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In vivo Toxicity Studies of Pristine Carbon Nanotubes: A Review

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1. Introduction

Discovered in 1991(Iijima, 1991), carbon nanotubes (CNTs) have attracted considerable attention in many fields of science and technology because of their unique structural, mechanical, and electronic properties. Their potential seemed paramount in the fields of materials science, including conductive and high-strength composites, energy storage and energy conversion devices, sensors, field emission displays and radiation sources, hydrogen storage, nanometer-sized semiconductor devices, probes and interconnects (Dresselhaus et al., 2004). Thus their studies progressed rapidly during the last two decades.

The chemistry of CNTs grew out from the efforts to open and fill the tubes (Ruoff et al., 1993; Sloan et al., 1998 as cited in Harris, 2009) and, indeed, to functionalize their sidewalls (J. Chen et al., 1998; Y. Chen et al., 1998). The first approach was driven partly for the study of matter in confined spaces and partly in order to use the tubes as templates for nanowires (Harris, 2009).

On the other hand, CNT functionalization was needed to disperse ("to solubilize") the tubes in aqueous media (Hirsh, 2002). Then, functionalization with biomolecules has become in vogue and many research groups have begun to investigate biological uses of these new types of nanostructures. According to some authors, CNTs could be possibly used as biosensors (Balavoine et al., 1999; Bekyarova et al., 2005; Lin et al., 2004; Richard et al., 2003; J. Wang et al., 2003; S. Wang et al., 2003), substrates for neuronal growth (Hu et al., 2004; Mattson et al., 2000; Lovat et al., 2005), supports for adhesion of liposaccharides to mimic cell membranes (X. Chen et al., 2004), delivery systems (Bianco et al., 2005), medical imaging agents (Ashcroft et al., 2007; Hartman et al., 2008; Sitharaman et al., 2005) and radiotherapeutics (Hartman et al., 2007a). Such potential uses remind those proposed for fullerenes and derivatives in the nineties (Jensen et al., 1996).

The growing use and mass production of CNTs raised concerns about their safety and environmental impact soon after the announcement of the national nanotechnology initiative by the US president in 2000 (http://www.nano.gov/). First toxicity studies addressed their safety at workplace (Oberdorster et al., 2005). Since then, the investigations of their toxicity first *in vitro* and then *in vivo* were reported in countless publications but their results remain contradictory (Kolosnjaj et al., 2007). As mentioned

by most authors, this is due to several factors including surface defects, sizes, and degree of aggregation of the tested material, and exposure protocols (Oberdorster et al., 2005, Kolosnjaj et al., 2007).

In this chapter we will try to present an uptake of the current knowledge on CNT toxicity as a function of the route of administration. Because of the great number of papers devoted to this subject, we are quite aware that we will miss quoting a number of works and we apologize to forgotten colleagues.

For a better understanding of biological behaviour of CNTs we will first describe their main general characteristics.

2. General characteristics

Carbon nanotubes are mainly composed of sp^2 bonds, similar to graphite, and are categorized as single-walled nanotubes (SWNTs) and multi-walled nanotubes (MWNTs).

2.1 Structure

2.1.1 Single-walled carbon nanotubes (SWNTs)

Single-walled carbon nanotubes, composed by a rolled monolayered graphene sheet (that might be end-capped by half a C60 molecule), exist in a variety of structures corresponding to the many ways a sheet of graphite can be wrapped into a seamless tube. Each structure type has a specific diameter and chirality, or wrapping angle (α). The "armchair" structures (Fig.1 a), with $\alpha = 30°$, have metallic character. The "zigzag" tubes (Fig.1 b), for which $\alpha = 0°$, can be either semi-metallic or semiconducting, depending on the specific diameter. Nanotubes with chiral angles intermediate between 0 and 30° (Fig.1 c) include both semimetals and semiconductors. The terms "armchair" and "zigzag" refer to the pattern of carbon–carbon bonds along a tube's circumference (Dresselhaus et al., 2004).
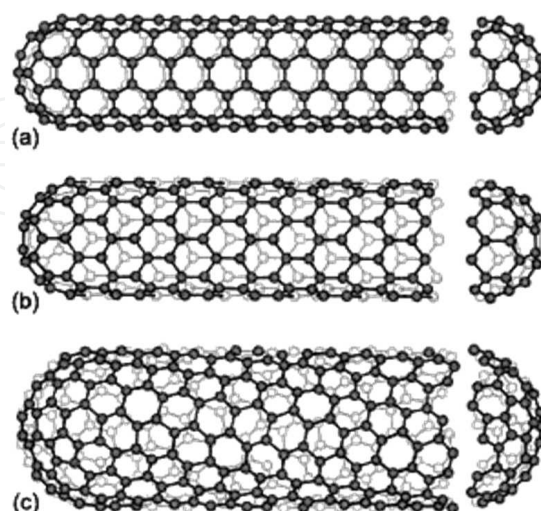

Fig. 1. Schematic representations of SWNTs in a variety of structures: (a) "armchair" (b) "zigzag" stricture (c) "intermediate" structure.

The diameter of the tubes generally varies from 0.4 to 20 nm, while the length usually reaches several micrometers. The tubes are often entangled and the ropes (Fig. 2) of SWNTs are held together by van der Waals forces (Popov et al., 2004).
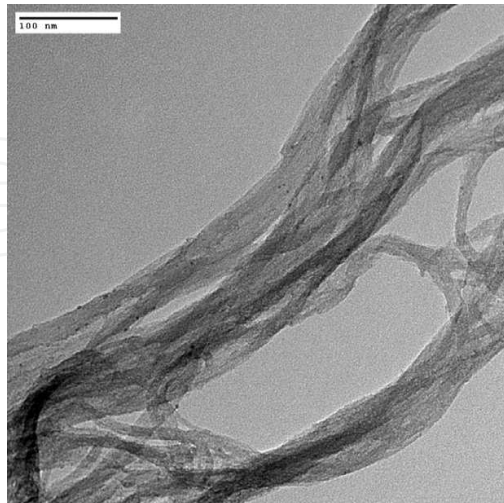

