RESEARCH ARTICLE

Tissue histology and physiology following intravenous administration of different types of functionalized multiwalled carbon nanotubes

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Background: Carbon nanotubes (CNTs) constitute one of the most important types of nanomaterials, increasingly gaining interest as tools for nanomedicine applications, such as sensors, implants or delivery systems. Our groups have reported previously that chemical functionalization of CNTs can lead to their almost complete elimination from the body of animals through the urinary excretion route. The administration of CNTs may, however, impact the physiological function of organs through which CNTs traverse or accumulate. **Aim:** The present study addresses the short-term impact (first 24 h) of intravenous administration of various types of multiwalled nanotubes (MWNTs) on the physiology of healthy mice. **Materials & methods:** Nonfunctionalized, purified MWNTs (pMWNTs) and different types of water-dispersible, functionalized MWNTs (*f*-MWNTs) were tail-vein injected. Histological examination of tissues (kidney, liver, spleen and lung) harvested 24 h post-administration indicated that organ accumulation depended on the degree of ammonium (NH_3^+) functionalization at the *f*-MWNT surface. **Results:** The higher the degree of functionalization of MWNT-NH_3^+, the less their accumulation in tissues. pMWNTs coated with autologous serum proteins prior to injection accumulated almost entirely in the lung and liver in large dark clusters. Moreover, various indicators of serum and urine analyses also confirmed that MWNT-NH_3^+ injections did not induce any physiological abnormality in all major organs within the first 24 h post-injection. Interestingly, no abnormalities were observed either for *f*-MWNTs highly functionalized with carboxylate groups (diethylentriaminepentaacetic-functionalized MWNTs) or by upscaling to the highest doses ever injected so far *in vivo* (20 mg/kg). **Conclusion:** The high degree of *f*-MWNT functionalization responsible for adequate individualization of nanotubes and not the nature of the functional groups was the critical factor leading to less tissue accumulation and normal tissue physiology at least within the first 24 h post-administration, even at the highest carbon nanotube doses ever administered in any study today.

Keywords: functionalized carbon nanotubes, histology, mice, nanomedicine, nanotoxicology, pharmacology, urine analysis

The toxicological profile of newly discovered nanomaterials and the environmental and health risks posed following their wider utilization is currently an issue of intense debate and interest [1–4]. There has been an explosive increase in the number of nanomaterials designed for biomedical applications that has generated extraordinary interest and expectations for effective, disease-eradicating therapeutic modalities [5]. At the same time, the toxicological burden of such novel nanomaterials remains largely unknown, further complicating the discussion for the need of a new regulatory framework for nanomaterials [6,7]. One such type of highly innovative nanomaterial is the carbon nanotube (CNT), which was first defined in the early 1990s by Iijima [8]. Extraordinary characteristics of this material, consisting only of a network of carbon atoms in the nanometer scale, include great tensile strength, as well as high electrical and thermal conductivity [9].

Our work has focused on the pharmacological development of functionalized CNTs (*f*-CNTs) using the 1, 3-dipolar cycloaddition reaction [10,11] to render the CNT surfaces water dispersible and therefore compatible with the biological milieu. Various biomedical applications of *f*-CNTs have been explored and encouraging proof-of-principle studies have indicated their effective role as delivery systems for genes, peptides, antimicrobial agents and cytotoxic drug molecules [12–16]. However, the clinical evaluation of any therapeutic or diagnostic agent based on *f*-CNTs will involve the administration or implantation of nanotubes and their matrices into patients. In order to design such clinical studies, preclinical development of *f*-CNTs is essential, particularly the determination of their *in vivo* pharmacological and toxicological profiles. Towards that goal, we first reported tissue biodistribution and blood circulation half-life

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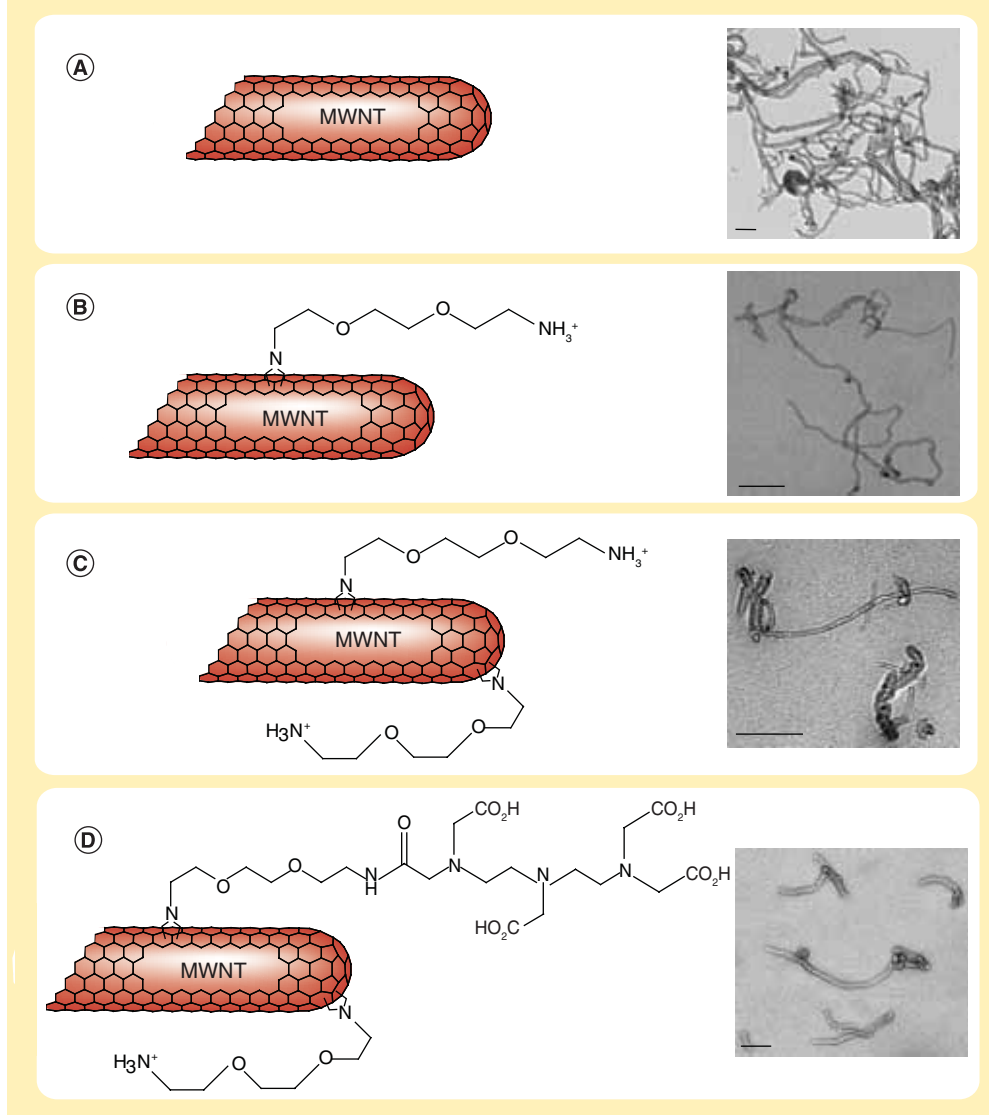
data following intravenous administration of single-walled CNTs (SWNTs) functionalized covalently with tracer radionuclides [17]. Other laboratories have also carried out *in vivo* studies following intraperitoneal [18], intratumoral [19] or intravenous [20–24] administration of different types of CNTs. However, none of these reports has studied the physiological and histological impact on the tissues that CNTs traversed or accumulated.

