Functionalised carbon nanotubes: high biocompatibility with lack of toxicity

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Abstract: Recent studies from our laboratories have clearly shown the utility of the carbon nanotubes (CNTs) in biomedical applications. Therefore, the behaviour of CNTs following in vivo administration is of high interest. In this paper, we summarise some of our previously published results towards the assessment of the health impact of CNTs. We will initially describe the *in vivo* distribution of radiolabelled multi-walled CNTs (MWNTs) once they reach systemic circulation using micro Single Photon Emission Tomography (microSPECT) imaging and scintillation counting. Our conclusions on the effect of CNT degree of functionalisation on the tissue accumulation will also be described. Finally, our proposed mechanism of CNT elimination from the body through the kidney glomerular filtration barrier will be shown. Overall, through this overview of our recent studies we offer a summary of why we believe the development of functionalised carbon nanotubes can be further explored since it increasingly offers biocompatible material with lack of toxic responses.

Keywords: carbon nanotubes; functionalisation; nanomedicine; toxicity; kidney.

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Maurizio Prato obtained his Laurea Degree in Chemistry in 1978 from the University of Padova, Italy, where he was appointed Assistant Professor in 1983. He moved to Trieste as an Associate Professor in 1992. He was therefore promoted to Full Professor in 2000. He spent a postdoctoral year in 1986–1987 at Yale University, was Visiting Scientist at the University of California, Santa Barbara, in 1991–1992, and was Professeur Invité at the Ecole Normale Supérieure in Paris, France, in July 2002. His research interests focus on the functionalisation chemistry of fullerenes and carbon nanotubes for applications in materials science and medicinal chemistry and on the synthesis of biologically active substances. His scientific contributions have been recognised by national and international awards, which include Federchimica Prize (1995, Association of Italian Industries), the National Prize for Research (2002, Italian Chemical Society), and the Gonzales-Ciamician Award (2008). He is a recipient of an ERC Advanced Research Grant in 2008.

1 Introduction

The new field of research defined as Nanomedicine concerns the study and the applications of materials that contain at least one dimension in the order of a billionth of a metre. Because of these small dimensions, nanomaterials can play an important role in different biological processes at the nanoscale level. Due to their inherent structural properties, carbon nanotubes, a new allotropic form of carbon constituted of a rolled graphitic network, are creating a great expectation in Nanomedicine. Currently, various biomedical applications have been reported where functionalised carbon nanotubes (f-CNTs) have been used as a novel delivery system for genes, peptides, antimicrobial agents and anticancer molecules [1]. Different reports have shown that CNTs provide a suitable framework for the introduction of various therapeutic molecules eventually delivered to cells, tissues and organs. To assess the use of CNTs for therapeutic purposes, a fundamental step consists of a thorough analysis of the mechanism of biodistribution, accumulation and elimination of these nanomaterials in a living organism. We and others have put many efforts to discern the behaviour of CNTs following in vivo administration. Recently, we have reported a tissue biodistribution and blood half-life study of covalently radiolabelled single-walled CNTs (SWNTs) administered intravenously [2]. Similarly, other groups have studied the impact of f-CNTs in vivo following different ways of administration including intraperitoneal and intratumoral routes [3]. There is general

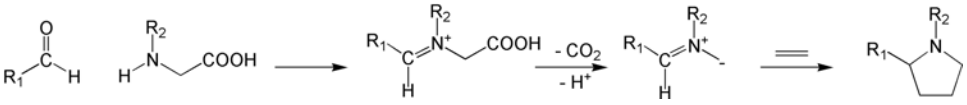
consensus that functionalisation plays a major role in tuning the toxic effects of carbon nanotubes [4]. As a consequence, the damages that these nanomaterials might provoke in the tissues and organs can be minimised. In the last decade, we have focused our research in the optimisation of a versatile methodology for the covalent modification of the surface of CNTs to create a water soluble material [5]. Indeed, the synthesis of f-CNTs compatible with physiological conditions is critical for their integration in living systems.

