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The effect of polymer coatings on the pulmonary toxicity of multi-walled carbon nanotubes

Lindsey M. Bishop

Thesis submitted to the School of Medicine at West Virginia University

In partial fulfillment of the requirements for the degree of Master of Science in Cellular and Integrated Physiology

Tim Nurkiewicz, Ph.D., Chair
Aaron Erdely, Ph.D., Mentor
Jim Antonini, Ph.D.
Vince Castranova, Ph.D.

Department of Cellular and Integrated Physiology

Morgantown, West Virginia
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Key Words: Multi-walled carbon nanotubes, toxicity, polymer coatings, life cycle

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Abstract

The effect of polymer coatings on the pulmonary toxicity of multi-walled carbon nanotubes

Lindsey M. Bishop

Nanotechnology is the science of developing and manipulating molecular structures smaller than one hundred nanometers in at least one dimension. These structures are collectively called engineered nanomaterials. A subgroup of these engineered nanomaterials are multi-walled carbon nanotubes (MWCNT). They are of great interest to industry because of their small size, large surface area, strength, and conductivity in comparison to materials currently used. Practical applications of MWCNT include improvements for water filtration systems, composites, and drug delivery. The same properties that make them advantageous for industry may also confer adverse health effects. For workers in the U.S. handling CNTs, the process of synthesizing and handling the dry powder form has been shown to be the greatest risk for inhalation exposure.

Companies synthesize or purchase as-produced multi-walled carbon nanotubes (AP-MW), apply a polymer coating (PC-MW), then embed the PC-MW into a composite (e.g. thermoplastic or fiberglass matrix). The intermediate step of polymer coating the AP-MW is for increased dispersion in order to use less material, improve bonding to epoxy matrices for increased strength, and better handling to reduce exposure. Workers are potentially exposed at each production stage especially from dry powder handling of the AP-MW and PC-MW. Comprehensive toxicological data on AP-MW and paired PC-MW remains sparse. The aim of this study was to understand the effect of polymer coatings on the toxicity of AP-MW *in vivo*. Two AP-MW, with different characteristics, were chosen for this study. Each AP-MW came from a distinct manufacturer, each with the polymer coated version for toxicological analyses.

All materials used for the *in vivo* toxicological studies were comprehensively characterized (e.g., length, width, surface area, etc.). Male C57BL/6J mice were dosed by oropharyngeal aspiration with vehicle (physiologic dosing medium), AP-MW or PC-MW from each company at 4 µg (workplace relevant) or 40 µg (known to induce pathology) and sacrificed at 4 h, 1, 7, 28 and 84 d post-exposure. Post-exposure analyses included: bronchoalveolar lactate dehydrogenase activity (cytotoxicity), cell influx (inflammation) and inflammatory proteins, and relative mRNA expression in pulmonary tissue. Qualitative pathological endpoints such as granuloma formation and fibrosis were examined in conjunction with pulmonary biodistribution primarily to address differences due to the polymer coating. Both AP-MW induced time- and dose-dependent measures of pulmonary cytotoxicity, inflammatory cell influx, and inflammatory proteins that resolved by 84 d post-exposure. The PC-MW from Company 1 had no additional effect on induced pulmonary cytotoxicity, inflammatory cell influx, or inflammatory gene expression and inflammatory protein production as compared to the respective AP-MW. However, pulmonary cytotoxicity, inflammatory cell and inflammatory protein production were attenuated in the PC-MW exposed groups from Company 2 in comparison to the respective AP-MW. Based on qualitative observations of AP-MW and PC-MW from Company 1, particles deposited mostly within the bronchioles, were generally cleared by 84 d while a small portion was persistent in alveolar regions. There was no difference in histopathology with respect to the materials compared from Company 1. Similar distribution of particle deposition in lung compartments was observed for AP-MW and PC-MW exposed groups from Company 2, however a larger fraction deposited into alveolar regions. Small granulomas were observed in the bronchioles of AP-MW exposed groups from Company 2. In contrast, granuloma formation was absent in the PC-MW exposed groups with slightly more particle aggregation. In conclusion, the two polymer coatings studied did not

increase the pulmonary toxicity compared to the AP-MW. In fact, inflammation and histopathology was attenuated in mice exposed to the polymer coating from Company 2. It is evident that pulmonary toxicity, from AP-MW to PC-MW, must be carefully considered within the occupational setting.

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Contributors:

M Orandle¹, L Cena¹, V Kodali¹, M Dahm², M Schubauer-Berigan², T Sager¹, J Scabilloni¹, D Schwegler-Berry¹, T Eye¹, L Battelli¹, J Kang¹, G Casuccio³, K Bunker³, A Stefaniak¹, PC Zeidler-Erdely¹, L Sargent¹, RR Mercer¹, A Erdely¹

¹NIOSH, Morgantown, WV; ²NIOSH, Cincinnati, OH; ³RJ Lee Group, Monroeville, PA

Disclaimer:

“The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.”

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2.0 Introduction

2.1 Nanotechnology and carbon nanotubes

Nanotechnology is the science of developing and manipulating molecular structures, called engineered nanomaterials, which are smaller than one hundred nanometers in at least one dimension for use in macroscale products (Donaldson et al., 2006). Within the last two decades, nanotechnology has become a major research and manufacturing field for the creation of stronger materials, longer-lasting fabrics and scratch resistant plastics for use in industrial and consumer sectors. Practical applications for nanotechnology have tremendous potential in consumer based products. These include water filtration systems, sunscreens, electronic microprocessors, and thermoplastics (Thostenson et al., 2002; Liu et al., 2005; Xiong et al., 2006). The benefits of incorporating engineered nanomaterials into composites allow manufacturers to tailor the molecular structure of these materials to achieve specific chemical properties for functionally distinct products. For example, the final product of an item reinforced with nanomaterials can be stronger, more reactive, or improve electrical conductivity (Martins et al., 2013). To date, over eight hundred commercial products rely on nanoscale materials and processes.

The carbon-based engineered nanomaterials are some of the most widely utilized for market applications. As their name suggests, carbon nanomaterials are composed of carbon with varying metal impurities, as a result of their formation and synthesis. Most carbon nanomaterials take the form of spheres or tubes. Spherical carbon nanomaterials are referred to as fullerenes, which were discovered by Robert Curl Jr., Harold Kroto and Richard Smalley (Liu et al., 1998). These individuals won a Nobel Prize in Chemistry in 1996 for their discovery of fullerenes (Royal Swedish Academy of Sciences, 1996). In the same type of process that formed fullerenes, Sumio Iijima discovered cylindrical concentric tubes, which today are referred to as multi-walled carbon nanotubes (MWCNT). A short time later, Iijima took the information about MWCNT and isolated

single walled carbon nanotubes (SWCNT) in the presence of a metal catalyst. SWCNT are hollow tubes composed of a single layer of carbon with diameters around one nanometer (Yudasaka et al., 1997; DeVolder et al., 2013). The formation of SWCNT is favored when the metal nanoparticles-to-carbon ratio are high, whereas, MWCNT can be formed with minimal amounts of metal catalyst, these catalysts act to align the nanotubes for further downstream applications (Zhang et al., 1999). However, the metal impurities utilized for the formation of SWCNT and MWCNT are generally unfavorable in commercial products (Baughman et al., 2002). For this reason, large batches of CNT are sold in purified forms, with metal contaminants removed. Once formed, both SWCNT and MWCNT have an incredible propensity to agglomerate. This is due to intrinsic van der Waals forces, and high hydrophobicity. Because these materials agglomerate easily, they are often incorporated into liquid medium for increased dispersion (Donaldson et al., 2006; Porter et al., 2008). An increase in dispersion for industrial purposes provides a more homogenous mixture for a stronger and durable end product. Without adequate dispersion techniques, an agglomerated heterogeneous blend could lead to a fragile product.

As shown in Figure 1, SWCNT become agglomerated tangles in liquid medium. SWCNT appear as very thin fibers having diameters of about one nanometer and being several micrometers long, which results in their high aspect ratio (length/diameter) (Donaldson et al., 2006; Ajayan et al., 1997). Rarely do SWCNT exist as individual tubes but rather as bundles. From a market perspective, SWCNT only make up a small subset of carbon-based nanomaterials (Future Markets Inc., January 2016). It is the larger MWCNT that dominate the current CNT market. In contrast to SWCNT, MWCNT have additional concentric layers with much larger diameters between five and two hundred nanometers (Donaldson et al., 2006; Oberdorster et al., 2016). In addition, the lengths of MWCNT can be much longer than that of SWCNT. MWCNT have weaker van der Waals forces

and can exist as single tubes and form fewer bundles, especially with increasing diameters as seen in Figure 1 (Yu et al., 2000).

2.2 SWCNT-induced pulmonary effects

While industry saw the potential for numerous applications, there was uncertainty about how CNT would affect biological systems. Initial field studies indicated that inhalation was the most likely route of exposure for individuals working with the dry powder form of CNT (Dahm et al., 2011). The dry powder exhibit a lower density. Therefore, it aerosolizes with ease and has the ability for deposition into the airways within the lungs (Muhlfeld et al., 2012). The pulmonary system is a highly branched network of tubules which culminate into a complex web of vascular alveoli. These alveoli are specialized for providing a large surface area in the lung for gas exchange of oxygen and carbon dioxide. Small inhaled particles, such as CNT, can travel through this network and into alveolar regions and may impede the efficiency of gas exchange, whereas larger particulates deposit within upper airways of the primary bronchi, and generally are cleared (Oberdörster et al., 2005; Simeonova et al., 2009). Normally, in these alveolar regions, particles

are blocked from entry into the systemic circulation by a plethora of host defenses. These defense mechanisms can range from a protective epithelial cell lining, to resident phagocytic cells, to the surfactant layer. Particle exposures are known to induce tissue injury and potentially affect pulmonary function (Deng et al., 2005; Bergamaschi et al., 2006; Simeonova et al., 2009). Because of their physicochemical characteristics, it was suspected that CNT would have the ability to induce pulmonary damage *in vivo*.

