CARBON NANOTUBES: CARBON-14 LABELING AND ECOLOGICAL AVAILABILITY

by

Elijah Joel Petersen

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Doctoral Committee:

Professor Walter J. Weber, Jr., Chair
Professor Gordon L. Amidon
Professor Kim F. Hayes
Associate Professor Nicholas Kotov

To all sentient beings

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Abstract

Carbon nanotubes comprise a class of nanomaterials having demonstrated promise for broad ranges of potential applications. The ecological and human health risks these nanomaterials may pose after release into environmental systems, however, are yet largely unknown.

The lack of an adequate method for quantifying carbon nanotubes in environmental media has been a principal challenge associated with determining their environmental behaviors. To address this problem, a modified chemical vapor deposition process employing carbon-14 labeled methane was used to synthesize radioactively labeled single- and multi-walled carbon nanotubes (SWNTs and MWNTs). These nanotubes were used to show that HeLa cells rapidly and apparently irreversibly assimilated unmodified MWNTs.

Given these results, previous qualitative detections of nanotubes in ecological receptors, and shared chemical properties with polycyclic aromatic hydrocarbons (PAHs), the bioaccumulation potential of nanotubes was investigated. The labeled nanotubes and a representative PAH, pyrene, were individually spiked to identical sediment and soil samples. The uptake and depuration behaviors of these compounds by the earthworm *Eisenia foetida* and the oligochaete *Lumbriculus variegatus*, potential entry points to

terrestrial and aquatic food chains, were then assessed. Bioaccumulation values determined for the nanotubes were almost two orders of magnitude smaller than those measured for pyrene, indicating that purified nanotubes, unlike pyrene, are not readily absorbed into organisms.

Carbon nanotubes are also commonly physically and chemically altered, and these modifications can change their physicochemical properties and possibly also their environmental behaviors. Purified MWNTs were treated with a 3:1 mixture of sulfuric to nitric acid, a process that made the nanotubes more hydrophilic. These nanotubes were similarly spiked to soils and sediments, but their ecological uptake was determined to be the same as that for the unmodified nanotubes.

The octanol-water distribution coefficient, k_{ow}, represents a chemical property known to relate to bioaccumulation and is frequently employed for predictions thereof. A modified shake-flask method was used to measure the distributions of purified and 3:1 acid modified MWNTs between water and octanol. While their bioaccumulation behaviors were similar, different distribution coefficients were found for these nanotubes thus suggesting that, unlike typical hydrophobic organic chemicals, k_{ow} coefficients may not predict such behaviors for nanotubes.

Chapter 1

INTRODUCTION AND BACKGROUND

1.1 Background

1.1.1 Overview

Carbon nanotubes (CNTs), first discovered by Iijima in 1991 (Iijima 1991), comprise one of the most promising classes of new materials to emerge from nanotechnology to date. Two principal types of CNTs have been fabricated. Single-walled carbon nanotubes (SWNTs) are one-layered graphitic cylinders having diameters on the order of a few nanometers, and multi-walled carbon nanotubes (MWNTs) comprise between 2 to 30 concentric cylinders having outer diameters commonly between 30-50 nm (see Figure 1.1). Their unique structures endow them with exceptional material properties with respect to electrical and thermal conductivity, strength, and high surface-to-mass ratios. These characteristics in turn make them suitable for numerous potential applications, including uses in composite materials, sensors, and hydrogen-storage fuel cells (Dillon et al. 1997; Dalton et al. 2003; Snow et al. 2005). A number of these applications have reached or are approaching their respective commercialization phases.

CNT research has been driven to date by potential applications, and extensive information regarding their relevant electrical, thermal and mechanical properties has been forthcoming. Their potential health and environmental impacts, on the other hand, have not been similarly characterized, and the risks they pose to the welfare of humankind and the environment are not well understood (Colvin 2003). If even a small fraction of their potential applications are realized, it is inevitable that they will enter such human and ecologically critical environments and media as the water we drink and the food we eat. The materials comprising the pure nanotubes may themselves pose environmental or human health risks, or they may act as adsorbents, concentrators, and durable sources and carriers of various environmental contaminants (Yang et al. 2006b). This research initiates an assessment of the extent to which SWNTs or MWNTs released into environmental systems may bioaccumulate in human and ecological receptors.

1.1.2 Novel Considerations Related to The Potential Environmental and Human Health Implications of Nanomaterials

Nanomaterials have been defined as particles possessing one characteristic dimension less than 100 nm. As described above for carbon nanotubes, materials on this scale possess surprising new properties that have given rise to numerous applications in a broad range of fields. Materials on this size scale, however, may also pose unique environmental and human health risks. Few new technologies have been without deleterious impacts

and nanotechnology is unlikely to be different (Colvin 2003; Masciangioli and Zhang 2003; Oberdorster et al. 2005; Wiesner et al. 2006).

A principal challenge for determining the potential ecological and human health risks posed by nanoparticles is the numerous differences between them and typical environmental pollutants, differences that may substantially limit the application of common environmental risk and/or fate and distribution models for nanomaterials. One such difference is the size of nanomaterials. Unlike most contaminants, nanoparticles are within the size range of many cellular organelles. If such materials are able to enter cells, as has been demonstrated for many types of nanoparticles (Scrivens et al. 1994; Marinakos et al. 2001; Jaiswal et al. 2003; El-Sayed et al. 2005; Kirchner et al. 2005), they could potentially alter cellular functioning in novel positive or negative ways. Also unlike common environmental pollutants, nanoparticles are known to agglomerate, and in this form may pose exacerbated or mitigated risks. The most striking example of this phenomenon has been observed with fullerene particles. After treatments similar to those that they could experience in environmental systems, some fullerene particles formed aggregates, often known as nC_{60}, that are cytotoxic at concentrations seven orders of magnitude less than other slightly modified fullerene particles (Sayes et al. 2004; Brant et al. 2005). Another difference between nanomaterials and most contaminants relates to surface coatings or functionalization of the nanomaterials. Carbon nanotubes, for example, have been solubilized/dispersed by a wide range of polymers, surfactants, and

macromolecules (O'Connell et al. 2002; Zheng et al. 2003; Sinani et al. 2005) and also by chemical treatments such as addition of functional groups to the nanotubes or shortening them with acid treatments (Liu et al. 1998; Ziegler et al. 2005; Kostarelos et al. 2007). Furthermore, nanotubes may also interact with compounds ubiquitous in environmental systems, such as naturally occurring organic matter (Hyung et al. 2007). As such, nanotubes modified with these different methodologies or impacted after their release into environmental systems could manifest distinctly different environmental behaviors. Some types of nanoparticles, such as carbon nanotubes, also differ from typical hydrophobic organic chemicals (HOCs) in that they are polydisperse; e.g., regardless of the synthesis procedure employed, carbon nanotubes vary widely in length and diameter each combination of which may dramatically or subtly influence their environmental behaviors. In this regard, CNTs more closely resemble natural organic matter than HOCs.

Lastly, the desorption behaviors of nanomaterials may differ from those of typical hydrophobic organic chemicals. After varying time periods, an equilibrium can be approached for organic chemicals between soils or sediments and water, a result that stems from the chemicals' ability to transfer between these phases. In other words, the attachment of the organic chemicals to the organic carbon fractions of the soil or sediments is to some extent reversible. For nanoparticles such as carbon nanotubes that typically require sonication prior to dispersal in aqueous solutions, it is unlikely that nanotubes could readily transfer back into the aqueous phase after sorption.

It is possible, however, that bioturbation or certain hydrodynamic conditions in water bodies could lead to mixing which could then resuspend the nanotubes. Alternatively, interactions with natural organic matter as described above could change the nanotube properties and cause their dispersal into aqueous systems. Even if nanotubes were not resuspended, nanotubes may be released from the soil or sediment particles in the guts of ecological receptors or the gastrointestinal tracts of humans after uptake and then be available for absorption into the organisms. The extent to which these various scenarios would influence the fate of nanotubes is yet unknown.

Given these significant potential and proven differences between the behaviors of nanoparticles and typical environmental pollutants, the terminology used to describe nanomaterials is important; this nomenclature may intentionally or unintentionally suggest certain environmental behaviors for these materials which may be misleading. Caution should be exercised in describing nanomaterials using terms such as molecules, macromolecules, or chemicals, as these terms implicitly suggest a similarity between nanomaterial behaviors and those of typical organic or inorganic pollutants or certain biomolecules. At the same time, the fundamental principles developed for environmental systems and processes will likely still serve as useful starting points for studying the environmental behaviors of nanomaterials, but the potential for different behaviors should be acknowledged and new paradigms developed when necessary. The most commonly used phrase in the reviews of the potential environmental and

human health risks to describe nanomaterials is “nanoparticle” (Colvin 2003; Masciangioli and Zhang 2003; Oberdorster et al. 2005; Wiesner et al. 2006). This term indicates differences between nanomaterials and typical pollutants as described above and also captures how such materials often act more similarly to particles than typical pollutants. The term particle, however, suggests certain features and behaviors in the field of pulmonary toxicology, an association that may be misleading (Colvin 2003).

1.1.3 Cellular Interactions with Carbon Nanotubes

Cellular interactions with carbon nanotubes have gained widespread research attention in recent years both with regards to using CNTs as a tool for biomedical studies (Strong et al. 2003; Cherukuri et al. 2004; Kam et al. 2004; Pantarotto et al. 2004; Barone et al. 2005; Gheith et al. 2005; Heller et al. 2005a; Singh et al. 2005; Cherukuri et al. 2006; Gheith et al. 2006; Kam et al. 2006; Singh et al. 2006; Liu et al. 2007a; Liu et al. 2007b) as well as with regards to the potential toxicological properties of carbon nanotubes (Shvedova et al. 2003; Correa-Duarte et al. 2004; Pantarotto et al. 2004; Cui et al. 2005; Ding et al. 2005; Heller et al. 2005a; Jia et al. 2005; Manna et al. 2005; Monteiro-Riviere et al. 2005; Sato et al. 2005; Bottini et al. 2006; Chen et al. 2006; Sayes et al. 2006; Smart et al. 2006; Becker et al. 2007; Kostarelos et al. 2007; Pulskamp et al. 2007; Wick et al. 2007). The toxicological literature on cellular interactions with CNTs is vast and often conflicting, with some researchers showing the biocompatibility of carbon nanotubes and others nanotubes’ cytotoxicity (Smart et al. 2006). Numerous

factors are likely involved in the toxicity of nanotubes, including the physicochemical properties of the nanotubes (Sayes et al. 2006; Becker et al. 2007) and the presence of metal catalysts in the nanotube mixtures (Shvedova et al. 2003; Kagan et al. 2006). Two factors that have not been readily characterized are the rates at which CNTs can enter cells and the masses of nanotubes that have entered the cells. Initial concentrations of carbon nanotubes dispersed in cell media are measures generally used for toxicological studies, but this approach does not readily indicate the quantity of those nanotubes that have attached to or entered the cells to cause cellular dysfunction and damage. Rates at which nanotubes can enter cells would be particularly important measures for assessing biodistributions of nanotubes in organisms. After oral ingestion of contaminated water, for example, nanotubes would be in transit through the digestive systems of organisms and the rate at which they interact with cells lining this system could partially determine their absorption into systemic circulation in the organisms.

Cherukuri et al. (2004) conducted the primary research investigation of the cellular uptake rates of CNTs. They used spectrofluorimetry to assess the uptake rates of single-walled carbon nanotubes dispersed with a noncytotoxic pluronic surfactant by mouse macrophage-like cells, and showed a linear increase in cellular nanotube concentrations with time. Other investigators have estimated nanotube concentrations in cells using a variety of spectroscopic methods or by bonding bulky fluorescent polymers on the nanotubes (e.g., Becker et al 2007 and Kam et al. 2006). These experimental

approaches, however, have significant limitations with regards to human and ecological toxicology and environmental investigations as discussed in section 1.1.6.

While uncertainty remains regarding the cytotoxicological properties of carbon nanotubes, many researchers have indicated that they can indeed enter cells (Cherukuri et al. 2004; Kam et al. 2004; Heller et al. 2005a; Monteiro-Riviere et al. 2005; Kam et al. 2006; Becker et al. 2007; Kostarelos et al. 2007). These nanotubes were typically modified chemically and/or bound by surfactants or biomacromolecules such as DNA. In particular, Kostarelos (2007) showed that a broad range of functionalized SWNTs and MWNTs could enter numerous types of cells including 3T6, HeLa, Jurkat human T-lymphoma, human keratinocytes, *Escherichidia coli,* and *Crpyptococcus neoformans*.

1.1.4 Interactions Between Carbon Nanotubes and Organisms

Given the apparent widespread ability for carbon nanotubes to enter cells during *in vitro* experiments, interactions between nanotubes and whole organisms becomes of increasing importance. The preponderance of *in vivo* toxicological investigations with CNTs have centered around inhalation risks (Lam et al. 2004; Warheit et al. 2004; Lam et al. 2006). This focus stems mainly from the morphological similarities between carbon nanotubes and asbestos. This research, however, does not address other potentially significant exposures pathways for ecological receptors after carbon nanotubes are released into environmental matrices, such as by oral

ingestion or dermal absorption. Under these conditions of prolonged exposure, the distribution of nanotubes within organisms and potential CNT accumulation in the organisms' fatty tissues would be of critical importance.

The biodistribution of carbon nanotubes has been studied using rats (Wang et al. 2004; Singh et al. 2006; Liu et al. 2007a) and rabbits (Cherukuri et al. 2006) to assess the applicability of CNTs for medicinal purposes. In contrast to the cellular studies showing facile cellular uptake, these researchers generally did not find significant concentrations of nanotubes in organisms after intravenous injection. One exception was for Liu et al. (2007) who found significant accumulation in the liver 24 hours after exposure, a difference they speculated to stem from the shorter length of their SWNTs. The cause of the discrepancies between these *in vivo* studies and the multitude of *in vitro* investigations showing significant cellular uptake is unclear. They are unlikely to be entirely due to nanotube length, because nanotubes having a range of lengths, diameters, and agglomeration states were used in the cellular studies. It is also uncertain how closely the behaviors of highly modified carbon nanotubes after intravenous injection would relate to the behaviors of nanotubes released into ecosystems. Organisms could possibly be exposed to CNTs in environmental settings through inhalation, dermal absorption, and oral uptake of contaminated water, soil, or food. The nanotubes in such systems could be present in either dispersed or aggregated forms and would be unlikely to possess the sophisticated surface coatings utilized in these *in vivo* experiments.

The influence of carbon nanotubes on aquatic organisms has also been investigated (Templeton et al. 2006; Cheng et al. 2007; Roberts et al. 2007; Smith et al. 2007). All of these studies indicated that CNTs exerted toxic effects on these organisms, although one investigator speculated that the metal catalysts in the mixture may have been the primary cause of the toxicity (Cheng et al. 2007). Templeton and co-workers (2006) showed that, while purified SWNTs did not cause acute or chronic toxicity, unpurified SWNTs and a fraction of shorter SWNTs did have significant toxic impacts, thus highlighting the importance that size could play in toxicological investigations. Lysophosphatidylcholine coated SWNTs were qualitatively detected in *Daphnia magna* using Raman spectroscopy (Roberts et al. 2007), a technique that can identify the presence of SWNTs but cannot give quantitative results. The presence of surfactant-stabilized SWNTs has also been identified in fish (Smith et al. 2007). These results suggest the potential for carbon nanotubes to accumulate in ecological receptors.

1.1.5 Bioaccumulation of Hydrophobic Organic Chemicals

One of the critical environmental risks associated with hydrophobic organic chemicals (HOCs) is bioaccumulation, a process by which compounds accumulate in organisms' fatty tissues with time. This phenomenon can be particularly dangerous when the chemicals are present in environmental systems at concentrations that do not cause immediate effects but do build up with time to levels that pose chronic toxicity (Neely et al. 1974). By the time these effects have been determined, it may be difficult

to limit the introduction of a compound to the environment to prevent its toxic impacts. Absorbed chemicals could also be transported throughout food chains, accumulating in organisms at higher trophic levels (i.e., humans) at increasing concentrations.

After the discovery of this phenomenon, characteristics to identify compounds that may have this potential were sought. A principal chemical property that has been linked to bioaccumulation is the octanol-water partitioning coefficient (k_{ow}) (Di Toro et al. 1991; Belfroid et al. 1996; Mackay and Fraser 2000). This coefficient represents a ratio of equilibrium concentrations of a chemical in water and octanol, a phase having chemical properties similar to those of fatty tissues. The mechanistic basis for use of this coefficient in ecosystems is equilibrium partitioning theory (Di Toro et al. 1991). This theory assumes that a compound reaches a thermodynamic equilibrium among the various phases present in a system through passive diffusion in response to chemical potential differences in those phases. This approach has a number of recognized limitations, however, including a linear sorption model and failure to account for biotransformation, chemical aging in organic soil phases, and behavioral differences among organisms (Belfroid et al. 1996).

The biota-sediment (or soil) accumulation factor (BSAF) is often used in this framework. This term represents the concentration of a compound in the organism normalized by its lipid content divided by that in a soil or sediment normalized by its organic carbon content. Although some authors

suggest a decrease in BSAF values for highly hydrophobic compounds (Thomann et al. 1992), a relationship between accumulation by organisms in terrestrial or sediment ecosystems and hydrophobicity has not been generally found (Belfroid et al. 1996). This finding can be explained by assuming a similar relative increase in affinity by the organisms' lipids and by the soil or sediment organic carbon for HOCs that possess higher octanol-water partitioning coefficients.

Although the octanol-water partitioning coefficients of carbon nanotubes have not previously been measured, the highly hydrophobic nature of nanotubes suggests they will also possess large k_{ow} values. Equilibrium partitioning behavior suggests that in the absence of sediments nanotubes would accumulate in organisms at high concentrations as a result of the greater affinity for lipid phases than for water. Numerous correlations have been formulated to link k_{ow} values, aqueous concentrations of organic chemicals, and the corresponding concentrations in fish (Neely et al. 1974; Mackay and Fraser 2000). It would thus appear tempting to use any measured partitioning coefficient of carbon nanotubes to predict their uptake by fish and other organisms. As described above, the use of equilibrium partitioning could also be used to predict the behaviors of nanotubes in systems with soils or sediments. Under these conditions nanotubes would be expected to exhibit similar BSAF values to typical HOCs given the predicted strong interactions with both the sediment or soil organic matter and the organism fatty tissues.

By this logic, one possible approach for predicting the uptake of carbon nanotubes by ecological receptors is by comparison with compounds that share chemical similarities. CNTs, for example, comprise molecular structures containing extensive sp^2 carbons arranged in fused benzene rings (Iijima 1991). Their respective smaller macromolecular counterparts having between two to seven aromatic rings, polycyclic aromatic hydrocarbons (PAHs), are known to readily accumulate in the fatty tissues of organisms, in large part as a result of their hydrophobicities and resistances to microbial degradation (Di Toro et al. 1991; Jager et al. 2003a). Based on this comparison and the detection of nanotubes in cells and aquatic organisms, the possibility that these CNTs may similarly bioaccumulate in ecological receptors and be transferred throughout food chains thus represents a broad ranging and serious concern.

The applicability of these theories and correlations developed for HOCs for predicting the environmental behaviors of nanotubes requires scrutiny though given the differences described above between the behaviors of nanotubes and typical chemical pollutants. Humic acids, for example, are highly hydrophobic but not known to be absorbed by rats or fish likely as a result of their inability to pass through the biological membranes in the gastrointestinal tracts of the organisms (Geyer et al. 1987). This result stands in contrast to what would be predicted with equilibrium partitioning behaviors. While nanotubes are known to enter cells, their ability to pass through the gut membranes has not yet been established. As such, it is necessary to

investigate the extent to which nanotubes exhibit behaviors similar to those of HOCs.

1.1.6 Previous Limitations with Quantifying Carbon Nanotubes in Biological and Environmental Medias

Direct measurements of the ecological uptake of carbon nanotubes have not yet performed, largely because methods have not been available to readily quantify them in complex environmental or biological systems. Such common experimental methods as optical counting, spectroscopic methods, and elemental carbon analysis can be used to measure carbon nanotubes in relatively pristine samples, but the presence of other carbonaceous materials severely hinders use of these methods. The polydisperse nature of carbon nanotubes makes chromatographic techniques inapplicable; e.g., regardless of the synthesis procedure employed, nanotubes vary widely in length and diameter. Detection techniques able to distinguish carbon nanotubes from background carbon materials also remain a challenge. Near-infrared spectrofluorimetry has been used to detect carbon nanotubes in cells and rabbits (Cherukuri et al. 2004; Cherukuri et al. 2006). This approach cannot however detect metallic SWNTs or carbon nanotube bundles, and changes in carbon nanotube surface chemistry, a likely phenomenon in most environmental or biological systems, can influence absorption readings (O'Connell et al. 2002). Raman spectroscopy has been used to determine the presence of SWNTS in *Daphnia magna* (Roberts et al. 2007), but this approach is best suited for SWNTs, and does not provide quantitative results.

The addition of fluorescing chemicals or polymers with radioactive metals to carbon nanotubes has been used to assess nanotube behavior in biological systems (Kam et al. 2004; Kam et al. 2006; Singh et al. 2006; Liu et al. 2007a). Such probes may however alter the physicochemical characteristics of the nanotubes, and thus likely also their environmental behavior.

1.2 Objectives

To overcome the many limitations associated with quantifying carbon nanotubes in biological and environmental samples, the first objective of this dissertation research effort was to synthesize carbon-14 labeled SWNTs and MWNTs. Specifically, the carbon-14 isotope was incorporated into nanotubes using a modified chemical vapor deposition method with combinations of regular and carbon-14 labeled methane gas. Beta emissions from this isotope can be detected in most samples following combustion of the material of interest and liquid scintillation counting. This allows for facile quantification of modified or unmodified individual or bundles of SWNTs or MWNTs.

Such a tool could then be used to assess the rate at which unmodified SWNTs and MWNTs become associated with organisms and human cells. As such, the second research objective was to measure carbon nanotube assimilation by human cells. Assimilation here refers to the combination of cellular uptake and strong attachment to the cell membrane. This was investigated by determining the rate at which purified MWNTs interacted with HeLa cells, epithelial cells from a human carcinoma cell line. The successful completion of this objective would outline a novel quantification approach that

would both hold many advantages for future toxicological investigations of carbon nanotubes and yield information regarding the extent to which cells may assimilate unmodified CNTs.

Motivated by previous reports of the detection of CNTs in human cells and ecological receptors, the third objective was to measure the bioaccumulation potential of nanotubes in environmental receptors. As previously stated, the ability for nanotubes to be absorbed by organisms and then transferred throughout food chains could pose significant environmental and human health risks. Radioactively labeled carbon nanotubes were thus spiked to soils and sediments, and their uptake assessed by the earthworm *Eisenia foetida* and the oligochaete *Lumbriculus variegatus*, representative ecological receptors for terrestrial and sediment ecosystems, respectively. For these experiments, the accumulation and depuration behaviors of purified and acid modified carbon nanotubes were tested. In addition to the potential risks caused by the nanotubes themselves, CNTs possess strong sorptive capacities for various metals including lead, cadmium, and copper (Li et al. 2003) and a broad range of hydrophobic organic chemicals (Long and Yang 2001; Yang et al. 2006a; Yang et al. 2006b). Carbon nanotubes could hypothetically act similarly to charcoals and other forms of black carbon by sequestering such compounds and limiting their bioavailability and mobility. It is also possible, conversely, that nanotubes loaded with highly elevated concentrations of toxic chemicals could transport these such chemicals into organisms exacerbating bioaccumulation and food chain transfer. While such

effects were not explicitly investigated here, the potential for organisms to accumulate carbon nanotubes would likely correspondingly increase the uptake of other environmental contaminants.

The fourth objective was to assess the extent to which distribution coefficients for carbon nanotubes between water and octanol could be used to predict CNTs' bio-uptake behaviors. As described above, this coefficient, called the octanol-water partitioning coefficient for typical HOCs, has been frequently used to estimate a chemical's accumulation by environmental receptors (Di Toro et al. 1991; Belfroid et al. 1996; Mackay and Fraser 2000). The physicochemical properties of carbon nanotubes, however, differ broadly from those of typical organic compounds, and it is thus unclear the extent to which such a value would relate to the biological uptake of the nanotubes. A modified shake-flask method was developed to determine the distribution coefficients for purified MWNTs and those modified by sonication in a 3:1 mixture of sulfuric to nitric acid for 2 hrs, and these values then compared against the BSAFs determined during the completion of the third objective.

