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Critical Review

Challenges in the Medicinal Applications of Carbon Nanotubes (CNTs): Toxicity of the Central Nervous System and Safety Issues

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Abstract

Carbon nanotubes (CNTs) are gradually emerging as a new option for possible use in neural prosthesis, drug delivery, cancer treatment, bioengineering, gene therapy and regeneration therapy. With an increase in the development and application of CNT-based medicinal products, the potential hazards of CNTs to biological systems are getting greater public attention. A lack of toxicity information and safety standards has hampered possible applications of the CNT-based products in humans and animals. Since many CNT-based products are designed for the central nervous systems (CNS) applications (neural prosthesis, drug delivery and regeneration therapy), it is important to understand the danger these nanotubes may pose to the CNS. Therefore, the aims of article is to review the adverse effects of different forms of CNTs on the CNS, the role of protein corona in CNTs' toxicity, the challenges the adverse effects pose in applications of the CNT-based products, and possible mechanism for the evolution of CNTs's neurotoxicity. The information presented in this review will be useful in designing more effective and safer CNT-based products.

Abbreviations

CNT: Carbon nanotubes;
CNS: Central nervous system;
PEG: Polyethylene glycol;
TEER: Trans-epithelial electrical resistance;
SWCNT: Single walled CNT;
MWCNT: Multi walled CNT;
COOH: Carboxylic group;
LDH: Lactic acid dehydrogenase;
DNA: Deoxyribose nucleic acid;
ADMA: Activity-dependent microglial apoptosis;
IL: Interleukin;
NFκB: Nuclear factor kappa B

Carbon nanotubes (CNTs) are cylindrical allotropes of carbon with less than 100 nm in diameter and up to several μm in length. In general, CNTs can be classified as narrow diameter (less than 1 to 2 nm), small diameter (greater than 2 to 10 nm), medium diameter (greater than 10 to 30 nm) and large diameter (greater than 30 nm). The size of metal nanoparticle substrates controls the CNT diameter during synthesis. The aspect ratio (length/diameter) of CNTs may range from 100:1 to 132,000,000:1 [1]. CNTs possess many unique structural, mechanical, and electronic properties such as large surface to volume ratio, electron confinement and high electrical and thermal conductivities. The unique properties of CNTs has prompted their applications in medicine including, but not limited to drug delivery, neural prosthetics (integration of CNTs with neurons to improve the functions of damaged neurons in vivo), gene therapy, tissue regeneration or biosensor based diagnosis [2-5]. However, lack of toxicity information and safety standards has prevented application of new CNT-based products outside the laboratory studies. Although extensive research is being conducted in the area of CNT toxicity in vitro and in vivo [6-20], the research is mostly focused on the peripheral organs such as lungs, liver, spleen, dermal tissues and heart, etc. Recently, the research work has shifted to address the CNTs' toxicity on the central nervous system (CNS), but different studies have used different types of CNTs, experimental design and end-points of toxicity that has added considerable disagreements in their results. The present article has reviewed the diverse in vitro and in vivo research data and proposed a unified hypothesis regarding the CNTs' CNS toxicity that will assist scientists develop effective and safer CNT-based devises.

The CNS consists of the brain, spinal cord and a network of neurons, astrocytes and microglial cells. A dynamic blood-brain barrier (BBB) separates blood supply to the CNS from the systemic circulation and regulates transport across the barrier (Figure 1) [21-25]. Recent observations that nanoparticles, including the CNTs, can pass through the BBB without damaging the tight junction has led to the development of CNT-based products for treatment of brain disorders [26-28]. Pristine CNTs, being highly hydrophobic, rapidly aggregate in aqueous solution, poorly biocompatible and exhibit high toxicity. Functionalization improves their water solubility, dispersion and biocompatibility, while reduces their toxicity [29-31]. However, possible defunctionalization of functionalized-CNTs in body may result in accumulation of pristine CNTs into the CNS and present a significant hurdle in clinical use of the CNT-based products. This suggests that pristine and functionalized CNTs both may pose significant risk to the CNS and that an understanding of possible adverse effects of CNTs will help develop new biocompatible and safe CNT-based devises.

Therefore, the aim of this article is to review the CNS toxicity of CNTs and elucidate the challenges that the adverse effects may pose in applications of the CNT-based products in humans. This review article consists of the following sections: (i) the central nervous system toxicity of CNTs, (ii) CNTs' modulation of microglial and astrocyte activities, (iii) an integrated pathway for CNT toxicity, (iv) protein-corona and CNTs' CNS toxicity, (v) human risk assessments and (vi) conclusions and future research.

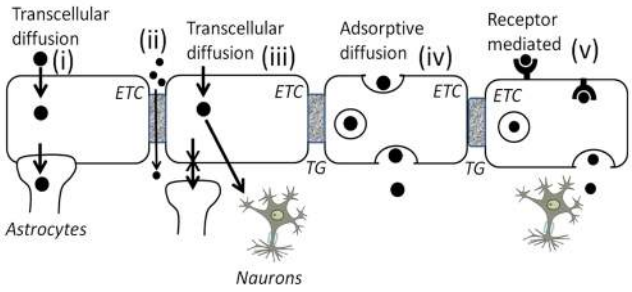

Figure 1. The blood-brain barrier consists of capillary endothelial cells surrounded by basal lamina, neurons and astrocytes (in some cases, astrocytic perivascular end feet may embed the endothelial cells). The endothelial cells express a number of transporters and receptors such as excitatory amino acid transporters, glucose transporter, L-system for large neutral amino acids and P-glycoproteins. The main routes for molecular traffic across the BBB are following: (i) Transcellular diffusion in endothelial cells embedded with astrocytes in which hydrophobic particles diffuse across the endothelium and directly enter the astrocytes. (ii) Pericellular transport in which hydrophilic particles may enter the brain via the tight-junction hydrophilic pores. (iii) Transcellular diffusion in endothelial cells separated from astrocytes in which the particles diffuse across the endothelial cells and then interact with neurons, astrocytes or microglial cells. (iv) Adsorptive diffusion in which particles are adsorbed onto the membrane and then enter the cell via endocytosis, a means of mass transport. (v) Receptor mediated transport in which the particles bind to a receptor or a transporter that translocate them into the cells via the receptor's configuration change. The particles may exit the cells via exocytosis. Some receptors/transporters facilitate active transport (against the concentration gradient) that requires ATP.

I. The Central Nervous System Toxicity of CNTs

In general, CNS toxicity is evaluated using in vitro and in vivo methods. In vitro models include organotypic explants and dissociated cultures such as brain endothelial cells [32-39]. The commonly measured toxicity indices are cell growth and proliferation, apoptosis, necrosis, neurotransmitter release or reuptake, inflammation, oxidative stress, frustrated phagocytosis, receptor and ion channel functions, signaling mechanisms, electrophysiology and morphological changes. In in vivo models, experimental animals (wild-type or genetically modified) are exposed to different doses of the toxins and, at different time intervals, the indices of toxicity (behavior, blood and tissue chemistry, toxins' plasma and tissue distribution, toxicokinetics, inflammation, oxidative stress and brain neurotransmitter, etc.) are measured.

I.1. In vitro Toxicity

The aim of this section is to review the effects of CNTs on cytotoxicity, integrity of the tight-junctions, oxidative stress, inflammation and apoptosis in CNS and non-CNS cells in vitro.

