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Carbon Nanotubes – Interactions with Biological Systems

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1. Introduction

Carbon nanotubes (CNT) are highly versatile materials, with an enormous potential for biomedical applications. Their properties are dependent upon production process and may be modified by subsequent chemical treatment.

Carbon nanotubes can be used to improve polymers' composites mechanical properties. Its tailoring allows for the creation of anisotropic nanocomposites (Kanagaraj et al, 2007; Koerner et al, 2004; Pulskamp et al, 2007; Sen et al, 2004). Due to their semi - conductive behaviour, its usage may provide electrical stimulation (Grunlan et al, 2004; Huang et al, 2003). The use of CNT as translocators in drug-delivery systems or in image diagnosis has also been suggested (Bianco et al, 2005; Cherukuri et al, 2004). High tumour accumulation of single-walled CNT (SWCNT) has been described, anticipating the possibility of further therapeutic uses (Liu et al, 2007). There are several studies on gas, temperature, pressure, glucose, chemical force and resonator mass sensors based on CNT (Barone et al, 2005; Barone et al, 2005b; Collins et al, 2000; Hrapovic et al, 2004; Kong et al, 2000; Lee et al, 2007; Lin et al, 2005; Perez et al, 2005; Wood et al, 1999; Yan et al, 2007; Yang et al, 2006; Yun et al, 2007).

In face of recent studies, special attention has been drawn into promising orthopaedic use of CNT for improving tribological behaviour and material mechanical properties. However, and considering the conductive properties of CNT the range of orthopaedic application may broaden up, since it is known that electrical fields as small as 0,1 mV/cm may enhance osteoblastic proliferation locally (Brighton et al, 1992). CNT based electrodes could be considered for integrating implantable orthopaedic devices. CNT have been reported to have direct and distinct effects on osteoblasts and osteoclasts metabolic functions (Narita et al, 2009; Sirivisoot et al, 2007; Tutak et al, 2009).

CNT have been discovered in 1991 (Iijima, 1991), but seem to have been around for quite a long time, since they were detected in gas combustion streams like the ones in normal households stoves (Murr et al, 2004). The fact that CNT are small enough to be inhaled has

raised the question of lung reaction to their presence. The impact on the skin of handlers and the environmental consequences of mass production are also pertinent interrogations, as it is the possibility of secondary organ dissemination.

2. Health hazards

2.1 Respiratory toxicity

Some authors described strong cytotoxic effects on guinea pig alveolar macrophages of SWCNT and, at a smaller extent, of multi-walled carbon nanotube (MWCNT), when compared to fullerenes (C60). The same authors also describe impairment of phagocytic activity (Jia et al, 2005). Cytotoxicity comparable to asbestos-particles induced on murine macrophages has been described by Soto (Soto et al, 2005). Experiments conducted by Magrez on three lung-tumor cell lines suggest CNT led to proliferation inhibition and cell death, although CNT showed less toxicity than carbon black nanoparticles and carbon nanofibers (Magrez et al, 2006). Davoren et al. assessed SWCNT cytotoxicity on a distinct lung-carcinoma cell line (A549) and describe SWCNT concentration - dependent toxicity and the protective effect of serum (Davoren et al, 2007).

Another study, conducted by Sharma, concluded that SWCNT induced oxidative stress in rat lung cells (Sharma et al, 2007). The same oxidative stress related changes are described by Herzog et al. in primary bronchial epithelial cells and A549 cells but the study points out that the length of the response is strongly dependent on the dispersion medium used (Herzog et al, 2009). Pulskamp also describes oxidative stress in two cell lines (rat macrophages NR8383 and human A549) cultured in contact with CNT. However, when comparing purified SWCNT and commercial CNT their findings suggested the biological effects were associated with the metal traces. They also describe puzzling divergent results between MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and WST (water soluble tetrazolium salt, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) viability assays, both dependent on the activity of mitochondrial dehydrogenases (Pulskamp et al, 2007). These discrepancies can only be explained based on interactions of non-soluble formazan crystals in MTT with CNT, as opposed to WST soluble final formazan salt.

