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

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2 ***FACE ON THE TOXIC RESPONSE TO HIGH ASPECT RATIO NA-***
3 ***NOMATERIALS (HARNS)"***

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26 ***Summary***

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

37

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

41

42 **1 INTRODUCTION**

43 **1.1 Nanosized materials as a double sword**

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

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

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

60 When it comes to nanomedicine only a correct balance between risks and benefits will allow to take
61 sound decisions. For instance at the American Chemical Society Fall Meeting held in Boston in 2010 it was
62 proposed to employ radioactive salts sealed inside carbon nanotubes for targeted radiotherapy. This is
63 obtained by chemical modification of the surface of the tubes with sugar or other targeting molecules.
64 The sugar could play a variety of roles, making the nanotubes soluble and stopping them from clumping
65 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

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

97 ***1.3 Why a specific study on HARNs***

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

102 The fibrous form has a specific role in toxicity as it is the cause of failed phagocytosis and of the trans-
103 location in various biological compartments, typically the parietal pleura. Macrophage clearance is one of
104 the major route whereby the body defences get rid of unwanted foreign materials. Inhaled particles which
105 do not damage the phagolysosome membrane may be easily engulfed by alveolar macrophages and
106 transported to the lymphatics. When the material is in fibrous form the macrophage attempts often end
107 up with frustrated phagocytosis and macrophage death, following the scheme reported in Figure 1, which
108 may apply also to inflammatory reactions occurring in body compartments other than the lung. Long fi-
109 bres cannot be phagocytosed, while the short ones are more easily uptaken and cleared, which explains the
110 higher toxic potency of long vs. short fibres [9]. However beside fibre dimensions also surface reactivity
111 determines the fate of the fibre and its ultimate toxicity [10] (Figure 1). In the case of isometric silica par-
112 ticles, for instance, surface reactivity determines reactions with the phagosome membrane and cell death
113 [11].

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

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

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

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

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

136 ***1.4 Not all fibres nor all HARNs are toxic***

137 Some fibrous materials such as asbestos, the zeolite erionite, artificial ceramic fibres and others are
138 highly toxic when micrometric fibres remain airborne and reach the lung alveoli and the pleura. Not all fi-
139 bres however are equally toxic, some, e.g. wollastonite (CaSiO_3), are even inert [14]. A comparison of the
140 toxicity of chrysotile asbestos with several other fibrous materials committed by WHO in 2005 [15] re-
141 ported great differences in toxicity among the different materials. The available data on HARNs are still
142 scarce to allow a similar study. In search of appropriate positive and negative controls for HARNs some of
143 us have recently reported that imogolite, a hydrated alumino-silicate with the formula $(\text{OH})_3\text{Al}_2\text{O}_3\text{SiOH}$
144 [16] – opposite to carbon nanotubes - appears inactive in cell viability, NO production, and epithelial bar-
145 rier permeability [17, 18].

146 **1.5 Materials considered**

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

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

156 The basic structures of the materials described are schematized in Figure 3 and their dimensional as-
157 pects depicted in Figure 4.

158 **2 CARBON NANOTUBES**

159 **2.1 Carbon nanotubes: forms and chemical requirements for their applica-** 160 **tions.**

161 CNTs are a form of elemental carbon. Like in fullerenes and graphite, carbon is organized in layers of
162 hexagonal rings having conjugated double bonds (Figure 5A). Because of their high length/diameter ratio
163 CNTs are comprised into the general term HARN. The discovery of CNTs is generally attributed to Iijima
164 [19].

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

175 CNTs exhibit high thermal and mechanical resistance, electrical conductivity or semiconductivity. Such
176 properties make CNTs interesting in a variety of industrial applications e.g. as component in electronics,
177 energy-storage devices, solar cells, sensors, or in mechanical applications as filler in polymeric composites
178 [21]. Their physico-chemical properties may be modulated by varying the method of synthesis and by ap-
179 plying post-synthesis treatments. Therefore a large variety of CNTs forms may be produced which exhibit
180 different chemical reactivity one from the other.

