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Determinants of carbon nanotube toxicity ☆

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ABSTRACT

In the last few years questions have been raised regarding the potential toxicity of carbon nanotubes (CNTs) to humans and environment. It is believed that the physico-chemical characteristics of these materials are key determinants of CNT interaction with living organisms, and hence determine their toxicity. As for other nanomaterials, the most important of these characteristics are the length, diameter, surface area, tendency to agglomerate, bio-durability, presence and nature of catalyst residues as well as chemical functionalization of the CNT. This review highlights the recent advancements in the understanding of the CNT properties which are essential in determining CNT toxicity. Hence the focus is on CNT dimensions, surface properties, bio-durability and corona formation as these fields have evolved greatly in recent years.
A deeper understanding of these events and their underlying mechanisms could provide a molecular explanation of the biological and physiological responses following CNT administration and therefore help in the development of safe by design materials.

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1. Introduction

Carbon nanotubes (CNTs) are unique nanomaterials as they possess inimitable physico-chemical properties, such as mechanical, thermal, or electrical conductivity. CNTs are widely utilised in numerous applications (computers, aircraft airframe, and sporting goods, bicycles, golf irons) and have also emerged as efficient drug delivery carriers in the biomedical field [1,2]. Due to the ever-increasing demand for these nanomaterials there is a potential for increased human exposure to them, hence there is a growing body of literature with the aim of evaluating the health effects of CNTs.

As for other nanomaterials, the biological reactivity and toxicity of CNTs have been shown to depend on numerous physicochemical characteristics, such as the length, diameter, surface area, tendency to agglomerate, dispersibility in solution, presence and nature of catalyst residues, as well as chemical functionalization of these nanomaterials [3–13].

This review will highlight the recent advancements in the understanding of which CNT properties are essential in determining their toxicity. We will specifically focus on CNT dimensions, surface properties,

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bio-durability and corona formation as this field of research has progressed greatly in the recent years, and how these properties can be exploited in the field of nanomedicine.

2. CNT properties as determinant of toxicity

2.1. Dimensions

CNTs can exist as compact tangles of nanotubes that can be considered as particles, or as longer, straighter "fibres". One could anticipate that the hazard from these two different forms of CNT would differ — for example; nanotube-particle effects would be confined to the lungs (as is the case for fibrosis and cancer), whilst nanotube-fibre effects, (similar to the adverse effects of asbestos), would affect the pleura.

Whatever the CNT appearance, their dimensions (*i.e.* diameter and length) are extremely important as they can affect the site of CNT deposition in the airways and pulmonary parenchyma [14]. It is understood that the clearance of CNTs from beyond the ciliated airways is dominated by slow, macrophage phagocytosis. As a consequence, fibres that deposit at these sites will have the potential to contribute to the largest accumulation in the lungs. Length impacts are less important on aerodynamic diameter for thin fibres [15] except when length is sufficient to cause interception (a mechanism of particle deposition that is confined to fibres). This involves the centre of gravity of the fibre that follows the airstream at a bifurcation, whilst the tip of the fibre makes contact with the wall, resulting in its deposition.

The mechanisms of toxicity of CNTs once in contact with cells is also heavily dependent on the length of the material as demonstrated for other high aspect ratio materials like asbestos fibres. This issue can be analysed in terms of the ability of CNTs to induce inflammation, which is a key factor in tissue remodelling and cancerogenesis. Poland et al. [16] performed one of the first studies investigating the role of length in CNT inflammation. In this study, the peritoneal mesothelium (as a convenient model for the pleural mesothelium) was exposed to CNT in order to determine whether there was asbestos-like, length-dependent toxicity. The authors revealed that long fibrous CNTs show a similar, or even greater, propensity to produce inflammation and fibrosis in the peritoneal cavity, as compared to long asbestos fibres [16]. In contrast neither short asbestos fibres nor short tangled CNTs caused any significant inflammation. These findings were recently confirmed by the same group, in a study investigating the response to CNT directly instilled into the pleural cavity of mice [17]. The authors suggest that there are two important factors in the pro-inflammatory effects in the peritoneal and pleural cavities, shared by both asbestos fibres and long multi-walled CNTs (MWCNTs): i) Failure of long fibres to negotiate the diaphragmatic or pleural stomata with their subsequent retention at the vicinity of the diaphragm or the pleural cavity. This is in contrast to smaller particles which can easily navigate the peritoneal and pleural cavities through the stomata to accumulate in the parathymic nodes [18]. Furthermore by the use of well calibrated silver nanotubes, 5 μm has been demonstrated as being the maximal length critical for a passage through the stomata in the pleural mesothelium in mice [19]. These data can be extrapolated to humans, since there is a remarkable consistency in the reported size of stomata on the diaphragm or parietal pleura across mammalian species [19]. ii) Macrophages attempt to phagocytose these long fibres at the site of accumulation (peritoneal face of the diaphragm). This can result in frustrated phagocytosis (as these long materials cannot be fully engulfed by the gathering macrophages) which can further stimulate recruitment of inflammatory cells and mesothelial cell damage, leading to chronic inflammation and granuloma development [16].