The initial studies to evaluate the toxicity of CNT were performed using SWCNT (Lam et al., 2004; Warheit et al., 2004; Shvedova et al., 2005; Mangum et al., 2006). In 2004, Lam et al., intratracheally instilled mice with SWCNT and found that exposure to these materials induced dose-dependent epithelioid granulomas, and in some cases interstitial inflammation 7 d post-exposure. They concluded that if SWCNT reached the lungs, these induced toxicities can be considered an occupational health hazard if chronically inhaled (Lam et al., 2004). Shvedova et al. (2005) introduced a large bolus dose (10-80 µg) of SWCNT by pharyngeal aspiration in mice. In this technique, a dose of material is deposited into both the conducting and respiratory zone of the lung, while the animal is under light anesthesia and breathing normally. Following exposure, this group described an inflammatory and granulomas response at sites of SWCNT agglomerate deposition in the distal bronchial and proximal alveoli. In addition, exposed mice showed signs of interstitial fibrosis 7 days post-exposure in the lung parenchyma, concluding that these nanotubes translocated to distal alveolar septa (Shvedova et al., 2005). Most of the scientific community agreed that pulmonary exposures to SWCNT induced varying degrees of inflammation, granuloma formation and interstitial fibrosis (Lam et al., 2004; Warheit et al., 2004; Shvedova et al., 2005)

2.3 SWCNT induces cardiovascular and systemic toxicities

In addition to SWCNT impacting the pulmonary tissue, a group of researchers studied the cardiovascular toxicity and systemic translocation of SWCNT following pulmonary exposure. Li et al. (2007) showed that pulmonary toxicity of SWCNT following respiratory exposure could be correlated with systemic toxicities. They were able to demonstrate a dose-dependent increase in vascular oxidative damage and mitochondrial dysfunction (Li et al., 2007). Furthermore, they found that mice exposed to SWCNT developed aortic mitochondrial DNA damage over 60 days post-exposure, lending support that respiratory exposure to SWCNT may exert prolonged adverse health effects. Mitochondrial dysfunction has been used as a factor to link a number of cardiovascular diseases, including atherosclerosis (Li et al., 2007; Simeonova et al., 2009). With the onset and progression of atherosclerosis, inflammatory cells are recruited to sites of insult by activated platelets. This in turn recruits leukocytes to the site of damage to clear debris and begin repair mechanisms. Pulmonary exposures to SWCNT induced platelet activation, and exacerbated atherosclerotic lesion formation, *in vivo* (Simeonova et al., 2009; Li et al., 2007). Erdely et al. (2009) explained that a chronic systemic response to pulmonary exposures of SWCNT can exacerbate cardiovascular dysfunction. This group established that respiratory exposure to SWCNT caused the development of a unique blood gene and protein signature, which confirmed the intricate occurrence of cross-talk between the pulmonary and systemic circulations following exposure (Erdely et al., 2009). Overall, the *in vivo* experiments detailing the adverse effects of pulmonary exposure to SWCNT were vital when assessing the potential health impact on a larger scale, such as the workers who may be at risk for inhaling CNT.

2.4 MWCNT-induced pulmonary effects

Over the years, our knowledge of the pulmonary and systemic exposures to SWCNT has led scientists to investigate the impact of MWCNT on pulmonary toxicity, seeing that this material dominates the current consumer market. In 2008, researchers at the University of Edinburgh described inflammation and formation of granulomas, in the mesothelial lining of the chest cavity in mice following intrapleural instillation of MWCNT (Poland et al., 2008). In addition, Muller et al. (2005) administered a bolus dose of MWCNT (unprocessed and ground material) to Sprague-Dawley rat lungs by intratracheal instillation. This group reported that 60 days post-exposure, pulmonary tissue had significant increases in inflammation, granulomas and fibrosis compared to control. In those exposures, the ground form of MWCNT were more dispersed in alveolar regions and induction of granulomas was observed with additional particle laden macrophages, similar to results reported for SWCNT (Lam et al., 2004; Shvedova et al., 2005; Warheit et al., 2004). Porter et al., (2010) showed that an aspiration of dispersed MWCNT caused time-and dose-dependent responses of granuloma formation and pulmonary fibrosis. Mercer et al. (2011) reported penetration of MWCNT through alveolar epithelial cells into the alveolar interstitial space after pharyngeal aspiration of dispersed MWCNT in mice. These findings suggested the need to evaluate chronic toxicity of MWCNT at sites within the deep lung.

Concerns of occupational exposures have arisen due to the understanding that adverse effects occur from MWCNT lung deposition which were observed during animal studies in previous years (Muller et al., 2005; Simeonova et al., 2009; Mangum et al., 2006; Li et al., 2007; Mercer et al., 2008; Porter et al., 2010; Sager et al., 2014). The results from an inhalation study conducted by Ma-Hock showed that inhaled MWCNT produced lesions in the lung and stressed the need to compare various MWCNT toxicities. Selection of MWCNT with the lowest risk for

applications would reduce the potential risk of inhalation exposure to humans (Ma Hock et al., 2009). A repeated 13-week inhalation study with male and female rats exposed to MWCNT showed particle laden alveolar macrophages, increased inflammatory cells and interstitial fibrosis (Pauluhn et al., 2010). Both of these exposure studies were essential for the development of a recommended exposure limit (REL) in order to protect and prevent lung injury to workers handling these types of MWCNT (Pauluhn et al., 2010; Ma-Hock et al., 2009; NIOSH CIB 65).

2.5 MWCNT induces cardiovascular and systemic toxicities

The nanotoxicology community began to recognize that pulmonary exposures to MWCNT have a unique impact on systemic toxicity. It has been shown that inhalation exposures to MWCNT suppressed immune function in mice. Suppression occurred through the activation of signals, beginning at the pulmonary tissue and ending with the activation of signals in the spleen (Mitchell et al., 2007, 2009). MWCNT exposures have led to serum increases of pro-inflammatory cytokines such as, interleukin-6 (*Il-6*), and the neutrophil chemoattractant, chemokine (C-X-C) ligand 1 (*Cxcl1*). Increased serum inflammatory proteins were a result of pulmonary exposure to MWCNT, which suggested a corresponding response by systemic tissue. This was verified by increased gene expression of *Il-6* in aortic tissue (Erdely et al., 2009). These studies demonstrated a close intersect between the pulmonary and systemic circulation following exposures to MWCNT. Oropharyngeal aspiration in MWCNT to mice promoted susceptibility of cardiac tissue to ischemia/reperfusion injury without a pulmonary inflammatory response, suggesting a risk to the cardiovascular system in the presence of MWCNT (Urankar et al., 2012). Another study evaluated arteriolar reactivity in response to MWCNT inhalation. Results indicated that MWCNT inhalation leads to impairments of endothelium-dependent dilation in coronary microcirculation at 24 h post-exposure (Stapleton et al., 2012).

Pulmonary exposures to MWCNT requires complex evaluations of cardiovascular toxicity, regardless of rodent model (Erdely et al., 2009; Stapleton et al., 2012). A long term assessment of systemic toxicity was conducted in 2009 with Wistar rats. This 90-day inhalation toxicity study concluded that inhalation exposure to MWCNT produced no systemic toxicity while minimal multifocal granulomas and neutrophilic inflammation was observed in lung tissue (Ma-Hock et al., 2009). In 2010, a study conducted by Jurgen Pauluhn reported that extrapulmonary translocation or systemic toxicity was absent in any biological specimens.

2.6 Post-processing MWCNT

As discussed, numerous toxicological studies have demonstrated the potential health implications following pulmonary exposures to MWCNT *in vivo* (Shvedova et al., 2005; Mercer et al., 2011; Mercer et al 2013a, 2013b; Erdely et al., 2009, 2013). Continued research has discovered that alterations to the physicochemical properties of CNT can lead to dramatically different ranges of functional end products (Kong et al., 2004; Vaisman et al., 2006; Sahoo et al., 2010; Martins et al., 2013). Post-processing MWCNT allows manufacturers to tailor large quantities of MWCNT for specific use. Post-manufacturing processes include, and are not limited to, acid washing, functionalization or polymer coating (Liu et al., 2005; Xiong et al., 2006; Wang et al., 2009). Velasco-Santos (2003) explained that functionalized carbon nanotubes bonded to a polymer matrix, exhibited enhanced the mechanical properties of a thermoplastic product. Functionalization can include, nitrogen doping, amination and esterification of the carboxylic groups bound to the surface of CNT (Li et al., 2013). An inverse relationship exists between particle size and surface expressed molecules as a result of functionalization. Importantly, the number of atoms or molecules attached to the surface of MWCNT may determine material reactivity in biological tissue (Nel et al., 2006; Sager et al., 2014). A study in 2013 assessed

covalently functionalized MWCNT to assess the impact of surface charge in a toxicological model for pulmonary fibrosis. The results demonstrated that surface charge is an important predictor of the pro-fibrotic potential of functionalized MWCNT in the lung, with highly negative MWCNT being less toxic and highly positive MWCNT being more toxic (Nel et al., 2009, 2013). Overall, post-processing results in dramatically different sizes, shapes, and chemical compositions of MWCNT, many of which, have unknown toxicities when compared to the AP-MW *in vivo*.

