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Lung Deposition Patterns of MWCNT Vary With Degree of Carboxylation

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Abstract

Functionalization of multi-walled carbon nanotubes (MWCNT) is known to affect the biological response (e.g., toxicity, inflammation) *in vitro* and *in vivo*. However, the reasons for these changes *in vivo* are not well described. This study examined the degree of MWCNT functionalization with regard to *in vivo* mouse lung distribution, particle retention, and resulting pathology. A commercially available MWCNT (source MWCNT) was functionalized (f-MWCNT) by systematically varying the degree of carboxylation on the particle's surface. Following a pilot study using seven variants, two f-MWCNT variants were chosen and for lung pathology and particle distribution using oropharyngeal aspiration administration of MWCNT in Balb/c mice. Particle distribution in the lung was examined at 7 and 28 days post-instillation by bright-field microscopy, CytoViva hyperspectral dark-field imaging, and Stimulated Raman Scattering (SRS) microscopy. Examination of the lung tissue by bright-field microscopy showed some acute inflammation for all MWCNT that was highest with source MWCNT. Hyperspectral imaging and SRS were employed to assess changes in particle deposition and retention. Highly functionalized MWCNT had a higher lung burden and were more disperse. They also appeared to be associated more with epithelial cells compared to the source and less functionalized MWCNT that were mostly interacting with alveolar macrophages (AM). These results showing a slightly reduced

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Author's Contributions

R. F. H. Jr., K.L.T., and A. H. prepared the manuscript. R. F. H. Jr. performed the experiments including CytoViva imaging. A. H. and S. M. conceived the experiments. Z. W and S. M. prepared and characterized the nanotube samples. K.L.T. was instrumental in the ImageJ transformation of CytoViva images and data analysis of Raman images. R. B and S. D. performed the Raman Spectroscopy. All authors read and approved the final manuscript.

Competing Interests

The authors declare no conflicts of interest.

Ethics Approval and Consent to Participate

All animal procedures were performed under protocols approved by the IACUC of the University of Montana. Nanoparticle

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pathology despite extended deposition has implications for the engineering of safer MWCNT and may establish a practical use as a targeted delivery system.

Keywords

MWCNT; macrophage; Stimulated Raman Scatter; functionalization; carboxylation

Introduction

Multi-walled carbon nanotubes (MWCNT) are currently used in a variety of applications and products ranging from composite materials, energy storage devices, drug delivery, optics, and various developing areas of engineering (Spitalsky *et al.*, 2010, De Volder *et al.*, 2013). Engineered nanomaterials (ENM), like MWCNT, are being studied for potential toxicities, as occupational and casual internalization in humans is likely to increase in the future (Castranova *et al.*, 2013). Of the potential routes of exposure, the lung is anticipated to be the most sensitive route for MWCNT exposure (Borm and Driscoll, 1996). The physical properties most associated with harmful MWCNT interactions include particle length and rigidity that mimics asbestos fibers (Hamilton *et al.*, 2009, Palomaki *et al.*, 2011, Hamilton *et al.*, 2013a, Murphy *et al.*, 2013, Hamilton *et al.*, 2017), metal contaminations, such as Ni and Fe, from catalytic reactions involved in the creation of these particles (Hamilton *et al.*, 2012, Hamilton Jr. *et al.*, 2013), surface area (Poulsen *et al.*, 2016), and particle agglomeration (Ntim *et al.*, 2012). *In vivo* lung exposures of MWCNT in mouse models showed increased fibrotic response over time and thickening of related connective tissue (Mercer *et al.*, 2011, Mercer *et al.*, 2013a). With extended MWCNT lung exposures, the particles appear to redistribute/accumulate throughout the body in secondary organ exposures in singlet and aggregate form (Mercer *et al.*, 2013b). The aggregation state of the CNT has been reported to determine the distribution of the particle in the lung tissue, particularly when the CNT is a single-walled CNT (Mercer *et al.*, 2008). One hypothesis of MWCNT bioactivity (defined here as potential to cause toxicity and/or inflammation), involves activation of the NLRP3 inflammasome in cells interacting directly with the particles (i.e., alveolar macrophages (AM)), creating acute inflammation, which can persist and result in sustained chronic inflammation and eventually pathology (Bunderson-Schelvan *et al.*, 2016).

Due to design concerns, and possible reduction of MWCNT toxicity, alternative forms of this ENM have been developed and are referred to as 'functionalized' MWCNT (f-MWCNT) (Klumpp *et al.*, 2006). Functionalization allows MWCNT to be modified to a purposeful property, such as delivering a pharmaceutical to a specific target (Klumpp *et al.*, 2006, Zhang *et al.*, 2010b). There are a variety of ways to change the surface characteristics of MWCNT including addition of amines, polysaccharides, hydroxyls, etc., but 'carboxylation' or adding -COOH groups on the surface is a common modification to MWCNT (Sun *et al.*, 2002, Terrones and Terrones, 2003). Following MWCNT carboxylation, other modifications to the MWCNT surface such as, metal doping or addition of pharmaceutical compounds can be accomplished (Zhang *et al.*, 2010a). Another reason to functionalize MWCNT is to make the particles less bioactive. Several studies have

demonstrated that functionalized MWCNT are less toxic, generate less inflammation, and are tolerated better by cellular and animal models (Hamilton *et al.*, 2013a, Hamilton *et al.*, 2013b, Hamilton *et al.*, 2014, Sager *et al.*, 2014, Fanizza *et al.*, 2015, Ruenraroengsak *et al.*, 2016, Sweeney *et al.*, 2016, Ursini *et al.*, 2016).