1.3 Overview

The results of this research are presented in eight chapters. The second chapter describes the experimental methods and materials. The third chapter details results of the synthesis, purification, and characterization of radioactively labeled SWNTs and MWNTs. In the fourth chapter, the radioactively labeled MWNTs were used to assess the assimilation rate for HeLa cells, a human cell line. The potential for nanotubes to bioaccumulate

in terrestrial and sediment ecosystems was investigated in chapters 5 and 6 with the oligochaete *Lumbriculus variegatus* and the earthworm *Eisenia foetida*. Based on the broad range of nanotube physicochemical properties for various applications, the potential for uptake of chemically modified nanotubes using acid mixtures was studied in chapter 7. Such modifications procedures have been previously shown to cause significant physicochemical changes to nanotubes with regards to their hydrophilicity and length. Also in this chapter, a modified shake-flask method was developed to assess the distribution of MWNTs between octanol and water phases. This marks the first time that such a coefficient has been measured for carbonaceous nanoparticles, and these values were compared against the uptake results for *E. foetida* and *L. variegatus* to assess the extent to which such coefficients can predict the bioaccumulation behaviors of MWNTs. Lastly, overriding conclusions are drawn and auspicious future research directions highlighted in Chapter 8.

Results from this dissertation comprise four articles that either have been submitted or are being prepared for submission to peer-reviewed journals. The results shown here have been presented at numerous conferences including three American Chemical Society conferences and one conference organized by the National Institute of Occupational Safety and Health. I will also be giving a presentation at the Fall 2007 American Chemical Society conference based on my receipt of one of the 2007 Graduate Student Paper Awards by the American Chemical Society's

Environmental Chemistry Division for a paper using the results from chapters 3 and 5. This research was recently highlighted in an article by the Michigan Record on March 27, 2006.

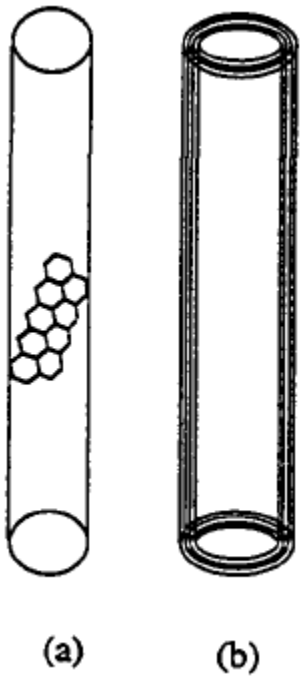

Figure 1.1: Schematic of carbon nanotube structures: a) single-walled carbon nanotubes and b) multi-walled carbon nanotubes (Adapted from (Lueking 2003).

Chapter 2

MATERIALS AND METHODS

2.1 Chemicals

Nickel nitrate hexahydrate (99%), magnesium nitrate hexahydrate (reagent grade), ferric nitrate (reagent grade), citric acid, n-octanol (ACS grade), sulfuric acid (technical grade; 93-98%), nitric acid (ACS grade; 68-70%), hydrochloric acid (37-38%), and 30% hydrogen peroxide (ACS grade) were purchased from Fisher. Alkaline magnesium carbonate was obtained from Sigma Aldrich. Helium gas (99.95%), argon gas (99.998%), and methane gas (99.97%) from Cryogenic Gases. Carbon-14 methane was obtained from American Radiolabeled Chemicals. Phosphate buffered saline (PBS) solution, dulbecco's modified eagle's medium, trypsin-EDTA, and fetal bovine serum were purchased from Gibco, while tryphan blue and penicillin/streptomycin were obtained from Sigma-Aldrich. Cell culture plates were from Falcon (78.5 cm^2).

2.2 Single- and Multi-Walled Carbon Nanotube Synthesis and Purification

2.2.1 Single-Walled Carbon Nanotube Synthesis

Single-walled carbon nanotubes (SWNTs) were synthesized using a methane chemical vapor deposition method (Li et al. 2002). Alkaline magnesium carbonate was annealed under Ar at 400 °C for 1 hr. One gram of iron nitrate was dissolved in 100 mL MilliQ water and mixed with 10 g annealed magnesium carbonate. This solution was bath sonicated for half an hour, dried at 115 °C, and the solids ground to a powder with mortar and pestle. One gram of the catalyst was heated to 850 °C under a stream of 250 mL/min Ar, and a mixture of regular and carbon-14 methane flowing at 60 mL/min mixed with 250 mL/min argon gas was flown over the catalyst for 15 minutes before cooling in Ar.

2.2.2 Multi-Walled Carbon Nanotube Synthesis

Multi-walled carbon nanotubes (MWNTs) were synthesized via chemical vapor deposition using methane as the feedstock gas (Chen et al. 1997). A 1.94-g quantity of nickel nitrate and a 2.56-g quantity of magnesium nitrate were thoroughly mixed, and 2 g of citric acid and 20 mL of Milli-Q water were then added. This solution was dried at 100 °C for approximately 40 hours, and the resulting green solid was calcined at 700 °C for 5 hours in air to produce a fluffy grey catalyst. One hundred milligrams of this catalyst was added to a quartz boat, and hydrogen gas was flown at a rate of 100 mL/min over the boat as the temperature in the reactor was raised to and held

at 600 °C. The flow of hydrogen gas was then stopped, and a mixture of carbon-14 methane and regular methane was introduced at a flow rate of approximately 300 mL/min for thirty minutes. After the methane gas flow was stopped, the reactor was cooled to room temperature in argon.

2.2.3 Carbon Nanotube Purification

For all experiments, except the HeLa cell assimilation one, the single- and multi-walled carbon nanotubes were purified by sonication in full-strength hydrochloric acid for 1 hr. For the HeLa experiments, the MWNTs were purified by bath sonication in 6-N nitric acid for 1 hr.

2.3 Carbon-14 Labeling Quantification

2.3.1 Solid Carbon Nanotubes

To determine the radioactivity of the synthesized nanotubes, the MWNTs were combusted in a biological oxidizer (OX 500, R. J. Harvey Instrumentation Corporation). This instrument was used to burn the nanotubes at 900°C for three minutes under a stream of oxygen gas running at 350 mL/min, the $^{14}CO_2$ released during the combustion process was captured in carbon-14 scintillation cocktail, and the radioactivity in the cocktail measured using a LS6500 liquid scintillation counter (Beckman, Fullerton, CA). Samples were generally counted for one hour or until the uncertainty in the measurement was less than 1% of the radioactivity. The direct addition of solid carbon nanotubes to scintillation cocktail followed by scintillation counting was found to consistently underestimate the radioactivity of the carbon nanotubes relative to combusting the nanotubes in the biological

oxidizer. The decreased measured radioactivity from samples in which the carbon nanotubes were directly added to scintillation cocktail likely stems from absorption of beta emissions by nanotube agglomerations, or from settling of insoluble nanotubes in the scintillation cocktail.

2.3.2 Carbon Nanotube Radioactivity Measurements in Solutions

The radioactivities of samples with MWNTs dispersed in water were determined by adding the solution to Ready Safe scintillation cocktail (Beckman Coulter Inc.). Blank samples with only water and cocktail showed that the measured background radioactivity is roughly constant for 0 to 3 mL of water per 20 mL of cocktail solution. As such, 2 mL of water was added to each scintillation vial for the settling experiments, and the water volume was evaporated from 20 mL to less than 3 mL for the phase distribution experiment. A total of 20 background samples were used to determine the average background radioactivity. The radioactivity of samples in which MWNTs were dispersed in octanol was determined by adding 2 mL of solution to a 20 mL borosilicate scintillation vial with ScintiSafe cocktail (Fisher Scientific). The background radioactivity was determined by the average of 10 blank samples. Preliminary experiments showed that the measured radioactivity in samples with water and cocktail remained relatively constant over time, but that the radioactivity measured in the octanol samples decreased dramatically during the first 24 hours and then roughly stabilized. As such, the radioactivities of octanol-cocktail samples were measured by scintillation counting approximately 24 hours after the initial mixing, and the

measured radioactivity then corrected to yield the radioactivity immediately after the octanol solution was mixed with the cocktail.

2.4 Carbon Nanotube Characterization

2.4.1 Transmission Electron Microscopy

Microscopic analysis of the carbon nanotubes was performed using transmission electron microscopy (TEM). TEM samples were prepared by sonicating multi-walled carbon nanotubes in dimethyl formamide or single-walled carbon nanotubes in water, dripping the solution onto holey carbon film grids (Ted Pella), and viewing the grids using a JEOL 3011 TEM operating at 300 kV.

2.4.2 Scanning Electron Microscopy

For scanning electron microscopy (SEM) analysis, HCl purified or 3:1 acid mixture treated multi-walled carbon nanotubes were dispersed in dimethyl formamide and added to silicon wafers. These wafers were assessed with a Philips/FEI XL30 FEG scanning electron microscope using an accelerating voltage of 15.0 kV.

2.4.3 Thermal Gravimetric Analysis

The quality of the purified carbon nanotubes was assessed using thermal gravimetric analysis (TGA) (Pyris 1 TGA, Perkin Elmer). TGA has been commonly used to measure the presence of amorphous carbon impurities and residual catalyst materials in carbon nanotube samples (Dillon et al. 1999; Chiang et al. 2001; Harutyunyan et al. 2002). Amorphous carbon impurities generally burn at lower temperatures than carbon nanotubes as a

result of their less stable chemical configuration. The presence of carbon impurities can thus be quantified by analyzing the derivative of the mass change with respect to temperature; peaks at lower temperatures represent the oxidation of carbon impurities, while the principal peak at a higher temperature is typically attributed to the carbon nanotubes. The mass remaining after oxidation indicates the fraction of residual catalyst in the sample.

2.4.4 Raman Spectroscopy

Carbon nanotube samples were prepared by pressing SWNTs onto carbon tape adhered to aluminum foil. The carbon tape was completely covered with a thick layer of SWNTs to prevent interference from the carbon in the carbon tape during the spectroscopy. Raman spectra were obtained using a Renishaw inVia Raman Microscope equipped with a Leica microscope, RenCam CCD detector, 785 nm diode laser, 1200 lines/mm grating and 50 μm slit. Due to the high variability in the D- and G-band peaks and areas indicated in the literature (Itkis et al. 2005), ten spectra were averaged for each spectrum shown here.

2.4.5 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) spectra were taken using a Kratos Analytical Axis Ultra X-ray photoelectron spectrometer. These spectra were then used to assess the elemental composition and changes to the functional groups of the MWNTs after acid treatment. A thick mat of mat of

nanotubes was pressed onto carbon tape to ensure that carbon from the carbon tape did not influence the results.

2.5 Carbon Nanotube Acid Modification

One common method to introduce defects in carbon nanotubes and to make them more hydrophilic is sonication in an aggressive acidic mixture composed of a 3:1 ratio of concentrated sulfuric and nitric acid (Liu et al. 1998). To assess to what extent such a modification would influence the bioaccumulation of MWNTs in environmental settings, purified MWNTs were bath sonicated for 2 hrs in this acid mixture. The carbon nanotubes were then filtered using 0.45 μm polytetraflouroethylene (PTFE) filter paper, and washed with boiling water after each step to remove residual acids and water-soluble aromatic impurities formed during the acid treatments. These nanotubes were labeled "3:1 MWNTs."

2.6 HeLa Cell Uptake Tests

2.6.1 Cell Culturing

HeLa cells were maintained at 37°C and 5% CO_2 in media consisting of Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and penicillin (100 U/mL)/ streptomycin (100 μg/mL). Cells were used at passage numbers 55-61, and seeded at roughly 1 x 10^6 cells per 78.5 cm^2 plate every 7 days.

2.6.2 Cell Assimilation

Cellular uptake of multi-walled carbon nanotubes (MWNTs) was quantified using carbon-14 multi-walled nanotubes. HeLa cells were seeded

at 8 x 10^5 cells per 78.5 cm^2 culture plate and maintained for 1 week. Cell growth approached confluency after this period, and the total number of cells was 38 ± 2 x 10^6 cells per plate. The MWNTs were sonicated at a concentration of 0.1 mg/mL in 200 mL of cell growth medium for 15 minutes at 262.5 watts administered at ten second on/off pulse intervals. The supernatant was slowly decanted into a second beaker to remove the non-dispersed carbon nanotube agglomerates. The solution was mechanically stirred when adding the aliquots to the cell culture plates. In a preliminary test, this process was shown to produce a homogeneous solution. In this test, aliquots were taken at the beginning, middle, and conclusion of the mixing period, their radioactivity was determined, and the standard deviation was found to be 7 percent. Preliminary results using live/dead counts did not show acute cellular toxicity in HeLa cells incubated for 24 hrs with MWNTs dispersed at the concentration described above.

MWNT uptake by adherent HeLa cells was measured after 15 minutes, 1 hr, 6 hr, 12 hr, and 24 hr. Triplicate plates were used at each time interval. The nanotube solution overlying the cells was decanted at the conclusion of each uptake period, and the plate cultures were washed with refrigerated phosphate buffered saline solution to arrest cellular activity and completely remove any unattached nanotubes. Washing of the cells with phosphate buffered saline solution for a second time did not reveal removal of additional unattached nanotubes. Two mL of trypsin-EDTA was then added to each plate to suspend the cells, and the plates were incubated for five minutes. A

3-mL quantity of DMEM media was added to each plate to neutralize the trypsin-EDTA, and the solution was then mixed thoroughly to fully suspend the cells. The cell solution was passed through filter paper (Whatman 1 Qualitative) to capture the cells. These filters were allowed to dry in air, combusted in the biological oxidizer, and their radioactivity measured by scintillation counting. Plates with cells that were not amended with carbon nanotubes were used to measure background radioactivity.

The release of carbon nanotubes after cellular uptake was also assessed. The nanotube solution was introduced to triplicate plates for 12 hours, the cellular media then decanted, and the plates washed with room temperature phosphate buffer saline solution. The plates were refilled with cell media and incubated for an additional 12 hours. To quantify nanotube release by the cells, the media overlying them on each plate and the phosphate buffered saline solution used to wash each plate were filtered (Whatman 1 Qualitative), and the filter papers combusted in the biological oxidizer. Radioactivity in the cells was measured as described above.

2.7 Lumbriculus variegatus Uptake and Depuration Experiments

2.7.1 Aquatic Worm Culturing

Lumbriculus variegatus were obtained from the Carolina Biological Supply Co. (Burlington, NC) and used to assess the availability of the carbon nanotubes to biological uptake and accumulation. The organisms were cultured in aquariums containing artificial freshwater (ISO 1996) and unbleached brown paper towels, and maintained at 21±2 ^{o}C under photo-

period (light:dark) ratios of 16:8 hrs. The overlying water was changed, and aquatic worms fed (daphnia food, Carolina Biological Supply Co.) at least two times per week.

2.7.2 Sediments

Carbon nanotubes or pyrene samples were added to mixtures of 90% sediment (Huron River, Ann Arbor, MI) with 10% Michigan Peat (by mass) or unamended sediment. The addition of 10% MI Peat allowed for a larger number of worms to be used for bioavailability experiments with a 50:1 ratio of sediment organic carbon to dry weight of the aquatic worms (Kukkonen and Landrum 1994). The sediment and peat samples were analyzed to ensure neither the soils nor sediment contained any traces of the target contaminants. The sediment was air-dried and passed through a 2-mm mesh sieve prior to ecological experiments. The organic carbon content of the sediment and peat were 0.66% and 45.1%, respectively.

2.7.3 Uptake Experiments

Uptake experiments were conducted according to a modified EPA method (U.S. EPA Office of Water 2000). Carbon-14 single-walled carbon nanotubes (0.03 or 0.003 mg/g dry sediment) and multi-walled carbon nanotubes (0.37 or 0.037 mg/g dry sediment) were dispersed by sonication in water prior to addition to the sediment. Carbon-14 labeled pyrene (positions 4,5, 9, and 10) in methanol and non-radioactive pyrene were dissolved in acetone and added to sediment to give a final concentration of 0.054 mg/g dry sediment. The samples were thoroughly tumbled, the acetone from the

pyrene samples allowed to volatilize, and the samples then refrigerated. Sediment samples were freeze-dried, combusted using the biological oxidizer, and the radioactivity determined using scintillation counting to determine the initial concentration of the compounds in the sediments and the homogeneity of their distribution. Occasionally, elevated nanotube concentrations would be detected likely as a result of carbon nanotube aggregates that were not fully dispersed during the sonication process. Samples for which sediment radioactivities were greater than two times the mean value were excluded from calculating the mean sediment concentration. Sediment samples spiked with non-radioactive carbon nanotubes or pyrene and unspiked sediment samples were prepared similarly as controls.

Six days after the samples were spiked with carbon nanotubes or pyrene, 50 g (dry weight) of amended or unamended sediment was added to 300 mL lipless beakers, and twice daily water renewal was initiated using artificial freshwater (ISO 1996). Aquatic worms were removed and placed in a tray for one day prior to the start of the experiment. On the following day, sixty aquatic worms were added to each container to achieve a 50:1 ratio of organic carbon in the sediment to dry mass of aquatic worms (Kukkonen and Landrum 1994). The aquatic worms were not fed during the experiment. At the beginning of the experiment and on a weekly basis thereafter, hardness, pH, dissolved oxygen, alkalinity, and conductivity measurements were taken

to ensure that the water quality remained relatively constant during the experiments (U.S. EPA Office of Water 2000).

Aquatic worms were sieved from the sediments after predetermined intervals to determine the uptake of the desired compound. The worms were collected from the sediment and placed in beakers with 500 mL of new artificial freshwater for 6 hours, a period that has been shown to allow the organisms to purge >98% of their gut contents but also minimizes tissue depuration of non-polar hydrophobic chemicals (Mount et al. 1999). The worms were blotted dry, weighed, and added to biological oxidizer boats with 100 mg of D-mannitol to aid combustion. After drying overnight, the worms were combusted in the biological oxidizer and the radioactivity measured using liquid scintillation counting.

On days 7, 14, and 28, aquatic worms were also removed from containers with non-radioactive nanotubes or pyrene and unmodified sediments. The number of living worms was compared between these containers and those with carbon-14 labeled compounds. The lipid content was measured using a spectrophotometric method for the aquatic worms from blank and spiked sediments (Van Handel 1985). Biota-sediment accumulation factors (BSAFs) were calculated as the ratio of the compound concentration in organism normalized by its lipid fraction to concentration in sediment normalized by its organic carbon fraction.

2.7.4 Depuration Experiments

On day 14 or 28, the aquatic worms from three containers were added to 600 mL beakers containing 500 mL of clean water or to 300 mL lipless beakers with 50 g dry mass clean sediment and filled with clean water. For the worms added to clean sediment, the worms were removed from the containers after the predetermined depuration interval and sediment particles removed. After depuration for 1, 2, or 3 d, the worms were removed from their containers, blotted dry, and then added to biological oxidizer boats with 100 mg of D-mannitol. The radioactivity remaining in the worms was determined via biological oxidation and scintillation counting as described above.

2.7.5 Statistical Analysis

Statistically significant differences among the means of triplicate samples were conducted using two-way t-tests or analysis of variance ($p<0.05$) (Microsoft Excel). Attempts were made to model the uptake data using a two-compartment, first-order coefficient model using nonlinear curve fitting with SAS (SAS Institute). This model did not provide a good fit for the single- and multi-walled carbon nanotube data, and the results are not included here.

2.8 *Eisenia foetida* Uptake and Depuration Experiments

2.8.1 Earthworm Culturing

Earthworms (*Eisenia foetida)* were obtained from the Carolina Biological Supply Co. (Burlington, NC), maintained on a worm bedding

(Carolina Biological Supply) at 21± 2°C, and kept moist with deionized water. The worms were fed twice a week with worm food comprising a mixture of crude proteins and carbohydrates (Magic Worm Products, Amherst Junction, WI).

2.8.2 Soils

Three soils were used for the bioaccumulation experiments. They were collected from Chelsea and Ypsilanti, Michigan and from the North Campus at the University of Michigan Ann Arbor, Michigan and are indicated as “Chelsea,” “Ypsilanti,” and “NC” soil, which respectively possess organic carbon fractions of 5.95%, 1.14%, and 2.17%. The soils were air-dried and passed through a 2-mm mesh sieve. The samples were previously analyzed to ensure the soils did not contain traces of pyrene.

2.8.3 Uptake Experiments

Nanotube and pyrene uptake by the earthworm *Eisenia foetida* from the test soils was determined using modified standard procedures (ASTM 1998). Carbon-14 SWNTs (0.03 mg/g dry soil) and MWNTs (0.3 or 0.03 mg/g dry soil) were dispersed by sonication in water at 262.5 watts for 30 minutes in an ice-water bath to prevent damage to the nanotubes during the sonication process (Heller et al. 2005b). Carbon-14 labeled pyrene (positions 4,5, 9, and 10) dissolved in methanol and non-radioactive pyrene were dissolved in methylene chloride and added to the soil to give a final concentration of 0.04 mg/g dry soil. All soil samples were thoroughly tumbled and the soil with pyrene air-dried overnight to allow the solvents to volatilize.

Soil samples taken from the SWNT, MWNT, and pyrene-spiked soils were freeze-dried, combusted using the biological oxidizer, and the radioactivity determined using scintillation counting. At least four samples were combusted for each soil. This allowed us to determine the initial concentration of the compounds in the soils as well as the homogeneity of their distribution. Samples with elevated nanotube concentrations would be detected on occasion likely as a result of carbon nanotube aggregates that were not fully dispersed during the sonication process. Samples for which soil radioactivities were greater than the mean value plus two standard deviations were excluded from calculating the mean soil concentration. Samples with non-radioactive carbon nanotubes or pyrene and unspiked soil samples were prepared similarly as controls.

Three adult worms with combined masses between 1.2 and 2.0 g were transferred to moist (20% water for Chelsea and Ypsilanti soils and 25% for NC soil) soil samples (30 g dry mass) in 250-ml glass jars, the jars loosely closed with a cap to prevent worm escape but allow air exchange, and then held in the dark at 21±2^{o}C. Worms were removed from triplicate containers after 1, 7, 14, and 28 d for the Chelsea soil and after 14 days for the Ypsilanti and NC soils. After removal, the earthworms were washed with Milli-Q water, transferred to wet filter paper in Petri dishes for 24 hrs in the dark to allow purging of gut contents, and again rinsed with clean Milli-Q water until the radioactivity of the water had a background radioactivity concentration. The worms were then transferred to glass centrifuge tubes, freeze-dried for 24

hrs, weighed, combusted in a biological oxidizer, and the radioactivity determined using liquid scintillation counting. Bioaccumulation factor (BAF) values were calculated as the ratio of the concentration of the compound of interest in the organism divided by that in the soil.

2.8.4 Depuration Experiments

After exposure for 14d, the earthworms were removed from three containers and added to containers with unspiked soils to allow for depuration. After depuration for 1, 2, or 7 d, the worms were removed from their containers and the radioactivity remaining in the worms determined as described above.

2.9 Phase Distribution Experiments

2.9.1 Settling Experiments

Settling experiments were conducted to assess the extent to which the dispersed MWNTs would settle during the equilibration phase of the octanol-water phase distribution experiments. A microbalance was used to weigh 2.5 mg of MWNTs which were added to 250 mL beakers with 100 mL of water or octanol. The samples were sonicated for 30 minutes in an ice-water bath to minimize damage to the carbon nanotubes during the sonication process. Immediately after sonication, two 2-mL samples of the liquid were removed and the radioactivity determined as described above and 50 mL of the remaining liquid was added to a test tube. A 2-mL aliquot was removed from each of the vials after 1, 4, 8, 10, 14, 17, and 21 days. Triplicate samples were tested for the HCl purified and 3:1 treated MWNTs dispersed in octanol or in water.

2.9.2 Octanol-Water Phase Distribution Experiments

Octanol-water partitioning coefficients (k_{ow}) have been previously used to predict the bioaccumulation of hydrophobic organic chemicals (HOCs). One possible approach for estimating the bioaccumulation potential of carbon nanotubes would be to treat them similarly to HOCs by using models and correlations developed for these organic compounds. There are numerous differences between nanotubes and HOCs though as described in section 1.1.2, and the applicability of such models to the environmental behaviors of CNTs is thus unclear. As such, the distribution coefficients measured using this methodology should not be carelessly equated with the k_{ow} values of typical organic chemicals.