2.7 Polymer-coated MWCNT

The abundance of research involving CNT and their impact on pulmonary and systemic health effects has focused mainly on AP-MW and is well known (Oberdorster et al., 2016; Bergamaschi et al., 2006; Borm et al., 2004; Donaldson et al., 2002, 2006). A recent advancement in the life cycle of MWCNT manufacturing involves polymer-coating MWCNT (PC-MW) for numerous reasons, some of which include increased dispersion into composites, ease in handling and reduced dustiness without affecting the properties of the native MWCNT (Liu et al., 2005; Xiong et al., 2006;). What remains unknown is the pulmonary toxicity of PC-MW from U.S. manufacturing companies in comparison to their AP-MW.

The French Institute of Health and Medical Research (INSERM) investigated the toxicity of MWCNT coated with a polystyrene polybutadiene polymethylacrylate (PMMA) polymer. Coating MWCNT with PMMA did not affect the intrinsic properties of the MWCNT and decreased cytotoxicity, inflammation and oxidative stress *in vitro* and *in vivo* (Tabet et al., 2011). Though this group found that a PMMA polymer-coated MWCNT reduced toxicity, it remains unclear if other types of polymer coatings affect toxicity of MWCNT *in vivo*. To more thoroughly understand the impact of polymer coatings on the pulmonary toxicity of MWCNT, the current

project utilized two PC-MW which are currently being handled in the U.S., and assessed the toxicity of these materials compared to their associated AP-MW counterpart.

2.8 CNT and potential human health implications

The U.S. National Science Foundation predicted that a large increase in workers would be needed to support the growing field of nanotechnology. They urged the importance of toxicological evaluation of nanoparticles before any adverse health consequences could arise as a result of handling these materials. Despite the toxicity findings to date regarding CNT, even the most modest predictions show continued growth of the industry through 2025 (Future Markets Inc., January 2016).

NIOSH issued a current intelligence bulletin (CIBs) that summarized toxicity research to date regarding occupational hazards of CNT. In this document, NIOSH reviewed short-term and sub-chronic animal studies involving CNT exposures, which resulted in early stage fibrotic and inflammatory lung responses. This compilation of studies was used to establish a recommended exposure limit (REL) for individuals handling these materials. NIOSH proposed a recommended exposure limit (REL) of $1 \mu\text{g}/\text{m}^3$ of elemental carbon as a respirable mass, based on an 8-hour time-weighted average (TWA) concentration. The proposed REL is a recommendation used to minimize the risk for pulmonary inflammation and fibrosis to workers handling CNT over a life time of exposure, or 45 working years (NIOSH CIB 65). Researchers at NIOSH used this recommendation when extrapolating the data from workplace exposures for the design of an *in vivo* inhalation study (Erdely et al., 2013). This inhalation study exposed mice to similar levels of MWCNT, based on extrapolations from exposure assessments of U.S.-based CNT facilities. Their findings showed limited pulmonary inflammatory potential of MWCNT at exposures reported in US worksites evaluated by NIOSH, and their estimates suggested a considerable amount of

working years (about 76) would be needed to induce significant pathology (Erdely et al., 2013). Since the proposed REL was established, an industrywide exposure assessment study was conducted to assess occupational exposures to CNT. Of the fourteen total sites visited within the study, epidemiologists concluded that all worker exposure samples, based on personal breathing zone measurements, were below the NIOSH REL for the respirable fraction (Dahm et al., 2015).

It is important to understand the interactions of CNT and their impact on pulmonary toxicity in rodents in order to generate data that can be used for risk assessment (Oberdorster et al., 2015). Our understanding of the toxicological impact can be used to develop predictive screening standards at occupational levels. To achieve this goal, toxicologists must utilize exposure assessment studies in order to design *in vivo* protocols where results accurately reflect current worker exposures within the U.S. (Xia et al., 2009; Liou et al., 2015).

3.0 Study AIM

The overall objective of the current study was to evaluate and describe the toxicological impact of two different AP-MW and their PC-MW counterparts *in vivo*. Based on previous animal studies, it is critical to understand how these materials impact occupational health. To date, little is known about the toxicities associated with PC-MW, most of which make their way into composite manufacturing for consumer products. Furthermore, research regarding the toxicities of these nanoenabled composites is sparse. Ongoing exposure assessment studies from U.S. CNT manufacturing facilities were vital to this research as a way to bridge the gap between occupational exposure assessments and *in vivo* animal studies. The research presented here, utilizes the same AP-MW and PC-MW currently handled in U.S. facilities which have participated in NIOSH field assessments.

This study focused on the combination of AP-MW and PC-MW from two separate companies (Figure 2). Each company had separate intentions for utilizing polymer coatings. Company 1, synthesizes their own branched AP-MW and uses an off the shelf polyurethane coating. This company wanted to reduce dustiness of the AP-MW in order for ease of handling and involuntary product loss downstream, as this material aerosolizes easily. Additionally, they use polyurethane to increase dispersion of MWCNT in liquid medium to improve thermoplastic manufacture. Conversely, Company 2 purchases a bulk supply of AP-MW, and coats this material with a proprietary poly(arylene ethynylene) (PAE) polymer (Figure 2). Company 2 utilizes this polymer to increase dispersion and adhesion to an epoxy matrix which will be used in the manufacture of composites.

Figure 2: AP-MW and PC-MW material overview. Each U.S. based company adopts different methods for polymer coating. This study focused on the pulmonary toxicity following the addition of an off the shelf polyurethane or proprietary polymer to AP-MW *in vivo*.

4.0 Methods

The general experimental approach to assess pulmonary toxicity *in vivo*, to both AP-MW and PC-MW is shown in Figure 3. The hypothesis was that PC-MW would alter toxicity in comparison to AP-MW *in vivo*. Time- and dose-dependent responses were assessed along with potential pathological outcomes following exposures. Each chapter herein describes the molecular and pathological effects of these pairs of AP-MW and PC-MW following oropharyngeal aspiration in a mouse model.

Figure 3: Endpoint analyses following exposure to either AP-MW or PC-MW. Analyses include: analysis of BAL for cytotoxicity as measured by LDH, pulmonary inflammatory cell influx and protein abundance, and quantitative measurement of relative mRNA expression of pulmonary tissue.

4.1 Exposure Assessment

Ongoing research has suggested that occupational exposure to CNT could result in a variety of adverse health effects. To address this concern, Dahm et al. (2015) conducted an industry wide exposure assessment study of CNT primary and secondary manufacturers. To assess worker exposure, this group utilized personal breathing zone (PBZ) samples. Briefly, PBZ samplers are attached to the collar of a worker, throughout normal working hours. Following each shift, PBZ samples were collected for analysis of elemental carbon at inhalable and respirable sized fractions by transmission electron microscopy. These fractions are assessed to understand the percentage of particles that can be inhaled, as well as the fraction that has the potential to reach deep within the lungs (respirable). Until more information is available about the effects of CNT on occupational health, PBZ elemental carbon (EC) samples are a way to limit potential health risk and to assess worker exposure at both the inhalable and respirable sized fractions (Dahm et al., 2015). Fractions of material that have the potential to reach larger airways and persist are known as inhalable, while respirable sized fractions have the potential to travel deeper within the lung. The representative electron microscopy images of PBZ samples collected from both workplaces that handle the materials tested for this study were evaluated (Figure 5 and 19). The PBZ TEM images for this study were used in order to determine proper dosing methodology for adaptation in a rodent model.

4.2 Characterization

Scanning transmission electron microscopy (STEM) images were obtained for all materials from Company 1 and 2 using an S-5500 ultrahigh resolution SEM with STEM capabilities (RJ Lee Group, Inc., United States). Length and diameter for each material was calculated based on an

average of $n=200$ nanotubes. All measurements for zeta potential, an indicator of surface charge in the dosing medium, were performed using a Nano ZS90 instrument (Malvern Instruments, UK). Viscosity of control medium was previously determined at room temperature using a VS-10 viscometer (Malvern Instruments) and used as the value for calculation of zeta potential. The agglomeration state of MWCNT can be different for each material depending on the structural arrangement of carbon atoms and their interaction with surrounding ions, in solution. Riddick (1968) used zeta potential to develop categories that describe the stability of these dispersions in liquid media. Essentially, a weak electrical force (-12mV) will allow the material to agglomerate, because the particles do not have enough surface charge to repel each other. Normally, a dispersion with a large electrical force (-61 to -80 mV) allows particles to remain suspended in liquid, or have high stability. X-ray photoelectron spectroscopy (XPS) was utilized to measure the elemental surface chemistry of each sample. In addition, hydrodynamic diameter was measured by dynamic light scattering (DLS). DLS characterizes nanoparticles by measuring the fluctuations in the scattering intensity of a solution over time (Ansell et al., 2011). The measured hydrodynamic diameter is a value to explain how a particle diffuses within a fluid, in this case, the physiologic medium for *in vivo* exposures. The hydrodynamic diameter represents the particle size of spherical structures once in the physiologic medium. Although this method is not optimal for fibrous structures, it can be used to qualitatively infer the degree of agglomeration of CNT. The endotoxin contamination was measured using the Limulus Amebocyte Lysate test. Metal content was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

4.3 Study design and particle aspiration

Male C57BL/6J pathogen-free mice weighing 20-25g were obtained from Jackson Laboratories, Bar Harbor, ME. All mice were housed in the assessment and accreditation of

laboratory animal care (AAALAC)-accredited NIOSH Animal Facility and afforded food and tap water *ad libitum* in ventilated cages, on autoclaved hardwood chip bedding in laminar flow cages and an environment of controlled humidity and temperature. This environment was equipped with 12 hours of light/dark cycles per day. Animals were allowed to acclimate for at least seven days prior to use in any experiments. Animal care and use procedures were conducted in accordance with the “PHS Policy on Humane Care and Use of Laboratory Animals” and the “Guide for the Care and Use of Laboratory Animals” (NIH publication 86-23, 1996). The procedures utilized in this study were approved by the National Institute for Occupational Safety and Health Institutional Animal Care and Use Committee.