Interestingly, direct exposure to long MWCNTs resulted in the significant release of IL-1ß, TNF-α, IL-6 and IL-8 from human macrophages (THP-1) but not from mesothelial cells (Met5a) [20]. This pro-inflammatory response, although modest, was length-dependent and was shown to be the consequence of frustrated phagocytosis. Moreover, incubation of mesothelial cells with a conditioned medium from macrophages exposed to CNT dramatically amplified their response to the same nanomaterials, showing a mechanism for the production of a pro-inflammatory environment in the pleural space following exposure to long CNTs.

It has to be noted that opposite results have been obtained in other cell types as demonstrated by Liu et al. which show that 3 to 14 μm MWCNTs exerted higher toxicity to a murine macrophage cell line than shorter materials (1.5 μm), while the inflammatory response was higher for the shorter CNTs. Although no characterization of the CNT surface was performed in this study, a surface-related effect (see also Section 2.2) seems unlikely as both CNTs were coated with the surfactant Pluronic F-127. Alternatively, rigidity differences could explain the discrepancies in this study [21]. Furthermore, Brown et al. demonstrated that straighter and shorter CNTs induced a higher TNF-α production from THP-1 macrophages compared with other CNT samples [22]. Interestingly, in the study by Liu et al., there was a divergence between the effect of length on cytotoxicity and on inflammation; longer CNTs were more cytotoxic but less inflammatory [21]. Such a distinct dissociation between cell viability and inflammatory and/or oxidative response has been described in the literature in many other cell types [11,23–25]. This phenomenon shows that at least in certain cell types, the determinants of cell viability and those for the induction of an inflammatory response are not necessarily the same.

The role of the diameter in the toxicity of CNTs has been far less well evaluated [26–28]. However in one such study, the authors analysed the toxicity of two highly purified MWCNT samples of similar hydrophobicity, surface reactivity, and length (<5 μm), but presenting markedly different diameters (9.4 *vs.* 70 nm). The toxicity of the CNTs was analysed *in vitro* in murine alveolar macrophages, in terms of cytotoxic activity (LDH release), CNT uptake, and ability to induce oxidative stress. Moreover toxicity was also analysed in rats in terms of LDH activity and total protein measurements as well as cellular responses in bronchoalveolar lavage after intratracheal instillation. The results of the study showed that both CNTs were internalized as aggregates in alveolar macrophages, however the thin MWCNTs appeared significantly more toxic than the thicker ones, both *in vitro* and *in vivo*, when compared on a mass basis [29]. In another similar study thin MWCNTs (diameter ~ 50 nm) with a high degree of crystallinity showed mesothelial cell membrane piercing (yet not internalized) and cytotoxicity *in vitro* and subsequent inflammogenicity and mesotheliomagenicity *in vivo*. In contrast, thick (diameter ~ 150 nm) or tangled (diameter ~ 2–20 nm) MWCNTs were less toxic, inflammogenic, and carcinogenic [13]. It is interesting to note that the authors suggest that despite sharing their needle like shape with asbestos — the thinner CNTs do enter the cells the same way as asbestos hence caution is needed when comparing CNT toxicity to that of asbestos. These results are in contrast to data from Wang et al., who reported a higher toxicity of short (1–5 μm) CNTs (diameter in the range between 40 and 100 nm), as compared to a sample with a diameter of 10–20 nm to alveolar macrophages [26]. In a similar study long thick (5–15 μm) CNTs induced greater DNA damage in A549 epithelial cells and inflammatory response in the lung of mice compared to their thinner counterparts [27]. However, it is important to note that in this study length changed simultaneously with diameter, hence the effects were not solely diameter related [27]. Collectively, these studies suggest that both the length and the diameter are important parameters to be considered in the toxicological assessment of CNT.