Fig. 2. Transmission electron micrograph of ropes of SWNTs

2.1.2 Multi-walled carbon nanotubes (MWNTs)

Multi-walled carbon nanotubes possess several graphitic concentric layers (Fig.3), made either by a single rolled graphene layer (resembling a scroll of parchment) or, more commonly, encased within one another (as Russian nesting dolls). The distance between each layer of graphene in a MWNT is about 0.34 nm (Iijima, 1991).
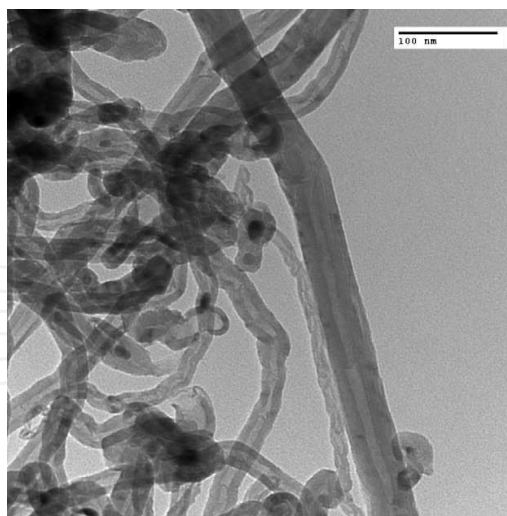

Fig. 3. Transmission electron micrograph of a bundle made of individual entangled MWNTs.

2.2 Impurities

Carbon nanotube powder often contains up to 30% metal (mainly iron and nickel) catalyst particles, as we can see on figure 2 (note the little round electron-dense particles present on

nanotube ropes), and some amorphous carbon. Carbon nanotubes should be purified prior to their administration, in order to avoid any metal catalyst-related toxicity (Valko et al., 2005).

Several techniques of purification have been reviewed elsewhere (Sinha & Yeow, 2005) and the most commonly used technique appears to be the oxidation using strong acid treatments, which allows solubilisation and removal of a large part of the metallic impurities. Nevertheless, this methodology has an impact on the tubes. Strong acid conditions cut the tubes in shorter pieces and generate carboxylic functions at the tips and around the sidewalls where the curvatures of the tubes present a higher strain (Ziegler et al., 2005).

2.3 Dispersibility

Pristine (chemically unmodified) CNTs are insoluble and hardly dispersible in water. In order to disperse CNTs several groups proposed covalent and non-covalent functionalization, which will be described in this chapter in terms of *in vivo* interactions.

3. Interfacing pristine carbon nanotubes with living organisms

The interaction of CNTs with cells has been described by many research groups and has been reviewed by several authors, all converging to the conclusions that the toxicity of CNTs *in vivo* depends on the type of CNTs, including the method of production, impurities and purification process (which might affect the sidewalls), length, aggregation state, surface coating, and chemical modification. Moreover, special care is needed for the choice of reagents used to evaluate the viability of the cells (Kroll et al., 2009), as these nanoparticles may interact with assays and even dispersion agents, potentially resulting in a secondary rather than primary toxicity (Casey et al., 2007).

These physical and chemical characteristics are, indeed, important in *in vivo* studies. However, we should also consider other phenomena that may occur in complex living systems, such as interaction of CNTs with several different cells at a time, biotransformation and innate foreign body reactions, etc.

Organism-CNT interactions were first described in *in vivo* toxicity studies, which were performed in order to assess the exposure risks to CNTs at workplace. Airborne CNTs might represent a danger to people handling these materials on daily basis either by crossing their skin barrier or entering and residing in their lungs. However, in recent years, potential applications of CNTs in the biomedical field intensified the research on the *in vivo* behaviour of these materials and increased the number of studies devoted to the evaluation of their potential toxicity after non-pulmonary routes of administration.

3.1 Pilot studies on carbon nanotubes in regards to workplace safety

In 2001 the potential of CNTs to induce skin irritation was evaluated by performing two routine dermatological tests (Huczko & Lange, 2001). Initially, 40 volunteers with allergy susceptibilities were exposed for 96 h to a patch test consisting in a filter paper saturated with a water suspension of unrefined CNTs synthesized by the arc discharge process. Secondly, a modified Draize rabbit eye test using a water suspension of unrefined CNTs

was conducted with four albino rabbits monitored for 72 h after exposure. Both tests showed no irritation in comparison to a CNT-free soot control and it was concluded that "no special precautions have to be taken while handling these nanostructures'' (Huczko & Lange, 2001).

In a second two-part study, other investigations have been made to seek for exposure routes and toxicity of SWNTs (Maynard et al., 2004). The study was undertaken to evaluate the physical nature of the aerosol formed from SWNTs during mechanical agitation. This was complemented by a field study in which airborne and dermal exposure to SWNTs was evaluated while handling unrefined material. Although laboratory studies indicated that unrefined SWNT material could release fine particles into air under sufficient agitation, concentrations generated while handling material in the field were very low. Estimates of the airborne concentrations of nanotube materials generated during handling suggest that concentrations were lower than 53 µg/m^3 in all instances. In another way, glove deposits of SWNTs during handling were estimated at between 0.2 mg and 6 mg per hand (Maynard et al., 2004).

3.2 Respiratory exposure: Pulmonary toxicity

Carbon nanotubes are very light and could become airborne and potentially reach the lungs; therefore the earliest *in vivo* studies tried to assess their pulmonary toxicity (Lam et al., 2004; Warheit et al., 2004).

For this purpose three kinds of SWNTs were studied, namely raw and purified HiPco and CarboLex CNTs. The first material is rich in iron impurities and the last one contains nickel and yttrium impurities. The particles were dispersed by brief shearing (2 min in a small glass homogenizing tube) and subsequent sonication (0.5 min) in heat-inactivated mouse serum. Mice were then intra-tracheally instilled with 0, 0.1, or 0.5 mg of CNT or carbon black or quartz particles used as negative and positive control, respectively. Seven and 90 days after this single treatment the animals were sacrificed for histopathological examination of the lungs. All CNT treatments induced dose-dependent epithelioid granulomas and, in some cases, interstitial inflammation in the animals euthanized after 7 days (Lam et al., 2004). These lesions persisted and were more pronounced in the group euthanized after 90 days. The lungs of some animals also revealed peri-bronchial inflammation and necrosis that had extended into the alveolar septa. The lungs of mice treated with carbon black were normal, whereas those treated with high-dose quartz revealed mild to moderate inflammation. These results show that, under these conditions and on an equal- weight basis, if carbon nanotubes reach the lungs, they are much more toxic than carbon black and can be more toxic than quartz, which is considered a serious occupational health hazard in chronic inhalation exposures (Lam et al., 2004).

