and commercial SWCNTs and MWCNTs. What they observed is that three commercial CNTs samples substantially increased the ROS that contributed to oxidative stress and decreased the mitochondrial membrane potential. However, when compared to acid-purified SWCNTs, with low metal content, they observed less toxic effects, similar to that of the negative controls, exhibiting little or no cytotoxicity. For all four CNTs cases, IL-8 and TNF-α levels in A549 epithelial cells or rat macrophages were not increased by their presence. However, another study showed this observation does not stretch across all cell lines. For example, loss of cell viability in human neuroblastoma cells (SH-SY5Y) was observed with MWCNTs treatment. Several MWCNT preparations were assessed ranging from of 97% purity with or without acid treatment; in comparison to MWCNTs of 99% purity, all CNT preparations showed no development of oxidative stress during prolonged cells culture. Only when the concentration of MWCNTs was increased did the effect of metal impurities or other contaminant became evident. Other reports that looked at in vivo toxicity, showed a correlated increase in CD8+ levels and CD4+/CD8+ ratio of peripheral T-cell in subcutaneous mice implanted with iron-contaminated MWCNTs. In addition, other cytokine levels were increased in comparison to MWCNTs purified at 1800 degrees °C and even more pronouncedly with those MWCNT purified by 2800 °C heat treatment. The group proposed that the release of water-soluble ions from iron or other metallic impurities induced the generation of ROS. It has also been
reported by Haniu et al., 2010 that impurities are involved in the inhibition of cell proliferation\textsuperscript{91}.

Those that argue against these studies point to a report by Cheng et al, 2009 performed on human macrophage cells instead of rat cells\textsuperscript{92}. The group did not find differences in cell death between purified MWNTs via heat and unpurified tubes. Another piece of evidence that supports this hypothesis or the lack of it, is the absence of toxicity in Fe2O3 treated tubes. Additionally, Haniu et al., 2010 study found that purified and unpurified MWCNTs significantly altered the level of expression of the DJ-1: a protein that may be involved in protection against oxidative damage\textsuperscript{91}. In general, from these studies and other similar to it we can observe that the metal impurities may have impact on the toxicity of CNTs. However, the amount, concentration, method of administration as well as the assay used to assess the impurity contribution towards toxicity should be carefully accounted for.

4.3 Impact of CNT Length on toxicity

Length of CNTs has been shown to affect the toxicity of CNTs mainly due to failure of their cellular internalization, which in part has been reported to be greatly affected by CNTs length, showing that the smaller the MWCNTs (sub 1µm), the easier the penetration to cell membrane\textsuperscript{93}. In fact, long CNTs can cause biopersistence\textsuperscript{94} or CNTs retention. Results of Murphy et al, 2011 clearly demonstrated this association\textsuperscript{95}. Their study found that short CNTs instilled into pleural cavity were cleared, while long CNTs didn't. Retention of long CNTs can lead to severe inflammation which causes progressive fibrosis\textsuperscript{95}. Similarly, longer CNTs with 825 nm length have been shown to induce more inflammation than 220 nm CNTs. A proposed illustration to these results is the poor macrophage engulfment with increase of length\textsuperscript{96}.
In addition, long MWCNTs (5–15 µm and diameter 20–60 nm) were found to be more genotoxic in human alveolar carcinoma epithelial cells (A549) compared to the shorter ones (length 1–2 µm and diameter 60–100 nm). Furthermore, the study also showed that long MWCNTs with the same length (5–15 µm) but with different diameters may exhibit different level of toxicity, suggesting that smaller diameter < 2 nm significantly reduces DNA damage and thereby the toxicity.\(^97\).

It has also been reported that the absorption of long CNTs in rat small intestine is lower than that for short ones.\(^98\) On the other hand, one study involving MWCNT indicated that smaller nanoparticles are more cytotoxic than larger nanoparticles.\(^99\) An in vitro model by Simon-Deckers et al, 2008 suggested no association.\(^100\) This emphasizes the involvement of other factors contributing to CNTs’ toxicity.

### 4.4 Effects of the Type of CNTs: Single Wall versus Multi-Wall

Single and multi-walled are two different structural characteristics of carbon nanotubes. They differ mainly in structure, size and chemical surface state. SWCNTs have one cylindrical graphene layer with diameter range (0.6 – 2.4 nm), whereas MWCNTs have many concentric cylinders of graphitic shells. MWCNTs have larger diameter ranging from 2.5 to 100 nm.\(^9\) SWCNTs have large surface area with strong van der Waals forces. This may lead to the tendency of forming bundles as well as reduced efficiency of their surface area. On the other hand, MWCNTs have lower tendency for bundle formation due to relatively lower surface area and the presence of many active defects in their side walls.\(^87,\ 101\) However, functionalization of CNTs can help in the reduction of aggregation state and therefore, reduce their toxicity.\(^102\).