2.2. Surface properties and functionalisation

Smooth surfaces without any hanging bonds make pristine CNTs chemically inert and incompatible with nearly all solvents. This is a massive problem as solution based CNTs are a must for any CNT applications in nanomedicine. Therefore great efforts have been taken to modify these materials to increase solubility by purifying the nanotubes by

covalent functionalisation through a multi-step acid treatment. Another method of surface modification of CNTs is non-covalent functionalisation. Here the nanotubes interrelate with various molecules through weak interactions such as surface adsorption onto the side walls of CNTs. These functionalisations can be with numerous proteins, DNA, PEG, surfactants or other polymers [30–32].

It is widely believed that the oxidation of the surface of the CNTs is a very important determinant of their toxicity. It has been demonstrated that surface oxidation of MWCNTs by acidic treatment affects cell viability of human neuroblastoma cells in a dose dependent manner [33]. In a different but corroborating study it has been reported that hydrophobic pristine CNTs appeared less toxic for T cells than oxidized CNTs, which induced cell apoptosis [34]. Furthermore it has been shown that while CNTs, carbon black, and carbon nanofibers all inhibited proliferation and induced cell death in different human lung-tumor cell lines, these effects were more pronounced as the aspect ratio of carbon based nanomaterials (with the presence of chemically functional groups (carbonyl)) decreased [35]. Similarly in a recent study the use of *in vitro* and *in vivo* mice models have demonstrated that MWCNTs coated with an acid-based polymer displaying a larger amount of carbonyl and acidic groups as compared with non-coated MWCNTs were readily internalized by macrophages and induced a higher inflammatory and oxidative response than non-coated MWCNTs [11]. In a similar study acid functionalized (concentrated sulfuric and nitric acid) single walled CNTs (SWCNTs) were more potent than pristine-SWCNT in inducing mouse lung epithelial cell cycle arrest and lung inflammation [36]. Using an identical functionalization method, the same group demonstrated that such acid-functionalizations also enhanced cardiac toxicity of SWCNTs after pulmonary exposure [37]. This increased toxicity could stem from either the greater bioavailability of the well dispersed functionalized SWCNT preparations or from the high negative charge on functionalized SWCNTs or potentially both aspects combined.

Defects or imperfections in the carbon framework of CNTs have also been suggested in playing a role in the overall toxicological properties of the nanomaterials. Muller et al. have recently observed an acute lung response to MWCNTs (LDH activity, total protein, cellular count and TNF-α and IL1-β levels in BALF, 3 days after CNT administration) as well as additional genotoxic effects which the team ascribe to the presence of structural defects in the CNTs [38]. These defects partially reduce oxidized functionalities at the surface of the CNTs. It is important to note however that the long-term responses (presence of granulomes and fibrosis) were not different in MWCNTs with and without structural alterations. The authors postulate that this phenomenon reflects a progressive passivation of the CNTs being resident in the lungs over time (*i.e.* through the deposition of endogenous proteins). Another explanation might be that the fibrotic process induced by CNT is determined by other physicochemical parameters than those determining acute inflammation and therefore less or not influenced by the modified parameter (structural alterations).

Since genotoxicity and inflammation are generally regarded as critical events in carcinogenesis [39], Muller et al., hypothesized that MWCNTs with structural defects would induce the formation of tumors whereas pristine MWCNTs would not [40]. To examine this an intraperitoneal bioassay (previously used to investigate the carcinogenic potential and mechanisms of action of several chemicals and materials [41,42], including asbestos and other man-made fibres [43–45]) was utilised. However the researchers were unable to verify their hypothesis — although crocidolite and MWCNTs with defects elicited similar early inflammatory responses in the peritoneal cavity of the rat, only crocidolite showed a carcinogenic activity [40]. Thus, the genotoxic properties [46] and the capacity of MWCNTs to induce an inflammatory reaction in the lung [47] and in the peritoneal cavity of rodents [16] may not be necessarily associated with a carcinogenic potential in this particular peritoneal bioassay. The authors speculated that this could be related to the different ability of crocidolite and MWCNT with defects to produce free radicals, which are key components in genotoxicity and inflammation. Although crocidolite fibres have shown to generate free radicals and cause mutations through an oxidative stress dependent pathway [48] the MWCNTs with defects in this study did not generate free radicals or even display radical quenching abilities.