In a similar way, a parallel pulmonary toxicity assessment of pristine SWNTs was described (Warheit et al., 2004). The aim of the study was to evaluate the acute lung toxicity of intratracheally instilled SWNTs in rats. The applied CNTs were produced by laser ablation and contained about 30 to 40% amorphous carbon (by weight) and 5% each of nickel and cobalt. The lungs of rats were instilled either with 1 or 5 mg/kg of the following control or particle types: SWNTs, quartz particles (positive control), carbonyl iron particles (negative control), and the vehicle - phosphate buffered saline (PBS) and 1% Tween 80, or graphite

particles (Warheit et al., 2004). Following exposure, the lungs of treated rats were assessed using bronchoalveolar fluid biomarkers and cell proliferation methods, as well as by histopathological examination of lung tissue at 24 h, 1 week, 1 month, and 3 months post-instillation. Exposures to high-dose (5 mg/kg) of SWNT produced mortality in approximately 15% of the instilled rats within 24 h post-instillation. This mortality resulted from mechanical blockage of the upper airways by the instilled particulate SWNT. In the surviving animals, SWNT produced temporary inflammatory and cell injury effects. Results from the lung histopathology indicated that pulmonary exposures to SWNT in rats produced a non-dose-dependent series of multifocal granulomas, which were evidence of a foreign tissue body reaction. However, they were non-uniform in distribution and not progressive beyond one month of post-exposure. The observation of SWNT-induced multifocal granulomas was inconsistent with the following: lack of lung toxicity by assessing lavage parameters, lack of lung toxicity by measuring cell proliferation parameters, apparent lack of a dose response relationship, non-uniform distribution of lesions, the paradigm of dust-related lung toxicity effects, and possible regression of effects over time. The observation of granulomas, in the absence of adverse effects measured by pulmonary endpoints was surprising, and did not follow the normal inflammogenic/fibrotic pattern produced by fibrogenic dusts, such as quartz, asbestos, and silicon carbide whiskers (Warheit et al., 2004).

While the first authors (Lam et al., 2004) concluded that SWNT were more toxic than quartz nanoparticles and crystalline silica particles, the second ones (Warheit et al., 2004) observed only a transient pulmonary inflammation and granuloma formation after SWNT exposure, contrarily to sustained lung inflammation, cytotoxicity, enhanced lung cell proliferation, foamy macrophage accumulation and lung fibrosis after exposure to quartz particles. The differences between these findings may be related in part to species differences (mouse vs. rat), but are more likely due to the differences in the experimental designs of the two studies (Warheit, 2006).

Respiratory toxicity of MWNTs has been also evaluated after intra-tracheal administration of MWNTs or ground MWNTs suspended and sonicated in sterile 0.9 % saline containing 1 % of Tween 80, at doses of 0.5, 2.0 or 5.0 mg, corresponding to approximately 2.2 mg/kg, 8.9 mg/kg and 22.2 mg/kg body-weight (bw) to Sprague-Dawley rats (Muller et al. 2005). The applied CNTs were still present in the lungs after 60 days (80% and 40% of the lowest dose) and both induced inflammatory and fibrotic reactions (Muller et al. 2005). At 2 months, pulmonary lesions induced by MWNTs were characterized by the formation of collagen-rich granulomas protruding in the bronchial lumen, in association with alveolitis in the surrounding tissues. These lesions were caused by the accumulation of large MWNT agglomerates in the airways. Ground CNTs were better dispersed in the lung parenchyma and also induced inflammatory and fibrotic responses. Both MWNTs and ground MWNTs stimulated the production of TNF-α in the lung of treated animals (Muller et al. 2005).

The physiological relevance of intra-tracheal instillation of CNTs is debatable since physiologically inspired particles would probably encounter several barriers in the upper respiratory tract before reaching the trachea and the lungs. Nevertheless, purified SWNTs elicited inflammation, fibrosis and granulomas formation in C57BL/6 mice even when administered by pharyngeal aspiration (Shvedova et al., 2005). The nanotubes used in this study were produced by HiPco and where further purified by acidic treatment. The analysis

also proved that CNTs accounted for more than 99% of carbon. The animals were treated with either SWNT (0, 10, 20, 40 μg/mouse) or two reference materials (ultrafine carbon black or SiO2 at 40 μg/mouse). The animals were sacrificed at 1, 3, 7, 28, and 60 days following exposures. A rapid progressive fibrosis found in mice exhibited two distinct morphologies: 1- SWNT-induced granulomas mainly associated with hypertrophied epithelial cells surrounding dense micrometer-scale SWNT aggregates and 2- diffuse interstitial fibrosis and alveolar wall thickening likely associated with dispersed SWNTs. These differences in fibrosis morphology were attributed to the distinct particle morphologies of compact aggregates and dispersed SWNT structures. Importantly, deposition of collagen and elastin was also observed in both granulomatous regions as well as in the areas distant from granulomas. Increased numbers of alveolar type II (AT- II) cells, the progenitor cells that replicate following alveolar type I (AT-I) cell death, were also observed as a response to SWNT administration. Moreover, functional respiratory deficiencies and decreased bacterial clearance (Listeria monocytogenes) were found in mice treated with SWNT (Shvedova et al., 2005).

In a mechanistically oriented study, the physicochemical determinants of the MWNTs' toxicity mechanism were investigated (Muller et al., 2008). In this experiment the toxicity of MWNTs was evaluated after the tubes were heated at 600°C (which allowed loss of oxygenated carbon functionalities and reduction of oxidized metals) or at 2400°C (which annealed structural defects and eliminated metals) or after the MWNTs heated at 2400°C were grinded (introduction of structural defects in a metal-deprived framework). The MWNTs were suspended in 1% Tween 80 and physiological saline and administered intra-tracheally (2 mg/rat). The results show that pulmonary toxicity (and genotoxicity of MWNTs, determined *in vitro*) were reduced upon heating but restored upon grinding, indicating that the intrinsic toxicity of the tubes was mainly mediated by the presence of defective sites in their carbon framework (Muller et al., 2008).