AM are the principle cells in the lung responsible for clearing particle debris, including uptake and removal of MWCNT of any variant (Bunderson-Schelvan *et al.*, 2016). However, the interaction of ENM and MWCNT, specifically, with epithelial cells *in vivo* is less well understood. Epithelial cells can take up ENM, but their phagocytic activities are considered more limited than the AM (Fytianos *et al.*, 2016). One study links the epithelial type 1 cell to f-MWCNT uptake in immortalized cell cultures (Ruenraroengsak *et al.*, 2016).

Unfortunately, most of the data on epithelial interaction with MWCNT and f-MWCNT are limited to *in vitro* studies, primarily using transformed cell lines (Ursini *et al.*, 2016). Consequently, the studies are restricted to a specific epithelial cell type and particle ignoring the complex environment of the lung.

Carboxylation of the MWCNT surface reduces aggregation, increases dispersion in hydrophilic environments, and increases stability of the f-MWCNT suspension (Fraczek-Szczypta *et al.*, 2012, Ntim *et al.*, 2012). Furthermore, increasing carboxyl groups on MWCNT amplifies these physiochemical characteristics (Fraczek-Szczypta *et al.*, 2012, Fanizza *et al.*, 2015). Since it has been established that carboxylated MWCNT (f-MWCNT) are less bioactive than the source MWCNT in various model systems, testing the effect of differing degrees of carboxylation *in vivo* is a logical step to possibly increase or maximize the potential benefits of carboxylation. The hypothesis of this study is that increasing the surface functionalization (adding carboxyl groups to the MWCNT surface) changes the deposition of the particle in the lung due to smaller size aggregates and altered surface properties such as lower carbon to oxygen ratios, more defects, and increased hydrophobicity. This would be predicted to result in a significant fraction of the f-MWCNT being taken up by lung epithelial cells as opposed to solely by AM.

Materials and Methods

Preparation of the functionalized-MWCNT

Multiwall carbon nanotubes (MWCNT) (OD 20-30 nm, length 10-30 μ m, purity >95%) were purchased from Cheap Tubes Inc. (Cambridgeport, VT), and all other chemicals were purchased from Sigma Aldrich (St Louis, MO) with purity higher than 95%. The synthesis of the functionalized MWCNT was described in greater detail in a previously published manuscript (Wu *et al.*, 2017). Additional information on characterization and relative bioactivity can be found in a recent publication (Hamilton Jr. *et al.*, 2018). In addition, an abbreviated characterization table (Table 1) is shown here for convenience, as a much more comprehensive characterization of f-MWCNT is available in reference stated above (Hamilton Jr. *et al.*, 2018).

MWCNT suspensions

All nanotubes were weighed and suspended in dispersion media (DM) (Porter *et al.*, 2008), which consisted of mouse serum albumin (Sigma, St. Louis, MO; 1 mg/mL or 0.1%) and 1,2 dipalmitoyl-sn-glycero-3-phosphocholine (DSPC, Sigma, 1 µg/mL or 0.0001%) diluted in phosphate-buffered saline (PBS). Nanotube suspensions were sonicated for 5 min at 1/3 max power in a Qsonica cup-horn sonicator (Q500, Newtown, CT) attached to a circulating water-bath (VWR International, Radnor, PA) at 500 watts and 20 Hz (8000 Joules) at a stock concentration of 1 mg/mL.

Characterization of the functionalized-MWCNT

Scanning electron microscope (SEM) images and elemental analysis data for samples were collected on a LEO 1530 VP scanning electron microscope equipped with an energy-dispersive X-ray analyzer. For length determinations, 1 mg f-MWCNT was added in to 500 mL DI water and sonicated with low power for 20 min, followed by stirring for 30 min. The resulting MWCNT suspension was dropped onto a silicon wafer and dried at RT. The f-MWCNT variants were then observed under SEM where the length was determined. Multiple measurements were taken and the average length and standard deviation is reported in Table 1. The specific surface areas (SSA) of the samples were measured using a Quantachrome NOVA 3000 series (Model N32-11) High Speed Gas Sorption Analyzer (Boynton Beach, FL) at 77.40 K. The samples were heated and degassed at 300°C in a vacuum oven for three hours before surface area assessment.

Particle size and zeta potential were measured in DM at 25°C using a Malvern Zetasizer nano Zen 3600 (Malvern Instruments, Worcestershire, UK) at a 90° detector angle. Endotoxin contamination was determined by washing/sonicating 1 mg/mL MWCNT in endotoxin-free water for 30 min followed by centrifugation at 16,000x *g* for 15 min prior to assay. The endotoxin assay (ToxinSensor) was performed on the isolated supernatant according to the manufacturer's protocol (GenScript, Piscataway, NJ). Aggregate size was expressed as mean nm ± the standard deviation. The zeta potential was expressed as mean mV ± the standard deviation. Complete results for all MWCNT variants can be found in earlier referenced material (Hamilton Jr. *et al.*, 2018). An abbreviated list of MWCNT particles, with regard to hydrodynamic size, zeta potential and endotoxin contamination, exclusively used in this study is available in Table 2. Transmission electron microscopy (TEM) was used to image the MWCNT variants. This was done with a Hitachi H—7100 transmission electron microscope set at 75kV (see Figure 1).