Results of Fraczek et al, 2008 showed that SWCNTs forms smaller aggregate compared with MWCNTs aggregates.\(^103\) The author linked the differences among CNTs toxicity in vivo to
surface structure and size. SWCNTs in the study contained hydroxyl –OH and carboxyl –COOH groups that made their surface more hydrophilic compared to MWCNTs. This hydrophobicity enables them to be uniformly dispersed inside tissues forming small aggregates ranging from 5 to 30 µm that can be phagocytosed. In contrast, MWCNTs form larger aggregates 300 µm that cannot be phagocytosed\textsuperscript{103}. The smaller size and length of SWCNTs makes them less aggregated in vivo and facilitate their uptake by macrophages\textsuperscript{102}. A study of Di Giorgio et al., 2011 showed the mechanism of toxicity by the fibrous surface of MWCNTs and SWCNTs, though the mechanisms are different. In case of MWCNTs, the plasma membrane damage and aberrant phagocytosis caused the toxicity due to the fibrous shape of MWCNTs. In case of SWCNTs, impurities may play the primary role in the induction of oxidative damage to cells\textsuperscript{104}. Results of Walker et al, 2009 study conducted in vitro indicate that both of the purified SWCNT and MWCNTs induce similar effects with no sign of toxicity at low concentrations (0.04–0.4 µg/ml)\textsuperscript{105}. As a result, we can conclude that differences among SWCNT and MWCNTs in their ability to form aggregates, variability in size and surface state can be expressed as variability in toxicity among CNTs types.

4.5 Toxicity effects of solubilizing agents

The attractive interaction between individual CNTs and their tendency to form bundles\textsuperscript{106}, require an agent to helps their dispersion in aqueous media. Results of a comparison between SWCNTs solubilized in natural dispersant (gum arabic, amylose, Suwannee River natural organic matter) and synthetic ones (polyvinyl pyrrolidone, Triton X-100) on both prokaryotic (\textit{E. coli}) and eukaryotic (WB-F344 rat liver epithelial cells) clearly indicated that the toxicity of solubilizing agent increases SWCNTs toxicity\textsuperscript{107}. Dispersion of MWCNTs have been successfully obtained using DNA. Such dispersions involve non-covalent π-π interaction between DNA strands and surface of MWCNTs resulting in high solubility and stability.
without loss of electrochemical properties. In contrast, use of sodium dodecyl sulfate (SDS) as solubilizing agent for SWCNTs induced growth inhibition in normal rat kidney epithelial cells (NRK-52E) at relatively low concentration whereas higher concentration of SDS solubilized SWCNTs were found to cause apoptosis and genotoxicity.

4.6 The effect of CNT functionalization on toxicity

Functionalization of CNTs involves the modification of their surface by introducing functional groups that may result in enhanced solubility in aqueous media. The process may be either a covalent modification or a non-covalent one, and can help in achieving greater compatibility with physiological systems by resulting in higher solubility in aqueous media. Study performed by Coccini et al, 2010 correlated the reduction of cytotoxicity of CNTs with chemical functionalization that increase the solubility, improve the dispersibility, and reduce the CNT’s agglomeration.

Another advantages of functionalization is the ability to conjugate CNTs with various groups that help in cell specific targeting, cellular processing and elimination. In the last few years, many promising studies demonstrating the reduction of toxicity by functionalization have been reported. Use of biological molecules such as proteins and antibodies in conjugation might help with the selective binding of CNTs to target biomolecules and reduce the amount of the material needed.

Functionalized MWCNTs were capable of crossing cell membrane in RAW 264.7 murine macrophages and non-phagocytic A459 human lung carcinoma cells. Additionally, functionalization with 220-kDa lectin protein was shown to reduce toxicity and apoptosis in J774A macrophage when compared with unpurified CNTs, purified CNTs and fluorescein isothiocyanate functionalized CNTs, suggesting the ability of the functional group (220-kDa lectin) to interact with receptors present on macrophages. On the other hand,
functionalization (COOH SWCNT) with carboxylic acid was shown to induce higher toxicity compared to non-functionalized SWCNTs in the HUVEC cell line\textsuperscript{117}. This proves the role of type of functionalizing group in the induction or reduction of toxicity and the importance of the right selection of functionalizing group for the intended application.

### 4.7 The effect of aggregation state

There is a direct relationship between the aggregation state of CNTs and their relative toxicity. The higher the agglomeration state of CNTs, the more toxic they are. The reason is higher agglomeration state results in bigger, stiffer and more solid CNTs agglomerates\textsuperscript{118}. The same concentration of SWCNTs with different agglomeration states were reported to have different impacts on toxicity in vitro, showing that toxicity increases with increase in the agglomeration state of SWCNTs (Figure 5)\textsuperscript{119}. In vivo, a high degree of agglomeration of MWCNTs was shown to result in increased accumulation of CNTs in vital organs like such as the lungs and livers, subsequently initiating an inflammatory response\textsuperscript{120}.

We can thus conclude that a number of factors affect the toxicity of CNTs including their physicochemical properties such as length, type and degree of agglomeration the amount of metallic impurities, the type of solubilizing agent and the type of functionalizing group on their surface. Modifying these factors would have a strong influence on the production of biocompatible and less toxic CNTs. Further, functionalization with specific favorable groups can be used in reduction of toxicity of CNTs.