The results of *in vitro* studies that suggest toxicity are partially supported by several *in vivo* studies but, once again, with often divergent conclusions.

Huczko instilled intratracheally a soot of CNT in guinea pigs and later measured tidal volume, breathing frequency, pulmonary resistance and bronchoalveolar fluid cell and protein content. These authors concluded that working with soot containing CNT was probably not a health hazard, but no histopathological study was referred (Huczko et al, 2001). Lam et al. conducted studies in mice and concluded the SWCNT could be toxic if they reached the lungs; Warheit et al. conducted a similar study in rats, describing granuloma formation and considering it as probably resulting from aggregates of CNT (Lam et al, 2004; Warheit et al, 2004).

Muller et al. compared asbestos, carbon black and MWCNT effects when instilled in the trachea of rats, at different doses. These authors described dose-dependent persistent inflammation and granuloma formation, more significant with MWCNT than with carbon black but less extensive than with asbestos (Muller et al, 2005).

Shvedova et al. described unusual acute inflammatory response, early granulomatous reaction and progressive fibrosis in mice exposed to SWCNT, leading to the conclusion of CNT intrinsic toxicity. This study used a technique of pharyngeal aspiration instead of the

intratracheal instillation used in the previous studies, and allowed aerosolization of fine SWCNT particles. These particles were associated with fibrogenic response in the absence of persistent local inflammation, suggesting health risks for workers (Shvedova et al, 2005). However, a more recent study describes significant changes in deposition pattern and pulmonary response when SWCNT are more evenly disperse in the suspension prior to pharyngeal aspiration (Mercer et al, 2008).

More recently, inhaled MWCNTs migration to the subpleura and associated increased number of pleural mononuclear cells and subpleural fibrosis was described in mice (Ryman-Rasmussen et al, 2009), further advising caution and appropriate security measures when handling CNT.

Wang et al. presented a study with dispersed SWCNT (DSWCNT) supporting data from previous reports, in the sense that they describe *in vitro* and *in vivo* stimulation of lung fibroblasts proliferation and collagen deposition, and metalloproteinase 9 increased expression, in the absence of inflammation (Wang et al, 2010).

It has also been hypothesized, and demonstrated for other types of nanoparticles, that following inhalation, nanoparticles may reach the central nervous system (CNS)(Elder et al, 2006). Nanoparticles enter the nervous system by transcytosis and are presented to neuron cells (Zensi et al, 2009). Studies showing that inhaled gold nanoparticles accumulate in olfactory bulb of rats and reach the cerebral cortex, as well as the lung and thereof other organs such as esophagus, tongue, kidney, aorta, spleen, septum, heart and blood (Yu et al, 2007).

These observations suggest that if there are high doses of nanoparticles in the air they can enter into the CNS via the olfactory nerve during accidental or prolonged environmental or occupational exposure to humans, and that nanoparticles may exert their effects not only on respiratory tract and neighboring organs but spread to distant organs.

2.2 Epidermal/dermal toxicity

Several studies have also been conducted on epidermal/dermal toxicity of CNT. Functionalized 6-aminohexanoic acid-derivatized SWCNT may cause dose-related rise in inflammatory cytokines (Zhang et al, 2007).

MWCNT induction inflammatory pathways may be similar to those of combustion-derived metals (Ding et al, 2005) and cause decreased cell viability, changes on metabolic, cell signalling, stress and cytoskeletal protein expression (Witzmann & Monteiro-Riviere, 2006). Other authors report presence of chemically unmodified MWCNT in cytoplasmic vacuoles of cultured human keratinocytes and induction of the release of interleukin 8 in a time dependent manner (Monteiro-Riviere et al, 2005) and SWCNT inhibition of HEK9293 cells growth through induction of apoptosis and decreased cell adhesion has also been described (Cui et al, 2005).

