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HIGHLIGHT

Making carbon nanotubes biocompatible and biodegradable

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Carbon nanotubes are promising nanomaterials with great potential in the field of nanomedicine for both therapeutic and diagnostic applications. Different approaches have been developed to render this material biocompatible and to modulate any ensuing toxic effects. In the context of medical use, although chemically functionalised carbon nanotubes display reduced toxicity, they are still considered with scepticism due to their perceived non-biodegradability. Recently, it has been demonstrated that functionalised carbon nanotubes can be degraded by oxidative enzymes. This finding is offering a new perspective for the development of carbon nanotubes in medicine. This article highlights recent advances that can act as paradigm-shifts towards the design of biocompatible and biodegradable functionalised carbon nanotubes and allow their translation into the clinic.

Introduction

In the last few years, the field of nanobiotechnology has been revolutionised with the emergence of a variety of novel nanomaterials such as quantum dots, fullerenes and carbon nanotubes that have been modified and engineered in order to improve their biocompatibility.[1–3] Carbon nanotubes (CNTs) consist exclusively of carbon atoms arranged in condensed aromatic rings, which in turn are organised in one (single-walled carbon nanotubes: SWCNTs)[4] or more (multi-walled carbon nanotubes: MWCNTs)[5] concentric graphene sheets rolled-up into cylinders. The nanometre-scale dimension of SWCNTs is in the range of 0.4–2.0 nm in diameter and a few μm in length, while MWCNTs have diameters up to 100 nm and lengths from 1 to several μm.[6] The existence of such nanomaterials was first reported in the 1950s but their structure was described at the atomic level only in 1991 by Sumio Iijima.[5] They belong to the family of fullerenes, the third allotropic form of carbon along with graphite and diamond. Their extraordinary and unique structural, electronic, mechanical and chemical properties have been a source of inspiration for many researchers attracted by the innovative characteristics of this material and their promising potential for applications in field emission, energy storage and molecular electronics.[6,7]

Research in the field of CNTs has reached a level of reasonable understanding of the basic properties these nanostructures possess, which allows the exploration of new applications, including biological and biomedical.[2] Such applications are intrinsically dependent on the solubilisation and dispersion of carbon nanotubes in biological, aqueous-based environments since, as prepared, they are completely insoluble in most organic solvents and aqueous buffers. In order to overcome this hurdle, the chemistry of CNTs has been extensively investigated through the functionalisation of their external tips and sidewalls. Several approaches to functionalise CNTs, including defect-group chemistry, covalent sidewall chemistry, non-covalent wrapping by polymers, biopolymers, surfactants and other amphiphilic molecules, have been explored.[8] The use of such functionalised carbon nanotubes (*f*-CNTs) compatible with aqueous environments has opened the door to their possible biomedical applications.

The main biological and biomedical applications of carbon nanotubes include their use as ultra-sensitive biosensors for glucose and DNA, nanocomposites for neural and orthopedic prosthetic devices and delivery systems.[9] Work from our laboratories has reported proof-of-concept studies that illustrated how *f*-CNTs can act as delivery systems for drugs, antigens and genes transported into prokaryotic and mammalian cells with minimal cytotoxicity.[10,11] In this highlight we will focus our attention on the important advances of such applications considering in particular CNTs that have become biocompatible through both covalent and non-covalent

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chemical functionalisation. We will also discuss the recent reports on the degradability of *f*-CNTs and emphasise its implication on the future therapeutic and diagnostic translation of the material closer to the clinic.