181 The number of studies devoted to the production of new tailored forms of CNTs for the different in-
182 dustrial 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-

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

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

219 ***2.3 Synthetic chrysotile asbestos nanotubes***

220 Stoichiometric chrysotile tubular nanocrystals have been recently, synthesized [56] as possible start-
221 ing materials for applications in nanotechnology and as a standard reference sample for the investigation
222 of the molecular interactions between chrysotile asbestos and biological systems. Each single nanocrystal
223 of pure chrysotile has a tubular shape of about 49 nm in outer maximum diameter, a hollow core of about
224 7 nm and a length of the order of some microns. The presence of iron does not change the tubular shape
225 of nanocrystals which appear just slightly longer than the iron free ones. Iron ions replace both Mg and Si
226 into the octahedral and tetrahedral sheets [57].

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

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

238 To gain direct experimental evidence of the chemical role of iron in asbestos reactivity a set of na-
239 nochrysotiles have also been synthesized with 0.6% and 0.9% (w/w) iron by means of the same procedure
240 [60]. Even the lowest iron-loading induced DNA strand breaks, lipoperoxidation, inhibition of redox me-
241 tabolism and alterations of cell integrity, i.e. the same toxic characteristics of natural chrysotile [59].
242 These results suggest that metal ions play a crucial role in the oxidative stress and genotoxic effects
243 caused by chrysotile asbestos.

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

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

254 ***2.4 Carbon Nanotubes vs. Asbestos***

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

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

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

274 Several reviews have recently been devoted to the comparison between CNT and asbestos [36, 39,
275 63-65]. They mostly consider the physical similarities such as shape and chemical stability in a physiologi-
276 cal environment and report several outcomes in various cell systems and in *in vivo* rodent models. Few re-
277 views [63, 64] also mention some chemical aspects - surface properties, presence of metal transition ions,
278 adsorptive potential- in relation with the toxicological properties reported (cell derived ROS , cytotoxicity,
279 DNA damage, physical interference with mitosis, stimulation of target cell proliferation and induction
280 chronic inflammation). Other focus on activation of macrophages and injury on epithelial / mesothelial
281 cells [65], and on the potential to activate signaling pathways modulating transcription factor activity, in-
282 ducing apoptosis and DNA damage [39] without considering at all the differences in chemical properties.

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

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

291 ***2.4.1 Chemical composition of CNTs and asbestos: differences and similarities***

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

298 CNTs are allotropes of carbon exhibiting a surface made up by a rolled hexagonal lattice of carbon at-
299 oms linked by σ and π covalent bonds.

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

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

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

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

324 ***2.4.2 Metal ions and surface generation vs. quenching of free radicals and ROS*** 325 ***on asbestos and CNTs***

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

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

340 Pioneer work by Pezerat and co-workers hypothesized more than two decades ago a crucial role for
341 iron in asbestos toxicity [80, 81]. The role of iron in the induction of oxidative stress and toxicity upon ex-
342 posure to asbestos fibres was confirmed by a number of studies [73, 82-84]. Iron ions involved in free rad-
343 ical generation are those present at the fibre surface in a poorly coordination state [85] [61, 86, 87] or
344 those easily removable (bio-available) [76, 88]. Since iron sealed within the graphene layers cannot be re-
345 leased in the medium [89], the amount of bio-available iron in CNTs varies greatly from sample to sample
346 and cannot be predicted from total iron content [78]. As a consequence of such variability, conflicting da-
347 ta are found with CNTs, as both ROS production [42, 90-92] and scavenging (see below) were described
348 [51, 93, 94].

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

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

355 fact when pure carbon nanotubes are administered to cultured cells, ROS generation did not occur [90,
356 94].