Phase distribution experiments were first attempted by sonicating a 2.5 mg sample of HCl purified or 3:1 modified multi-walled carbon nanotubes in 100 mL of water or octanol; the distribution behaviors of SWNTs were not similarly categorized due to the instability of the SWNT dispersion. After allowing the sample to sit overnight, 25 mL of the sample was combined with 25 mL octanol in a 50-mL test tube for samples initially sonicated in water and the converse for those first sonicated in octanol. All experimental conditions were tested in triplicates. After 21 days, two 2-mL aliquots of each sample were taken from the octanol phase, and the radioactivity determined as described above. To determine the radioactivity in the water phase, water was removed from the test tubes using a syringe. Air was bubbled out of the syringe during passage through the octanol phase to prevent octanol from

entering the syringe. Typically, 15 to 20 mL of water were drawn up into the syringe while attempting to avoid any potential areas of octanol remaining on the side of the test tube. The needle of the syringe was then removed to avoid octanol uptake during the removal of the needle from the sample, and all of the liquid except for the last one to two milliliters was added to preweighed scintillation vials. These vials were weighed, added to an oven at 80 °C, the samples heated typically from 24-30 hrs until the volume of water remaining in the vials was less than 3 mL, and the radioactivity then measured using scintillation counting.

These results, however, did not reveal an ability of carbon nanotubes to transfer across the water-octanol interface. For the samples in which the nanotubes were initially sonicated in water, the radioactivity of the octanol phase was at the background levels and likewise for the water phase when the MWNTs were sonicated originally in octanol. As such, a modified OECD shake flask method was developed (Organization for Economic Cooperation and Development. 1981. Partition coefficients. OECD Guideline 107. Paris). A 2.5 mg sample of MWNTs was weighed and dispersed in water as described above. After allowing the sample to sit overnight, 25 mL of the sample was combined with 25 mL octanol in a 100 mL beaker. The sample was sonicated for 30 minutes with the probe 0.3 inches from the bottom of the beaker. This height was slightly above the octanol-water interface and allowed for thorough mixing of the two phases. The samples were then added to 50 mL test tubes, which were inverted in an attempt to remove

residual octanol from the bottom of the water phase. After 4, 8, 14, and 21 days, the MWNT distribution between the two phases was assessed using triplicate measurements and the procedures described above.

The impact of dispersing the carbon nanotubes first in octanol and then measuring their partitioning behavior was also assessed. These experiments were conducted analogously to those described above except for that the carbon nanotubes were initially sonicated in octanol and 25 mL of water was combined with 25 mL of this octanol phase prior to the second sonication. The octanol-water partitioning data were assessed after 16 days using triplicate vials.

Chapter 3

CARBON-14 SINGLE- AND MULTI-WALLED CARBON NANOTUBE SYNTHESIS AND CHARACTERIZATION

3.1 Introduction

As discussed in the introduction of this thesis, the exciting properties of carbon nanotubes promise a broad range of future application. This expected widespread usage will inevitably lead to their release into environmental systems. The understanding of the environmental fate and behaviors of nanotubes is as yet largely unknown, however, in large part as a result of the lack of a method to quantify carbon nanotubes in environmental and biological settings.

Common experimental methods such as optical counting, spectroscopic methods, and elemental carbon analysis can be used to measure carbon nanotubes in relatively pristine samples, but the presence of other carbonaceous materials severely hinders the use of these methods. The polydisperse nature of carbon nanotubes makes chromatographic techniques inapplicable; e.g., regardless of the synthesis procedure employed, nanotubes vary widely in length and diameter, which would be

very challenging to be resolved by HPLC. Appropriate detection techniques that can distinguish carbon nanotubes from background carbon materials also remains a challenge for the use of HPLC. Near-infrared spectrofluorimetry has been used to detect carbon nanotubes in cells and rabbits (Cherukuri et al. 2004; Cherukuri et al. 2006). However, this approach cannot detect metallic SWNTs or carbon nanotube bundles, and changes in the carbon nanotube surface chemistry, a likely phenomena in many environmental or biological systems, can influence absorption readings (O'Connell et al. 2002). Raman spectroscopy has been used to determine the presence of SWNTS in *Daphnia magna (Roberts et al. 2007)*, but this approach can only detect SWNTs, and cannot provide quantitative results. The addition of fluorescing chemicals or polymers with radioactive metals to carbon nanotubes has also been used to assess how carbon nanotubes would interact in biological systems (Kam et al. 2004; Singh et al. 2006). The addition of such a probe, however, may well change the physicochemical characteristics of the nanotubes and thus likely also its environmental behaviors.

This chapter describes a novel carbon synthesis approach for radioactively labeling carbon nanotubes and the robust characterization of nanotubes synthesized through this chemical vapor deposition process. Synthesis and purification procedures were designed to minimize the presence of amorphous carbon and catalyst impurities. The presence of significant concentrations of amorphous carbon impurities would undermine the quantification of carbon nanotubes present in cellular or environmental

medias using measurements of their radioactivities. Thermal gravimetric analysis (TGA), electron microscopy, and Raman spectroscopy were thus performed to assess the presence of this impurity. Given that most potential applications of carbon nanotubes would likely utilize highly purified nanotubes, efforts were also made to limit the quantity of catalyst materials remaining in the carbon nanotubes samples used in the following cellular and environmental investigations. The fraction of the metal impurities remaining in the samples was assessed using TGA. Lastly, the specific radioactivity and the homogeneity of the carbon-14 isotope distribution were assessed for the radioactively labeled nanotubes using biological oxidation followed by scintillation counting.

3.2 Experimental Methods

Single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) were synthesized, purified, modified and characterized as described in Chapter 2. Briefly, SWNTs and MWNTs were synthesized using the modified methane chemical vapor deposition processes as described in section 2.2. Streams of regular and radioactive methane gas were combined in controlled ratios and flown over metal catalysts at elevated temperatures. The nanotubes were then purified as described in section 2.2.3 to remove metal catalyst impurities remaining from the synthesis procedure.

Non-radioactively labeled carbon nanotubes were similarly synthesized and purified. These nanotubes were then assessed with a broad range of

analytical instruments to determine the purity of the nanotubes with regards to amorphous carbon impurities as well as catalyst impurities as described in section 2.4. Some MWNTs were also modified by strong acid treatments as described in section 2.5 and their chemical properties investigated using X-ray photoelectron spectroscopy.

The radioactive labeling of the carbon nanotubes was assessed using biological oxidation followed by scintillation counting as described in section 2.3.1. In brief, the carbon nanotubes were carefully weighed, combusted in the biological oxidizer, the carbon-14 dioxide emitted captured in scintillation fluid, and the radioactivity of that fluid assessed using scintillation counting.

3.3 Results and Discussion

3.3.1 Carbon-14 Labeling Quantification

The radioactive labeling of both single- and multi-walled carbon nanotubes was affirmed. While the specific radioactivity of the carbon nanotubes varied from batch to batch depending upon the quality of the catalyst and the relative flow rates of the radioactive and regular methane gases, typical specific radioactivities were 1.35 ± 0.03 mCi/g and 0.122 ± 0.004 mCi/g for the SWNTs and MWNTs, respectively. The low standard deviations suggest that the ^{14}C atoms are homogenously distributed throughout the nanotubes. The rationale for the higher specific radioactivity for the SWNTs mainly relates to the lower total methane flow rate for the synthesis procedure; for the same carbon-14 methane flow rate, the radioactive fraction of the total flow rate would be much higher for the

SWNTs. It is certainly possible to obtain similar specific radioactivities for both kinds of nanotubes by modifying the methane flow rates. The high price of the carbon-14 methane, however, makes significantly increasing the radioactivity of the MWNTs prohibitively expensive especially given that the nanotube yield using the MWNT catalyst often varies considerably.

3.3.2 Transmission Electron Microscopy

As shown in Figures 3.1 and 3.2, transmission electron micrographs indicated that the SWNTs had diameters typically from one to two nanometers, while the diameters for the MWNTs generally ranged from 30 to 70 nanometers. The lengths also varied but were generally several micrometers. High-resolution transmission electron microscopy (Figure 3.3) indicated that the multi-walled nanotubes have a fishbone configuration. This result accords with those of other studies (Zhang et al. 1999). Micrographs were also taken from MWNTs after treatment with the 3:1 acid mixture as shown in Figure 3.4. The open end of the nanotube is representative of the damage that this aggressive solution causes to the nanotubes opening up their ends (Liu et al. 1998).

3.3.3 Scanning Electron Microscopy

Scanning electron microscopy was used to assess the length distributions for the different types of MWNTs as shown in Figures 3.5 through 3.8. Surprisingly, the length distribution did not significantly differ between the nanotubes modified with the 3:1 acid mixture and those only purified with hydrochloric acid. It was previously shown that mixing single-

walled nanotubes with this acid solution for 1 hr decreased the average size from 280 nm to 150 nm (Liu et al. 1998). The average diameter for the purified MWNTs was 386 nanometers while that for the acid modified nanotubes was 407 nanometers. The length distributions were very broad though and this result should not be taken as an indication that this procedure increased the length of the nanotubes. Perhaps the greater diameter of the multi-walled carbon nanotubes typically ranging from 30 to 70 nanometers and their composition of numerous concentric carbon layers made them more resistant to size shortening.

3.3.4 Thermal Gravimetric Analysis

Thermal gravimetric analysis is a procedure commonly used in the carbon nanotube literature to assess the presence of amorphous carbon impurities and residual catalyst materials in carbon nanotube samples (Dillon et al. 1999; Chiang et al. 2001; Harutyunyan et al. 2002). None of the samples indicated the presence of an amorphous carbon peak. Figures 3.9 through 3.12 represent example graphs for each of the various carbon nanotubes samples used in this thesis. Table 3.1 shows the carbon purity (fraction of carbon in the sample compared to catalyst materials) for all of the CNT samples. The MWNTs had significantly higher purities than the SWNTs, a result hypothesized to come in part from the higher purity of the multi-walled carbon nanotubes prior to acid purification. The yield of the multi-walled carbon nanotubes was significantly higher than that for the single-walled

carbon nanotubes per mass of catalyst resulting in a much higher ratio of carbon to metals in the initial samples.

3.3.5 Raman Spectroscopy

Raman spectroscopy is among the most common technique used to assess the purity of SWNT samples by investigating the relative heights of or areas under the G-band and D-band peaks as shown in Figure 3.13. The areas underneath the G-band peaks compared to those for the D-band was 19.6 ± 0.2 (n=3), a ratio that corresponds to minimal amorphous carbon impurities (Itkis et al. 2005).

3.3.6 X-ray Photoelectron Spectroscopy

Survey spectra taken for the HCl purified and 3:1 acid mixture treated MWNTs did not indicate the presence of metal catalysts (Figures 3.14 and 3.15). This result agrees with the lower metal concentrations measured using TGA. It should be noted though that XPS only measures the top few nanometers of the sample, and the lack of detection of the metal catalysts indicates the low quantity of metal catalysts present and not their absence. The 3:1 acid modification increased the oxygen content in the carbon nanotubes samples from 1.4 ± 0.2% to 6.8 ± 0.3% (n=3) thus indicating the damage to the nanotubes and the increase in functional groups on the nanotubes as a result of the acid treatment. This oxygen content for the HCl purified MWNTs agrees with that for an XPS spectrum (1.57%) collected using a different, but similarly purified, MWNT sample, which suggests the reproducibility of this technique with different samples.

3.4 Summary

Carbon-14 labeled SWNTs and MWNTs were synthesized for the first time to the best of our knowledge. Radioactivity measurements of these nanotubes indicated a low standard deviation for the nanotube samples even though the samples burned had very small masses (typically less than 1 mg) thus indicating the uniform distribution of this isotope throughout the nanotube samples. Non-radioactively labeled nanotubes were thoroughly characterized and shown to possess high purity with regards to amorphous carbon and catalyst impurities as measured using electron microscopy, Raman spectroscopy, and thermal gravimetric analysis. The application of these nanotubes for environmental and biological applications will be investigated in the following chapters.

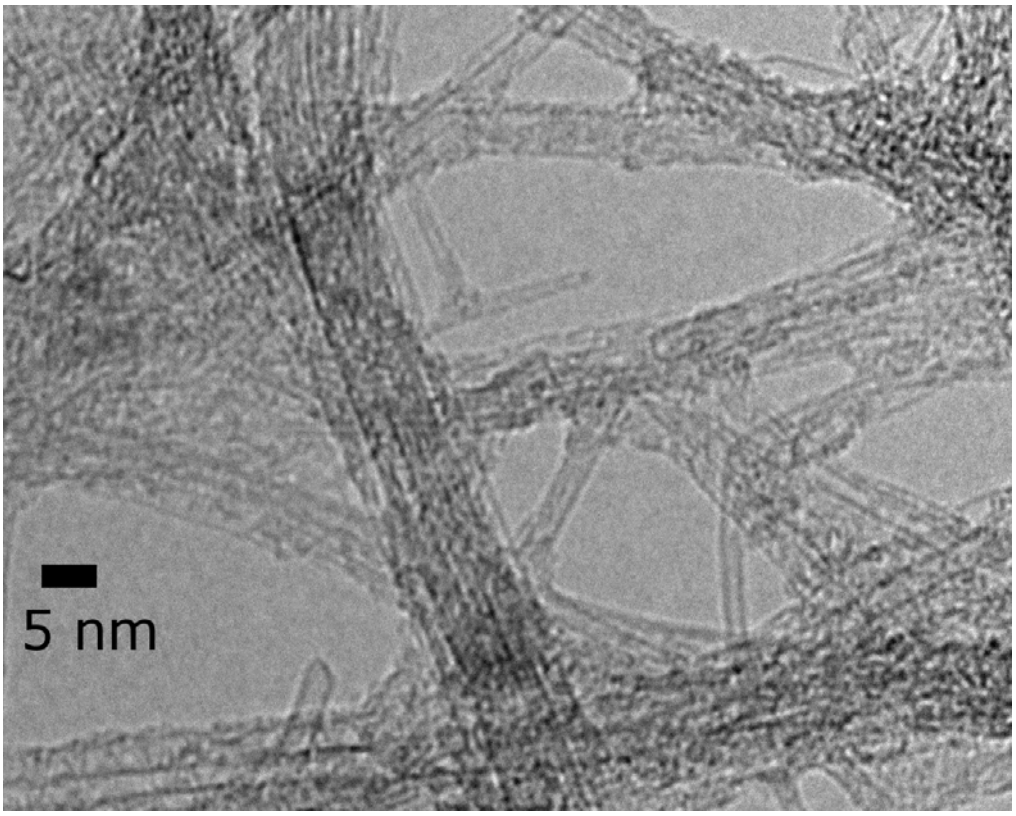

Figure 3.1: Transmission electron micrograph of single-walled carbon nanotubes (250 kx magnification).

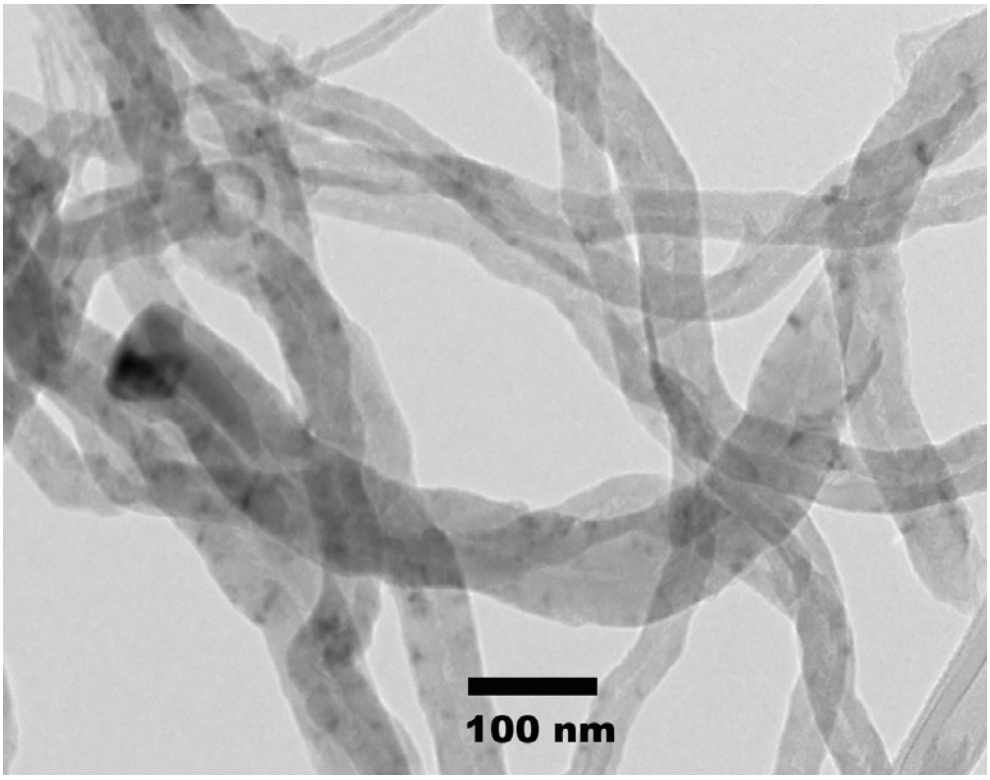

Figure 3.2: Transmission electron micrograph of multi-walled carbon nanotubes (30 kx magnification).

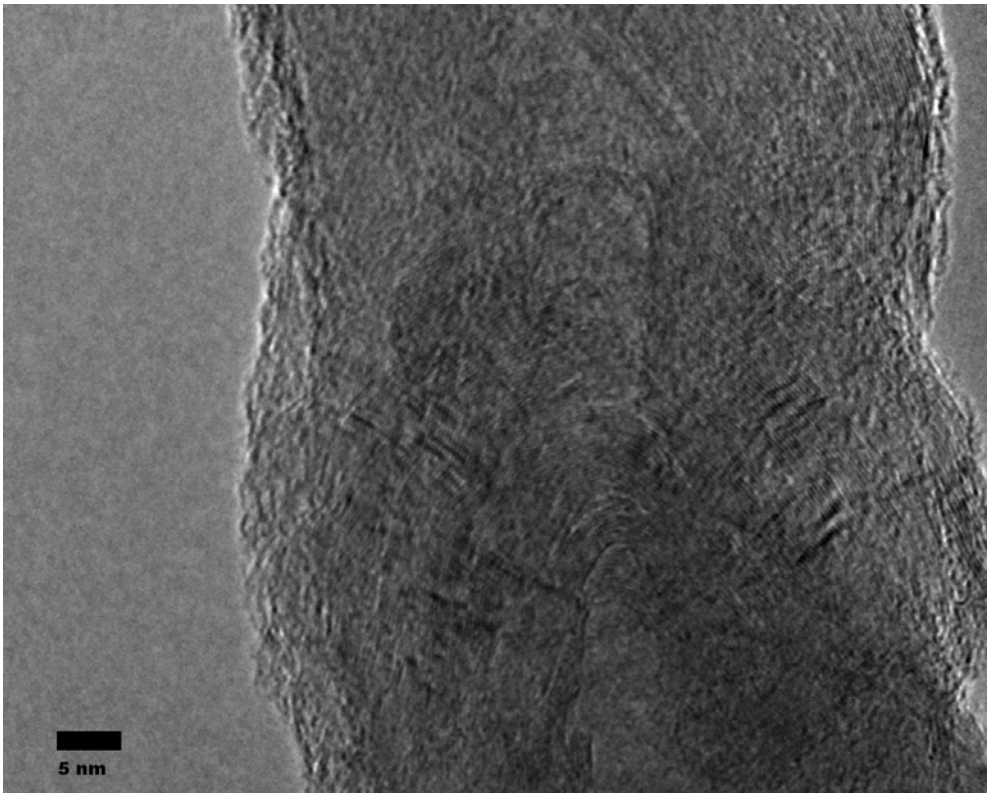

Figure 3.3: High-resolution transmission electron micrographs of multi-walled carbon nanotubes (300 kx magnification).

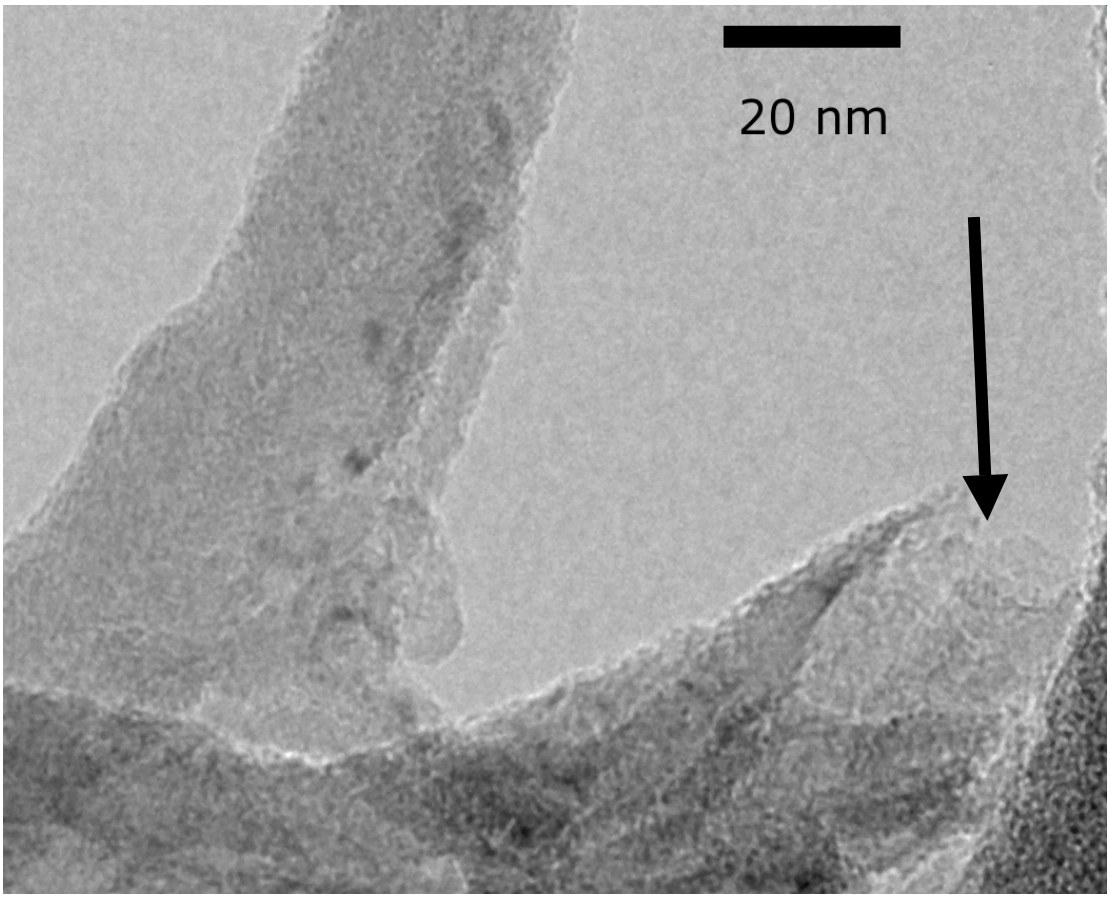

Figure 3.4: Transmission electron micrograph of multi-walled carbon nanotubes treated with a 3:1 mixture of sulfuric to nitric acid (150 kx magnification). The arrow points to an opened end of one of the multi-walled carbon nanotubes.