In the present work, we have investigated whether tissue accumulation following intravenous administrations of functional multiwalled nanotubes (*f*-MWNTs) in comparison to purified MWNTs (pMWNTs) in mice occurred and its histological and physiological impact on those animals. The MWNTs were made water dispersible by insertion of ammonium–triethylene glycol chains onto the sidewalls and tips of the MWNT backbones (Figure 1). We observed that intravenous administration of nonfunctionalized pMWNTs led to lung, liver and spleen accumulation, whereas *f*-MWNT accumulation in organs was dependent on the degree of functionalization, but was independent of the characteristics of the functional group.

Figure 1. Molecular structures and transmission electron microscopy images of multiwalled carbon nanotubes.



(A) Nonfunctionalized, purified MWNT (pMWNT), **(B)** ammonium-functionalized MWNT (MWNT-NH_3^+) with 0.2 and **(C)** 0.9 mmol/g of MWNT-NH_3^+ and **(D)** diethylentriaminepentaacetic-functionalized MWNT (MWNT-DTPA). Scale bars correspond to 200 nm.

Materials & methods

MWNTs

Nonfunctionalized pMWNTs were purchased from Nanostructured & Amorphous Materials Inc. (Houston, TX, USA). Regular pMWNTs used in this study were 94% pure, stock number 1240XH. Outer average diameter was between 20 and 30 nm and length was between 0.5 and 2 µm. The pMWNTs and *f*-MWNTs used in this study have been fully characterized and reported previously. For detailed information regarding the characterization of the nanotubes before and after functionalization, please see [25]. Briefly, the level of elemental impurities was 10.6% (Fe and Ni) in the pMWNTs and 8.1% in *f*-MWNTs, as determined by atomic absorption analysis and thermogravimetric analysis. A mixture of closed- and open-ended nanotubes was found in all samples by transmission electron microscopy (TEM) and cryo-TEM. To perform the toxicological studies in mice, pMWNTs were suspended in mouse serum (Sera Laboratories International Ltd., UK). The serum suspensions were bath sonicated (45 kHz) for 10 min. The black homogeneous suspensions obtained were administered *in vivo* without any further treatment. Water-dispersible ammonium-functionalized MWNTs (MWNT-NH_3^+) and diethylentriaminepentaacetic-functionalized MWNTs (MWNT-DTPA) were prepared as described in detail elsewhere [10,11,17]. The number of free NH_3^+ groups remaining on the MWNTs was measured by the quantitative Kaiser test. Two samples of MWNT-NH_3^+ were prepared with different amounts of NH_3^+ groups: 0.2 and 0.9 mmol/g of material. MWNT-DTPA have been prepared starting from MWNT-NH_3^+ with 0.9 mmol/g of amines, 55% of NH_3^+ remained unreacted. The total MWNT-DTPA charge should therefore be negative. The MWNT-NH_3^+ were suspended in 5% dextrose solutions and MWNT-DTPA were suspended in phosphate-buffered saline (PBS) solutions prior to *in vivo* studies performed in mice.

TEM analysis

For the TEM analysis, the different MWNTs, dispersed in organic solvent (pMWNTs) or in water (*f*-MWNTs), were deposited in 300-mesh copper grids coated with formvar/carbon support film (Euromedex, France). After drying, images were collected using a Philips 208 TEM working at different accelerating voltage. Digital images were captured using a CCD high-resolution camera AMT (Eindhoven, The Netherlands).

Animal-handling procedures

Female BALB/c mice (Harlan, UK), 6–8 weeks old, were housed in groups of five, bedded on wood shavings and maintained on a standard rodent chow diet with mains drinking water *ad libitum*. A temperature of 19–22°C was maintained, with a relative humidity of 45–65% and a 12-h light/dark cycle (lights on at 7:00 am). Animals were acclimatized for 7 days before each experiment. All procedures were approved by the Home Office (1989) Code of Practice for the Housing and Care of Animals used in Scientific Procedures (UK).

Administration of MWNTs in mice

BALB/c mice were separated randomly in groups of five mice and injected intravenously by the tail vein with 200 µl (per mouse) of the following suspensions: mouse serum only, 200 µg of pMWNTs in mouse serum, 5% dextrose only and 200 µg of MWNT-NH_3^+ in 5% dextrose. MWNT-NH_3^+ in 5% dextrose had two different functionalization degrees: 0.2 mmol of NH_3^+ per gram of material and 0.9 mmol/g of NH_3^+ per gram of material. The mice were placed individually into metabolic cages (Tecniplast, UK) and deprived of food for 24 h. Urine production and water consumption were monitored. At 24 h post-injection, the mice were sacrificed and necropsied. Kidneys, liver, spleen, heart and lungs were harvested and weighed. In an additional study, BALB/c mice were separated randomly in groups of four mice and injected intravenously by the tail vein with 200 µl of the following suspensions per mouse: mouse serum only, 400 µg of pMWNTs in mouse serum, PBS only and 400 µg of MWNT-DTPA in PBS. The mice were placed by group into metabolic cages (Tecniplast, UK) and monitored over 24 h. Pooled urine production and water consumption were monitored. At 24 h post-injection, the mice were sacrificed and necropsied. Kidneys, liver, spleen, heart and lungs were harvested and weighed.