357 Oxygenated free radicals easily react with CNTs similarly to fullerenes. Such reactivity makes CNTs
358 promising as antioxidant agents. Several studies report an antioxidant activity of CNTs in polymeric com-
359 posites, which preserves the polymeric matrix from degradation [104]. Purified MWCNTs were reported
360 by some of us to scavenge hydroxyl radicals and superoxide anion [105]. A decrease in reactive oxygen
361 species found in vivo was assigned to the scavenging potential of purified MWCNTs [94]. Recent studies
362 report an antioxidant activity of pristine and modified SWCNTs [106]. The antioxidant properties of CNTs
363 could find applications in medicine. An antioxidant therapy may be suggested in several diseases where
364 mitigation of oxidative stress is beneficial, e.g. cardiovascular diseases and neurodegenerative disorders
365 [107]. To be employed in such way, however, CNTs need to be efficiently delivered to the organ/tissue of
366 interest, then cleared when their function is over. Theoretical calculations reported that the scavenging
367 activity of SWCNTs may be modulated by introducing defects [20] or by varying their diameter, length,
368 and chirality [108-110]. Experimental studies are needed to confirm such hypothesis.

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

372 ***2.4.3 Biopersistence, biodistribution and translocation***

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

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

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

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

390 In vivo experiments showed that MWCNTs persist in the lung for some months [41, 44, 117]. Because
391 of their graphitic structure CNTs are highly insoluble [118] and it has been suggested that they may be as
392 biopersistent as amphiboles [64]. However, the CNT durability may vary depending to surface defects or
393 functionalization, e.g. surface carboxylation reduced CNT durability [73]. Insertion of COO groups, in fact,
394 causes collateral damage to the graphenic structure introducing active sites that provide points of attack
395 for oxidative degradation. Oxidative degradation may take place in cellular compartments e.g. reactive
396 oxygen species produced by alveolar macrophages following phagocytosis [73]. A possible degradation of
397 SWCNT by myeloperoxidase, an enzyme involved in the generation of reactive oxygen species in neutro-
398 phils, was also reported [119]. However as the physiological oxidizing environment is not very harsh
399 chemical degradation may require long times. Using an in vitro flow through assay with phagolysosomal
400 simulated fluid at pH 4.5, SWCNTs have been shown to persist for several months [120]. Conversely,
401 strong oxidants such as nitric acid or hydrogen peroxide may cause a partial degradation of CNTs and have
402 been used for shortening processes [121]. If confirmed in vivo, these studies may open the door to a safe
403 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 ($> 10 \mu\text{m}$) 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 phagocytosed [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 ($< 2\mu\text{m}$) 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 $10 \mu\text{m}$ 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]

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

430 **2.4.4 Final remarks**

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

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

441 **3 CARBON NANOFIBERS**

442 Carbon nanofibers (CNFs) is a general term used to describe filaments comprised of graphene layers
443 stacked at an angle to the fibre axis. They have lengths in the order of micrometers (up to 200 μm), and
444 diameters ranging from some tens of nanometre up to ca. 200 nm. Graphene layers may be arranged as
445 stacked cones, cups [133] or plates. In the last case three types of CNFs may be recognized, depending on
446 the size and orientation of the graphene layers within their structure: platelet (alignment perpendicularly
447 to the fibre axis), tubular (alignment parallel to the axis), and herringbone (alignment angled to the axis)
448 [134, 135].

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

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

457 Adsorption, translocation, excretion of CNFs are expected to be close to what happens with CNTs
458 [64]. Note that a high variability was observed for CNFs [103]. In a biological system, in fact, the absorp-
459 tion, distribution, metabolism, and toxicity of carbon nanomaterials depend on the inherent physical and
460 chemical characteristics such as functionalization, coating, length, and agglomeration state which is influ-
461 enced by external environmental conditions [137]. Exposure to CNFs could take place following bio-
462 medical applications or occupational exposure [136]. Airborne CNFs were found in several manufactures
463 [138] and the exposure preferably occurs through inhalation, although dermal exposure cannot be ex-
464 cluded. To date, however, there is a lack of information on whether CNFs can be absorbed across the
465 skin's stratum corneum barrier. Several reports indicate that carbon fibres may cause dermatitis [139,
466 140], suggesting that carbon nanomaterials may entry into the viable epidermis after topical exposure
467 [141]. The stratum corneum is the outermost layer of the epidermis and consists of several layers of com-
468 pletely keratinized dead cells, which form a barrier between the "milieu interieur" and the outside envi-
469 ronment. However, disease or occupational conditions that cause damage to the stratum corneum barrier
470 (e.g. abrasion, solvent exposure) may abrogate these protective functions.