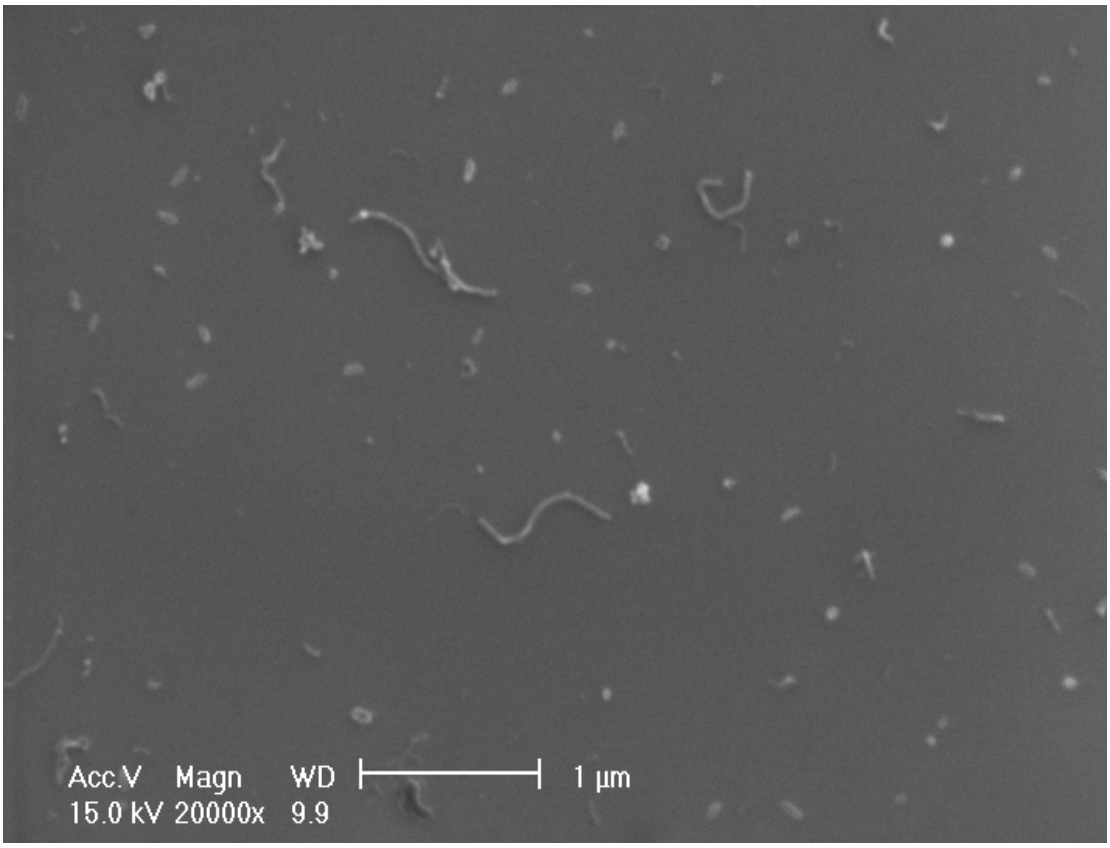

Figure 3.5: Scanning electron micrograph of HCl purified multi-walled carbon nanotubes (20 kx magnification).

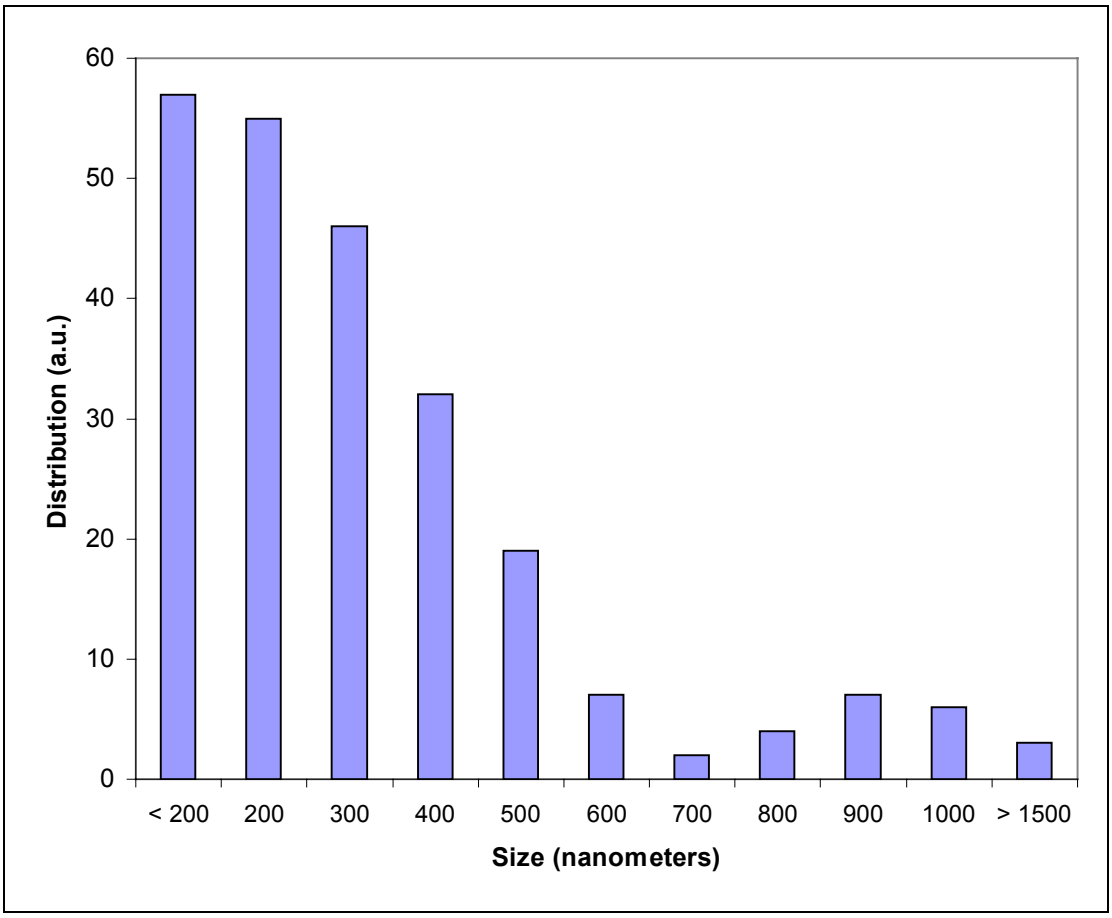

Figure 3.6: Length distribution plot of HCl purified multi-walled carbon nanotubes (n=239).

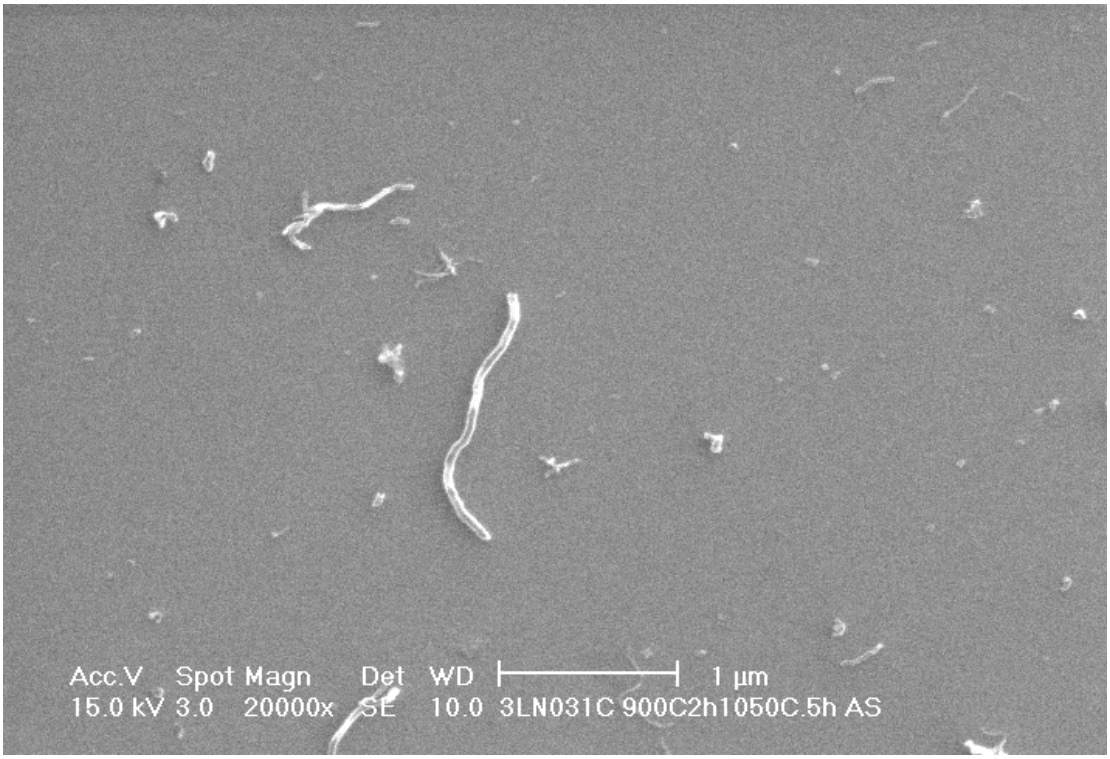

Figure 3.7: Scanning electron micrograph of 3:1 acid mixture treated multi-walled carbon nanotubes.

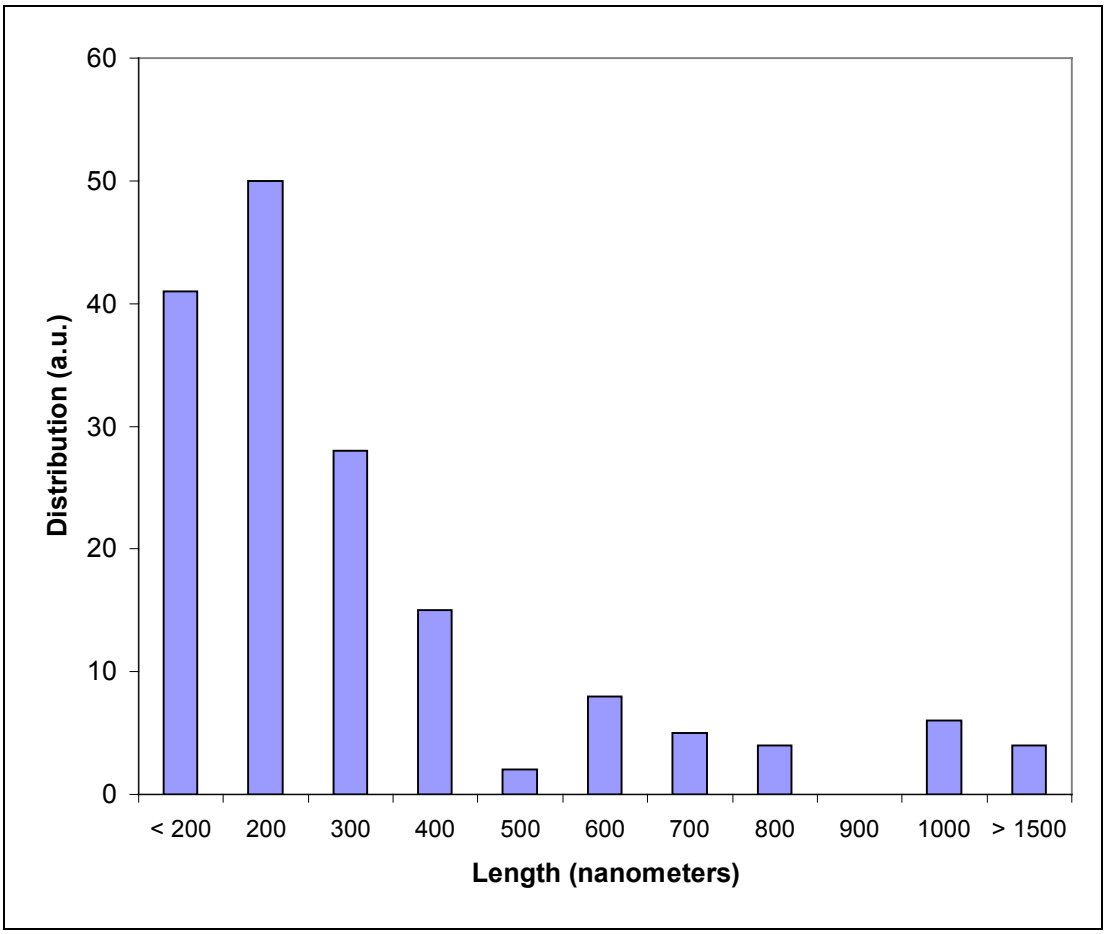

Figure 3.8: Length distribution plot of 3:1 acid mixture treated multi-walled carbon nanotubes (n=165).

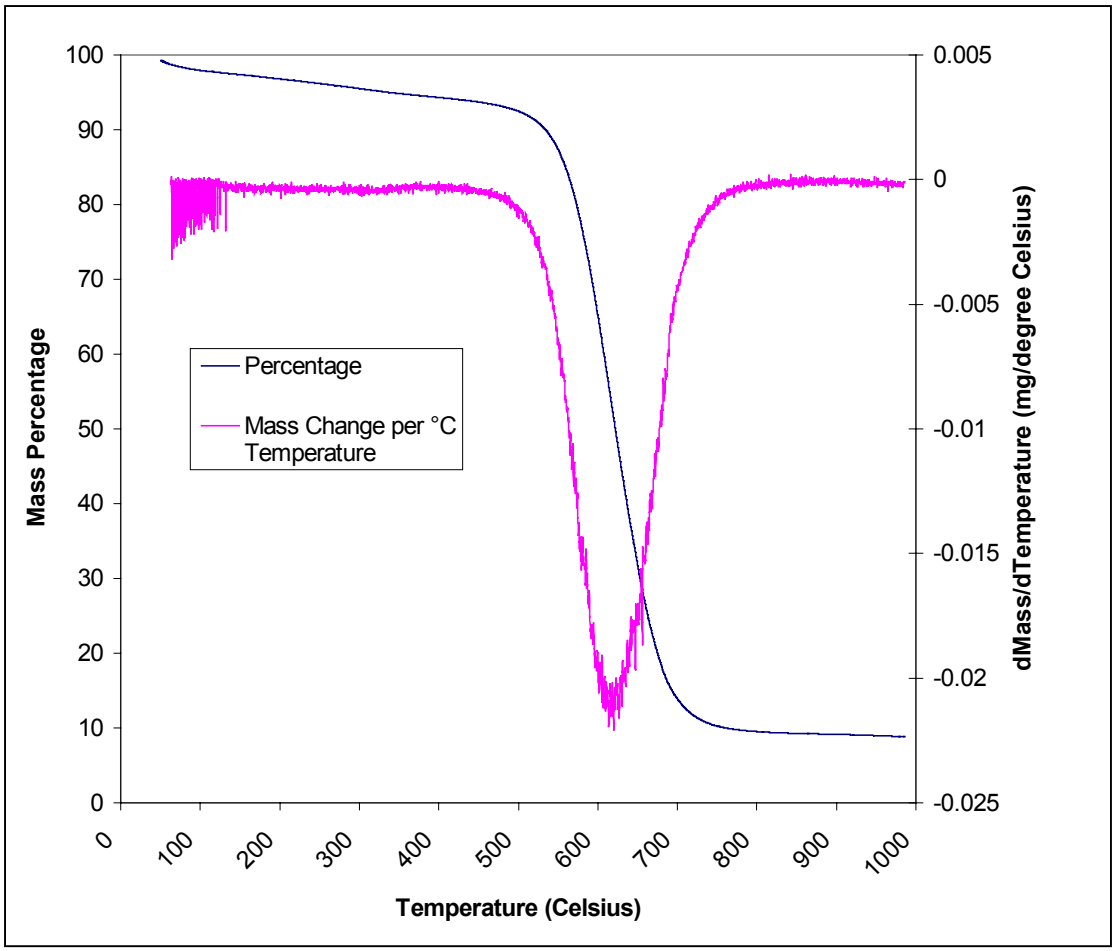

Figure 3.9: Thermal gravimetric analyzer graph of purified single-walled carbon nanotubes.

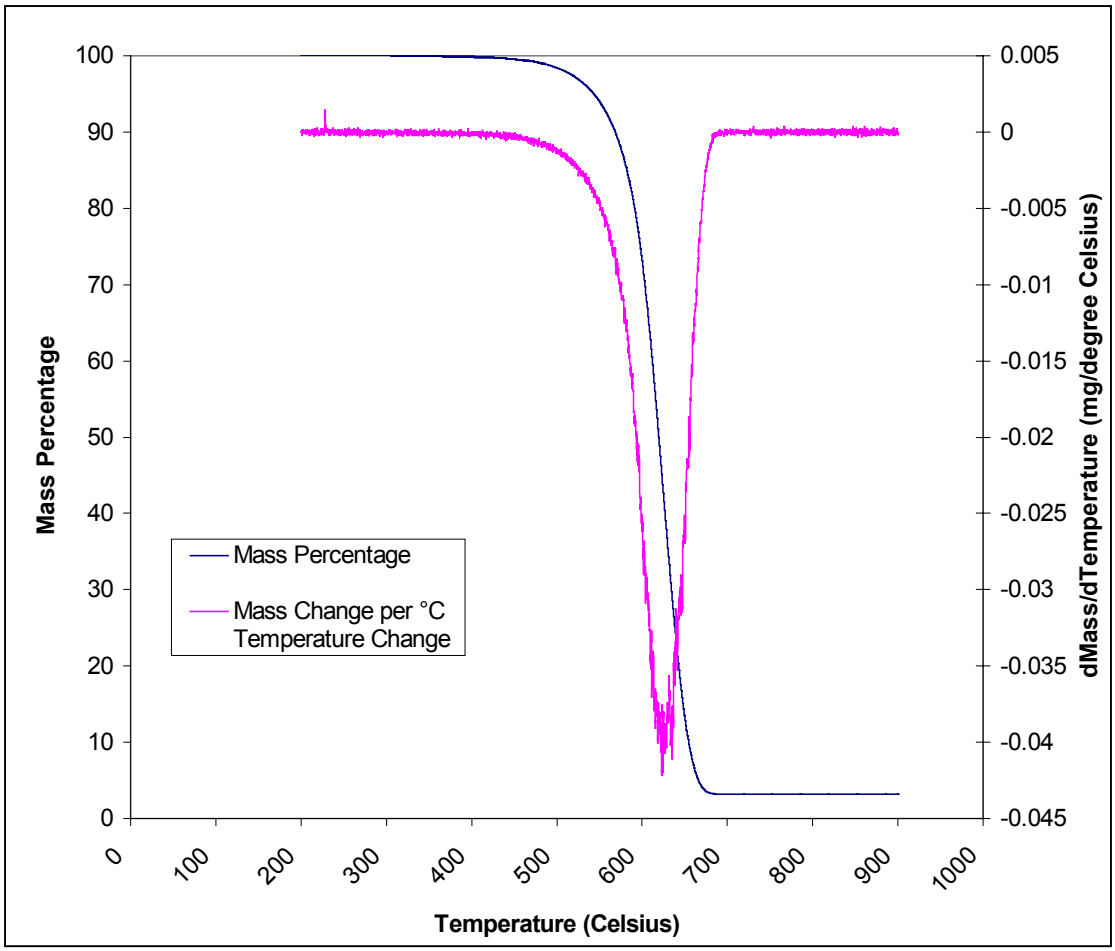

Figure 3.10: Thermal gravimetric analyzer graph of 6N nitric acid purified multi-walled carbon nanotubes.

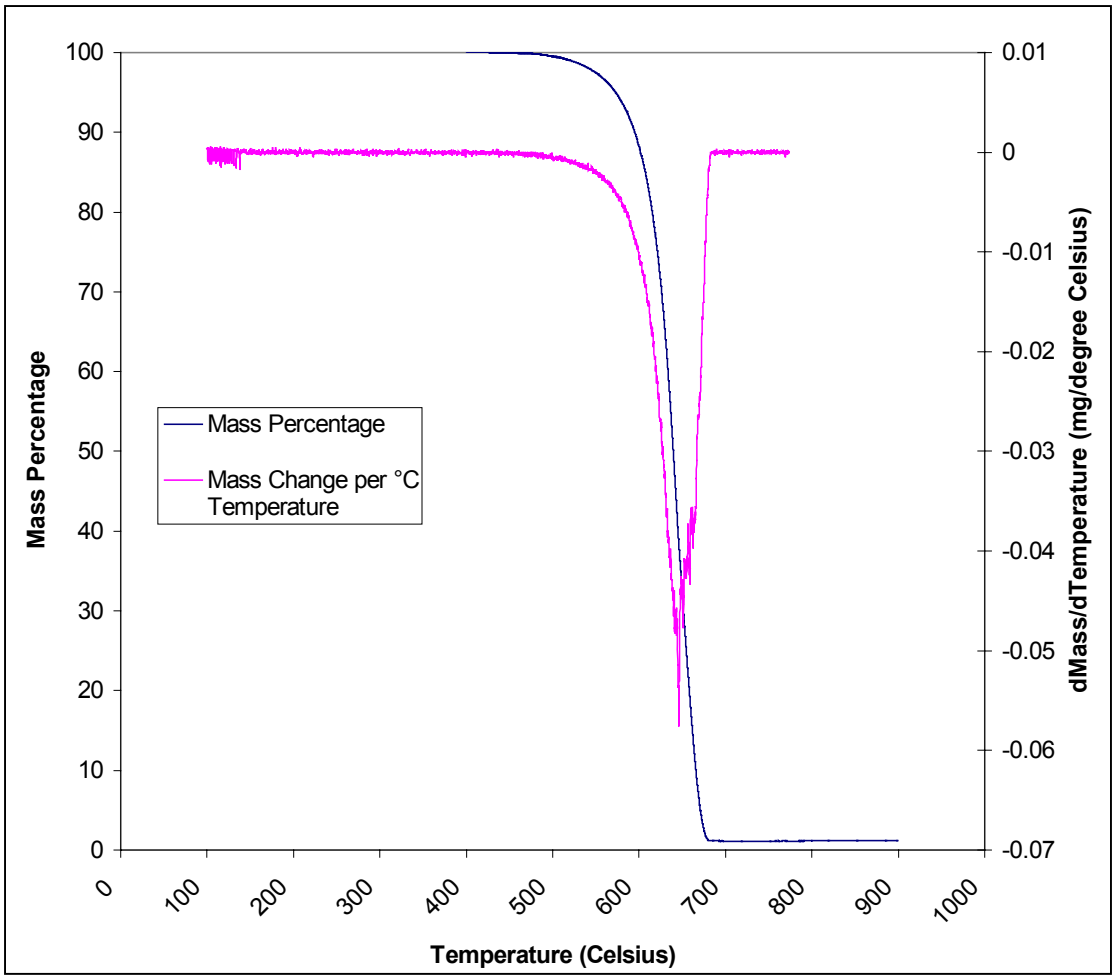

Figure 3.11: Thermal gravimetric analyzer graph of HCl purified multi-walled carbon nanotubes.

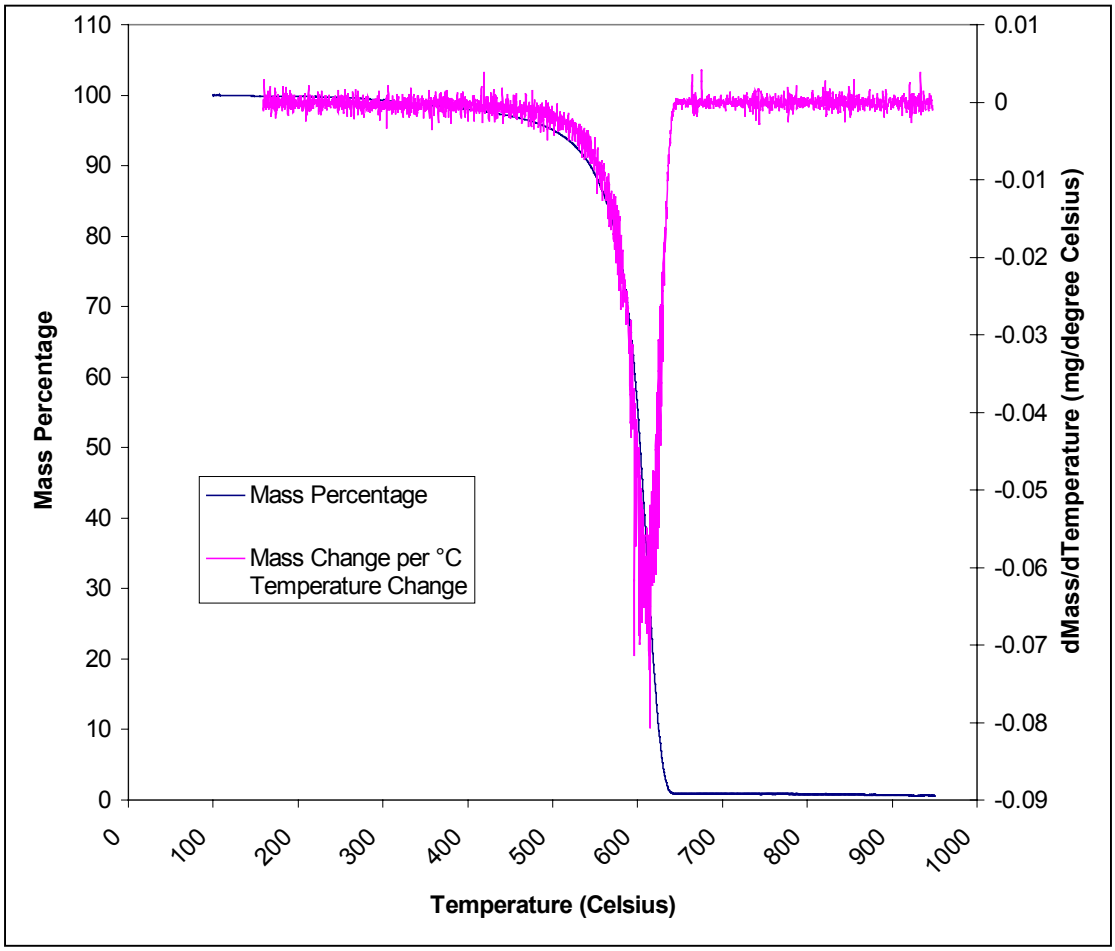

Figure 3.12: Thermal gravimetric analyzer graph of 3:1 modified multi-walled carbon nanotubes.

