VIP Nanotube Toxicology

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Asbestos-like Pathogenicity of Long Carbon Nanotubes Alleviated by Chemical Functionalization**

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Carbon nanotubes (CNTs) are considered one of the most popular types of nanomaterials and in the last few years have gained tremendous interest in a wide range of applications due to their unique physical, chemical, and electronic properties. Multi-walled carbon nanotubes (MWNTs) consist of sheets of carbon atoms rolled up into multiple concentric hollow tubular structures.[1] The lack of dispersibility of pristine MWNTs in most solvents is owing to strong inter-tube van der Waals forces and this has been an obstacle for their effective use in biological applications and material sciences (i.e. composites).[2,3] This may be largely overcome by surface modification of the nanotube backbone, allowing application of CNTs in biomedical applications.[4]

Some types of chemically functionalized CNTs have shown great advantages for use as delivery systems because of their capacity to pierce cellular membranes and translocate directly into the cytoplasm, providing a method for effective drug and macromolecule intracellular transport.[5–7] Moreover, chemical surface-functionalization strategies can improve the colloidal properties of the CNT dispersions and result in populations of individualized MWNTs in physiological environments that have the capacity for glomerular translocation, leading to rapid urinary excretion.[8–11] Such biokinetic processes are also extremely important to determine the biopersistence and ultimately the potential risk from medical use of carbon nanotubes.

The use of CNTs—particularly in mass-scale, industrial applications—is currently considered with apprehension owing to their yet undefined safety profile and their potential environmental and health risks, especially given their structural resemblance to asbestos fibers.[12] Several research groups have attempted to determine the carcinogenic risks that may be associated with intended or unintentional exposure to CNTs using various in vivo models.[13–18] The first study that highlighted the importance of carbon nanotube length characteristics was carried out by Poland et al.[14] using pristine (non-functionalized), long CNTs in a structure–toxicity study, which was originally validated with asbestos fibers.[19–21] According to this method, which relates length and biopersistence of asbestos fibers to the development of mesothelioma (cancer of the pleural membrane), non-functionalized MWNTs longer than 20 μm were found to trigger an inflammatory response and result in granuloma formation seven days after intra-peritoneal exposure, similar to long asbestos fibers (LFA, long fiber amosite). This was thought to be due to induction of a process termed "frustrated phagocytosis" as resident and recruited macrophages attempt unsuccessfully to remove the long fibers from the mesothelium.[14,22] Similar conclusions regarding the risk of unwanted

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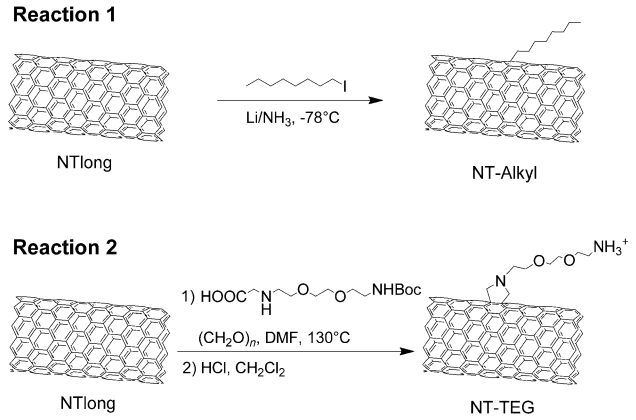
reaction from pristine MWNT exposure were described by Takagi et al.[13] using a heterozygous p53 (a tumor suppressor protein) knockdown rodent model. In a longer term exposure study, Muller et al.[16] investigated the effect of MWNTs with and without structural defects over 24 months and found no mesothelioma formation with both types, which was thought to be due to their shorter length of about 1 μm and in this way illustrating that reactogenicity can also be avoided by selecting the appropriate material design characteristics.

Taking a step further herein, we investigated whether chemical functionalization of long, reactogenic MWNTs could diminish or altogether abolish the inflammatory response and granuloma formation associated with this material, and therefore chemically modulate its risk profile.[14,23] The same previously studied long MWNTs (NTlong)[14] were chemically functionalized using two different chemical approaches, namely the Billups functionalization and the 1,3-dipolar cycloaddition reaction.[24,25] Each of these functionalization reactions allowed the covalent linkage of different types of organic chains (Supporting Information, Figure S1). The Billups functionalization introduced an octyl chain on the side walls of MWNTs (NT-Alkyl), while the 1,3-dipolar cycloaddition of azomethine ylides generated pyrrolidine rings bearing an ammonium-terminated tri(ethylene glycol) chain (NT-TEG; Scheme 1).