Serum biochemistry analysis

Whole blood was collected from the inferior vena cava of BALB/c mice following inhalation of a terminal dose of isoflurane (Abbott, UK). The blood was allowed to clot on ice for at least 30 min prior to centrifugation at 4000 rpm for 15 min at room temperature. The serum was collected and stored at -80°C. Serum biochemistry analysis was conducted by the Laboratory Diagnostic Service of the Royal Veterinary College

(London, UK) for the following parameters: total protein, albumin, sodium, potassium, chloride, urea, creatinine, total bilirubin, lactate dehydrogenase, alanine aminotrasferase (ALT), aspartate aminotrasferase (AST), creatine kinase and alkaline phosphatase (ALP).

Urinalysis

Urine collected from mice at 24 h post-injection was analyzed using Multistix 10 SG reagent strips (Bayer, UK) for the following parameters: glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrite and leukocytes.

Histopathology

The tissues harvested from mice were fixed in 10% buffered formalin and processed for routine histology with hematoxylin and eosin stain by the Laboratory Diagnostic Service of the Royal Veterinary College (London, UK). Microscopic observation of tissues was carried out with a Nikon Microphot-FXA microscope coupled with a digital camera (Infinity 2).

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Data were analyzed for differences by Student's t-test and considered to be statistically significant $p < 0.05$ for comparison against the 5% dextrose-treated group. Additionally, the control serum- and pMWNT-treated groups were also analyzed for differences by Student's t-test and considered to be statistically significant $p < 0.05$. F-tests were also performed to evaluate the variance differences and validate the statistical analysis.

Results

In the present study, we examined whether the intravenous administration of different MWNTs (Figure 1) induced any tissue injury or other histological or physiological abnormality on the organs that have been shown previously to interact with the nanotubes *in vivo* during the initial 24 h following injection. Nonfunctionalized pMWNTs and water-dispersible *f*-MWNTs were compared in order to verify the impact of functionalization on the *in vivo* profile of these nanostructures. We injected pMWNTs and MWNT-NH_3^+ with two different degrees of functionalization through the tail vein of female BALB/c mice. These mice were kept for 24 h in metabolic cages, their behavior was monitored and urine and blood samples were collected to examine the function of all major organs. At 24 h post-administration of MWNTs, mice were sacrificed and organs were harvested and examined.

Nonfunctionalized pMWNTs are extremely hydrophobic materials that are difficult to disperse in aqueous-based environments owing to the van der Waals forces, leading to aggregation in bundles. However, dispersions of such pMWNTs have been described by others [26] by simple pre-mixing with serum proteins. For the purposes of the present study, we followed a similar protocol for the preparation of pMWNTs dispersions that acted as controls, by pre-incubation and sonication of the nanotubes in autologous mouse serum to obtain aqueous suspensions of pMWNTs. As can be seen from Figure 1, TEM examination of all the MWNTs samples used in this study revealed that, even though water dispersibility was improved for the serum-coated pMWNTs, the *f*-MWNTs were much more individualized. Functionalized MWNT-NH_3^+ are highly water dispersible and aqueous dispersions in 5% dextrose were prepared with such nanotubes with two different degrees of functionalization: 0.2 and 0.9 mmol of NH_3^+ (functional groups) per gram of material. Along with the vehicle controls (serum and 5% dextrose), the nanotubes were injected intravenously in female BALB/c mice through the tail vein. After recovering from anesthesia, the group of mice injected with pMWNTs (200 µg/animal) exhibited subdued behavior, hunched posture and signs of respiratory distress, including tachypnea. Additionally, these mice were less active than the groups injected with the vehicle controls and MWNT-NH_3^+ (200 µg/animal).

However, the signs of distress observed initially in the group injected with pMWNTs diminished over the 24 h period. Finally, 24 h post-administration, blood was collected to run biochemical analysis, the mice were killed and the kidneys, liver, spleen, heart and lungs were harvested. In Table 1, the serum biochemistry data are shown, whereas Table 2 shows the urine analysis data, performed to assess the function of the hepatic and renal systems of the mice treated with different MWNTs. Comparison of the serum levels of enzymes indicating hepatocellular injury, ALT, AST, ALP, total protein, albumin and bilirubin levels between the 5% dextrose control and MWNT-NH_3^+ groups have only shown statistically significant differences for the total protein of MWNT-NH_3^+ with a loading of NH_3^+ of 0.2 mmol/g ($p < 0.05$). Comparison of the serum control versus the pMWNT groups

Table 1. Serum biochemical analysis in BALB/c mice at 24 h post-administration of multiwalled carbon nanotubes.

	Dextrose control	Serum control	pMWNTs	MWNT-NH_3^+ (0.2 mmol/g)	MWNT-NH_3^+ (0.9 mmol/g)
Total protein (g/l)	49.9 (± 1.77)	51.0 (± 1.54)	50.2 (± 1.23)	47.8* (± 0.67)	48.3 (± 2.44)
Albumin (g/l)	32.8 (± 1.26)	33.7 (± 0.46)	32.7 (± 0.91)	32.2 (± 0.42)	32.7 (± 1.37)
Sodium (mmol/l)	158.0 (± 2.69)	154.1 (± 0.98)	154.4 (± 3.11)	157.7 (± 1.11)	155.9 (± 1.13)
Potassium (mmol/l)	4.77 (± 0.438)	4.48 (± 0.269)	5.07§ (± 0.151)	4.98 (± 0.246)	4.47 (± 0.247)
Chloride (mmol/l)	117.1 (± 3.47)	116.3 (± 1.38)	116.0 (± 2.21)	116.3 (± 1.79)	116.0 (± 0.95)
Urea (mmol/l)	7.7 (± 1.26)	10.0* (± 1.36)	10.2 (± 2.83)	13.0 (± 4.93)	8.9 (± 1.23)
Creatinine (µmol/l)	47 (± 3.4)	48 (± 2.9)	49 (± 2.6)	50 (± 5.7)	47 (± 1.5)
Total bilirubin (µmol/l)	1.5 (± 0.30)	1.3 (± 0.51)	1.1 (± 0.31)	1.6 (± 0.11)	1.6 (± 0.58)
LDH (U/l)	1428 (± 540.4)	647* (± 204.4)	808 (± 213.4)	1022 (± 217.7)	1302 (± 554.2)
ALT (U/l)	46 (± 25.6)	28 (± 8.2)	33 (± 17.7)	55 (± 16.7)	29 (± 4.9)
AST (U/l)	135 (± 58.3)	78 (± 11.7)	95 (± 33.9)	155 (± 53.6)	77 (± 40.7)
CK (U/l)	624 (± 489.8)	119 (± 31.3)	229 (± 236.8)	508 (± 469.0)	564 (± 577.4)
ALP (U/l)	294 (± 15.6)	202‡ (± 5.4)	172‡¶ (± 14.3)	310 (± 22.1)	281 (± 27.2)

All groups n = 5. Values of average (± standard deviation).