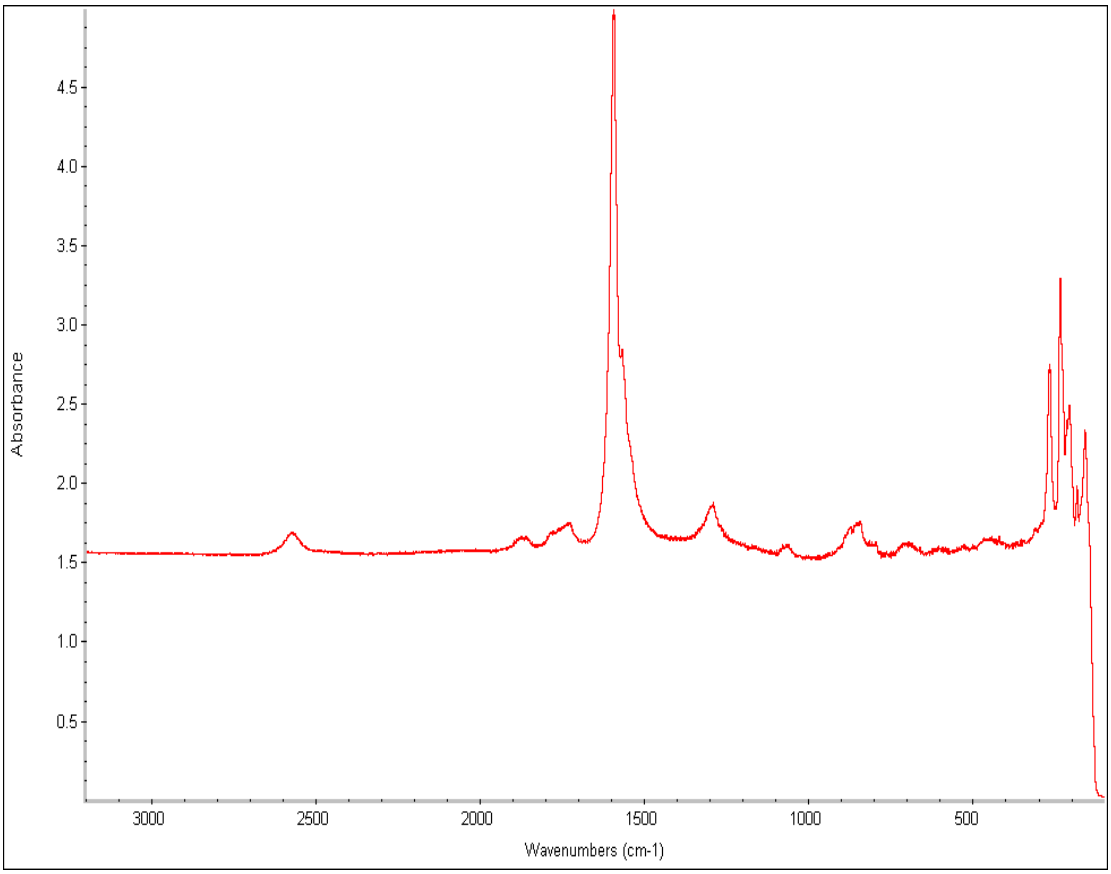

Figure 3.13: Raman spectrum of single-walled carbon nanotubes.

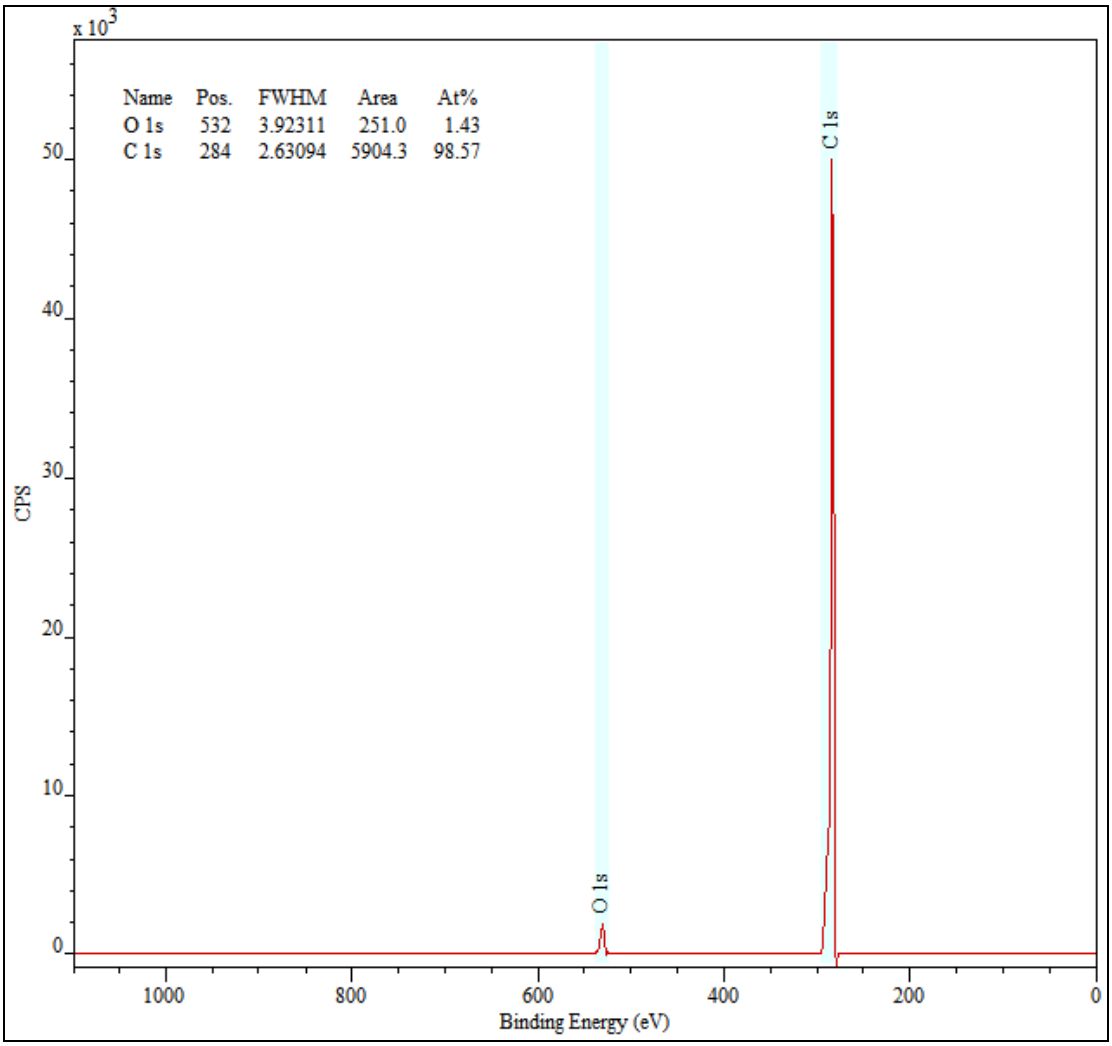

Figure 3.14: X-ray photoelectron spectrum of HCl purified multi-walled carbon nanotubes with elemental analysis.

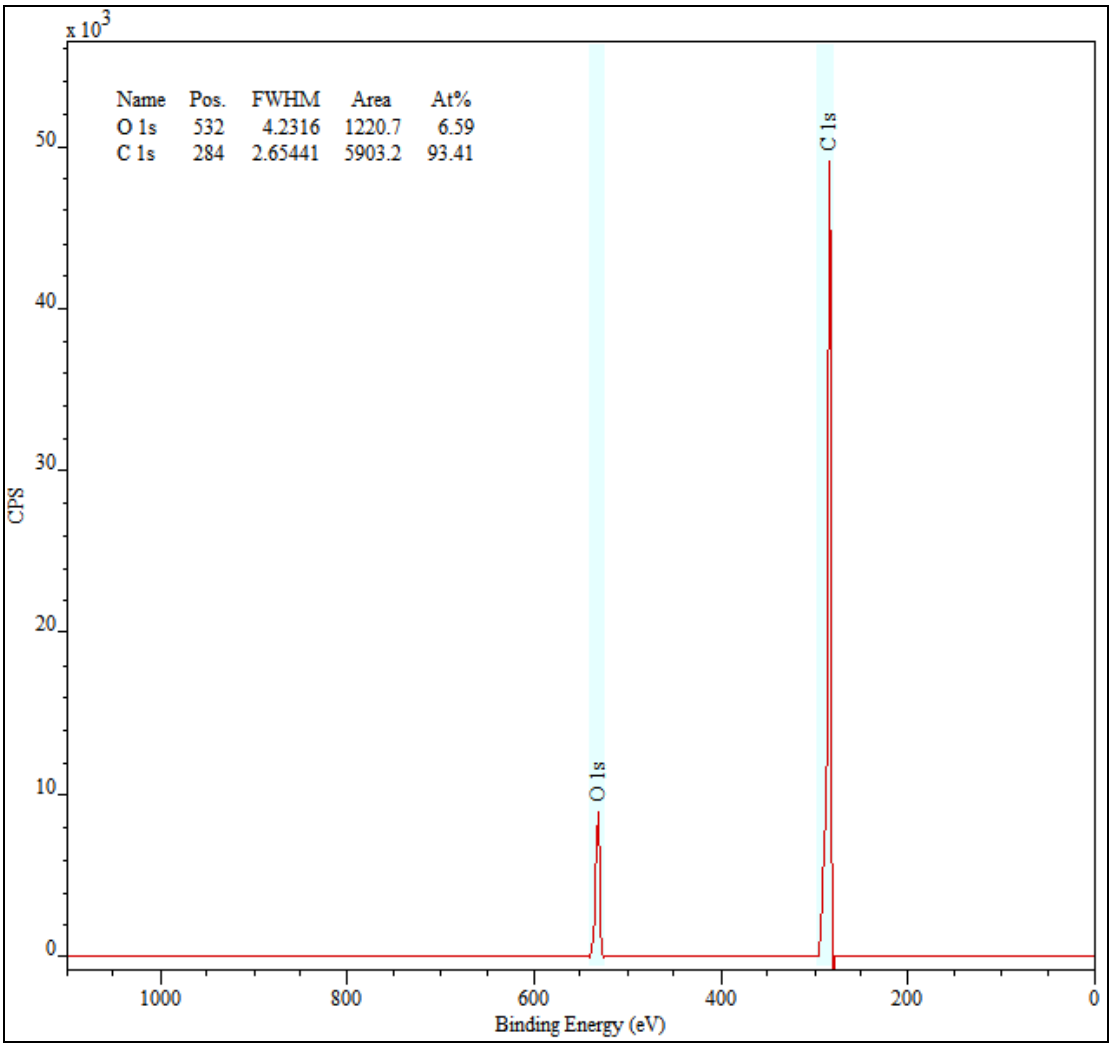

Figure 3.15: X-ray photoelectron spectrum of 3:1 acid mixture modified multi-walled carbon nanotubes with elemental analysis.

	Carbon Purity (Percent)
6N Nitric Acid Purified MWNTs	96.9 ± 0.1
HCl Purified MWNTs	98.9 ± 0.01
3:1 Acid Mixture Modified MWNTs	99.86 ± 0.24
HCl Purified SWNTs	92.1 ± 0.4

Table 3.1: Summary of carbon purity for different carbon nanotube samples determined using thermal gravimetric analysis.

Chapter 4

MULTI-WALLED CARBON NANOTUBE ASSIMILATION BY HELA CELLS

4.1 Introduction

Synthesis procedures for carbon-14 nanotubes were described in Chapter 2 and nanotubes synthesized using these chemical vapor deposition methods thoroughly characterized in Chapter 3. These radioactively labeled carbon nanotubes promise a critical new tool in assessing the cytotoxicity of carbon nanotubes by allowing the exact quantification of nanotubes in cells.

There have been numerous toxicological investigations on carbon nanotubes including inhalation (Lam et al. 2004; Warheit et al. 2004), dermal (Shvedova et al. 2003; Manna et al. 2005; Monteiro-Riviere et al. 2005), and cellular exposure effects (Shvedova et al. 2003; Cui et al. 2005; Jia et al. 2005; Manna et al. 2005; Monteiro-Riviere et al. 2005; Bottini et al. 2006; Sayes et al. 2006; Becker et al. 2007). Much of this work has been severely hindered, however, by an inability to accurately quantify masses of carbon nanotubes in biological systems, thus preventing direct linkages of toxicological responses to carbon nanotube concentrations in cells or organs.

The current limitations of analytic techniques for carbon nanotube quantification were described at length in sections 1.1.6 and 3.1.

Several researchers have attempted to investigate the cellular uptake of modified or functionalized carbon nanotubes (Cherukuri et al. 2004; Kam et al. 2004; Heller et al. 2005a; Monteiro-Riviere et al. 2005; Kam et al. 2006; Becker et al. 2007; Kostarelos et al. 2007). Most of these researchers have assessed the extent to which carbon nanotubes can enter cells instead of quantifying their accumulation by cells. The sole research investigation on the uptake rates of CNTs by cells showed that the uptake rate of surfactant-modified SWNTS was relatively constant across a 24-hour uptake period (Cherukuri et al. 2004). These results yielded valuable evidence for those hoping to use such nanotubes for biological applications, but unmodified nanotubes will also likely be released into environmental systems and the extent to which their cellular interactions would differ from those with various functionalizations is of critical interest for those investigating the potential environmental and human health risks of this nanomaterial.

Here I show that carbon-14 MWNTs can be successfully used to precisely measure CNT concentrations in biological samples. MWNTs were dispersed in cellular solution and their assimilation rates determined for HeLa cells, a widely used epithelial cell from a carcinoma cell line; assimilation here refers to the combination of strong attachment and cellular internalization. Results using these cells give indications for cellular interactions of carbon nanotubes with other environmentally critical cells such as the epithelial cells

in organism's digestive tracts, especially given the similar uptake behaviors shown by Kostarelos et al. (2007) using a broad range of cells. Unlike the highly modified nanotubes used in the broad majority of previous cellular investigations, the MWNTs used here were only briefly treated by purification in 6N nitric acid prior to cell tests. As such, this investigation provides crucial evidence for how unmodified carbon nanotubes would interact with cells.

4.2. Methods

As described in detail in section 2.6, MWNTs were suspended in cellular media consisting of Dulbecco's modified eagle's medium, fetal bovine serum, and antiobitics. This solution was decanted to remove the undispersed nanotubes, and the solution then mechanically mixed to produce a homogenized slurry. Eight milliliter aliquots of this solution were added to plates with confluent HeLa cells and the cells incubated with this solution for various times. After the predetermined exposure interval, the cell media was decanted and the cells washed with ice-cold phosphate buffered solution (PBS). A second rinsing with this solution did not show the removal of additional nanotubes, and preliminary tests indicated that washing with PBS solution was sufficient to remove carbon nanotubes from the polystyrene plates. The cells were then removed from the plate using trypsin-EDTA, captured on filter paper, combusted, and their radioactivity assessed using scintillation counting. The extent to which carbon nanotubes would be released from the cells after the replacement of the nanotube solution with clean cell solution was also assessed.

4.3 Results and Discussion

As shown in Figure 4.1, HeLa cells rapidly accumulated the multi-walled nanotubes. The fraction of carbon nanotubes that entered the cells versus the fraction that were strongly bound to the cell surface was not investigated here, and these results represent the combination of cellular uptake and attachment. Seventy-four percent of the nanotubes added to each plate were assimilated within the first 15 minutes, and after 6 hrs, the cellular concentration reached a maximum at eighty-nine percent of the nanotubes added. The uptake of the nanotubes by the HeLa cells appeared also to be nearly irreversible. Only 0.9 ± 0.5% of the carbon nanotubes accumulated by the cells after the 12 hr uptake period were released during a subsequent 12 hr period during which the cells were incubated with regular media. Despite differences in the dispersion techniques and carbon nanotube types involved, this result agrees with those obtained by Strano and coworkers (Heller et al. 2005a), who determined that single-walled carbon nanotubes wrapped with DNA remained in murine myoblast stem cells for the duration of a three month time period. These researchers utilized raman spectroscopy to qualitatively confirm the continued presence of nanotubes within these cells.

This rapid and nearly complete uptake of multi-walled carbon nanotubes by the HeLa cells differs substantially from that estimated for single-walled carbon nanotubes solubilized using a Pluronic surfactant and mouse peritoneal macrophage-like cells (Cherukuri et al. 2004). The

investigators in that study observed a relatively constant rate of carbon nanotube accumulation by the cells over a 24 hr time period. While numerous factors (e.g., cell type, carbon nanotube type, and carbon nanotube concentration) might have influenced these results, the disparities in uptake rates could also stem, at least partially, from different analytical approaches used to measure nanotube concentrations. Labeling carbon nanotubes with the carbon-14 isotope provides a straightforward means for quantitatively measuring carbon nanotubes at minute concentrations in a wide variety of media, regardless of changes in nanotube agglomeration state, physical or chemical properties, or aquatic conditions. Conversely, aqueous conditions and dispersion states of the carbon nanotubes can impact their detection using spectrofluorimetry (O'Connell et al. 2002). That approach is capable only of detecting semiconducting single-walled carbon nanotubes and neglects metallic ones, and is unable to detect agglomerations containing semiconducting and metallic nanotubes because the latter quench fluorescence from the former. Thus, to quantify carbon nanotube concentrations in phagocyte cells, the authors extrapolated the spectral activity of the semiconducting nanotubes to both semiconducting and metallic nanotubes.

Another potential difference between these two studies is that there did appear to be some MWNT settling during the first fifteen minutes after addition to the cell culture plates. The ability of the PBS washings to completely remove carbon nanotubes from the culture plates, however,

suggests that these settled nanotubes were internalized or strongly attached to the cells. Nevertheless, this rapid settling may have introduced more contact between the cells in carbon nanotubes than as experienced in the experiments by Cherukuri et al. (2004), for which the nanotubes were indicated to remain fully dispersed. This difference may also partly explain the more rapid initial cellular assimilation of the carbon nanotubes in this study.

These results also accord with those by Kostarelos et al. (2007) in that these unmodified nanotubes appeared to have strong cellular interactions. While future work is necessary to quantify the extent to which the broad array of nanotubes used by those authors would enter cells, it appears that cellular assimilation of carbon nanotubes is not dependent upon surface modifications. Nevertheless, uptake mechanisms for carbon nanotubes are still unclear and may vary based on the nanotube modification and cell type. The assimilation rates and capacities of different types of cells for various types of CNTs are unknown, but the radioactive nanotubes developed here would be ideal for such investigations.

Upon release into environmental systems, these results suggest rapid cellular attachment of nanotubes or uptake by dermal or digestive cells of humans or ecological receptors and hence the potential for significant bioaccumulation. Passage through cells and tissues is necessary though for the nanoparticles to enter systemic circulation in the organisms, and the extent to which and the rate at which nanotubes travel across these various

tissues is unknown. If nanotubes only become attached to or entered the outermost cells, the periodic sloughing off of these cells may mitigate the nanotubes' toxic effects and bioaccumulation would not be expected. Another complicating factor between cellular investigations and the actual ecotoxicological impacts of nanotubes is the state at which nanotubes would be present in environmental systems. Nanotubes may settle out of aqueous solutions in aquatic ecosystems, become strongly attached to soil or sediment organic matter, or may interact with natural organic matter (Hyung et al. 2007). Any of these changes to the physicochemical properties or aggregation state of the nanotubes may affect their environmental behaviors. Nevertheless, the results shown here for unmodified nanotubes and those by others for nanotubes with various physical and chemical properties demonstrate strong interactions between nanotubes and cells and indicate that understanding the bioaccumulation potential of nanotubes represents a critical research topic.

4.4 Summary

Purified multi-walled carbon nanotubes were shown to rapidly become assimilated with HeLa cells in time intervals as short as 15 minutes. These carbon nanotubes also appeared to be relatively irreversibly bound to the carbon nanotubes with less than 1% of the accumulated carbon nanotubes being released by the cells after 12 hours of exposure to clean cell media. These results and those by others (Cherukuri et al. 2004; Monteiro-Riviere et al. 2005; Kam et al. 2006; Kostarelos et al. 2007) suggest that a broad range

of cells can internalize or strongly attach single- and multi-walled carbon nanotubes. As such, the potential for accumulation of carbon nanotubes by humans and ecological receptors represents a significant research concern. Some researchers have investigated the biodistribution of intraveneously injected functionalized carbon nanotubes (Wang et al. 2004; Cherukuri et al. 2006; Singh et al. 2006; Liu et al. 2007a) and have generally found them not to accumulate in the organisms, although Liu et al. (2007) found significantly slower clearance and accumulation in the liver after 24 hours. The impact of functional groups and exposure pathway (i.e., intravenous injection versus oral exposures through contaminated water, soil, or food) is unclear, and the applicability of these studies to the biological uptake of carbon nanotubes in environmentally relevant settings is thus likely quite limited.

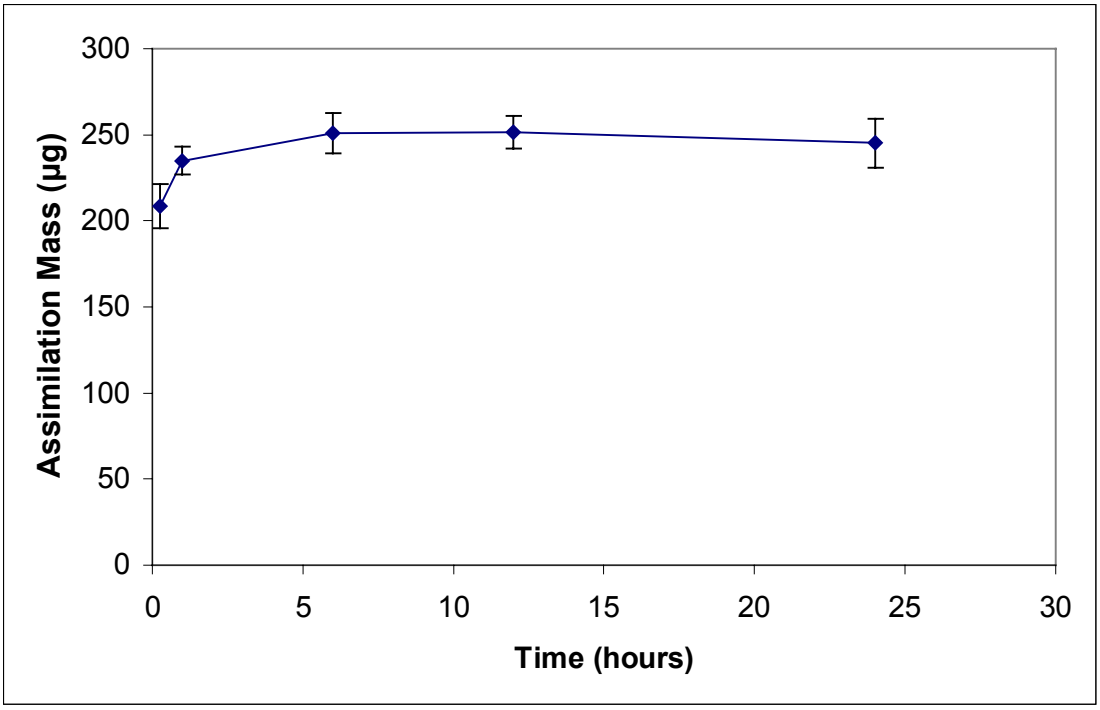

Figure 4.1: Assimilation of multi-walled carbon nanotubes by HeLa cells. An 8-mL volume of cellular media having a carbon nanotube concentration of 313 µg/ plate was added to each plate. Results are mean ± SD of triplicate plates.