### 5. Mechanisms of CNT Toxicity

The type and physical properties of CNTs mentioned in the section above have a great influence on the type and degree of toxicity. The literature reporting the mechanisms of toxicity present three possible mechanisms of CNTs cytotoxicity that include oxidative stress, cell membrane injury and genotoxicity as discussed below.
5.1 Oxidative stress

Oxidative stress is defined as imbalance between the production of reactive oxygen species (ROS) or levels of oxygen free-radicals that overcome the antioxidant defense system\textsuperscript{121}. This imbalance can be expressed as diminished levels of antioxidants, such as GSH or increase in the production of reactive oxygen species. The resultant ROS can easily oxidize proteins, nucleic acids and fats resulting in loss of function of these molecules that end with disturbed homeostasis\textsuperscript{102, 122}. In vitro studies show both decrease in GSH levels and increase in ROS production as a function of increased SWCNT concentration\textsuperscript{123}. Similarly, decrease in GSH was proposed to be involved in \textit{E. coli} loss of viability induced by SWCNT\textsuperscript{124}. Similar pattern of cytotoxicity was observed for MWCNT in dose-dependent manner for human embryonic kidney cells (HEK)\textsuperscript{99}. Jiang et al. 2013 showed recently that surface functionalization of MWCNTs is a determining factor for ROS cytotoxicity inside the cell\textsuperscript{125}. In vivo, it has been shown that intravenously injected SWCNTs in mice showed low toxicity\textsuperscript{126}. On the other hand, pretreatment of neuronal PC12 cells with different concentrations of vitamin E (VE), has been shown to protect against SWCNTs induced toxicity in a dose-dependent manner by increasing cell viability, reducing cell apoptosis and reducing ROS formation\textsuperscript{127}. The induction of oxidative stress by CNT has shown to be influenced greatly by their physical and chemical properties\textsuperscript{104} and by the presence of reactive species and transition metals\textsuperscript{88, 128}. In fact, accumulation of nanomaterials inside the cell or at higher concentrations, can by itself participate in oxidative stress induction or cell death\textsuperscript{129}. Oxidative stress was shown to interfere with cellular signaling which in turn may exert apoptosis. In addition, oxidative DNA damage, cytotoxicity and necrosis were reported as consequences of CNTs induced oxidative stress\textsuperscript{104}. Gene expression analyses of lung and skin cells treated with MWCNTs have been linked with serious retardation of some of the major cell functions. These affects range from reduction of cellular growth and metabolism at
low doses to reduced proliferation and induction of innate immune responses at higher ones. Apoptosis, necrosis and a possible G2/M cell cycle block all may be involved in the reduction of proliferation. Furthermore, Cheng et al, 2011 reported that SWCNTs delay the cell cycle phase and inhibit cell growth that could result in loss of cell proliferation. Pathways dependent on NADPH-oxidase activity have been primarily implicated in pulmonary responses to SWCNT oxidative stress and lung toxicity, most likely caused by mitochondrial dysfunction. Others, have also shown that SWCNT induced hydroxyl and oxygen radical generation can activate several molecular pathways that include MAPK, AP-1, NF-κB, and Akt signaling. Finally, oxidative biodegradation pathways such as enzyme generated hypochlorite and myeloperoxidase reactive intermediates might play a role in ROS response to SWCNT. Oxidative stress induced by MWCNTs in rat lung epithelial cells (LE) resulted in apoptosis and immense DNA breakage by inhibiting cell viability, increased level of tumor suppressor proteins p53; p21 and bax proteins. NF-κB activity increased with significant reduction of IκBα. Additionally, ATP and ADP/ATP ratio shows marked decline in cellular ATP content and cytochrome c release. Increased lipid peroxidation, which is one of the major manifestations of oxidative damage was also reported in association with reduced GSH levels after MWCNTs treatment.

5.2 Membrane injury

Many reports have considered membrane damage as another possible way in which CNTs can induce cytotoxicity. Among these reports, the study of Hirano et al, 2008 demonstrate the role of membrane injury and not oxidative stress in cytotoxicity when exposing highly purified MWCNTs (67 nm) to mouse macrophages (J774.1). These results were confirmed by loss of both oxidative stress signal transduction and absence of the effect antioxidant N-acetylcysteine (NAC) as well as glutathione synthesis inhibitor, buthionine sulfoximine BSO.
on cytotoxicity. In addition, both of the apoptotic and MAP kinases pathways were not activated significantly by the treatment. The study proposes that membrane extension in the course of MWCNTs engulfment results in membrane injury. In another study, dendritic cells (DCs) were able to phagocytose MWCNTs and show no cytotoxicity or immune activity. In fact, macrophages extension along the CNTs fiber to engulf particles may cause lysosomes damage and induce cell death by necrosis. In addition, ROS can be produced as result of membrane injury. Another report shows that double wall CNTs (DWCNTs) affect membrane integrity through induction of potassium efflux that leads to Nlrp3 inflammasome activation, with no role of ROS in cytotoxicity.

5.3 Genotoxicity of CNT

Genotoxicity represent any chromosomal or DNA damage which includes gene mutations, chromosome breaks and rearrangements. There are many factors that may assist CNTs’ interaction with genetic material and development of genotoxicity, including, its ability to penetrate nuclear membrane, the similarity in size to those to microtubules and high affinity of SWCNTs to G-C rich region in DNA sequences. Lindberg et al. showed that SWCNTs and MWCNTs induced DNA damage in mesothelial cells in vitro. Genotoxicity of MWCNTs was reported not only to human and mammalian cells, but also found to be genotoxic on plant cells (Allium cepa) and plasmid DNA (pBR322). Carbon nanomaterials may contribute directly (primary genotoxicity) or indirectly to genotoxicity. The direct way can take place when nanomaterials interact with DNA or mitotic apparatus and the indirect way may result from oxidative stress and inflammatory responses.