Animals

Balb/c mice (2-months old, male) were housed in controlled environmental conditions (22 ± 2°C; 30-40% humidity, 12-h light: 12-h dark cycle) and provided food and water *ad libitum*. All procedures were performed under protocols approved by the IACUC of the University of Montana. Mice were exposed to nanoparticles by oral pharyngeal aspiration. Briefly, the mice were anesthetized using inhalation isoflurane and a volume of 50 µL of particle suspension (50 µg/25 g mouse) was delivered into the back of the throat. The stock MWCNT suspension concentration of 1 mg/mL was determined by experimentation to be the most concentrated we could get the stock concentration before aggregation issues

became problematic. At the predetermined time, mice were euthanized by sodium pentobarbital (Euthasol™). The 50 µg dose is known to induce rapid (1 week) lung pathology for unmodified MWCNT exposures (Porter *et al.*, 2010, Hamilton *et al.*, 2012, Hamilton *et al.*, 2013a, Porter *et al.*, 2013). The 7- and 28-day particle exposures were selected for different reasons. The 7-day MWCNT exposure typically shows maximal pathological anomalies (e.g. lesions, extra-cellularity, influx of inflammatory cells, and collagen deposition) associated with MWCNT exposures. In contrast, the 28-day exposure typically shows a reduction or resolution of particle-induced inflammation and a clearance of particle burden in the lung (Porter *et al.*, 2010). The selection of the MWCNT dose in this study (50 µg/25 g mouse) was based on previous publications suggesting that it is relevant to simulate occupational exposures. For example, Snyder-Talkington, et.al., (2016) state that 10 – 80 µg is reasonable for MWCNT exposures and that a 10 µg dose in a mouse mimics a one month exposure in humans (Snyder-Talkington *et al.*, 2016). Furthermore, other studies have used 40 µg and 80 µg as their 'high dose' for bolus exposures (Porter *et al.*, 2010, Mercer *et al.*, 2011).

Histology

The lungs from each mouse were inflation-fixed through the trachea with 3% paraformaldehyde-PBS and submerged in the same fixative overnight at 4°C. The lungs were washed with cold PBS, dehydrated, and embedded in paraffin. Tissue sections (7 µm) were stained with hematoxylin-eosin (RAS Harris Hematoxylin and Shandon Alcohol Eosin) for histological analysis using a Leica automated stainer (Thermo Shandon, Thermo Fisher Scientific, Houston, TX). Note 1: for CytoViva hyperspectral analysis, the lung sections were processed in a similar manner, but without staining. They were cover-slipped with Permount (Thermo Fisher Scientific, Houston, TX) media and allowed to dry. Note 2: for Raman spectroscopy the sections were cut thicker (10 µm) and left unstained and uncovered. All lung sections were random mid-coronal sectioning, 150 µm apart.

Lung Pathology Evaluations

Mouse lung tissue sections were imaged at 1000x (100x oil objective) using a Zeiss Axioskop 2 plus microscope attached to Zeiss AxioCam ERc 5s digital camera and processed using Zeiss AxioVision software (Zeiss, Oberkochen, Germany). Lung sections were scored on the basis of lung disease visible in the lung sections using a 5-point scale (0, 1, 2, 3 and 4) with zero being normal lung morphology and 4 being the worst possible lung pathology evident. There were 3 exposed mice per MWCNT condition. The values shown in this paper are the median value for each condition.

CytoViva hyperspectral imaging

The MWCNT deposition was documented by using a hyperspectral dark-field imaging system made by CytoViva (Auburn, AL). Carbon nanotubes, especially ones without metal contamination, are difficult to isolate with conventional Spectral Angle Mapping (SAM) techniques, as the spectral libraries generated from the MWCNT do not differentiate well from the background tissue. An alternative process known as Spectral Feature Fitting (SFF) was developed to make carbon particles more easily detectable in this system. SFF differs from SAM in that a single spectrum most representative of a MWCNT in lung tissue was

used as opposed to a library of spectra. Then, spectral features most similar to this single spectrum were isolated and the rest removed from consideration. Pixel intensity was plotted on a X-Y scatter-plot with error (*MSE*) on X and intensity on Y. A gate was drawn manually on this scatter-plot to determine positive hits for the carbon material represented in the dark-field image (the vehicle control was used to determine the signal floor and false positives). The same gate was used for all samples. This gate was determined in an iterative process that minimizes the *MSE*. Positive pixels were superimposed on the image as colored specs or clusters in the case of aggregation. This SFF technique can be done on stained or unstained tissue in bright or dark-field microscopy using ENVI 4.8 software (CytoViva, Auburn, AL).

Using hyperspectral dark-field imaging, the optimal wavelength characteristics were established for detection of all MWCNT variants used in this study. Figure 1A illustrates the noisy tissue hyperspectral peak contrasted with the equally noisy background signal (no peak). In contrast, figure 1B shows the optimal hyperspectral peak for MWCNT (~ 600 nm) as the tissue and background are shown well below the particle signal. The spectral characteristics for tissue and particle are almost identical with the exception of signal strength. This illustrates the problem identifying unique spectra for MWCNT in tissue using a spectral angle mapping (SAM) approach. Therefore, signal intensity was used in the Spectral Feature Fitting (SFF) application identifying MWCNT in mouse lung tissues for 7- and 28-day exposures.