Scheme 1. Functionalization of CNTs following the Billups approach (Reaction 1) and the 1,3-dipolar cycloaddition of azomethine ylides (Reaction 2). DMF = dimethylformamide; Boc = *tert*-butyloxycarbonyl.

The pristine, non-functionalized MWNTs (NTlong) and the two types of functionalized MWNTs (*f*-MWNTs) were dispersed in 0.5% bovine serum albumin (BSA), and full characterization of these dispersions was obtained using transmission electron microscopy (TEM) and atomic force microscopy (AFM), as shown in Figure 1.

Because the bioassay used to study reactivity to the material was based on the length of the fibers, we attempted a semi-quantitative analysis of the nanotube length distribution from the TEM images using Image J software. The pristine MWNTs were found to contain the highest fraction of very long nanotubes ranging from 10 μm to 40 μm (Figure 1; Table S1). The alkyl functionalized *f*-MWNT sample contained nanotube lengths in the range of 5–15 μm, while the MWNTs functionalized by the 1,3-dipolar cycloaddition contained a nanotube population with a length distribution ranging from 2 μm to 4 μm. Such differences in length could be due to the differences in the hydrophilicity and hydrophobicity of the appended chemical groups, the alkyl group being more hydrophobic than the tri(ethylene glycol) group, leading to larger effective length by bundling of multiple tubes during aqueous dispersion.

The nanotube samples were dispersed under mild sonication in BSA/saline, and 50 μg of each type of MWNTs was injected intraperitoneally in C57BL/6 mice. The abdominal cavity was then lavaged with a physiologically compatible saline solution, 24 hours and seven days post-injection, as previously described by Poland et al.[14] The ensuing inflammatory response was studied based on the change in protein levels and the polymorphonuclear leukocytes (PMN) in the peritoneal fluid lavaged at 24 hours. The pristine, long MWNTs and the alkyl functionalized MWNTs caused a significant increase in PMN and protein levels (Figure 2), similar to the inflammatory response obtained from exposure to LFAs. The 1,3-dipolar cycloaddition functionalized MWNTs did not lead to significant elevation in protein levels and total PMNs, suggesting the absence of an inflammatory response. The contribution to reactivity from soluble contaminants can be excluded because both functionalized MWNTs were prepared from the same starting pristine long MWNTs (which have previously been shown to be free from soluble contaminants).[14] A similar assessment was conducted here and no contribution from these contaminants to the inflammatory reaction was observed with the alkyl-functionalized MWNTs was found (data not shown).

After seven days, reactivity to material exposure is mainly manifested by formation of granulomas (collections of macrophages and giant cells with deposition of collagen) on the mesothelial membrane, as the total PMN levels in the peritoneal cavity decrease (Figure S2). This common pathogenic response to long fibers, like LFA and NTlong, is evident on mesothelial membrane surfaces, confirmed also here by hematoxylin and eosin (H&E) staining of the tissue and scanning electron microscopy (SEM) of the membrane surface (Figure 3). SEM images after treatment with pristine and alkyl *f*-MWNTs show a granular surface, observed at low magnification, that is absent with the vehicle and the 1,3-dipolar cycloaddition *f*-MWNTs (Figure 3a). At higher magnification, an accumulation of cells is evident on the diaphragm surface, as it attempts to eliminate the nanotube material. H&E staining in Figure 3b confirmed the development of granuloma lesions following alkyl *f*-MWNT exposure, similar to that observed with the long pristine MWNTs and LFA.

Based on the experimental data obtained, we can suggest that chemical functionalization of pristine NTlong with octyl chains following the Billups reaction did not lead to a significant reduction in the effective length (after aqueous dispersion) of the starting material. Therefore, the inflammatory response observed and subsequent granuloma formation was similar to those from exposure of the peritoneum to NTlong and LFA fibers. Aqueous dispersion of the same