Chapter 5

ECOLOGICAL UPTAKE AND DEPURATION OF CARBON NANOTUBES BY *LUMBRICULUS VARIEGATUS*

5.1 Introduction

Although carbon nanotubes have drawn widespread research attention in recent years, their potential environmental and human health impacts have not been well characterized, and the risks they may pose to the welfare of humankind and the environment are largely unknown (Colvin 2003). Carbon nanotubes are molecules containing extensive sp^2 hybrized carbons arranged in fused benzene rings. One potential approach for predicting their environmental behaviors would be through comparison to their counterparts of smaller sizes having between two to seven aromatic rings, often called polycyclic aromatic hydrocarbons (PAHs). These compounds are known to readily accumulate in organisms' fatty tissue in large part as a result of their hydrophobicity and resistance to microbial degradation (Di Toro et al. 1991; Jager et al. 2003a). Experimental evidence from the biouptakes of PAHs by 27 species of benthic organisms suggests that biota-sediment accumulation factors (BSAFs) of PAHs do not widely vary based on their octanol-water

partitioning values (Tracey and Hansen 1996). This leads to the conjecture that carbon nanotubes may be highly bioaccumulable molecules, which would have profound implications in an ecological and human health context.

Additional support for the bioaccumulation potential of carbon nanotubes comes from the cellular uptake literature and the work conducted in Chapter 4 investigating cell assimilation by HeLa cells. Numerous studies have indicated that carbon nanotubes can enter cells (Cherukuri et al. 2004; Kam et al. 2004; Heller et al. 2005a; Monteiro-Riviere et al. 2005; Kam et al. 2006; Kostarelos et al. 2007) and cause toxic damage to cells (Shvedova et al. 2003; Cui et al. 2005; Ding et al. 2005; Jia et al. 2005; Manna et al. 2005; Monteiro-Riviere et al. 2005; Bottini et al. 2006; Sayes et al. 2006; Pulskamp et al. 2007). Carbon nanotubes have also been shown to cause toxic effects to aquatic organisms (Templeton et al. 2006; Cheng et al. 2007; Roberts et al. 2007; Smith et al. 2007), and SWNTs, for example, have been qualitatively detected in *Daphnia magna* (Roberts et al. 2007) and fish (Smith et al. 2007). The extent to which carbon nanotubes released into the environment may be accumulated by ecological receptors, however, is unknown. If organisms uptake these compounds, they could then be transferred throughout food chains and could enter organisms at higher trophic levels such as humans at significant concentrations, thus posing profound ecological and human health risks.

In order to accurately evaluate the extent to which carbon nanotubes released into the environment bioaccumulate in organisms, ^{14}C-labeled

carbon nanotubes (both single- and multi-walled) were synthesized using modified chemical vapor deposition procedures as described in Chapter 3. This radioactive labeling process overcomes a formidable challenge in determining the uptake of nanotubes: the current lack of a method to quantify both individual and bundles of unmodified single- or multi-walled carbon nanotubes in environmental or biological systems. The ability of these radioactively labeled nanotubes to give quantitative results for nanotube concentrations in biological samples was determined in Chapter 4. In this chapter, these nanotubes were spiked to sediments, and assessed with respect to their uptakes by *Lumbriculus variegatus*, a sediment-burrowing oligochaete. Oligochaetes have been used extensively as bioindicators of pollution (Lauritsen et al. 1985) and *L. variegatus* has been selected by the U.S. Environmental Protection Agency as the freshwater organism for assessing bioaccumulation (U.S. EPA Office of Water 2000).

5.2 Methods

Full experimental details are described in section 2.7. Briefly, radioactively labeled multi-walled carbon nanotubes (MWNTs) and single-walled carbon nanotubes were synthesized via modified chemical vapor deposition methods using mixtures of unlabeled and ^{14}C-labeled methane as feedstock gases (Chen et al. 1997; Li et al. 2002). The nanotubes so produced were then purified by bath-sonication in full-strength hydrochloric acid for 1 hr. The radioactivity of the synthesized nanotubes, spiked sediments, and aquatic worms were determined via combustion in a

biological oxidizer (OX 500, R. J. Harvey Instrumentation Corporation), and the cocktail then analyzed using a LS6500 liquid scintillation counter (Beckman). Carbon-14 labeled SWNTs, MWNTs, or combinations of regular and carbon-14 pyrene dissolved in acetone were added either to mixtures of 90% sediment (Huron River, Ann Arbor, MI) with 10% Michigan Peat (by mass) or to unamended sediment. These sediment samples were then thoroughly mixed, the acetone evaporated for the pyrene samples, and the sediments then added to beakers with *Lumbriculus variegatus*. After predetermined intervals for the uptake experiments, the worms were removed and placed into containers with clean water, and allowed to purge their guts for 6 hrs. After drying, the radioactivity in the worms was measured as described above. For depuration experiments, the worms were removed from the spiked sediments and placed in beakers either with fresh water or fresh water and clean sediment. Radioactivity in the worms was measured after each depuration interval. Containers with sediment spiked with non-radioactive SWNTs, MWNTs, or pyrene were used as blank controls to assess acute toxicity and measure lipid content (Van Handel 1985). Biota-sediment accumulation factors (BSAFs) were calculated as the ratio of the compound concentration in organism normalized by its lipid fraction to concentration in sediment normalized by its organic carbon fraction.

5.3 Results and Discussion

5.3.1 Uptake Experiments

Despite compositions of fused benzene rings similar to those of pyrene, the BSAF values for *L. variegatus of the* SWNT and MWNT were almost an order of magnitude lower than those for pyrene, as illustrated in Figure 5.1. The uptake data do not indicate systematic differences between the SWNTs and MWNTs. The SWNTs may have been present as bundles, as indicated in Figure 3.1, with apparent diameters approaching those of the MWNTs thus causing similar uptake. BSAF values for worms exposed to sediments spiked with SWNTs, MWNTs, and pyrene for 28d were 0.28 ± 0.03, 0.40 ± 0.1, and 3.6 ± 0.2, respectively. BSAF values for 16 different PAHs of broadly varying hydrophobicities exposed to sediments for 28d and with a depuration interval of 12 hrs have been shown to range from 0.4 to 5 (Ingersoll et al. 2003), thus confirming low uptakes for carbon nanotubes relative to those for PAH compounds.

Subsequent experiments in which the organic carbon content of the sediment was decreased by a factor of 8 by removal of the Michigan Peat amendment were performed. In these experiments a decrease in BSAF values after 14 days of exposure from 0.51 ± 0.09 to 0.035 ± 0.015 was observed. Assuming that partitioning processes leading to an eventual bioaccumulation of such compounds by organisms depend upon a thermodynamic equilibrium between sediment organic carbon and organism lipid phases being reached, the fraction of organic carbon in the sediment

would not be expected to affect BSAF values for nonionic organic chemicals (Di Toro et al. 1991). The observed changes in BSAF values are inconsistent with this understanding of hydrophobic organic chemical uptake, suggesting that the nanotubes detected have not been absorbed into organism tissues, but rather are associated with sediment matter remaining in the organism's gut.

Interestingly, standard deviations of BSAF values for the carbon nanotubes are all significantly larger than those for pyrene. This result may support the notion that a significant fraction of the radioactivity detected in the aquatic worms was from sediment-associated nanotubes not purged from the organisms after 6 hrs of depuration, a parameter that would reasonably vary over a greater range than that of absorption by tissues. This variability may also stem from greater heterogeneities of carbon nanotube distributions in the sediment. While all pyrene was dissolved in acetone prior to spiking, some carbon nanotubes were not fully dispersed by sonication. Larger aggregates of carbon nanotubes may then have caused small regions of elevated nanotube concentration.

Increases in the mortality of *L. variegatus* exposed to sediments containing SWNTs, MWNTs, or pyrene compared to unspiked sediments were not observed at the concentrations and exposure durations investigated here. Measurement of acute toxicity across a broad range of nanotube concentrations was not attempted.

5.3.2 Depuration Experiments

How rapidly the organisms studied purged carbon nanotubes from their bodies was also investigated (Figure 5.2). After roughly three days of depuration in beakers containing only water, the organisms had purged over 80% of the single- or multi-walled nanotubes remaining in the worms after the initial 6 hrs of depuration, while only 13% of the pyrene was excreted after the same interval. The relatively slow depuration of pyrene is attributed to low rates of clearance from organism tissues compared to rates of sediment gut purging. Conversely, the rapid elimination of the carbon nanotubes suggests that the major fraction of carbon nanotubes present in the worms after the initial 6 hrs of depuration comprised nanotubes associated with residual gut sediment. Depuration rates of MWNTs in beakers containing both water and clean sediment were significantly faster than those in beakers containing only water, suggesting that the worms would almost completely purge the carbon nanotubes from their systems after a few days of exposure to water and clean sediments. Concentrations of nanotubes detected in organisms were below background concentration levels after two days of depuration in clean sediment dispersions in water.

5.4 Summary

We show here that biota-sediment accumulation factors for purified CNTs of both single- and multi-walled nature by a common aquatic oligochaete, *Lumbriculus variegates,* are shown here to in fact be lower by nearly an order of magnitude than those for pyrene, a three-ringed PAH.

CNTs detected in the test organism appear to be associated predominantly with sediment materials accumulated in its gut, rather than being absorbed into its tissues, importantly suggesting that unmodified carbon nanotubes released into sediment ecosystems may, unlike PAHs, not readily bioaccumulate in aquatic organism tissues and thus not magnify in associated food chains. Explanations for the limited nanotube uptake compared to that of pyrene are explored in detail in Chapter 6.

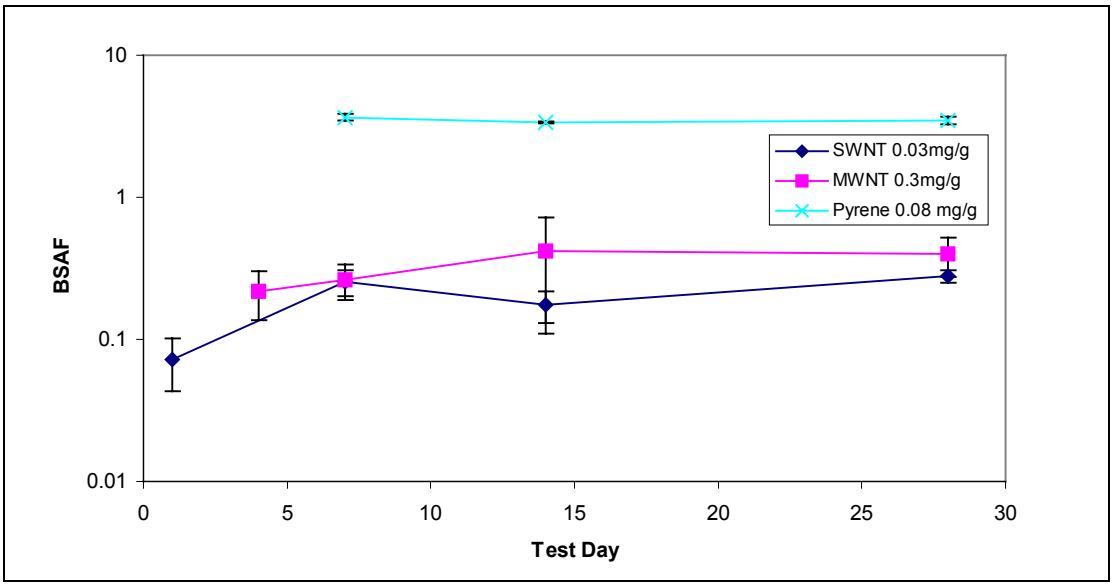

Figure 5.1: Biota-sediment accumulation factors (BSAFs) of single-walled carbon nanotubes (SWNT) (0.03 mg/g dry sediment), multi-walled carbon nanotubes (MWNT) (0.37 mg/g dry sediment) and pyrene (0.054 mg/g dry sediment) uptake by *L. variegatus.* All compounds were spiked to mixtures of 90% sediment (Ann Arbor, MI) with 10% Michigan Peat (by mass). Error bars represent one standard deviation (n=3).

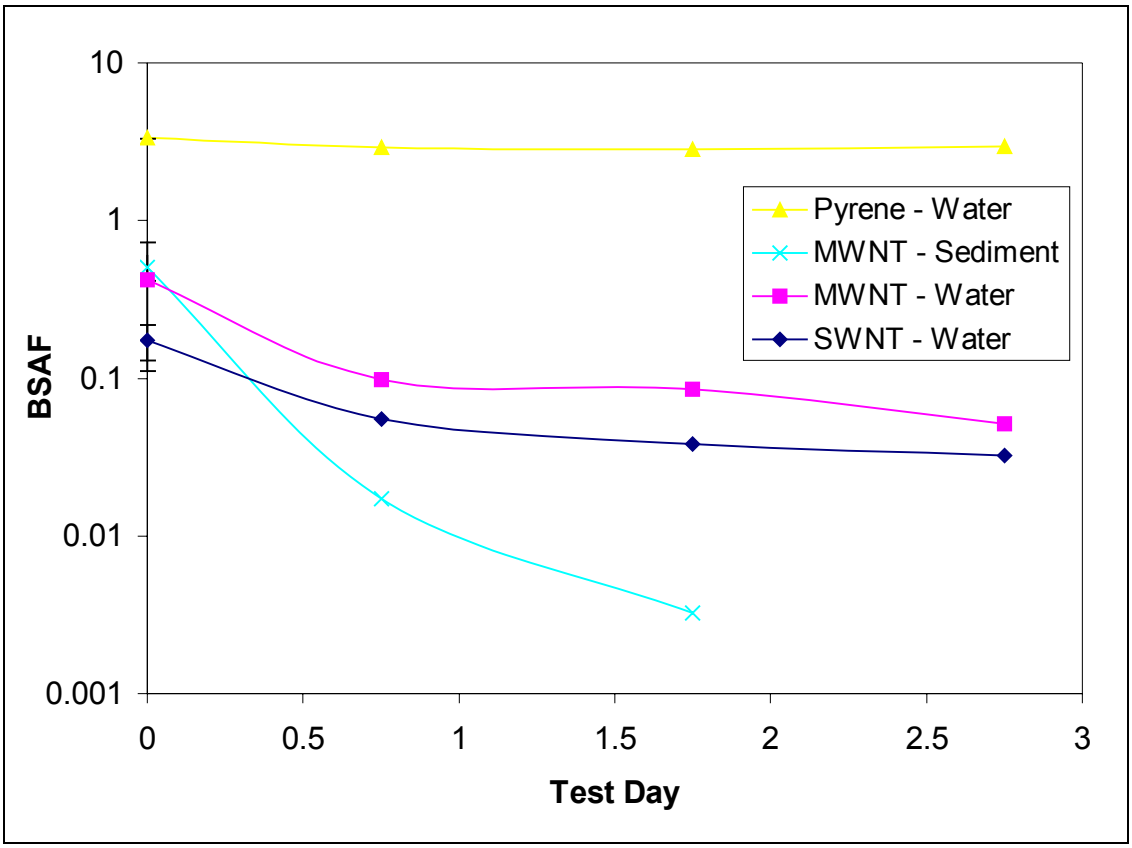

Figure 5.2: Biota-sediment accumulation factors (BSAFs) of single-walled carbon nanotubes (SWNT) (0.03 mg/g dry sediment), multi-walled carbon nanotubes (MWNT) (0.37 mg/g dry sediment) and pyrene (0.054 mg/g dry sediment) depuration by *L. variegatus.* "Water" indicates samples for which the depuration was conducted in beakers with only water, while "Sediment" indicates that the depuration was conducted in beakers with water and 50g clean sediment. Times represent the depuration period after the standard 6 hrs for gut clearance. Error bars represent one standard deviation (n=3).

	BSAF
Pyrene (0.05 mg/g)	3.353 ± 0.050
MWNT #1 (0.37 mg/g)	0.418 ± 0.308
MWNT #2 (0.37 mg/g)	0.506 ± 0.092
MWNT (0.037 mg/g)	0.370 ± 0.093
SWNT (0.03 mg/g)	0.174 ± 0.045
SWNT (0.003 mg/g)	0.141 ± 0.006
MWNT Sediment Only (0.37 mg/g)	0.035 ± 0.015

Table 5.1: Biota-sediment accumulation factors (BSAFs) for *L. variegatus* uptake after 14 days of exposure. The term "Sediment Only" refers to a sample where the oligochaetes were exposed in sediment without the amendment of 10% (by dry mass) Michigan Peat. Means and standard deviations are the result of triplicate measurements.

Chapter 6

BIOACCUMULATION OF RADIOACTIVELY LABELED CARBON NANOTUBES BY *EISENIA FOETIDA*

6.1 Introduction

Given the widespread interest in carbon nanotubes for new technologies, their release into terrestrial ecosystems is inevitable. Rationale for the potential uptake of carbon nanotubes by organisms in terrestrial ecoystems mirror those discussed in Chapter 5 for aquatic sediment ecosystems. Although *L. variegatus* was not shown to bioaccumulate nanotubes in chapter 5, it is possible that differences in the feeding between earthworms and aquatic worms, and the unknown behaviors of carbon nanotubes in environmental systems could lead to different results for earthworms exposed to soils spiked with carbon nanotubes. Earthworms are often used to assess the bioaccumulation of chemicals in terrestrial ecosystems due to their intimate contact with and ingestion of soil and their frequent consumption by many vertebrate species (Ma et al. 1998; Jager et al. 2000; Krauss et al. 2000). Given these factors, the potential for carbon

nanotubes to enter the earthworm *Eisenia foetida,* a potential entry point to terrestrial food chains, was explored.

6.2 Methods

The uptake of carbon nanotubes or pyrene by *Eisneia foetida* was conducted using a modified ASTM method (ASTM 1998) as described in section 2.8. Carbon-14 labeled multi-walled carbon nanotubes (MWNTs) and single-walled carbon nanotubes (SWNTs) were synthesized and purified as described in section 2.2 according to Chen et al. (1997) and Li et al. (2002). Biological oxidation followed by scintillation counting was used to assess the radioactivity of the synthesized nanotubes, spiked soils, and earthworms. Carbon-14 labeled SWNTs, MWNTs, or combinations of regular and carbon-14 pyrene dissolved in methylene chloride were added to soils from Chelsea or Ypsilanti, Michigan and referred to as “Chelsea” and “Ypsilanti” soils, soils that possessed organic carbon fractions of 5.95%, 1.14%, respectively. These soil samples were then tumbled, solvent volatilization allowed overnight, and three adult earthworms added to moist containers (20% moisture content) with 30 g dry weight soil. Worms were removed from triplicate containers after 1, 7, 14, and 28 d for the Chelsea soil and after 14 days for the Ypsilanti soil. After removal, the earthworms were washed with Milli-Q water, transferred to wet filter paper in Petri dishes for 24 hrs in the dark to allow purging of gut contents, and again rinsed with clean Milli-Q water until the radioactivity of the water had a background radioactivity concentration. The worms were then transferred to glass centrifuge tubes,

freeze-dried for 24 hrs, weighed, combusted in a biological oxidizer, and the radioactivity determined using liquid scintillation counting. After exposure for 14d, the earthworms were removed from three containers and added to containers with unspiked soils to allow for depuration. After depuration for 1, 2, or 7 d, the worms were removed from their containers and the radioactivity remaining in the worms determined as described above. Containers with sediment spiked with non-radioactive SWNTs, MWNTs, or pyrene were used as blank controls to assess acute toxicity. No toxicity was found with the nanotube and pyrene concentrations and exposure durations used here. Bioaccumulation factor (BAF) values were calculated as the ratio of the compound concentration in organism to concentration in soil.

6.3 Results and Discussion

6.3.1 Uptake Experiments

Bioaccumulation factors (BAF; concentration of the chemical in the worm divided by that in the soil) of the SWNTs and MWNTs by *E. foetida* were almost two orders of magnitude lower than those for pyrene, as shown in Figure 6.1 and Table 6.1. These low levels of uptake may be largely accounted for by carbon nanotubes in soil mass remaining in the worms' guts after depuration. Gut loading (dry weight egesta per dry weight worm) for *Eisenia foetida* was found to be 0.63 ± 0.022 for mineral soil (Hartenstein et al. 1981). A 0.05-fraction of gut content remaining after 24 hours depuration has been reported for *E. foetida*, a value similar to the fraction of gut content (0.056 ± 0.021) remaining for *E. Andrei* after 24 hours depuration (Jager et al.

2003b) . Assuming 5% gut content remaining in *E. foetida* after 24 hours, the BAF for a non-bioaccumulating chemical would be 0.0315 ± 0.001 for *E. foetida*. None of the earthworm uptake data points for SWNT and MWNT (Figure 6.1 and Table 6.1) were significantly greater than this level ($P > 0.05$), while all pyrene values exceeded this value. Gut loading of soil for *E. foetida* was previously found to vary based on the properties of the substrate and moisture content (Hartenstein et al. 1981). As such, differences in experimental conditions could account for the fact that the nanotube BAFs measured here were generally slightly less than 0.03.

This finding suggests that any apparent differences between the SWNT and MWNT uptakes stemmed largely from differences in the gut contents of the worms and are not indicative of differences in accumulation behaviors between these two types of carbon nanotubes. The earthworm bio-uptake of MWNTs after 14 days was larger than that for SWNTs in the Chelsea soils, but the BAF values for the SWNTs were higher than the MWNTs for exposure in Ypsilanti soil. Uptakes after 28 days of exposure to the Chelsea soil, however, were almost identical for the SWNTs and MWNTs. The differences in BAF values after 14 days are difficult to explain or rationalize, and may relate to unapparent differences in the health of worms during worm selection at the beginning of the experiments.

The pyrene BAFs shown in Table 1 were, unlike those for carbon nanotubes, strongly correlated to the organic carbon content of the soils, consistent with equilibrium partitioning expectations. The BAF for the

Ypsilanti soil after exposure for 14 days was a factor of 4.8 higher than that for the Chelsea soil, while the organic carbon content of the Chelsea soil was a factor of 5.2 greater than that of the Ypsilanti soil.

6.3.2 Depuration Experiments

Rates of carbon nanotube purging after earthworm exposure to clean soils were also investigated (Figure 6.2). Unlike the depuration of pyrene, which exhibited an expected exponential decay behavior, the depuration behaviors of the SWNTs and MWNTs did not exhibit a clear pattern. This is again consistent with a conclusion that the majority of the carbon nanotubes measured were in the guts of the earthworms. Similar depuration behaviors were previously found with the oligochaete, Lumbriculus variegatus, for polydimethylsiloxane-spiked sediment (Kukkonen and Landrum 1995). The apparent uptake of this chemical was suggested to be a result of sediment residing in the organism's gut after the initial depuration period, and not of chemical absorption into the tissues of the organism.