Biocompatibility of carbon nanotubes

Biocompatibility of a nanomaterial can be an elusive and challenging term to define accurately, very much dependent on the context of application and use. In the present context and field of study, we refer to nanomaterial biocompatiblity as: (a) the ability to interact with the biological milieu in the absence of triggering acute adverse reactions (*e.g.* apoptosis, cell detachment, tissue necrosis); (b) naive immunoreactivity and absence of acute inflammatory responses; (c) absence of intoxication from metabolism of chemical components; (d) harmless or without long-term tissue accumulation leading to material deposits in the body. The physicochemical characteristics of any nanomaterial are evidently the fundamental parameters that will determine the biological responses obtained upon interaction with biological matter. Modulation of the nanomaterial chemical nature by covalent bonding can lead to dramatic changes in its physical and biological properties. In terms of biocompatibility, based on knowledge generated from a few laboratories during the last decade it is now well accepted that chemical functionalisation of carbon nanotubes can lead to a dramatically improved biocompatibility profile.[12] We have taken advantage of this improved profile to explore various aspects of *f*-CNTs in biomedical sciences. In Fig. 1 different examples from our work with carbon nanotubes[13] are shown to illustrate some of the chemical modifications carried out. Different types of organic reactions have been applied to generate a library of diverse CNT conjugates.[13,14] The combination of oxidative treatment to generate carboxylic functions followed by the cycloaddition reaction allowed us to obtain doubly functionalised *f*-CNTs that were further functionalised with therapeutic agents and imaging probes (structures **4** and **5**).[14,15] Alternatively, a double 1,3-dipolar cycloaddition reaction allowed introduction of two orthogonally protected amino groups, subsequently exploited to also generate doubly *f*-CNTs (structures **3** and **4**).[15] Besides the 1,3-dipolar cycloaddition, direct addition of multiple functional groups has been recently explored obtaining triple-functionalised carbon nanotubes (structure **7**).[16] These types of chemical modifications will be further explored and will be essential in the design and synthesis of biodegradable carbon nanotubes.

Chemically and physically modified CNTs and their interaction with other macromolecules can be studied in the context of cell biology investigations.[2] The fundamental interactions between CNTs and cells, intracellular compartments and other cellular components (*e.g.* biomembranes) are gradually being revealed.[17] Fig. 2 illustrates part of our current knowledge about *f*-CNTs and their interaction with mammalian cells. Therapeutically useful mechanisms of binding to plasma membranes and internalisation into cells have been shown that can be further enhanced, dependent on the structure and surface chemistry of the *f*-CNTs. Results from ours and other laboratories have shown that *f*-CNTs are able to translocate directly into the cytoplasm of mammalian cells.[17]

The development of CNTs as platforms for the delivery of therapeutic modalities is pursued by various laboratories around the world now. Our work has shown that amino-functionalised carbon nanotubes can: (a) deliver peptides intracellularly;[18] (b) be covalently functionalised with

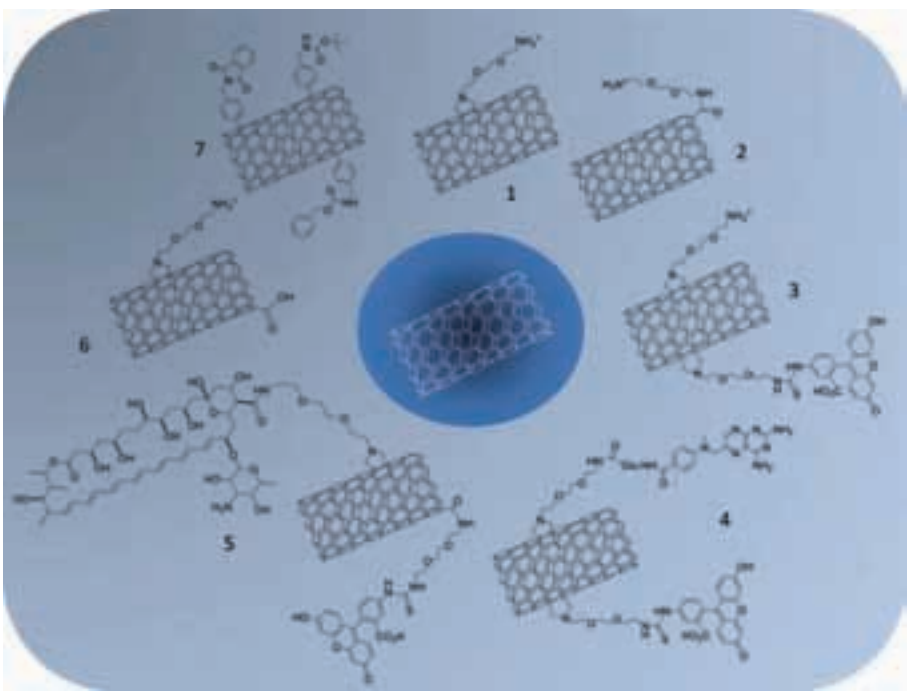

Fig. 1 Examples of chemical modifications on carbon nanotubes. Structures **1** and **2** correspond to mono-functionalised CNTs; structures **3–6** correspond to bi-functionalised CNTs; and structure **7** corresponds to tri-functionalised CNTs.