Patlolla et al. describe dose and time-dependent cytotoxicity, genotoxicity and induction of apoptosis by purified MWCNT in normal human dermal fibroblasts cells. The MWCNT used in this study had been treated for extraction of metal (Fe) impurities and then, by treatment with sulfuric/nitric acid, functionalized in very high degree. The authors report that 2 to 7% of final weight was due to carboxyl groups (Patlolla et al, 2010).

3. Biological response and mechanisms of toxicity

Whilst assessing *in vitro* cytotoxicity of SWCNT on fibroblasts and trying to bring some light on the issue of how the removal of catalytical metal would influence the toxicity, Tian et al. concluded that the refined SWCNT were more toxic, inducing significant changes on

cytoskeleton and cell morphology, probably because of the enhancement of the hydrophobic character by the refinement treatment, the toxicity seemingly directly related to surface area (Tian et al, 2006). Sayes et al. reported decreased SWCNT cytotoxicity in dermal fibroblasts with higher functionalization density (Sayes et al, 2006). However, other authors compared pristine and oxidized MWCNT effects on human T lymphocytes and described increased toxicity of oxidized CNT, with high doses, even if oxidation increased solubility (Bottini et al, 2006).

Koyama et al. reported time-dependent changes in T lymphocytes by measuring CD4 and CD8, associated with local granuloma formation after subcutaneous implantation in mice, although overall toxicological changes were in absolute lower then with asbestos (Koyama et al, 2006). These results might seem somehow in conflict with the findings by Dumortier et al. that concluded that functionalized SWCNT did not affect B and T lymphocytes viability. However, the authors emphasized that absence of functional changes was only observed in the CNT functionalized via the 1,3-dipolar cycloaddition reaction, in non - oxidized nanotubes (Dumortier et al, 2006). Brown et al. conducted *in vitro* studies that suggested monocytic cells' response is strongly dependent of morphology and state of aggregation of the CNT. Long, straight well-dispersed nanofilaments induced the production of more TNF- α and ROS than highly curved and entangled aggregates; incomplete uptake or frustrated phagocytosis of CNT was also described (Brown et al, 2007). Barillet et al. showed that short (0.1-5 nm) and long (0.1-20 nm) CNT, and the presence of metal residues, induced different cell response and toxicity (Barillet et al, 2010).

The same mechanisms of frustrated phagocytosis, increased production of proinflammatory cytokines and oxidative stress apparently justified the *in vivo* findings described by several authors. They conducted studies with longer implantation times and these effects may eventually lead to carcinogenesis (Fraczek et al, 2008; Poland et al, 2008; Takagi et al, 2008).

Whilst several authors describe low cellular uptake of SWCNT (Barillet et al, 2010; Davoren et al, 2007; Herzog et al, 2007), others describe high cellular uptake (Jia et al, 2005; Pulskamp et al, 2007). As suggested by Shvedova et al. and Mercer et al., the degree of uptake and the cell response may be dependent on the carrier of the CNT (Mercer et al, 2008; Shvedova et al, 2009). Protein adsorption to CNT surface and its subsequent structural change may trigger phagocytosis, and Barillet et al. suggest CNT may induce membrane damage by mechanical action (Barillet et al, 2010).

Bihari et al. reported SWCNT thrombogenic and platelet activation effects in mice and point out this could cause possible systemic problems, along with hindering the use of these materials for drug delivery (Bihari et al, 2010).

These recent studies stress the need of careful re-evaluation and research before enlarging the field of CNT application. However, as Fraczek et al, Lacerda et al, Schipper et al and Sitharaman et al, among others, findings suggest, CNT behaviour *in vivo* depends on their length, functionalization and degree of agglutination (Fraczek et al, 2008; Lacerda et al, 2008; Schipper et al, 2008; Sitharaman et al, 2008). Up to now, several studies report low or undetected liver and systemic toxicity in mice, although CNT presence has been shown in the liver, lung and faeces after intravenous injection (Deng et al, 2007; Fraczek et al, 2008; Schipper et al, 2008).