Differences between the uptake and depuration behaviors of carbon nanotubes and pyrene by *E. foetida* can be attributed to one or more of several factors, factors which also largely explain the related behaviors demonstrated with *L. variegatus*. *E. foetida* can potentially uptake chemicals directly from interstitial waters by dermal absorption or oral ingestion, and from uptake of soil particles and subsequent release of the chemical of interest to the interstitial fluids (Belfroid et al. 1996). Given the limited solubility of carbon nanotubes in water, their uptake by earthworms from

interstitial water is likely minimal. This nanotube behavior also suggests that dermal absorption of nanotubes would be highly limited. The potential for uptake of soil particles, desorption of the chemicals in the organisms' gut, and absorption into the organism represents a more likely potential source of bio-uptake. This exposure pathway has been shown to be significant for chemicals having high octanol-water partitioning coefficients (log k_{ow} > 5) (Belfroid et al. 1996). The results shown here though do not suggest significant absorption through this exposure route, at least after 28 days of exposure. The hydrophobic nature of the nanotubes would suggest strong sorption to organic matter associated with the soil or sediment particles, and it is unclear as to what extent the carbon nanotubes would desorb from the organic matter in the gut of the worms. If some fraction of the nanotubes had indeed desorbed, cellular uptake of these nanotubes across the body wall of the worms may have limited absorption into worm bodies. Cellular uptake of a variety modified SWNTs and MWNTs by a broad range of cells has been recently determined (Kostarelos et al. 2007), although the cellular uptake mechanisms for carbon nanotubes is still debated. The extent to which nanotubes would pass through these cells and enter systemic circulation in the bodies of the worms has not yet been established, but the cellular assimilation of unmodified carbon nanotubes was shown to be rapid in Chapter 4.

The results presented here clearly indicate that absorption of purified SWNTs and MWNTs into the tissues of *E. foetida* is minimal in comparison to

that of a representative PAH counterpart, pyrene. Nonetheless, carbon nanotubes can undergo modification by acid treatment, numerous chemical reactions, and/or adsorption of polymers or biomolecules. Each event can significantly change such physicochemical properties such as nanotube length and solubility in water. Compounds readily available in environment systems (natural organic matter, for example) have been shown to disperse MWNTs, a change which could significantly influence their ecological behaviors (Hyung et al. 2007). Development of better understandings of the extents to which such alterations may impact nanotube toxicokinetics represents a crucial area for ongoing research. The carbon-14 labeled nanotubes developed here clearly provide a promising tool for further environmental investigations along these and other lines.

6.4 Summary

The uptake and depuration behaviors of the spiked carbon nanotubes and pyrene by the earthworm *Eisenia foetida*, a potential entry point to terrestrial food chains, were then assessed. Bioaccumulation factors determined for the nanotubes were almost two orders of magnitude smaller than those measured for pyrene, indicating that purified carbon nanotubes, unlike pyrene, are neither readily absorbed into organism tissues nor manifest equilibrium partitioning thereto. These results mirror those determined in chapter 5 for the uptake of sediment-spiked nanotubes by the oligochaete *Lumbriculus variegatus*.

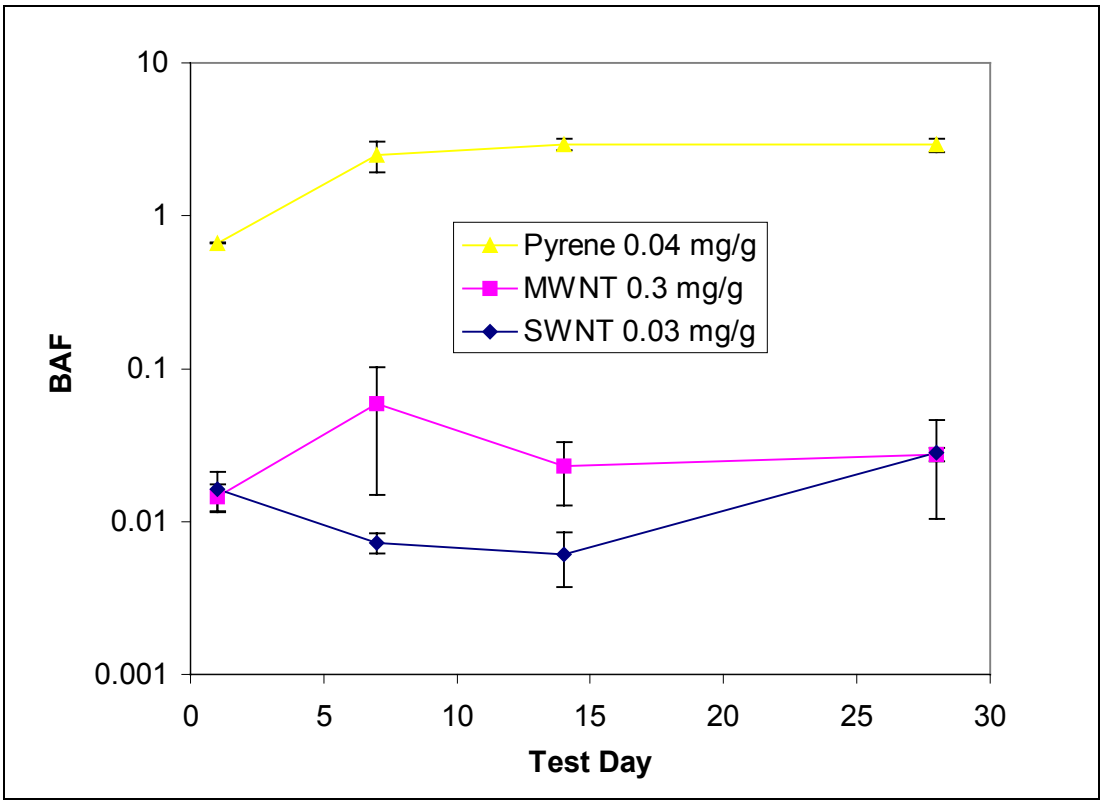

Figure 6.1: Bioaccumulation factors (BAFs) of single-walled carbon nanotubes (SWNT) (0.03 mg/g dry soil), multi-walled carbon nanotubes (MWNT) (0.3 mg/g dry soil) and pyrene (0.04 mg/g dry soil) spiked to Chelsea soil. Error bars represent one standard deviation (n=3).

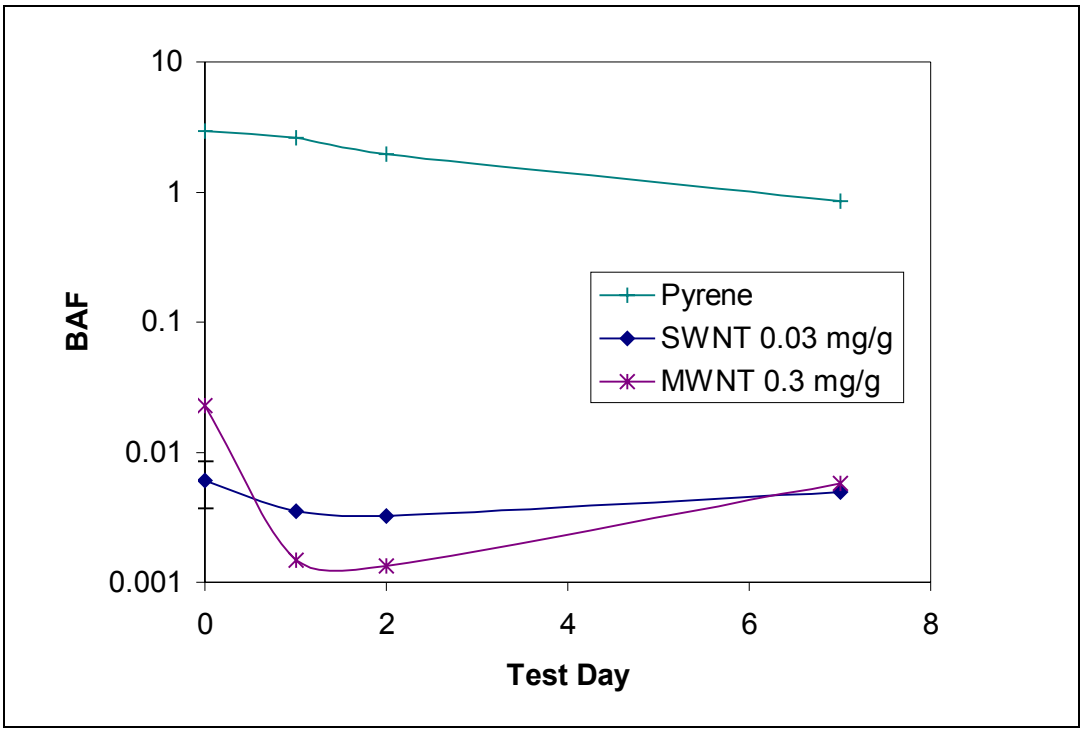

Figure 6.2: Bioaccumulation factors (BAFs) for the depuration behaviors of single-walled carbon nanotubes (SWNT) (0.03 mg/g dry sediment), multi-walled carbon nanotubes (MWNT) (0.3 mg/g dry sediment) and pyrene (0.04 mg/g dry sediment) spiked to Chelsea soil after 14 days exposure. Test day refers to the depuration time in fresh soil. Error bars represent one standard deviation (n=3).

	BAF
Pyrene Chelsea Soil (0.04 mg/g)	2.94 ± 0.25
Pyrene Ypsilanti Soil (0.04 mg/g)	14.0 ± 0.9
MWNT Chelsea Soil (0.3 mg/g)	0.023 ± 0.01
MWNT Chelsea Soil (0.03 mg/g)	0.016 ± 0.001
MWNT Ypsilanti Soil (0.3 mg/g)	0.014 ± 0.003
SWNT Chelsea Soil (0.03 mg/g)	0.0061 ± 0.002
SWNT Chelsea Soil (0.1 mg/g)	0.0078 ± 0.005
SWNT Ypsilanti Soil (0.03 mg/g)	0.022 ± 0.003

Table 6.1: Bioaccumulation factors (BAFs) after 14 days exposure for single-walled carbon nanotubes (SWNT), multi-walled carbon nanotubes (MWNT), and pyrene uptake by *E. foetida*. Mean and standard deviation values were calculated from triplicate measurements.

Chapter 7

ECOLOGICAL UPTAKE AND PHASE PARTITIONING OF PURIFIED AND ACID-MODIFIED MULTI-WALLED CARBON NANOTUBES

7.1 Introduction

As discussed in Chapter 1, one major difference between carbon nanotubes and most environmental contaminants is the potential for surface coatings on or chemical functionalization of the CNTs. Nanotubes, for example, have been solubilized/dispersed by a wide range of polymers, surfactants, and macromolecules (O'Connell et al. 2002; Zheng et al. 2003; Sinani et al. 2005) and also by chemical treatments, such as by adding functional groups to the nanotubes or shortening them with acid treatments (Liu et al. 1998; Ziegler et al. 2005; Kostarelos et al. 2007). Furthermore, nanotubes may also interact with compounds ubiquitous in environmental systems such as natural organic matter (Hyung et al. 2007). It is unknown though to what extent these changes to the nanotubes would affect their fate and distribution in environmental systems.

One potential approach for estimating the extent to which such modified nanotubes would accumulate in ecological receptors would be to test the applicability of approaches that have been previously used for similar purposes with hydrophobic organic chemicals (HOCs). Predictive models for bioaccumulation of HOC in a wide range of terrestrial and aquatic ecosystems have often been based on the octanol-water partitioning coefficient (Di Toro et al. 1991; Belfroid et al. 1996; Mackay and Fraser 2000). This coefficient is used to relate partitioning via passive diffusion processes between contaminated media such as sediments, soils, and water and the lipid tissue in organisms, an approach based on Equilibrium Partitioning Theory. This theory has many recognized limitations though including reliance on a linear sorption model and the failure to account for biotransformation, aging, and differences in behavior among various organisms (Belfroid et al. 1996). The application of octanol-water partitioning values for estimating bioconcentration behaviors, nevertheless, still holds value for screening wide databases of chemicals for potentially bioaccumulating chemicals (Mackay and Fraser 2000).

Although purified SWNTs and MWNTs were not shown to accumulate in the earthworm *Eisenia foetida* and the aquatic oligochaete *Lumbriculus variegatus* in Chapters 5 and 6, CNTs with a wide range of sizes and chemical properties are being investigated for their future applications. The potential for some forms of this class of nanoparticles to accumulate in

organisms remains a distinct possibility and a critical topic for elucidating the potential human and environmental risks, if any, posed by nanotubes.

As such, we tested the ecological uptake of MWNTs modified by sonication in a 3:1 acid mixture of sulfuric to nitric acid using *E. foetida* and *L. variegatus*. The phase distribution behaviors of purified and 3:1 acid-modified MWNTs between water and octanol were also investigated, and these values and the extent to which these nanotubes were absorbed by ecological receptors were then compared against the bio-uptake behaviors of HOCs with similar partitioning coefficients. This investigation was intended to help clarify the relationship between the accumulation behaviors of carbon nanotubes and HOCs in two primary ways: i) the extent to which the uptake of carbon nanotubes would resemble that of other organic chemicals with similar octanol-water distribution coefficients and ii) to what extent previous approaches for modeling the environmental behaviors of HOCs can be used to predict how various forms of CNTs would behave in environmental systems.

7.2 Experimental Methods

MWNTs purified with hydrochloric acid for 1 hr were bath-sonicated in a mixture of 3:1 concentrated sulfuric to nitric acid for 2 hrs. These nanotubes were filtered, dried, and robustly characterized as described in Chapter 3. They were found to maintain high purity with regards to amorphous carbon and to possess minimal traces of metal catalysts from the synthesis process. X-ray photoelectron spectroscopy was used to confirm a

significant increase in the elemental percentage of oxygen in the MWNT sample after the 3:1 acid treatment process, a change which indicates a greater density of functional groups on the nanotubes. Length distributions of the nanotubes determined using scanning electron microscopy (SEM) did not indicate changes in the nanotube lengths as a result of this modification procedure.

Given these changed physicochemical properties of the nanotubes, the extent to which these alterations influenced nanotubes' bio-uptake was assessed. These modified MWNTs were sonicated, spiked to soils or sediments, and their uptake and depuration behaviors then measured using the earthworm *E. foetida* and the oligochaete *L. variegatus*. These procedures mirrored those used for the purified MWNTs as described in sections 2.7 and 2.8. Also similarly to the purified nanotubes, the amendment of 3:1 acid mixture modified MWNTs to the soils and sediments did not cause acute toxicity to the organisms for the concentrations and exposure durations examined here.

A modified shake-flask method was used to assess the nanotubes' distribution coefficients as described in section 2.8. MWNTs purified with HCl for 1 hr or those also modified by the 3:1 acid mixture were dispersed in water or n-octanol, 25 mL of the solution added to test tubes, and then 25 mL of the complimentary phase added. This sonication treatment was similar to that which the MWNTs received prior to their addition to the soils or sediments. The octanol/water mixtures were allowed to equilibrate for three weeks after

which period the radioactive nanotube concentrations were measured in both the water and octanol phases. The results, however, indicated that minimal transport across the water-octanol interface occurred for nanotubes originally dispersed in either phase. As such, additional experiments were conducted in which a second round of ultrasonication was used to vigorously intermix the two phases by positioning the probe slightly above the interface. Given this different methodology as compared to the traditional octanol-water partitioning coefficient measurements, the term “distribution coefficient” is used here when referring to values determined using this method.

The stability of the MWNT dispersion for both types of MWNTs in octanol and water was also assessed. MWNTs were sonicated in both solutions at similar concentrations to those used in the distribution experiments, 50 mL of the solutions added to test tubes, and the nanotube concentrations in the liquid phases measured at various times.

7.3 Results and Discussion

7.3.1 Ecological Uptake and Depuration

Modifying the carbon nanotubes by sonication with the 3:1 sulfuric to nitric acid mixture did not significantly change their uptake and depuration behaviors by either *E. foetida* or *L. variegatus*. The bioaccumulation factor (BAF) values for both organisms with the 3:1 treated MWNTs resembled those for the purified MWNTs as shown in Figures 7.1 and 7.2. Depuration behaviors for *L. variegatus* after 14 days of exposure in sediment amended with 10% by mass Michigan Peat resembled those for the purified MWNTs as

illustrated in Figure 7.3*;* these results are generally similar to those found for *L. variegatus* and polydimethylsiloxane, a compound believed not to significantly absorb into the organisms' tissues (Kukkonen and Landrum 1995). As shown in Figure 7.4, the concentration of the 3:1 nanotubes in the worms after depuration in clean sediment for two days was found to be below the background level indicating the rapid rate at which the nanotubes can be purged from these organisms. These results taken together suggest that the 3:1 acid-mixture modified MWNTs measured in the organisms were again associated with soils or sediments remaining in the guts of the organisms and not absorbed into their tissues. The BAF values for the aquatic worms with 3:1 acid mixture modified unamended sediments and those amended with 10% Michigan Peat were 0.67 ± 0.26 and 0.39 ± 0.08, similar values as to those shown for the purified nanotubes. These results strongly suggest that the uptake of these nanotubes did not follow equilibrium partitioning. If the nanotubes uptake did indeed followed the behaviors described by Equilibrium Partitioning Theory, the BAF value should be a factor of 8 greater for the worms exposed to nanotubes spiked to unamended sediments given that the percent of organic carbon decreased from 5.1 to 0.66 % without the amendment of the Michigan Peat.

The lack of a change in the bio-uptake behaviors for the modified MWNTs was unexpected largely because these nanotubes were more stable in water, and as such, more likely available for biological uptake through oral ingestion or dermal absorption. It is possible that the modified MWNTs still

strongly interacted with the soil and sediment particles and were thus not present in the water phase at significant concentrations. The nanotubes were thus not available through ingestion of pore water, or, after the uptake of soil or sediment particles by the organisms, the nanotubes may not have appreciably desorbed from these materials in the guts of the organisms. It has also been shown though that some chemical compounds are less likely to bioaccumulate in human's adipose tissue if they become more hydrophilic by biotransformation and can then be more readily excreted (Geyer et al. 1987). Another possible explanation for the lack of uptake is that these MWNTs were still unable to pass through the gut lining of the organisms or through dermal absorption to enter systemic circulation in the organisms' bodies. This would stand in contrast to the facility in which nanotubes are known to readily enter cells.

7.3.2 Phase Partitioning Behaviors

One of the most intriguing findings from this set of experiments was the apparent inability of MWNTs to cross the interfacial boundary between the octanol and water phases. Nanotubes were not detected in either the octanol or water phases when the nanotubes were initially dispersed in the complimentary phase. This behavior differs from that of typical HOCs which would readily transfer between these phases although in larger quantities from the water to the octanol phase. The cause of this behavior is yet unclear, but may be in part a result of the surface properties of the carbon

nanotubes that possess both hydrophobic and hydrophilic sections, and thus share chemical similarities with surfactants.

After sonication, the measured distribution coefficient for the 3:1 acid mixture modified MWNTs was approximately 2.7. Measurements of this coefficient for different time periods are shown in Table 7.1. The distribution coefficient for the HCl purified MWNTs, conversely, could not be determined using this methodology as the concentration of MWNTs in the aqueous phase was below the detection limit. Given that the 3:1 acid modification only changed the nanotubes' hydrophilicity, differences in the octanol/water distribution behaviors are attributed to the increase in the quantity of functional groups on the nanotubes after this treatment.

The extent to which settling of nanotubes in the aqueous phase could be responsible for the 1 hr MWNT results was assessed as shown in Figure 7.5. Both types of MWNTs appeared to be relatively stable in water for the duration of the distribution experiments. This suggests that the inability to measure the distribution coefficient of the HCl purified MWNTs was not a result of settling in the aqueous phase. Additionally, settled aggregates of MWNTs were not visually evident in the bottoms of the test tubes for the HCl purified MWNTs. Comparisons can be made between the settling behavior of the various types of carbon nanotubes comparing their stability in water and octanol, but such discussions are beyond the scope of this thesis.

The extent to which MWNTs' distribution coefficients and bio-uptake behaviors mirror those for HOCs with similar coefficients may help

demonstrate the extent to which empirical relationships developed for the bioaccumulation of HOCs would be applicable for nanotubes. The distribution coefficient for the 3:1 MWNTs was near the octanol-water partitioning coefficients (k_{ow}) for some chemicals known to bioaccumulate in organisms. The k_{ow} coefficients for toluene and chlorobenzene, for example, are 2.69 and 2.84, respectively (ATSDR 1994). Other chemicals with similarly low k_{ow} coefficients may not bioaccumulate to an appreciable extent if they are readily metabolized within organisms. Biotransformation of chemicals has also been shown to influence bioaccumulation behaviors especially if chemicals become hydrophilic and are thus more readily excreted from the organisms (Geyer et al. 1987).

The difference in the distribution coefficients measured here for two different types of MWNTs but their similar bioaccumulation by organisms indicates that distribution coefficients for MWNTs may not be a useful predictor of their uptake by ecological receptors. As such, the distributions coefficients determined here should not be equated with octanol-water partitioning coefficients for typical HOCs, and specifically, these values should not be misused by predicting the bio-uptake of different types of nanotubes using empirical equations developed for organic chemicals with their k_{ow} values. It should be noted though that, in the experiments conducted here, the nanotubes came into contact with soil or sediment particles prior to interactions with ecological receptors, and the corresponding sorption of the nanotubes may have determined the lack of uptake regardless of the

nanotube properties. The uptake behaviors of nanotubes in ecological systems in the absence or sediment particles though is unknown, may differ from those shown here, and represents an important topic for future research investigations as described in Chapter 8.

7.4 Overview

Modifying MWNTs with a 3:1 mixture of sulfuric to nitric acid did not significantly modify their bioaccumulation behaviors by either *E. foetida* or *L. variegatus*. The nanotubes appeared to remain in the guts of the organisms instead of being absorbed into their tissues. Investigating the distribution behaviors of multi-walled carbon nanotubes between water and n-octanol indicated that the nanotubes do not readily transfer between these two phases. After mixing the phases via sonication, the distribution coefficient for the 3:1 modified MWNTs was approximately 2.7, while that for the HCl purified MWNTs could not be determined as a result of low nanotube concentrations in the water phase. The similar ecological behaviors of these compounds though suggest that the distribution coefficients are not indicative of their bioaccumulation behavior. This stands in contrast to the usage of octanol-water partitioning coefficients for organic chemicals to predict their concentrations in organisms. As such, the distribution coefficients measured here should thus not be confused with octanol-water partitioning values for typical hydrophobic organic chemicals, and empirical relationships developed for HOCs should not be used to predict the environmental behaviors of these nanotubes.

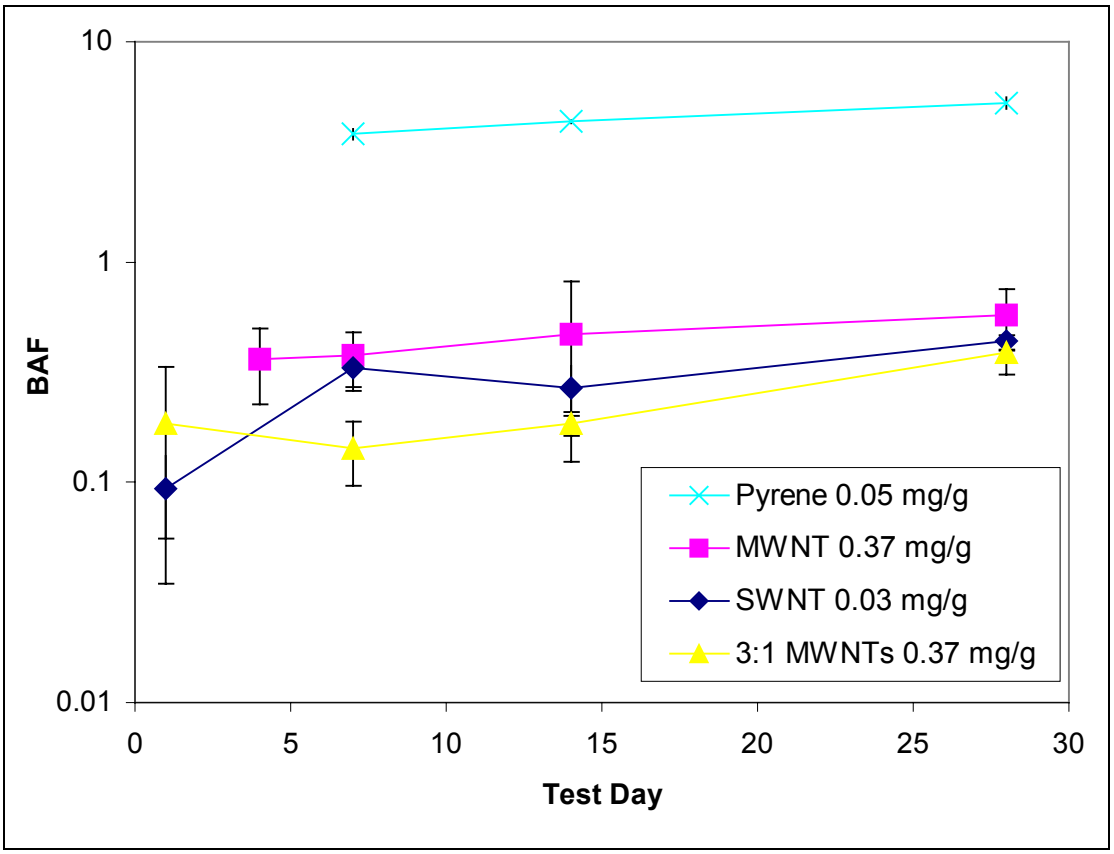

Figure 7.1: Bioaccumulation factors (BAFs) for *L. variegatus* uptake of 3:1 modified MWNTs spiked to sediment amended with 10% by mass Michigan Peat. All data points are from triplicate measurements and error bars represent the standard deviation of those measurements.