**p < 0.05 and ‡p < 0.001 indicate statistical significance compared with control 5% dextrose-treated group.*

§p < 0.05 and ¶p < 0.01 indicate statistical significance compared with control serum-treated group.

ALP: Alkaline phosphatase; ALT: Alanine aminotrasferase; AST: Aspartate aminotrasferase; CK: Creatine kinase; LDH: Lactate dehydrogenase; MWNT-NH_3^+: Ammonium-functionalized multiwalled nanotube; pMWNT: Nonfunctionalized, purified MWNT.

verified a significant difference in the decrease of ALP for the pMWNT group ($p < 0.01$). Renal function may be monitored using a combination of the serum levels of urea nitrogen and creatinine (Table 1) and urine analysis parameters, such as pH and the presence of erythrocytes, leukocytes, protein and bilirubin in urine (Table 2). No differences were found between groups for the various parameters that act as indicators of renal function, suggesting no adverse effects on physiological function.

Histological examination of tissues 24 h post-MWNT administration, using hematoxylin and eosin-stained sections, indicated that no tissue degeneration, inflammation, necrosis or fibrosis had occurred in any of the different groups. In Figures 2 & 3, representative images from sectioned tissues after injection of nonfunctionalized pMWNTs and functionalized MWNT-NH_3^+ compared with the tissues after injection of control vehicles (serum and 5% dextrose, respectively) are shown. Figure 2 shows hematoxylin and eosin-stained sections of the lung, liver, spleen and kidney injected with mouse serum (Figure 2A, C, E & G) and 200 µg of pMWNTs in serum (Figure 2B, D, F & H). The accumulation of pMWNT clusters in the lung (Figure 2B) and liver (Figure 2D) could be observed, whereas the spleen and kidney did not contain any deposits. Figure 3 shows the hematoxylin and eosin-stained sections of the lung, liver, spleen and kidney injected with 5% dextrose (Figure 3A, D, G & J),

Table 2. Urine analysis of BALB/c mice.

	Dextrose control	Serum control	pMWNTs	MWNT-NH_3^+ (0.2 mmol/g)	MWNT-NH_3^+ (0.9 mmol/g)
Glucose (mmol/l)	Neg.	Neg.	Neg.	Neg.	Neg.
Bilirubin	Neg.	Neg.	Neg.	Neg.	Neg.
Ketone (mmol/l)	Trace	Neg.	Neg.	Neg.	Trace
Specific gravity	1.025	1.025	1.020	1.020	1.020
Blood (erythrocyte/µl)	Neg.	Neg.	Neg.	Neg.	Neg.
pH	6.5	6.0	6.5	6.5	6.5
Protein (g/l)	30	30	30	Trace	30
Urobilinogen (µmol/l)	0.2	0.2	0.2	0.2	0.2
Nitrite	Pos.	Pos.	Pos.	Pos.	Pos.
Leukocytes (cell/µl)	Neg.	Neg.	Neg.	Neg.	Neg.

Samples of urine collected at 24 h post-administration of MWNTs. All groups n = 5.
MWNT-NH_3^+: Ammonium-functionalized multiwalled nanotube; Neg.: Negative; pMWNT: Nonfunctionalized, purified MWNT; Pos.: Positive.

200 µg of MWNT-NH_3^+ with 0.2 mmol/g of NH_3^+ (Figure 3B, D, H & K) and 0.9 mmol/g of NH_3^+ (Figure 3C, F, I & L) in 5% dextrose. Tissue histology appeared normal for all tissues, except accumulation of nanotube clusters observed in the liver and spleen of animals injected with the *f*-MWNT, which had a low degree of surface functionalization (0.2 mmol/g of NH_3^+). By contrast, it was not possible to detect MWNT-NH_3^+ with a high functionalization degree (0.9 mmol/g) in any of these tissues.

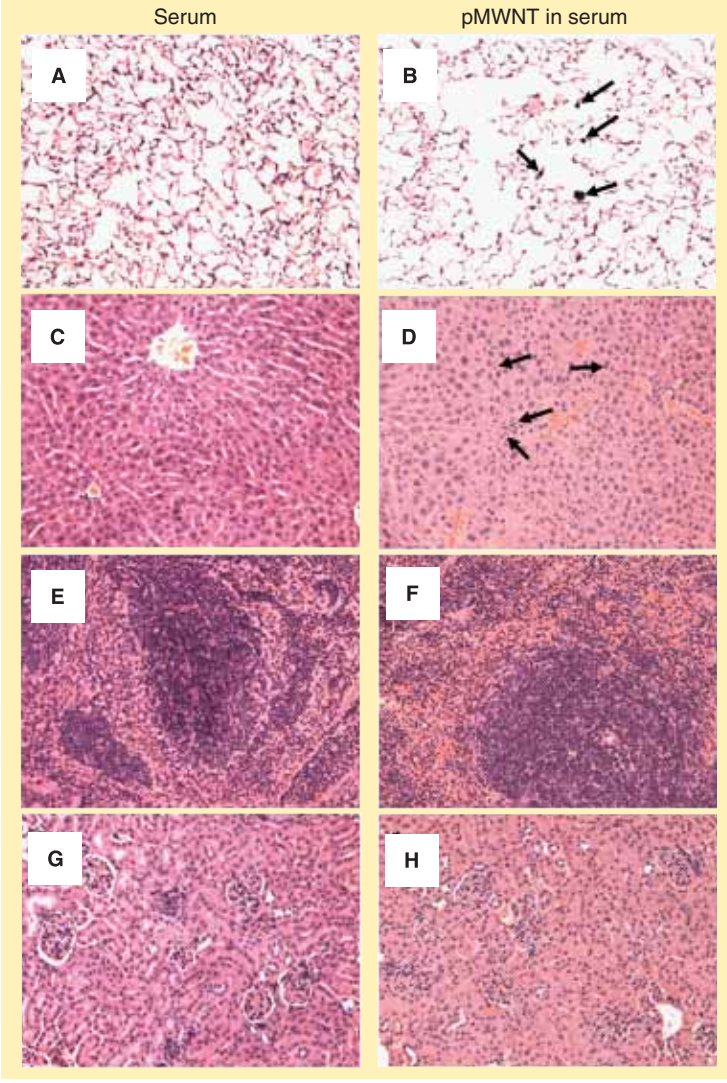
The tissues where accumulation of nanotubes was observed were further investigated in an attempt to determine the exact location of MWNTs in those tissues. Figure 4 depicts higher magnification images of the sections for lung, liver and spleen of BALB\c mice at 24 h post-administration of 200 µg pMWNTs in serum (Figure 4A, C & E) and 200 µg MWNT-NH_3^+ with 0.2 mmol/g of NH_3^+ in 5% dextrose (Figure 4B, D & F). Interestingly, it was possible to observe accumulated large clusters in the sections of animals that received pMWNTs in the lung (arrows in Figure 4A), smaller clusters in liver sinusoids (arrows in Figure 4C) and a small amount of punctuated accumulations in the spleen (arrows in Figure 4E). Low-functionalized MWNT-NH_3^+ (0.2 mmol/g) showed liver (arrows in Figure 4D) and spleen (arrows in Figure 4F) but not lung (Figure 4B) accumulation. These nanotubes appear in small punctuated accumulations inside Kupffer cells (liver) and in the intermediate zone of the spleen.

