Effect of chemical composition and state of the surface on the toxic response to high aspect ratio nanomaterials (HARNS)

"EFFECT OF CHEMICAL COMPOSITION AND STATE OF THE SURFACE ON THE TOXIC RESPONSE TO HIGH ASPECT RATIO NANOMATERIALS (HARNS)"

Summary

Nanomaterials often act as a double sword. On the one hand they offer new exceptional properties, on the other one show signs of toxicity. High Aspect Ratio Nanomaterials (HARNs) cause more concern than isometric nanoparticles because of their physical similarity with asbestos. Many compounds may be prepared in fibrous shape with nano-sized diameter differing one from the other in various ways. This review reports a comparative picture of the chemical features and related toxic responses to a variety of HARNs, namely carbon nanotubes, asbestos, carbon nanofibres, oxide and metal wires and rods. In spite of similarities in form, durability and several biological responses elicited in vitro and in vivo, carbon nanotubes, - opposite to asbestos - quench radicals, are hydrophobic and may be fully purified from metal impurities. Most of the other HARNs produced so far are metal or metal oxide compounds, less biopersistent than carbon nanotubes.

Key words: HARNs, Carbon Nanotubes, Carbon Wires, Asbestos, Gold Nanorods, Nanowires, Nano-Chrysotile, Free Radicals, Hydrophilicity/Hydrophobicity, Free Radical Generation, Free Radical Quenching, Nanotoxicology.

1 INTRODUCTION

1.1 Nanosized materials as a double sword

Nanotoxicology - the new discipline which parallels the enthusiastic development of nanotechnology - stems from the experience of different groups of scientists. When - about a decade ago - it became clear that appropriate methods in nanotechnology would have allowed the synthesis of nano particles in controlled shape and size, biomedical scientists were quite excited by the idea that such materials could become versatile devices for diagnostic, drug delivery or “intelligent” cancer cells killers. Beside medical applications several industries looked at the new materials such as carbon nanotubes as an excellent way to improve various productions by means of an extremely strong, nearly inert and light material.

In the mean time particle toxicologists and pathologists were alarmed by the possible exposure of workers and users to particles of unknown toxicity (which could be more pronounced on smaller particles than those traditionally studied), let alone the idea of a biopersistent particle being injected on purpose in the body [1-5]. On several occasions the media stressed the (potential) toxicity of nanoparticles but most unfortunately a general idea that any nanoparticle is hazardous just because of its size was retained, in spite of what reported in several books, reviews and experimental studies.

Information on the hazard associated to each kind of nanoparticles is much required in order to decide whether to develop its production, stop it, or at least provide sufficient precautionary procedures during production, use and disposal.

When it comes to nanomedicine only a correct balance between risks and benefits will allow to take sound decisions. For instance at the American Chemical Society Fall Meeting held in Boston in 2010 it was proposed to employ radioactive salts sealed inside carbon nanotubes for targeted radiotherapy. This is obtained by chemical modification of the surface of the tubes with sugar or other targeting molecules. The sugar could play a variety of roles, making the nanotubes soluble and stopping them from clumping together as well as providing a site for proteins to recognize. Sealed up carbon nanotubes with radioactive

66 salts inside would provide an excellent tool in targeted radiotherapy [6]. Under such circumstances any
67 potential toxicity of the nanotube itself is not relevant, when compared to the benefit to reduce the tissue
68 injury arising from traditional radiotherapy.

69 Clearly nano sized materials may act as a double sword as, on the one hand, they may fulfil several
70 tasks never thought before, on the other one - because of several factors including their size - they may
71 turn out to be a very hazardous material.

72 *1.2 What makes a particle or a fibre toxic?*

73 Particle and fibre toxicology is nowadays a relatively large field of toxicology involving several occupa-
74 tional and environmental issues. It is somehow an ancient discipline which has been deeply investigated in
75 the last decades. Silicosis, the disease caused by crystalline silica dusts, is one of the most ancient occupa-
76 tional pathologies, reported by Hippocrates in 400 BC and by Plinio in 70 AD. Following a large number of
77 studies the mechanism of action of silica at the molecular level has been partly clarified, even if some of
78 the steps yielding the disease are still obscure or controversial because of the complexity of the physico-
79 chemical features involved when the toxicant is in the solid state [7]. Beside silicosis several other particle
80 or fibre associated pathologies are well known – e.g. asbestosis, mesothelioma, lung cancer, hard metal
81 diseases - while there is not yet any medical evidence of “nanopathologies”, i.e. a pathology caused by a
82 material because it is in nano-size.

83 Many signs of toxicity appear from in vitro and in vivo studies on some nanomaterials which suggest
84 caution in their use, before they might damage human life and the environment. Traditional particle toxi-
85 cology has clearly evidenced that the pathogenic response to an inhaled fibre does not concern a single
86 step but is the sum of several subsequent events, each of which is determined by different physico-
87 chemical features of the particle considered. Three major factors act together, namely the “form” of the
88 particle, its crystal and surface composition and its biopersistence [8]. Form stands for fibrous vs isomet-
89 ric, nano vs. micron sized, smooth vs. indented, crystalline vs. amorphous particle. Biopersistence deter-
90 mines the time the particle remains unaffected into a given biological compartment, thus together with

the administered dose or the exposure determines the correct “dose”, meaning the extent of interaction of the body with that given material. Finally surface composition determines the nature of the contact of the particle with living matter, i.e. fluids cells and tissues. Surface reactivity, the potential to adsorb biomolecules, to disrupt cell membranes, to adhere to a given substrate are features derived from the chemical composition of a surface, which ultimately determine safety, biocompatibility or toxicity of a given kind of (nano) material.

1.3 Why a specific study on HARNs

HARNs is the term employed to indicate high aspect ratio - or fibre shaped – nanoparticles, a group of nanomaterials which deserve a specific approach [9]. They share with asbestos an elongated fibrous shape, one of the factors (associated to biopersistence and surface composition) which contributes to the high carcinogenic potential of asbestos.

The fibrous form has a specific role in toxicity as it is the cause of failed phagocytosis and of the translocation in various biological compartments, typically the parietal pleura. Macrophage clearance is one of the major route whereby the body defences get rid of unwanted foreign materials. Inhaled particles which do not damage the phagolysosome membrane may be easily engulfed by alveolar macrophages and transported to the lymphatics. When the material is in fibrous form the macrophage attempts often end up with frustrated phagocytosis and macrophage death, following the scheme reported in Figure 1, which may apply also to inflammatory reactions occurring in body compartments other than the lung. Long fibres cannot by phagocyted, while the short one are more easily uptaken and cleared, which explains the higher toxic potency of long vs. short fibres [9]. However beside fibre dimensions also surface reactivity determines the fate of the fibre and its ultimate toxicity [10] (Figure 1). In the case of isometric silica particles, for instance, surface reactivity determines reactions with the phagosome membrane and cell death [11].

There are nowadays several nanomaterials exhibiting one or two dimensions in the nanosize range - a typical example are respectively graphene sheets and carbon nanotubes - which make them different from “regular” isometric nanoparticles, having all three dimensions at the nanosize level.

Asbestos are the typical example of a material with exceptional properties, allowing an extremely large variety of employments (Figure 2), which turned out to be one of the greatest occupational tragedies of the XX century, still going on in the present days because of both, the latency of the asbestos associated diseases and the still increasing trade of this material worldwide (Russia, Canada, India, Brazil, China and other countries). In the case of HARNs no one would repeat what happened with asbestos, so that particular attention should be given to those HARNs who also share with them a high biopersistence and their other properties, including their surface reactivity.

The role of form and biopersistence in determining the pulmonary hazard of HARNs has been extensively reviewed by Donaldson et al. [9] and will here be just mentioned, while we will concentrate on the chemical aspects which may modulate the potential hazard of these materials caused by their shape.

It has to be pointed out that whilst in some cases materials non toxic at the micron size level may become toxic when synthesized at the nanolevel, there are few indications in the literature on what happens when well known solid toxicants are reduced from the micron size level to the nano-size one. In the case of crystalline silica only two studies have been performed so far, which indicate a lower toxicity of nanoquartz [12]. Titania nanorods turned out to be not more significantly toxic than isometric micron-sized particles [13].

In the case of asbestos there are no published studies; from an ongoing study in our laboratory we may anticipate that tests performed on natural chrysotile nanofibres indicate a reduction in toxic potency when passing from the micron to the nano size [unpublished results].

1.4 Not all fibres nor all HARNs are toxic

Some fibrous materials such as asbestos, the zeolite erionite, artificial ceramic fibres and others are highly toxic when micrometric fibres remain airborne and reach the lung alveoli and the pleura. Not all fibres however are equally toxic, some, e.g. wollastonite ($CaSiO_3$), are even inert [14]. A comparison of the toxicity of chrysotile asbestos with several other fibrous materials committed by WHO in 2005 [15] reported great differences in toxicity among the different materials. The available data on HARNs are still scarce to allow a similar study. In search of appropriate positive and negative controls for HARNs some of us have recently reported that imogolite, a hydrated alumino-silicate with the formula $(OH)_3Al_2O_3SiOH$ [16] – opposite to carbon nanotubes - appears inactive in cell viability, NO production, and epithelial barrier permeability [17, 18].