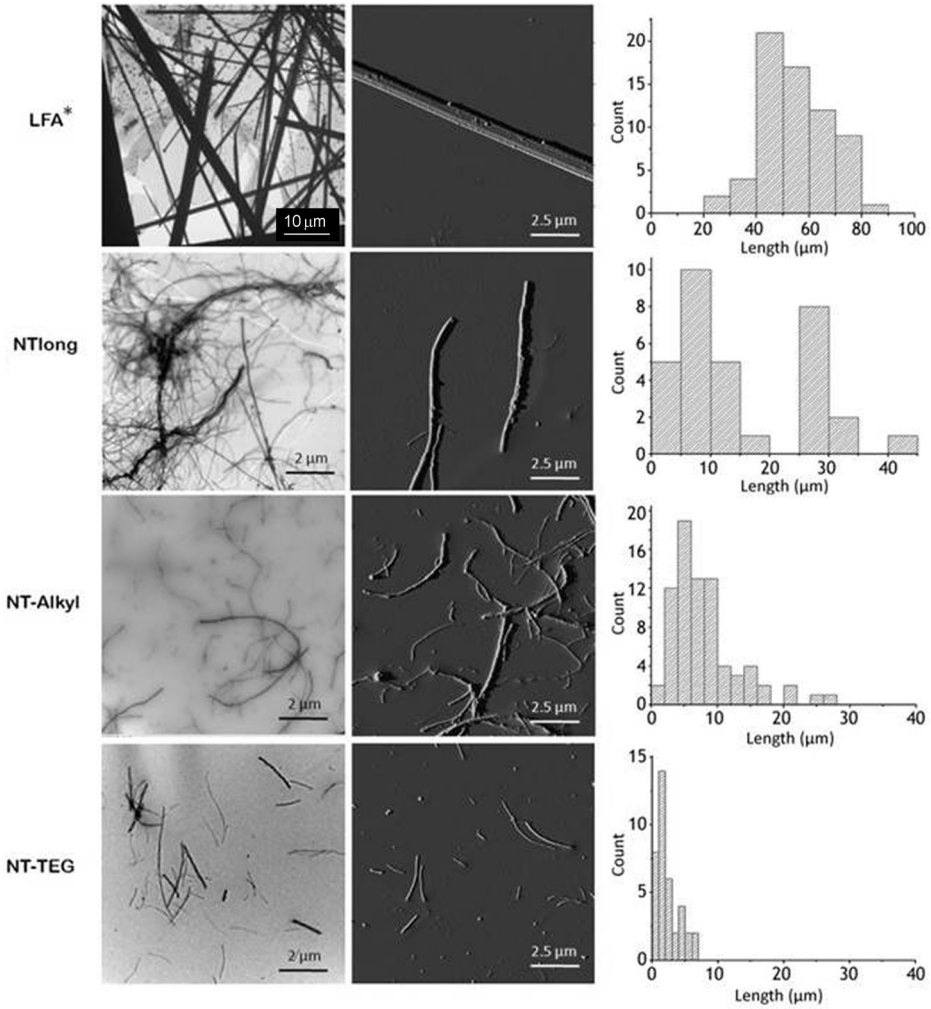


Figure 1. Fiber characterization using transmission electron microscopy and atomic force microscopy. Samples were dispersed in 0.5% BSA/saline prior to visualization with TEM and AFM. Pristine MWNTs (NTlong) and the alkyl functionalized MWNTs (NT-Alkyl) samples contain longer MWNTs compared to NT-TEG, as shown by TEM (left) and AFM (center) images, and confirmed by the size-distribution analysis (right) that was based on the TEM images at low magnification. * indicates that the size distribution was too wide because the LFA fibers were longer than the TEM grid. The size distribution shown is thought to be underestimating the length of the fibers.

starting pristine NTlong functionalized with TEG chains using the 1,3-dipolar cycloaddition reaction led to reduction of their effective length, most likely because of efficient debundling and disaggregation of individual nanotube fibers. These observations are in good agreement with the long–short fiber hypothesis in particle toxicology[22] and lead us to propose that the alkyl *f*-MWNTs interacted with the tissue primarily as long bundles of nanotubes, while the 1,3-dipolar cycloaddition TEG-functionalized material interacted as shorter, individualized fibers. We therefore suggest that only chemical functionalization reactions and appended functionalities that lead to shortening or untangling/debundling of aqueous dispersions of *f*-MWNTs will help to resolve toxicological risks associated with long-fiber exposure. Such functionalized-nanotube dispersions can have a biological activity similar to the (non-functionalized) tangled MWNTs described by Poland et al.[14] or the (as-prepared) shorter MWNTs used by Muller et al.,[16] both of which reported no carcinogenic risk from exposure to such materials even 24 months post-injection. Chemical functionalization strategies on the surface of long nanotubes that are not able to improve their effective length and aqueous dispersibility will most likely maintain the reactogenicity of the nanotubes, although more such studies are needed to confirm this hypothesis.