471 Because of their graphitic structure CNFs are highly insoluble thus highly biopersistent. Due to their
472 strong chemical stability CNFs are not expected to be broken down when inhaled [142]. Nevertheless
473 Yokoyama [143] studied a new hydrophilic type of carbon nanofiber for application to biomaterials. Such
474 nanofibers, named hat-stacked CNFs, because of the novel arrangement where the singles carbon layer of
475 the graphite structure are similar to stacked hats (graphene hats), implanted in the subcutaneous tissue of
476 rats showed a shortening with time, likely due to delaminating of graphene layers. Delamination, which
477 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 $O_2^{\cdot-}$ 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.

503 Metal impurities induce ROS generation by CNFs. Formation of OH[•] radicals was observed in macro-
504 phages after few minutes of exposure to CNF containing about 1% of iron. Radical release increased upon
505 addition of H₂O₂ suggesting a metal-dependent Fenton reaction [148].

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

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

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

521 ***4 METAL/OXIDE HARNs***

522 With the tremendous advances on the capacity to manipulate the unique physicochemical properties
523 of nanoscaled systems with varied composition we may foresee that soon many metals and oxides will be
524 prepared in form of HARNs for different applications, including diagnostic or therapeutic purposes [27].
525 An increasing number of nanorods and nanowires of several metals and metal oxides have now been
526 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

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

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

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

569 **4.2 Metal and oxide nanowires**

570 Nanowires (NWs) are one dimensional nanostructures, with diameter constrained to tens of nanome-
571 tres or less and an unconstrained length. The aspect ratio is usually very high and NWs with AR > 1000 are
572 currently produced. Many chemically different types of nanowires exist: metallic (e.g., Ni, Pt, Au), semi-
573 conducting (e.g., Si, GaN) and oxides (e.g., SiO₂, TiO₂). NWs include single-crystalline homostructures as
574 well as heterostructures of at least two single-crystalline materials having different chemical composi-
575 tions. Generally, there are two basic morphologies in nanowire heterostructures: radial, such as
576 core-shell nanowires, and axial heterostructures, comprising multisegment nanowires. The peculiar elec-
577 trical properties due to their size make NWs suitable to build the next generation of computing devices.

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

587 As for the other HARNs, size, surface charge and surface coating are important parameters in deter-
588 mining how NWs uptake occurs in mammalian cells. The cellular uptake of NWs can occur through one of
589 the following cell-dependent pathways: phagocytosis, receptor-mediated endocytosis and pinocytosis
590 [168]. Macrophages are specialized cells able to perform phagocytosis of NWs. To date, the only available
591 study on phagocytosis of NWs was performed by Muller et al. [169]. They observed that ZnO NWs from 4
592 to 10 μm in length, with at high tendency to form aggregates, are easily phagocytosed by human monocyte
593 macrophages. Internalization via receptor-mediated endocytosis was proposed by some authors [170,
594 171]. Chou [171] also observed that NWs with length longer than the diameter of cells are internalized by
595 a single cell if in bundles and by multiple adjacent cells if well dispersed and in straight form.

596 Finally, Song and co-workers observed that short Fe NWs ($< 2 \mu\text{m}$), with positive surface charge, in-
597 troduced into the cell culture medium, migrate to the cell surface under electrostatic attraction [172] and
598 are internalized via a pinocytosis process. Instead, long Fe NWs (5 μm) are internalized only if perpendicu-
599 lar to the cell membrane. The authors speculated that the long NWs were similar to nanoneedles, which
600 could perforate and diffuse through the lipid bilayer of cell membrane without inducing cell death. This
601 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 biocompatibility
603 [174-177]. Exposure of human and bovine epithelial cells to SiO₂ 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 SiO₂ NWs only modestly reduced cell viability in different cell
606 types [175-177]. Examination of the mechanisms responsible for SiO₂ 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 SiO₂ NWs revealed a lower cyto-
609 toxicity than amorphous SiO₂ 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, SiO₂ 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 SiO₂ 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].