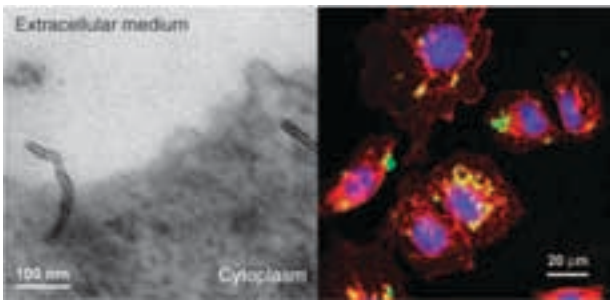

Fig. 2 Functionalised MWCNT plasma membrane translocation leading to cell internalisation. Left panel, multi-walled carbon nanotubes (MWCNT-NH_3, structure **1** in Fig. 1) translocate the plasma membrane of a human cell line (HeLa) imaged by transmission electron microscopy. Right panel, confocal laser scanning microscopy of single-walled carbon nanotubes (SWCNT-NH_3^+, structure **1** in Fig. 1) trafficking to the perinuclear region of epithelial human lung carcinoma cells.

antigenic peptides presented *in vivo* to raise antibodies;[19] (c) non-covalently bind, condense and deliver biologically active nucleic acids (plasmid DNA and siRNA).[20] Moreover, it has also been shown that the charge ratios between the cationic CNTs and plasmid DNA during complex formation are critical in constructing effective CNT-based non-viral gene delivery vectors.[21] Regarding the possibility for adverse effects due to such behaviour, reports in the literature are encouraging. Pantarotto *et al.* demonstrated that cell-internalised peptide-functionalised CNTs are immunogenic, eliciting antibody responses of the right specificity, while at the same time *f*-CNTs alone were non-immunogenic.[22] Cherukuri *et al.* found that macrophage cells can actively ingest significant quantities of single-walled CNTs without showing toxic effects[23] and Dumortier *et al.* reported that *f*-CNTs are not cytotoxic against the cells that regulate the immune system. Indeed, *f*-CNTs induce neither cell death nor activation of lymphocytes and macrophages and do not alter cell functions of these immunoregulatory cells.[24]

In pharmacological studies, the profile of CNT time-dependent tissue distribution, residence and pharmacokinetics following passive exposure or intended use (as in the case of a therapeutic or diagnostic intervention) will determine to a large extent the applicability and regulatory framework around such novel nanomaterials.[2] Understanding of the specific physicochemical and pharmacological parameters that are intricately involved in determining the observed pharmacokinetic (*e.g.* blood circulation half-life) and pharmacodynamic (tissue distribution, absorption) profiles of *f*-CNTs is imperative for clinical translation. We have systematically studied the structure–function relationships that determine the *in vivo* profile of *f*-CNTs using dynamic small animal imaging techniques (such as single photon emission computed tomography, SPECT).[25,26] Some basic rules in the design of CNTs to achieve deposition in specific tissues have been revealed, such as ways to achieve: (a) rapid urinary excretion after intravenous (i.v.) administration;[27] (b) accumulation mainly in the lung after i.v. administration;[28] (c) predominant liver and spleen deposition after i.v. administration (Fig. 3). Our current knowledge suggests that tissue distribution is critically dependent on a mix of parameters between the structural and surface characteristics of *f*-CNTs and the route of administration or exposure.

Despite considerable progress, widespread applications of CNTs are still considered with apprehension due to the reported risks that may be associated with their use. One of the most serious concerns is associated with possible, yet still unknown, environmental or health risks given their structural resemblance to carcinogenic, long asbestos fibres.[29] Several research groups have studied the structure–toxicity relationships of CNTs using several *in vivo* models and paradigms. One of the studies that highlighted potential risks of pristine (non-functionalised) CNTs was carried out by Poland *et al.* using a structure (length)–toxicity paradigm which was originally validated with asbestos fibres.[30] The CNT length, tissue accumulation and bio-persistence of asbestos fibres in the peritoneal cavity were found as critical contributing factors in the possible development of mesothelioma (cancer of the pleura). Non-functionalised, rigid MWCNTs longer than 20 µm were found to lead to the increased risk of carcinogenesis.[30] On the other hand, Muller *et al.*[31] investigated the effect of MWCNTs with and without surface defects over a longer term study (24 months) and observed no risk of mesothelioma from both CNT types, thought to be due to their short length (about 1 µm). Most toxicological studies to date have focused on effects from CNT exposure *via* inhalation, dermal contact or ingestion, predominantly using non-functionalised CNTs in an attempt to mimic possible workplace exposure levels. Chemical functionalisation of CNTs has been shown to improve dramatically the toxicological burden. Sayes *et al.*[12a] reported that increased functionalisation renders SWCNTs less toxic, and similar such observations were also