Male C57LB/6J mice, eight to ten weeks of age, were exposed by oropharyngeal aspiration to either vehicle control or selected MWCNT at two different concentrations, 4 or 40 μg per mouse dependent on study design (Figure 4). Physiologic medium, the vehicle, was prepared fresh each morning and contained mouse serum albumin (0.6 mg/ml) and pulmonary surfactant (10 $\mu\text{g}/\text{ml}$; 1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine, DPPC; Sigma Life Science) prepared in United States pharmacopeia (USP) grade-phosphate buffered saline (PBS) without calcium and magnesium. Low (4 μg) and high (40 μg) dose concentrations were chosen from previous studies in order to mimic workplace exposures. The lowest dose was chosen to accurately reflect relevant human exposure levels as reported by Erdely et al. (2013). The low dose is equivalent to 7.6 working years, a time point representative of the years in operation of these studied facilities within the U.S., while the highest dose (40 μg) will elicit pathology within a short time frame (84 d) (Erdely et al., 2013). According to the manufacturers, the polymer coatings are 7 and 15% of the total weight from Company 1 and Company 2, respectively. Extrapolations were made in order to account for the weight of the coating, which was then dispersed in the liquid medium for control

dosing purposes. Each dry AP-MW and PC-MW was weighed equally by mass. Each AP-MW had a final concentration of 1.6 mg/mL in physiologic dosing medium while the PC-MW samples were suspended in 850 or 930 μ L of physiologic dosing medium (Company 1 and Company 2 respectively). Thus, the mass of MWCNT in each sample, regardless of coating, remained similar.

Figure 4: Experimental Design. Two sets of mice were used for each company, one for histopathology as well as pulmonary and systemic tissue gene expression and a second set for analyses of BAL samples to determine inflammatory cell influx and cytotoxicity.

Each mouse was anesthetized in a bell jar containing a tissue-tek mega-cassette (Sakura) packed with gauze, which was moistened with USP grade isoflurane (0.5 mL) (Piramal Healthcare, Andhra Pradesh, India). When fully anesthetized, as noted with unsteady gate, decreased mobility and tail drop, the mouse was taken out of the bell jar and placed on a slant board (Saffiotti et al., 1968; Rao et al., 2011). The animal was positioned in such a way that its back, neck and cranium rests on the board. The mouth was gently opened to suspend the mouse by its incisor teeth along a thin braided cord. The tongue was pulled aside and extended away from the oral cavity by small forceps. A fifty microliter suspension of dosing medium was pipetted at the base of the tongue, while tongue restraint continued for five normal breaths to inhale the droplet. Once all the exposure

solution was aspirated by normal respiration, the tongue was released and the mouse was lifted from the board, and placed back into the housing unit. Each mouse was observed for a short time following this procedure to ensure full recovery from anesthesia. At 0 (4 hours), 1, 7, 28 and 84 d post-exposure, mice were euthanized for evaluations of pulmonary responses using two sets of mice for each company. The first set of mice was used to determine molecular parameters, pulmonary inflammatory cell influx and cytotoxicity using bronchoalveolar lavage fluid (BALF). In the second set of mice, right lung lobes were fixed and embedded in paraffin wax, and sectioned for determination of lung burden of MWCNT and histopathology. The left lung lobe was ligated and frozen in liquid nitrogen for relative mRNA expression.

4.4 Bronchoalveolar lavage (BAL):

Mice were anesthetized with an i.p. injection of sodium pentobarbital (100mg/kg body weight) at 4 hr, 1, 7, 28 and 84 d post-exposure. Whole blood was collected from the inferior vena cava and saved for later analysis. Following euthanization by exsanguination, BAL was performed by cannulating the trachea and lavaging the lungs with physiological saline, first at 0.6 mL and two subsequent lavages at 1.0 mL for a total of 2.6 mL of BAL fluid (BALF). Lavage collections were centrifuged for acellular and cellular fractions. The acellular fraction (< 0.6 ml) of the first lavage was kept separate for BALF inflammatory protein analysis (Myriad/ RBM; Austin, TX) by multiplex immunoassay RodentMAP v3.1. The acellular BALF fraction was also used to determine the cytotoxicity of MWCNT as measured by lactate dehydrogenase activity (LDH). Lactate dehydrogenase is a cytoplasmic enzyme present in most cells. A rupture of the cell membrane can cause cellular contents, including LDH, to be released to the surrounding fluid. Therefore, measuring LDH activity is an indicator of cell damage following MWCNT exposure *in vivo*. The Cobus 400 analyzer series (Roche Diagnostics, North America) quantitatively

determines the catalytic activity of LDH in non-frozen samples by measuring the reduction of pyruvate to form L-lactate in the presence of NADH.

The cellular BALF fractions from both the first and second lavage were combined in a total of 800 μ L of USP grade PBS. Total cell number was measured using a hemocytometer. A hemocytometer is a chamber, that when loaded, allows an individual to tally the number of cells in a precise volume of fluid. Using this method, the concentration of cells in the total fluid was calculated to determine the total number of cells harvested by BAL. The reconstituted cellular fraction of the BALF was loaded into funnels for the ThermoScientific Cytospin 4 (Fisherbrand-Colorfrost Plus Microscope Slides-Precleaned) and spun (800 g for 5 min) onto slides. Differentials from the cellular fraction were made from counts of 300 cells per slide following staining by a modified Wright-Giemsa stain. Cells were identified as neutrophils, eosinophils, lymphocytes and macrophages. The number of each cell type was determined as the percent cell type multiplied by the total number of cells. Pulmonary inflammation is indicated by an elevation in neutrophils harvested by BAL.

4.5 Pulmonary gene expression

Lung tissues were dissected and immediately frozen in liquid nitrogen. All samples were stored at -80° C prior to analysis. RNA was isolated from pulmonary tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Pulmonary gene expression was determined by standard 96-well technology using the StepOne Plus (Applied Biosystems, Carlsbad, CA, USA) with pre-designed Assays-on-Demand TaqMan probes and primers including *Il-6* (Mm00446190_m1), *Ccl22* (Mm00436439_m1), *Cxcl2* (Mm00436450_m1), *Ccl2* (Mm00441242_m1), and *Spp1* (Mm00436767_m1) (Applied Biosystems). Using 96 well plates, RNA (1 μ g) was reverse transcribed using random hexamers (Applied Biosystems) and Superscript III (Invitrogen,

Carlsbad, CA, USA). Dilute cDNA (1/10) was used for gene expression determination. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as the endogenous reference. Relative gene expression was calculated using the comparative threshold method ($2^{-\Delta\Delta C_t}$) with vehicle-treated mice serving as the reference group (Schmittgen et al., 2008).

4.6 Particle distribution

To understand particle deposition following exposure, one hematoxylin and eosin (H&E) stained section of lung was examined at 10X magnification. Based on the relative amount of particle/size of the deposit (small, medium, large and extra-large) each focused deposition was scored individually from 1-4. Minimal depositions or small deposits were scored with a one. This included small clusters of particle-laden macrophages, or particularly small extracellular deposits of particles. Individual alveolar macrophages containing particles were difficult to view at low magnification. Scores incrementally increased with increasing deposition; a score of four included extensive deposition or extremely large deposits. This score included airways with entangled particles or extracellular aggregates. This score does not account for plugged airways in each sample. To normalize the data and compensate for different amounts of aspirated particles, burden in each lung region was expressed as a percent of the total lung burden. Percent of particle burden was tabulated for bronchioles, terminal bronchioles, alveolar ducts and alveolus. By choosing to represent particle burden as a percent of the total lung burden, it is easy to make comparisons between lung regions and understand location of particle distribution following exposure.

4.7 Histopathology

Mice were euthanized and the left lung lobe was excised and frozen, while the remaining right lung lobes were inflated with 10% buffered formalin. Following fixation, right lung sections were cut, stained with H&E and interpreted by a board certified veterinary pathologist, who was

blinded with respect to treatment group. Presented in this thesis are initial qualitative observations of histopathology with respect to AP-MW vs PC-MW. The pulmonary pathology that MWCNT can induce *in vivo* is well-established, the particular goal was to report any unique observations of effect due to polymer coatings. Quantitative measures of inflammation, granuloma formation, and fibrosis are in process.

4.8 CytoViva hyperspectral imaging

CytoViva technology integrates enhanced darkfield-based microscopy with high resolution hyperspectral imaging. Sirius Red staining consists of immersing slides in 0.1% picosirius solution, the left lung tissue stained with Sirius Red were mounted and cover slipped for use in the dark field imaging. This unique preparation helps distinguish MWCNT from normal tissue. Nanoparticles within the lung sections were imaged using a darkfield-based illumination on an Olympus BX-41 microscope (CytoViva, Auburn, AL) at 100X oil immersion. The combination of the CytoViva hyperspectral imaging and CytoViva microscope system has been used previously to quantify the presence of MWCNT in tissues (Mercer et al., 2013). Staining of extracellular matrix components helps to understand tissue remodeling and connective tissue pathologies (Mercer et al. 2011, Lattouf et al., 2014). This technique enhances the contrast between biological tissue and MWCNT (Mercer et al., 2011). Carbon nanotubes have properties that allow them to scatter light very differently than biologic tissues or mounting medium. These materials scatter light to a greater extent than surrounding tissue, thus nanomaterials in a given section stand out as bright white structures. Using this method, lung sections can be assessed through low magnification to identify areas of CNT in tissue that would not normally be detected by other traditional light microscopy techniques (Mercer et al., 2011).