In this contribution we summarise of our previously published results towards the assessment of the health impact of functionalisated CNTs [6]. We will initially summarise the in vivo distribution of radiolabelled multi-walled CNTs (MWNTs) once they reach systemic circulation using microSingle Photon Emission Tomography (microSPECT) imaging. Then, we will describe the effect of CNT degrees of functionalisation. And finally, we will show a possible mechanism of elimination through the kidney glomerular filtration barrier. The toxic effects of these nanomaterials are strictly related to the capability of the organism to eliminate them. Our results have clearly demonstrated that the excretion route depends on the shape, backbone structure, surface character and degree of individualisation of CNTs. The last property increases following the increase of CNT aqueous solubility. As a consequence, the toxicity of CNTs is likely inversely proportional to the degree of functionalisation of the surface of the CNTs.

2 Results and discussion

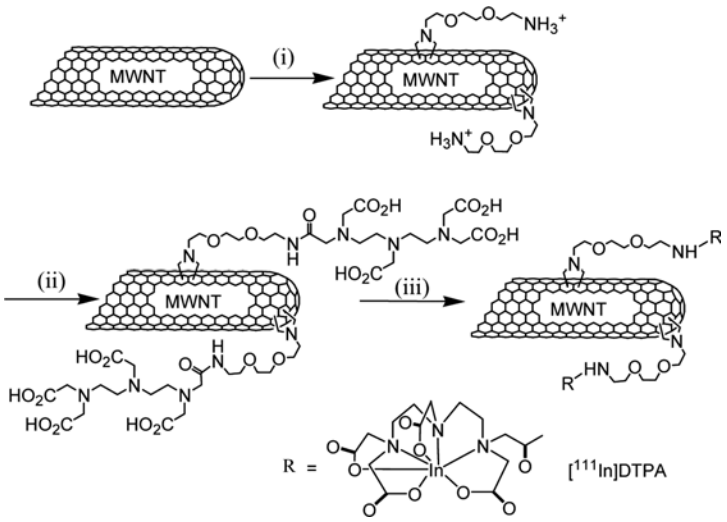
An important enhancement of water solubility of carbon nanotubes was reached following 1,3-dipolar cycloaddition reaction to CNTs [6]. In a similar way to the known 1,3-dipolar cycloaddition of azomethine ylides to fullerenes [7], we have developed a strategy for functionalising and solubilising CNTs [5]. The azomethine ylides are generated in situ by the condensation of α-amino acids and aldehydes (Scheme 1). In our first application, the triethylene glycol group was used as *N*-sustituent group of the α-amino acid (Scheme 1). The reaction is very versatile and useful, not only for the easy attachment of pyrrolidine rings substituted with chemical functions to the sidewalls of the CNTs, but also for the possibility of the construction of novel materials with diverse applications. This reaction has been performed satisfactorily with different types of nanotubes, such as pristine HiPco-SWNTs, purified and oxidised SWNTs and MWNTs. We used in this case MWNTs. A variety of techniques helps us to calculate the degree of functionalisation: the change in the solubility, the decrease of the van Hove transitions (NIR bands), the amount of material lost during thermogravimetric analysis and the increase of the D-band in Raman spectroscopy. Microscopy techniques such as transmission electron microscopy (TEM) and atomic force microscopy (AFM) give us important information about the purity and morphology of the CNTs. SWNTs and MWNTs have been functionalised using *N*-substituted α-amino acid characterised by the presence of a terminal *tert*-butoxycarbonyl (Boc) protected amino group [8] and paraformaldehyde, leading to functionalised materials (Scheme 1). The ammonium salt was obtained using gaseous hydrochloric acid. Kaiser test is another tool available for characterisation that gives us a measure of the number of free amino groups per weight of material.

Scheme 1 Generation of the azomethine ylides. 1,3-dipolar cycloaddition with nanotubes and deprotection of the amino groups



The solubility in aqueous solutions of the pristine material rendered it suitable for the subsequent biological studies. After the generation of covalent bound amino groups around the external surface of the tubes, these focal points were further modified with the chelating agents DTPA (diethylentriaminepentaacetic dianhydride). CNT conjugates were then labelled with the γ-emitting radionuclide ^{111}In. Scheme 2 shows the chemical modification performed on the surface of MWNTs.