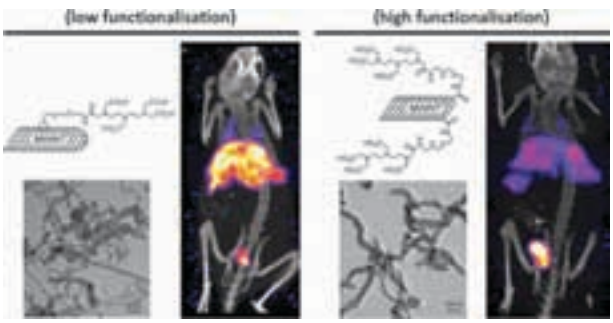

Fig. 3 Functionalised CNT biodistribution in mice (with low and high degree of functionalisation). Chemical structure, transmission electron microscopy images of the *f*-MWCNTs and SPECT/CT images of live animals injected with radiolabelled *f*-CNTs indicate that high liver accumulation (left) can be modulated leading to increase in urinary excretion (high bladder signal) as degree of functionalisation increases (right) (*unpublished results*).

reported *in vivo* for different animal models. Studies conducted in our laboratories[12b] showed that intravenously injected ammonium-functionalised MWCNTs did not cause any physiological or pathological abnormalities after 24 hour post-injection even with high injected MWCNT doses (20 mg kg^{-1}) in mice. Moreover, very recent toxicological investigations carried out using the chemically functionalised CNTs from our laboratories administered intrapleurally indicated that functionalisation chemistries that can lead to individualised nanotubes below 1 μm in length were not retained in the pleural cavity (drained to the lymph nodes), therefore did not present any toxicological risk.[32]

It must be stressed that toxicological profile and risk depend heavily on the tissue distribution, accumulation and retention (biopersistence) of any nanomaterial. Regulatory authorities (FDA, EMA, EPA, and HPA) place considerable attention to such behaviour, since biopersistence is commonly associated with long-term toxicity risks. Having identified that toxicological risks can be averted by shortening carbon nanotubes, enhanced safety can be achieved by either excretion or biodegradation of the material that comes in contact with living tissues.

Biodegradability of carbon nanotubes

Health impact, biopersistence and environmental accumulation are currently considered as key issues to determine the widespread application of carbon-based nanomaterials and in particular the future usage of carbon nanotubes in mass-scale applications.[33] The increasing industrial production of CNTs is raising many concerns about their fate. It is currently difficult to estimate the amount of nanotubes that will come in contact with air, soil, water and living species in the near future. Moreover, in the context of medical applications, approval for clinical use of carbon nanotubes will be greatly facilitated on demonstration that the material is either eliminated or degraded in the body. As a consequence, it is of fundamental importance to explore the possibility that CNTs could be degraded under certain conditions, ideally by the action of microorganisms or at the cellular level.[34] Although the design of their biocompatibility is actively pursued as described above, very little is known regarding their degradation once a living organism is administered or exposed to CNTs.

Nanomaterials in general, and CNTs in particular, have been assumed to be resilient and persistent due to their rigid and strong structure, characterised by increased resistance to different chemical treatments. As a consequence, their biopersistence into organs and tissues has been expected to be very high. Non-biodegradable nanomaterials can accumulate in tissues causing harmful side-effects. Moreover, the degradation products of nanomaterials could also provoke adverse responses.[33,34] One such example is cadmium released from the core of quantum dots, once they are administered *in vivo*.[35] Therefore, "*controlled biodegradation*" represents an important and challenging objective, since the development of nanomaterials for biomedical applications will be strongly related to their programmed degradation and clearance from the body. For example, mesoporous silica nanoparticles are degraded intracellularly and the derived products (mainly orthosilicic acid) are eliminated through the kidney without apparent toxic effects.[36] Such knowledge needs to be demonstrated and eventually applied also to CNTs.