I.1.1. Effects of CNTs on endothelial tight junction and cytotoxicity

Studies have used Caco2 intestinal endothelial cell lines, Calu-3 airway epithelial cell lines and brain endothelial cells to study possible effects of CNTs on their permeability and cytotoxicity. MWCNTs and SWCNTs both decreased trans-epithelial electrical resistance (TEER) without causing cytotoxicity, even at the highest dose used [40-42]. Coyuco et al [43] showed that SWCNT-COOH, but not PEG-SWCNT, disrupted the organization of ZO-1, a protein marker of tight junction, resulting in disruption tight junctions (as discussed later, SWCNT-COOH, but not PEG-SWCNT, form protein corona). Rotoli et al [44] showed that long-MWCNTs and long-SWCNTs, but not short CNTs, reduced the cells' TEER values, while the short-SWCNTs caused cytotoxicity (Figure 2) by increasing oxidative stress, activating catalase, superoxide dismutase and glutathione peroxidase (an indication of free radical production) and increasing lactate dehydrogenase (LDH) leak into the cytoplasm in Caco-2 cells [45, 46]. The decrease in TEER may be attributed to the development of oxidative stress (Figure 3) and ensuing abnormalities in the tight junction proteins [47]. Listed below are two unanswered questions with regard to the penetration of CNTs into the CNS.

(1) Whether CNTs permeate the tight-junction barriers without damaging its integrity or damages the barrier, resulting in migration of proteins from systemic blood into the brain and causing toxicity

(2) Do the CNTs interact with the brain's influx and efflux receptors/transporters?

Figure 2

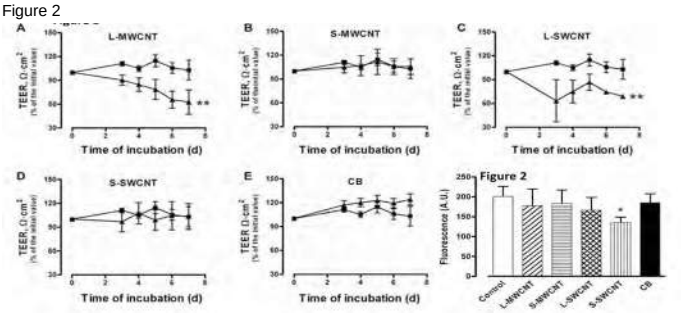

Figure 2. Cytotoxicity of different forms of CNTs. A: Short-SWCNTs, but not long- SWCNTs, long-MWCNTs or short-MWCNTs cause cytotoxicity in Calu-3 cells. B and D: Time-course of change in TEER values in Calu-3 cells exposed to long-MWCNT and long-SWCNT, respectively. Square: control values and triangle: CNT exposed values. **significant when compared from control values. C and E: Time-course of change in TEER values in Calu-3 cells exposed to short-MWCNT and short-SWCNT, respectively. Square: control values and triangle: CNT exposed values. The control and exposed values did not differ significantly. F: Time-course of change in TEER values in Calu-3 cells exposed to carbon black. Square: control values and triangle: carbon black exposed values. Reprinted from Rotoli et al [44] with permission.

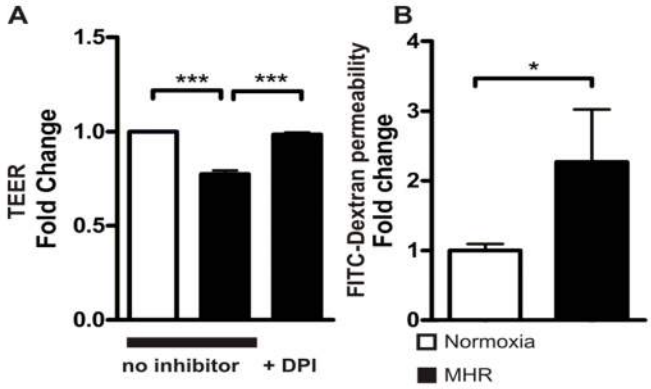

Figure 3. Oxidative stress induced decrease in TEER values (A), increase in dextran permeation (B) and decrease in expression of the tight junction proteins (C and D) in brain endothelial cells. This study is based on the principle that transient hypoxia followed by reperfusion significantly activated NADPH oxidase activity resulting in an increase in oxidative burst that is blocked by diphenyleneiodonium (DIP) exposure. As shown in Figure 3A, C and D: the hypoxia-induced oxidative stress decrease the TEER values associated with decrease expression of Claudin-5 (C) and zonula occludens 1 (ZO-1) (D) tight junction proteins. B: Hypoxia significantly increased dextran permeation. These observations suggest that oxidative stress may be causally related to the disruption of the blood-brain barrier. Reprinted from Zehendner et al [47] with permission.

I.1.2. Effects of CNTs on electrophysiology of the neuro-glial cultures

Belyanskaya et al [33] have compared sensitivities of dissociated spinal cord (SC) cultures (consisting of astrocytes >> glial cells, oligodendrocytes > neurons) and dorsal root ganglia (DRG) cultures (glial cells > neurons >> astrocytes, oligodendrocytes) to aggregated SWCNTs (SWCNTa) and dispersed SWCNTs (SWCNTd) in vitro. Their studies yielded the following results.

1) In SC cells, SWCNTd and SWCNTa caused 18% and 32% decrease, respectively, in total DNA contents, possibly due to a decrease in glial cell population, while neuronal population remained unchanged. In DRG cells, the two CNTs caused 33% and 58% decrease, respectively, in total DNA content, possibly due to decrease in glial and, to some extent, neuronal cells. In DRG cells, in addition to the glial cells, SWCNTa also affected neurons.

2) The electrophysiology of DRG neurons, but not SC neurons, was significantly modulated

by SWCNTd that resulted in relatively positive resting membrane potential (V_{mem}), lower capacitance (C_{mem}) and slightly suppressed width of action potential (AP_{width}) and the current needed for triggering action potentials (I_{thresh}).

3) In DRG cultures, but not SC cultures, SWCNTd reduced the Ca^{2+} conductance and the Ca^{2+} resting potential, thus suppressing the inward currents. The adverse effects of SWCNTs on neurons may be either due to either accumulation of SWCNTs in neurons or secondary to their effects on astrocytes.
Unlike the results of Belyanskaya et al [33], Gavello et al [48] showed that (i) Na^{+} and Ca^{2+} voltage-activated currents were not affected by MWCNTs and (ii) in the activated cells, the membrane potential was more depolarized, firing frequency was increased, and action potential amplitude reduced. These differences may be attributed to the differences in the type of CNTs and the cells used in the two studies.

In vitro electrophysiological studies provide insights into the mechanisms of action of toxins and drugs on the subcellular, cellular, and network levels. Electrophysiological recordings in the in vivo models have been hampered by many shortcomings including selection of appropriate recording sites to assess consciousness, cognitive functioning, perception, memory, etc. There is an urgent need to refine the in vivo models to establish relationship between electrophysiological measurements and higher brain functions.

I.1.3. CNS Toxicity of pristine and functionalized CNTs

In general, pristine CNTs exhibit relatively lower bioavailability than functionalized CNTs. Zhang et al [49] studied the adverse effects of pristine SWCNTs (pSWCNTs) and PEG functionalized SWCNTs (PEG-SWCNTs) in PC-12 cells in vitro and showed that pSWCNTs and, to a lesser extent, PEG-SWCNT decreased cell viability, damaged mitochondria, increased cytoplasmic LDH, increased ROS and decreased GSH concentration. The proteomic studies showed that pSWCNTs and PEG-SWCNTs modulated different sets of genes: pSWCNTs and PEG-SWCNT both down-regulated expressions of anti-oxidative enzymes, while pSWCNT, but not PEG-SWCNT, up-regulated expressions of pro-oxidative enzymes. These observations present a controversial picture of the toxic effects of CNTs on cells having tight junction. However, extensive variations in the nanoparticle groups, functionalization and experimental conditions do not allow direct comparison of different results.