Other important design parameters of chemically functionalized nanotubes (surface charge, flexibility, molecular weight of appended groups) also need to be studied and correlated with their induced toxicological burden, using more complex in vivo models. The effect of chemical functionalization on nanotube stiffness (as expressed by Young's modulus), being perhaps one of the most important parameters, has been reported to vary with the degree of functionalization,[26] and might be implicated in the reactogenicity of these materials.[18] Further systematic investigations could determine the possible limitations from exposure to different types of nanotubes and reveal the appropriate chemical functionalizations to allow their safe use. Our study offers an illustration of how chemical functionalization can alleviate the reactivity profile of a specific type of long, pristine MWNT, making them safer to use.

Experimental Section

Chemical functionalization of MWNTs: Long pristine MWNTs, which were previously used by Poland et al.,[14] are denoted as NTlong and were functionalized using two different chemical reactions. The NTlong were functionalized using octyl iodide (octyl functionalization)[24] generating NT-Alkyl as shown by Reaction 1 in Scheme 1 and through the 1,3-dipolar cycloaddition[25] affording NT-TEG (Reaction 2 in Scheme 1). Thermogravimetric analysis is reported in the Supporting Information, Figure S1.

Preparation of MWNT aqueous dispersions: For biological purposes, all MWNT samples and long asbestos amosites, used as a positive control, were dispersed at a final concentration of 100 $\mu g\,mL^{-1}$ in 0.5% bovine serum albumin/saline solution by bath sonication for 2 h.

Toxicological effects of intraperitoneally injected MWNT samples: Six- to eight-week-old C57L6 mice were obtained from Harlan (Oxfordshire, UK), allowed to acclimatize for 1 week, kept in groups

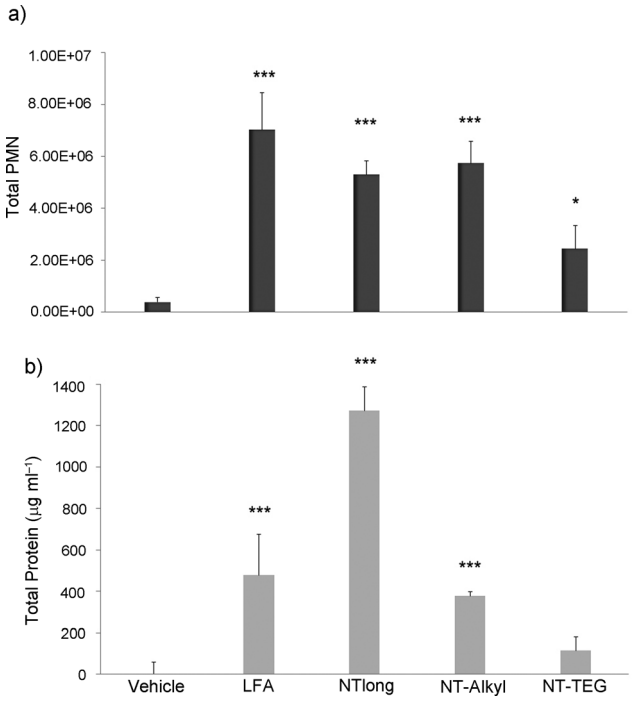


Figure 2. Inflammatory reaction in the peritoneal cavity 24 h post-injection with nanotubes. Female C57Bl/6 mice were intraperitoneally injected with 50 μg of vehicle control (0.5% BSA/saline), pristine MWNTs (NTlong), the two chemically functionalized MWNTs (NT-Alkyl and NT-TEG), or LFA, as a positive control. The peritoneal cavity was lavaged with saline and the inflammatory response was evaluated by: a) total PMN leukocytes; and b) total protein (protein exudation). Data represent the mean of four animals ± standard deviation. * $P<0.05$; *** $P<0.005$ versus the vehicle control.