The hue of CytoViva dark-field images tends to vary from one image to the next. Therefore, images were normalized for side-by-side comparison by exporting separated image channels from ENVI for the SFF overlay and dark-field, followed by conversion in ImageJ to green and grayscale, respectively. ImageJ image calculator was used to normalize tissue brightness intensity if needed and to subtract overlapping regions of SFF channel from the dark-field image. Finally, the channels are merged into the resulting images presenting in the Results section of this manuscript.

Stimulated Raman Scattering

Stimulated Raman Scattering (SRS) was used in order to further confirm CytoViva results and to analyze larger regions of tissue at higher resolution. The images of unstained lung tissue sections were acquired using a SRS imaging setup built in house, which is based on a two-photon laser scanning microscopy system (Kole, 2017). The home built SRS microscope is integrated with a dual-output (1064 nm/532 nm, 80MHz) ultrafast oscillator (Lumera, Coherent Germany) coupled into an optical parametric oscillator (OPO) (Levante Emerald, APE Germany) to provide tuneable (750nm – 970nm) ~6 picosecond pulse trains. The 1064 nm output from the oscillator is used as the Stokes beam and the output from the OPO is used as the pump beam, which is tuned to match a Raman mode of interest. The Stokes pulse train (1064nm) is amplitude-modulated at 7 MHz by an electro-optic modulator (EOM, Conoptics) and beams are spatiotemporally overlapped and sent collinearly to the SRS microscope. The images were acquired using a 50x (0.95 NA, Zeiss) objective and a custom-built large area photodiode (PS100-6, First sensor) detector. A high OD band-pass filter (Chroma Technology, 890/220m) was used to selectively transmit the pump beam and

to block the Stokes beam for stimulated Raman Loss (SRL) detection (Freudiger *et al.*, 2008). The SRL signals (based on transferred modulation from the Stokes beam to the pump beam), thus acquired, were demodulated and amplified by a lock-in amplifier (HF2LI, Zurich Instruments) with a time constant of 5 μ s. Images were acquired with 20 μ s pixel dwell time at two different Raman frequencies with different laser powers: 2700 cm^{-1} at <1.5mW at sample plane and 2930 cm^{-1} at 30 mW total power. The 2700 cm^{-1} frequency was at very low power because higher power caused significant photo-damage. These same settings are used for all samples. At 2700 cm^{-1} (white regions, Figures 8 and 9), numerous specks are visible in MWCNT-exposed tissues, while the background signal in dispersion media control tissue is minimal. The 2930 cm^{-1} frequency represents -CH₃ symmetric stretch and allows the tissues to be clearly visible (blue regions, Figures 8 and 9). The 2930 cm^{-1} spectrum corresponding to tissue can be seen in Figure 1C. In addition to the tissue-only control, a MWCNT-only control was examined to confirm positive signal at 2700 cm^{-1} and negative signal at 2930 cm^{-1} .

SRS images were generated for 7- and 28-day exposure groups including the source, minimally carboxylated, and maximally carboxylated MWCNT. A minimum total image field area of 0.25 mm^2 was quantified for each experimental replicate, with $n = 3$ replicates per treatment group. Images were processed and analyzed using MATLAB v9.2 and ImageJ v1.51 software. First, images were separated into two channels representing signals identified by SRS as tissue and MWCNT. Each channel was segmented into regions of interest using automated thresholding methods selected as appropriate based on pixel intensity histograms: Li method (Li and Tam, 1998), for tissue channel and inter-modes (Prewitt and Mendelsohn, 1966), for MWCNT. Sample images from each group were tested to qualitatively confirm automated methods were consistent with manual visual thresholding. Autothresholding was used to avoid variability and subjectivity of a human observer. Next, the areas of tissue and MWCNT regions in each segmented channel were quantified. Quantifying relative projected areas of particle (MWCNT) and tissue identified by SRS determined the particle burden. The expression “% Particle Burden” is defined as MWCNT-positive areas normalized by tissue-positive areas in experimental groups minus background in the DM control group. In order to compare relative agglomerate sizes of MWCNT, the mean particle cluster size was measured in each image.

Statistical analyses

Statistical analyses involved comparison of means using a one or two-way ANOVA followed by Dunnett's test or Sidak's adjustment to compensate for increased type I error resulting from pair-wise mean comparisons. All probabilities were two-tailed unless otherwise stated. Nonparametric analysis of pathology scores was evaluated by Kruskal-Wallis followed by Dunn's test. Statistical power was greater than 0.8. Statistical significance was defined as a probability of type I error occurring at less than 5% ($P < 0.05$). The minimum number of experimental replications was 3. Graphics and analyses were performed on PRISM v.7.0 (GraphPad, San Diego, CA).

Results

Functionalized Particle Characterizations

In this study, the f-MWCNT with different treatment times (5 min and 120 min) were referred to as f-MWCNT_{38.4}, and f-MWCNT_{14.7} based on the C:O ratio (free carbon atoms per oxygen atom, see Table 1). The morphologies of MWCNT_{source}, f-MWCNT_{38.4} and f-MWCNT_{14.7} are shown in TEM images (Figure 2) and show that there was minimum morphological change with treatment time. Additional detail of these processes can be found elsewhere (Wu *et al.*, 2017). Hydrodynamic size, diameter and zeta potential in DM can be found in Table 2. Endotoxin levels in all particles were below 1 ng/μg. Additional information on these particles with regard to basic toxicity and inflammation can be found elsewhere (Hamilton Jr. *et al.*, 2018).