Finally, in order to check the hypothesis linking lung toxicity to CNT aggregates (Mutlu, Budinger et al., 2010), the authors instilled intratracheally unpurified aggregated or highly dispersed SWNTs in 1% Pluronic F 108NF to mice. As-produced HiPco SWNTs were either dispersed in PBS or highly dispersed in Pluronic solution in a dose of 40 μg, which was chosen to match or exceed those previously reported to cause pulmonary fibrosis in mice (Mutlu, Budinger et al., 2010; Shvedova et al., 2005). According to the authors, lung inflammation induced by SWNTs is minimal compared to that induced by urban particulate matter or asbestos fibers (used as positive control). Aggregated SWNTs in PBS caused areas of chronic inflammation, while highly dispersed SWNTs do not cause any inflammation or fibrosis. Moreover, nanoscale dispersed SWNTs are taken up by alveolar macrophages and cleared from the lung over time (Mutlu, Budinger et al., 2010). Besides, by administering unpurified CNTs, the authors (Mutlu, Budinger et al., 2010) avoided a potential effect due to surface defects of tube sidewalls (Ziegler et al., 2005), which might contribute to an increase in collagen deposition (Mercer et al., 2008).

In a study where rats were instilled with 0.04, 0.2, or 1 mg/kg of individually dispersed MWNTs in Tween 80 (Kobayashi et al., 2010), it has been observed that pulmonary inflammatory responses occur only in the lungs of the group treated with the highest dose. Moreover, the authors did not find any evidence of chronic inflammation, such as angiogenesis or fibrosis, induced by MWNT instillation (Kobayashi et al., 2010). Light

microscopic examination indicated that MWNT aggregates deposited in the lungs were phagocytized by alveolar macrophages and were accumulated in the lungs until 6-month post-exposure. These aggregates were located in the alveolar or interstitial macrophages, but individual MWNTs were not present in the cells of the interstitial tissue (Kobayashi et al., 2010). However, in the light micrograph panels, provided by the authors (Kobayashi et al., 2010) MWNTs seem to extend outside the macrophages in several directions, which is commonly referred to as incomplete or frustrated phagocytosis that is known to be a pro-inflammatory condition (Balkwill & Mantovani, 2001). According to the authors, the MWNTs used in this study were less than 20 μm long, but after measuring the tubes inside the alveolar macrophages, they concluded that median length was approximately 1.5 μm, although some tubes were measuring up to 6 μm (Kobayashi et al., 2010).

In contrast, a recent study reported that highly dispersed MWNTs could, depending on the way of administration (i.e. intratracheal instillation or inhalation) and dose, produce pulmonary lesions (Morimoto et al., 2011). The MWNTs that were used in this study were ground in a fructose mold - the fructose was rinsed afterwards with water and hydrogen peroxide. According to the authors this process slightly oxidized the tubes, which were subsequently dispersed in a 0.05% Triton X-100 solution (Morimoto et al., 2011). Triton X-100 is often used in cell biology to digest the cell membrane and cytoplasm to access the cell nucleus (http://fr.wikipedia.org/wiki/Triton_X-100). In the experiment of intratracheal instillation, two single doses (0.2 mg or 1 mg, 1.1 μm of mean particle length) were administered to rats and the study was conducted up to 6 months. While only a transient infiltration of inflammatory cells was observed for 0.2 mg treated animals, the high dose caused small granulomatous lesions and transient collagen depositions. In parallel, the authors conducted an inhalation study of dispersed MWNTs in a daily average mass of 0.37 ± 0.18 mg/m³ (Morimoto et al., 2011). The rats were exposed to aerosol particles for 6h per day, 5 days a week for 4 weeks. At the end of the experiment, the dispersed MWNTs with the average length of 1.1 μm caused only a minimal transient inflammation, which did not cause neutrophil infiltration into alveolar space. Moreover granulomatous lesions or collagen depositions were not observed (Morimoto et al., 2011).

In conclusion, the studies performed thus far indicate that due to van der Waals interactions individual SWNTs are prone to form large aggregates, in air or in aqueous solutions, which can be more than one hundred micrometers in diameter. While accidental industrial exposure is the most probable risk and might have a serious impact at workplace, toxicity was not observed after the intratracheal instillation of nanoscale dispersed SWNTs at a dose of 1.6 mg/kg (Mutlu, Budinger et al., 2010), or nanoscale dispersed MWNTs at a dose of 0.66 mg/kg (Morimoto et al., 2011). It is worthy to note that these doses would be the equivalent to a single instilled dose of approximately 112 g or 46 g, respectively, in an average weighting human.

3.2.1 Respiratory exposure and health risks

The analogy between CNTs and asbestos fibres was pointed out in the late nineties (Service, 1998). The term asbestos refers to a variety of fibrous silicates, which were exploited commercially in past for their desirable physical properties, such as sound absorption, average tensile strength, and resistance to fire, heat, electrical and chemical damage (http://en.wikipedia.org/wiki/Asbestos). Asbestos fibres have a high aspect ratio and they

are characterized by high chemical stability in physiological environment (Kane & Hurt, 2008). The pathologies related to asbestos exposure, especially lung fibrosis (asbestosis) and lung cancer (mesothelioma) that most often originates in the pleura, the outer lining of the lungs, have caused a major worldwide occupational health disaster (Donaldson & Poland, 2009) and founded reasonable fear of these airborne fibres.

Each lung is invested by a membrane, the pleura, which is arranged in the form of a closed invaginated sac. The membrane lining on the lung with its 'visceral' mesothelial layer is the visceral pleura and the membrane attached to the chest wall, covered by a continuous 'parietal' mesothelial cell layer is the parietal pleura. The pleural mesothelium is the primary mesothelial target for inhaled fibres (Donaldson et al, 2010). The space between the visceral and the parietal pleura contains the pleural fluid and a population of pleural macrophages. Pleural liquid is derived mainly from capillaries in the parietal pleura and is principally removed by lymphatic stomata in the parietal pleura (Lai-Fook, 2004), which drains the pleural fluid to the lymph nodes. This turnover is important for clearance of particles and fibres that reach the pleural space (Donaldson et al, 2010). While the exact mechanism of fibre deposition in the pleural mesothelium remains unclear, research indicates that retention of biopersistent fibres at the parietal pleura initiates mesothelial injury and inflammation that, overtime, lead to mesothelioma. When the fibre diameter is small, the fibre will align with the flow and deeply penetrate the lungs. The fibres are more or less cleared by macrophages, depending on their length. If the fibers are too long they cannot be entirely phagocytized. This unachieved – 'frustrated' phagocytosis is pro-inflammatory condition, characterized by the release of inflammatory mediators into the environment. These mediators may recruit other cells (for example collagen synthesizing fibroblasts) or cause DNA damage and mutations to proliferating cells, which may in term cause tumour development (Balkwill & Mantovani, 2001). Carbon nanotubes are fiber-shaped, however, for what concerns SWNTs, they are flexible and bendable, and often entangled. These particle-sized tangles would not obey the fibre toxicity paradigm because of their non-fibrous geometry (Donaldson et al, 2010; Kane & Hurt, 2008). Multi-walled carbon nanotubes, on the other hand, are much stiffer and generally less entangled; therefore, if long enough, they might present a risk (Donaldson et al, 2010).