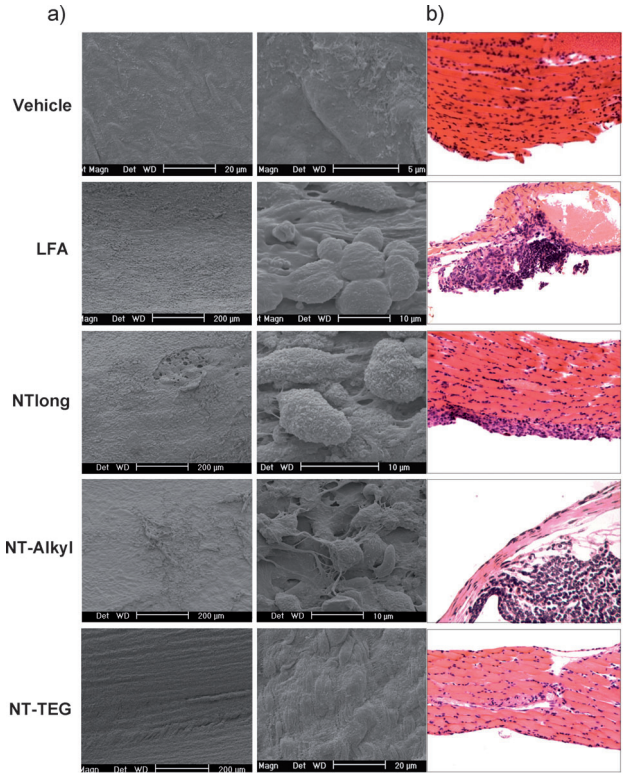


Figure 3. The effect of fibers on the diaphragms after 7 days. Female C57Bl/6 mice were intraperitoneally injected with 50 μg of vehicle control (0.5% BSA/saline), pristine MWNTs (NTlong), the two chemically functionalized MWNTs (NT-Alkyl and NT-TEG), or LFA as a positive control, the mice were killed after 7 days and the diaphragms excised, fixed, and prepared for visualization. a) SEM images of the diaphragm surface and b) histology using H&E staining shows the presence of granulomatous inflammation with NTlong, LFA, and NT-Alkyl but not with NT-TEG. Low and high magnification of the SEM images are shown.

of five for the duration of the experiments, and given food and water. All experiments were conducted with prior approval from the UK Home Office. Two groups of animals ($n=4$–6) were intraperitoneally injected with 0.5 mL containing 50 μg of NTlong, NT-Alkyl, NT-TEG, or LFA in 0.5% BSA/saline (vehicle control; 0.5 mL). One group of animals was sacrificed after 24 h and the other group 7 days post-injection.

Inflammatory reaction after injections of MWNT samples: 24 h and 7 days post-injection, mice were sacrificed by cervical dislocation and the peritoneum lavaged three times using 2 mL washes of sterile ice-cold PBS. The lavages were pooled together and placed on ice for the duration of the processing. The lavage fluid was then centrifuged at 1000 rpm for 5 minutes at 4 °C in a Hettich Universal 320R centrifuge (Hettich Zentrifuger, UK) and an aliquot of the supernatant was retained for total protein measurement. The remaining supernatant was discarded and the cell pellet resuspended in 0.5 mL of 0.1% BSA/sterile saline solution and mixed with fluorescently labeled Ly-6C/G (Gr-1) antibody (Invitrogen, UK), which is used to differentiate polymorphonuclear (PMN) leukocytes from other cells. The Gr-1 positive PMN leukocytes were quantified using flow cytometry. The assay with the Gr-1 antibody was performed according to the manufacturer's instructions. It is recommended that between 0.1 μg and 0.25 μg of antibody be used per 1×10^6 cells in a 100 μL staining volume.

Total protein in the lavageate: Total protein concentration of the peritoneal lavage fluid was measured using the bicinchoninic acid (BCA) protein assay (Thermo Scientific Pierce, UK). The colorimetric assay was performed according to the manufacturer's instructions.

Assessment of granuloma formation: After 7 days post-injection, the diaphragm was carefully dissected by cutting through the ribs and chest wall with care taken not to puncture the diaphragm, as previously described by Poland et al.[14] The diaphragm was gently rinsed three times by emersion in ice-cold sterile PBS and placed overnight into: a) methacarn fixative (60% methanol, 30% chloroform, and 10% glacial acetic acid) for histological staining; or b) 3% glutaraldehyde/0.1M sodium cacodylate (pH 7.2) buffer for scanning electron microscopy of the diaphragm surface. After overnight incubation in fixative, the diaphragm was carefully excised from the surrounding ribs prior to further processing for either histological or SEM analysis.

Histological examination of diaphragms: The diaphragm was then removed from the ribs and a similar section of the upper quadrant of the diaphragm was removed from each animal. This excised tissue was dehydrated through graded alcohol (ethanol) and imbedded on-edge in paraffin.[14] Sections of the diaphragm (4 μm) were stained 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 to an Infinity 2 digital camera.

Surface examination of diaphragms using SEM: The excised diaphragm was stained with osmium tetroxide prior to critical point drying, mounted, gold sputter coated, and then viewed by scanning electron microscopy (FEI XL30 TMP, Eindhoven, The Netherlands).

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