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Oxidative enzymatic response of white-rot fungi to single-walled carbon nanotubes

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ABSTRACT

Although carbon nanomaterials such as single-walled carbon nanotubes (SWCNT) are becoming increasingly prevalent in manufacturing, there is little knowledge on the environmental fate of these materials. Environmental degradation of SWCNT is hindered by their highly condensed aromatic structure as well as the size and aspect ratio, which prevents intracellular degradation and limits microbial decomposition to extracellular processes such as those catalyzed by oxidative enzymes. This study investigates the peroxidase and laccase enzymatic response of the saprotrophic white-rot fungi *Trametes versicolor* and *Phlebia tremellosa* when exposed to SWCNTs of different purity and surface chemistry under different growth conditions. Both unpurified, metal catalyst-rich SWCNT and purified, carboxylated SWCNTs promoted significant changes in the oxidative enzyme activity of the fungi while pristine SWCNT did not. These results suggest that functionalization of purified SWCNT is essential to up regulate enzymes that may be capable of decomposing CNT in the environment.

1. Introduction

Single-walled carbon nanotubes (SWCNTs), formed from single-atom thick sheets of carbon wound into nanometer scale tubes (Iijima, 1991), display a number of remarkable properties ranging from superior tensile strength, thermal and electrical conductivity, and relative ease of chemical modification (Georgakilas et al., 2002; Collins et al., 1997). These properties make CNTs promising components in next-generation thermopolymers, electronics, and drug delivery systems (Bianco et al., 2005; Trojanowicz, 2006). As a result of their wide range of uses and the rapidly advancing production methods, carbon nanotubes are increasingly prevalent in manufactured products. Despite the increase in CNT production, very little is known about the eventual fate of CNTs once introduced into the environment through accidental release, dispersal in landfills, and as a part of biosolid waste for land application (Wiesner et al., 2009; Turco et al., 2011; Holden et al., 2013).

As with the vast majority of industrial products, the environmental fate of CNTs is partially dependent on degradation by microorganisms found in soils, sediments, and landfills (Klaine et al., 2008). Commercially produced CNTs have lengths on par, or much larger than, many biological cells with aspect ratios of up to 1,000,000 to 1 making intracellular degradation unlikely. Instead, the most likely mechanism by which environmental CNTs are microbially transformed and degraded are via extracellular redox processes, such as those catalyzed by oxidative enzymes (Allen et al., 2008; Zhao et al., 2011). Of particular interest are enzymes of the peroxidase and polyphenol oxidase groups (Collins et al., 1996; Novotny et al., 1999). These lignin-modifying enzymes catalyze the oxidation of aromatic structures by generating highly reactive radicals, which interact with aromatic structures in a variety of ways (Blanchette, 1991; Leonowicz et al., 1999; Rabinovich et al., 2004). Laccase, for example, oxidizes phenolic compounds into their corresponding phenoxy radicals; following radical formation, ring fission can be caused by spontaneous rearrangement and reaction with other nearby compounds (Thurston, 1994; Leonowicz et al., 2001). Peroxidase enzymes utilize heme cofactors in the presence of peroxides to facilitate a wide range of redox reactions (Dunford and Stillman, 1976). Environmentally important peroxidase enzymes include manganese peroxidase, which generates reactive Mn(III)-chelates (Forrester et al., 1988; Wariishi et al., 1988, 1992), in addition to more versatile peroxidases that are able to reduce and oxidize a variety of substrates (Camarero et al., 1999). Peroxidase and polyphenol oxidase enzymes, such as laccase, may also interact synergistically. For example, reactive species generated by oxidation of phenolic

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compounds by laccase are able to serve as substrates for versatile peroxidases; this synergy allows lignin-modifying enzymes to function as powerful degraders of highly condensed compounds (Leonowicz et al., 2001), including functionalized SWCNT (Allen et al., 2008).

As a result of their repertoire of degradative enzymes, saprotrophic fungi, such as the wood-rotting Basidiomycetes *Trametes versicolor* and *Phlebia tremellosa*, are considered excellent candidates for the degradation of a wide range of industrially-produced xenobiotics (Rabinovich et al., 2004; Riva, 2006). However, there is limited research demonstrating enzymatic or direct microbial decomposition of manufactured carbon-based nanomaterials. Allen et al. (Allen et al., 2008) demonstrated that pristine CNT where unreactive toward purified solutions of horse radish peroxidase, while mildly carboxylated, analogs exhibited chain shortening and oxidation under the same conditions. Previous research by our group has demonstrated that lab-cultured white rot fungi are able to successfully degrade partially hydroxylated C60 fullerenes, i.e., C60 fullerols (Schreiner et al., 2009). The distinction between pristine and functionalized, hydroxylated or carboxylated, may be crucial when estimating the potential for microbial decay of CNT's as the highly condensed nature of unfunctionalized CNTs, may dramatically impede microbial decomposition and increase the potential for long-term environmental accrual as has been seen for pristine fullerenes.