Scheme 2 Synthesis of [^{111}In]DPTA carbon nanotubes. (i) paraformaldehyde, Boc-NH-$(CH_2CH_2O)_2$-CH_2CH_2-$NHCH_2COOH$ in DMF followed by 4M HCl in dioxane; (ii) DTPA dianhydride and diisopropylethylamine in DMSO and (iii) $^{111}InCl_3$ in sodium citrate

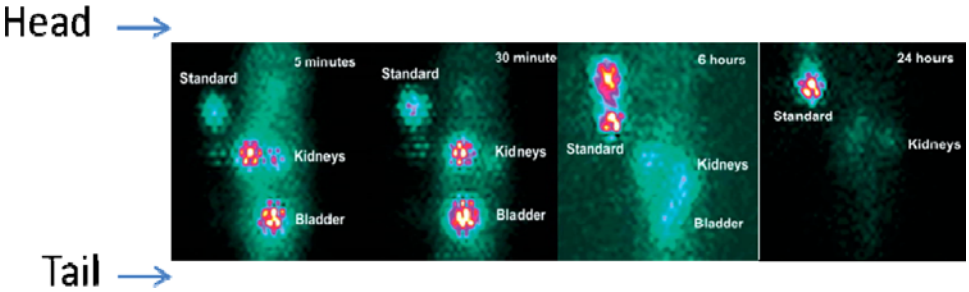


These nanomaterials were fully characterised by the use of different techniques aforementioned such as Kaiser Test, TGA, electron microscopy techniques and Raman spectroscopy. While TGA clearly shows a weight loss that correspond to the organic molecules attached onto the surface of MWNTs, Raman spectroscopy shows that those groups are covalently linked to the nanotubes. TEM studies show the debundling and individualisation of the nanotubes as a consequence of the introduction of polar groups in these materials.

The obtained conjugates were intravenously administered to rats and dynamically traced *in vivo* using a microSPECT scanner. Figure 1 shows how f-CNTs move along the rat body. As the time increases, the CNTs are eliminated. [^{111}In]DTPA-MWNTs reached the kidneys and the bladder within 1 min after entering systemic blood circulation. f-CNTs are mostly located in the kidneys and the bladder after 30 min. After 6 h, the amount of remaining radiolabelled material into the different organs is scarce and located in the kidneys. Most of the radioactive MWNTs have been eliminated through

the urinary system. After one day, there is hardly a residual amount of MWNTs in the kidneys.

Figure 1 Images of the whole body distribution of [^{111}In]DTPA-MWNTs in rats after 5 min, 30 min, 6 h and 24 h post-injection (see online version for colours)



The data reported in Figure 2 confirm that most of the radioactive nuclide is found in the urine after 24 h. Kidneys also show a noticeable concentration of f-CNTs. From these results, we can conclude that

- CNTs leave the organism through the urinary system
- the lifetime of the CNTs within the organism is short, being the nanomaterials evacuated in a brief period of time.

In the next step of our study, after the identification of the pathway that the nanotubes follow to be eliminated, we have evaluated their effect when they entered the body [6b]. A comparative analysis of non-functionalised purified MWNTs (pMWNTs) and functionalised MWNTs (f-MWNTs) with two different degrees of functionalisation (0.2 mmol and 0.9 mmol of amino groups per gram of nanotubes) was carried out. Our goal was to establish the influence of the nature of the functional groups and the degree of CNT functionalisation on tissue accumulation and damage. Transmission Electron Microscopy (TEM) analysis of the different samples revealed that the functionalisation of the CNTs induced debundling and improved the individualisation of the nanomaterials (Figure 3). The decoration of the surface with pendant fragments that incorporate triethylene glycol moieties increases remarkably the solubility of CNTs in water.

Following the intravenous administration in mice of the different unmodified and modified nanotubes, the animals were sacrificed 24 h after the injection. The kidneys, liver, spleen, heart and lungs were harvested and examined. We have analysed the different tissues to detect injury or abnormality during the first 24 h post-injection of the CNTs.

Figure 4 shows different optical microscopy images of organs such as lung, liver, spleen and kidney. While a, c, e and g represent control experiments with mouse serum, b, d, f and h are representative pictures of organs of mice treated with 200 μg/animal of purified MWNTs. Arrows in pictures b and d highlight the presence of pMWNTs in those tissues. This analysis proves the accumulation of CNTs, very likely due to the lack of individualisation and solubility that are characteristics of non functionalised MWNTs.