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

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

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

648 **5 Conclusions**

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

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

656 Carbon nanotubes, both MWCNTs and SWCNTs are hazardous in most of the forms examined. More-
657 over several studies agree on a large number of similarities between CNTs and asbestos, in spite of rele-
658 vant chemical differences which have been here highlighted.

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

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

666 **6 *Future Perspectives***

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

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

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

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

692 **7 *Executive summary***

693 ➤ **High Aspect Ratio Nanomaterials**

- 694 ▪ HARNs are a large variety of materials with different chemical composition and shape.
- 695 ▪ Concerns about their safety are mainly related to their fibrous form and high biopersistence,
696 but their chemical nature may also play an additional role.

697 ➤ **Carbon nanotubes**

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

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

702 ▪ New investigations aimed to identify the physico-chemical determinants of toxicity should be
703 carried out considering that the variability in the toxic responses elicited stems from the dif-
704 ferences in physico-chemical features.

705 ➤ **CNT modifications**

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

708 ▪ surface modifications may modulate the biological responses elicited

709 ➤ **Asbestos vs. CNTs**

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

712 ▪ Their chemical nature is however remarkably different as far as free radical release / quench-
713 ing or hydrophilicity is concerned.

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

716 ➤ **Nanorods and nanowires**

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

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

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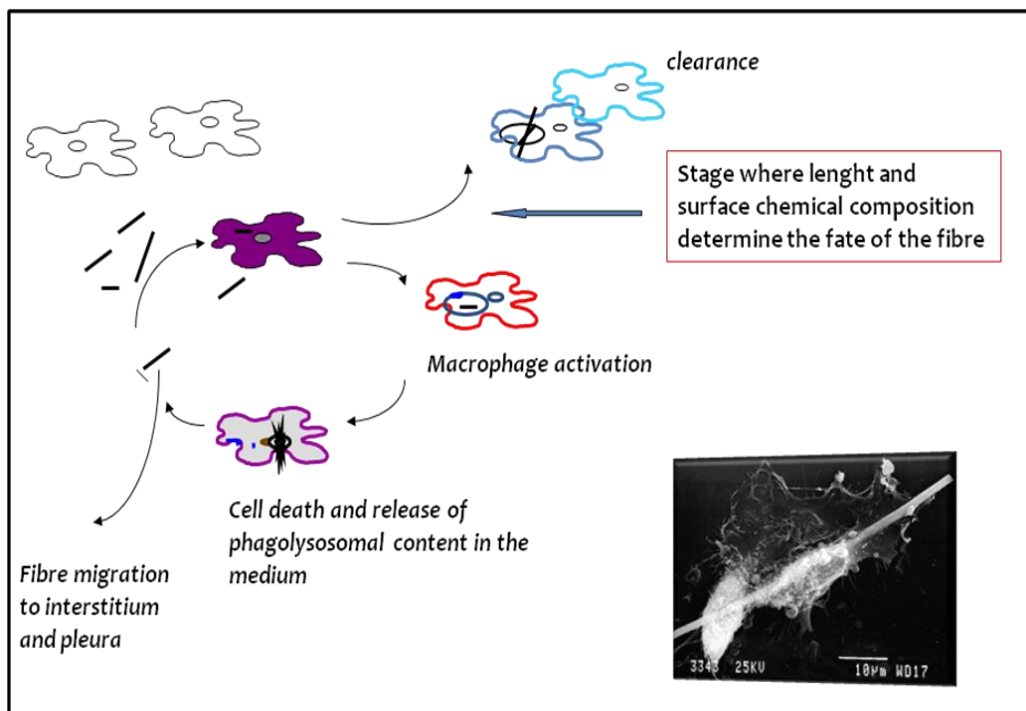
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1156 **FIGURES, CAPTIONS and TABLE**

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1158 **FIGURE 1. Scheme of the expected events in the alveoli upon inhalation of fibrous particles.** Frustrated
1159 phagocytosis mainly occurs with long fibers, but is determined not only by fiber length but also by the
1160 chemical composition of the fibers. Short fibers which do not react deleting the phagolysosomal mem-
1161 brane are phagocitized and cleared by macrophages. Conversely long fibers and those reacting within the
1162 phagolysome activate and ultimately kill the cells and are released again in the medium.