Lung pathology following 7- and 28-day exposure

Two time points were chosen (7 and 28 days post-exposure) to examine changes in lung pathology and particle deposition. A seven-day exposure was selected because it is typically around the time when significant lung abnormalities attributed to MWCNT exposure are readily apparent (Porter *et al.*, 2010, Hamilton *et al.*, 2012, Hamilton *et al.*, 2013a). Day 28 was selected since lung pathology starts to resolve and particles disappear following MWCNT exposure in the lung. The pathological features most encountered include airway thickening, presence of inflammatory cells, lesions, and granulomas. The extent of these features throughout the lung section was also factored in the pathology scoring. Three MWCNT variants were selected to represent the full range of MWCNT types. The source MWCNT was used as the positive control, in addition to the minimally and maximally carboxylated f-MWCNT. Figure 3 shows the pathology scoring result. The source MWCNT, did not result in excessive pathology in this mouse exposure model, as seen before using other MWCNT (Hamilton *et al.*, 2012, Hamilton *et al.*, 2013a). The most typical morphological abnormality was the presence of extra cells (inflammatory cells) and tissue thickening. A median score of 2 (although significant) was indicative of mild to moderate lung changes. There are typically a range of responses to MWCNT, that often reflect the purity of the material (Hamilton *et al.*, 2012). In this case, however, there was not much acute pathology and the little detected did not appear to diminish with time. The f-MWCNT was not significantly different from the source MWCNT exposure. There was a slight non-significant trend suggesting less pathology with the f-MWCNT. Only the particle exposures compared to DM were significantly different. It was concluded that the lack of a difference in lung pathology was attributed to the mild inflammatory effect of the parent material limiting the ability to significantly detect changes as a result of functionalization or time.

Figures 4A – D show high magnification images of selected lung sections from the seven-day particle exposures. Figures 5A – D, in contrast, represent the 28-day particle exposures. Figures 4A and 5A illustrate normal lung morphologies. The presence of inflammatory cells was the primary lung abnormality, and it is featured in several of the exposed lung section images (Figures 4B, 4C, 4D, 5C and 5D). Large particle aggregates were visible and appeared to be primarily taken up by alveolar macrophages. However, the presence of smaller aggregates was not clearly visible even with high magnification, particularly for the

f-MWCNT particle. Hence, specialized imaging was necessary to detect these fine particles, as described below.

CytoViva hyperspectral dark-field detection of MWCNT

Due to the difficulties encountered in imaging small f-MWCNT in the high magnification bright field microscopy, an alternative method was used, which was designed for the specific application of ENM detection. Unfortunately, elemental carbon, in any form, is one of the more difficult particles to confidently image in tissue using this technique. Using the Spectral Angle Mapping (SAM) approach, often used when studying gold or silver deposition, produced spectral libraries that were difficult to distinguish with confidence from the lung tissue. While this is not always an issue with every MWCNT (metal contaminated for example, are easy to detect), it was a problem with the MWCNT used in this study. Instead, SFF was successfully used with this material. This approach uses a single “ideal” spectrum (brightest area from the tissue background), and creates a signal based on the deviation, or more importantly lack thereof, from that ideal spectrum seen in Figure 1B. Figures 6 and 7 show representative lung tissue sections analysed by the SFF approach on days 7 and 28, respectively. Figures 6A and 7A are the no particle DM controls. The lack of green (pseudo-color) signal indicated the absence of particle as expected. Figures 6B and 7B show the source MWCNT exposures, and Figures 6C and 7C are images from lungs exposed to the minimally carboxylated MWCNT. Both the source and minimally carboxylated MWCNT showed some minor indication of small particles in epithelial cells, but most of the material appeared to be sequestered in the AM. Figures 6D and 7D are images from lungs exposed to maximally carboxylated MWCNT. At 7 days, these maximally f-MWCNT were visible as numerous fine particles, which appeared to be in lung epithelial cells in addition to within AM. At 28 days, there was a reduced overall particle burden in maximally f-MWCNT samples, which is suggestive of clearance occurring over time. Overall, the SFF analysis confirmed the hypothesis that the degree of carboxylation affected the lung burden following *in vivo* exposures.

Stimulated Raman Spectroscopy detection of MWCNT

The presence of MWCNT in lung sections from mice exposed for 7- and 28-days was also assessed by SRS. Figures 8 (7 day) and 9 (28 day) show computer-colored images from SRS with blue indicative of lung tissue, white indicative of MWCNT, and black representing empty space. Figures 8A and 9A were vehicle controls for 7 and 28 days post-instillation respectively. As expected, there was no indication of particle in these lung sections (see supplemental Figure 1A for wide-angle image). Figures 8B and 9B were source MWCNT instillations at 7 and 28 days post-instillation (see supplemental Figure 1B for wide-angle image). The particle deposition in both images was sparse and usually clustered to some degree (see quantitation below). Figures 8C and 9C illustrate the f-MWCNT with the lowest degree of functionalization at 7- and 28-day post-instillation, respectively. At both time points, a majority of particles were tightly clustered, most likely indicative of AM uptake. Figures 8D and 9D show the effect of maximal functionalization. This f-MWCNT was more distributed throughout the lung tissue in addition to containing clusters that suggest uptake by AM. Whole tissue section scans indicated that the particle deposition was often associated with terminating bronchi, while the distal parts of the lung section were

occasionally absent of particle (supplemental Figure 1C). It appeared that smaller particle depositions were slowly disappearing with time from 7 to 28 days (see quantitation below). It may be easier to visualize the particles in the absence of tissue (blue tissue channel removed), and that can be found in Supplemental Figures 2 and 3 corresponding to day-7 and day-28 particle exposures respectively.