1.5 Materials considered

We will report here what is known on most of the HARNs whose toxicity has been largely investigated: carbon based materials, such as carbon nanotubes (CNTs) and carbon nanofibers (CNFs) and metal / oxide based nanowires (NWs) and nanorods (NRs). CNTs, which are by far the most studied because of their potential applications on the one hand and of their close similarity to asbestos on the other one will be considered in detail. Because of a recent fear that CNTs might become the “asbestos” of the present century, we will compare physico-chemical features relatable to toxicity in CNTs and asbestos, highlighting not only similarities but also chemical differences.

Among the large variety of nanowires and rods prepared so far we will concentrate on the most investigated and applied ones - typically gold - and on a variety of metal/metal oxide compounds

The basic structures of the materials described are schematized in Figure 3 and their dimensional aspects depicted in Figure 4.

2 CARBON NANOTUBES

2.1 Carbon nanotubes: forms and chemical requirements for their applications.

CNTs are a form of elemental carbon. Like in fullerenes and graphite, carbon is organized in layers of hexagonal rings having conjugated double bonds (Figure 5A). Because of their high length/diameter ratio CNTs are comprised into the general term HARN. The discovery of CNTs is generally attributed to Iijima [19].

CNTs are made of one (single-walled SWCNT) or more (multi-walled MWCNT) graphene sheets rolled-up to form tubes. Depending upon the synthesis procedure CNTs may exhibit external diameters ranging between 1 to 200 nm. Their length may vary from nanometres to micrometers, depending upon the method of synthesis and may be modified by mechanical or chemical shortening. The graphene layers contain various amount and degree of defects [20] which may arise directly from the synthesis or may be introduced or eliminated *ad hoc.* After synthesis CNTs generally contain amorphous carbon, metals deriving from the catalyst used in their synthesis and inert materials (alumina or silica) used as support for the catalysts up to 20-30% w/w of the product. Only in extreme conditions a purification yields a 99% carbon content. Often metal ions remain on or within the carbon framework acting as a catalytic centre for free radical release or other reactions.

CNTs exhibit high thermal and mechanical resistance, electrical conductivity or semiconductivity. Such properties make CNTs interesting in a variety of industrial applications e.g. as component in electronics, energy-storage devices, solar cells, sensors, or in mechanical applications as filler in polymeric composites [21]. Their physico-chemical properties may be modulated by varying the method of synthesis and by applying post-synthesis treatments. Therefore a large variety of CNTs forms may be produced which exhibit different chemical reactivity one from the other.

The number of studies devoted to the production of new tailored forms of CNTs for the different industrial applications has exponentially increased, as shown in Figure 5B. Many forms of CNTs have shown

183 distinctive signs of toxicity, but the number of studies on their health effects compared in the histogram
184 keeps well below that of their production.

185 The possibility to introduce functionalities at the surface of CNTs through radical reactions has at-
186 tracted the interest of several scientists. Surface functionalization of CNTs in fact opens a wide range of
187 applications [22, 23], e.g. by making CNTs compatible with aqueous media, by increasing their dispersion
188 in polymeric matrixes, or by binding specific molecules to their surface.

189 MWCNTs find application mainly as components in high strength composites while SWCNTs are cur-
190 rently studied for their conducting/semiconducting properties in electronic devices, and as biosensors.

191 CNTs also attract a great interest for several applications in medicine [24-26]. CNTs rapidly cross the
192 cell membranes like fullerenes, which makes them apt to act as nanovectors [27, 28]. In such application
193 chemical modifications are required to impart hydrophilicity and bind drugs or biomolecules [29]. CNTs in
194 bulk materials have been proposed as alternative artificial hard tissues [30], as tissue scaffold materials
195 for bone formation [31], as microcatheters [32] or as substrates for neuronal growth in nervous system
196 disorders [33, 34].

197 *2.2 Toxicity / biocompatibility of carbon nanotubes*

198 CNTs are currently object of a large debate on their toxicity/biocompatibility. Several extensive re-
199 views on this topic have been published. We refer to them for a detailed discussion [35-40].

200 Overall a substantial consensus has not been reached yet, mainly because of controversial data ob-
201 tained in the different studies. Such variability in the toxic effect elicited by CNTs has mostly to be as-
202 cribed to the differences in shape and chemical composition/modifications of CNTs employed in the dif-
203 ferent studies [17, 41-47].

204 It is generally accepted that physical and chemical properties modulate the cell responses toward
205 CNTs. Differences in toxic responses have been observed to be related to length [28, 48], presence of
206 metals [42], oxidation of the surface [49, 50], presence or absence of defects [50, 51], tube diameter di-

mensions [52, 53]. However very few studies have been designed to relate physico-chemical determinants to the toxic response to CNTs by testing a large set of samples differing for one single property at the time. This kind of approach allowed the assignment to defects - among other properties – of lung toxicity and genotoxicity [50, 51]. For the time being therefore toxicity needs to be assessed for each kind of nanotube employed and the clues to synthesize safe CNT materials have not yet been disclosed.

The major alarm raised on CNTs toxicity in the past decade concerns their physical similarity with asbestos, namely form and durability. Several studies have been devoted to assess whether CNTs are able to induce neoplastic transformations in mesothelial cells similarly to asbestos fibers. Conflicting data were obtained [54], as both ability to induce mesothelioma [45, 46] and absence of carcinogenic response [55] were reported. Such variable outcomes may be ascribed to different physical chemical properties among the examined specimens. The next two paragraphs are therefore devoted to describe synthetic asbestos and to a systematic comparison between asbestos and CNTs.

2.3 Synthetic chrysotile asbestos nanotubes

Stoichiometric chrysotile tubular nanocrystals have been recently, synthesized [56] as possible starting materials for applications in nanotechnology and as a standard reference sample for the investigation of the molecular interactions between chrysotile asbestos and biological systems. Each single nanocrystal of pure chrysotile has a tubular shape of about 49 nm in outer maximum diameter, a hollow core of about 7 nm and a length of the order of some microns. The presence of iron does not change the tubular shape of nanocrystals which appear just slightly longer than the iron free ones. Iron ions replace both Mg and Si into the octahedral and tetrahedral sheets [57].

Free radical generation and the effect of pure nano-chrysotile on human lung epithelial A549 cells have been compared to that elicited by a well known toxic natural chrysotile (UICC A, from Rhodesia). After a 24-h incubation, the natural, but not the synthetic form exerted a cytotoxic effect, detected as leakage of lactate dehydrogenase. Generation of carbon centred radicals in cell free tests and lipoperoxidation on lung epithelial cells took place in the presence of the natural, but not of the synthetic chrysotile. Anti-

oxidant systems were also affected differently. The pentose phosphate pathway and its regulatory enzyme glucose 6-phosphate dehydrogenase were markedly inhibited only by the natural specimen, which also caused a depletion of intracellular reduced glutathione in A549 cells [58]. Similarly, synthetic chrysotile nanofibers, devoid of iron, did not exert genotoxic and cytotoxic effects nor elicited oxidative stress in a murine alveolar macrophage cell line [59]. Briefly all the properties relatable to toxicity were absent in the synthetic form.

To gain direct experimental evidence of the chemical role of iron in asbestos reactivity a set of nanochrysotiles have also been synthesized with 0.6% and 0.9% (w/w) iron by means of the same procedure [60]. Even the lowest iron-loading induced DNA strand breaks, lipoperoxidation, inhibition of redox metabolism and alterations of cell integrity, i.e. the same toxic characteristics of natural chrysotile [59]. These results suggest that metal ions play a crucial role in the oxidative stress and genotoxic effects caused by chrysotile asbestos.

Accurate analysis over a set of iron loaded samples revealed that generation of hydroxyl radical and carbon centred radicals are catalyzed by iron ions in specific crystallographic sites [61]. Even the smallest iron contamination may impart radical reactivity. The most reactive surface sites in carbon centred radical generation are isolated iron ions in octahedral coordination in both axial and rhombic distortion. Conversely, the mechanism of $OH^{\bullet}$ generation seems to be independent of the iron lattice distortion. Aggregated iron ions and/or extra-framework clustering are less reactive in both mechanisms. Moreover carbon centred radical generation requires iron in low oxidation state or iron in high oxidation state but easily reducible to iron(II) by endogenous reducing agents, e.g. ascorbic acid.

The studies above described constitute the first direct experimental confirm of the role played by isolated iron ions within the asbestos framework in free radical release and cell damage.

2.4 Carbon Nanotubes vs. Asbestos

Carbon nanotubes share with asbestos some relevant properties relatable to asbestos pathogenicity such as their “fibrous” habit and a high biopersistence (reviewed by Donaldson et al. [9]). The possibility

that carbon nanotubes would show asbestos-like behaviour in the human body was raised several years ago [62]. More recently, some experiments in vivo have been performed to evidence any asbestos-like pathogenic response, such as a persistent inflammation or the induction of mesothelioma, which is a disease only caused by asbestos and few other mineral fibres [45-47].