4. Mechanisms of interaction of CNT

The questions related to possible interactions between CNT and various dye markers, pointing out the difficulties in the interpretation of the obtained results are raised by several

authors, pointing out the need for careful interpretation of the results (Casey et al, 2007; Davoren et al, 2007; Pulskamp et al, 2007).

The commonly used MTT assay, used to assess cell viability and proliferation, has been described to falsely lower results due to attachment of insoluble formazan to CNT (Pulskamp et al, 2007).

Guo et al. describe SWCNT dose-dependent adsorption and depletion of over 14 amino acids and vitamins from RPMI cell culture medium. This implies that indirect mechanisms of toxicity may influence the results of *in vitro* studies, since some of these molecules are essential for cell viability and proliferation. SWCNT cause dose-dependent adsorption of culture medium amino acids and vitamins, showing higher affinity for planar aromatic or conjugated structures, and for positively charged solutes (Guo et al, 2008).

Functionalization of SWCNT and MWCNT with terminal or surface specific groups alters solubility and protein adsorption, including of cytokines IL6 and IL8, in a dose-dependent manner (Tian et al, 2006). In the absence of specific chemical affinity between the nanotube surface and the protein, one cause of interference would be the seizing of the molecule inside the nanotube, dependent on molecule size, unless CNT are functionalized with specific groups that promote chemical binding. CNT's active surface issues are equally important, as in a composite CNT surface available for interaction is reduced because nanotubes are embedded in a matrix.

There are several possible mechanisms of interaction. Molecule adsorption is probably strongly dependent on charge and molecule size, and also on the CNT surface available for interaction.

The authors explored protein adsorption to non-functionalized and functionalized multi-walled CNT (MWCNT) and to ultra high molecular weight polyethylene (UHMWPE)/MWCNT composite and with UHMWPE polymer alone.

Two different proteins were chosen, bovine serum albumin (BSA, Promega) and histone (Histone, calf thymus, Merck). Histones are a group of small proteins, with molecular weights varying from around 21 500 Dalton to 11 200 Dalton; at neutral pH, histones are positively charged (Panyim & Chalkley, 1971). Bovine serum albumin (BSA) has a molecular weight of around 66 700 Dalton and its isoelectric point is 4.7, thus being negatively charged at pH 7, due to the domination of acidic groups over amine groups (Tsargorodskaya et al, 2004).

Solutions of both proteins (concentration 200 µg/mL) were prepared through agitation in PBS (without calcium and magnesium), and the pH adjusted to 7 with HCl 1 N.

MWCNT (range of diameter 60-100 nm, length of the tubes 5-15 µm), non-functionalized and functionalized with carbonyl, carboxyl and hydroxyl groups were added to the solutions (n=6), with a concentration of 100 µg/mL, and mixed by vortexing. Bulk samples of composite (0.2% MWCNT) and polymer were also incubated in the solutions, maintaining the same weight/volume rate.

After 12 hours at room temperature, solutions were filtered using 0.2 µm polyethersulfone low protein binding syringe filters (VWR). Initial albumin and histone solutions were also filtered.

Protein content in the filtrates was assessed, in triplicate, by the bicinchoninic acid assay (BCA Protein Assay, Calbiochem), accordingly to the manufacturer's instructions. PBS was used as blank. The protein content in solutions incubated with the materials is expressed in percentage of histone and BSA filtered solutions, assumed as 100%.

The normal distribution was verified by the Kolmogorov-Smirnov test, homogeneity of variance by the Levene test and means compared ANOVA (Tukey test). The statistical analysis was done using software OriginPro 8 (OriginLab Corporation, USA).

Figure 1 shows the percentages of BSA and histone left in filtrate after incubation with functionalized and non-functionalized CNT, assuming filtered solutions of both proteins as 100%; bars represent mean and error bars represent standard deviation.

When considering BSA and histone adsorption to UHMWPE and MWCNT/UHMWPE composite, no differences were found between both materials and protein solutions.