Quantification of particle burden indicated the deposition and/or retention of MWCNT in the lung was highest in the maximally functionalized group compared to the minimally functionalized and source MWCNT (Figures 10A and 10B). Also, quantification of the mean particle cluster size indicated highest dispersion in the maximally functionalized group (Figures 10A and 10B). These results support the hypothesis that MWCNT carboxylation leads to smaller particle agglomerates in lungs following instillation, which may lead to increased deposition. The maximally carboxylated MWCNT showed the highest lung burden at 7 days, but is decreasing at the later 28 day time-point. The other MWCNT did not show any significant changes in lung burden between time-points (Figure 10). This suggests that the increased dispersion due to functionalization may result in higher initial deposition, but is later cleared more rapidly. The large agglomerates were most likely MWCNT in AM as illustrated in earlier Figures.

Discussion

Detailed characterization of the series of MWCNT used in this study demonstrated subtle, but clear shifts in the surface chemistry and properties as a result of carboxylation that plateaued after varying degrees of carboxylation time (Wu *et al.*, 2017). In a recent publication with these same materials, it was evident that even the lowest amount of carboxylation eliminated toxicity or activation of THP-1 macrophage-like cell IL-1 β release *in vitro*, compared to the source MWCNT (Hamilton Jr. *et al.*, 2018). However, toxicity and IL-33 release using A549 epithelial cells changed gradually with carboxylation and the effects plateaued at approximately the same point as the change in surface properties (Wu *et al.*, 2017, Hamilton Jr. *et al.*, 2018). Induction of autophagy after 7 days also varied *in vivo* with carboxylation (Hamilton Jr. *et al.*, 2018). Consequently, the current study examined how variation in MWCNT carboxylation affected distribution, particle retention and resulting pathology in a mouse model to determine the relevance of the previous *in vitro* studies.

Based on our prior experience and that from a number of other laboratories (Hamilton *et al.*, 2013a, Hamilton *et al.*, 2013b, Li *et al.*, 2013, Sweeney *et al.*, 2016, Hamilton Jr. *et al.*, 2018), we proposed that carboxylation would decrease toxicity and *in vivo* inflammation. Since suspension of carboxylated MWCNT results in smaller agglomerates than unmodified MWCNT, we anticipated that there would be better dispersion following instillation. What was less clear was whether there would be differences in the cell types that the carboxylated MWCNT would be interacting with and how that might affect clearance of carboxylated MWCNT compared to unmodified MWCNT. In order to have more confidence in detecting the presence of smaller agglomerates in the lungs, two approaches were used. Both CytoViva (SFF method) and Raman (SRS) imaging gave similar results and detected MWCNT with few false positives. There was a quite high signal-to-noise ratio when

comparing the minimal background in control tissues versus the high signal in particle-exposed tissues, which indicates a high specificity of these methods for detection of MWCNT. SFF provided better images of tissues to observe in which cells the particles were present, while SRS provided higher resolution of each individual particle and captured a larger area of the lung section. Therefore, both methods were complementary for general observation of tissue localization and particle resolution for quantification.

The hypothesis of this study was that adding carboxyl groups to the MWCNT surface would change the deposition of particles in the lung due to smaller size (less aggregated) and altered surface chemistries. Combined with the *in vitro* results with A549 cells (Hamilton Jr. *et al.*, 2018), smaller agglomerates would be predicted to result in an increased probability that some of the f-MWCNT would appear in the lung matrix associated with epithelial cells as opposed to solely being taken up by AM. The results of the current study supported this hypothesis. Based on all of the imaging methods, the key observations were that as carboxylation increased, there was less pathology (albeit the source material MWCNT produced only moderate pathology), better dispersion in the lungs, and increased presence in the lung tissue matrix. This finding is consistent with other studies using various *in vitro* models of epithelial cells (Ruenaroengsak *et al.*, 2016, Hamilton Jr. *et al.*, 2018). Nevertheless, our study is one of the first to provide evidence of this change in cell association *in vivo*. Additional studies are needed to further confirm these suggested changes in particle uptake among AM and epithelial cells, such as experiments that utilize specific markers for these cell types.

However, it is unlikely that the agglomerate size as a result of carboxylation was solely responsible for any or all of the changes in cell association. It is well known that ENM form a corona on their surfaces that will affect which receptors and cells they interact with and rates of phagocytosis (Mahon *et al.*, 2012, Wang *et al.*, 2013, Bai *et al.*, 2016). In this study, bare MWCNT were associated primarily with macrophages and rarely with epithelial cells, but that changed as carboxylation increased on the f-MWCNT. The explanation for this change is not simply explained by the surface properties of the agglomerates since all of them, whether bare MWCNT or carboxylated, would be coated (corona) by proteins and lipids present in the DM used to suspend the various particles as well as proteins and lipids in the lung lining fluid (Marchesan and Prato, 2015).