Recently Kumarathasan et al [50] showed that CNT cytotoxicity in vitro in human lung epithelial cells (A549) and murine macrophages (J774) was assay-dependent. The cell-titer blue (CTB) reduction and BrdU incorporation tests indicated that the polar oxidized CNTs (high defects) exhibited higher cytotoxicity compared to the pristine CNTs. In contrast, cellular ATP and LDH tests showed that pristine CNTs were more cytotoxic than oxidized CNTs. Thus, metal content and surface properties affected ATP and LDH assays, while surface polarity affected CTB and BrdU assays. They proposed that the tests used for toxicity assessment of CNTs in vitro should be well characterized to reliably estimate their risk potential.

I.1.4. Beneficial and adverse effects of CNTs on stem cell proliferation and differentiation

Experimental studies have shown that stem cell implantation can be a promising therapy for stroke and other brain injuries since they can sprout into healthy neurons and may be able to re-establish normal activity in brain-injured patients [51, 52]. CNTs modulate differentiation of mesenchymal stem cells and the survival of bone, cartilage, and adipose cells [53-55]. In addition, neural stem cells differentiate on CNT substrates [56, 57]. Despite promising results, the prospect of CNTs' adverse effects on stem cells hampers their application in stem cell based regeneration therapy. Zhu et al [58] have shown that MWCNTs accumulate, induce apoptosis and increase the mutation frequency by 2-fold compared with the spontaneous mutation frequency in mouse endothelial cells. Taken together, these observations provide strong evidence for beneficial effects of CNTs, but the procedures shall be carefully scrutinized for CNT toxicity.

I.1.5. Factors affecting in vitro toxicity of CNTs

Unlike bulk carbon particles' toxicity, CNTs' toxicity depends upon particle size and aggregation state [59, 60], particle shape and rigidity [16, 61], surface charge [62, 63] and composition of protein corona (section II).

I.1.6. Significance of the in vitro studies

In vitro studies provide information regarding inherent toxicity of nanoparticles unaffected by the secondary factors such as transport barriers, metabolic degradation, excretion, etc. This study showed the following:

(i) pCNTs and PEG-CNTs both cause cytotoxicity (characterized by LDH release), although pCNTs were more potent than PEGCNTs.

(ii) Oxidative stress may be the key determining event for development of CNT toxicity. Studies have shown that CNT exposure, in vitro or in vivo, rapidly increased generation of reactive oxygen species (ROS) and consequent oxidative stress [18, 64] that triggers cell signaling pathways resulting in increased expression of proinflammatory and fibrotic cytokines [65-68].

(iii) CNTs compromise the endothelial tight junctions by interfering with the junction proteins. Whether the permeability increases is not yet established.

(iv) CNS cells respond to the dispersed CNTs (dispersed >> aggregated), while PNS cells respond to both aggregated and dispersed CNTs (aggregated >> dispersed).

(v) Despite large variations in types of CNTs, dose regimes, cell models and duration of interaction, the in vitro studies reflect a common theme that functionalization of CNTs improved their aqueous dispersion and biocompatibility, and reduced their toxicity.

These observations suggest that CNTs are inherently toxic to biological systems. However, the particles' overall toxicity, determined via in vivo studies, may be different from their inherent in vitro toxicity due to the body's secondary factors listed above.

I.2. In vivo Toxicity

The application of CNTs as drug transporter is well established and most appealing because of their unique physicochemical properties [69]. In addition, more exciting applications of CNTs' are (i) their integration with neural cells that form artificial synapses, thus allowing possible use of CNTs in neuro-electrical interfacing in vivo [2-4] and (ii) their used in regenerative medicine since CNTs facilitate proliferation and differentiation of stem cells [70]. These novel applications may allow treatment for many diseases that are currently untreatable. Since many of the prosthetic and regenerative applications require implantation of CNTs into the CNS, their safety is of serious concern because of poor and conflicting information regarding biocompatibility of CNT within the brain. Similar to the in vitro situations, in in vivo situations also, functionalized CNTs may exhibit improved biocompatibility than pristine CNTs. However, the prospect of defunctionalization (dissociation of functional groups) and ensuing accumulation of pristine CNTs in the brain may further complicate the safety of functionalized CNTs in vivo. Therefore, an understanding of possible neurotoxic effects of CNTs in vivo must be resolved before the nanotubes are used safely in human medicine. Some of the important issues associated with CNTs' neurotoxicity in vivo are described below.

I.2.1. CNTs and the blood-brain barrier in vivo

CNTs can negotiate the BBB via multiple pathways, each designed for a particular function (Figure-1). There also are certain minor pathways such as travelling through nasal passage and nerve terminals or diffuse through the neuronal/glial cytoplasmic membrane that bypass the barrier [71]. In general, permeation of a toxin across the BBB is directly related to their hydrophobicity, but inversely related to their size including diameter, length and molecular weight [72]. CNTs and other nanoparticles, functionalized with ligands such as transferrin can bind to the blood-brain transporters/receptors and cross the barrier without damaging the tight junction. Pristine CNTs, being highly hydrophobic, has been shown to aggregate into different size particles, thus may not cross the BBB. In comparison, functionalized CNTs remain dispersed and can negotiate the barrier.

There are substantial but not compelling evidence showing that CNTs may induce oxidative stress that could disrupt the blood-brain barrier, thus allowing their entry into the brain [73]. Short CNTs and other nanoparticles deposited in the lungs may translocate into the circulatory system and eventually reach the brain [74, 75], while nanoparticles inhaled through the nose can translocate directly to the brain via the olfactory neurons [76, 77]. Accumulation of CNT-filled macrophages in the alveoli increased permeability of the epithelial cell barrier and particle translocation to the alveolar interstitial fluid, thus increasing the probability of CNTs reaching the brain [78-81]. However, more research is needed to establish possible translocation of CNTs from lungs into the brain.

I.2.2. Possible defunctionalization of CNTs in the brain

As discussed above, a key problem associated with long-term application of functionalized CNTs is their defunctionalization [82-84]. Since defunctionalized CNTs may be relatively more hydrophobic and stable, their accumulation in tissues may pose significant health hazard. Earlier studies have shown defunctionalization of PEG-SWCNT in liver, but not in spleen under similar conditions [83]. Recently, Nunes et al [85] have shown that the brain microglia may effectively engulf and defunctionalize the amino-functionalized CNTs in vivo. This initial observation of CNT defunctionalization within the brain necessitates further investigations to establish their pharmacological and toxicological consequences.

I.2.3. Possible mechanisms underlying CNT toxicity in the brain in vivo

As discussed above, CNTs may cause toxicity via development of oxidative stress [86], although oxidative stress-independent inflammation mechanisms also exist [87, 88]. The intranasal exposure may be relevant to CNTs since intranasal instillation of ultrafine carbon black significantly induced the expression of pro-inflammatory cytokines and chemokines [89-91]. Unique to CNTs is the relationship between their aspect ratio (diameter to length) and potential adverse health effects in the lungs and the brain in vivo [92]. MWCNTs developed systemic inflammation, progressive fibrosis and granulomas [79, 93] in a length-dependent manner. MWCNTs with greater than 20 μm length generated frustrated phagocytosis and ensuing polymorphonuclear cells and granulomas [94]. There is some evidence for development of carcinogenesis via frustrated phagocytosis [95-97].