Poland and co-workers [47] showed that MWCNTs injected directly into the abdominal cavity of mice induce inflammation, formation of granulomas and early fibrosis or scarring in the mesothelial lining. Shorter nanotubes had much less of an effect, as did carbon black nanoparticles used as a non-fibrous reference material. Inflammation and granulomatous lesions elicited by long CNTs were similar to those induced by long fibres of amosite asbestos. Tagaki and co-workers [45] even showed that MWNTs injected, in a large volume, into the abdominal cavity of mice induce malignant mesotheliomas in p53+/– heterozygous mice — a common genetically engineered mouse model with an increased propensity to toxicity induced cancer. Mesothelioma induction was finally observed also after a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats [46]. At the opposite Muller et al. [55] reported that crocidolite induced a clear carcinogenic response while MWCNT with or without structural defects did not induce any mesothelioma in male Wistar rats. It has to be pointed out that the above experiments were performed with CNT preparations differing in form, length and level of contaminants, which might account for the different outcomes.

Several reviews have recently been devoted to the comparison between CNT and asbestos [36, 39, 63-65]. They mostly consider the physical similarities such as shape and chemical stability in a physiological environment and report several outcomes in various cell systems and in *in vivo* rodent models. Few reviews [63, 64] also mention some chemical aspects - surface properties, presence of metal transition ions, adsorpitive potential- in relation with the toxicological properties reported (cell derived ROS , cytotoxicity, DNA damage, physical interference with mitosis, stimulation of target cell proliferation and induction chronic inflammation). Other focus on activation of macrophages and injury on epithelial / mesothelial cells [65], and on the potential to activate signaling pathways modulating transcription factor activity, inducing apoptosis and DNA damage [39] without considering at all the differences in chemical properties.

Most comparisons of CNTs are made with crocidolite asbestos [39]. Interestingly Jaurand et al report that chrysotile is the asbestos form more close to CNTs. In fact most CNTs are curled and flexible in similar way to chrysotile, but different from amphiboles.

In conclusion while the CNTs - asbestos analogy was mainly raised because of some points of physical similarity, we will here give a detailed description of the chemical differences and similarities between the two entities. We recall here that in the case of isometric nanoparticles surface chemistry and particularly the formation of Reactive Oxygen Species (ROS) plays a crucial role in toxicity, thus by the same token, chemical aspects in HARNs behavior need to be considered in detail [4,8,13,17].

2.4.1 Chemical composition of CNTs and asbestos: differences and similarities

Asbestos are naturally occurring hydrated silicates. They belong to two mineralogical groups: serpentines (chrysotile) and amphiboles (actinolite, amosite, anthophyllite, crocidolite, and tremolite). The amphibole minerals are composed of octahedrally coordinated cations sandwiched between two double silicate layers. The oxygen atoms of the silicate chains coordinate both Si and other cations (Mg^{2+}, Fe^{2+}, Fe^{3+}). Chrysotile is composed of an octahedral magnesium hydroxide layer, the so-called brucitic layer, intercalated between silicate tetrahedral layers which form tightly rolled sheets [66].

CNTs are allotropes of carbon exhibiting a surface made up by a rolled hexagonal lattice of carbon atoms linked by σ and π covalent bonds.

Both CNTs and asbestos have a thin and elongated shape, compatible with a fibrous morphology according to the WHO definition. The CNTs diameter ranges from 0.4 to 3 nm for SWCNT and from 2 to 200 nm for MWCNT [2, 35, 67]. The diameter of single chrysotile fiber falls below 100 nm whereas in crocidolite and amosite (amphiboles) is about 200 nm [68].

One of the most prominent chemical difference between the two materials lies in their opposite degree of hydrophilicity /hydrophobicity. Asbestos are all very hydrophilic materials because of both the metal ions exposed (positive surface charges) and the silicon-oxygen bonds, giving rise to silanols (SiOH)

when exposed at the surface in presence of moisture. Silanols coordinate water molecule and establish strong H-bonding with several other molecules [69]. The presence of metal ions exposed to the surface creates an uneven distribution of charges, resulting in acid Lewis sites of variable strengths, which strongly attract polar molecules and constitute the active site where catalytic generation of free radicals might take place.

At the opposite CNTs, unless oxidized or functionalized, are highly hydrophobic therefore incompatible with water [51]. Few hydrophilic surface sites may originate from metal traces from the catalyst exposed at the surface or upon strong oxidation. As prepared CNTs are tightly bound in aggregates or bundles because of Van der Waals attractive forces among graphene sheets [70, 71]. In aqueous media they form large agglomerates [72]. Dispersion in water may be improved introducing a sufficient number of charged functionalities at the surface to generate repulsion among particles. In the past few years, *in vivo* bio-distribution studies have been carried out by a number of groups using different tracking methodologies [73]. As observed for molecular substances the hydrophilic character and the presence of charged functionalities modified the pharmacokinetic profile of nanoparticles. Additionally, for nanoparticles, diameter, length/diameter ratio and tendency to form aggregates are expected to play a role [74]. We recall here that protein adsorption, cell uptake and translocation depend upon the hydrophilic/hydrophobic degree [75].

2.4.2 Metal ions and surface generation vs. quenching of free radicals and ROS on asbestos and CNTs

Metals are considered important elements to account for fibre toxicity [76, 77]. All asbestos fibres contain iron, either structural (crocidolite, amosite), as a consistent part of the crystal framework, or as contaminant (chrysotile, tremolite) substituting e.g. Mg^{+2} ions, which share with Fe^{+2} size and charge. Iron in asbestos fibres can be present in both ferrous (Fe^{+2}) and ferric (Fe^{+3}) form within the asbestos crystal structure.

Depending on the method of production CNTs may also contain iron and other different redox active metals (e.g. Co, Ni, Mo) as a residue of the catalyst employed in their synthesis [35]. The amount is highly variable, and may reach 20% in unpurified CNTs [78]. Metals may be present in different oxidative states as ions, clusters or even organized in metal nanoparticles. Kim and co-workers analyzed some samples of CNTs in which iron was found as a mixture of α-Fe^0, γ-Fe^0, and carbide phases [79]. The metal residues may be extracted from CNT, e.g. by an acidic treatment, but often few traces remain. A full elimination of any metal trace may be achieved by heating at extremely high temperatures where the metal vaporize. Such purified samples were successfully employed to distinguish the effect of metals or framework defects in causing lung toxicity and genotoxicity in vitro [50, 51].

Pioneer work by Pezerat and co-workers hypothesized more than two decades ago a crucial role for iron in asbestos toxicity [80, 81]. The role of iron in the induction of oxidative stress and toxicity upon exposure to asbestos fibres was confirmed by a number of studies [73, 82-84]. Iron ions involved in free radical generation are those present at the fibre surface in a poorly coordination state [85] [61, 86, 87] or those easily removable (bio-available) [76, 88]. Since iron sealed within the graphene layers cannot be released in the medium [89], the amount of bio-available iron in CNTs varies greatly from sample to sample and cannot be predicted from total iron content [78]. As a consequence of such variability, conflicting data are found with CNTs, as both ROS production [42, 90-92] and scavenging (see below) were described [51, 93, 94].

Fiber-derived free radicals contribute with cell-derived free radicals to the overall asbestos-induced oxidative stress. Asbestos have been shown to induce ROS generation in cell cultures [82, 95] as consequence of both highly reactive surface iron ions [84] and of frustrated phagocytosis [96, 97].

Several in vitro studies using different cell lines suggest that also CNTs may induce ROS generation and oxidative stress in cellular system models [42, 98-102]. Frustrated phagocytosis was observed by Brown and coworkers with CNT [103] but metal ions play the most important role in oxidative stress [42, 100]. In

fact when pure carbon nanotubes are administered to cultured cells, ROS generation did not occur [90, 94].

Oxygenated free radicals easily react with CNTs similarly to fullerenes. Such reactivity makes CNTs promising as antioxidant agents. Several studies report an antioxidant activity of CNTs in polymeric composites, which preserves the polymeric matrix from degradation [104]. Purified MWCNTs were reported by some of us to scavenge hydroxyl radicals and superoxide anion [105]. A decrease in reactive oxygen species found in vivo was assigned to the scavenging potential of purified MWCNTs [94]. Recent studies report an antioxidant activity of pristine and modified SWCNTs [106]. The antioxidant properties of CNTs could find applications in medicine. An antioxidant therapy may be suggested in several diseases where mitigation of oxidative stress is beneficial, e.g. cardiovascular diseases and neurodegenerative disorders [107]. To be employed in such way, however, CNTs need to be efficiently delivered to the organ/tissue of interest, then cleared when their function is over. Theoretical calculations reported that the scavenging activity of SWCNTs may be modulated by introducing defects [20] or by varying their diameter, length, and chirality [108-110]. Experimental studies are needed to confirm such hypothesis.

Conversely an uncontrolled antioxidant activity may damage cells. Reduction of the physiological free radical levels may in fact lead to impairment of the cellular physiological functions since free radicals have a key role in cellular proliferation and in host defence [111].