Very recently, proof-of-concept studies have reported that strong oxidative enzymes may be able to degrade *f*-CNTs. Star and co-workers reported the catalytic biodegradation of carboxylated SWCNTs through natural, enzymatic catalysis by the oxidative activity of horseradish peroxidase (HRP) in the presence of low concentrations of H_2O_2.[37,38] Within ten days nearly all the nanotubes were degraded. Oxidised aromatic fragments were detected in the course of nanotube degradation which eventually evolved to carbon dioxide. Alternatively, it has been also demonstrated that SWCNTs can be biodegraded by fluids mimicking the phagolysosome milieu (PSF; phagolysosomal simulating fluid).[39] PSF is a medium designed to simulate the acidic oxidising environment typically present into late-stage endosomes and phagolysosomes of macrophages. Following the addition of hydrogen peroxide, necessary to create the typical physiological conditions, carboxylated SWCNTs underwent degradation generating extensive amount of nanotube debris. On the contrary, pristine, non-functionalised SWNTs were resistant (no morphological changes observed) when exposed to the same biological oxidative conditions (HRP or PSF).[37–39] It can therefore be hypothesised that the degraded CNTs may be more readily eliminated from organs and tissues and may pose less toxicological risks compared to intact nanotubes.

We have recently expanded the study of enzymatic degradation to multi-walled nanotubes derived from different commercial sources.[40] In a comparative analysis we assessed the capacity of HRP and PSF to degrade carboxylated MWCNTs (*ox*-MWNTs) (Fig. 4). After two months in both biological oxidative environments, *ox*-MWNTs were dramatically affected structurally and clearly degraded although not completely. Distribution of lengths showed that nanotubes were significantly shortened in comparison to the starting material. Thus, the possibility that CNTs undergo a degradation process is not only unique to SWCNTs but it is also demonstrated

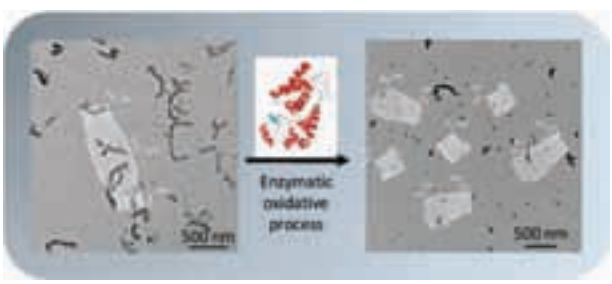

Fig. 4 Functionalised multi-walled carbon nanotubes are degraded by oxidative enzymes. TEM images of carboxylated-MWCNTs before and after treatment with HRP in the presence of hydrogen peroxide for 60 days.

for MWCNTs. In a subsequent study by Star and co-workers, investigation of the effect of HRP/H_2O_2 on different types of MWCNTs (purified, oxidised and nitrogen-doped) allowed more precise assessment of the mechanisms of degradation, proposed as an occurring layer-by-layer exfoliation likely facilitated by side-wall defects.[41] The rate of degradation was associated to the degree of carboxylation on MWCNTs, eventually liberating CO_2 as a final product. Because of their concentric graphitic structure, MWCNTs were more resistant and required longer time to degrade in comparison to SWCNTs. We have also evidenced an extensive shortening of the nanotubes by enzymatic action, therefore both exfoliation and further shortening are likely active processes in the presence of HRP.[40,41] More defective tubes like *N*-doped MWCNTs, which consist on concentric hat-stacked nanotubes in which some of the carbon atoms are replaced by nitrogen, are degraded much faster.[41]

Functionalised SWCNTs were shown to be degraded inside cells such as neutrophils and macrophages by myeloperoxidase (MPO) activity.[34] The products of biodegradation generated *in vitro* and aspirated into the lungs of mice did not trigger any toxic effect. This finding has important implications on the possible inflammatory responses induced by unintentionally inhaled *f*-CNTs and to their intended use as vehicles for pulmonary drug delivery. Another study also reported the possibility of nanotube degradation within cells.[42] Intracellular morphological changes of oxidised double-walled CNTs coated with RNA were monitored using the Raman signature of these nanotubes. Defects seemed to accumulate on their external graphene sheet after cellular internalisation by human prostate adenocarcinoma cells; however no experimental evidence of the mechanism of degradation or the possible enzymes involved in the process were assessed neither discussed. Moreover, no data are currently available on the capacity of specific phagocytic cells or a specific tissue to induce CNT degradation following their *in vivo* administration in animal models. This could be partly associated with the difficulty of phagocytic cells to recognise CNTs. Although several reports have shown extensive uptake of CNTs,[17a,23] an appropriate functionalisation might accelerate their elimination. In this direction, Kagan and colleagues have demonstrated *in vitro* that phosphatidylserine coating of SWCNTs offers to macrophages, primary monocytes, dendritic cells and microglia, a signal that initiates "digestion" of nanotubes.[43] A similar behaviour was also observed *in vivo* by alveolar macrophages. Such strategies to improve targeting and uptake of CNTs by professional phagocytes can be extremely beneficial and can be considered the initial step to enhance the intracellular degradation of functionalised CNTs.