II. CNTs' modulation of microglial activities

Microglial cells are the innate immune cells of the CNS [98]. When inactive, microglial cells exists as branched ramified cells ($CD11b^{+}$, $CD45^{low}$) and remains quiescent. In response to injury, microbial infection or toxin exposure, the ramified microglia proliferate into rod-like reactive microglia that migrate to the site of injury where they (i) release protective and pro-inflammatory factors and (ii) phagocytose damaged cells and debris [99-102]. Factors secreted by activated microglia include superoxides, nitric oxide, glutamate, matrix metalloproteinases (MMPs), interleukins and tumor necrosis factor [103-111]. An excess of some of the factors (superoxides, MMPs, glutamate, etc.) listed above can amplify inflammation and contribute to neuropathy. To prevent the microglia-mediated neuropathy, body regulates the population of activated microglia via 'activity-dependent microglial apoptosis' (ADMA).

In a recent study, Sauer et al [112] have shown that MWCNTs (NM-400: 1.5 μm length, NM-401: 15 μm length and NM-402: 10 μm length) induced inflammation in length dependent manner. NM-401 and NM-402 caused cytotoxicity at 500 μg/ml and 1000 μg/mL. Both NM-401 and/or NM-402 further induced oxidative stress and ensuing pro-inflammatory cytokines such as CINC1, M-CSF, TNF-α and CINC1. This suggests that long-MWCNTs, but not small-MWCNTs, induced inflammation and toxicity in lungs that has been attributed to the frustrated phagocytosis [94, 113-115]. Konduru et al [116] have suggested that SWCNTs, depending upon their functionalization, may enter the brain via the resident microglial cells and that long SWCNTs, may result in frustrated phagocytosis and ensuing inflammatory activity in the brain (Figure 4).

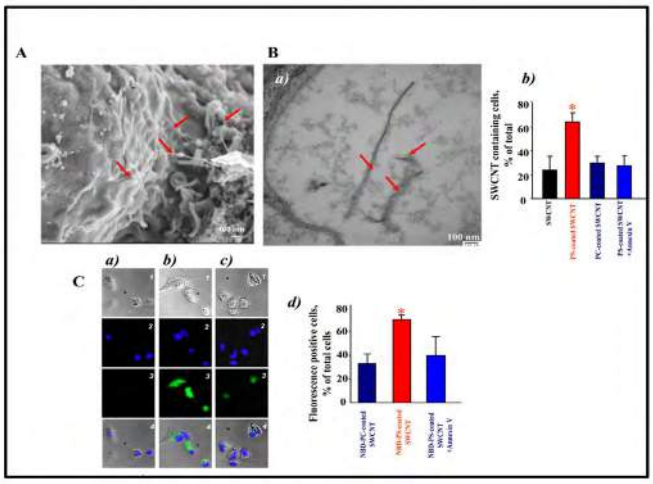

Figure 4. Interaction between microglia and functionalized SWCNTs. A. Scanning electron micrographs of microglia treated with phosphatidylserine functionalized SWCNTs (PS-SWCNT) in vitro (phosphatidylcholine functionalized SWCNTs (PC-SWCNTs) are not shown). The red arrows point to SWCNTs that are visible outside the cells, an indication of frustrated phagocytosis. B. Transmission electron micrographs of primary microglia exposed to PS-SWCNT in vitro. a: Endothelial cells containing PS- SWCNT. b: Quantitative assessments of SWCNT phagocytosis by RAW 264.7 macrophages. The PS-SWCNT positive cells were present in highest abundance. C(a-c). In vitro uptake by microglial cells of florescent PS- or PC-SWCNTs. (a) PS-SWCNT, (b) Annexin V treated PS-SWCNTs and (c) merging of the two images. Column description for a to c: 1: bright field image, 2: blue fluorescence image, 3: green fluorescence image of florescent dye-labeled phospholipids and 4: overlap of blue and green fluorescence images with image under bright field. Cd: Quantitative evaluation of cell number with engulfed SWCNT. Annexin V treatment of PS- SWCNT prevented their engulfment by microglia. Data are mean ± s.d., n = 4. *p<0.05, NBD-PS-coated SWCNT vs NBD-PC-coated SWCNT and NBD-PS-coated Annexin V treated SWCNT. Reprinted from Konduru et al [99] with permission.

In addition to the development of oxidative stress and inflammation, multi-nucleation has also been reported in microglia exposed to MWCNTs for 70 hours . Figure 5a shows a confocal image of microglial cells displaying two nuclei after 70 h incubation with MWCNTs. Figure 5b shows quantification of mono-nucleated versus multi-nucleated cells showing a statistically significant rise (approximately22%) in multi-nuclear microglial cells. Although mechanism for poly-nucleation is not fully understood, MWCNTs may biomimetically interact with the mitotic spindle microtubules [117] or with the actin filaments of the contractile ring and imped the separation of the two daughter cells, resulting in G2 arrest [118, 119]. The existence of small compact-DNA aggregates in cytoplasm suggests microglial apoptosis following MWCNT exposure.

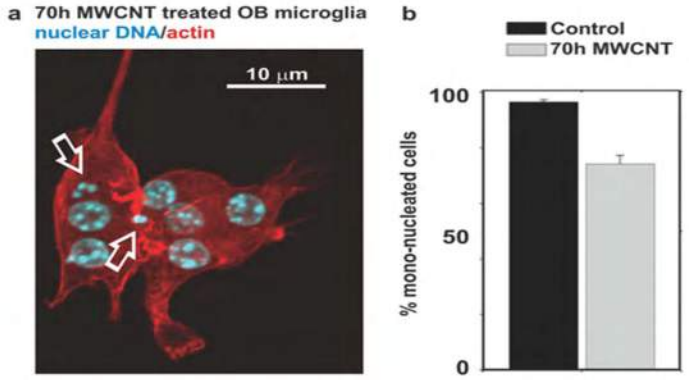

Figure 5. (a) Confocal imaging of MWCNT treated microglial cells stained for actin (red channel) and nuclear DNA (blue channel). Each cell contains two nuclei and shows features of cell death by apoptosis (F-actin disorganization and the presence of small intracellular compact DNA masses). (b) Quantification of the percentages of mono-nucleated versus multi-nucleated cells revealed a significant increase in microglial cell multi-nucleation in primary brain cultures treated with MWCNTs. Reprinted from Villegas et al [113] with permission.

III. An integrated pathway for CNT toxicity

Despite considerable diversity, one common theme of the in vitro and in vivo studies is that oxidative stress is the hierar-

chical event leading to the development of CNT toxicity in the CNS. As shown in Figure 6, an injury, pro-inflammatory agents, bacterial toxin or nanoparticles activate the microglial cells, resulting in an increase oxidative stress and ensuing release of the pro-inflammatory factors that evolve inflammation. Inflammation eliminates the initial cause of injury, removes the necrotic cells and tissues resulting from the original insult, and then initiates the process of repair. After an appropriate level of inflammatory response, the active microglial population decline due to ADMA. Activation of astrocytes (by yet undetermined mechanisms) releases the anti-inflammatory factors (IL-10, IL-4) via activation of anti-inflammatory NFκB, p50-p50 [109, 120] that resolves inflammation. A sequential evolution and resolution of inflammatory activity is essential for appropriate injury healing [120]. CNTs, although have been claimed to be inherently nontoxic [121], activate the pro-inflammatory and suppress the anti-inflammatory signaling, thus deregulating inflammation. This may delay healing and increase the potential for neuropathy. This hypothesis is supported by the following observations:

(i) SWCNTs can penetrate the blood-brain barrier via inter-cellular mechanism that may induce pro-inflammatory activity similar to the concept of frustrated phagocytosis [122].