A study performed with nickel containing milled pluronic-suspended MWNTs (Ryman-Rasmussen et al., 2009) with a length ranging between 100 nm and 10 μm showed that the nanotubes are observed inside the sub-pleural tissue macrophages after a single 6 h inhalation exposure of 30 mg/m^3. Fibrotic lesions, which increase 2 and 6 weeks after exposure, remain focal and regional. This effect did not occur after exposure to a dose of 1 mg/m^3, which according to the authors corresponds to 0.2 mg/kg. While the authors did not find MWNTs-loaded macrophages inside the pleura, they did notice an increased number of pleural mononuclear cells.

A study published about the same time reported that MWNTs could reach the pleura after pharyngeal aspiration (Porter et al., 2010). The inflammation extended from lungs to pleura in half of the MWNT-exposed mice. At 56 days post exposure, MWNTs penetrated the pleura in two out of four mice treated with the highest MWNT dose. The inflammation induced by the nanotubes was transient at low doses but persistent through day 56 at a dose of 40 μg.

In another study performed by the same group (Mercer et al., 2010), the authors reported an initial high density of penetrations into the sub-pleural tissue and the intra-pleural space one day following aspiration of MWNTs (80 μg per mice). The kinetics of penetration decreased due to the clearance by alveolar macrophages by day 7 and reached steady state levels in the sub-pleural tissue and intra-pleural space from days 28 to 56. The majority of the tubes (62% of the dose) resided in alveolar macrophages, while 0.6% of tubes, reached the visceral pleura region (sub-pleura and intra-pleural space).

As it has been already emphasized (Donaldson et al, 2010), the question that we should ask with regard to any fibre in relation to mesothelioma is not "Do fibres reach the pleura?" but "Are fibres retained in the parietal pleura", which is the site of origin of pleural mesothelioma.

When long and short CNTs (as well as long and short asbestos fibres) were injected directly into the pleural space, the authors found (Murphy et al., 2011) evidence of length-related inflammation, with no significant inflammation when short tubes (fibres) were administered. While no short samples were visible at day 1 or day 7, the mesothelium, that was thicker on day 1, returned to its normal thickness by day 7. While short tubes and fibres cleared from the pleura through stomatal openings, long tubes and fibres remained inside the pleura near stomata, where they persisted and caused inflammation and progressive fibrosis.

3.3 Effects of carbon nanotubes after intra-peritoneal administration

While pulmonary toxicity studies clearly indicate that inhalation of CNTs aggregates represents a possible occupational health hazard, the toxicity of CNTs after *in vivo* administration through bio-medically relevant routes is still a matter of debate. Among different routes of administration, the intra-peritoneal way has several advantages, firstly it offers the possibility to administer larger doses of suspended nanoparticles and secondly, the peritoneal cavity has a recognized particle-clearance mechanism. Particles leaving the peritoneal cavity pass *via* the retrosternal route through stomata (pore like structures) to the parathymic (mediastinal) nodes to the upper terminal thoracic duct or right lymphatic duct (Abu-Hijleh et al., 1995). Moreover, the peritoneal cavity and its mesothelium-covered viscera were recognized as a convenient substitute for pleural cavity mesothelium in fibre toxicity studies (Donaldson et al., 2010).

The first study of *in vivo* toxicity of CNTs after intra-peritoneal administration was conducted in our laboratory in collaboration with the Department of Chemistry of Rice University (Hartmann et al., 2007b). We compared the acute toxicity of full-length and ultra-short CNTs suspended in a Tween 80 aqueous solution, under the same conditions we used since 1996 to study the acute and sub-acute toxicity of [60]fullerene in mice (Moussa, F. et al, 1996). Our preliminary results showed that irrespective of the length of the administered CNT material, CNT aggregates induced a granulomatous response inside the organs like that which occurs in lungs after inhalation or intra-tracheal instillation (Hartmann et al. 2007).

One year later, in a comparative study of MWNTs and asbestos fibres, it was reported that MWNTs exhibit a length-dependent pathogenic behaviour (Poland et al., 2008) including granuloma formation and inflammation. In order to assess the role of fibre length, samples of long and short asbestos fibres and MWNTs with length ranges less than 5 μm, less than 20 μm- referred to as short, tangled MWNTs; and long tubes of the mean of 13 μm (24% of

them were larger than 15 μm) and maximum 56 μm have been suspended in bovine serum albumin and saline and administered intra-peritoneally to mice in a dose of 50 μg per mouse. The MWNTs samples differed in the source, preparation and purification method (the short ones being purified by acid treatment). At day 7 after injection, the authors reported that only the samples containing long fibres (asbestos or MWNTs) caused significant polymorphonuclear leukocyte infiltration, protein exudation and granulomas. However, the mesothelial lining on the pleural side of the diaphragm was normal in every case (Poland et al., 2008) and the inflammation decreased by day 7. Short fibres of any kind did not cause significant inflammation, neither at day 1 nor at day 7 after administration, except for one mouse out of three in the group treated with short tangled MWNTs of the length < 20 μm. The overall conclusion of the study was that short MWNTs do not mimic the behaviour of long asbestos, but that their data cannot preclude the possibility that short MWNTs may be by some other mechanism that was not assessed in this study. Long MWNTs produced inflammation, foreign body giant cells and granulomas that were qualitatively and quantitatively similar to the foreign body inflammatory response caused by long asbestos. However, for the specimen treated with shorter MWNTs that did exhibit granuloma, the authors concluded that it was maybe due to the fact that the sample they injected was contaminated with long fibres, caused by some other unidentified component specific for the precise MWNTs sample, or the granulomas could have arisen spontaneously by chance (Poland et al., 2008).