The importance of surface properties is further illustrated by studies modulating this characteristic of CNTs in order to decrease or suppress toxicity and make these materials biocompatible. In a recent study MWCNTs dispersed in serum bovine albumin were shown to be a potent inducer of pulmonary fibrosis (by initiating a sequence of cooperative cellular events that play a role in the pathogenesis of pulmonary fibrosis). However when the same materials were dispersed in pluronic F108 (PF108) the same detrimental effects were not observed. The authors demonstrated that the PF108 coating protected the lysosomal membrane from CNT damage. This study further demonstrates the critical role of surface properties on CNT toxicity [49].

As already discussed the CNT dimensions and surface properties are important determinants of their overall toxicity, however the interplay between the two characteristics is still unclear. In a recent study, we investigated this issue by comparing cytotoxicity and inflammatory properties of long and short MWCNTs (mean lengths of 9.5 and 4.8 μm respectively). These materials had a similar diameter and catalyst metal (iron) content but differed in the following characteristics: i) increased iron oxide nanoparticle residues (two-fold increase of the iron oxide/CNT ratio), ii) increased structural defects (increase of 1% of the sp3/sp2 ratio), together with iii) COOH and OH functionalization (4.4 at.% of increase for surface O amount) in short *vs.* long CNTs [50]. These experiments show that the *in vitro* exposure of a murine macrophage cell line to the short and long MWCNTs elicited similar reduction in macrophage viability, but only short CNTs induced marked dose-dependent pro-inflammatory and pro-oxidative responses. This inflammatory response was linked to oxidative stress since they were abrogated by incubation with an anti-oxidant. Furthermore, the short CNTs were more readily internalized by the macrophages, which is similar to previous data demonstrating that hydrophilic acidic polymer-coated MWCNTs were significantly more internalized than the hydrophobic polymer-coated ones [11]. However, a more in depth analysis of the data suggests that the enhanced inflammatory and oxidative responses to short CNTs were not only a consequence of a higher uptake of the materials by the cells, but could also be a result of the material's intrinsic characteristics (length, surface features). A pro-inflammatory role of the COOH functions described previously [51], could also be proposed in this context.

In another study long pristine CNTs used by Poland et al. [16] where chemically functionalised with tri(ethylene glycol) (TEG) and an alkyl group. The authors observe that the functionalisation affected the length of the CNTs. They hypothesize that these changes could be due to differences in the hydrophilicity and hydrophobicity of the appended chemical groups — with the alkyl group being more hydrophobic than the TEG group. These materials were then injected intraperitoneally into mice. The data demonstrated that the pristine, long MWCNTs and the alkyl functionalized MWCNTs caused a significant increase in PMN and protein levels. However the TEG functionalized MWCNTs did not lead to an elevation in inflammatory response. Based on their findings the authors suggest that chemical functionalization of pristine CNTs with octyl chains did not significantly reduce the length of the nanomaterials. On the other hand the pristine CNTs functionalized with TEG chains were greatly reduced in length most likely due to efficient de-bundling and disaggregation of individual nanotube fibres. The authors furthermore hypothesize that the alkyl modified MWCNTs interacted with the tissue primarily as long bundles of nanotubes, while the TEG-functionalized materials interacted as shorter, individualized fibres [52]. These studies stress that surface properties alongside the length of the nanomaterials should be considered as essential determinants in CNT-induced oxidative stress and inflammation.