(ii) SWCNTs attenuated the LPS-induced activation of SIRT3, caspase-3 and caspase-7 expression, but suppressed cleavage of p53 in mice microglial cells. Since p53, SIRT3 and Caspase-3 and -7 are critical for apoptosis of eukaryotic cells, SWCNT's may also suppress the ADMA, resulting in prolongations of the pro-inflammatory activities (Figure 6) [123].

(iii) SWCNTs, depending on their functionalization, differentially modulate the anti-inflammatory activity. Pristine or phosphatidylcholine coated SWCNTs either suppressed or did not modulate, while phosphatidylserine coated SWCNTs activated the anti-inflammatory activity [116].

(iv) MWCNTs, because of their structural similarities to the protofilaments that constitute the microtubules (4 nm) [124], may intermingle with the intracellular microfilaments and cause proliferation defects and necrosis [118]. In addition to microglial cells, SWCNTs also produce multi-nucleation in HeLa cells [125].

Recently, Arora et al [126] have published an excellent review stressing importance of in vitro studies in toxicity testing of nanoparticles. More toxicology research is needed before the CNTs' medical application is approved. Development and standardization of high throughput screening is desired for rapid screening. Future toxicology studies will determine the fate of nanotechnology in medicine and drug delivery.

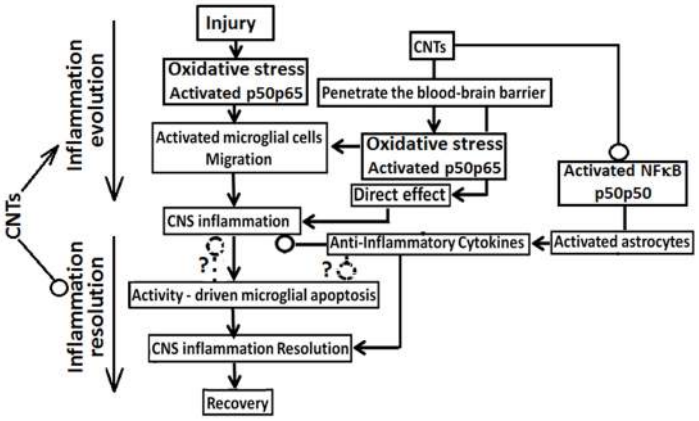

Figure 6. Flow diagram showing effects of CNTs on injury-induced oxidative stress, inflammation and injury healing. The details are described in the text. ——>Stimulation, ——o Inhibition.

IV. CNTs' Protein Corona and CNS Toxicity

Nanoparticles, including CNTs, in contact with biological fluids rapidly interact with the proteins and lipids, resulting in formation of multilayered corona (Figure 7). The innermost corona layer is due to protein-nanoparticle (Figure 7-1) interaction (hard corona) characterized by the nanoparticle surface (example: hydrophobic surface may attract hydrophobic moieties of the proteins, while the negatively charged particles attract positively charged proteins, etc.). The protein-protein interactions (Figure 7-2 to 5) characterize the outer layers (soft coronas) in which the penultimate layer determine the proteins of the next outer layer (example: negatively charged penultimate layer will attract positively charges proteins). Gasser et al [127] have shown that different proteins and lipids bind to different types of CNTs without any distinct pattern, indicating nonspecific adsorption of proteins onto a CNT. Functionalization of nanoparticles that modulate their hydrophobicity and/or steric hindrance may also modulate their corona composition (Figures 7 and 8) [127]. Ge et al [128], using experimental and theoretical approaches, investigated interactions between SWCNTs and bovine fibrinogen (BFG), γ globulin, transferrin (Tf) and bovine serum albumin (BSA). As shown in Figure 9 [128], the proteins exhibited competitive binding onto the SWCNT surfaces and the proteins' unique structure and the amount of hydrophobic residues in each protein binding governed their binding. Pristine, functionalized and lipid-bound CNTs exhibited differential composition of protein corona. The corona's proteins are in equilibrium with their surrounding and undergo dynamic exchange processes as the CNTs travel through different body fluids (example: the nanoparticle corona in blood may be considerably different from the corona in the cerebrospinal fluid).

Recent studies have proposed a new paradigm for consequences of protein–nanoparticle interactions [129-131]. According

to the new paradigm, the outermost layer of protein corona, not the nanoparticle's surface, determines their interaction with biological systems (Figure 10). The protein-nanoparticle interaction alters conformation of the bound proteins, resulting in their misfolding [132]. The misfolded proteins do not exhibit their intended biological properties. In case of fast exchange time with the surface, the misfolded proteins may be released into the surrounding fluid, while, in case of a slow exchange time with the surface, the stable misfoded proteins may remain bound to the surface [129, 133, 134]. The nanoparticle surface may also induce abnormal unfolding of the bound proteins, resulting in formation of novel conformational epitopes (Figure 11) that may elicit unwanted responses, resulting in development of adverse effects. This suggests that the nature of corona's outer layer may change rapidly as the particles encounter different bodily fluids or undergo conformational change. The rapidly changing protein corona may also change the particles' biological properties including their toxicity [131, 135].

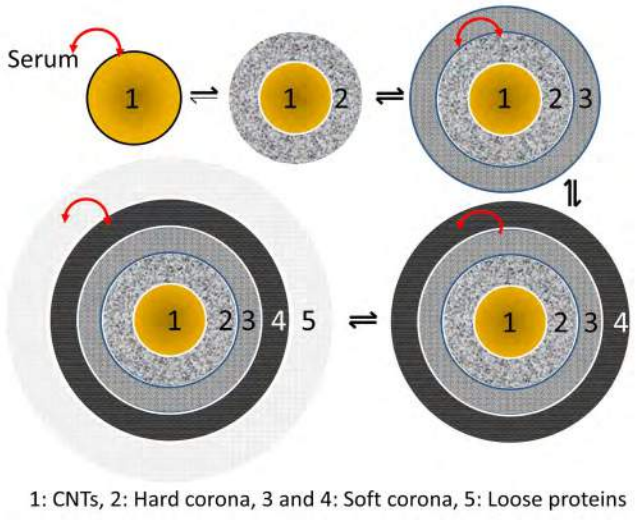

Figure 7. Sketches describing formation of protein corona. When nanoparticles encounter serum or other biological fluids, the nanoparticle's surface determine the composition of proteins adsorption onto the surface (hydrophobic surface will attract hydrophobic sites of a protein, cationic surface will attract anionic site of the proteins and vice versa). This results in formation of 'hard' corona (Figure 7-2). The properties of the 'hard' corona protein then attracts another layers of proteins (Figure 7-3) called soft corona. The three layers of corona are shown in Figure 7-2,-3 and-4. There may be some proteins loosely associated (-5) with the outermost layer. The characteristics of outermost layer are determined by the nanoparticle surface properties.

If the nanoparticle corona contains the complement peptides C3a, C4a and C5a, it may serve as a neutrophil chemotactic factor [136, 137]. The corona containing the opsonins C3b and iC3b will result in accumulation of neutrophils around the phagocytic cells. In case of long-CNTs, frustrated phagocytosis may result in an increase in oxidative stress, pro-inflammatory cytokines and degradative enzymes, causing tissue damage. In addition, CNTs may also activate C3b, C4b, iC3b and C3d that act as adjuvants, thus increasing the immune response to foreign materials [138].