5.3.1 Primary genotoxicity of CNTs

Primary genotoxicity of CNTs ensued by the direct interaction of particles with the DNA. Muller et al, 2008 demonstrate the direct induction of genotoxicity by MWCNTs through
aneugenic and clastogenic effects\textsuperscript{148}. The aneugenic effect or chromosome loss, can be explained by physical interaction of CNTs with components of the mitotic spindle during cell division or the interaction with proteins that are directly or indirectly involved in chromosome segregation like tubulin and actin resulting in genetic instability, in the form of micronuclei chromosomal imbalances, or aneuploidy in daughter cells. On the other hand, the clastogenic effect involves the formation of adducts or chromosomal aberrations. Additionally, physical association between SWCNTs and DNA, microtubule and centrosome fragments were reported to cause multipolar mitotic spindles and aneuploidy\textsuperscript{149}.

5.3.2 Secondary genotoxicity of CNTs

CNTs indirect genotoxicity arises by the generation of ROS by inflammatory cells, when they interact with different cell components that might result in clastogenic effect of CNTs\textsuperscript{129}. In fact, the generated ROS are able to interact with DNA causing oxidation of DNA, DNA breakage, or lipid peroxidation mediated DNA adducts\textsuperscript{150}. The link between oxidative stress and genotoxicity has been described in many reports\textsuperscript{104, 129, 151, 152}. Interestingly, purity and dispersion properties exert non genotoxic effect in SWCNTs suggesting that minimizing factors associated with ROS production such as purity of CNT, may reduce the chance of genotoxicity\textsuperscript{153}.

5.4 Interaction with the immune system

It is interesting that CNTs can be recognized by vertebrate innate immune system. The recognition takes place via the complement system, which is composed of over 35 proteins and glycoproteins present in cell surface body fluids\textsuperscript{154}. Salvador Morales et al in 2006, were the first to identify the pathway of activation of human serum complement system, which may generate the inflammatory peptides C3a, C4a and C5a(c)\textsuperscript{155}. Although non-functionalized SWCNTs, DWCNTs and MWCNTs and most functionalized CNTs in vitro
can activate complement systems, there are other ways in which some types of CNTs can activate the complement including the lectin pathway. Functionalization of CNTs does not prevent complement activation, but, it can minimize it. Additionally, exposure of CNTs in some cell types has been associated with inflammation. High concentration of MWCNTs has been shown to induce an over expression of genes related to immune response including innate immune response and inflammation in human skin fibroblast. HEK293 cells exposed to MWCNTs for 48 h cause a time-dependent significant increase in IL-8 level.

In summary, physical properties of CNT (length, type) metal catalyst impurities, solubilizing agent, functionalization of CNT, cell type and aggregation state play an important role in the biological responses to CNTs. The toxicity mechanisms may involve oxidative stress; some studies propose membrane injury. Genotoxicity may also result.

6. Conclusions

Carbon nanotubes (CNTs) are novel class of nanomaterials which have enormous potential for applications in biomedical systems including delivery of therapeutics, biomedical imaging, biosensors and as scaffolds for tissue engineering. With their unique physicochemical properties, CNTs can be functionalized with various biomolecules either covalently or non-covalently to increase their biocompatibility and decrease the cytotoxicity. The strategy of functionalization has been shown to be effective in various studies for drug delivery and gene delivery systems. However, the cytotoxicity has still remained as the limiting factor for use of CNTs in biological systems. As short-walled or multiwalled nanotubes are developed, numerous studies have been conducted to understand the relation between their physical properties and cytotoxicity. Bioactivity of MWCNTs has been shown to be affected by their diameter, length and functionalization in vitro and in vivo. Similarly, SWCNTs’ toxicity is shown to be affected by their size and length. These
parameters are found to result in cytotoxicity by causing changes in the ROS generation \(^{158}\). The method of CNT synthesis influences the viability and behavior of cells and the interaction of CNTs with cells. The presence of metal impurities in CNTs has substantial role on response of cells, and it has been shown that metal impurity such as iron can induce cytotoxic and genotoxic effects \(^{159}\). Understanding the factors responsible for the CNT toxicity is required for further development of safe CNTs based applications. Different ways in which CNTs cause toxicity has been recognized, but the mechanisms involved are still in investigational stages. Recently, genotoxic effect of CNT by direct contact with DNA was shown to induce mutations in DNA \(^{160}\). Also, genome wide gene expression study has been performed in vivo in animal models to understand the molecular mechanism of CNTs’ toxicity at organism level \(^{161}\). Although several studies have been performed employing new approaches for toxicological characterization of CNTs in biological systems, complete understanding of the cellular uptake, internalization and changes in gene expression associated with the CNT toxicity has still remained elusive. Such understanding will be required for future development of CNTs for biomedical applications.