2.4.3 Biopersistence, biodistribution and translocation

Biopersistence may be related to the toxic potential of particulates. The longer a hazardous fiber or particle remains unaffected into a given biological compartment, the longer the biological response elicited might persist over time. Somehow biopersistence would thus enhance the “dose”, not the nature of the caused damage. Well-known particulate toxicants such as asbestos or quartz are characterized by a high biopersistence which exacerbates their toxic effects [8]. Biopersistence is also crucial for nanoparticles used in diagnosis and therapy. On the one hand a safe material should not be modified in the body,

on the other one, as a foreign body should be eliminated, as quickly as possible, once its task is completed. In this case a controlled pharmacokinetic profile is needed.

Note that at the opposite biopersistence is searched when nanomaterials are used as permanent prosthetic devices.

Biopersistence in the lung is the result of the clearance mechanisms and interactions with the biological medium, both related to the structure and chemistry of the material. For amosite and crocidolite estimated clearance half-times are measured in years to decades, whereas for chrysotile the majority of fibres are cleared within months, although some fibres may be sequestered and slowly cleared [112]. The brucitic layer makes the chrysotile acid-sensitive. Fibres that reach the lung can undergo focal fibre fragmentation [113, 114] in the acidic (pH 4.5) phagolysosome of macrophages [115]. Conversely, amphiboles exhibit a more complex chemical composition and a higher stability [116].

In vivo experiments showed that MWCNTs persist in the lung for some months [41, 44, 117]. Because of their graphitic structure CNTs are highly insoluble [118] and it has been suggested that they may be as biopersistent as amphiboles [64]. However, the CNT durability may vary depending to surface defects or functionalization, e.g. surface carboxylation reduced CNT durability [73]. Insertion of COO groups, in fact, causes collateral damage to the graphenic structure introducing active sites that provide points of attack for oxidative degradation. Oxidative degradation may take place in cellular compartments e.g. reactive oxygen species produced by alveolar macrophages following phagocytosis [73]. A possible degradation of SWCNT by myeloperoxidase, an enzyme involved in the generation of reactive oxygen species in neutrophils, was also reported [119]. However as the physiological oxidizing environment is not very harsh chemical degradation may require long times. Using an in vitro flow through assay with phagolysosomal simulated fluid at pH 4.5, SWCNTs have been shown to persist for several months [120]. Conversely, strong oxidants such as nitric acid or hydrogen peroxide may cause a partial degradation of CNTs and have been used for shortening processes [121]. If confirmed in vivo, these studies may open the door to a safe use of CNT in medicine. However, whether such degradation processes would be sufficient to prevent ad-

404 verse side effects of CNTs as well as the efficiency of the process on the various types of CNTs (SW vs.
405 MW, short vs. long) remains to be clarified.

406 Asbestos fibres and CNTs are both subjected to macrophagic and lymphatic clearance [44, 122, 123].
407 Long asbestos fibres (> 10 µm) are slowly cleared as they cannot be easily enclosed by macrophages lead-
408 ing to frustrated phagocytosis. Frustrated phagocytosis was observed in cells engulfing long and well dis-
409 persed CNTs [47, 103]. Conversely, short CNTs or long CNTs in tangled forms do not pose a problem to
410 macrophages [28]. Likewise, CNT aggregates are usually easily phagocyted [28]. Note that single walled
411 CNTs are more likely to tangle while multi walled ones tend to maintain a rigid shape.

412 Inhaled asbestos fibres are detected in lung, lymph nodes, pleura and peritoneum of the exposed
413 peoples. The shorter fibres (< 2µm) were observed in the lymph nodes and in the pleural plaques [124].

414 Biodistribution of CNTs after deposition in the lung has been poorly investigated. As macrophage
415 clearance, both translocation and biodistribution of CNTs are modulated by the aggregation state [43,
416 125-127]. Short MWCNTs were observed in the lung and in the lymph nodes after intratracheal instillation
417 like short asbestos fibres [44]. No passage from the alveolar space to the systemic circulation and systemic
418 organs (liver, spleen, and kidneys) was detected for CNTs [126].

419 One controversial point on CNTs toxicity is their ability to reach the pleura. Asbestos fibres are able to
420 translocate to the pleural cavity, causing pleural effusion, fibrosis and mesothelioma. Ryman-Rasmussen
421 [128] showed that MWCNTs reach the subpleura in mice after inhalation exposure. Mercier [129] and co-
422 workers observed a distribution of MWCNTs into the subpleural tissue and the intrapleural space after
423 pharyngeal aspiration.

424 Kane and co-workers [130] noted that long asbestos fibres accumulated preferentially at the perito-
425 neal face of the diaphragm around the stomata (pore like structures less than 10 µm in diameter linking
426 the peritoneal cavity to the underlying lymphatic capillaries). Retention of long fibres at the diaphragmatic
427 mesothelial surface could initiate inflammation, proliferation and granuloma formation [131]. Poland [47]

showed that also long CNTs accumulate at the diaphragm, following instillation in the peritoneal cavity, which would suggest that they are too long or bulky to exit through the stomata [132].

2.4.4 Final remarks

In conclusion CNTs are a rapidly growing family of carbon based materials having large differences in their physico-chemical properties. Moreover most of their applications require surface modifications (co-valent grafting, surface functionalization, coatings) which increase the variety of physico-chemical characteristics, by fully modifying surface properties. The relationship between the above mentioned physico-chemical features of CNTs and the toxic responses at the molecular level is still an area of large interest, being the basis of the possible design of CNT-based biocompatible materials.

As to CNTs similarity with asbestos the above data clearly show several differences at the chemical level, in spite of similar physical features which vary from one to the other CNT specimen considered. Table 1 compares asbestos and CNTs as far as the physico-chemical features most relevant to toxicity are concerned.

3 CARBON NANOFIBERS

Carbon nanofibers (CNFs) is a general term used to describe filaments comprised of graphene layers stacked at an angle to the fibre axis. They have lengths in the order of micrometers (up to 200 μm), and diameters ranging from some tens of nanometre up to ca. 200 nm. Graphene layers may be arranged as stacked cones, cups [133] or plates. In the last case three types of CNFs may be recognized, depending on the size and orientation of the graphene layers within their structure: platelet (alignment perpendicularly to the fibre axis), tubular (alignment parallel to the axis), and herringbone (alignment angled to the axis) [134, 135].

Excellent mechanical, electrical and surface properties make CNFs ideal candidates for a wide range of applications such as structural materials, field emission displays, hydrogen storage materials, tips for scanning probe microscopy, nanometre sized semiconductor devices and sensors. Several applications in

biomedical and regenerative medicine are known. The presence of many reactive sites allows selective functionalization to immobilize proteins, enzymes, and DNA, for biosensor preparation. CNFs have also been considered for hard (e.g., orthopaedic and dental) and soft (e.g., cartilage, tendon, vascular) tissue implants and they have been already used as regenerative scaffolds for neural and bone regeneration or as drug and gene delivery vehicles [136].

Adsorption, translocation, excretion of CNFs are expected to be close to what happens with CNTs [64]. Note that a high variability was observed for CNFs [103]. In a biological system, in fact, the absorption, distribution, metabolism, and toxicity of carbon nanomaterials depend on the inherent physical and chemical characteristics such as functionalization, coating, length, and agglomeration state which is influenced by external environmental conditions [137]. Exposure to CNFs could take place following biomedical applications or occupational exposure [136]. Airborne CNFs were found in several manufactures [138] and the exposure preferably occurs through inhalation, although dermal exposure cannot be excluded. To date, however, there is a lack of information on whether CNFs can be absorbed across the skin's stratum corneum barrier. Several reports indicate that carbon fibres may cause dermatitis [139, 140], suggesting that carbon nanomaterials may entry into the viable epidermis after topical exposure [141]. The stratum corneum is the outermost layer of the epidermis and consists of several layers of completely keratinized dead cells, which form a barrier between the ''milieu interieur'' and the outside environment. However, disease or occupational conditions that cause damage to the stratum corneum barrier (e.g. abrasion, solvent exposure) may abrogate these protective functions.

Because of their graphitic structure CNFs are highly insoluble thus highly biopersistent. Due to their strong chemical stability CNFs are not expected to be broken down when inhaled [142]. Nevertheless Yokoyama [143] studied a new hydrophilic type of carbon nanofiber for application to biomaterials. Such nanofibers, named hat-stacked CNFs, because of the novel arrangement where the singles carbon layer of the graphite structure are similar to stacked hats (graphene hats), implanted in the subcutaneous tissue of rats showed a shortening with time, likely due to delaminating of graphene layers. Delamination, which occurred in lysosomes and cytoplasm, could have originated from the intercalation of hydrophilic sub-

478 stances such as enzymes and proteins. Intercalations is possible because of the rich functional groups at
479 the edges of the graphene hats [143].

480 CNFs may subjected to macrophagic clearance. Long CNFs may induce frustrated phagocytosis, escape
481 clearance by normal mechanism and persist in the lung. Although they may exhibit different structure
482 (Figure 3), CNFs usually show a strong tendency to agglomerate and form bundles in aqueous media main-
483 ly because of their hydrophobicity. Agglomerates of small dimension (few micrometers) might be easily
484 engulfed by macrophage or other cells. Interestingly monocytic cells treated with CNFs entangled into ag-
485 gregates of about ten microns in diameter do not exhibit signs of incomplete uptake, unlike monocytic
486 cells treated with straight and well dispersed CNTs [103].