Urine production, weight body loss and organ weight at 24 h post-administration of vehicles and MWNTs were also monitored and these data are shown in Table 3. The weight of organs can indicate signs of inflammation, commonly evidenced by increased organ weight. As can be seen, no significant differences were found among groups with the exception of spleen weight for serum control and pMWNT groups in comparison to the 5% dextrose group ($p < 0.05$).

In order to study whether the nature of the chemical moiety on the functionalization groups could lead to different interactions with the organs or to any tissue injury, we performed a further study by maintaining the high degree of functionalization at 0.9 mmol per gram of material, but using a different functional group (DTPA) that contains four -COOH groups, and upscaled the injected dose. The functionalized MWNT-DTPA were suspended in PBS and a higher dose of 400 µg of nanotubes per mouse was administered through the tail vein. For comparison, a group of animals injected with a higher dose of 400 µg of nonfunctionalized pMWNTs suspended in mouse serum were used as a control. Following the intravenous administration of 400 µg pMWNTs, all treated mice adopted a hunched posture, exhibited piloerection and presented acute signs of respiratory distress including tachypnea. Furthermore, these signs were persistent over the 24 h period of observation indicating that, as expected, the distress symptoms were dose dependent compared with the previous study of lower pMWNT doses (200 µg/animal), in which those symptoms lapsed soon after administration. In the case of the functionalized MWNT-DTPA group, none of these signs were observed at any time within the course of the experiment.

The animals were also killed and organs harvested and fixed in formalin 24 h following administration. The lung and liver seemed to be the main tissues where accumulation of pMWNTs occurred. Figure 5 shows representative hematoxylin and eosin-stained histological sections of lung and liver from the PBS control group (Figure 5A & B) and the groups injected with a high dose (400 µg/mouse) of pMWNTs (Figure 5C & D) or MWNT-DTPA (Figure 5E & F). In addition, the insets on each image in Figure 5 show the macroscopic appearance of the whole organ at necropsy.

Figure 2. Histology of mice injected with purified (nonfunctionalized) multiwalled carbon nanotubes.



Hematoxylin and eosin-stained sections of lung **(A & B)**, liver **(C & D)**, spleen **(E & F)** and kidney **(G & H)** of BALB/c mouse tissues at 24 h post-administration with mouse serum **(A, C, E & G)** and 200 µg of pMWNT in serum **(B, D, F & H)**. Magnification ×10.
pMWNT: Nonfunctionalized, purified multiwalled nanotube.

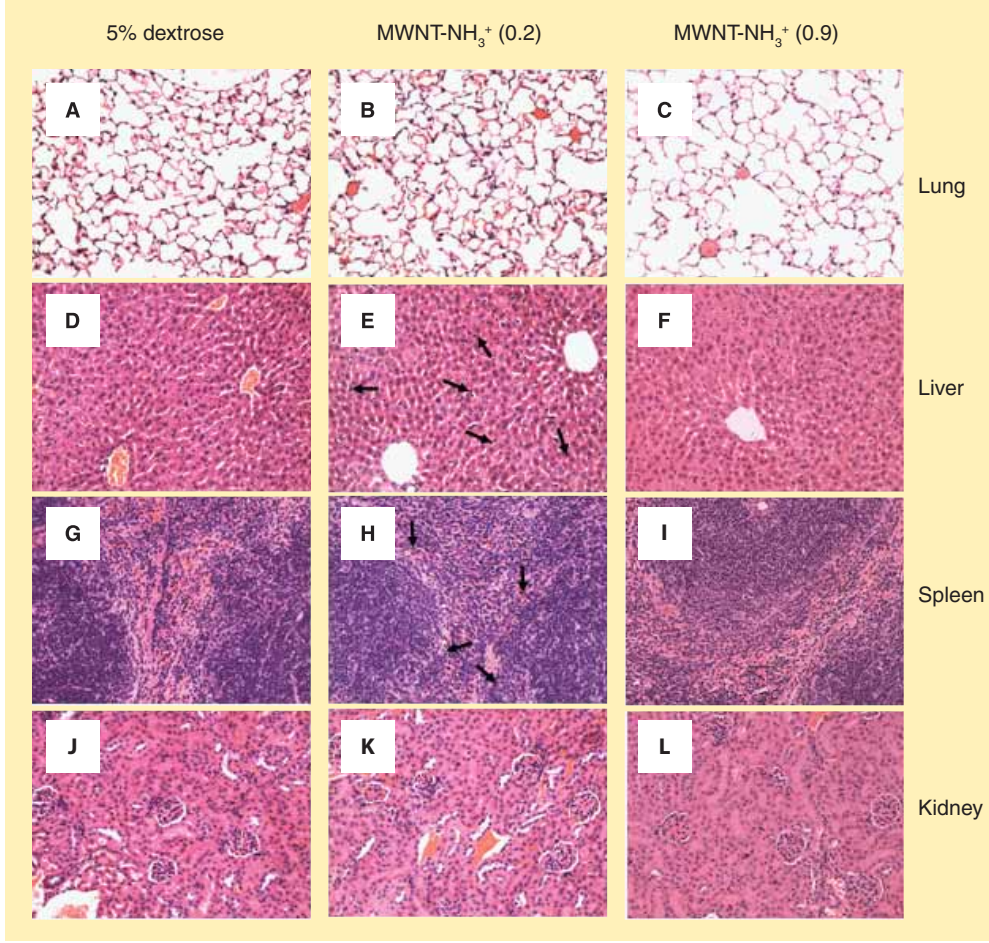
The histology sections of all organs confirmed the absence of cell degeneration, necrosis, inflammation or fibrosis as had been observed in the previous study using lower injected doses. However, the lung and liver sections from mice injected with pMWNTs contained large dark clusters distributed throughout the tissues (Figure 5C & D). Gross differences were observed between the organs at necropsy, manifested as a general discoloration (a darker overall color) of the lungs (inset in Figure 5C) and livers (inset in Figure 5D) of animals in the pMWNT group as compared with the control and MWNT-DTPA groups. Interestingly, even at this high dose in mice, MWNT-DTPA did not appear to accumulate or induce any tissue injury (Figure 5E & F).