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Figures Legends

**Figure 1.** Structures of carbon nanotubes and their field of use. A) Schematic representation showing the structures of SWCNTs and MWCNTs. B) Schematic illustration showing the different applications of Carbon Nanotubes such as drug delivery, diagnostics, biosensors, biomedical imaging, as well as tissue engineering and regenerative medicine.

**Figure 2.** Schematic illustration showing different synthesis methods of carbon nanotubes. A) Chemical vapor deposition (CVD) method used for carbon nanotubes synthesis. B) The laser-ablation technique used in the synthesis of carbon nanotubes. C) The carbon arc-discharge technique performed to synthesize carbon nanotubes. D) Different functionalization strategies of CNTs showing. i) Cycloaddition with in situ generated dichlorocarbene. ii) Photoinduced generation of reactive nitrenes. iii) Functionalization by nitrenes iv) 1,3 dipolar cycloaddition of azomethine ylides. v) Reaction scheme for fluorination of nanotubes, defunctionalization and further derivatization. vi) 1,3 dipolar cycloaddition of nitrile amines vii. Reaction pathway for obtaining water-soluble ammonium-modified nanotubes. The latter can be used for the delivery of biomolecules.

**Figure 3.** Different types of carbon nanotubes based biosensors. (a) PEGylated SWNTs with RGD conjugation and radiolabelling used for in vivo tumour targeting. Copyright 2015 Nature Publishing Group. (b) Scheme of SWNTs with three different isotope compositions (13C-SWNT, 12C12/13C-SWNT, 12C-SWNT), conjugated with different targeting ligands. (c) Experimental setup of the two coupled single wall carbon nanotube quantum dots in a multiple quantum dot system. (d) Schematic illustration showing different types of CNT-Biosensors, Enzymatic CNT Biosensors and DNA-Functionalized CNT Biosensors, as well as the combination of nanoparticles with CNTs and enzymes to...
form specific type of biocomposites for biomedical applications. (e) CNT electrode array used for sensitive detection of DNA hybridization in the presence of Ru(bpy)$_3$$^{2+}$ mediated with guanine oxidation $G_{ox}$ $^{167}$. (f) Schematic of a SWCNT field-effect transistor (FET). SWCNTs are being coated to a biotin layer which allows streptavidin to bind and thereby change the characteristics of FET $^{168}$. (g) Schematic representation of the CNT electrically contacted glucose oxidase electrode where GOx is constituted on the FAD units. Reprinted with permission from $^{169}$

**Figure 4.** Factors affecting carbon nanotubes toxicity on blood cells. a) an illustration showing how the structure of CNTs can affect phagocytosis by macrophages and tissue clearing; low aspect ratio MWCNTs can be engulfed by macrophages while it is not the case with high aspect ratio MWCNTs. b) besides their dimensions, other considerations should be taken into consideration when it comes to safety of CNTs, such as preventing their aggregation and increasing their solubility, which makes excretion of urine easier and thereby preventing accumulation of tissue. Reprinted with permission from $^{170}$ c) Schematic illustration of the factors affecting CNT toxicity; it shows how these factors such as functionalization, impurities, size and shape have the ability to mediate CNT toxicity $^{171}$.

**Figure 5.** Pulmonary toxicity of single-wall carbon nanotubes in mice 90 days after intratracheal instillation with 0.5mg of a test material per mouse. a) Serum control. b) Carbon black. c) CNT. Abnormal appearances were present in parts of the lung that were exposed to NT. d) PNT. Even distribution of particles. e) RNT. Granulomas may be resulted due to the presence of clusters of black pigment. f) RNT. Dorsal view shows some necrotic changes. g) Carbon black, h) Quartz, and it shows lymphocytes gathered around the area where macrophages, containing quartz particles, are present; i) CNT. Granulomas contained black particles, j) RNT. Granulomas at low magnification. K) RNT. Granulomas at high
magnification, 1) PNT. Necrosis of a large granuloma. Reprinted with permission from 172. m)

Schematic figure showing the different effects of CNTs addition to various tissues. It causes some modulations in the immune system which may lead to cancer; they also affect platelets formation as well as destruction of the red blood cells membrane; in some cases, they can cause cell death.
Biography of Corresponding Authors

Adnan Memic graduated summa cum laude with a BSc in Chemistry. He received his Ph.D. in Chemistry/Biochemistry from Wayne State University with Mark Spaller. He was a postdoctoral fellow in Chicago with Brian Kay at the University of Illinois. He was previously a visiting assistant professor of toxicology and pharmacology at Dartmouth’s Geisel Medical School. He joined King Abdulaziz University in 2010, was promoted to associate professor of nanotechnology and is concurrently a part-time Lecturer on Medicine at Harvard Medical School. His research focuses on bioactive molecule discovery and development including generation of biomaterials, carbon nanomaterials, chemical and peptide analog libraries, protein and antibody engineering towards solving challenges in targeted drug delivery, biosensing, and tissue engineering and regenerative medicine applications.

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Biography of Co-authors

Reem Alshehri received her BSc and M.Sc in Biochemistry from King Abdulaziz University, Jeddah, Saudi Arabia. Her research area concentrated on the mechanisms of mercuric
compound toxicity and their role in initiating of oxidative stress, autoimmunity, and response of autistics to organic forms of mercury. Her research interests include molecular mechanisms contributing to autism, neuroscience, and using nanotechnology tools in the treatment and diagnosis of neurodegenerative diseases.