487 Several studies show that the cytotoxicity of CNFs is very low [144, 145] or even absent [103]. Cyto-
488 toxicity might be related to the presence of few dangling bonds - i.e. unpaired electrons in a free orbital
489 arising from the homolytic rupture of the carbon-carbon bonds. As they may easily form new bonds they
490 constitute highly reactive sites [146]. Analysis of the dose-dependent toxicity of different carbon based
491 materials (CBN) in human lung-tumour cell lines revealed that the number of viable cells decreases as a
492 function of dose for all CBN tested. The number of viable cells decreased in the sequence carbon black >
493 CNFs > CNTs. Dangling bonds are present in carbon black with a high density, whereas in carbon nano-
494 tubes they preferentially occur at the lattice defects and at end caps [146]. CNFs exhibit more reactive
495 sites than CNTs. In fact, they have more graphene edge planes, which are ledges of carbon that protrude
496 from the surface at regular intervals, that could lead to easier physical bonding with other materials [147].
497 Surface modifications to improve dispersion in biological media, which results in adding carbonyl, carbox-
498 yl, and/or hydroxyl groups, increase CNF cytotoxicity [146].

499 CNFs did not significantly produce ROS in mouse keratinocytes [144], nor in acellular tests. A weak in-
500 crease dose dependent of $O_2^{-\bullet}$ production was observed in monocyte cells by Brown and co-workers
501 [103]. Dose response was higher for platelet CNFs than for a platelet/herringbone CNF mixed sample,
502 suggesting implication of the graphene structure.

Metal impurities induce ROS generation by CNFs. Formation of $OH^{\bullet}$ radicals was observed in macrophages after few minutes of exposure to CNF containing about 1% of iron. Radical release increased upon addition of H_2O_2 suggesting a metal-dependent Fenton reaction [148].

CNFs have a low inflammatory potential. Platelet and platelet/herringbone CNFs did not stimulate inflammatory cytokines such as TNF-a (tumour necrosis factor) in monocyte cells [103]. Hat stacked CNFs (H-CNFs), modified with carboxyl groups in order to improve their dispersion showed only a induction of TNF-α [30]. One week after implantation in the subcutaneous tissue of rats, H-CNFs caused granulomatous inflammatory change, but not an acute severe inflammatory reaction [30].

Lindberg and co-workers [145] compared the potential genotoxic of CNTs, containing < 5%wt of Co and Mo, and graphite nanofibers containing < 3% wt of Fe. Genotoxicity was assessed by the comet and the micronucleus assay in human bronchial epithelial cells. While CNTs induced a dose-dependent increase in DNA damage at all dose and treatment times, graphite nanofibers induced DNA strand breaks and chromosomal damage in human bronchial epithelial only after long time of treatment with no dose dependence.

Conversely CNFs containing iron impurities (<1.4% wt) showed a genotoxicity comparable with asbestos and stronger than SWCNT (Fe < 0.23%) [148]. The authors hypothesized that CNFs cause genotoxicity via two different mechanisms: i) by production of ROS, likely via Fenton reaction, which in turn react readily with DNA and ii) by physically interfering with DNA/chromosomes and/or the mitotic apparatus.

4 METAL/OXIDE HARNs

With the tremendous advances on the capacity to manipulate the unique physicochemical properties of nanoscaled systems with varied composition we may foresee that soon many metals and oxides will be prepared in form of HARNs for different applications, including diagnostic or therapeutic purposes [27]. An increasing number of nanorods and nanowires of several metals and metal oxides have now been studied and sometime already available to the market. Among the nanorods, we will report here only on

527 the most studied ones with intended applications in nanomedicine: i.e. gold nanorods. The paragraph de-
528 voted to nanowires has been more generally focused on the many studies dealing with metal and metal-
529 oxide nanowires relevant in nanomedicine.

530 *4.1 Metal nanorods: the case of gold*

531 Gold nanostructures are often used as a model system since their physical and chemical properties
532 can be easily manipulated [149]. Gold nanorods (Au NRs) are one kind of most promising and widely uti-
533 lized materials owing to their biocompatibility and optical tunability [150]. Au NRs are usually synthesized
534 with relative low aspect ratio but high aspect ratio (length/diameter > 11) have recently been prepared.
535 Many applications of high aspect ratio Au NRs have been proposed [151] as useful object in nanomedi-
536 cine: tools for cellular imaging, molecular diagnosis and targeted thermal therapy.

537 Wet chemical synthesis of gold nanorods is the most popular route to prepare these nanomaterials
538 and it requires the use of cetyltrimethylammonium bromide (CTAB) as shape-directing surfactant, which
539 forms a bilayer on the surfaces of gold nanorods [152, 153]. Au nanorods toxicity, uptake, circulation and
540 distribution has been thoroughly investigated in different labs and from different points of view [154,
541 155]. Surface modification of cetyltrimethylammonium bromide (CTAB)-stabilized gold nanorods is pri-
542 marily reported as a method to demote inherent toxicity of Au NRs [156]. CTAB is indeed a well-
543 established agent promoting cellular toxicity of Au NRs, thus many studies claim to substitute it with other
544 less toxic coatings [154, 157]. There is not a clear consensus, however, on the mechanism of CTAB-
545 induced cytotoxicity, which can be due either to free CTAB in solution (Connor [157, 158], or to the simul-
546 taneous effect of CTAB molecules in solution and at the Au NRs surface [154]. Unfortunately the removal
547 of the CTAB bilayer results in suspension instability and nanorod agglomeration. Some strategies - still to
548 be found- are required to replace, stabilize CTAB or coating Au with other less toxic surfactants. A great
549 attention has been devoted to the formation of specific chemical interfaces surroundings Au NRs. The
550 modification of each of these interfaces provides strategies for altering nanorod properties such as stabil-
551 ity against aggregation, toxicity, and ease of assembly. Murphy and co-workers [159] clarify that three in-
552 terfaces are relevant in tailoring Au NRs properties: i) the gold−surfactant interface, ii) the hydrophobic

surfactant bilayer, and iii) the surfactant interface with bulk water. The last one - the solvent-accessible interface - dictates nanorod interactions with other particles, macromolecules, and living cells.

According to several authors, the effect of the Au NRs aspect ratio is relevant in terms of cellular uptake, rather than being the actual cause of cytotoxicity. In human breast adenocarcinoma cells (MCF-7) shorter Au NRs seem to be easier to internalize than longer ones [154]. This is believed to be a protein receptor mediated endocytosis process and appears to be energy dependent [160, 161]. A set of Au NRs with different aspect ratio and surface charge was tested on human colon cancer cell line (HT-29) [157]. The authors report that serum proteins from the culture media - most likely bovine serum albumin - adsorb to Au NRs leading to all nanorod samples bearing the same effective charge, regardless of the initial surface charge. This confirms that surface properties of nanomaterials change substantially after coming into contact with protein-rich solution media [162, 163]. A clear consensus has not yet been achieved about the role of surface charge in cellular uptake. Some studies report plays a significant role [154], while for others surface charge seems not to bear the expected importance in driving uptake and modulating toxicity [157, 164].

The promising synthesis of nanoscale hybrid HARNs, e.g. Au-nanorod/SWCNT/Au-nanorod [165], will make the assessment of molecular mechanism of toxicity of these nano-objects a further entangled maze.

4.2 Metal and oxide nanowires

Nanowires (NWs) are one dimensional nanostructures, with diameter constrained to tens of nanometres or less and an unconstrained length. The aspect ratio is usually very high and NWs with AR > 1000 are currently produced. Many chemically different types of nanowires exist: metallic (e.g., Ni, Pt, Au), semiconducting (e.g., Si, GaN) and oxides (e.g., SiO_2, TiO_2). NWs include single-crystalline homostructures as well as heterostructures of at least two single-crystalline materials having different chemical compositions. Generally, there are two basic morphologies in nanowire heterostructures: radial, such as core–shell nanowires, and axial heterostructures, comprising multisegment nanowires. The peculiar electrical properties due to their size make NWs suitable to build the next generation of computing devices.

NWs may also be readily functionalized with various biomolecules including enzymes, antibodies or nucleic acids, thus being good candidates for several biomedical applications. NWs with different segments along the length provide the opportunity to introduce multiple chemical functionalities by exploiting the selective binding of different ligands to the various segments. Such functionalization imparts catalytic and recognition/binding properties onto NWs, that can be used as nanosensors for detection of biological and chemical species (metal ions, viruses, proteins etc) and as nanocarriers (gene carriers for non-viral gene delivery, antibodies conjugated carriers for specific binding to malignant cells and in vivo targeting of breast tumours) [166, 167]. Unfortunately little is known to date regarding the potential toxicity of this type of nanomaterial.