In our laboratory (Kolosnjaj-Tabi et al., 2010) we administered Tween-suspended ultra-short (20-80 nm long) and full length SWNTs in a dose up to 1000 mg/kg. Our results indicated that regardless of the administered dose (50-1000 mg/kg b.w.), length, or surface state of the administered material, large aggregates of CNTs (>10 μm) irremediably induce granuloma formation (Fig. 4).
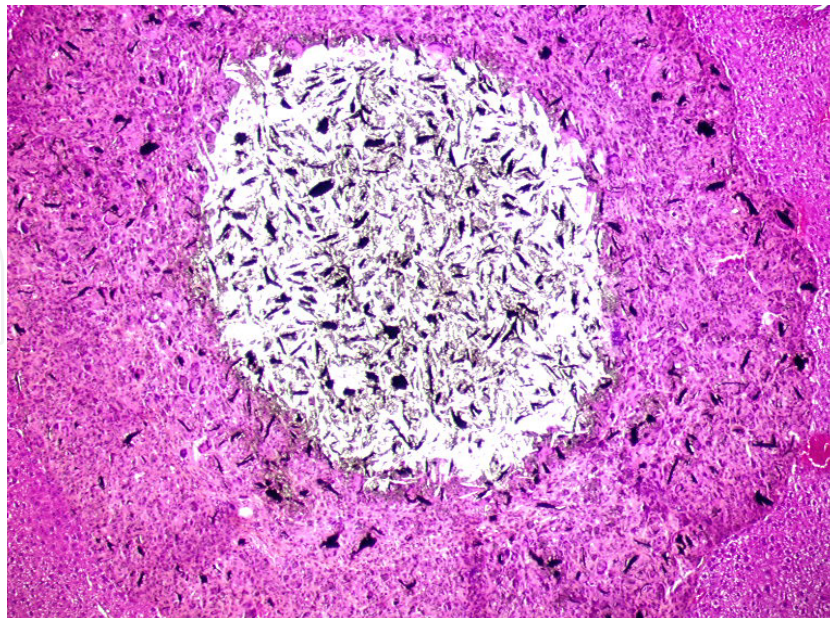

Fig. 4. Light micrograph after hematoxylin-eosin staining of a liver section from mice i.p. injected with a single dose of ultra-short SWNTs at 90 days post-administration showing a US-tube-laden granuloma. (Magnification = 10).

The administered doses were high, yet necessary to ascertain a sufficient circulating dose of administered material. The bolus dose was responsible, in set terms, for granulomas that were formed after aggregation of intra-peritoneally administered tubes. Smaller agglomerates (< 300 nm), on the other hand, did not induce granuloma formation nor did they cause any major life-threatening condition under our experimental conditions. A large portion of well-dispersed CNTs was eliminated through the kidney and the bile ducts. However, the aggregated part of the administered dose was not cleared from the body and persisted inside cells 5 months after administration (Fig. 5).
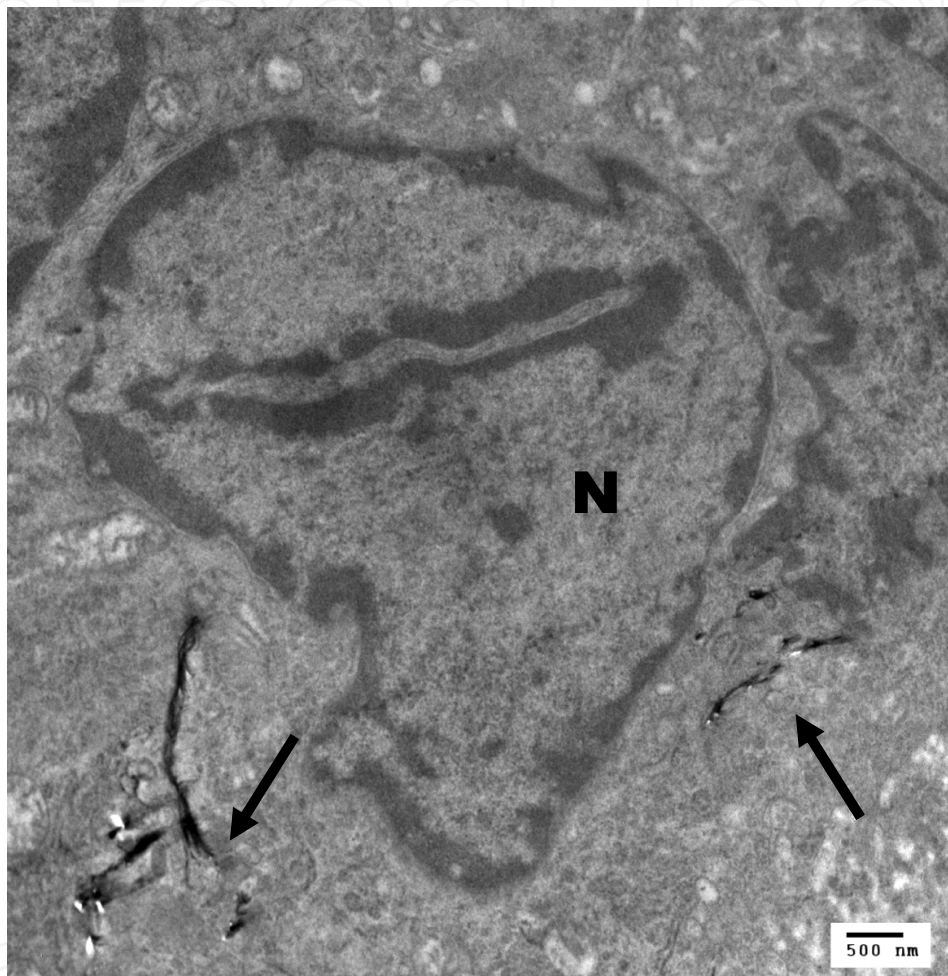

Fig. 5. Transmission electron micrograph of bundles of SWNTs in a Kuppfer cell, found in the liver of a mouse 5 months after treatment

The persistence of the nanotube aggregates inside the cells was probably due to the slow disaggregation and slow elimination of larger aggregates.

3.3.1 Carbon nanotubes and mesothelioma

The ability of MWNNTs to induce mesothelioma, a deadly cancer, in experimental models in rodents is still a matter of debate. This cancer is a highly specific response to bio-persistent fibres and may occur in the pleura (outer lining of the lungs and internal chest wall), peritoneum (the lining of the abdominal cavity), pericardium (a sac that surrounds the heart), or tunica vaginalis (a sac that surrounds the testis) (Moore et al., 2008).