The maximally carboxylated MWCNT had the highest lung burden, especially at the earlier 7 day time point. This could be explained by more initial deposition due to better particle dispersion, decreased clearance, or a combination of both factors. Carboxylated MWCNT were better distributed within the lung compared to the source MWCNT and the distribution was dependent on the amount of carboxylation. It could be argued that broader distribution would challenge the ability of lung macrophages to clear the finer and more broadly distributed materials. However, macrophages did effectively phagocytise and take up all of the MWCNT variants used in this study. The rates of macrophage phagocytosis of the different MWCNT were not examined in this study, but this could provide an explanation for the changes in lung burden. Nevertheless, a more likely explanation for the increased lung burden of carboxylated MWCNT was the internalization of carboxylated MWCNT by epithelial cells, which would restrict and reduce clearance by lung macrophages. The uptake

of carboxylated MWCNT by various epithelial cells could have a secondary effect on the production of lung lining lipids and proteins (surfactant and mucus) and/or mucociliary escalator efficiency that may provide an alternative explanation for the reduced clearance of carboxylated MWCNT.

Yet, another possibility is suggested by work done on well-dispersed single-walled carbon nanotubes (SWCNT), which showed that 90% of the non-aggregated SWCNT deposited in the lung interstitium as opposed to 20% for the aggregated MWCNT (Mercer *et al.*, 2008). In addition, this work reported the presence of SWCNT in alveolar macrophages and below type II pneumocytes by TEM (Mercer *et al.*, 2008). Taken together, these results closely mimic the deposition of f-MWCNT and suggest the particle deposition can be the result of aggregation state with the more aggregated CNT being cleared more efficiently by the AM. Regardless, more work is needed to provide clear evidence of the effects of carboxylation on MWCNT deposition and retention.

Results indicating carboxylated MWCNT tend to localize to epithelial cells with higher overall lung burden and low pathology suggests that they may be useful as potential carriers to deliver therapeutic agents. Carboxylated MWCNT have been demonstrated in many laboratories to have no or minimal toxicity, do not induce activation of the NLRP3 inflammasome in macrophages decreasing the potential of chronic inflammation from these materials. However, future studies would have to determine how long the carboxylated MWCNT remain in the epithelial cells and whether they might traverse into the circulation.

Conclusions

MWCNT have been generated in a wide variety of sizes and many laboratories have been able to functionalize MWCNT adding to the wide diversity of this important class of ENM. As the diversity of materials increases it is important to fairly quickly determine the relative safety of new materials in order to protect public health and increase the potential usefulness of these materials. The potential to cause toxicity has been related to properties such as length, rigidity and presence of contaminating metals (Hamilton *et al.*, 2009, Palomaki *et al.*, 2011, Hamilton *et al.*, 2012, Poland *et al.*, 2012). Furthermore, many laboratories have demonstrated that the surface of MWCNT can be functionalized to increase their utility for a variety of engineering and biomedical applications (Sun *et al.*, 2002, Zhang *et al.*, 2010b, Hamilton *et al.*, 2013a, Hamilton *et al.*, 2013b, Allegri *et al.*, 2016). Unfortunately, the amplified diversity increases the challenge to quickly determine relative safety in relevant biological settings, especially *in vivo*.

The observation that maximally functionalized MWCNT distribute more uniformly throughout the lung is novel, and taken together with the absence of cellular damage, and extended deposition, may establish a practical use for these particles as a potential drug-delivery mechanism targeting lung epithelial cells for an extended period of time. However, any tissue exposed to fibers over an extended period of time, such as f-MWCNT, run the risk of interstitial fibrosis. This risk still needs to be evaluated, along with other potential pathologies before any particle-associated therapeutic can be considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MWCNT	multi-walled carbon nanotubes
f-MWCNT	functionalized-multi-walled carbon nanotubes
ENM	engineered nanomaterials
AM	alveolar macrophage
DM	particle dispersion media
PBS	phosphate-buffered saline
SEM	scanning electron microscopy
SSA	specific surface areas
SAM	spectral angle mapping
SFF	spectral feature fitting
SRS	stimulated Raman scattering
MSE	error mean squared
H & E	hematoxylin and eosin
C	carbon
O	oxygen
NLRP3	NLR pyrin domain 3

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Figure 1:
Spectra used in SFF hyperspectral imaging (Cytoviva). **A)** Background tissue intensity (green) versus non-tissue background (yellow). **B)** Particle signal (red) versus background tissue intensity (green) and non-tissue background (yellow). **C)** Raman spectra for tissue background.

Figure 2:
TEM images of A) source MWCNT, B) minimal f-MWCNT_{38.4}, and C) maximal f-MWCNT_{14.7} demonstrating little visible difference (20 nm diameters in all instances).

Figure 3:
Relative lung pathology 7 and 28 days post-particle exposure contrasting the source MWCNT with minimally and maximally carboxylated MWCNT. Pathology scores range from 0 to 4, with 0 indicating no pathology and 4 being the worst possible indication of disease. Data expressed as median scores $\pm$ range. Asterisks ** indicate statistical significance at $P < 0.01$, or * $P < 0.05$ compared to the corresponding DM control.