As for the other HARNs, size, surface charge and surface coating are important parameters in determining how NWs uptake occurs in mammalian cells. The cellular uptake of NWs can occur through one of the following cell-dependent pathways: phagocytosis, receptor-mediated endocytosis and pinocytosis [168]. Macrophages are specialized cells able to perform phagocytosis of NWs. To date, the only available study on phagocytosis of NWs was performed by Muller et al. [169]. They observed that ZnO NWs from 4 to 10 μm in length, with at high tendency to form aggregates, are easily phagocyted by human monocyte macrophages. Internalization via receptor-meditated endocytosis was proposed by some authors [170, 171]. Chou [171] also observed that NWs with length longer than the diameter of cells are internalized by a single cell if in bundles and by multiple adjacent cells if well dispersed and in straight form.

Finally, Song and co-workers observed that short Fe NWs (< 2 μm), with positive surface charge, introduced into the cell culture medium, migrate to the cell surface under electrostatic attraction [172] and are internalized via a pinocytosis process. Instead, long Fe NWs (5 μm) are internalized only if perpendicular to the cell membrane. The authors speculated that the long NWs were similar to nanoneedles, which could perforate and diffuse through the lipid bilayer of cell membrane without inducing cell death. This kind of uptake process has been observed for cellular uptake of carbon nanotubes [173].

602 Some studies examining different NWs indicate a low cytotoxic potential and high biocompability
603 [174-177]. Exposure of human and bovine epithelial cells to SiO_2 NWs at low concentrations (40 µg/ml)
604 resulted in no cytotoxicity [174], which is quite relevant considering that most silicas are indeed cytotoxic
605 [11, 178] High concentration (>100 µg/ml) of SiO_2 NWs only modestly reduced cell viability in different cell
606 types [175-177]. Examination of the mechanisms responsible for SiO_2 NW-induced cytotoxicity indicates
607 that apoptotic pathways are not activated and that cytotoxicity appears to be primarily due to increased
608 necrosis [176, 177]. However, even at the highest concentration tested SiO_2 NWs revealed a lower cyto-
609 toxicity than amorphous SiO_2 NPs ad hoc synthesized in the same lab [176]. These results indicate that
610 structural differences between silica nanomaterials can have dramatic effects on interaction of these na-
611 nomaterials with cells.

612 Structurally a NW surface differs from the surface of a isometric NP mainly for the presence of a large
613 number of edges induced by the few nanometre small curvature radius. The poorly bound atoms at the
614 edge are a primary source of lattice defects, which are usually reported to enhances surface reactivity.[17]
615 If compared to planar native oxides, SiO_2 NWs are capable of much larger surface hydroxylation when ex-
616 posed to aqueous media. The high hydroxyl group concentration on the surface makes NWs more hydro-
617 philic and less prone to aggregation than SiO_2 nanoparticles [179]. Differences in the aggregation may also
618 contribute to the observed differences in cytotoxicity.

619 Low cytotoxicity have been observed also on several metal NWs. Iron NWs (average diameter and
620 length 50 nm and 2-5 µm) have no significant effect on cell proliferation of human epithelial cells from
621 cervical carcinoma. Nickel NWs (20 µm long and 200 nm in diameter) do not affect the viability of human
622 monocytic leukaemia cells [180], nor of osteoblast and osteosarcoma cells [170]. The authors suggest that
623 a critical factor for the high survival rate may be the presence, in both cases, of the 3–4 nm oxide layers.
624 The oxide layer reduces metal ions release in the cells that may be responsible for the toxic effects elicited
625 [181].

ZnO NWs were found to be toxic to human monocyte macrophages [169]. However, ZnO NWs dissolved very rapidly in a simulated body fluid at lysosomal pH, whereas they were comparatively stable at extracellular pH. NWs dissolution was observed also after phagocytosis, triggered by the acidic pH within the phagolysosome. The authors indicated Zn^{2+} release as responsible for ZnO NWs cytotoxicity. Conversely, no toxicity - measured as cell viability and morphological changes - was observed in human cervical epithelial cells and in cell from subcutaneous connective tissue cultured with ZnO NWs (average diameter and length 1 µm and 200 µm) [182] where the pH is expected to be close to neutral. Therefore, the ZnO NWs toxicity is clearly linked to their solubility, which is in turn pH-dependent, making the toxicity of this material strictly related to the biological environment.

The effect of surface charge on cytotoxicity to fibroblasts and neoplastic tissue cells has been examined for several gold NWs of few micrometer in length. NWs have been functionalized by a monolayer of thiols with amino, alkyl, or carboxyl end groups, or coated with serum. Amino-modified NWs exhibit positive zeta potential, whereas a negatively charged surface is obtained for the mercapto-acid-modified NWs. Mercapto-acid modified NWs, which exhibit the more negative zeta potential, have been found to be the most toxic ones [183] on both fibroblast cells and HeLa cell. Several aspects could account for this surface charge dependent cytotoxicity, e.g. a better dispersion in culture media, a more specific interaction with cell membranes. However, contradictory data are still reported in the literature and an ultimate reason has not yet been found.

Similar results have been obtained with 5µm long silver NWs with different coatings. At low concentration, the higher cytotoxicity was observed for negatively charged surfaces. At the higher concentrations, all NWs were cytotoxic [183]. Finally, mercapto-acid modified gold NWs with length from 0.5 to 9 µm and aspect ratios from 1:2, 1:10, 1:25, and 1:50 exhibited the same degree of cytotoxicity [184].

5 Conclusions

The mere fibrous form is not sufficient to establish the toxicity of a given type of HARNs. The chemical nature of HARNs varies remarkably form one to the other materials and covers all types of chemical bond-

ing, from covalent to metallic and ionic. The most relevant characteristics of the materials described are illustrated and compared in Table 2. Many of them may be prepared in different forms and modified in their surface properties, which further expands the chemical varieties. Length, flexibility and surface modifications appear to modify the potential toxicity of many of the substances examined in the present review.

Carbon nanotubes, both MWCNTs and SWCNTs are hazardous in most of the forms examined. Moreover several studies agree on a large number of similarities between CNTs and asbestos, in spite of relevant chemical differences which have been here highlighted.

With metal and metal oxide materials the potential to release metal ions in the biological environment is one property of concern, with the only exception of gold and likely of other noble metals which might be considered in the future. As a whole the oxide layer protects from cytotoxicity and the elongated form does not appear to enhance toxicity.

We are proceeding but still far from finding a clue to disclose what makes a nano fibre toxic, which will be the first step to establish the requirements for a design of new safer materials. In the meanwhile each new material will need to be tested for toxicity before being produced and used.

6 Future Perspectives

As not all what is nano is dangerous, with the large number of studies appearing on nanomaterials and their potential toxicity the list of hazardous materials and of the physico-chemical properties involved in the adverse biological responses will be progressively implemented. On the basis of such data possible associations between given physico-chemical properties and toxic effects may be sorted out. Finally by preparing and testing a large number of given HARN – e.g. MWCNTs -differing one from the other in one single physical chemical characteristic, it will be possible to disclose what may make such material dangerous and how to prepare safe ones of similar kind. Appropriate positive and negative controls will also be required in such procedures.

More studies on CNT toxicity are needed considering the industrial interest in their usage and the fear associated to their health effects. Common protocols (sample preparation, endpoint, markers) and full physical-chemical characterization will be required for any future toxicological study. New in vitro and ex vivo studies on tailored CNTs will shed more light on their toxicity and potential carcinogenicity. Eventually in vivo studies where CNTs will be administered to experimental animals with different and reliable procedures will allow establishing or ruling out CNTs carcinogenicity and confirming damage to lung functions. Hopefully we will not need epidemiology to establish toxicity. Under such circumstances it will be possible to verify which of the large kind of CNTs, if any, are carcinogenic to animals and possible human carcinogens.

Tailored chemical modification of the exposed surface may be found to convert potentially hazardous HARNs in non toxic entities. However such modifications will need to be persistent over long periods of time in biological environments. Therefore the research will be addressed more on irreversible chemical modifications than on any sort of coatings.

Considering the vast chemical nature of HARNs so far synthesized, any new one will need to be tested for toxicity before being produced and used. However once toxicity tests will have been performed for such a large variety of materials, it might be possible to draw general hypothesis on the chemical characteristics to avoid and those which yield safe products

7 Executive summary

- **High Aspect Ratio Nanomaterials**
  - HARNs are a large variety of materials with different chemical composition and shape.
  - Concerns about their safety are mainly related to their fibrous form and high biopersistence, but their chemical nature may also play an additional role.
- **Carbon nanotubes**

- SWCNT and MWCNT are both covalent solids which may retain some metals in a variable oxidation state as a residue of the catalysts used in their synthesis.

- Overall, a clear indication of toxicity came out of studies on currently available CNTs. Caution in handling is therefore necessary.

- New investigations aimed to identify the physico-chemical determinants of toxicity should be carried out considering that the variability in the toxic responses elicited stems from the differences in physico-chemical features.

➢ **CNT modifications**

- Due to the nature of the carbon-carbon bonds CNTs may relatively easily oxidized and functionalized with a large variety of molecules, giving rise to very different entities.

- surface modifications may modulate the biological responses elicited

➢ **Asbestos vs. CNTs**

- The similarity between asbestos and CNTs concerns not only some physical features but also several cellular responses and in vivo damages.