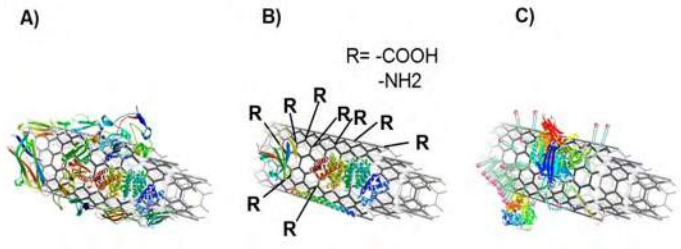

Figure 8. Adsorption of proteins to pristine (A) and functionalized (B and C) MWCNTs. Figure 8A shows binding of plasma proteins onto the pristine-MWCNT surface. Figure 8B shows alterations in the protein corona pattern when MWCNTs are functionalized. Figure 8C shows that further alteration in protein binding is observed when lipids from surfactant are bound to the MWCNTs. Reprinted from Gasser et al. [124] with permission.

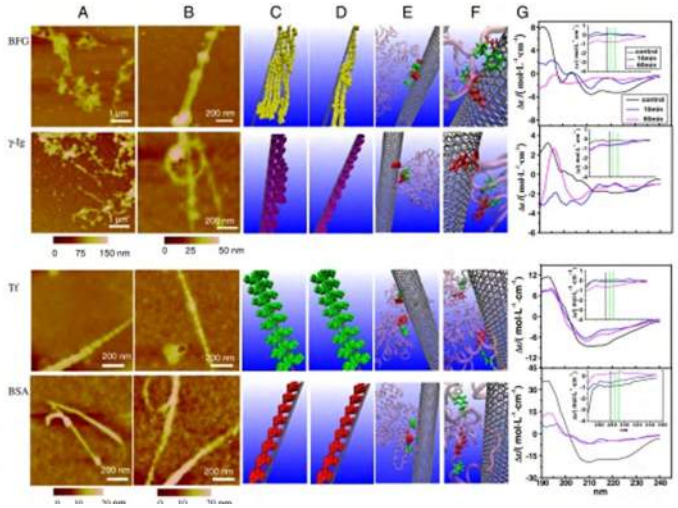

Figure 9. SWCNTs interaction with plasma proteins such as bovine fibrinogen (BFG), γ globulin, transferrin (Tf) and/or bovine serum albumin (BSA) that results in formation of protein corona. A and B: AFM images of proteins after incubation with SWCNTs for 10 min (A) and 5 h (B). C and D: Molecular modeling for the proteins' binding to SWCNTs after incubation for 10 min (C) and 5 h (D). E: Locations of the binding sites on proteins for SWCNTs (tyrosine is colored red and phenylalanine is colored green). F: The detailed orientations of aromatic rings of tyrosine and phenylalanine residues interacted to six-member rings of SWCNTs. G: The far-UV CD and the near-UV CD (inset) spectrum of proteins after incubation with SWCNTs. Reprinted from Ge et al [125] with permission.

The misfolded surface proteins may not bind their intended receptors or exhibit enzyme activity, thus, they may trigger various signaling abnormalities, resulting in the development of toxicity. Loss of enzyme activity may be due to the conformational changes in the active site. Turci et al [139] showed that RNAse and lysozymes retained their native structures on

silica NP, while albumin and lactoperoxidase underwent an irreversible conformational change that increased the accessibility of the active site for its substrate. Contrarily, covalently bound horseradish peroxidise and chicken egg white lysozyme on SWCNTs retained their activity and their native structure even under denaturing conditions [140].

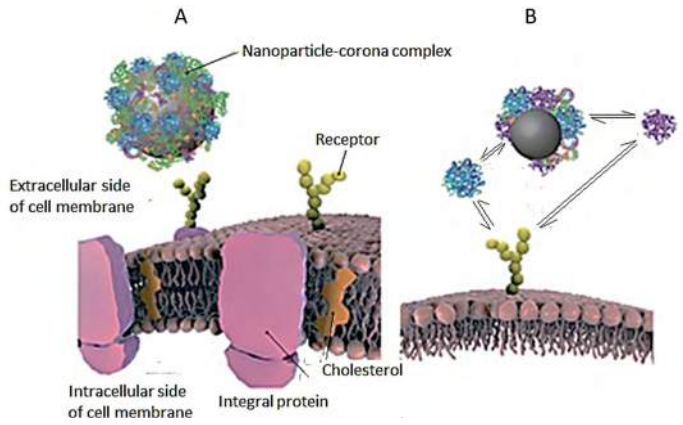

Figure 10. Biological significance of CNT-protein interaction.
A: Cell membranes consist of a lipid bilayer embedded with integral proteins, receptors, transporters and ion channels. When a nanoparticle encounters biological fluid, different proteins attach to the nanoparticle surface depending upon the surface properties. However, the outermost protein layer interacts with the cell membrane. If the corona contains a receptor-ligand, then the particle may bind the receptor, while, if the corona contains hydrophobic amino acids, it may bind to the nanoparticle surface. Thus, the outermost layer of the corona may determine the nanoparticles' biological properties.
B: The proteins in the outer corona are in equilibrium with the surrounding fluid. As the surrounding fluid changes, the corona composition also changes. For example, if nanoparticles move from blood into the brain, the corona proteins may equilibrate with the CSF proteins, resulting in formation of new corona and a different biological property. The equilibration also introduces new plasma-derived proteins into the CSF that may have their own adverse effects. Reprinted from Monopoli et al [126] with permission.

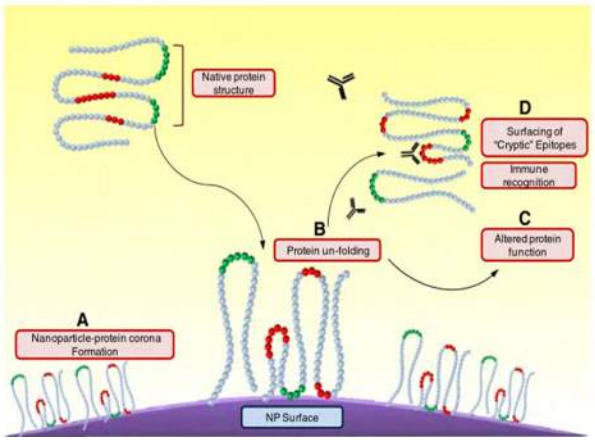

Figure 11. Schematic representation of the functional consequences of the nanoparticle-protein interactions. When proteins bind to the nanoparticle surface (A), the interactive forces may induce conformational changes to the protein's native structure and/or unfolding of the interacting protein molecules (B). The protein conformational changes may either (C) alter the function of the native protein molecule and/or (D) expose certain "cryptic" (hiddean/unknown functions) epitopes which may result in immunological recognition of the complex. As described in Figure 10, the modified proteins may be released into the surrounding fluid and initiate adverse effects (example: the plasma proteins having "cryptic" epitomes may be released in the CSF and induce abnormal signaling). Reprinted from Saptarshi et al [143] with permission.