5.0 Statistics

Data from rodent studies were log transformed prior to analysis to meet the assumptions of the statistical tests. Analysis of Variance (ANOVA) was done using JMP 11 with a Least Squares Means table by Student's t-test as a post-hoc test. Pairwise comparisons were performed using Student's t-test. Differences between experimental groups were considered significant with p-values less than 0.05.

6.0 Chapter 1: Company 1

6.1 Introduction

Manufactured MWCNT have the attention of physical chemists and scientists because of their vast potential for industrial application. However, the occupational information regarding the health impacts from handling these materials along their life cycle, is lacking. The first aim of this project involved characterizing, preparing and exposing mice to an as-produced, branched MWCNT (AP-MW) or the post-processed version, a polyurethane coated AP-MW (PC-MW). Company 1 utilizes a unique proprietary process to create the branched MWCNT, which are then coated with polyurethane in order to ease handling and aide in composite manufacturing. The pulmonary toxicities of these materials remain unknown. When PC-MW are added to polymer matrices (e.g. thermoplastics), the end result is a product with enhanced properties such as increased strength and conductivity. This company offers a MWCNT-infused product that is highly sought out by automotive, aviation, and construction industries.

The branched AP-MW is produced, utilized, and handled on a daily basis by facility workers. Figure 5 shows the type of material these workers have been exposed to during normal working hours. The material from Company 1 exists in a tangled web-like form, comprised of several hundred entangled MWCNT as seen in personal breathing zone (PBZ) collections. A goal of this study was to prepare the materials for animal studies that closely mimicked the PBZ samples (Figure 5). With that in mind, toxicological responses following *in vivo* exposures, can be directly correlated to human health exposures.

Figure 5: Representative TEM images of MWCNT. Images obtained from personal breathing zone sampling from facilities (left panels) mirrored samples prepared in dispersion medium for *in vivo* exposures (right panel). Facility relevant exposures are crucial when extrapolating the effects of *in vivo* studies to human health relevance.

Following bulk synthesis, the branched AP-MW are packaged by facility workers and transported to an offsite facility for post-production modifications. Company 1 selected to coat their base material, the branched AP-MW, with a thin layer of polyurethane. This company chose to utilize a polyurethane coating in order to reduce the dustiness associated with dry powder handling. Polyurethane makes up 7% of the total weight of bulk material (AP-MW plus polymer) and was accounted for so that the mass of MWCNT in each AP-MW and PC-MW sample remained similar. Continuing along the occupational life cycle for MWCNT, the PC-MW are incorporated into composites. These composites are manipulated by facility workers (sanding or grinding) to generate the final product. *In vivo* exposures are ongoing to determine the toxicological impact of particles generated from sanding composites containing PC-MW.

6.2 Results

6.2.1 Characterization

Prior to *in vivo* exposures, extensive morphological characterization of AP-MW and PC-MW was evaluated. Images obtained from PBZ were utilized to compare *in vivo* material preparations (Figure 5). Material was dispersed in organic solvent and visualized with a scanning transmission electron microscope (STEM), to verify their width distribution (Figure 6). Since the polymer coating makes up 7 % of the weight AP-MW and PC-MW were weighed by mass and dispersed in physiologic dosing medium, at either 1.6 mg/mL for AP-MW or 1.6 mg/930 μ L for PC-MW. Each dimension was calculated through an average of n=200 nanotubes. Mean widths of both materials were similar, approximately 16 nanometers in diameter, regardless of coating. In

Figure 6: The diameter distribution of both particles is shown (left). STEM images confirm that the widths of both types of particles, regardless of coating, are similar.

addition to STEM images, Table 1 shows the characterization data for each material obtained for Company 1.

Table 1: Material characterization. Regardless of coating, mean widths were around 16.0 nm. However, lengths between the two materials ranged from 0.22-50.9 μm .

Material	Mean Length (μm) (SD)	Length Range (μm) (SD)	Mean Width nm (SD)	Width Range (nm)	Zeta Potential (mV)	Hydrodynamic Diameter (nm)
Company 1 AP-MW	2.08 (3.8)	0.22-50.9	16.5 (18.1)	6.3-216	-12.1 $\pm$ 0.6	771 $\pm$ 33
Company 1 PC-MW	1.71 (1.6)	0.01-8.5	16.2 (22.0)	6.3-283	-11.9 $\pm$ 0.5	676 $\pm$ 8

The branched AP-MW from Company 1 is slightly longer with relatively the same mean width as the PC-MW. Both materials obtained from Company 1 agglomerated easily in solution (-11 to -12 mV). XPS also confirmed that a high degree of oxygen molecules were bound to PC-MW in comparison to AP-MW (Figure 7) indicating the polyurethane coating. Endotoxin levels

were below the level of detection (data not shown) and the percent weight of metal catalyst (1.7% iron) was similar between the AP-MW and PC-MW. Following characterization, animals were exposed to either: physiologic dosing medium (control), 4 μg , or 40 μg of AP-MW or PC-MW

(Figure 4). The lowest dose (4 μ g) equates to a cumulative deposited dose of exposures seen in evaluated workplaces to date (about seven years) (Erdely et al., 2013). In fact, this dose was calculated based on information gained from an exposure assessment study of workers exposed to elemental carbon at 10.6 μ g/m³ over an 8 hour day. The high dose (40 μ g) was chosen to understand pathological outcomes.

6.2.2 Company 1: Pulmonary cytotoxicity, inflammatory cell influx and protein expression

Following exposure, both AP-MW and PC-MW elicited pulmonary cytotoxicity, inflammatory cell influx and increases in mRNA expression of proinflammatory cytokines, which was time-and dose-dependent (Figure 8-10).

Figure 9: Polymorphonuclear leukocyte (PMN) inflammatory cell influx following exposure to AP-MW or PC-MW. Inflammatory cells peaked at 7d post-exposure but return to baseline by 84d. No significant difference was seen between AP-MW and PC-MW exposure groups. PC-MW did not increase inflammatory cell influx compared to AP-MW. N=6 per group. *p < 0.05 vs DM.

Company 1 elicited cytotoxicity, measured by lactate dehydrogenase activity, which was significantly different from control and resolved with time. The high dose of PC-MW was not significantly different from AP-MW (Figure 8). An increase in total cells, polymorphonuclear leukocytes (PMN) (neutrophils and eosinophils), and alveolar macrophages (AM) was observed in high dose exposures but no difference was observed as a result of coating over time (Figure 9, 10, 11). The influx of PMN was highest at 4 hr post-exposure and resolved by 84 d. Total cells, a result of increased macrophages, peaked at 7 d post-exposure. The lowest dose (4 μ g), regardless of coating, did not result in any significant PMN cell influx.

Figure 10: Total alveolar macrophages (AM) following exposure to AP-MW or PC-MW. AM from high dose exposures peaked at 7 d and resolved over time. At 4 hr, PC-MW was significantly different from AP-MW at similar dose. N=6 per group. *p < 0.05 vs DM, #p < 0.05 vs AP-MW of similar dose.

Figure 11: Total Cells following exposures to AP-MW or PC-MW. Increased total cell influx was noticeable in the high dose exposure groups compared to sham and peaked at 7 d post-exposure. PC-MW exposed groups were not significant from AP-MW at a similar dose, except at 4 hr post-exposure. N=6 per group. *p < 0.05 vs DM, #p < 0.05 vs AP-MW of similar dose.

Figure 12: Attempted engulfment of material by alveolar macrophages (AM) at 1 d is shown (AP-MW- left; PC-MW – middle and right). All particles remained as web-like aggregates *in vivo*.

Figure 12 shows isolated alveolar macrophages (AM) in direct contact with both AP-MW and PC-MW at 1 d post-exposure. Large entanglements of AP-MW and PC-MW made it difficult for AM engulfment. AP-MW and PC-MW continued to exist as tangled clusters following exposures (Figure 12). It is possible that the AM interact with AP-MW and PC-MW and were unable to separate these large bundles. Evidence of large bundles in contact with AM confirmed that these materials remained agglomerated *in vivo*, which directly correlated with how these materials exist in the workplace (Figure 5).

These materials adversely affected inflammatory BAL proteins and gene expression in pulmonary tissue (Figure 13 and 14). Pulmonary gene expression for inflammatory mediators, including interleukin-6 (*Il-6*), were significantly different from control at 4 hr, 1 and 7 d post-exposure in low and high dose AP-MW and PC-MW exposed groups (Figure 13). Relative mRNA expression of chemokine (C-C motif) ligand 2 (*Ccl22*; also referred to as macrophage derived chemokine) was significantly different in the high dose groups at 7 d post-exposure, regardless of dose. Additionally, mRNA expression for mediators including, interleukin 1 beta (*Il-1b*), chemokine (C-X-C motif) ligand 2 (*Cxcl2*), chemokine (C-C motif) (*Ccl2*), and secreted phosphoprotein 1 (*Spp1*) increased expression and resolved over time (data not shown). Overall, the relative mRNA expression of proinflammatory cytokines in PC-MW exposed groups was not significantly different from AP-MW.

A multiplex protein profile was generated to understand the regulation of inflammatory proteins from BALF (Figure 14). BAL proteins were analyzed with rodent multi-analytic profile (rodent MAP v3.1, Myriad, RBM).