A statistically significant (at a 0.05 level) higher amount of histone was retained by functionalized CNT, when comparing to non-functionalized CNT. A significantly higher amount of histone was retained in both groups of CNT when comparing to BSA. The difference in protein adsorption between the two groups of CNT incubated in BSA solution was not significant.

Fig. 1. Percentage of bovine serum albumin (BSA) and histone after incubation with CNT.

The results show that protein charge and size are paramount for the interaction with CNT, likewise CNT surface functional groups and the surface area available for interaction. Smaller, positive charged particles are more likely to bind in significant amounts to both functionalized and non-functionalized CNT, as the present results suggest, and in agreement with the conclusions from previous studies (Guo et al, 2008). Functionalizing CNT may further enhance adsorption.

The need to consider possible interactions between CNT and the substances evaluated and used in assays is, thus, clear. Positive advances are being pursued and achieved in better understanding and predicting the interactions between CNT and other nanoparticles and biological molecules (Xia et al, 2010). As it is also the authors' opinion, and as recent literature emphasizes, there is an urgent need for standardization of assays involving CNT (Ren et al, 2010).

5. Potential medical applications of CNT

CNT may be used in orthopedics as mechanical reinforcement, to tailor surface properties and provide a nanostructured surface that promotes bone cell adhesion and function, or

by exploring CNT conductive properties in the view of the development of smart implants.

Few studies are available on the cytocompatibility of composites using CNT as reinforcement. Chlopek et al. reported cytocompatibility of MWCNT similar to that of polysulfone, after culturing osteoblasts and fibroblasts in with polysulfone alone and with polysulfone plus MWCNT. These authors described a slight increase of collagen I production, in the absence of induction of IL6 and free radicals (Chlopek et al, 2006).

George et al. studied the adhesion behaviour of osteoblast-like cells MG63, primary osteoblasts and A549 cells on MWCNT surfaces. They determined that all cell types were capable of attaching and proliferating but not able to penetrate the mesh work. These findings suggested cell spreading and migration were affected (George et al, 2006). However, in a more recent study conducted by Meng et al., fibroblasts had improved growth and collagen synthesis on a nanofibrous scaffold made of composite MWCNT/polyurethane, when comparing to controls and polyurethane alone (Meng et al, 2008).

Shitaraman et al. described in vivo good tissue response to ultra-short SWCNT, propylene fumarate diacrylate (PPF) composite scaffolds, similar to the response to PPF alone (Shitaraman et al, 2008). Other studies report bone cell growth along CNT and plasma-sprayed carbon nanotube reinforced hydroxyapatite, suggesting CNT conductive properties may be explored (Balani et al, 2007; Zanello et al, 2006).

Sirivisoot's study concluded that osteoblast (bone forming cell) functions (specifically alkaline phosphatase activity and calcium deposition) are significantly greater on MWCNT grown by chemical vapor deposition on anodized porous Ti than on anodized Ti without CNT and currently-used Ti for up to 21 days (Sirivisoot et al, 2007).

Usui et al. implanted highly crystalline MWCNT, with purity around 98%, in subperiosteal and tibial defects in mice. These authors describe MWCNT incorporation into bone marrow tissue and bone matrix. When combined with rhBMP-2, MWCNT-collagen composites accelerated bone formation when compared to collagen and rhBMP-2 alone after implantation in dorsal musculature of mice (Usui et al, 2008).

The results of a study conducted by Tutak et al also describe a rise in alkaline phosphatase activity, collagen I synthesis and total protein content in MC3T3-E1 cells grown on thin film SWCNT substrates, along with a decrease in the number of viable cells, assessed by the MTT assay (Tutak et al, 2010). The authors suggest this may be due to initial toxicity and release of cytoplasmatic growth factors by injured cells.

Osteoblast response is probably dependent on surface energy density and the roughness of the SWCNT surface, both cell adhesion and proliferation being affected. Hydrophilic and medium rough films provoked higher cell adhesion and proliferation (Tutak et al, 2010).