- Their chemical nature is however remarkably different as far as free radical release / quenching or hydrophilicity is concerned.

- Evidence for the development of mesothelioma following CNTs exposure is still weak and needs to be assessed with different administration routes and long term animal experiments.

➢ **Nanorods and nanowires**

- Gold nanorods are by far the most studied and widespread. The biological responses elicited much depends upon their coating.

- A large variety of oxides and metal are available as nanowires. Metals have few oxide layers at their surface. Their toxicity appears not to exceed what found with isometric nanoparticles of the same composition. In the case of silica a nanowire was even less cytotoxic than isometric silica nanoparticles.

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FIGURES, CAPTIONS and TABLE

FIGURE 1. Scheme of the expected events in the alveoli upon inhalation of fibrous particles. Frustrated phagocytosis mainly occurs with long fibers, but is determined not only by fiber length but also by the chemical composition of the fibers. Short fibers which do not react deleting the phagolysosomal membrane are phagocitized and cleared by macrophages. Conversely long fibers and those reacting within the phagolysome activate and ultimately kill the cells and are released again in the medium.

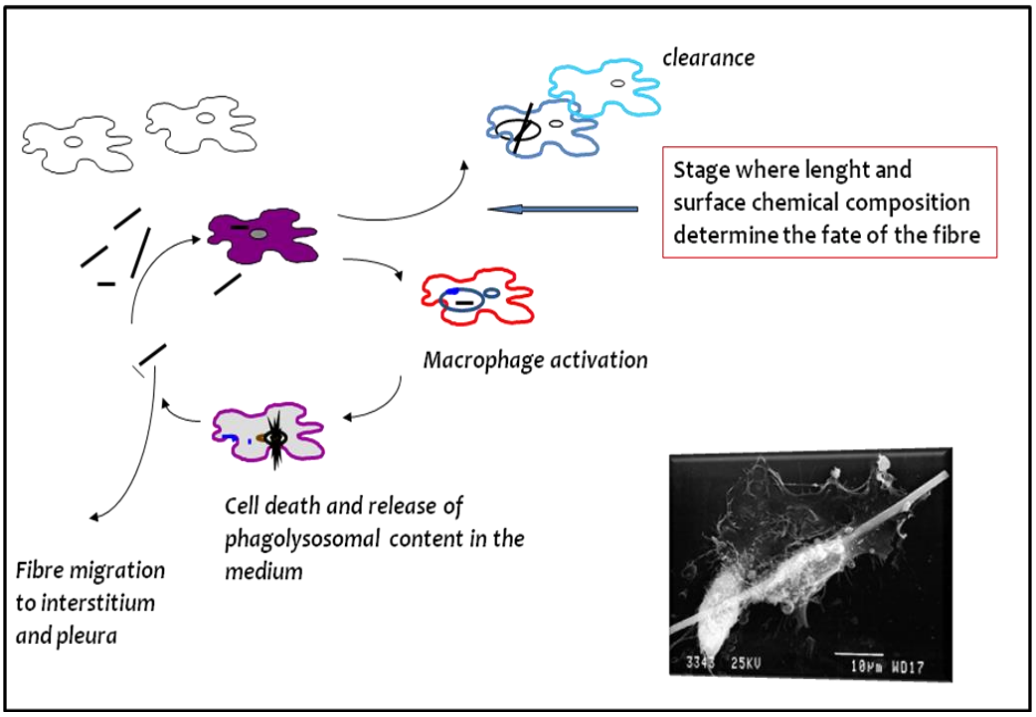
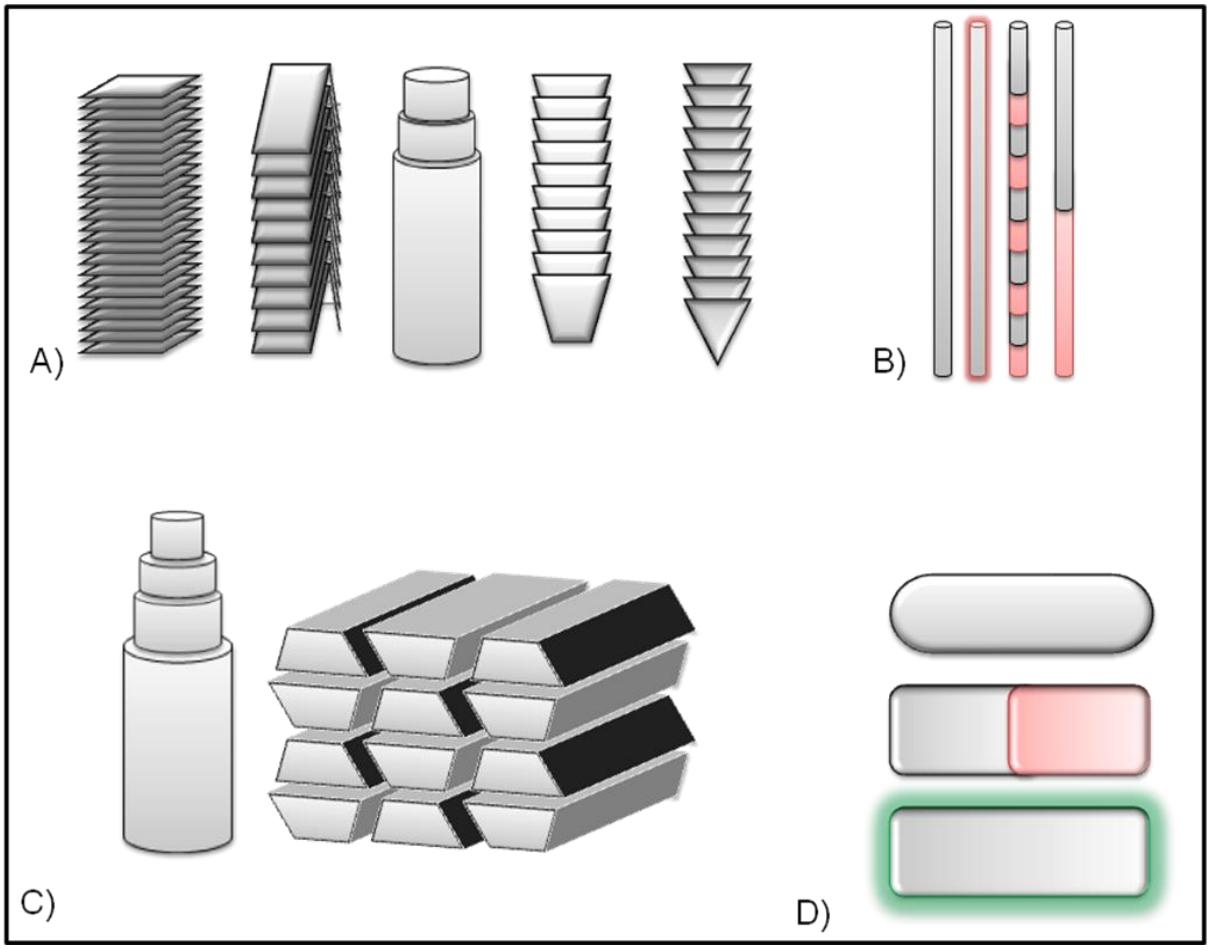


FIGURE 2. Magazine advertisings from different asbestos product manufacturers in the beginning of 20th century.

Asbestos is the typical example of a versatile material with exceptional properties which turned out to be one of the largest occupational tragedies. With appropriate studies on the new HARNs we have the possibility to develop safe material design and manufacturing strategies before a large scale commercialization takes place. Media source: 1) Turner Brothers Asbestos Company, Manchester, UK, 1918; 2) Keasbey & Mattison Company, Amblier, PA, 1928; 3) L. W. Kerney, Chicago, IL, 1905; 4) Johns-Manville Company, New York, NY 1925; 5) Industrial Gloves Company, Danville, IL, 1946.

FIGURE 3. Scheme of the structure of most common HARNs.

Schematic representation of the structures of A) carbon nanofibers with different arrangement of the graphene layers: (from left to right) platelets stacked perpendicularly to the fiber axis (platelet CNF); platelets angled to the axis (herringbone CNF); carbon nanotubes; stacked cups CNF; stacked cones CNF. B) nanowires: (from left to right) single segment or single crystal NW, two components radial or core shell NW, two components multisegment axial NW, two components axial or two segment NW; C) asbestos: (from left to right) serpentine and amphibole; D) nanorods: (from top to bottom) single metal NR, bi-component NR (e.g., Au-Pt), coated NR.