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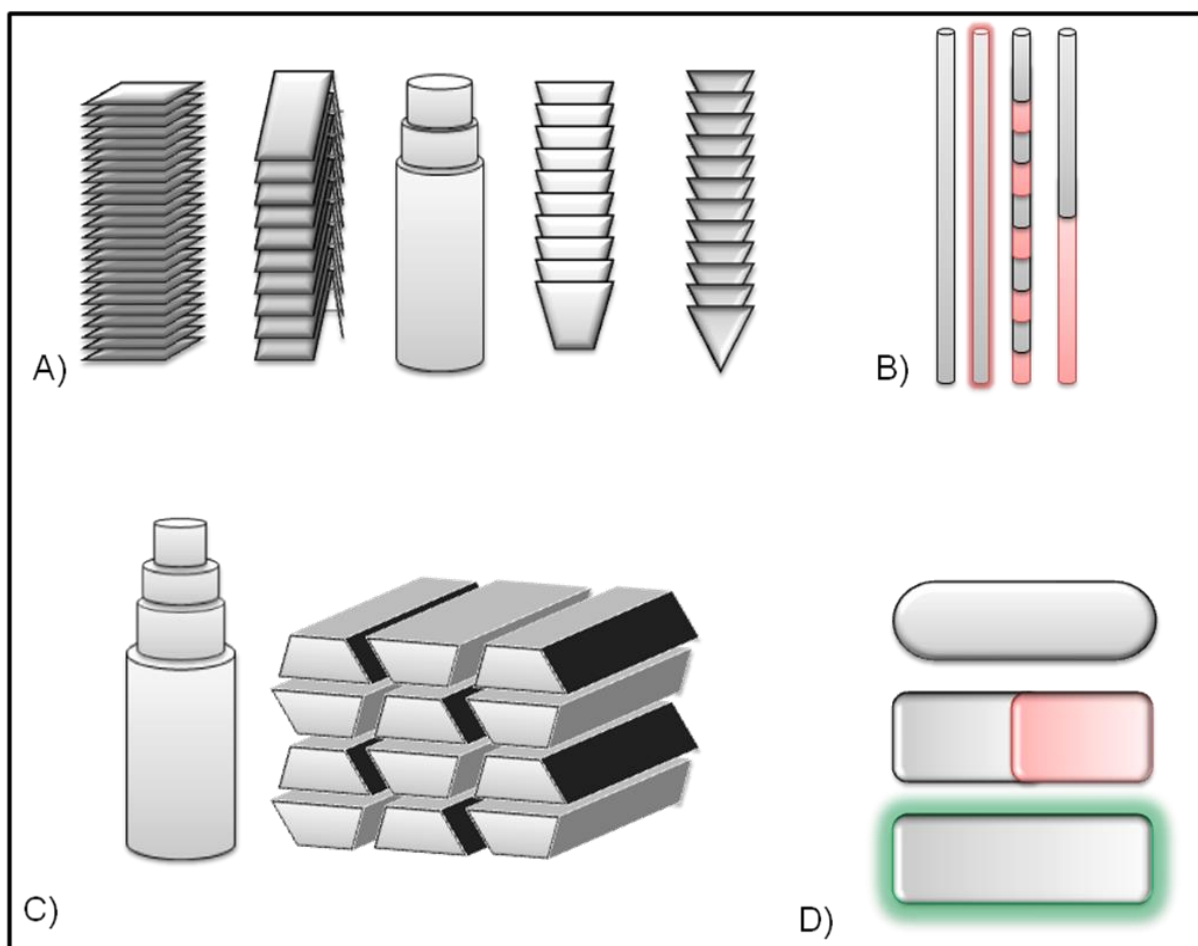
1165 **FIGURE 2. Magazine advertisings from different asbestos product manufacturers in the beginning of**
1166 **20th century.**

1167 Asbestos is the typical example of a versatile material with exceptional properties which turned out to be
1168 one of the largest occupational tragedies. With appropriate studies on the new HARNs we have the possi-
1169 bility to develop safe material design and manufacturing strategies before a large scale commercialization
1170 takes place. Media source: 1) Turner Brothers Asbestos Company, Manchester, UK, 1918; 2) Keasbey &
1171 Mattison Company, Ambler, PA, 1928; 3) L. W. Kerney, Chicago, IL, 1905; 4) Johns-Manville Company,
1172 New York, NY 1925; 5) Industrial Gloves Company, Danville, IL, 1946.