There is, also, growing evidence that MWCNT may inhibit osteoclast differentiation and activity in vivo and *in vitro* and do not affect osteoblasts negatively (Narita et al, 2009). In this study, the authors implanted rhBMP-2/collagen/MWCNT or rhBMP-2/collagen composites in the dorsal musculature of mice. They reported a significantly lower number of osteoclasts in the neo-formed ectopic bone in the rhBMP-2/collagen/80n-MWCNT group than in the rhBMP-2/collagen group.

Yadav et al described the use of MWCNT for reinforcement of hydroxyapatite and gelatin composites with the aim of improving mechanical properties of a potential nanocomposite for bone replacement. The authors describe absence of skin, kidney and liver changes after subcutaneous injection in mice of composites with 0, 1, and 2% non-functionalized MWCNT. However, in the group that received injection of a 4% MWCNT, mild changes

were observed, such as hydropic changes and ballooning of hepatocytes and glomerular abelation and swelling of renal tubules. Although a significant increase in the flexural strength of the composite was achieved through the addition of the MWCNT, the authors describe a lowering of the Young modulus, and final elasticity is still far from the one of natural bone (Yadav et al, 2009).

UHMWPE is one of the mostly used materials in biomedical applications such as acetabular cups as bearing surface in total hip arthroplasty. Coupled with a metal or ceramic femoral head, UHMWPE has shown excellent resistance to wear. However, despite the success of the total joint arthroplasty, wear is the major obstacle limiting the long-term performance of the UHMWPE implants. It has been estimated that billions of particles are produced yearly from the surface of a total hip replacement (Kurtz et al, 1999). Although UHMWPE components are in no imminent danger of wearing during patient's lifetime, osteolysis and loosening of the implants are attributed to the debris generated from the articulating surface. Moreover, the mechanical and physical properties of UHMWPE still need to be improved; in particular, enhancing friction and wear resistance can satisfy the material requirement in joint replacement surgeries (Jacobs et al, 2002).

Ruan et al (2003) reported an enhancement of toughness in UHMWPE films with the addition of 1 wt% MWCNT. Their results revealed an increase in strain energy density of ~150 % for the composites as compared with pure UHMWPE. They also reported an increase of ~140 % in ductility and up to 25 % in tensile strength. An analysis by nanoindenter and atomic force microscopy (AFM) of UHMWPE/MWCNT composites has been reported by Wei et al (2006). They have observed a decrease of the friction coefficient with MWCNT content increase.

With such promising results, the authors prepared UHMWPE/CNT composites processed through optimized compression moulding process in order to obtain a suitable reinforced polymeric material for the production of the acetabular cup component used as hip joint implants.

The effective use of carbon nanotubes in composites depends upon its homogeneous dispersion throughout the polymer matrix, without/less destroying their integrity. To prevent the CNT agglomeration and to improve the interfacial bonding between the polymer and the CNT, it was performed a chemical treatment to the MWCNTs following the methodology proposed by Esumi et al (1996). It is believed that the polymer spreads over the MWCNT due to their clean nature and seamless structure of the CNT, leading to an interface coating of the polymer on CNT of about few micron thickness which is due to wettability characteristics of polymer and thus the interfacial bonding between them increases which leads to enhancement of load transfer from polymer to MWCNTs (Ding et al, 2003; Potschke et al, 2002; Kanagaraj et al, 2007). Figure 2 clearly shows the homogeneous distribution of the MWCNT in the polymeric matrix.

The prepared 0.2% MWCNT/UHMWPE composite and pure UHMWPE were subjected to tensile tests for mechanical characterization and some tribological tests, using a ball-on-plate configuration (Kanagaraj et al, 2010). In Figure 3 it can be observed the variation of the wear volume for pure polymer and for composite against the sliding distance

Kanagaraj et al. developed a UHMWPE/MWCNT composite that presents superior wear behaviour (decreased wear volume and wear coefficient) when compared to conventional UHMWPE. Reinforcement of UHMWPE by adding multiwalled carbon nanotubes (MWCNT) allows improvement of mechanical characteristics for biomedical applications.