Figure 2 % ID radioactivity per gram tissue at 24 h after intravenous administration of [^{111}In]DTPA-MWNTs quantified by gamma counting (n = 3 and error bars for standard deviation) (see online version for colours)

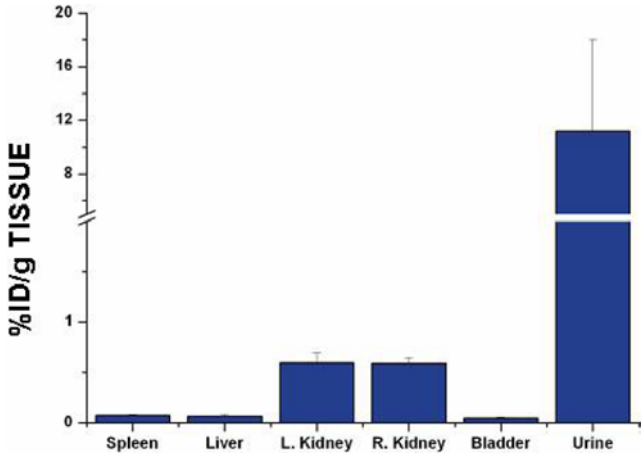
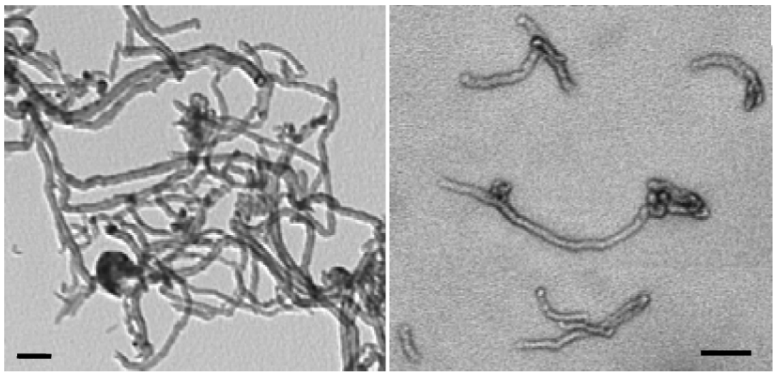


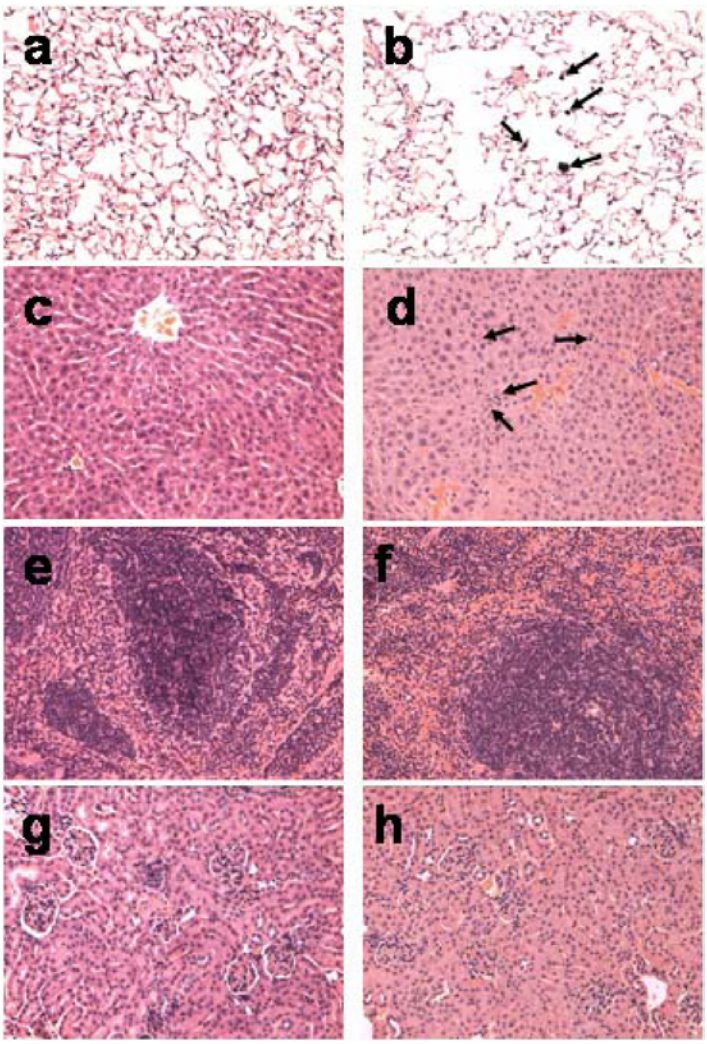
Figure 3 TEM images of the pristine CNTs (left) and functionalised CNTs (right). Scale bars correspond to 200 nm