Figure 14: Heat map of inflammatory proteins at 1 d and 28 d post-exposure. Significant downregulation is represented green, while red is significant of up regulation (Log₂ fold change). Time-and dose-dependent responses were observed. Additionally, polymer coating from Company 1 had no additive effect on BAL inflammatory protein abundance.

The rodent MAP analyzed fifty-two proteins within a sample of BALF. A time- and dose-dependent response of BAL inflammatory proteins was noted. In addition, the abundance of proinflammatory cytokines (*Il-6* and *Ccl22*) were upregulated at the high doses of AP-MW and PC-MW (Figure 14); these data complemented the increase of relative mRNA expression in pulmonary tissue. Areas of red color correlate with an increase in protein abundance ($\log_2$ fold change) while green was indicative of down regulation, with corresponding gradients in between. Based on the heat map, the PC-MW had similar effects on BALF protein levels when compared to the AP-MW of Company 1, which resolved by 28 d post-exposure.

6.2.3 Histopathology observations

Tissues from mice were harvested at 1 and 84 d post-exposure for pulmonary pathology. At 84 d post-exposure, both AP-MW and PC-MW deposited in pulmonary tissue as large extracellular aggregates within the airways (Figure 15). In particular, the high dose of AP-MW was found primarily within the bronchioles, entwined with resident cells. Mild neutrophilic inflammation was seen in the bronchioles of high dose exposure groups.

Figure 15. Histopathology of lung sections from exposed mice. For both materials, the majority of particles were seen within the lumen of small bronchioles. Qualitative observation confirmed particle location within nodular aggregates of inflammatory cells (black arrows).

Figure 16: Representative pulmonary tissue 84d post-exposure (0.1% picosirius red staining; nuclei counterstained with hematoxylin and eosin (H&E)). Both AP-MW (left) and PC-MW (right) were persistent in pulmonary tissue as clusters (>20 microns) in airways. Minimal to no alveolar fibrosis was observed.

Sirius Red staining confirmed the deposition of AP-MW and PC-MW in bronchioles at 84 d (Figure 16) and enhanced dark field microscopy validated this finding (Figure 17). The deposition of nanotubes was clearly seen in enhanced dark field imaging as the bright white regions within the bronchiolar space. Taken together, the images from both Sirius Red staining and enhanced dark-field imaging show that there were little signs of a fibrotic response and most particles deposited in the bronchioles were cleared by 84 d post-exposure. The effects of PC-MW exposure was not significantly different compared to AP-MW exposed groups.

Figure 17: Enhanced darkfield imaging of pulmonary tissue 1d post-exposure. Initial distribution of particle deposition in the lungs were similar between AP-MW (left) and PC-MW (right). Large dense clumps (>20 microns) were concentrated in small airways and alveolar regions proximal to the terminal bronchiole. (Oil immersion 100X).

For the AP-MW exposed groups, deposited particles were observed primarily in the bronchioles and were mostly cleared by 84 d (Figure 18). The fraction of AP-MW and PC-MW that deposited in alveolar space was persistent, when comparing 1 and 84 d post-exposure.

6.3 Discussion

Overall, both the AP-MW and PC-MW from Company 1 induced pulmonary cytotoxicity, increased inflammation and induced pathology. The response to these materials elicited a time- and dose-dependent response of pro-inflammatory cytokines as well as increased inflammatory protein abundance in BAL. The PC-MW did not increase the pulmonary toxicity compared to the base material. Pulmonary responses between both AP-MW and PC-MW groups were generally

similar including altered pathology. The material was comprised of web-like bundles and continued to exist in this form following exposure (Figure 5, 12, 15, 16). The results from these exposures correlated with previous rodent models in which exposures to MWCNT caused pulmonary inflammation, as well as a foreign body response to larger agglomerates (Ma-Hock et al., 2009; Pauluhn et al., 2010; Mercer et al., 20113a). Qualitative assessments affirmed primary particle deposition within the bronchioles and was mostly cleared by 84 d post-exposure.

Company 1 chose to coat their branched AP-MW with polyurethane (PC-MW) for various reasons. This includes, increased dispersion into composites and thermoplastics as well as increased the ease of handling. When incorporated into composites, the PC-MW have a high elastic modulus (stiffness) which is advantageous for structural material development. Another reason is to alter the density and organize PC-MW into a uniform network. This technique aides compounding processes to create multifunctional thermoplastics. Most polyurethanes are thermosetting polymers that do not melt when heated (Xiong et al., 2006; Wohlleben et al., 2013). As a result of this mixture, the coating exploits the inherit natures of MWCNT to dissipate heat. It has been shown that composites with 0.5-1% weight of MWCNT increased thermal conductivity by 60% when compared to plastics alone (Zhao et al., 2011). For this reason, 3% PC-MW by weight in composites is utilized by this particular company. More recently, the manufacturing of thermoplastics has become extremely efficient. Smaller quantities of PC-MW are needed to maintain the same level of thermal enhancement.

While incorporation of PC-MW has many manufacturing benefits, dry powdered AP-MW can present adverse health effects to workers handling this material. Dahm et al. (2011), reported that the highest amount of exposures to elemental carbon occurred during tasks of dry powder handling at facilities manufacturing CNT. The addition of polyurethane to AP-MW provides

additional occupational benefits as the “highly-entangled” PC-MW prevent individual MWCNTs from being released thereby reducing the risk to workers. The potential inhalation exposure is further diminished when smaller quantities of PC-MW are needed for composite manufacture (e.g. thermoplastics), which reduces the amount of dry powder handling done by facility workers. The company claims that PC-MW are easier to handle, presumably by the reduced dustiness, and this benefits packaging processes. It is clear that PC-MW infused products can provide new opportunities in consumer products as well as benefiting workers by reducing the potential for occupational exposures (Wohlleben et al., 2013).

Qualitative evaluations of lung sections from AP-MW and PC-MW exposed groups revealed adverse effects within the bronchioles (Figure 15, 16, and 17). These effects were similar for AP-MW and PC-MW. Particle deposition was primarily confined to the upper airways. Of the particles that were deposited in the bronchioles (AP-MW or PC-MW), most had been cleared from pulmonary tissue by 84 d post-exposure (Figure 18). Qualitative observations of alveolar fibrosis and collagen formation at 84 d post-exposure were absent likely due to the fact that most of this material was deposited in the upper airways and was subsequently cleared (Figure 16 and 17). Though the results above were qualitative, similar histopathological studies were described previously using another type of MWCNT (Mercer et al. in 2011). Following exposure to MWCNT 56 d post-aspiration, a significant interstitial increase of collagen fibers at high doses (40-80 μg) was observed. A dose-responsive increase in average thickness of alveolar connective tissue at 56 d post was noted. They concluded that a lower accumulation of MWCNT in the alveolar interstitium resulted in a lower fibrotic related response. Based on the results from Mercer et al. (2011), it is likely that quantitative assessments would reveal an absence of a fibrotic reaction at later time points (28 and 84 d) due to lower alveolar deposition (Figure 18).

Of the particles that remained in the lung at 84 d post-exposure, several (AP-MW or PC-MW) particles were contained in nodular agglomerates of inflammatory cells within the lumen of small bronchioles (Figure 15- black arrows). No significant differences were observed when comparing the AP-MW and PC-MW groups. Since most material was cleared from the upper airways, the presence of granuloma formation in alveolar regions was reduced due to low material persistence. In 2011, Mercer et al. explained that the occurrence of MWCNT in the airways at 7, 28 or 56 days post-aspiration was minimal (Mercer et al., 2011). In that same study, granulomatous lesions in alveolar airspace accounted for only 20% of the total lung burden. At similar mass doses of 40 µg, granulomatous lesions were relatively rare but, when present, were located near alveolar regions close to terminal bronchioles. If deposits of MWCNT in airspaces are not effectively cleared, a network of highly rich connective tissue can form an outgrowth in order to separate these poorly cleared particles. This type of response is observed in lungs where insoluble and/or particulate matter is poorly cleared (Mercer et al., 2011).

A large occupational exposure assessment study conducted in 2015 was able to quantify both the inhalable and respirable fractions of EC in a number of facilities producing CNT (Dahm et al., 2015). It was determined that the AP-MW from Company 1 has a respirable fraction of 3% (Dahm et al., 2015). If the percent deposition *in vivo* is correlated to occupational health, only a small fraction of that 3% would reach the alveolar region. Therefore, if a worker were exposed during normal working hours, it is likely that most material would deposit in upper bronchioles, with high probability of clearance according to our results. Ongoing assessments must consider the impact of both inhalable and respirable sized particles on occupational health.

7.0 Chapter 2: Company 2

7.1 Introduction

The second company taking part in this study purchases bulk master batches of MWCNT from a primary manufacturer. The base material is coated with a proprietary poly(arylene ethynylene) (PAE) polymer (Figure 2). This proprietary coating makes up 15% of the total weight after being processed (AP-MW plus proprietary coating). Similar to Company 1, Company 2 has introduced innovative materials to customers in aerospace, automotive and sporting equipment industries. One of the key reasons they utilize a proprietary polymer coating is to improve the interaction between MWCNT and resin systems. Although MWCNT are incredibly strong and used in distinct applications, they aren't easily incorporated into resins because of their inherent qualities to agglomerate in solution (Pegel et al., 2008). The unique PAE coating creates a non-covalent interaction between the MWCNT. Next, the incorporation of this PC-MW into a resin makes a stronger, more durable material. Additionally, this method effectively reduces the amount of base material needed to reach the same endpoints of strength and durability. While many companies use 1-5 % CNT by weight in composites, this company only utilizes 0.15%.