2.3. CNT bio-durability

Another critical issue in the understanding of CNT toxicity is their durability in the organism. It had been observed that pristine CNTs are relatively stable materials and can be detected up to 24 months after the initial exposure, at the site of exposure or distributed throughout the body [40,53–55]. Clusters of CNTs often surrounded by macrophages (subsequent granuloma formation) were still detectable by optical microscopy up to two years after an initial pulmonary exposure — demonstrating the durability of these nanomaterials [40]. Despite this clear indication of CNT bio-persistence and wide spread evidence of their cellular internalization [56,57], little information is available on the bio-persistence or modifications of CNTs after cellular uptake. Experiments by Allen et al., were the first to demonstrate the degradation of SWCNTs through enzymatic catalysis in abiotic conditions [58]. The authors incubated CNTs with horseradish peroxidase and low concentrations of hydrogene peroxide (40 μM), for 12 weeks, at 4 °C. TEM images illustrated that CNT length decreased by 45% at week 8, with some globular materials visible, while at 12 weeks, mostly globular material were present. UV–vis–NIR spectroscopy measurements further demonstrated a diminution of CNT diameter over time. In contrast to these findings, the same group observed that pristine SWCNTs failed to degrade when incubated in the same conditions (hydrogen peroxide, 4 °C), which leads the authors to hypothesize that defects or functionalization sites are important facilitators of enzymatic action [59]. These findings were further corroborated by a study showing the faster degradation of carboxylated MWCNTs when they contained more defects [60]. It is proposed that the presence of carboxylic functions as well as defects on the graphitic surface of the CNTs is likely to offer sites for interactions with the oxidative agents which are responsible for the degradation of the CNTs, therefore acting as facilitators of enzymatic action. Similar indications of CNT degradation were reported by Kagan et al., after *ex vivo* incubation of SWCNTs with myeloperoxidase (MPO) [61]. The authors observed a decrease in the quantity of graphitic material as early as 24 h after the initial incubation. With the exception of these reports of enzymatic degradation *ex vivo*, the literature is very sparse regarding the eventual fate of CNT *in vitro* or *in vivo*. However two recent studies buck this trend by reporting the partial degradation of MWCNTs functionalized with amine groups, as early as 2 days post cortical administration [62] and the degradation of SWCNT in the lung of mice after pharyngeal administration [63].

These studies pin point the importance of CNT functionalization for the effective degradation of the carbon lattice. However, the exact mechanisms underlying these effects are currently unclear. A recent study attempting to answer this question demonstrates that the addition of anti-oxidants (namely L-ascorbic acid and glutathione) significantly decrease the bio-degradation of SWCNTs by MPO. The authors therefore suggest that CNT biodegradation is not only enzyme-dependent but also needs strong oxidants to take place [64], the antioxidants being therefore secondarily important as their amount will determine the level of degradation possible. Finally it seems crucial to note the importance of the fate and biological impact of the by products of CNT bio-degradation as they might very well contribute to the overall adverse properties accredited to CNT.

2.4. The protein corona

When nanoparticles are in a physiological environment, they can selectively adsorb biomolecules, leading to the formation of a bio-corona. As with any other nanomaterial, such dynamic exchanges between CNT surfaces and bio-fluids (*i.e.* proteins or lipids) have been described [65–69]. This corona may lead to a modified biological "identity" of CNTs (new biological effects as well as potential modifications of the material by the biomolecules present in the corona — in terms of structure and/or functions). The formation of the bio corona is now considered as crucial in the determination of CNT toxicity and will be described below.

Several characteristics of the corona are extremely important and are routinely measured such as the thickness, density, identity, quantity, conformation and affinity [65]. These characteristics allow for a comprehensive overview of the interaction of a nanomaterial with a physiological environment. These measurements are achievable *via* numerous techniques including dynamic light scattering (DLS), transmission electron microscopy (TEM), polyacrylamide gel electrophoresis, circular dichroism, computational simulation, proteomics, or surface plasmon resonance usually performed *in situ* or *ex situ*. Generally, *in situ* techniques measure the corona with the nanomaterial still dispersed in the physiological environment. These techniques are considered the most relevant in the field of nanotoxicology. However these *in situ* studies are rare and provide the least amount of information. On the other hand, data from *ex situ* techniques are numerous, but remain less relevant because of the need to isolate the nanomaterials from the physiological environment.