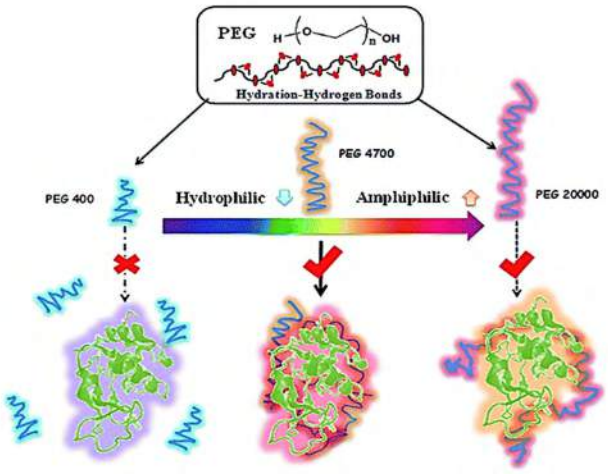

Figure 12. Protein binding to the PEG molecules. PEGs, depending upon its size, can bind to proteins and form stable complex. PEGs ≤ 400 MW that are mostly hydrophilic exhibit poor affinity, while PEGs ≥ 4,700 MW that are mostly amphiphilic exhibit high tendency to interact with proteins. Reprinted from Wu et al [139] with permission.

Polyethylene glycol (PEG) functionalization is commonly used to suppress protein binding onto the SWCNT's surface [141]. However, a recent study [142] has demonstrated that PEG itself can interact with proteins in aqueous solution. An interaction between proteins and long-chain PEGs induce structural changes in PEGs, resulting in formation of a relatively open structure with increased hydrophobic cavities. In this conformation, PEGs bind multiple proteins. The short-chain PEG, however, lacked the protein-binding sites and protein binding (Figure 12). Wu et al [142] suggested that high molecular weight PEG could change the conformation of proteins and the activity of PEG-coupled proteins. However, prevention of protein corona by PEGs may also prevent some of the beneficial effects attributed to the corona. Ge et al [128] showed that the SWCNTs coated with BFG, BSA, Tf, and IgG modulated the adverse effects of SWCNTs in vitro. The BFG-coated SWCNTs were essentially non-toxic, while BSA- Tf- and Ig-coated SWCNTs were relatively less effective in suppressing SWCNT toxicity (Figure 13 A and B). The fluorescence-based LIVE/DEAD assays showed presence of dead cells in uncoated and BSA-, Tf-, and Ig-coated SWCNTs, not in BFG-coated SWCNTs (Figure 13 C). In addition, nanoparticles with protein corona penetrat-

ed the blood-brain barrier without damaging the endothelial cells' tight junctions. The following observations support this hypothesis.

• The corona proteins interact with the blood-brain barrier surface and promote translocation of nanoparticles across the barrier via different mechanisms shown in Figure 14 [143].

• Adsorbed apolipoproteins, via interacting with low-density lipoprotein receptors [144], facilitate nanoparticle transport across the blood-brain-barrier [145, 146].

• Ttransferrin (Tf)-coupled nanoparticles, via a receptor-mediated transcytosis, can reach the brain parenchyma from systemic administration [147].

• Other proteins such as insulin, anti-glucose transporters, and certain gut hormones may also transport nanoparticles across the barrier, but their application is not yet demonstrated [29, 148].

Accumulation of CNTs in brain may be therapeutically important (in drug delivery and regenerative therapy), but may also enhance their toxicity potentials, especially if the corona proteins are misfolded. This aspect needs further characterization.

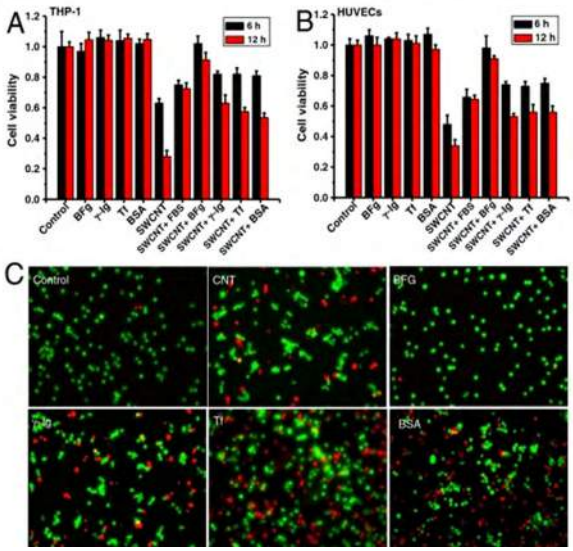

Figure 13. Effects of protein corona on proliferation and viability of cells in the presence of SWCNTs with/without protein coatings. The human acute monocytic leukemia cell line (THP-1) and umbilical endothelial cells (HUVECs) were exposed to SWCNTs (30 μg/mL) with or without proteins. The differential cytotoxicity was measured after treatment of the cells for 6 and 12 h. A and B: Quantitation of viable THP-1 (A) and HUVEC (B) cells exposed to SWCNTs for 6 and 12 hours. C: The live and dead stains for THP-1 cells after 12 h of SWCNT treatment. SWCNTs (top left), SWCNT+FBS (top middle), SWCNT+BFG (top right), SWCNT+γIG (bottom left), SWCNT+TI (bottom middle) and SWCNT+BSA (bottom right) caused 50%, 20%, 3%, 25%, 30%, 30% decrease in live cells, respectively. This suggests that although all of the protein-bound CNTs provided some protection, only BFG provided complete protection against CNT cytotoxicity. The reason for BFG's complete protection is its unique interaction that results in complete coverage of the nanotubes. Reproduced from Ge et al [125] with permission.

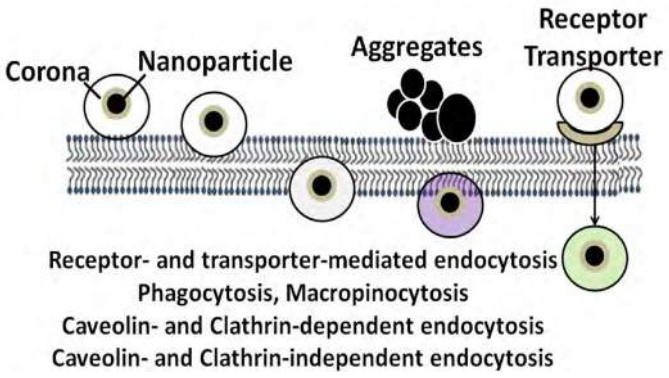

Figure 14: Interaction of nanoparticles with the cellular interface. Nanoparticles interact with cells via the protein corona. (A) Aggregated nanoparticles without protein corona remain attached to the membrane surface but do not internalize. (B) Non-specific uptake of extracellular fluid containing nanoparticle-corona complex may be taken up by cells via macropinocytosis which involves ruffling of the plasma membrane to form vesicles which ultimately fuse to form intact membrane. The corona composition may change as the particles move from extracellular fluid into the intracellular fluid. (C) Nanoparticle-protein complex may be ingested by specialized cells such as macrophages and neutrophils via phagocytosis. It involves folding of the plasma membrane over the nanoparticle complex to form the phagosome. (D) Endocytosis of nanoparticle complexes may be directed by specific receptors involving formation of caveolae that are plasma membrane indentations consisting of cholesterol binding proteins called caveolins or clathrin-coated vesicles. Apart from these other endocytic mechanisms, independent of clathrin or caveolae may also facilitate uptake of nanoparticles.

V. Human Risk Assessments

The risk assessment procedures commonly followed for the bulk chemicals include the following: Exposure assessment including identification and characterization of the populations exposed and determination of the magnitude, frequency and duration of the exposures. Hazard assessment includes hazard characterization (dose–responses and mechanisms of toxicity) and hazard identification. Final risk assessment based on exposure and hazard assessments. Is the procedure for bulk particles applicable to determine the risk for development of CNT toxicity in humans? The following factors must be considered to answer this question.

• The adverse effects of CNTs, in addition to their dose in terms of mass (such as mg/kg), are also determined by their size, surface area and surface reactivity. Thus, the dose-response curves constructed using the mass-based dose may yield in-

accurate prediction of toxicity. Inclusion of the surface area based (total surface area/kg) or the surface activity based doses may provide accurate information regarding CNT toxicity.