Fig. 2. SEM pictures of 0.2% MWCNT/UHMWPE composite. The presence of MWCNT is highlighted.

Fig. 3. Wear volume variation for pure polymer and UHMWPE/MWCNT composite against sliding distance.

It is clearly observed that the wear volume of the composite is lower than the one observed for pure polymer, which can be correlated with an increase of toughness of the composite when compared with the pure polymer. The decrease of wear volume was: 28%, 28% and 35 % for sidings’ distances of 43.2m, 86.4m and 232.2m, respectively. This decreasing results in a good load transfer effect from the nanotube to the polymer.

The decreasing of wear volume decreasing with incorporation of the MWCNT is consistent with an increasing of toughness for the composite sample as compared with pure polymer. In Table 1 it is shown the mechanical properties for both studied samples (UHMWPE and UHMWPE/MWCNT composite).

	Young's modulus		Tensile strength		Toughness		Breaking elongation	
	GPa	% inc.	MPa	% inc.	J/g	% inc.	%	% inc.
UHMWPE	0.718	0	21.17	0	50.84	0	280.73	0
UHMWPE/CNT	0.754	5.0	25.97	22.7	68.53	34.8	354.7	26.3

Table 1. Mechanical properties of UHMWPE and UHMWPE/MWCNT.

It is clearly observed an increase of the mechanical properties for the composite when compared with pure polymer. The observed increasing in toughness was of about 34.8%. The developed UHMWPE/MWCNT composite seems to present superior mechanical properties when compared to a conventional UHMWPE, but there is still some controversy when it comes to carbon nanotubes toxicity. To analyze the latter the authors assessed human osteoblast-like MG63 cells viability and proliferation and interleukin-6 (IL-6) production in contact with this composite’s particles (Figure 4).

Fig. 4. MG63 after 3 days culture in contact with UHMWPE/MWCNT composite particles.

The results suggest cytocompatibility similar to that of conventional UHMWPE (Table 2). The viability and proliferation were assessed by the WST-1 assay, known to be not influenced by the presence of CNT and the results expressed as percentage of control standard culture plate $\pm$ standard deviation. The IL6 production was assessed in culture medium by ELISA assay (Peprotech) and total protein content (Calbiochem) and results expressed as mean $\pm$ standard deviation.

Assay	UHMWPE/MWCNT	UHMWPE
WST-1	97.92 $\pm$ 8.29%	96.19 $\pm$ 7.92%
IL6 (pg/mL)	108.99 $\pm$ 9.90	92.52 $\pm$ 11.02
Total Protein (μ g/ mL)	163.29 $\pm$ 11.81	137.07 $\pm$ 6.17

Table 2. *In vitro* results: MG63 cells after 6 days in contact with UHMWPE/MWCNT composite and UHMWPE

Naresh et al. (2011) has observed hydroxyapatite coating over UHMWPE-CNT composites with time during the simulated body fluid test (SBF), where the test was carried out for a week. The thickness of the hydroxyapatite layer was also increased with time where approximately 55% of weight was increased on 7th day of SBF testing (Figure 5). From the optical images, it is also confirmed (Figure 6).

Fig. 5. Hydroxyapatite coating on UHMWPE/CNT composite with time during SBF test.

Fig. 6. Optical microscope images of Hydroxyapatite grown on UHMWPE/CNT 2.0 wt% on day 0, 3 and 5.

As Balani et al. have suggested (2007), the presence of CNT may assist in hydroxyapatite crystals nucleation and growth, promoting higher crystallinity.

Recently, formation of hybrid CNT-protein nanofibers was reported. These nanofibers contained fibronectin that promoted the nucleation of crystalline nuclei and the CNT

templates controlled the orientation of nuclei and the crystal growth of hydroxyapatite to form flake-like crystals (Wei et al, 2011). Nucleation and crystal growth occur in a biomimetic way.