Discussion

Several studies have reported the toxicological and physiological effects of nonfunctionalized CNTs *in vivo*, following local administration through the tracheal, nasal or subcutaneous routes in mice and rats [27]. Most of these studies reported adverse effects of CNTs resulting from organ accumulation, leading to tissue fibrosis and inflammatory responses. However, the critical factors responsible for the observed nonfunctionalized CNTs toxicity remain difficult to interpret owing to the different animal species, types of CNT and dosing regimes that have been used. Recently, we have shown the rapid passage of *f*-CNTs through the systemic circulation and the renal excretion of both single- and multiwalled *f*-CNTs [17,25]. In addition, other groups have reported the biodistribution of different types of covalently functionalized CNTs [18,21,23] and of noncovalently coated CNT dispersions with lipids and surfactants [20,22,24] administered intravenously in mice. Irrespective of the data presented in these studies, and whether the injected CNTs accumulated in tissues or not, the physiological and histological impact of intravenously administered CNTs was lacking. In this study, we attempted to study any histological and physiopathological effect of intravenous administrations with *f*-MWNTs compared with pMWNTs in healthy mice. This study focused on the impact of such MWNT administrations during the first 24 h post-injection.

The observed accumulation of pMWNTs in the capillaries of the pulmonary vascular bed (Figures 2B & 4A) is considered responsible for the respiratory distress these animals exhibited following administration. Moreover, in the liver, Kupffer cells in the sinusoidal walls contained the

Figure 3. Histology of mice injected with functionalized multiwalled carbon nanotubes.



Hematoxylin and eosin-stained 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 MWNT-NH_3^+ with 0.2 mmol/g of NH_3^+ **(B, D, H & K)** and 0.9 mmol/g of NH_3^+ **(C, F, I & L)** in 5% dextrose.
Magnification ×10.
MWNT-NH_3^+: Ammonium-functionalized multiwalled nanotube.

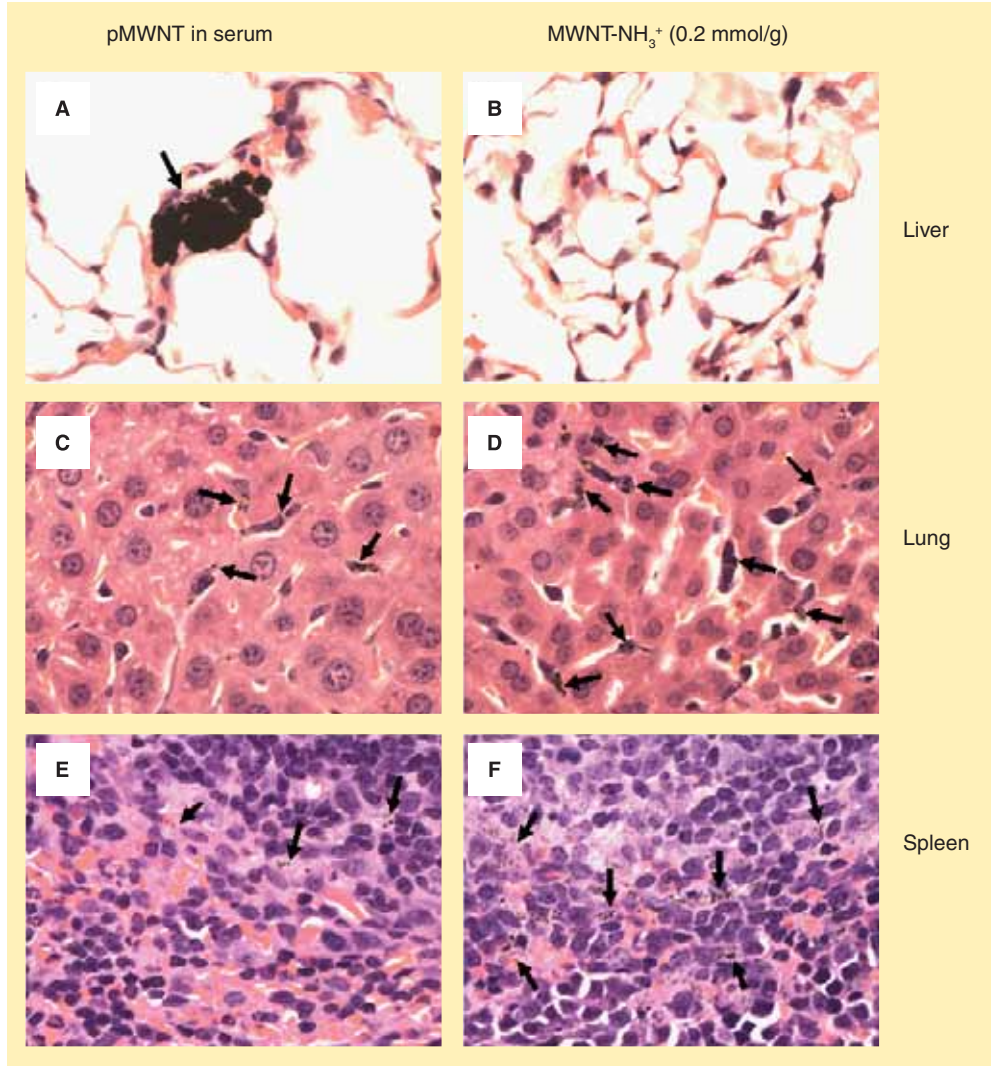
accumulated pMWNTs (Figure 4C). Liver accumulation of pMWNTs has not been shown before histologically in mice (our groups reported pMWNT accumulation in the liver of rats recently [25]) because all previous toxicity studies reporting histological data administered nonfunctionalized CNTs locally (through intranasal, intratracheal, intradermal routes) and reported nonfunctionalized CNTs accumulation in the alveoli and airway spaces [26,28–30]. pMWNT clusters accumulated in three main tissues: lung, liver and spleen, in what appeared to be correlated with increased dosing up to the highest dose ever injected in an animal (400 µg/mouse) (Figure 5C & D). Interestingly, no severe or acute response, such as allergy like, complement activation effects were observed in any of the animals or dose regimes in this study, however, more studies are warranted in order to determine any such effect.