• The electronic, optical and chemical properties of CNTs change as their diameter and length changes. CNT of a 10 nm diameter may be functionally different from the CNT of a 50 nm diameter, thus they may exhibit different bio-uptake, distribution, biological response and toxicity.

• CNTs, depending on their folding, may acquire either metallic or semi-conductor properties. Thus, chemically identical CNTs may exhibit different toxicity.

• Functionalization alters the biological properties of CNTs.

• Metal ion impurities alter the CNT cytotoxicity. CNTs are produced by chemical vapor deposition, carbon arc discharge, laser ablation, or electrolysis methods, using metals such as Ni, Co, Mo, Fe, Cr, Cu, and Al as catalysts [18]. Metal catalyst residuals are the main impurities in the as-produced CNTs. [18] Despite extensive cleaning, trace levels of metal contamination remain in CNT preparations that modulates their toxicity [128,149]. Mwangi et al [150] , using different sources of CNTs, showed that (i) metal contents in CNTs correlated with the decrease in survival of amphipods (Tables 1 and 2) and (ii) EDTA that chelated the metals, significantly improves survival in CNT exposed amphipods. These observations provide strong evidence that metal impurities modulate the toxicity of CNTs.

• The variance in toxicological information prevents comprehensive risk assessment of CNTs.

Taken together, the procedures developed for bulk particles may not accurately determine the CNTs' risk to humans. A new paradigm is needed to assess the toxicity of CNTs and then use the toxicity data in risk assessment.

Sample	Carbon	Oxygen	Silicon	Iron	Cobalt	Molybdenum	Nickel	Total metals
SWCNT-S	93.4	ND	ND	0.3	4.9	1.5	ND	6.7
MWCNT-S	89.7	11.8	0.9	ND	0.2	1.2	3.0	5.3
NAM MWCNT-S	86.1	13.5	ND	0.4	ND	ND	ND	0.4
MWCNT-H	70.9	15.3	0.1	10.1	2.5	0.6	0.5	13.3

Values are mean of four determinations

Table 1. Elemental compositions (percentage of wt) of single-wall carbon nanotubes from Shenzhen (SWCNT-S), multiwall carbon nanotube from Shenzhen (MWCNT-S) or Helix USA (MWCNT-H) and nitric acid modified (NAM) MWCNT-S samples analyzed with an energy dispersive spectrometer. Reprinted with permission from Mwangi et al [150].

Test	Treatment	Amphipod		Midge		Mussel		Oligochaete
		Survival (%)	Biomass (mg)	Survival (%)	Biomass (mg)	Survival (%)	Length (mm)	Biomass (mg)
1	Control	88 (5.0)x	1.2 (1.0)	80 (8.2)x	9.3 (2.6)x	98 (5.0)x	2.2 (0.1)	2.8 (0.2)x
	Nonsonicated MWCNT-H	5.0 (10)y	NR	43 (9.6)y	3.7 (0.6)y	23 (17)y	NR	1.2 (1.0)z
	Sonicated MWCNT-H	2.5 (5.0)y	NR	60 (8.2)z	1.4 (0.2)z	43 (19)y	NR	2.3 (0.4)y
2	Control	100 (0)x	NR	83 (5.0)x	NR	NT	NT	3.7 (0.8)x
	Nonsonicated SWCNT-S	20 (12)y	NR	10 (8.2)y	NR	NT	NT	1.4 (0.4)z
	Sonicated SWCNT-S	0 (0)z	NR	0 (0)z	NR	NT	NT	2.8 (0.2)y
3	Control	100 (0)x	NR	67 (7.5)x	NR	80 (28)x	NR	5.2 (4.0)x
	Nonsonicated MWCNT-S	8.0 (10)y	NR	55 (3.0)	NR	35 (26)y	NR	0.8 (0.2)z
	Sonicated MWCNT-S	5.0 (10)y	NR	8.0 (5.0)	NR	3.0 (10)z	NR	2.2 (0.6)y
4	Control	100 (0)x	0.8 (0.2)x	75 (19)x	5.5 (2.6)x	100 (0)x	1.3 (0.1)x	3.7 (1.2)x
	Nonsonicated NAM MWCNT-S	95 (5.8)x	0.3 (0.1)y	60 (14)x	2.6 (0.9)y	98 (5.0)x	1.1 (0.04)y	1.3 (0.4)y

Standard deviations in parenthesis, n = 4. Different letters for survival, length, or biomass in a column for a test indicate a significance difference among treatments ($p < 0.05$). The biomass is based on dry weight for the amphipod and midges and ash-free dry weight for the oligochaete.

NR = not reported because recovered organisms were used for photographs or transmission electron microscopy imaging or due to <50% survival in CNT treatments; NT = not tested.

Table 2. Mean response of the amphipod Hyalella azteca, the midge Chironomus dilutus, the mussel Villosa iris, and the oligochaete Lumbriculus variegatus amphipod Hyalella azteca, exposed to sonicated or nonsonicated single- or multiwall carbon nanotubes from Shenzhen (SWCNT-S or MWCNT-S) or from Helix (MWCNT-H) or nitric acid–modified (NAM) MWCNT-S in 14-d toxicity test. Sonication increased the CNT's dispersion (diameter 89 nm to 48 nm). All CNT forms, except nonsonicated NAM MWCNT-S, caused 100% to 60% decrease in survival of each species. Nonsonicated NAM MWCNT-S, exhibited 5% decrease. The toxic effects of CNTs were related to their metal concentrations since EDTA increased survival. Reprinted with permission from Mwangi et al [150].

VI. Conclusions and Future Research

Recently, there has been an exponential increase in the development of CNT-based products for drug-delivery and other medical use [2]. Although experimental studies have yielded promising results, the CNT-based products are not yet approved for clinical use possibly due to uncertainties associated with their toxicity. Although many excellent review articles characterizing the systemic effects of CNTs are available, their adverse effects on the CNS are not fully understood. In the present article, different aspects of the CNT-induced CNS toxicity have been reviewed in vitro and in vivo. Oxidative stress may be the hierarchical event leading to the development of , while protein corona may play a critical role in modification of the CNT toxicity. Listed below are some of the data-gap in existing literature and the possible research that will lead to

better understanding of the CNS toxicity of CNTs.

1. It is important to understand the fate of engineered CNTs in the CNS. Since CNT skeleton is not easily biodegradable, it will either remain into the brain for extended periods or exit the brain via efflux transporters. As discussed above, the corona may contain proteins with abnormal epitopes that may be recognized as 'foreign' particles and initiate immune reaction. This is especially important in determining the long-time applicability of CNTs as neural prosthetic.

2. There is a need to develop new methodologies for identification and removal of the 'misaligned' proteins from the outermost layer of corona.

3. If the benefits of CNTs are their tubular structure, can we develop nanotubes that will be more biocompatible then CNTs?

4. There is an urgent need to conduct acute and chronic toxicities in experimental animals exposed to more relevant exposure routs such as ambient air inhalation, contaminated food and injectable preparations. The role of 'aspect ratio' should be further investigated for organs other than lungs.

5. To develop appropriate safety standards, new pharmacological models need to be developed to evaluate neuro-pharmacokinetics and neuro-toxicokinetics of CNTs.

6. More research is needed to explain the mechanisms by which CNTs suppress apoptosis and migration of activated microglial cells, but induce apoptosis of other cells including neurons and astrocytes [146].

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