Asad Muhammad Ilyas graduated with BSc in Biology. He received his master’s degree from University of Skovde, Sweden with major in Molecular Biology in 2010. He completed his Ph.D in Biochemistry with major in Molecular Biology from King Abdulaziz University in 2016. His research focuses on targeting multiple signaling pathways for the treatment of Acute Myeloid Leukemia, including interventions using nanotechnology. In addition, his research focuses on studying the mechanism of cancer related genes in the development of leukemic hematopoiesis.

Adnan Arnaout is a fourth year Mechanical Engineering student at the American University of Beirut, minoring in Biomedical Engineering, with special interest in Tissue Engineering. He is an undergraduate researcher at the Nano Micro-technologies and Tissue Engineering Lab at the American University of Beirut. His research focuses on developing hydrogels for Tissue Engineering, as well as Microfluidics.

Dr. Farid Ahmed completed his Ph.D. in human biology from the Ludwig Maximilians University, Munich, in 2006. He performed his post-doctoral studies at the Clinical Cooperative Group-Leukemia in Munich. Dr. Ahmed’s current research interests is studying the molecular regulation of normal and malignant hematopoiesis and HSC self-renewal with the hope to identify underlying processes that lead to leukemic transformation and progression. Projects in Dr. Ahmed’s group focus on identification and characterization of candidate leukemic stem cell (LSC) in hematopoietic malignancies and therapeutic targeting of the leukemic cells that drive human leukemia. Dr. Ahmed is currently a member of several
scientific societies such as: International Society for Stem Cell Research (ISSCR), European Hematology Association (EHA) and ISEH - Society for Hematology and Stem Cells.

**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>BSO</td>
<td>Buthionine Sulfoximine</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
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<tr>
<td>CP3</td>
<td>Pro-apoptotic protein caspase-3</td>
</tr>
<tr>
<td>CVD</td>
<td>Chemical vapor deposition</td>
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<tr>
<td>DCs</td>
<td>Dendritic cells</td>
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<tr>
<td>DHP</td>
<td>Dihexadecylphosphate</td>
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<tr>
<td>DWCNT</td>
<td>Double wall CNTs</td>
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<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
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<tr>
<td>FET</td>
<td>Fluoroethyl-L-tyrosine</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>DNA M Dane</td>
<td>DNA methyltransferase</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled nanotube</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>PDA</td>
<td>Polydopamine</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PLFA</td>
<td>Poly lactic co-glycolic</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>SWCNT</td>
<td>Single-walled nanotube</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>WST-1</td>
<td>Water-soluble tetrazolium salt</td>
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</tbody>
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of polyethylene-glycol functionalized multi-walled carbon nanotubes in mice. Nanotechnology 2010,
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Figure 2

A. Thick film catalyst heating CVD

B. Thin film catalyst heating CVD

C. Pulsed UV laser (KRF excimer laser)

D. Conjugation of Biomolecules:

i. $\text{PhHgCl/Br/tris(amine) or CHCl}_3$/Na$_2$H

ii. $\text{F}_2$, 25-600 °C

iii. $\text{NH}_2$(CH$_2$)$_3$NH$_2$

iv. $\text{ROOC-N$_2$A}$

v. $\text{HO-}$

vi. $\text{HO-}$

vii. $\text{NH}_2$
Figure 4

(a) Short or tangled MWNT
Effective phagocytosis
Clearance through lymphatic system

Long and rigid MWNT
Incomplete phagocytosis
Mutagenesis caused by MWNT accumulation may cause cancer

(b) Functionalize with small hydrophilic groups
Ensure stable dispersions of individual CNTs

<10 μm
<30 nm
Red blood cells

(c) Impurities
Factors affecting CNT toxicity
Functionalisation

Nickel
Cobalt
Molybdeum
Iron
Octa-ammonium POSS
PEG
COOH

Ammonium

Shape
Size
Spherical
Fibrous
Rod

ACS Paragon Plus Environment
Figure 5

- Modulates immune response (1)
- Inflammatory cytokine secretion (2)
- Affects clot formation (3)
- Membrane Destruction and Morphological Changes (3)
- Apoptosis, Inflammation and Fibrosis (4, 5)

(a) (b) (c) (d) (e) (f) (g) (h) (i) (j) (k) (l) (m)
Table 1. Comparison of CNT Toxicity in vitro using Biological Model Systems