A previous study by Deng *et al.* using water-dispersible ^{14}C-taurine-functionalized MWNTs reported high affinity for the liver with an accumulation of more than 80% of the injected dose after intravenous administration in mice [31]. Liver biochemistry and histology sections showed no acute liver toxicity and gradual body elimination of intact nanotubes was observed within 3 months. While the present study was in press, Liu *et al.* reported that PEGylated lipid-coated SWNTs accumulated overwhelmingly in the liver and spleen of mice after intravenous administration and a slow elimination of the nanotubes from the liver up to 3 months

post-administration [32]. We believe that high liver and spleen accumulation occurs owing to poor stability and poor individualization of these nanotubes *in vivo*. Earlier studies by Carrero-Sanchez *et al.* [33] and Elias *et al.* [34] using pure MWNTs and nitrogen-doped MWNTs have also suggested that the dispersion in aqueous-based solutions and the biocompatibility of MWNTs was greatly improved after functionalization. In this study, we showed that the degree of tissue accumulation was greater as the degree of functionalization decreased, such that MWNT-NH_3^+ with lower number of functional groups accumulated in the liver and spleen. No tissue accumulation or injury was observed after intravenous administration of MWNT-NH_3^+ with high density of functional groups, which have been described recently to be excreted from blood circulation through the urine [25]. This is considered to be a reflection of the high degree of individualization obtained with higher degrees of functionalization.

Lastly, the kidneys have normal glomerular morphology without any MWNT accumulation or injury for all types of MWNTs studied here, indicating that nanotubes did not have an adverse effect on the kidney. Passage through the kidney,

Figure 4. Accumulation of multiwalled carbon nanotubes in tissues.



Hematoxylin and eosin-stained sections of lung **(A & B)**, liver **(C & D)** and spleen **(E & F)** of BALB/c mouse tissues at 24 h post-administration of 200 µg of pMWNT in serum **(A, C & E)** and 200 µg of MWNT-NH_3^+ with 0.2 mmol/g of NH_3^+ in 5% dextrose **(B, D & F)**.
Magnification ×40.
MWNT-NH_3^+: Ammonium-functionalized multiwalled nanotube; pMWNT: Nonfunctionalized, purified MWNT.

as is the case with the highly functionalized MWNT-NH_3^+, did not cause any damage to the glomerular filter or alter histologically the tissue in any way indicating that rapid translocation from the blood compartment and urinary clearance can occur without side effects to renal function. Interestingly, despite the fact that several studies have now reported urinary elimination of water-dispersible *f*-CNTs [17,18,21,25,32,35], the impact of the *f*-CNT passage through the glomerular filter on renal function had not been previously determined.

We believe that the most striking finding in the present study is the critical importance of the degree of MWNT functionalization compared with the type of functional group (in this case -NH_3^+ compared with -COO^- [in DTPA] groups) in the ensuing pharmacological profile following intravenous administration. The higher the degree of functionalization, the better the individualization of the nanotubes, therefore, the more extensive the clearance through the kidneys. If the individualization of CNTs leads to their rapid excretion from the body, as we have observed and reported, the risk from accumulated CNTs and their long-term toxicity will be minimized. However, we still believe that more toxicological markers and parameters, such as produced levels of cytokines, should be studied in the future following administration of CNTs. This study is considered just the beginning in terms of toxicological assessment of *f*-CNTs in a systematic structure–function manner. Moreover, the issue of long-term impact on the pathophysiology of tissues in which CNTs accumulate or traverse is an unresolved issue of principal concern that will have to be addressed if these exciting nanomaterials are to move closer to the clinic.

Conclusion

In recent years, CNTs have been explored intensively for a variety of biomedical applications. The toxicological and pharmacological profile of such carbon nanomaterials will have a determinant role in their transformation into clinically viable and effective therapeutics. It is now becoming established knowledge that covalent functionalization, irrespective of functional group and chemistry, offers significant improvements in the toxicity profile of CNTs *in vitro* and *in vivo*. The present work indicated that highly functionalized and water-dispersible MWNTs did not accumulate in or injure any tissues on passage. By contrast, nonfunctionalized pMWNTs and *f*-MWNTs with a low functionalization degree can interact and accumulate in different tissues. From this work, highly functionalized water-dispersible MWNTs are thought to constitute toxicologically naive materials that can be further developed for pharmacological applications that may involve their systemic administration.

Future perspective

The discovery, development and large-scale manufacturing and production of novel nanomaterials that have never been studied in the context of a

Table 3. Weight of the organs of BALB/c mice 24 h post-administration of multiwalled carbon nanotubes.