In addition, the mice injected with unmodified nanotubes presented symptoms such as subdued behaviour, hunched posture and signs of respiratory distress including tachypnea. These signs were faded away after 24 h. Functionalised MWNTs with different amount ammonium groups were also injected in mice. Figure 5 shows the haematoxylin and eosin-stained sections of the four aforementioned organs injected with 5% dextrose, 200 µg of ammonium MWNTs with 0.2 mmol/g and 0.9 mmol/g of NH_3^+ in dextrose.

The influence of the degree of functionalisation is evident in the images of the second and third column of Figure 5. In the case of functionalised MWNTs with a low degree of functional groups, it was observed an accumulation of MWNTs (see arrows in Figure 5) as clusters in the liver and spleen while we could not detect the presence of MWNTs in any of the tissues with the highly functionalised MWNTs. These data show a clear influence of the functionalisation over the excretion capability of MWNTs.

Figure 4 It shows haematoxylin and eosin-stained optical microscopy images of sections of the lung, liver, spleen and kidney injected with mouse serum (a, c, e, g) and 200 µg of pMWNT in serum (b, d, f, h) (see online version for colours)



Another open question concerned the influence of the nature of the functional groups. Two different samples of MWNTs functionalised with opposite charged groups have been used to ascertain this topic. Indeed, ammonium functionalised MWNTs with 0.9 mmol/g of functional groups reacted with DTPA to generate negatively charged CNTs. In both cases, the treated mice with high doses of oppositely charged nanotubes did not show any symptom. Lungs and liver of mice treated with pMWNTs show a dark colour, signal of accumulation of pMWNTs as we notice in the optical microscopy images where the aggregates were present (highlighted with arrows, Figure 6).

After the finding that functionalised carbon nanotubes with high water solubility are quickly eliminated from the body without any toxic effect or any evidence of accumulation, we have studied in detail possible routes involved in the excretion. It is

indeed of fundamental importance to study the mechanism used by the nanotubes for their removal from the body. In the case of pristine nanotubes, their aggregation led to an accumulation in the liver and lung. On the contrary, MWNTs with DTPA are cleared from blood circulation through the urinary system as previously shown. Because of an absence of f-MWNTs in the kidneys after 6 h (Figure 1), it seemed plausible to conclude that the clearance of this type of material followed the glomerular filtration system. The morphology of the kidney filtration barrier suggests that carbon nanotubes should traverse it longitudinally (Figure 7) [6c]. The cross section of nanotubes used in our studies was in the range of 20–30 nm, enough small to access to the filtration slits of podocytes (40 nm) and the fenestra (30 nm).

Figure 5 Haematoxylin and eosin-stained optical microscopy images of sections of lung (a–c), liver (d–f), spleen (g–i) and kidney (j–l) of BALB/c mouse tissues at 24 h post-administration of 5% dextrose (a, d, g, j), 200 µg of ammonium MWNTs with 0.2 mmol/g of NH_3^+ (b, e, h, k) and 0.9 mmol/g of NH_3^+ (c, f, i, l) in 5% dextrose (see online version for colours)