Until recently, studies on the interactions between bio-molecules and nanomaterials (including CNTs) have been limited to the use of single purified proteins (chymotrypsin, fibrinogen, albumin, *etc.*) or of the so-called "representative media" [67,68,71–73]. However as CNT applications in nanomedicine are ever increasing, the blood has gained extra relevance as it is the first physiological environment that CNTs will come across after intravenous administration. It is not then surprising to find that a large proportion of studies have focused on isolated blood proteins, isolated plasma or serum as models for blood protein adsorption (fibrinogen, gamma globulin-transferrin, serum albumin, complement proteins, *etc.*) [66–69]. From these studies, it is clear that CNTs can adsorb proteins to their surface, although this binding depends on protein-specific as well as CNT-specific characteristics. In a recent study the crystallization of proteins of the innate immune system (namely C1 complex, component of the complement system) on different types of CNTs was investigated [70]. The authors focused on the recognition protein C1q and the catalytic subunit C1s-C1r-C1r-C1s. TEM images clearly demonstrate that both C1q and C1s-C1r-C1r-C1s bind to the surface of MWCNT, while DWCNT bind to C1s-C1r-C1r-C1s and SWCNTs didn't bind to any component of C1 complex. Interestingly, the stacking of proteins on the surface of the CNTs was continuous, resulting in the depletion of the proteins in solution. Despite the binding C1 protein, the authors were unable to show any activation of the complement system [70]. This data is contradictory to another study in which the activation of the complement system by SWCNT and DWCNT was described [67]. In this study both CNTs were able to activate the human serum complement system *via* the classical pathway, while the DWCNTs only activated the alternative complement pathway. The use of whole serum as the source of complement in the latter study could explain the discrepancies between the two studies. It is possible that other serum proteins could form a stable barrier on the CNT surface triggering indirect C1 binding and further activation of the complement. The authors show that both SWCNTs and DWCNTs were able to bind to various quantities of human serum and plasma proteins [67]. As stated above, CNT-specific characteristics are important in their interaction with their biological surroundings. This is best exemplified in CNT interactions with proteins. With this in mind, Shannahan et al., assessed the protein corona associated to SWCNTs and MWCNTs presenting various functionalized groups (polyvinylpyrrolidone (PVP) or COOH), after a one hour incubation period in culture medium supplemented with 10% foetal bovine serum [69]. The authors identified and quantified 366 different protein components in the various CNT coronas, (total of 2507 present in the culture medium). The highest number of proteins were bound to COOH-SWCNT > COOH-MWCNT > MWCNT-PVP > raw SWCNT and raw MWCNT. The large number of proteins bound to COOH-CNT is likely due to the abundance of protein amines in the medium, which could associate with the carboxyls through electrostatic interactions. Interestingly, only 14 proteins bound to all CNTs. The authors also noted a small number of proteins specific to each CNT (3 for Raw SWCNT, 6 for MWCNT-PVP, 7 for Raw MWCNT, 16 for COOH-MWCNT, and 34 for COOH-SWCNT). Furthermore with only a few exceptions, all these

CNT-specific proteins were in low abundance (compared to proteins bound to all CNTs), and were of intracellular origin, with few or no extracellular domains [69]. In a similar experiment utilising the proteomic approach with HeLa cell homogenates, as archetypal of human proteomes, it was demonstrated that cytoskeletal proteins were dramatically over-represented on the MWCNT bio-corona [71]. By contrast, nuclear proteins (chromatin, ribosomal and sliceosomal proteins) were heavily represented in the fraction of proteins that did not bind effectively to CNTs. The authors suggest that the preferential binding of CNTs to cytoskeletal proteins might represent a biochemical basis for CNT toxicity. Interestingly, CNT diameter was also an important factor of efficiency for proteins: CNTs with a diameter of 20–40 nm or above bound a greater amount of proteins than materials with a diameter less than 10 nm.

Surprisingly in spite of being the major route of entry for nanomaterials [72], only a few corona studies have been conducted with proteins relevant to pulmonary exposure [66,67,72]. To the best of our knowledge, only one study has been published so far analyzing CNT coronas in the context of a pulmonary exposure [73]. In this study, the authors exposed mice to SWCNTs by pharyngeal aspiration, and recovered the bronchoalveolar lavage (BAL) fluid of the animals 24 h post exposure. The CNTs were then extracted from the BAL. The team then utilised liquid chromatography mass spectrometry to investigate the adsorption of phospholipids by SWCNTs. The data demonstrated that there was a selective adsorption of phosphatidylglycerols and phosphatidylcholines onto the surface of the CNTs. Importantly, the presence of surfactant proteins, and more specifically, that of surfactant protein (SP)-A, -B, and -D were identified. These proteins are secreted by airway epithelial cells and play an important role in the first line of defence against infection within the lungs. In a corroborating study *ex vivo* incubation of human BAL with DWCNTs resulted in a preferential binding of DWCNTs to SP-A, *via* a Ca^{2+} dependent mechanism [74].