Figure 4:

Representative H&E bright-field photomicrographs of Balb/c mouse lung sections exposed to various MWCNT for 7 days post-particle exposure. Dark areas indicate MWCNT deposition. **A)** Dispersion media control for 7 days. **B)** The source MWCNT for 7 days with particle isolated primarily in the alveolar macrophages. **C)** Particle f-MWCNT_{38.4} (5 min) for 7 days isolated exclusively in numerous alveolar macrophages. **D)** Particle f-MWCNT_{14.7} (120 min). Magnification at 1000x for all images and micrometer scale bar = microns.

Figure 5:

Representative H&E bright-field photomicrographs of Balb/c mouse lung sections exposed to various MWCNT for 28 days post-particle exposure. Dark areas indicate MWCNT deposition. **A)** Dispersion media control for 28 days. **B)** The source MWCNT for 28 days **C)** Particle f-MWCNT_{38.4} (5 min) for 28 days. **D)** Particle f-CNT_{14.7} (120 min) for 28 days. Magnification at 1000x for all images and micrometer scale bar = microns.

Figure 6:

Representative CytoViva hyperspectral dark-field photomicrographs (SFF) of Balb/c mouse lung sections exposed to various MWCNT for 7 days post-particle exposure. Bright green areas indicate MWCNT deposition. **A)** Dispersion media control for 7 days. **B)** The source MWCNT for 7 days with particle that appears to be isolated primarily in alveolar macrophages within the alveolar spaces of the lung section. **C)** Particle f-MWCNT_{38.4} (5 min) for 7 days that appears to be isolated exclusively in numerous alveolar macrophages in a lung airway. **D)** Particle f-MWCNT_{14.7} (120 min) for 7 days dispersing throughout the epithelial tissue matrix in addition to alveolar macrophage uptake. This particle deposition is not limited to particular lung areas, but freely disperses throughout the lung tissue. Magnification at 400x (40x objective) for all images and micrometer scale bar = 20 microns.

Figure 7: Representative CytoViva hyperspectral dark-field photomicrographs (SFF) of Balb/c mouse lung sections exposed to various MWCNT for 28 days post-particle exposure. Bright green areas indicate MWCNT deposition. **A)** Dispersion media control for 28 days. **B)** The source MWCNT for 28 days **C)** Particle f-MWCNT_{38.4} (5 min) for 28 days. **D)** Particle f-MWCNT_{14.7} (120 min) for 28 days. Magnification at 400x (40x objective) for all images and micrometer scale bar = 20 microns.

Figure 8:
Particle deposition in lung sections assessed by stimulated Raman scattering after 7-day exposure. Representative regions of merged tissue (blue) and MWCNT (white) channels. **A)** Dispersion media control for 7 days. **B)** The source MWCNT for 7 days. **C)** Particle f-MWCNT_{38.4} (5 min) for 7 days. **D)** Particle f-MWCNT_{14.7} (120 min) for 7 days. Magnification equal for all images; scale bar = 50 μm .

Figure 9: Particle deposition in lung sections assessed by stimulated Raman scattering after 28-day exposure. Representative regions of merged tissue (blue) and MWCNT (white) channels. **A)** Dispersion media control for 28 days. **B)** The source MWCNT for 28 days. **C)** Particle f-MWCNT_{38.4} (5 min) for 28 days. **D)** Particle f-MWCNT_{14.7} (120 min) for 28 days. Magnification equal for all images; scale bar = 50 µm.

Figure 10:

Particle quantification from stimulated Raman scattering for 7- and 28-day MWCNT lung exposures. A) Mean $\pm$ SEM percent particle burden (pink) in the lung sections (blue). B) Mean $\pm$ SEM particle cluster size in the lung tissue expressed as microns squared. Asterisks indicate statistical significance at *** $P < 0.001$ or at * $P > 0.05$ compared to corresponding control particle (no -COOH MWCNT). Daggers ††† indicate significance at $P < 0.001$ or † $P < 0.05$ compared to corresponding f-MWCNT. Symbol § indicates significance at $P < 0.05$ compared to day-7 for the same particle. Sample size was 3 mice per exposure.

Table 1.

Elemental analysis, C:COOH ratio, SSA of MWCNT and f-MWCNT

MWCNT	Treatment time (min)	% by Weight			C:O free C atoms per O atom	SSA (m ² /g)	Length ($\mu\text{m} \pm SD$)
		C	O	Ni			
MWCNT _{source}	0	93.9	4.90	1.20	N/A	174	10-30 *
f-MWCNT _{38.4}	5	90.1	9.06	0.84	53	220	3.4 $\pm$ 1.2
f-MWCNT _{14.7}	120	84.4	15.6	-	29	271	2.2 $\pm$ 0.9

* range determined by manufacturer (mean and variance data not available)

Table 2.

Agglomerate size (mean $\pm$ *SD*), zeta potential (mean $\pm$ *SD*) and diameter of MWCNT variants in dispersion media (DM).

MWCNT	Hydrodynamic Size (nm)	Zeta Potential (mV)	Diameter [*] (nm)
MWCNT _{source}	396 $\pm$ 22.0	-10.3 $\pm$ 0.39	20 - 30
f-MWCNT _{38.4}	169.1 $\pm$ 50.9	-12.7 $\pm$ 0.34	20 - 30
f-MWCNT _{14.7}	41.8 $\pm$ 1.2	-11.5 $\pm$ 1.2	20 - 30

^{*} range determined by manufacturer and confirmed by TEM (mean and variance data not available)