1175 **FIGURE 3. Scheme of the structure of most common HARNs.**

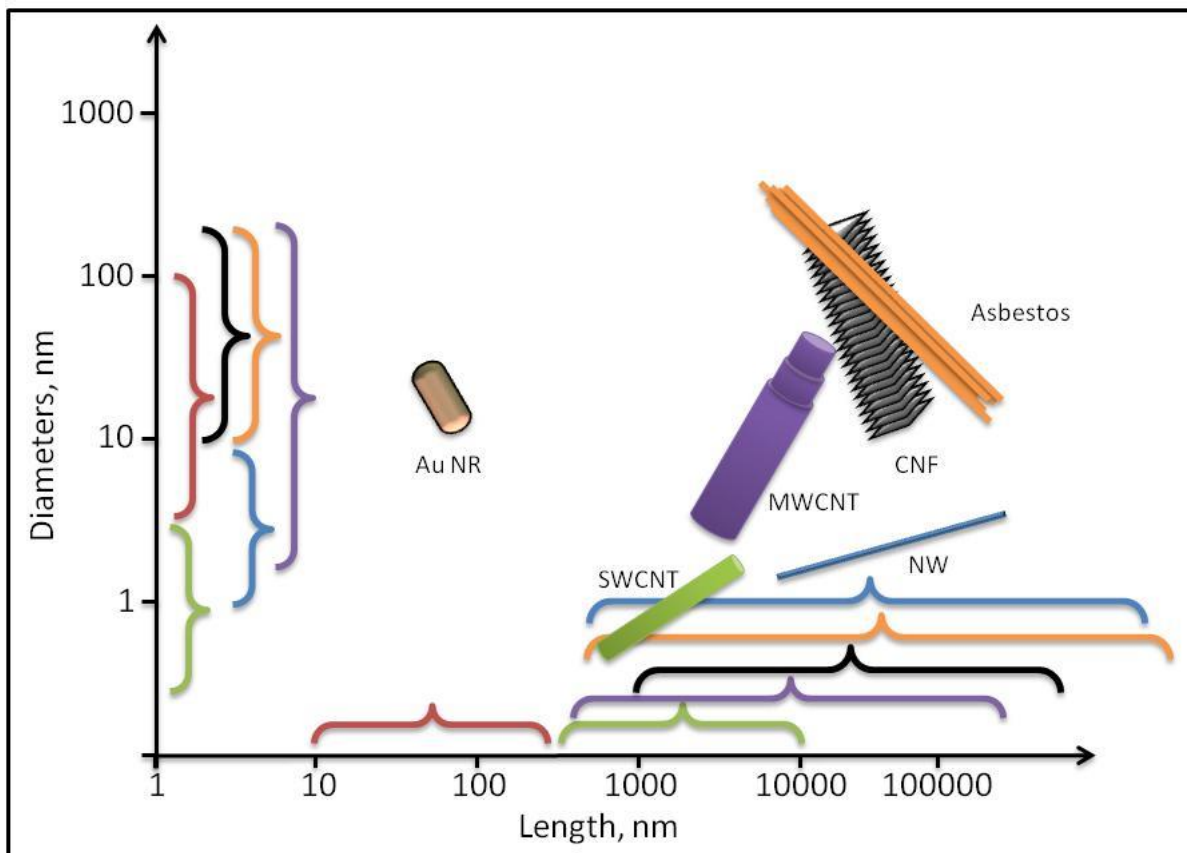
1176 Schematic representation of the structures of A) carbon nanofibers with different arrangement of the
1177 graphene layers: (from left to right) platelets stacked perpendicularly to the fiber axis (platelet CNF);
1178 platelets angled to the axis (herringbone CNF); carbon nanotubes; stacked cups CNF; stacked cones CNF.
1179 B) nanowires: (from left to right) single segment or single crystal NW, two components radial or core shell
1180 NW, two components multisegment axial NW, two components axial or two segment NW; C) asbestos:
1181 (from left to right) serpentine and amphibole; D) nanorods: (from top to bottom) single metal NR, bi-
1182 component NR (e.g., Au-Pt), coated NR.