Research on the impacts of carbon nanomaterials on microorganisms has been largely focused on the impact of nanomaterials on bacterial monocultures where both CNT and fullerenes have demonstrated antimicrobial properties (Lyon et al., 2006; Arias and Yang, 2009; Kang et al., 2007, 2009). In a study using a variety of different fullerene suspensions, the nanomaterials were found to function as potent antibacterial agents against the gram-positive bacteria *Bacillus subtilis* (Lyon et al., 2006), while unfunctionalized multiple-walled carbon nanotubes (MWCNT) were found to significantly decrease sporulation of the fungus *Paecilomyces fumosoroseus* in pure culture but had no effect on hyphal growth (Gorczyca et al., 2009). Kang et al. (2007, 2009) found that SWCNT were effective in inactivating pure cultures of *Escheria coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus epidermis* in defined media, though the authors found that antimicrobial activity against pure cultures was a poor indicator of microbial deactivation in more complex environmental samples (Kang et al., 2007, 2009).

It has been suggested that in more complex environmental systems, natural organic matter aids in the sorption of nanoparticles, diminishing their apparent toxicity (Li et al., 2008; Navarro et al., 2008). Studies of fullerene toxicity in soil have found little impact on soil respiration or on bacterial and fungal communities (Tong et al., 2007). Additionally, a recent study found that unpurified CNTs that contain residual amorphous carbon and catalysts from synthesis are able to influence microbial community composition in soils with low organic matter content, diminishing certain fungal and bacteria groups, while having less impact in soils with higher organic matter content (Tong et al., 2012). Such findings are also consistent with reports of the microbial toxicity of carboxylated SWCNT in soils with low organic content (Rodrigues et al., 2013).

The surface chemistry of CNT may not just control their chemical “lability” with respect to microbial enzyme decay, but may also play an important role controlling cytotoxic interactions of CNTs with soil microbial communities (Karakoti et al., 2006; Rodriguez-Yanez et al., 2013). For example, the cytotoxicity of both SWCNTS and MWCNTs has been reported to significantly increase once their surface has been oxidized (Fenoglio et al., 2008; Bottini et al., 2006). Other studies, however, have demonstrated that increased density of surface functionalization of SWCNTs may actually decrease cytotoxicity (Sayes et al., 2006). These seemingly contradictory responses are most likely a function of the propensity of functionalized CNTs under certain conditions to either bind to cells, or to homo and heteroaggregate and thus self-mitigate certain toxicological effects (Arias and Yang, 2009; Pasquini et al., 2012; Handy et al., 2008).

Impurities in CNTs derived from the manufacturing process, e.g. amorphous carbon and metal catalysts, must also be considered as potential influences on soil microbial activity. Although a broad range of synthesis methods exist for commercially available CNTs, many rely on formation of the nanomaterials around metallic nanoparticles catalysts (Melechko et al., 2005). As a result, the majority of CNTs contain metal impurities (Ge et al., 2011). For example, one popular method of large-scale SWCNT synthesis, electric arc discharge, uses nickel and yttrium catalysts (Journet et al., 1997). Liu et al. (2007) reports that catalytic nickel from a variety of SWCNT synthesized by this method is readily bioavailable, more so than the nickel salt $NiCl_2$ used as a reference (Liu et al., 2007). As is the case with the carbon nanomaterials themselves, bioavailability of the metal nanoparticles is highly dependent on environmental factors such as presence of soil organic matter and local redox conditions (Degryse et al., 2009; Auffan et al., 2010). One recent study in agricultural systems found that dissolution of natural organic matter by surfactants greatly increased mobility of heavy metals (Hernandez-Soriano and Jimenez-Lopez, 2012). Mitigation of metal toxicity by organic matter has previously been thought to render the catalyst's toxicity negligible in short-term soil incubations, although the authors acknowledge that some evidence suggests CNTs act to compound the toxic effects of metals by increasing the mobility of catalytic metals (Tong et al., 2012; Liu et al., 2007).

To improve our understanding of the interaction between growth environment, in this case the nutrient richness of growth media, and fungal response to SWCNT of different purity and surface chemistry we compare the measured changes in oxidative enzymatic activity of the saprotrophic white-rot basidiomycetes *Trametes versicolor* and *Phlebia tremellosa* in inoculation experiments on both a simple, defined minimal media and a complex malt media high in organic and phenolic compounds. To minimize variables we use one commercial source of SWCNT, all prepared using the same electric arc process and an yttrium nickel catalyst, but treated to different levels of purity and surface functionalization.

We expect that purified SWCNT will induce a minimal enzymatic response in either media treatment given its lack of chemical functionality, low metal content and large aspect ratio. However, SWCNT with either high metal content or surface functionalization will induce oxidative enzymes activity, either as a detoxification response or an induction of aromatic/lignin-like decay processes. Growth media nutrient content, which may be considered as a proxy for soil nutrient status available to microbes in a natural system, should modulate the ability of fungi to produce oxidative enzymes and thus we expect fungi on minimal media to have significantly reduced responses to metals or functionalized SWCNT.

2. Materials and methods

2.1. Carbon nanomaterials

Carbon nanotubes used in this study were purchased from Carbon Solutions Inc. (Riverside, CA, USA). Carbon Solutions uses an electric arc discharge method with an yttrium/nickel catalyst to produce the nanotubes (Niyogi et al., 2002). Transmission electron microscopy (TEM), using a FEI/Philips CM-100 Transmission Electron Microscope (FEI Company, Hillsboro, Oregon, USA) was employed to investigate the physical form, relative proportion of amorphous materials, and bundling of the SWCNT and compared with the manufacturers specifications to ensure conformity of the products. A summary of the chemical and physical characteristics of the