PBZ images obtained from facilities handling this AP-MW shows MWCNT in tight bundles and clusters. Preparations of AP-MW closely mimics workplace exposures (Figure 19). Each exposure was identical to the methods carried out for Company 1 (Figure 3 and 4). It was hypothesized that PC-MW would alter toxicity compared to AP-MW, *in vivo*. Following time- and dose-dependent exposures, it was verified that exposures to PC-MW does in fact alter pulmonary toxicity in comparison to AP-MW *in vivo*.

7.2 Results

7.2.1 Characterization

Representative PBZ TEM images of material obtained from Company 2 detail characteristics of this material. When in dispersion medium, this material tends to form small clusters around 500 nm (Figure 19). In addition, both particles (AP-MW or PC-MW) remain similar in their diameter distribution (16 nm) and STEM images confirmed that polymer coating does not increase particle diameter (Figure 20). AP-MW are longer in length and higher in hydrodynamic diameter compared to PC-MW (Table 2). The surface chemistry of the PC-MW is different than AP-MW and analysis by XPS confirms a higher amount of oxygen bound to PC-MW compared to AP-MW, confirming the polymer coating (Figure 21). The catalytic metals were similar (2% aluminum and 1.7% iron) and endotoxin was below the level of detection (data not shown).

Table 2: Particle characterization for AP-MW and PC-MW. Mean widths of all materials remain similar, regardless of polymer coatings while hydrodynamic diameter is altered in PC-MW.

Material	Mean Length (μm) (SD)	Length Range (μm) (SD)	Mean Width nm (SD)	Width Range (nm)	Zeta Potential (mV)	Hydrodynamic Diameter (nm)
Company 2 AP-MW	1.28 (1.1)	0.10-8.51	25.5 (34.9)	7.8-275	-11.1±0.6	608±30
Company 2 PC-MW	0.77 (0.4)	0.15-2.44	15.2 (3.50)	8.6-32.0	-10.7±0.4	360±12

7.2.2 Cytotoxicity, pulmonary inflammation and protein analysis

Both materials were cytotoxic and induced an inflammatory cell influx compared to control groups (Figure 22-25). Cytotoxicity was attenuated following PC-MW exposure and resolved over time (Figure 22). No significant effect was observed for the low dose groups. Total PMN influx was increased at 4 hr, and 1 d post-exposure, and both high dose groups were significantly different from sham. By day 7, only the AP-MW exposed groups was significant. The high dose AP-MW groups at 4 hr post-exposure, was significantly different than the PC-MW at the same dose (Figure 23). Likewise, total cell and alveolar macrophage influx were attenuated in the high dose PC-MW groups at 7 and 84 d post-exposure (Figure 24 and 25). Alveolar macrophages were observed engulfing particles of AP-MW and PC-MW (Figure 26).

Figure 22: Cytotoxicity of AP-MW and PC-MW as measured by lactate dehydrogenase activity over 4 hr, 1d, 7d, 28, and 84d post-exposure. All materials were cytotoxic; however, differences are seen in PC-MW exposed groups compared to similar dose of AP-MW. N=6 per group.*p < 0.05 vs DM, #p < 0.05 vs AP-MW of similar dose.

Figure 23: Polymorphonuclear leukocyte (PMN) Inflammatory cell influx following exposure to AP-MW or PC-MW. Inflammatory cells peaked at 7d post-exposure to PC-MW, but return to baseline by 84d. A significant difference was seen between AP-MW and PC-MW exposure groups at 4 hr post-exposure. PC-MW did not increase inflammatory cell influx compared to AP-MW. N=6 per group.*p < 0.05 vs DM, #p < 0.05 vs AP-MW of similar dose.

Figure 24: Total alveolar macrophages following exposure to AP-MW and PC-MW. High dose exposures caused the greatest increase in alveolar macrophages at 7 d with significance in AP-MW exposed groups.*p < 0.05 vs DM.

Figure 25: Total pulmonary cell influx following exposures to AP-MW and PC-MW over time. As expected, high dose exposure groups elicited the greatest responses at 7 d post-exposure and PC-MW was significantly different than AP-MW at similar dose at this time point.*p < 0.05 vs DM, #p < 0.05 vs AP-MW of similar dose.

MRNA for inflammatory cytokines (*Il-6* and *Ccl22*) were significantly increased from control in the high dose AP-MW and PC-MW exposed groups (Figure 27). Similar results were found for relative mRNA expression of *Il-1b*, *Cxcl2*, *Ccl2* and *Spp1* (data not shown). At 1 and 7 d post-exposure, relative mRNA expression for these cytokines were attenuated in the high dose PC-MW groups compared to AP-MW (Figure 27). In addition to relative mRNA expression, BAL inflammatory protein levels were elevated at 1 d post-exposure with attenuation by 28 d post-exposure. At 1 d post-exposure, pro-inflammatory proteins, in general, associated with PC-MW exposure were lower compared to the AP-MW, at the same dose (Figure 28). Together, relative mRNA expression was time-and dose-dependent and complementary to BAL protein regulation (Figure 27 and 28).

Figure 26: Alveolar macropages (AM) successfully engulfing AP-MW and PC-MW. Often particles were seen as dense clumps within AM (AP-MW-left and middle; PC-MW- right).

Figure 28: BAL inflammatory protein regulation at 1 and 28 d post-exposure to AP-MW and PC-MW. Time- and dose-dependent responses were observed for all materials. Overall, BAL inflammatory proteins were altered in PC-MW groups.

Following exposure, inflammation, granuloma formation and particle distribution were evaluated in pulmonary tissue samples. Clusters of AP-MW and PC-MW were located around terminal bronchioles (Figure 29). Alveolar fibrosis was qualitatively analyzed and no pronounced increase was observed (Figure 30). At 84 d post-exposure, small clumps of AP-MW were found in macrophages in regions of alveolar airspace.

Figure 29: Qualitative pathological evaluation showed majority of AP-MW (left) near terminal bronchioles. In contrast, PC-MW (right) were seen as large aggregates and do not elicit the same pathological consequences consistent with AP-MW exposed lungs.

Figure 30: Representative pulmonary tissue 84d post-exposure (0.1% picosirius red staining; nuclei counterstained with hematoxylin and eosin (H&E)). Images of collagen fibers in AP-MW (left) and PC-MW (right) exposed lungs at 84 d post-exposure. Sections of alveolar regions laden with AP-MW (left) and minimal collagen fiber formation.

Figure 31: Enhanced darkfield microscopy of AP-MW at 24 hr (left) and 84 d (right) post-exposure. Large particles were seen deposited at bronchiole to alveolar duct junctions (left) while 84 d post-exposure particles were viewed inside alveolar macrophages (right) (oil immersion 100X).

Figure 32: Particles mostly deposited both the bronchiole and alveolar regions. Particles that remained at 84 d exhibited persistence in alveolar regions. The fraction deposited in the bronchiolar regions were effectively cleared.

Mild inflammation of neutrophils was associated around particle deposits. In contrast to AP-MW, fewer particles of PC-MW were present within alveolar macrophages. Some of the material existed as extracellular aggregates and lacked associated inflammatory cells (Figure 29). Granuloma formation was apparent in AP-MW groups at 84 d (Figure 29 - left) but absent in PC-MW groups (Figure 29 – right). High dose exposures were associated with particle deposition around terminal bronchioles and alveolar ducts as seen in EDM (Figure 31). Minimal to no inflammation was noted and particle abundance of PC-MW was significantly less than the AP-MW.

Biodistribution of particles was assessed to understand deposition in the bronchiole and alveolar regions. Depositions of both particles were observed in the bronchiole and alveolar space. Of the total percent deposited at 1 d post-exposure, the high dose of AP-MW and PC-MW mainly deposited in alveolar regions (both 75%). At 84 d post-exposure, the largest fraction of the total deposition remained in the alveolar space for both AP-MW and PC-MW. Particle clearance occurred in the bronchioles while the remaining percent deposition in alveolar regions persisted over time (Figure 32).

7.3 Discussion

A purchased bulk supply of MWCNT was coated with a proprietary PAE polymer in order to enhance dispersion for composite material application. Overall, exposures to AP-MW and PC-MW induced time- and dose-dependent responses of pulmonary cytotoxicity, inflammation, and increased inflammatory protein abundance in BAL compared to sham treated mice. Based on results, the pulmonary toxicity of PC-MW exposed groups was attenuated in comparison to AP-MW treated groups. Primary particle deposition occurred within the alveolar regions which persisted at 84 d post-exposure. Qualitative observations of pathology indicated AP-MW exposed

groups exhibit signs of granuloma formation, whereas pulmonary tissue from PC-MW exposed groups do not. This was consistent with the hypothesis that PC-MW decreases toxicity compared to the respective AP-MW (Figure 29).

Company 2 recognized the benefits of using MWCNT in composite material because of their high mechanical strength and high electrical and thermal conductivity and was prepared to expand their division of composite materials. However, their difficult manipulation into host matrices imposed enormous limitations to their use in large-scale material production. Engineers and polymer scientists developed, a surface treatment and functionalization protocol to improve dispersion and interaction of MWCNT in various matrices. This surface treatment is their proprietary PAE polymer coating. This coating is a non-covalent, non-wrapping approach in which rigid conjugated PAE polymers adhere to the surface of the MWCNT. Side chains are added to this backbone for enhanced compatibility, increased adhesions to host matrices, and limited MWCNT induced aggregation. The major interaction between the PAE backbone and nanotube surface creates a stabilizing energy and eases their incorporation into a large array of diverse matrices for downstream products.