1185 **FIGURE 4. A dimensional view of the HARNs whose structure is depicted in Fig 3.**

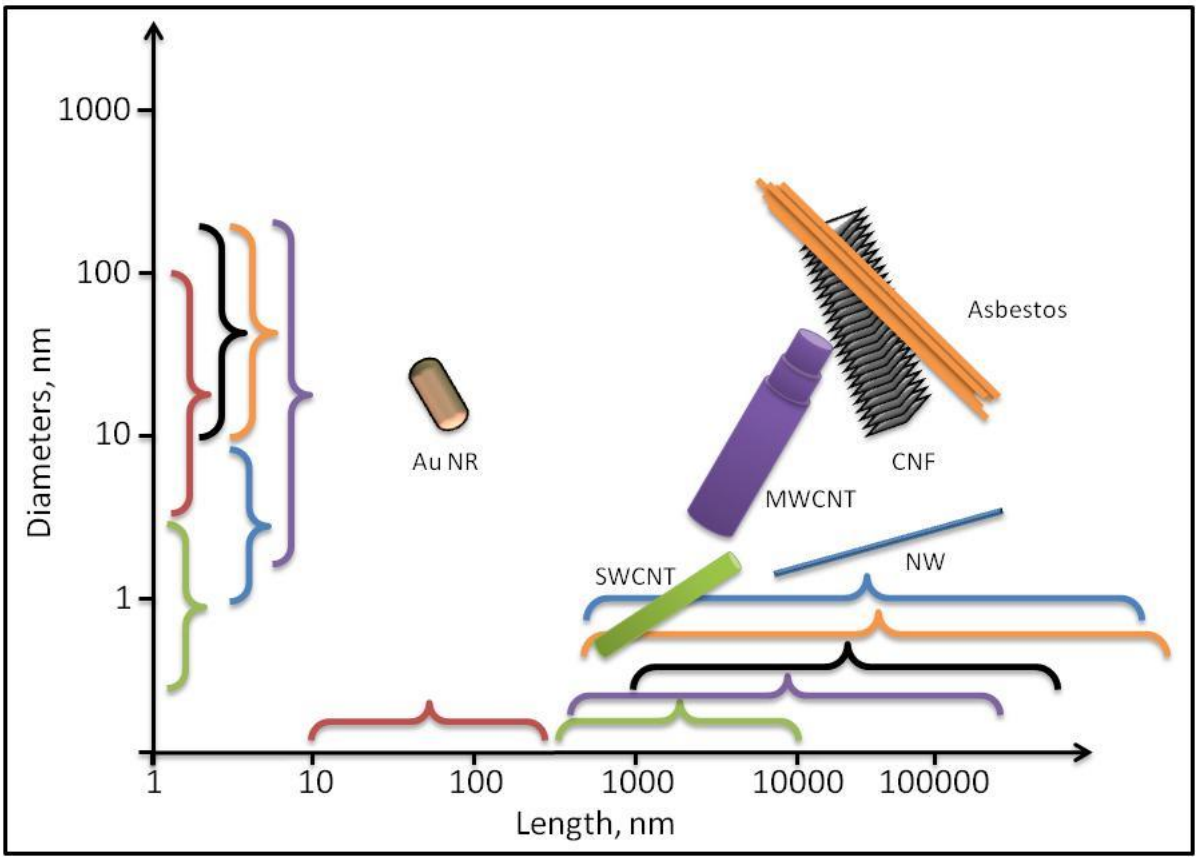


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FIGURE 5. Carbon-nanotubes: hype or hope?

A) Schematic structure of the carbon-carbon bonds and a scanning microscopy image of carbon nanotubes. B) The rate of growth of publications on carbon nanotubes (red) compared to those devoted to their toxicity (pale blue). Clearly studies on the synthesis and application of CNTs pay more than those devoted to the hazard associated to their production and use.

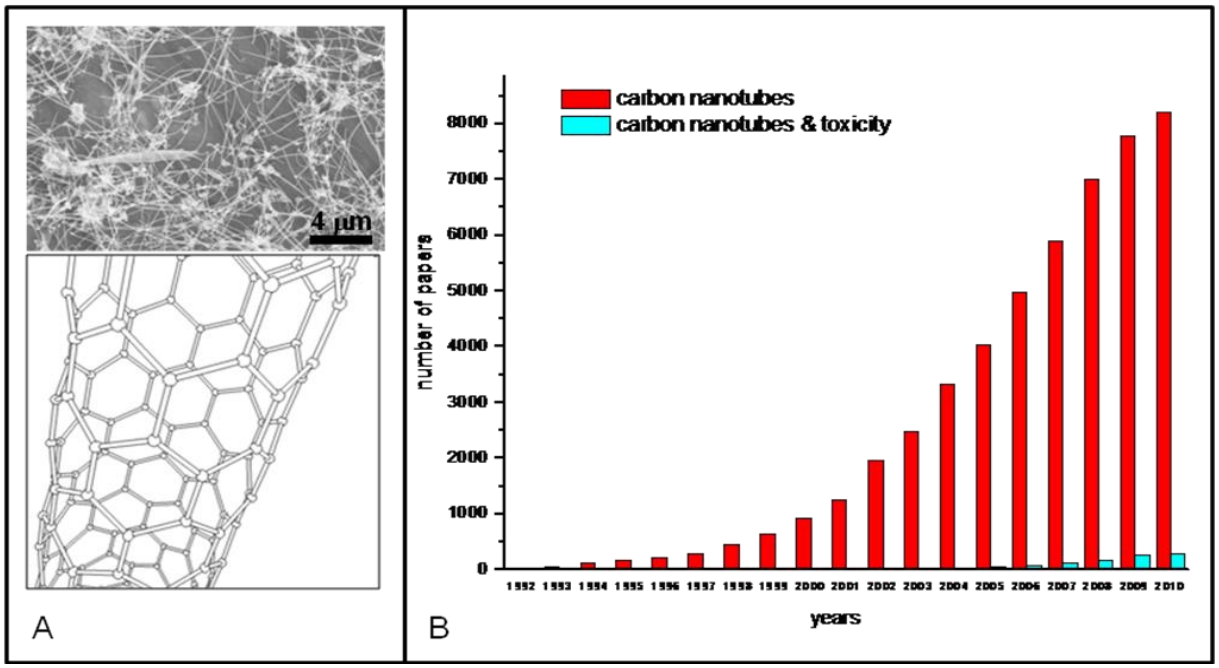

TABLE 1. CNT vs. Asbestos.

A comparison of the chemical properties of CNTs and asbestos which are - or may be - implied in their toxicity. The chemical nature of the two materials, at least in their "native" form, are quite different.

	Carbon nanotubes	Asbestos	
		Amphiboles	*Chrysotile*
bio-available metals	highly variable (Co, Ni, Fe – metallic or ionic)	stoichiometric Fe^{2+} and Fe^{3+} ions in crystal structure	substitutional Fe^{2+} ions
hydrophilicity hydrophobicity	highly hydrophobic if not functionalized	highly hydrophilic	
surface charge (physiological pH)	very low, negative if not functionalized	high, negative	high, positive
free radicals	free radicals and ROS scavenging	free radicals and ROS generation	
dissolution/degradation	enzymatic degradation in neutrophils (SWCNT) and degradation in phagolysosomal fluid (carboxylated SWCNT)	selective leaching of iron ions only in presence of strong chelators	

1201 **TABLE 2. Comparison of the most relevant physical and chemical properties of asbestos and HARNS potentially involved in the toxicity**

		Carbon nanotubes	Carbon nanofibers	Nanowires	Nanorods	Asbestos
Structure		single (SWCNT) or multi (MWCNT) graphene layered rolled sheets	graphene layers arranged as stacked cones, cups or plates, aligned perpendicularly (platelet); parallel (tubular) or angled (herringbone) to the fiber axis	single segment (single-crystal) or multi segment (at least two different single-crystal) with radial (core–shell) or axial alignment	single metal, bi-component, coated NR	amphiboles: octahedrally coordinated cations layers sandwiched between tetrahedral silicate layers chrysotile: multi-layered brucitic layers intercalated with silicate rolled sheets
Defects	*amount*	yes, variable depending upon synthesis procedures	high	yes, variable depending upon size	variable, low	largely variable in natural minerals
	kind	ring shapes other than hexagon, sp^3 hybridized C, dangling bonds at the lattice defects and at end caps	dangling bonds at the edges of the grapheme layers; defects similar to CNT ones	stacking faults and twins in the core shell, incomplete bond at the edges, presence of oxide layer on metal NW surface	faceting, twinning, vacancies	defects in the framework, absence or substitution of metal ions
Chemical composition		carbon	carbon	metals (Ni, Pt, Au), semiconductor (Si, GaN, etc.) and oxides (SiO_2, TiO_2)	metal (Au, Ag), oxide (TiO_2)	silicate sheet (SiO_2) including Mg^{2+}, $Fe^{2+/3+}$, Na^+ Ca^{2+}as structural or substitution ions
Nature of the chemical bond		covalent	covalent	covalent polar ionic metallic	covalent polar metallic	mixed: covalent polar + ionic
Presence of metals or metal ions		highly variable (Co, Ni, Fe). Clusters in different redox states	highly variable. Clusters in different redox states		always	Fe, higher from amosite and crocidolite
Hydrophilicity / hydrophobicity		hydrophobic	hydrophobic	variable, mostly hydrophilic depending upon composition	variable, depending upon coating	fully hydrophilic
Aggregation / agglomeration		yes	yes	variable, depending upon composition	variable, depending upon coating	naturally in bundles
Durability		high	high	variable, depending upon solubility of the metal/oxide constituting the wire	variable, depending upon the coating dissolution	amphiboles: high in all media chrysotile: generally high, low stability in acidic media

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