<table>
<thead>
<tr>
<th>Unmodified SWCNTs</th>
<th>Model System</th>
<th>Dosage and Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pristine SWCNTs</td>
<td>human vein endothelial cells (HUVEC)</td>
<td>5-50 µg/ml. they observed damage caused by cell fiber contact and oxidative stress probably due to contaminant metals</td>
<td>173</td>
</tr>
<tr>
<td>pristine SWCNTs</td>
<td>RAW264.7 cells</td>
<td>At concentration of 100 µg/mL they induced apoptosis through mitochondrial dysfunction and ER stress</td>
<td>174</td>
</tr>
<tr>
<td>Pristine SWCNTs</td>
<td>Mesothelioma cell line MSTO-211H</td>
<td>7.5 µg/mL water 10% decrease in cell proliferation and activity</td>
<td>118</td>
</tr>
<tr>
<td>SWCNTs</td>
<td>HeLa cells</td>
<td>100 µg/mL No effect on growth rate</td>
<td>175</td>
</tr>
<tr>
<td>0.5 DMSO pristine SWCNT</td>
<td>Human embryo kidney (HEK 293) cells</td>
<td>25 µg/mL G1 cell arrest and apoptosis</td>
<td>176</td>
</tr>
<tr>
<td>Functionalized SWCNTs</td>
<td>A549 lung epithelial cells</td>
<td>1.56-800ug/ml, cytotoxic at 400 and 800ug/ml</td>
<td>177</td>
</tr>
<tr>
<td>acid-functionalized single-walled carbon nanotubes (AF-SWCNTs)</td>
<td>three cell lines, LA4, MHS, and JAWSII</td>
<td>1 mg/ml significantly lowered the ability of these cell lines to present α-Glactosylceramide antigen</td>
<td>178</td>
</tr>
<tr>
<td>2-methacryloyloxy ethyl phosphorylcholine SWCNTs and phosphoryl choline grafted SWCNTs</td>
<td>RAW264.7, HUVEC and L929 cells</td>
<td>5, 10, 20, 40 and 80 µg/mL had greatly reduced but cannot completely eliminated cytotoxicity</td>
<td>179</td>
</tr>
<tr>
<td>fluorescein-labeled poly(ethylene glycol)-modified single-walled</td>
<td>HeLa, KB-3-1, and KB-8-5 cells</td>
<td>exhibited low toxicity at concentration of 0.001 to 100 mg/L</td>
<td>180</td>
</tr>
<tr>
<td>Material</td>
<td>Cell Type</td>
<td>Concentration</td>
<td>Effect</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>----------------------------------</td>
<td>---------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>RNA-polymer SWCNT conjugate</td>
<td>MCF-7 breast cancer</td>
<td>1 mg/mL</td>
<td>No significant cell damage</td>
</tr>
<tr>
<td>FITC-SWCNT</td>
<td>HeLa cell lines</td>
<td>5–10 mg/mL</td>
<td>50% survival of HeLa cells</td>
</tr>
<tr>
<td>Streptavidin-SWCNT</td>
<td>HL60 and Jurkat cells</td>
<td>0.025 mg/mL</td>
<td>No adverse effects</td>
</tr>
<tr>
<td>6-aminohexanoic acid-derivatized SWCNTs</td>
<td>human epidermal HEK keratinocytes</td>
<td>0.0000005–0.05 mg/ml</td>
<td>Dose dependent decrease in viability</td>
</tr>
<tr>
<td>Unmodified MWCNTs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pristine-multi-wall carbon nanotubes</td>
<td>human alveolar (A549) epithelial cells and normal bronchial (BEAS-2B) cells</td>
<td>1 -100 µg/ml</td>
<td>Induced membrane damage, particularly in BEAS-2B cells</td>
</tr>
<tr>
<td>Pristine MWCNTs,</td>
<td>A549 human lung epithelial cells</td>
<td>At doses of 12.5–100 µg/ml, pristine-MWCNT caused a time- and dose-dependent ROS increase and higher levels of lipid hydroperoxides compared to the controls</td>
<td></td>
</tr>
<tr>
<td>Pristine MWCNTs,</td>
<td>murine alveolar macrophages (MH-S)</td>
<td>1mg/ml</td>
<td>Toxicity observed</td>
</tr>
<tr>
<td>Pristine MWCNTs,</td>
<td>T lymphocytes</td>
<td>40µg/ml</td>
<td>Low toxicity was observed using the concentrations</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>human platelets</td>
<td>100µg/ml</td>
<td>No toxicity was observed</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>human macrophage cells</td>
<td></td>
<td>Toxicity observed on the cells using the MWCNTs</td>
</tr>
<tr>
<td>Functionalized MWCNTs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carboxylated-MWCNTs</td>
<td>Human pulmonary epithelial cells (A549)</td>
<td>5-20 µg/mL for up to 24 h incubation showed cell surface changes</td>
<td></td>
</tr>
</tbody>
</table>
four types of functionalized multi-walled CNTs

- Jurkat and THP1 cells
- At 100 µg/ml, three of the tested nanotubes activated immune-related pathways in THP1 but not in Jurkat cells

Plasmid DNA-MWCNT

- HeLa cell lines in vitro
- 10 mg/mL 50% survival of HeLa cells

MWCNTs-polyglycerol (PG)

- HT1080 cell line (human Fibrosarcoma)
- 1mg/ml, no toxicity observed

MWCNTs functionalized by carboxylation

- THP-1 cells and primary alveolar macrophages from C57BL/6 mice
- Functionalization completely eliminated inflammasome activation at concentrations from 6.25 to 50 µg/ml

surface-functionalized multiwalled carbon nanotubes

- human bronchial epithelial cells, BEAS2B cells
- The order of toxicity to BEAS2B cells was determined to be COOH > O > NH2 > pristine MWCNTs at 5 to 200 mg/L concentration.