The toxicological evaluation of the base AP-MW was similar to previous results that demonstrated an increase in the inflammatory potential of MWCNT following pulmonary exposures *in vivo* (Warheit et al., 2004; Shvedova et al., 2005; Mercer et al., 2011). Qualitative observations of lungs exposed to the high dose AP-MW, show particle clearance, by 84 d post-exposure. AP-MW remaining in the lung at 84 days post-exposure were present in small granulomas at terminal bronchioles, or within alveolar macrophages (Figure 26, 29-31). As these are qualitative observations, numerous studies have discussed an increase in granuloma formation following similar doses of MWCNT *in vivo*. In addition, Mercer et al. (2011) reported the frequent

incidence of highly loaded alveolar macrophages in MWCNT-exposed lungs, which was consistent with our findings (Figure 26, 29-31).

Tabet et al. (2011) showed that a polystyrene-coated CNT was protective against pulmonary toxicity. In conjunction with their results, we were able to conclude that PC-MW induced reduced effects on cytotoxicity, inflammatory cell recruitment, and inflammatory protein production (Figure 22-25; 27 and 28). Enhanced darkfield imaging and qualitative assessment of pulmonary deposition confirmed primary particle deposition within the alveolar regions for both AP-MW and PC-MW exposed groups and particles persisted at 84 d post-exposure (Figure 31 and 32). However, granuloma formation was absent in the high dose PC-MW exposed groups. In fact, PC-MW particles were qualitatively reported as large aggregates located at terminal bronchioles with minimal inflammation (Figure 29). Several mechanisms may explain these results and were further supported by Tabet et al. (2011). First, the coated MWCNT contained 15% polymer by weight and may have affected the delivered dose by altering the density of total nanotube material. Therefore, the lack of a granulomas response was simply due to a reduction in total particle load to the lung. Ongoing assessments are being performed to confirm that a reduction in MWCNT mass was not responsible for absent pathology and inflammation. Pauluhn et al. (2010) reported that the potential of MWCNT particles to cause reactions within the lung were dependent upon particle size, composition, and retained dose, which lends support to the lack of pathological responses in groups exposed to PC-MW. Second, perhaps the PC-MW were more hydrophobic than their counterpart, AP-MW, which could reduce cell adherence and explain the lack of inflammation surrounding large aggregates (Figure 32) (Tabet et al., 2011). A combination of these factors may contribute to the altered responses observed in the PC-MW exposed groups and ongoing studies are evaluating this phenomena. The primary aim for utilizing a proprietary PAE

coated MWCNT was to improve dispersion into epoxy matrices, and not necessarily reduce worker exposure. However, based on the results of this study, the reduction in pulmonary toxicity associated with PC-MW may improve the occupational safety of workers handling these coated tubes.

Overall, it is clear that the proprietary PAE polymer reduced pulmonary toxicity in comparison to AP-MW exposed groups *in vivo*. Ongoing quantitative assessments are underway to confirm absent granuloma formation and reduced inflammation in pulmonary tissue of the PC-MW exposed groups. Additionally, experiments are in progress to explain the underlying biochemical mechanism for such large particle aggregation and inflammatory response (Figure 29).

8.0 General Discussion

The main findings from this study support the hypothesis that pulmonary exposures to PC-MW cause varying degrees of pulmonary toxicity when compared to the base AP-MW. In neither instance did the polymer coating increase the toxicity of MWCNT. In fact, inflammation and adverse histopathology were attenuated by the PAE coating used by Company 2. There was remarkable consistency between PBZ collections, *in vivo* dosing preparations, and material observations in alveolar macrophages isolated from BALF (Figure 5, 12, 15, 19, 26, and 29), suggesting that our general toxicity finding can be reasonably extrapolated to human health outcomes.

The BAL inflammatory cell and protein results detailed in this study qualitatively agree with other MWCNT studies. Porter et al. (2010) reported a dose response to increased polymorphonuclear leukocytes (PMN) following MWCNT exposures *in vivo* at similar doses (40 µg). Pro-inflammatory cytokines were increased at high dose exposures, and this was in agreement with previous studies involving CNT exposure (Erdely et al., 2009, 2011; Ma-Hock et al., 2013). However, different types polymer coatings, such as the proprietary coating (PAE) from Company 2, appears to attenuate inflammation and developing pathology.

It should be noted that the PAE coating from Company 2 can have any number of side chain combinations added to the backbone which makes predicting the pulmonary toxicity of other PAE polymer-coated MWCNT difficult. From a dosimetry perspective, the low dose caused some inflammation that was acute, while the high dose caused significant inflammation that resolved with altered histopathology. The low dose was extrapolated to represent 7.6 years and the high dose, 76 years, of an average exposure to workers in a U.S. facility (Erdely et al., 2013). The first study of humans exposed to MWCNT, from a single facility, measured increased markers of

oxidative stress in inhaled breath condensates (Lee et al., 2015). These workers had a similar exposure compared to U.S. facilities with an average time of employment of four years. The general results (cytotoxicity, pulmonary inflammation) of this current study was consistent with past outcomes of inflammation *in vivo* and extrapolations to the general workforce.

How does the polymer coating from company 2 attenuate toxicity? It was observed from the histopathology that this particular material, once coated, tends to form a few large agglomerations in the conducting airways, unlike the uncoated counterpart. These large agglomerates seemed to be devoid of surrounding inflammatory cells. The formation of agglomerates would theoretically reduce the amount taken up by macrophages. This assumption would be consistent with a previous study showing reduced macrophage uptake of PMMA-coated MWCNT (Tabot et al., 2011). The reduced uptake by macrophages and resultant lower cell accumulation result in the observed attenuated inflammatory response seen in both this study and by Tabot et al., 2013. Also, the lack of inflammatory cells surrounding the agglomerates and absence of granulomas, would suggest a decreased surface activity for PAE-coated MWCNT. Another potential contributing factor was the general appearance of reduced deposition. This is likely a product of the large agglomerates (difficult to know how much CNT was contained) but may also be a product of altered density of the coated material. Ongoing studies are looking at macrophage accumulation and whether the coating affected the density of the material.

The results of this *in vivo* study closely related to findings of field assessment studies of facilities manufacturing and handling CNT. Preparations of MWCNT that mimic workplace exposures are paramount for occupationally-relevant interpretations (Figure 5 and 19). Our nanomaterial preparation and degree of dispersion mimics PBZ measurements from exposure assessment studies conducted in the U.S. (Dahm et al., 2015). Most biological studies take

considerable measures to disperse tangled MWCNT to produce a respirable material. The question can be raised whether the generated material is representative of human exposures or if the toxicological findings are the product of a different material structure size. To date, our research group is the only one to integrate exposure and toxicity assessments. This integration provides human health context to the toxicological outcomes.

Despite the potential attenuation of toxicity due to some polymer coatings of MWCNT (this study and Tabet et al., 2011), caution should be taken in workplaces that handle large quantities of AP-MW and PC-MW. The scientific community relies heavily on animal models to evaluate results from exposures and assess their impact on the working population, as epidemiological studies in the past had not been conducted (Birch et al., 2011). Future toxicological studies involving PC-MW must incorporate extensive chemical characterizations, paired with *in vivo* and *in vitro* studies, in order to make predictions of their impact on human health (Oberdorster et al. 2016; Simeonova et al., 2009). From the results of this study and other toxicological evaluations *in vivo*, pulmonary exposures to PC-MW have the potential to induce significant toxicity and should not be assumed as non-toxic.

An area of emphasis not currently addressed was the toxicologic impact of PC-MW following their addition into thermoplastics or composite material. Compared to composite material alone, these nano-enabled composites are more resistant to strain and have greater tensile strength (Cena et al., 2011). This study has addressed the toxicity of CNT in the first two stages along their life cycle from AP-MW synthesis, and post-processing (e.g. polymer coating). However, the next step of manufacturing involves incorporating the PC-MW into a polymer matrix (e.g. epoxy resin) and mechanically processing the PC-MW embedded composites. These processing steps include sanding or grinding activities, another task of manufacturing with risk of

inhalation exposure to particulates (Dahm et al., 2015). However, it remains unknown if the particulates generated as a result of sanding the PC-MW nanocomposites pose a toxicological risk to workers manipulating these materials. Additionally, it is unknown how different types of polymer coatings, like the ones addressed here, affect particulate generation. Ongoing studies are characterizing the aerosol generated following an industrial sanding process and complementary *in vivo* studies are addressing the toxicological impact of the respirable fraction generated from composites containing the PC-MW. To date, there has been no indication that free PC-MW are released following sanding of the composites. In support, previous studies that processed CNT-nanocomposites reported that respirable sized particles are generated but generally lack the presence of free nanoparticles (Cena et al., 2011). Also, it was concluded the probability of adverse health effects due to the inhalation of free PC-MW from sanded particulates generated from composites remains extremely low (Wohlleben et al., 2011). To date, no studies have shown enhanced toxicity from particulates collected from CNT-enabled composites compared to particles collected from the base composite with no CNT added.

The issue of health and wellness is not new to manufacturers of engineered nanomaterials, many of whom, have introduced engineering controls to reduce worker exposure. There are tasks within any workforce where individuals face a host of challenges that could result in reduced stamina, flexibility and strength. Considering all the variables to maintain optimal worker health, the easiest to control is exposure by preventative measures such as ventilation, filtration and enclosure. By understanding the toxicity of CNT along their life cycle, from AP-MW to PC-MW, companies like evaluated here, can use this information to establish protocols that reduce worker exposure and toxicity, while continuing to make advancements in nano-enabled products.

9.0 References

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