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1184

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

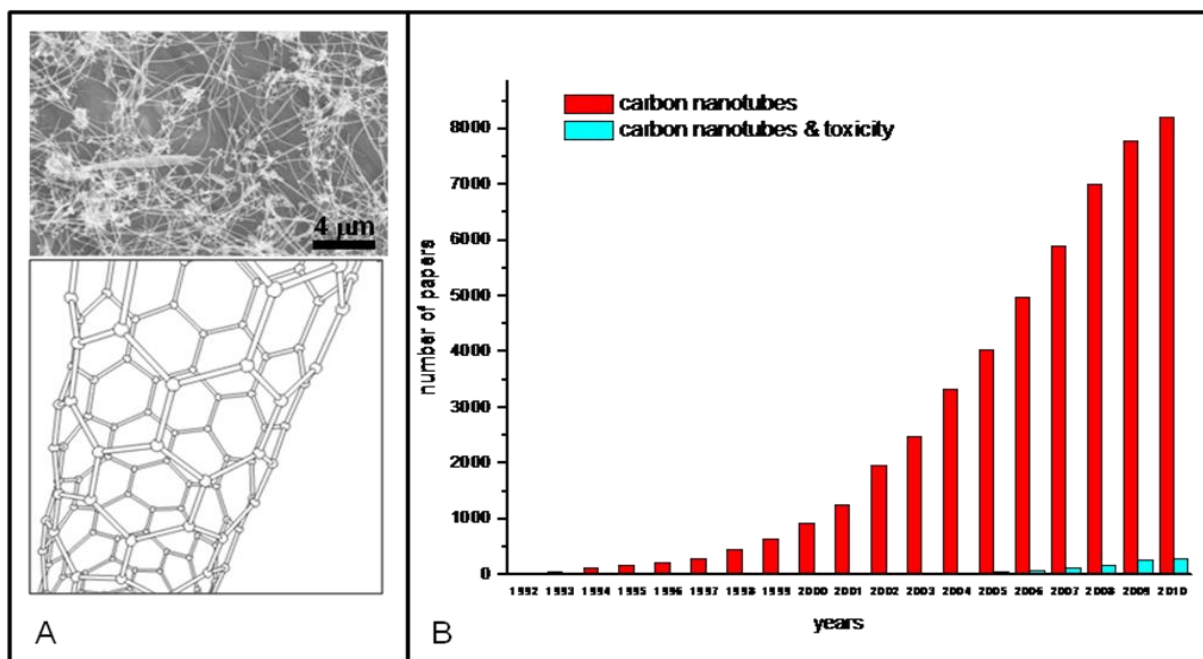


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1188 **FIGURE 5. Carbon-nanotubes: hype or hope?**

1189 A) Schematic structure of the carbon-carbon bonds and a scanning microscopy image of carbon nano-
1190 tubes. B) The rate of growth of publications on carbon nanotubes (red) compared to those devoted to
1191 their toxicity (pale blue). Clearly studies on the synthesis and application of CNTs pay more than those de-
1192 voted to the hazard associated to their production and use.



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1196 **TABLE 1. CNT vs. Asbestos.**

1197 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
1198 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 | |

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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 | <u>amphiboles</u> : octahedrally coordinated cations layers sandwiched between tetrahedral silicate layers <u>chrysotile</u> : multi-layered brucitic layers intercalated with silicate rolled sheets | |
| Defects | <i>amount</i> | yes, variable depending upon synthesis procedures | high | yes, variable depending upon size | variable, low | largely variable in natural minerals |
| | <i>kind</i> | 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 graphene 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 | <u>amphiboles</u> : high in all media <u>chrysotile</u> : generally high, low stability in acidic media | |