An important issue of protein corona formation is the resulting modification, conformation and/or activity of the bound proteins. In order to address this question a recent study by Ge et al., demonstrated that the rate of adsorption varies greatly amongst blood proteins (the equilibrium was rapidly reached for transferrin and serum albumin, but took much longer for fibrinogen and gamma globulin). Furthermore it was noted that there were competitive adsorptions amongst all proteins investigated [66]. Fibrinogen and gamma globulin secondary structures were modified because of their interaction with SWCNTs, but not that of transferrin or serum albumin. This alteration of secondary structure can have important functional consequences, as demonstrated in two studies with chymotrypsin and soybean peroxidase [75,76]. The soybean peroxidase retained only 30% of its native activity after binding with SWCNTs, whereas chymotrypsin preserved only 1% of its native activity. Infra-red spectroscopy measurements revealed that these alterations of protein activity were linked to severe modifications of secondary protein structures after their binding to SWCNTs [76]. This was confirmed by atomic force microscopy (AFM) with the unfolding of chymotrypsin on the surface of SWCNTs. These alterations of protein catalytic activity could have considerable consequences *in vivo*, depending on the targeted protein. Despite uncertainties in the underlying mechanisms of protein CNT interactions, there is no doubt that this will result in the modification of toxicity *in vitro* [66], as well as CNT uptake by macrophages [73], and targeting to professional phagocytes *in vitro* and *in vivo* [77].

2.5. Concerns with CNT toxicology testing

To adequately address the need of the industrials, engineered nanomaterials including CNT can have a very diverse range of physicochemical characteristics such as size, shape, surface charge, surface reactivity, solubility or impurities. As already discussed (these) different material properties can hugely influence the final biological effect of a nanomaterial, requiring that a varied range of dimensions should be evaluated for a comprehensive toxicity profile. Likewise, standardisation of protocols such as nanomaterial preparation and exposure conditions are essential to be able to compare studies carried out in different laboratories — ensuring that any differences in toxicological responses are due to the materials only and not the methodology. As hinted to within the review the toxicological profiles observed in the different studies cannot be solely attributed to the materials — other factors including the cells utilised, exposure doses/times and the end point investigated are all critical and need to be thoroughly scrutinized in order for the correct conclusions to be reached. A step towards addressing these issues, and one of the most important reasons for conducting nanotoxicology studies is to provide a knowledge base towards assessing the risks and any health implications associated with realistic nanomaterial exposures.

It might be obvious but experimental design is also crucial for correct interpretation of the findings. For instance the role of the CNT dispersant is vital in any corona study as the nanotube will no doubt be coated before it encounters any proteins in the biological environment. Another example of an apposite experimental design is the appropriate selection of the assay as CNT interference has been widely reported with many assay components and detection tools.

3. CNT and biomedical applications

As already hinted, CNTs have great potential in the field of nanomedicine. It had been shown that these nanomaterials can act as drug carriers in targeted delivery, as well as promising candidates for imaging, diagnosis and treatment tools. The authors of a bio-distribution study highlighting the benefits of CNT utilisation observed functionalized SWCNTs in the bone, kidney and stomach of mice (with no adverse effects) and finally excreted in the urine. The team propose that these nanomaterials can be modified for imaging applications in the future [78]. In a recent set of trials PEG modified SWCNTs were conjugated to a fluorescent photosensitizer. The authors manage to image different cancer cell lines *in vitro*. Furthermore the conjugated CNTs were capable of killing the cancer cells. Hence it is proposed that these materials could be great candidates for cancer imaging and therapy [79]. Collagen and polymer functional carbon nanotube-based matrices have successfully been used for tissue generation [80]. Another very interesting study has reported functionalised carbon nanotubes involved in the proliferation of osteoblastic cells [81]. Finally, MWCNTs functionalized with polyurethane have been implicated in cardiovascular applications — these materials contain oxygen-containing functional groups on the surface, and showed increased adhesion to the platelets. Furthermore the authors suspect the CNT involved in the amendment of the anti-coagulation activity, making them better biomaterials for implants for blood-related environments [82].

With an ever-increasing demand for CNTs there is a necessity for an in-depth toxicity analysis of these materials. As demonstrated CNTs with modifiable surfaces, a large surface area and desirable length, as well as unique physical properties can have many biomedical applications.

4. Conclusion

Altogether, this review highlights the role of CNT dimensions, surface properties, bio-durability and corona formation as determinants of CNT toxicity. An important conception is that any or a combination of these properties can be modified simultaneously for a given CNT, with a change in its overall toxicity. A deeper understanding of these events and their underlying mechanism could provide a molecular explanation of the biological and physiological responses to CNT administration and therefore help in the design of safer/less cytotoxic materials which could be extremely beneficial in the expansion of biomedical CNT applications.

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