In 2008, a Japanese team observed for the first time that MWNTs could induce mesothelioma after intra-peritoneal administration to p53 heterozygous mice (Takagi et al., 2008a). This type of mice are, however, more sensitive and have a shorter tumour onset rate than standard wild-type rodents. Indeed, different groups contested this study (Donaldson et al., 2008, Ichihara et al., 2008), but Takagi et al. explained and justified their experimental choices letting other studies to confirm mesothelium threats (Takagi et al., 2008b, c). The authors administered intra-peritoneally 3 mg of CNTs per mouse suspended in 1% Tween 80 and 0.5% methyl cellulose- aqueous solution. While the samples of crocidolite asbestos were evenly dispersed, MWNTs contained aggregates and fibres. The mice were monitored until one of the groups (MWNTs treated group) reached 100% mortality, which happened on day 180. The mice treated with MWNTs exhibited moderate to severe fibrous peritoneal adhesions, slight ascites, fibrous peritoneal thickening and a high incidence of macroscopic peritoneal tumours. Similar, but less severe findings were noted in the asbestos group. As the authors emphasized, it is important to limit the significance of this study to the monitoring of biological activity of a compartment of MWNTs longer that 5 µm. There is no information that this study method would be sensitive to pure nanometer-sized particles within the same timeframe (Takagi et al. 2008a).

An analogous study was made, in time, on wild-type rats (Muller et al., 2009) in a two years exposure period, where up to 20 mg of MWNTs with and without sidewall defects (induced by grounding of the raw material) suspended in phosphate buffer were intraperitoneally injected to a large number of rats. After 24 months, crocidolite induced mesothelioma in 34.6% animals whereas mesothelioma occurred only in 3.8% in the vehicle-treated rats. MWNTs with or without structural defects did not induce significant mesothelioma in this study, as mesothelioma occurrence was detected in up to 6% of MWNT-treated rats, which is in line with the spontaneous incidences of mesothelioma in rats (up to 6%). The incidence of tumours other than mesothelioma was not significantly increased across the groups (Bignon et al., 1995).

In contrast to what was observed after intra-peritoneal administration to wild type rats (Muller et al., 2009), MWNTs injected in a single intra-scrotal dose to rats, induced mesothelioma 37 to 40 weeks after treatment (Sakamoto et al., 2009). In the latter experiment, MWNTs were suspended in a 2% carboxymethyl cellulose aqueous solution and administered to rats at a dose of 0.24 mg/animal. According to the authors this dose corresponds to the maximal value recommended by the guideline for man-made mineral fibers (Bernstein & Riego Sintes, 1999, as cited in Sakamoto et al., 2009).

In conclusion, these findings also show that under some experimental conditions, MWNTs may induce mesothelioma formation. Thus, further investigations are urgently needed.

3.4 Effects of carbon nanotubes after sub-cutaneous administration

Subcutaneous implantations of clusters of MWNTs of different lengths (220 nm and 825 nm) in rats showed that the degree of inflammatory response around the shorter MWNTs was slighter than that observed around the longer ones, thus indicating that macrophages could envelop the shorter nanotubes more readily than the longer ones (Sato et al., 2005). However, no severe inflammatory response such as necrosis, degeneration or neutrophils infiltration was observed around both types of MWNTs.

These biological responses have also been described after subcutaneous implantations in mice of 2 mg/animal of SWNTs, two different types of MWNTs (20 and 80 nm of average diameter) and cup-stacked carbon nanotubes (CSNTs made with stacked truncated cones), for up to 3 months (Koyama et al., 2006). The nanotubes used in this study were purified by thermal treatment process during which the metal particles evaporate. After 1, 2, 3 weeks, 1 month, 2 months and 3 months post-implantation, the animals were sacrificed, blood was collected for CD4+ and CD8+ T-cells counting by flow-cytometry and tissue of skin (including muscle layers) were collected for histo-pathological examination. All mice survived, and no large changes in their weights were observed during the experimental period. After one week of implantation, only SWNTs activated the major histocompatibility complex (MHC) class I pathway of antigen-antibody response system (higher CD4+/CD8+ value), leading to the appearance of an oedematous aspect. After two weeks, significantly high values of CD4+ without changes in CD8+ signified the activation of MHC class II for all samples. The authors noted that antigenic mismatch becomes less evident with time, notably one month post-implantation, indicating an establishment of granuloma formation. Furthermore, the toxicological response of CNTs was absolutely lower than that of asbestos.

3.5 Effects of carbon nanotubes after intra-venous administration

3.5.1 Effects of SWNTs

The first report on the effects of SWNTs after intravenous administration to mice (3 mg/kg) monitored over four months indicated normal blood chemistries and normal histological examinations (Schipper et al., 2008). The animals did not show significant inflammatory lesions and SWNTs accumulated in liver and spleen as evidenced by Raman spectroscopy. The CNTs used in this study were highly dispersed with polyethylene glycol (PEG). CNT aggregates were eliminated with ultracentrifugation before administration to the animals.

Another study used highly dispersed pristine HiPco SWNTs with different PEGylated phospholipids (Liu et al., 2008). Big bundles and impurities were removed by centrifugation, and individually suspended tubes or small bundles were administered intravenously at a dose of approximately 20 µg or 100 µg per mouse. Blood, tissue and organ distribution and elimination in urines and faeces were evaluated by Raman spectroscopy, by assessment of the tangential graphite-like phonon mode (G band). Administered SWNTs accumulated mainly in liver and spleen, however the quantity decreased over a 3-month period. The authors concluded that SWNTs were mainly eliminated by the biliary pathway; only a small portion of short tubes (< 50 nm in length) was eliminated in the urines. Finally, the authors did not report any obvious sign of toxicity.

Other authors also reported low toxicity of SWNTs in mice over a 3-month period (Yang et al., 2008). Purified SWNTs were suspended in 1% Tween 80 aqueous solution and sonicated for 30 minutes prior administration to animals at various doses from 40 µg to 1 mg per mouse. Some of the serum biochemical parameters (ALT, AST and LDH) were higher in SWNT-treated animals compared to the control group 90 days post-exposure, indicating that induced hepatic injury and tissue breakdown were dose-dependent. The long-term accumulation of aggregated SWNTs was evidenced in histological sections of lungs, livers and spleens and was confirmed by Raman spectroscopy and transmission electron microscopy in organ lysates. However, no fibrosis was detected in the organs.