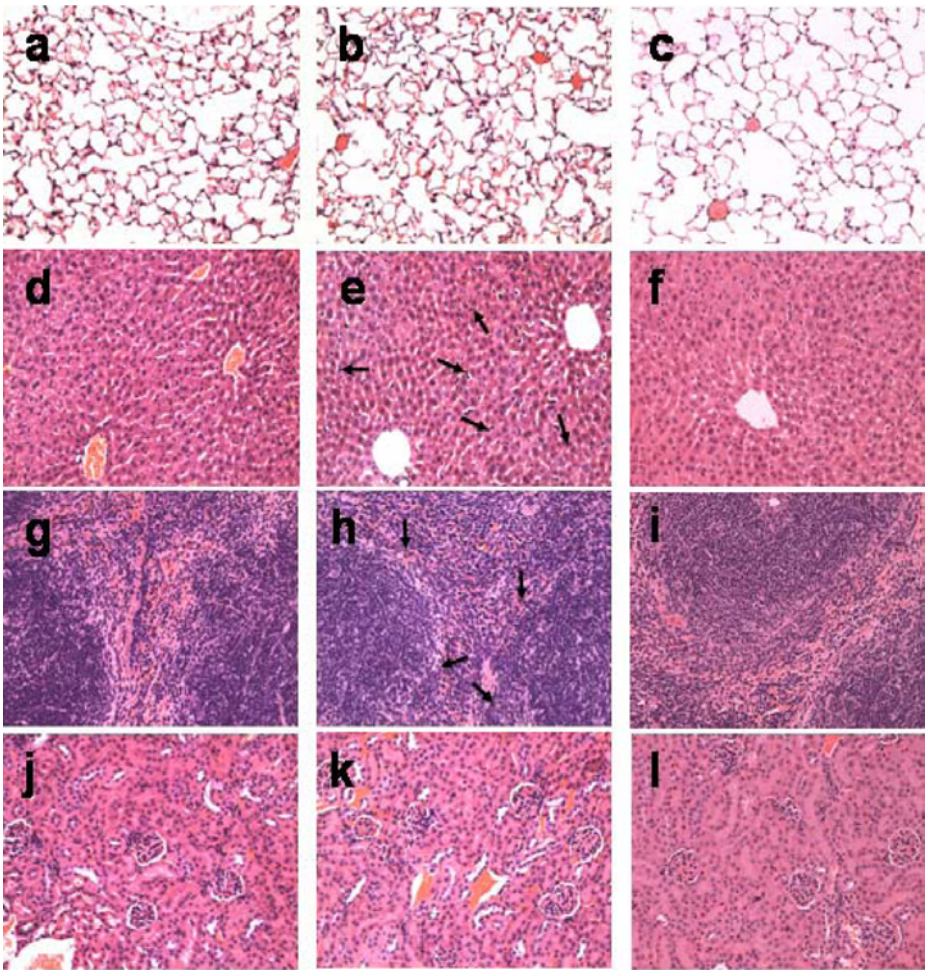
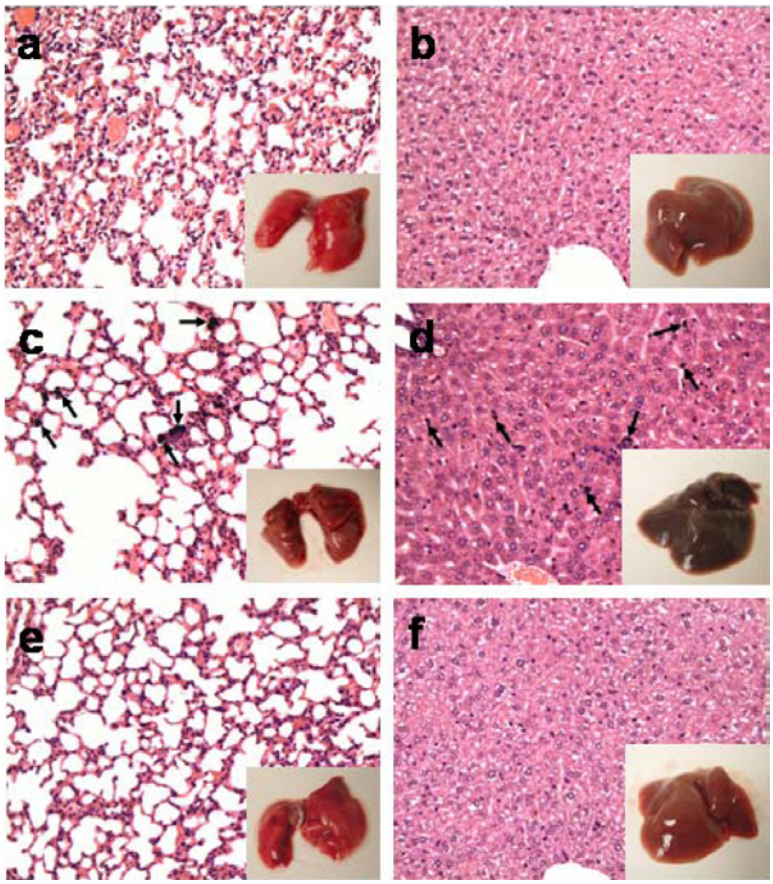
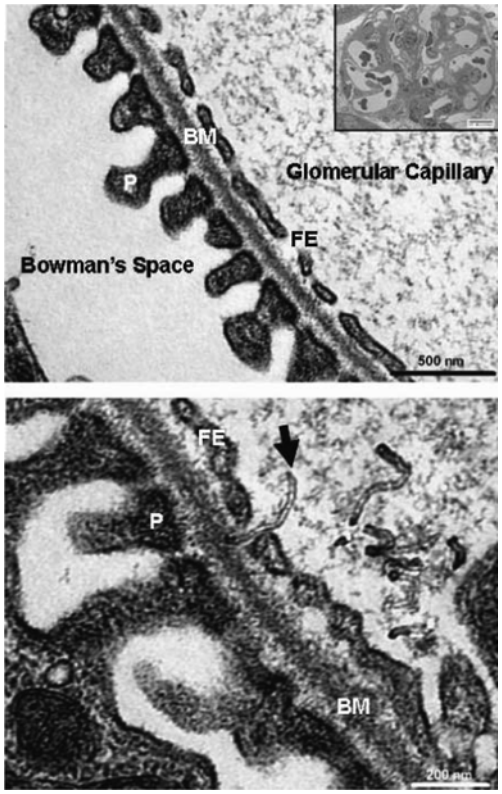