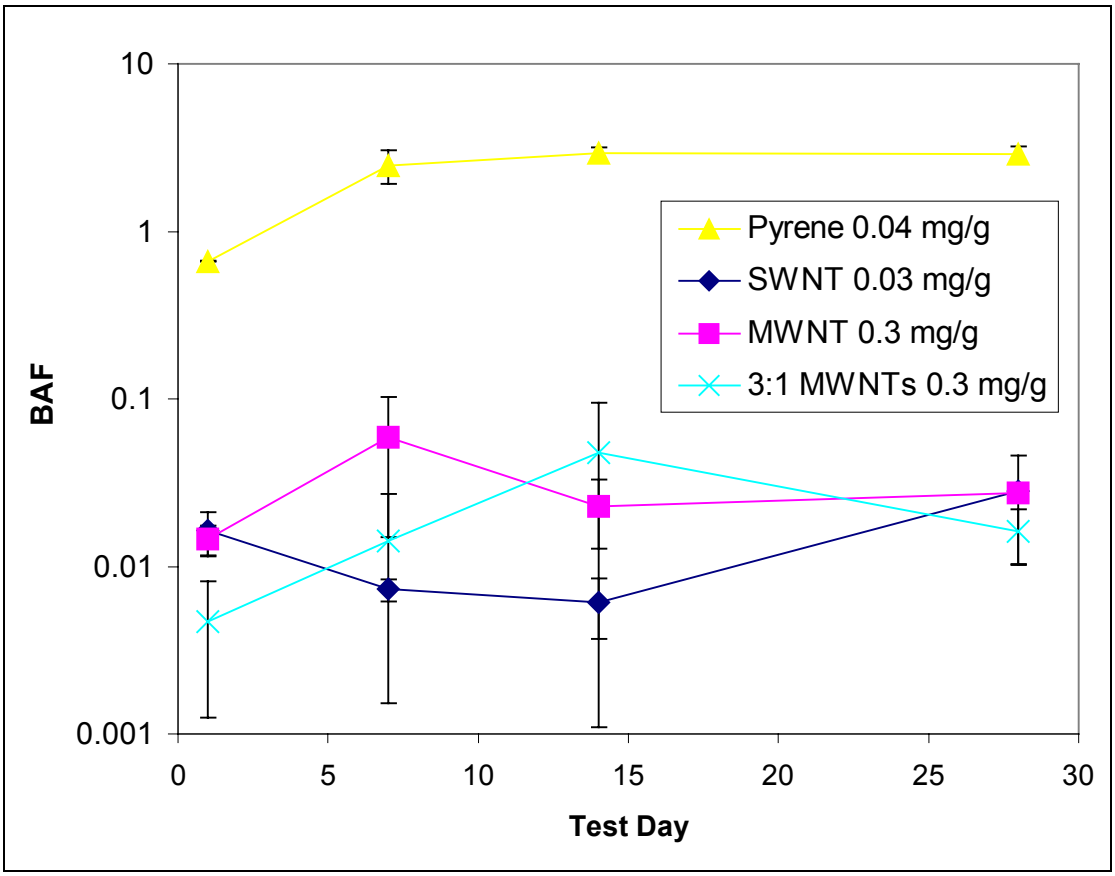

Figure 7.2: Bioaccumulation factors (BAFs) for earthworm uptake of 3:1 modified MWNTs spiked to Chelsea soil. All data points are from triplicate measurements and error bars represent the standard deviation of those measurements.

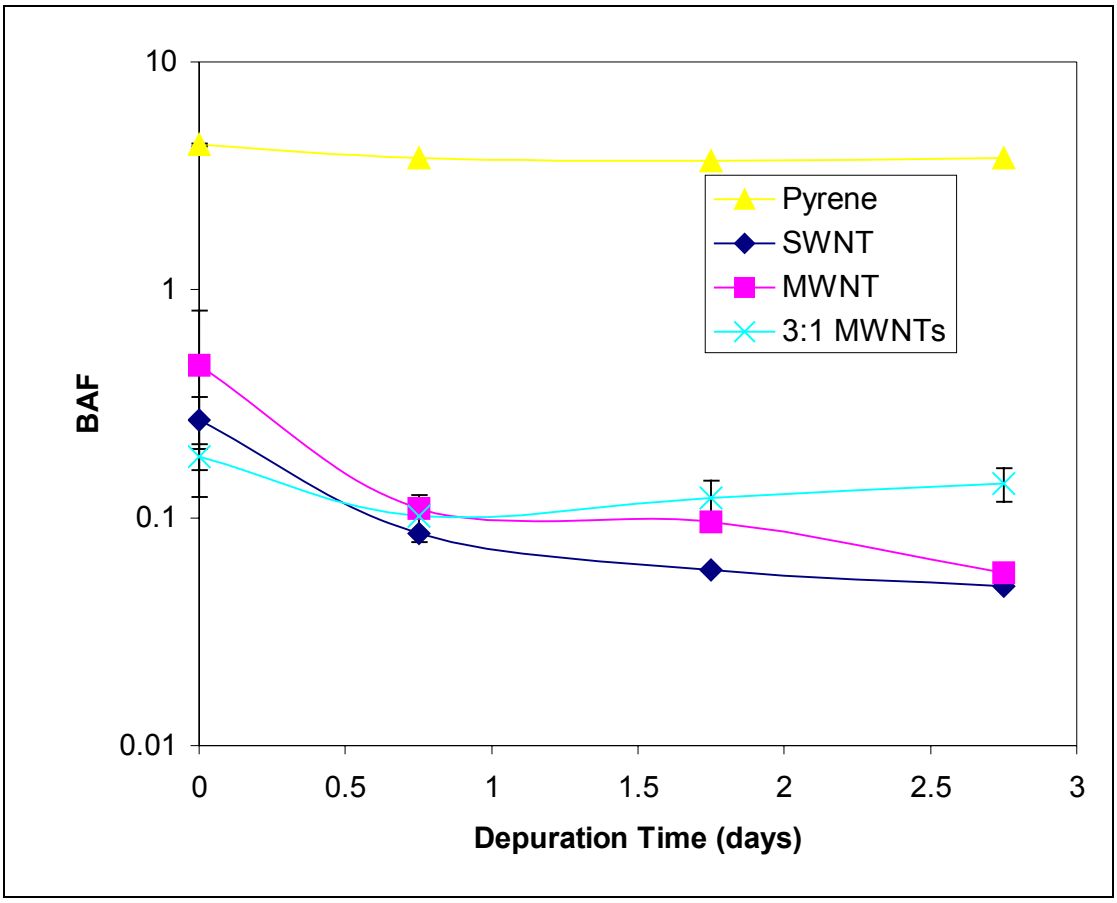

Figure 7.3: Bioaccumulation factors (BAFs) for *L. variegatus* depuration of 3:1 modified and HCl purified MWNTs, SWNTs, and pyrene spiked to sediments amended with 10% by mass Michigan Peat after 14 days of exposure. Depuration time refers to the amount of time passed after the initial 6 hours of purging.

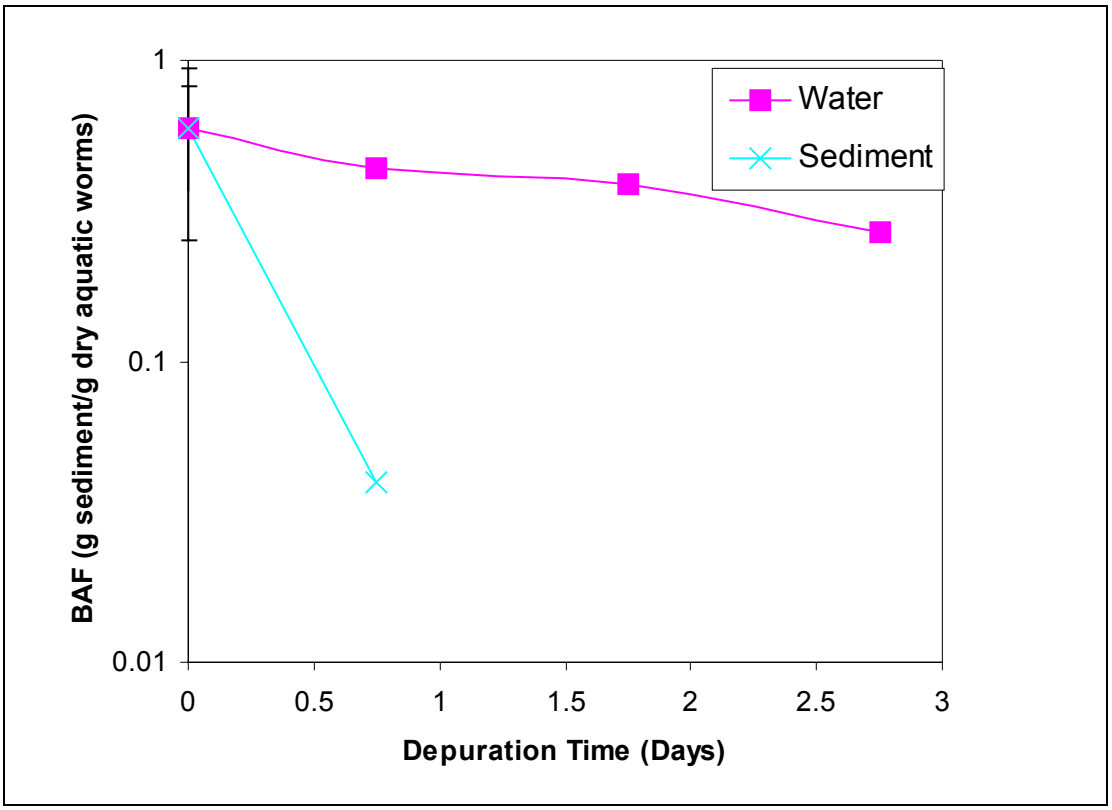

Figure 7.4: Bioaccumulation factors (BAFs) for *L. variegatus* depuration of 3:1 modified spiked to Michigan sediment after 28 days of exposure. Aquatic worm depuration data in beakers with only water are marked as "Water" and those in beakers with water and clean sediment are marked as "Sediment." Depuration time refers to the amount of time passed after the initial 6 hours of purging.

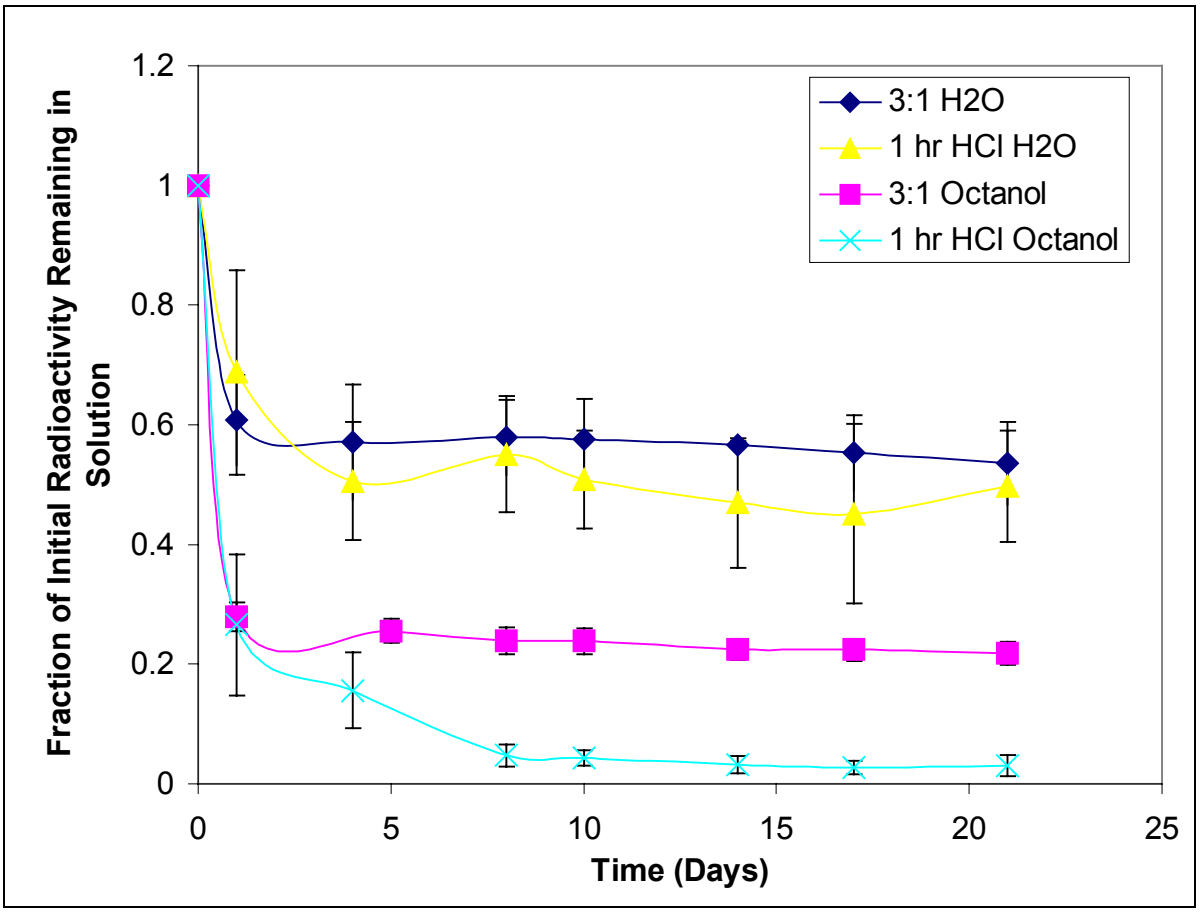

Figure 7.5: Settling of 3:1 treated or HCl purified multi-walled carbon nanotubes in water (H_2O) or octanol.

MWNT Type	Initial Solution	Time After Sonication	logCoefficient
3:1 MWNT	H_2O	4d	2.77 ± 0.06
3:1 MWNT	H_2O	8d	2.70 ± 0.13
3:1 MWNT	H_2O	14d	2.42 ± 0.14
3:1 MWNT	H_2O	21d	2.69 ± 0.05
HCl MWNT	H_2O	4d	2.95 ± 0.08
HCl MWNT	H_2O	8d	ND
3:1 MWNT	Octanol	16d	3.00 ± 0.12
HCl MWNT	Octanol	16d	ND

Table 7.1: Octanol/water distribution coefficients for multi-walled carbon nanotubes (MWNTs) dispersed in either water (H_2O) or octanol. Initial solution refers to whether the nanotubes were first dispersed in water or octanol. Time after sonication refers to the duration the samples were allowed to equilibrate after the sonication period used to mix the two phases. LogCoefficient is the logarithm of the octanol/water distribution coefficient (concentration in octanol divided by the concentration in water). ND indicates that the value could not be determined as a result of insufficient radioactivity in the aqueous phase. All means and standard deviations are from triplicate samples.

Chapter 8

CONCLUSIONS AND FUTURE WORK

8.1 Conclusions

The main conclusions evident from the results presented in this thesis are succinctly summarized in the five paragraphs presented below.

Carbon-14 labeled single- and multi-walled carbon nanotubes were synthesized using a modified methane chemical vapor deposition method. Measurements of the radioactivity of small (> 1mg) masses of nanotubes consistently revealed low coefficients of variation (generally < 5%), indicating a relatively uniform distribution of the carbon-14 isotope throughout the nanotube samples. Analytical measurements of the nanotubes using transmission electron microscopy, thermal gravimetric analysis, and Raman spectroscopy confirmed high nanotube purity with respect to amorphous carbon and catalyst materials. The synthesis and application of such carbon nanotubes overcomes serious prior experimental difficulties related to their quantification in biological and environmental samples, thus allowing a broad range of future research investigations for these materials.

Purified multi-walled carbon nanotubes were assimilated rapidly and apparently irreversibly by HeLa cells. Seventy-four percent of the nanotubes added to each plate were assimilated within the first 15 minutes, and the cellular concentration after 6 hrs reached a maximum at eighty-nine percent of the nanotubes added. Uptake of the nanotubes by the HeLa cells appeared also to be essentially irreversible, with only 0.9 ± 0.5% of the nanotubes accumulated after the 12 hr uptake period being released during a subsequent 12 hr period of cell incubation in regular media. These results combined with those of other researchers who have qualitatively shown nanotube uptake by a broad range of cells (Kostarelos et al., 2007) suggest that nanotubes released in water bodies may have strong dermal attachment to skin cells or gastrointestinal cells after water ingestion. The ability of nanotubes to pass through biological membranes though is a critical step for entering systemic circulation in organisms, but was not explored here and represents a topic for future work.

Purified single- and multi-walled carbon nanotubes were not readily bioavailable to model ecological receptors. Uptake and depuration results using the earthworm *Eisenia foetida* and the oligochaete *Lumbriculus variegatus* suggested that SWNTs and MWNTs spiked to soils or sediments were not accumulated by the organisms at significant concentrations. Concentrations of SWNTs and MWNTs in the organisms were relatively similar, both typically one to two orders of magnitude less than those for pyrene, a chemical known to bioaccumulate in organisms. Thus,

unlike pyrene, carbon nanotubes did not appear to adhere to simple equilibrium partitioning theory behavior among aqueous, soil or sediment organic carbon, and organism lipid phases.

Modifying the MWNTs by sonication in a 3:1 mixture of sulfuric and nitric acids for 2 hours did not change their bioavailability to *E. foetida* or *L. variegatus*. As indicated by an increased density of functional groups measured using x-ray photoelectron spectroscopy, such treatment made the nanotubes more hydrophilic, but did not change their length distribution as determined using scanning electron microscopy. Uptake and depuration behaviors by *E. foetida or L. variegatus* in environmentally relevant settings, however, were not changed by these modifications.

Phase distribution coefficients between octanol and water for purified and acid modified multi-walled carbon nanotubes were not indicative of their bioaccumulation behaviors. The experimental method used here significantly differed from the conventional shake flask method in that the carbon nanotubes required vigorous ultrasonication to enable redistribution between the two phases. The logarithm of the phase distribution coefficient for MWNTs modified by sonication in a 3:1 sulfuric to nitric acid mixture was approximately 2.7, a value that would suggest bioaccumulation by organisms based on the behaviors of organic chemicals with a similar octanol-water partitioning coefficient. The value for the purified MWNTs, conversely, could not be accurately determined as a result of the low nanotube concentration in the aqueous phase. This difference in the

distribution coefficients is attributed to an increase in density of functional groups on the 3:1 treated MWNTs as determined using x-ray photoelectron spectroscopy. Despite the changed distribution coefficient, the 3:1 acid modified MWNTs exhibited the same uptake and depuration behaviors as described above. As such, these nanotubes manifested distinctly different accumulation behaviors as compared to typical hydrophobic organic chemicals (HOCs). The nanotube coefficients measured here should not be carelessly equated to octanol-water partitioning coefficients measured for HOCs, and related predictive models and correlations developed using HOCs should not be used with MWNTs.

8.2 Future Work

While much remains unknown about the potential human and ecological risks associated with nanomaterials, the results presented here suggest several promising avenues for additional environmental investigations related to the fate and distribution of carbon nanotubes.

Ecological uptake and depuration of carbon nanotubes in aqueous systems in the absence of sediment particles. The experiments conducted here tested the uptake of carbon nanotubes after their spiking to soils or sediments. It appeared that the concentration of carbon nanotubes in the interstitial or overlying water was minimal, as a result of their sorption to soil or sediment particles and subsequent settling. If carbon nanotubes were to remain dispersed in a water body for extended time periods, their uptake by organisms such as fish might exhibit different bioaccumulation behaviors than

those determined for *L. variegatus*. Roberts et al. (2007), for example, qualitatively measured SWNT uptake by *Daphnia magna* using containers with suspended nanotubes but in the absence of sediment.

Additional investigations of the extent to which various physicochemical modifications may impact carbon nanotube bioavailability in different ecosystems. In addition to the acid modifications utilized in this dissertation, the nanotechnology literature is replete with physical and chemical approaches that can be used to modify carbon nanotubes. Given that researchers are assessing the application potentials of these altered nanotubes, elucidating the environmental behaviors of nanotubes with a broader range of physical and chemical properties becomes a clear research need. The carbon-14 nanotubes synthesized here can serve as a foundation for a survey of the bioaccumulation behaviors of carbon nanotubes having an array of sizes, functional groups, and adsorbed biomacromolecules or polymers. Such research will likely highlight those nanotube properties, if any, that most significantly impact their environmental behaviors and toxicities, thus guiding the safe manufacturing and production of devices incorporating carbon nanotubes.

The impact of carbon nanotubes on the fate and distribution of other organic and inorganic pollutants. Carbon nanotubes have been shown to possess strong sorptive capacities for various metals including lead, cadmium, and copper (Li et al. 2003) and a broad range of hydrophobic organic chemicals including polycyclic aromatic hydrocarbons and

polychlorinated biphenyls (Long and Yang 2001; Yang et al. 2006a; Yang et al. 2006b). Hypothetically, carbon nanotubes might act similarly to charcoals and other forms of black carbon by sequestering such compounds and limiting their bioavailability and mobility. Conversely, it is also possible that nanotubes could serve as concentrators, durable sources, and transporters of such chemicals into organisms, thus exacerbating bioaccumulation and food chain transfer. Carbon nanotubes have been shown to enter ecological receptors, although they did not accumulate within the organisms, and the passage of materials loaded with highly elevated concentrations of toxic chemicals through organisms could be pose serious environmental and human health risks.

Biological degradation of carbon nanotubes by microorganisms or fungi. While carbon nanotubes have highly inert chemical structures, it is possible that some type of microorganisms of fungi will be able to degrade or biotransform them. White rot fungi, for example, has been shown to mineralize numerous recalcitrant environmental pollutants (Bumpus et al. 1985). White rot fungi may also be able to introduce defects to carbon nanotubes structures, degrade functional groups already on the nanotube structures, or metabolize macromolecules bound to the surface of the nanotubes. Such modifications could change the environmental behaviors of the nanotubes as well as reveal the potential for biotic mineralization of the nanotubes. The radioactively labeled nanotubes developed here present a unique opportunity for assessing the extent to which different organisms can

metabolize carbon nanotubes. Measuring the carbon-14 dioxide released to experimental reactors would reveal the mass of nanotubes degraded. Such investigations would reveal the expected persistence of carbon nanotubes in environmental systems.

In vivo and *in vitro* toxicological and biodistribution studies using radioactively labeled CNTs. Compared to the current nanotube identification techniques established in the literature, radioactively labeled nanotubes hold many advantages with regards to elucidating cellular uptake rates of carbon nanotubes, studying the biodistribution of nanotubes in organisms, determining mechanisms of nanotube cytotoxicity, and evaluating the ability for nanotubes to cross biological membranes. Carbon-14 labeled nanotubes also allow for quantification of a wider range of nanotube types and dispersion states (i.e., agglomerated or individually dispersed), thus covering the broad range of conditions that humans or ecological receptors could realistically be exposed to nanotubes in environmental systems and biomedical applications. The ability to combine toxicological data with nanotube concentrations in tissues or cells will also likely facilitate identification of mechanisms behind nanotube toxicity. By determining nanotube concentrations that cause various acute, subchronic, and chronic toxic responses, professionals will be able to develop, as has been previously determined for numerous chemicals of potential human or ecological health concern, acceptable nanotube concentrations for various critical media such as water, food, and air.

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