Embriotoxicity was recently reported as an effect caused by intravenous administration of SWNTs in mice (30 µg/mouse) (Pietroiusti et al., 2011). The authors used pristine, oxidized or ultra-oxidized by acid treatment SWNTs. Cobalt was the only impurity that was released in the medium in which the tubes were dispersed (DMEM cell culture medium with fetal bovine serum). Before the end of gestation, the animals were sacrificed, and uteri, placentas, and foetuses were examined. A high percentage of early miscarriages and foetal malformations were observed in females exposed to oxidized SWNTs, while lower percentages were found in animals exposed to the pristine material. The lowest effective dose was 100 ng/mouse. Extensive vascular lesions and increased production of reactive oxygen species (ROS) were detected in placentas of malformed but not of normally developed foetuses. Increased ROS levels were likewise detected in malformed foetuses. No increased ROS production or evident morphological alterations were observed in maternal tissues (Pietroiusti et al., 2011).

3.5.2 Effects of MWNTs

MWNTs dispersed in mouse-serum (10 min of sonication) and injected to mice at a dose of 200 or 400 µg per mouse showed no severe acute response, 24 hours after administration (Lacerda et al., 2008). However, mice treated with aggregates of pristine MWNTs exhibited subdued behavior, hunched posture, and signs of respiratory distress. While serum biochemistry data did not show significant increase, optical microscopy revealed aggregate accumulation, mostly in livers and lung vessels, which were probably responsible for respiratory distress. Nevertheless, no tissue degeneration, inflammation, necrosis or fibrosis occurred 24 hours after injection.

The effect of Tween-suspended pristine MWNTs and PBS-suspended acid oxidized MWNTs up to 2 months were investigated after administration to rats at a dose level of 10 or 60 mg/kg (Ji et al., 2009). The authors reported an impact on body weight gain of the highest dose. Severe inflammatory cell infiltration in the portal region, cellular necrosis and focal necrosis were seen at a dose of 60 mg/kg in the MWNT-treated group 15 and 60 days following the treatment. Moreover, severe mitochondrial swelling, bile canaliculi expansion, mitochondrial destruction, loss and lysis of mitochondrial crest were also observed. In the acid treated (oxidized) MWNTs group only slight inflammatory cell infiltration was observed after 2 months. A slight increase of AST activity used as biochemical marker of liver injury was also reported in treated animals, but it did not increase more than twofold. Interestingly, 329 genes were up-regulated and 31 genes that were down-regulated more than twofold in MWNT-treated mice and 1139 genes were up-regulated and 505 genes were down-regulated over twofold in the mice treated with oxidized MWNTs.

In order to avoid mechanical blockage by the administered material, it is of capital importance to administer only individually suspended, short CNTs. Thus, further studies with individually suspended CNTs have to be made in order to confirm the direct effect of these materials after administration by the intravenous route.

3.6 Effects of carbon nanotubes after oral administration

Oral administration of 1000 mg/kg of body weight of SWNTs to mice (Kolosnjaj-Tabi et al.) resulted in neither animal death nor behavioral abnormalities. Compound-colored stool was

found 24 h after gavage in all treated groups. Two weeks after treatment, regardless of the length or of the iron content, the nanotube materials did not induce any abnormalities after pathological examination, indicating that under these conditions, the lowest lethal dose (LDLo) is greater than 1000 mg/kg b.w. in Swiss mice.

The potential effects of MWNTs after oral administration were also investigated on pregnant dams and embryo-fetal development in rats (Lim et al., 2011). MWNTs were administered to pregnant rats by gavage at 0, 40, 200, and 1,000 mg/kg/day. All dams were subjected to Cesarean section on day 20 of gestation, and the foetuses were examined for morphological abnormalities. A decrease in thymus weight was observed in the high dose group in a dose-dependent manner. However, maternal body weight, food consumption, and oxidant-antioxidant balance in the liver were not affected by treatment with MWNTs. No treatment-related differences in gestation index, foetal deaths, foetal and placental weights, or sex ratio were observed between the groups. Morphological examinations of the foetuses demonstrated no significant difference in incidences of abnormalities between the groups.

Intriguingly, it has also been reported that CNTs may be involved in oxidative stress with oxidative damage of DNA in the colon mucosa, liver, and lung of rats after oral administration of SWNTs in a dose of 0.064 or 0.64 mg/kg b.w. suspended in saline solution or corn oil (Folkmann et al., 2009). Suspensions of particles in saline solution or corn oil yielded a similar extent of genotoxicity. However, corn oil per se generated more genotoxicity than the particles (Folkmann et al., 2009).

4. Conclusion

Considered together, these diverging results highlight the difficulties in evaluating the toxicity of CNT materials. While the toxicity is certainly governed by the state of aggregation, length and stiffness of CNTs, other parameters might be involved. Most of them probably depend of the method of production of the sample, the method of purification and the method of preparation of the tested formulations. As the reactivity and the general behaviour of CNTs in biological media are not completely understood, assessing the safety of these nanoparticles should also include a careful selection of appropriate experimental methods. Thus, more studies are needed in order to determine the safety of CNTs. For the time being, precaution is necessary notably in case of CNT-exposure at workplace.

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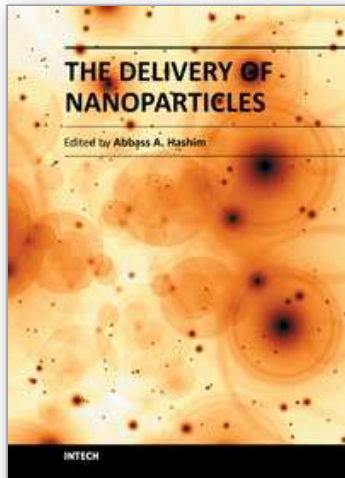
The Delivery of Nanoparticles

Edited by Dr. Abbass A. Hashim

Nanoparticle is a general challenge for today's technology and the near future observations of science. Nanoparticles cover mostly all types of sciences and manufacturing technologies. The properties of this particle are flying over today scientific barriers and have passed the limitations of conventional sciences. This is the reason why nanoparticles have been evaluated for the use in many fields. InTech publisher and the contributing authors of this book in nanoparticles are all overconfident to invite all scientists to read this new book. The book's potential was held until it was approached by the art of exploring the most advanced research in the field of nano-scale particles, preparation techniques and the way of reaching their destination. 25 reputable chapters were framed in this book and there were alienated into four altered sections; Toxic Nanoparticles, Drug Nanoparticles, Biological Activities and Nano-Technology.

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