Figure 6 Haematoxylin and eoxin-stained optical microscopy images of sections of lung (a, c, e) and liver (b, d, f) of BALB/c mouse tissues at 24 h post-administration of PBS (a, b), 400 µg of pMWNTs (c, d) and 400 µg of MWNT-DTPA (e, f). Insets show organs after treatment with the samples aforementioned (see online version for colours)



The nanotubes were 0.5–2 nm long and the glomerular basal membrane is in the range of 200–400 nm [9]. For steric reasons, any other possibility of passage is difficult to imagine. The data also indicate that the functionalised nanotubes are flexible to adopt the right conformation to reach the glomerular system and readily pass the Bowman's space. TEM images allowed confirming the presence of nanotubes in the lumen of glomerular capillary. The bottom image of Figure 7 clearly shows a nanotube crossing the filtration membrane. MWNTs have been then observed intact in the excreted urine of the rats. One of the main conclusions derived from this study is that the individualisation of the CNTs is critical to reach the urine for elimination. pMWNTs that form bundles accumulated in different organs, especially in the liver. Even if they manage to arrive to the lumen of glomerular capillary, they cannot be excreted because of the size of aggregate. Individual CNTs can be obtained by an increase in their functionalisation.

Figure 7 Top picture shows the glomerular filtration barrier of BALB/c mice: P, podocyte; BM, basal membrane; FE, fenestrated endothelium. Bottom figure shows the individualised MWNT (black arrow) 5 min after intravenous (tail vein) injection



3 Conclusions and perspectives

Nanomaterials designed for applications in medicine are bringing a lot of interests. However, before envisaging any clinical application of CNTs, a systematic analysis of their toxicity as well as of the mechanisms of elimination is mandatory. In this direction, we have found that:

- an increment in the functionalisation leads to a better individualisation of the CNTs and this leads to the absence of CNT aggregates in tissues
- the nature of the functional groups is not critical for the urinary excretion of CNTs, as long as these groups enhance the solubility in water of these nanomaterials
- CNTs use the glomerular filter barrier to reach the urine and to be excreted.

We have reviewed here the major breakthrough we have previously reported towards the evaluation of the toxicity aspects and the behaviour of CNTs for future applications in biomedicine. We are currently introducing other modifications on CNTs to extend and ameliorate our current panel of functionalised carbon nanotubes.

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