6. Conclusion and future remarks

The immense potential of CNT for biomedical applications is evident. Either as sensors, drug carriers, imagiology aids, bioelectrodes or reinforcement for composites, these are highly versatile and promising molecules.

The acetabular cup in hip prosthesis and the tribologic surfaces in knee prosthesis is often lined with ultrahigh-molecular-weight polyethylene (UHMWPE). It is accepted that debris particles derived from UHMWPE weight-bearing surfaces are the main cause for debris-associated aseptic loosening. Apart of the sometimes catastrophic consequences for the integrity of the implant, debris particles attract macrophages, causing their activation and the secretion of proinflammatory mediators such as interleukine (IL) 1, IL6, IL17, receptor activator of nuclear factor- κ B ligand (RANKL) and proteolytic enzymes.

Better wear behavior and less particle generation would help lowering the numbers of revision arthroplasty procedures due to material failure and aseptic loosening.

Kanagaraj et al. developed a UHMWPE/MWCNT composite that presents superior wear behavior (decreased wear volume and wear coefficient) when compared to conventional UHMWPE. The preliminary *in vitro* studies in human osteoblast-like MG63 show the cell response to particles of this novel composite is similar to that provoked by conventional medical grade UHMWPE. The viability and IL6 production do not show significant differences. More throughout studies, using different cell lines and a deeper look on cell response, can be developed.

However, the research carried out is far from having reached a consensus on CNT toxicity, although further studies are being conducted on the subject. Nevertheless, the main issue is that probably it is being compared what cannot be compared. As neatly indicated by the studies conducted by Herzog et al. (2009) and Barillet et al. (2010), among others, different CNT, produced by different methods, yielding different amounts of metal residues, with varying sizes and surface area, functionalized or not, different degrees of functionalization, dispersed in different mediums, elicit different responses, in different cell lines, assessed by different methodologies! Some of which are known to suffer from interference when CNT are present.

When performing either *in vitro* or *in vivo* studies, a deep knowledge on the type of CNT used and exploring possible interactions with experimental methods is mandatory. A thorough in depth study should be conducted, allowing establishment of experimental methodology guidelines. Studies conducted up to now advice precaution when handling these materials due to possible epidermal and respiratory detrimental effects.

In fact, the same characteristics that make nanomaterials so promising for drug delivery purposes also may be source of concern, if distant spread and accumulation in organs cannot be controlled.

It is clear that CNT surface characteristics are determinant for interaction with other molecules. As inferred from the histone and BSA adsorption assay here presented, adsorption is not just dependent on charge but also on molecule size. The degree in which it occurs is dependent on the surface area available for interaction, and the fact that it can occur in high degree (more than 50% of histone on solution was retained in the

functionalized CNT) may have dramatic effects on interaction with cells and cell response, and with assays used to assess this same response. Mechanisms of interaction are slowly being explored and revealed, but tools for predicting adsorption behavior are not yet widely available. Developing these tools and wide spreading their use call upon a interdisciplinary approach, putting together knowledge and technological resources traditionally handled by biologists, chemists, physicists, material science engineers, among others. A non-traditional and unique material demands a non-traditional and custom-made approach, as in the last decades a new era has begun.

The development of knowledge on CNT interactions with biological systems gives hope to fully explore the CNT potential as reinforcement component in composites for orthopedic applications. It makes a better use of their ability to promote biomimetic nucleation and growth of hydroxyapatite crystals and exceptional strength. The growing interest on bone electrophysiology and piezoelectricity, and CNT conductive properties, can anticipate their use in smart implants, able to adapt their performance to the mechanical environment and as constituents of materials that mimic bone natural properties and support osteoblast proliferation and differentiation. CNT applications are almost unlimited, and we can expect to see further research on their application as drug carriers and in imagiology, due to their capacity to cross biological membranes, near-infrared intrinsic fluorescence and biodistribution. The biodistribution and pharmacokinetics may be tuned by controlling the size, the surface chemistry, and the targeting ligand, and CNT can be loaded with a variety of drugs, being a specially promising tool in the fight against cancer.

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