All these results also underline the importance of the characteristics of the starting material, including the extent of existing structural defects on the carbon backbone, and the role of chemical functionalisation on the capacity to design biodegradable CNTs. It is important to highlight that biodegradability will depend on the type of functional groups chemically introduced on the surface or at the tips of CNTs. Indeed, not all types of functionalisation seem to favour nanotube degradation. Table 1 offers a correlation between the different chemical moieties used to functionalise nanotubes and their reported degradation in oxidative environments *in vitro* leading to complete decomposition.[34,37–41]

The demonstration of biodegradability for *f*-CNTs has important implications for the long-term toxicological profiles of these materials, particularly related to any potential clinical application. Further studies will need to also address the pharmacological toxicity and possible risks associated with the degradation products of nanotubes rather than the intact nanomaterials. It is impossible at this stage to predict whether the molecules derived from the degraded nanotubes will trigger toxic responses. Several factors might be responsible, such as the kinetics of degradation and the rate of body excretion of these species. This was partly addressed by Kagan *et al.* who demonstrated that the degradation products did not lead to any inflammatory responses in comparison to intact nanotubes, however further systematic studies will certainly be needed.[34] In the event that the biodegradability of CNTs becomes established, we believe that the toxicological issues associated with the long, fibre-shaped, biopersistent nature of the materials will become almost obsolete.

Table 1 The effect of surface functional groups on CNT oxidative degradation

Material[a]	Functionalisation	Biological oxidative environment[b]	Degradation	Ref.
SWCNT/ MWCNT	Pristine (no functional groups)	HRP/PSF	No	37–39
SWCNT	COOH	HRP/MPO	Yes	34, 37, 38
SWCNT	COOH/Taurine	MPO	No	43
SWCNT	COOH/Phosphatidyl serine	MPO	Yes	43
SWCNT	COOH/Phosphatidyl choline	MPO	No	43
SWCNT	COOH	PSF	Yes	39
SWCNT/ MWCNT	Aryl-sulfonation	PSF	No	39
SWCNT/ MWCNT	COOH	HRP/PSF	Yes	40
MWCNT	COOH	HRP	Yes	41
MWCNT	*N*-doped	HRP	Yes	41
MWCNT	CONH-$(CH_2CH_2O)_2CH_2CH_2$-NH_3^+	HRP/PSF/MPO	Yes	—[c]
MWCNT	COOH + 1,3-dipolar cycloaddition	HRP/PSF	Yes	—[c]

[a] SWCNT: single-walled carbon nanotubes; MWCNT: multi-walled carbon nanotubes. [b] HRP: horseradish peroxidase; PSF: phagolysosomal simulating fluid; MPO: myeloperoxidase. [c] Bianco, Kostarelos and Prato, *unpublished results*.

Conclusions and perspectives

CNTs are emerging nanomaterials with great potential for diagnostic and therapeutic applications in medicine. Among others, CNTs have been widely used as novel delivery agents for drugs, antigens and genes. However, a general scepticism has accompanied the enthusiastic research

carried out so far in this field, mainly emanating from some initial toxicity studies, that have implicated the intrinsic structural (shape, length, surface) properties of CNTs with unwanted cytotoxic responses and the risks for carcinogenesis. In addition, the assumption that CNTs are indestructible materials has led to a general conviction that CNTs are dangerous, not only for living species but also for the environment.

In this report, we illustrate by highlighting a few pieces of the increasingly growing body of evidence that CNTs can be designed to be made biocompatible and biodegradable. Biocompatibility can be built upon surface functionalisation: the introduction of hydrophilic moieties renders CNTs less biologically reactive and easier to transport along the physiological milieu (*e.g.* circulatory system, tissue parenchyma). Biodegradability can also be built by chemical functionalisation and the introduction of structural defects that can allow oxidative enzymes to degrade CNTs. More work along these lines is needed and will surely appear, but achievement of an improved biocompatibility and biodegradability profile reinforces optimism for the future development of CNTs in biology and medicine.

Acknowledgements

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