Table 2. Comparison of CNT Toxicity in vivo using Biological Model Systems

<table>
<thead>
<tr>
<th>Unmodified SWCNTs</th>
<th>Model</th>
<th>Dosage and outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pristine SWCNTs</td>
<td>male ICR mice</td>
<td>At concentration of 100 µg/mL they induced acute inflammatory response in the lungs of mice</td>
<td>174</td>
</tr>
<tr>
<td>Pristine SWCNTs</td>
<td>development of the mouse embryo</td>
<td>10 ng to 30 µg/mouse. At concentrations above 100ng/mouse reactive oxygen species (ROS) were detected in placentas of malformed but not of normally developed fetuses</td>
<td>193</td>
</tr>
<tr>
<td>Pristine SWCNT</td>
<td>Intravenous injection, systemic, rabbit in vivo</td>
<td>7.5 mL of 20 µg/kg body mass. They observed little to no toxicity.</td>
<td>196</td>
</tr>
<tr>
<td>Compound Type</td>
<td>Methodology</td>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
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<td>------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Pristine SWCNT</td>
<td>Intravenous injection, systemic, mice in vivo</td>
<td>Single 600µg injection showed no observable toxicity.</td>
<td></td>
</tr>
<tr>
<td>Functionalyzed SWCNTs</td>
<td>Swiss-Webster mice</td>
<td>0.064mg/kg oral administration, genotoxic effect</td>
<td></td>
</tr>
<tr>
<td>Functionalized SWCNTs</td>
<td>Swiss-Webster mice</td>
<td>0.25, 0.5, and 0.75 mg/kg the enhanced ROS including increased DNA damage, and decreased the mitotic index</td>
<td></td>
</tr>
<tr>
<td>oxidized single-wall carbon nanotubes</td>
<td>Development of the mouse embryo</td>
<td>10 ng to 30 µg/mouse. At concentrations above 100ng/mouse Cytotoxic effects to the mouse development were observed</td>
<td></td>
</tr>
<tr>
<td>DTPA-SWCNT</td>
<td>with female BALB/c mice in vivo</td>
<td>20 µg/µL PBS No acute toxicity after single 200 µL dose</td>
<td></td>
</tr>
<tr>
<td>DOTASWCNT</td>
<td>mouse model</td>
<td>0.012mg, low toxicity</td>
<td></td>
</tr>
<tr>
<td>SWCNT functionalized using phospholipids conjugated to hyaluronan</td>
<td>C57BL/6 mice</td>
<td>100 µl at 1 mg/kg were intravenously injected into the tail vein of C57BL/6 mice and did not alter the total number of leukocytes nor increased liver enzyme release</td>
<td></td>
</tr>
<tr>
<td>Unmodified MWCNTs</td>
<td>male Wistar rats</td>
<td>0.20 or 0.55 mg via intratracheal instillation were not cleared from lungs of rats following 364 day follow-up suggesting</td>
<td></td>
</tr>
<tr>
<td>Material</td>
<td>Description</td>
<td>Toxicity</td>
<td>Notes</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>------------------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>MWCNTs</td>
<td>lungs of Sprague-Dawley rats in ViVo and macrophages in Vitro</td>
<td>Observable toxicity</td>
<td></td>
</tr>
<tr>
<td>Purified MWCNTs</td>
<td>subcutaneous tissue of rats with MWCNTs with an average length of 220 and 825</td>
<td>Low toxicity</td>
<td></td>
</tr>
<tr>
<td>MWCNTs</td>
<td>Swiss albino mice model</td>
<td>60 and 100mg/kg, liver toxicity observed</td>
<td></td>
</tr>
<tr>
<td>MWCNTs</td>
<td>mouse model</td>
<td>40g, acute lung toxicity</td>
<td></td>
</tr>
<tr>
<td><strong>Functionalized MWCNTs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Several functionalized MWCNTs</td>
<td>C57Bl/6 mice</td>
<td>surface charge in determining the pulmonary fibrogenic effects of MWCNTs at a with final dose at 2 mg/kg using pulmonary aspiration</td>
<td></td>
</tr>
<tr>
<td>carboxyl group functionalized MWCNT</td>
<td>male Swiss-Webster mice</td>
<td>C-mwcnts were administered at doses of 0.25-0.75 mg/kg/day for 5 days body-weight gain of the mice decreased, induced reactive oxygen species (ROS), and enhanced the activities of serum amino-transferases (ALT/AST), alkaline phosphatases (ALP) and concentration of lipid hydro peroxide compared to control.</td>
<td></td>
</tr>
<tr>
<td>Glucosamine-MWCNT</td>
<td>Intraperitoneally into female Kunming mice</td>
<td>300 µL single dose, suspension concentration unknown had good biocompatibility</td>
<td></td>
</tr>
<tr>
<td>DTPA-MWCNT</td>
<td>Intravenous injection, systemic, female BALB/c mice</td>
<td>20 µg/µl PBS No acute toxicity after single 200 µL dose</td>
<td></td>
</tr>
<tr>
<td>14C-taurine-MWCNTs,</td>
<td>intravenous administration</td>
<td>10ug, low level acute toxicity</td>
<td></td>
</tr>
<tr>
<td>PEGylated MWCNTs</td>
<td></td>
<td>10 and 60mg/kg, induce toxicity in liver</td>
<td></td>
</tr>
</tbody>
</table>
Table of Contents Graphic
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