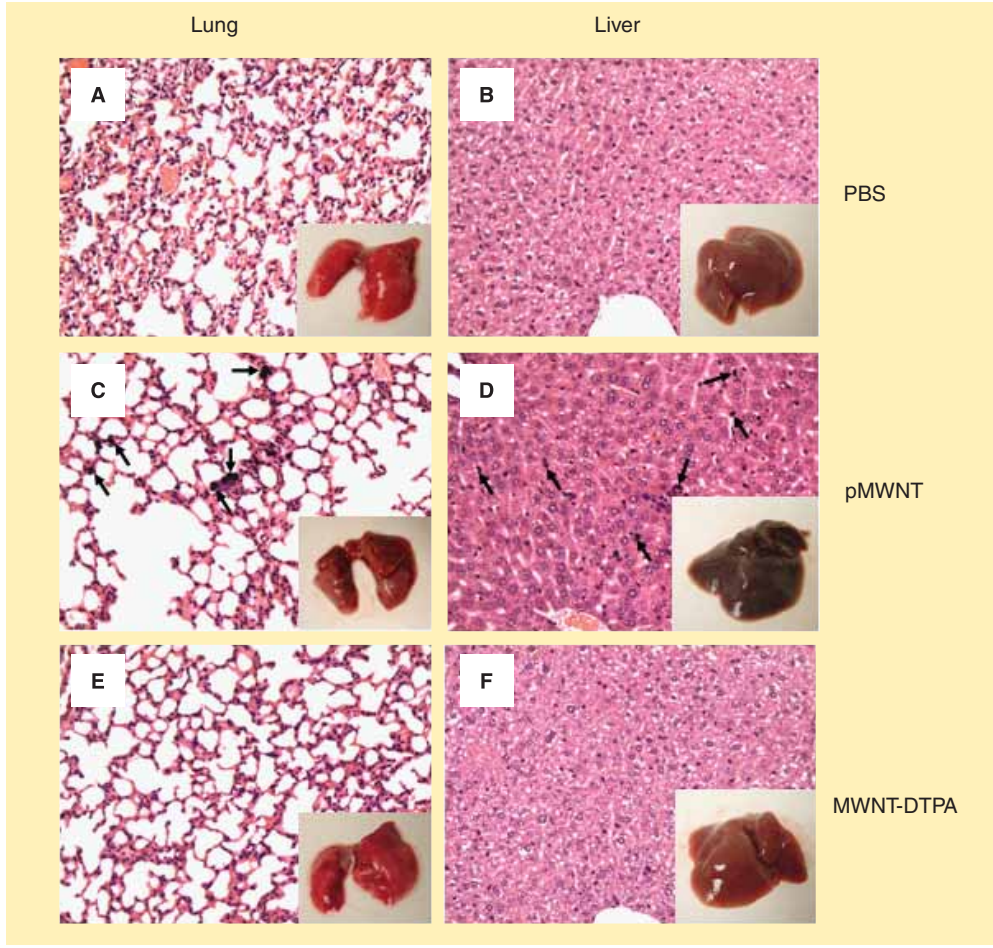
	Dextrose control	Serum control	pMWNTs	MWNT-NH_3^+ (0.2 mmol/g)	MWNT-NH_3^+ (0.9 mmol/g)
Urine produced (g)	0.64 (± 0.187)	0.67 (± 0.444)	1.16 (± 0.914)	1.54 (± 0.774)	1.69 (± 1.045)
Weight body loss (g)	3.2 (± 0.47)	3.3 (± 0.31)	3.6 (± 0.31)	3.4 (± 0.38)	3.8 (± 0.44)
Left kidney (g)	0.098 (± 0.008)	0.096 (± 0.005)	0.100 (± 0.007)	0.100 (± 0.012)	0.096 (± 0.009)
Right kidney (g)	0.104 (± 0.011)	0.104 (± 0.009)	0.096 (± 0.005)	0.106 (± 0.009)	0.098 (± 0.008)
Liver (g)	0.668 (± 0.055)	0.654 (± 0.042)	0.664 (± 0.030)	0.624 (± 0.032)	0.626 (± 0.044)
Spleen (g)	0.052 (± 0.004)	0.070* (± 0.012)	0.076* (± 0.013)	0.060 (± 0.024)	0.052 (± 0.004)
Heart (g)	0.078 (± 0.004)	0.076 (± 0.005)	0.076 (± 0.005)	0.080 (± 0.010)	0.080 (± 0.000)

All groups n = 5. Values of average (± standard deviation).

**p < 0.05 indicate statistical significance compared with control 5% dextrose-treated group.*

MWNT-NH_3^+: Ammonium-functionalized multiwalled nanotube; pMWNT: Nonfunctionalized, purified MWNT.

Figure 5. Histology of mice injected with a high dose of multiwalled carbon nanotubes.



Hematoxylin and eosin-stained 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 pMWNT **(C & D)** and 400 µg of MWNT-DTPA **(E & F)**. Digital macroscopic images of each organ harvested at necropsy are included as insets.
Magnification ×10.
MWNT-DTPA: Diethylentriaminepentaacetic-functionalized multiwalled nanotube; PBS: Phosphate-buffered saline; pMWNT: Nonfunctionalized, purified MWNT.

pharmaceutical/biomedical application dictate the need for systematic studies to identify and assess their toxicological and pharmacological profiles. *In vivo* administration of CNTs as components of therapeutic or diagnostic agents involves a multiscale, multistep process from the initial administration to trespassing the tissue endothelium and into the interstitial space of tissues, through the cell membrane into intracellular compartments and even through the perinuclear membrane into the nucleus of cells. The impact on the cell and organ function of such processes will have to be determined.

In the next few years and in order to enable the development of CNT-based constructs for biomedical applications, their *in vivo* fate after administration by several routes will have to be studied. It will be essential not only to describe the characteristics of the CNTs that determine their biocompatibility, but also their impact on tissues, possible detachment and metabolism of the surface functionalization in the body and the degree of body excretion. Efforts should also be directed to the investigation of the long-term toxicity, multiple exposure and accumulation *in vivo* at therapeutically relevant doses. However, all of this will be important in relation to their therapeutic efficacy. If CNTs can offer dramatic improvements in the therapy of untreatable diseases, the risk–benefit ratio will have to turn in favor of their utilization, provided significant improvements are achieved in the clinic.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Chemically functionalized carbon nanotubes (*f*-CNTs) injected systemically have been previously shown to be excreted in large amounts through the kidney into the urine.
- This study investigated the effect of different degrees of functionalization of multiwalled carbon nanotubes (MWNTs) and the nature of the functional group (-NH_3^+ vs -DTPA) on the tissue function and histology at early time points (up to 24 h) after intravenous administration.
- At the doses and time scales studied, the intravenous administration of MWNTs did not cause inflammation, fibrosis or necrosis in any major organ.
- Nonfunctionalized, purified MWNTs accumulated in the lung and liver as large dark aggregates and caused short-term respiratory distress that was more severe (but never lethal) at higher doses.
- Water-dispersible ammonium-functionalized multiwalled carbon nanotubes (MWNT-NH_3^+) with low degree of functionalization were not found in the lung but accumulations of small clusters were observed in the liver and spleen.
- Highly functionalized and water-dispersible functionalized MWNTs were excreted in urine and did not accumulate in or injure any tissue, regardless of the chemical moiety of the functional group.
- Further longer-term toxicological studies of *f*-CNTs are needed to elucidate the pathophysiological effects following tissue accumulation or trespass.
- Highly functionalized water-dispersible MWNT-NH_3^+ are thought to constitute toxicologically naive materials that warrant further development as promising materials for biomedical applications.

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