Application Note

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Understanding the Mechanism of Carbon Nanotube Degradation in Macrophage Cells

Introduction

Carbon nanotubes are a promising vehicle for drug delivery and cellular targeting. However, before they can be implemented for medical use, their physiological lifecycle and clearance pathways must be better understood. As with many other foreign materials, macrophage cells play a central role in the degradation and elimination of carbon nanotubes from the body of an organism. Several mechanisms of intracellular, oxidative biodegradation of carbon nanotubes by reactive oxygen species (ROS) have been identified. For example, peroxidase enzymes, present in neutrophil and goblet cells, convert hydrogen peroxides to a variety of specialized acids which attack and degrade the walls of carbon nanotubes.

Recently, researchers from the University of Paris, Diderot utilized Protochips' Poseidon system in an effort to further understand the mechanism of carbon nanotube breakdown by living organisms. New insights provided by the Poseidon system enabled the group to bridge the structural changes observed using traditional microscopy techniques with known biochemical pathways. During the study they were able to identify two mechanisms of hydroxyl radical induced multi-wall carbon nanotube (MWCNT) biodegradation by macrophage cells. In order to identify these mechanisms, the researchers combined data using two approaches: *in vitro* biological studies and, for the first time, *in situ* TEM monitoring of carbon nanotube degradation in liquid. Dr. Damien Alloyeau used the Poseidon Liquid TEM platform to image the process of oxidative attack on MWCNT in real time. The mechanisms that they observed correlated perfectly with the degradation pathway observed in carbon nanotubes during long-term ROS exposure in macrophage cells.

These dynamic observations of nanomaterials under oxidative stress are an important step in studying the behavior and reactivity of nanomaterials in a physiological environment. Mechanistic insights into nanomaterial transformations are important to both material scientists interested in optimizing the reactivity and/or stability of nanostructures under oxidizing conditions and to biologists evaluating the effects and potential risks of nanomaterials the body.

Experiment

In order to study the mechanism by which macrophage cells break down MWCNTs, researchers employed both traditional methods of electron microscopy and cutting edge *in situ* TEM studies. Using a traditional approach to study their intracellular breakdown, MWCNTs were either introduced into the lung tissue of male Sprague-Dawley rats via intratracheal instillation or incubated directly with THP-1 macrophage

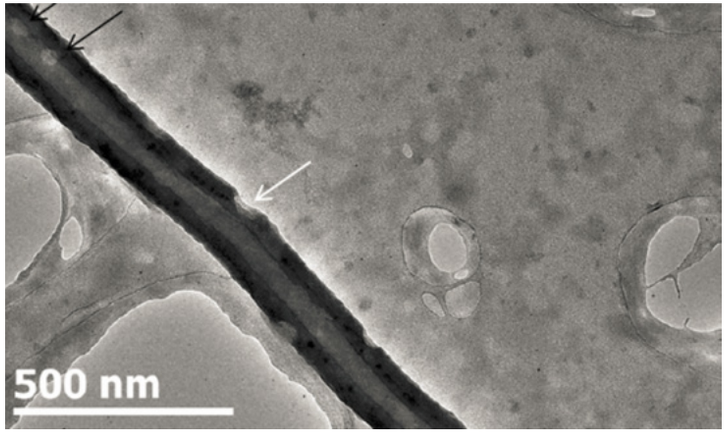

Figure 1: Traditional thin section TEM image of a multiwall carbon nanotube inside a mouse macrophage cell. Seven days after exposure, the structure of the nanotube exhibits scarring and the presence of perforations. Arrows indicate the locations of holes in the carbon nanotube.

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cells. After seven days, *ex vivo* TEM analysis of thin tissue sections showed the accumulation of MWCNTs within the macrophage cells as shown in Figure 1. After accumulation in the macrophage cells, the graphitic structure of the MWCNTs exhibited significant scarring. Perforated areas or holes in the graphite wall; (indicated in Figure 1 with arrows) increased with the length of time that the MWCNTs were incubated within the macrophage cells. Thinning of the graphite wall also increased as a function of exposure time. Luminescent studies using the dye, 2',7'-dichlorodihydrofluorescene diacetate, indicated that the concentration of ROS within the macrophage cells increases three-fold after 24 hours of exposure to the MWCNTs. Addition of an ROS scavenger, N-acetyl-L-cysteine, to the cell media reduced the production of ROS within the macrophage cells and prevented the degradation of the MWCNTs, which indicated that ROS produced by the macrophage cell after MWCNT update play a central role in the biodegradation of MWCNTs.

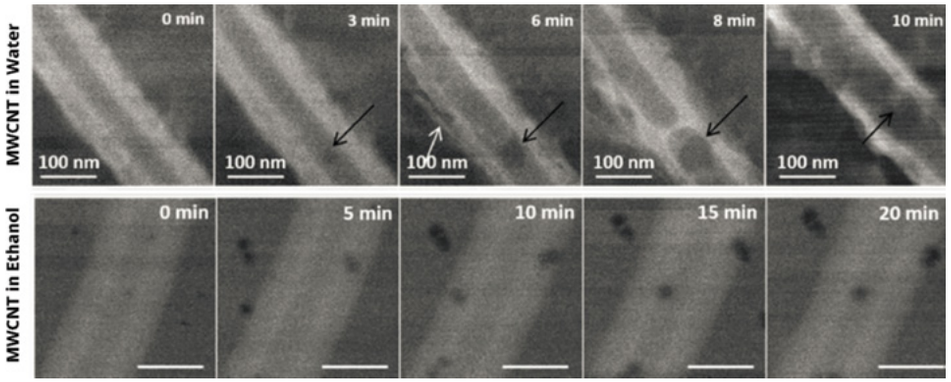

Figure 2: *In situ* liquid STEM images of carbon nanotube degradation in water in the presence of hydroxyl radicals. **Top Row:** Time series of images showing the progression of damage in the graphite structure of a carbon nanotube. Black arrows show the location of holes forming in the carbon nanotube and white arrows denote tears along the axis of the carbon nanotube. **Bottom Row:** Time series of images showing carbon nanotubes imaged in ethanol (in the absence of hydroxyl radicals). Even after extended imaging time, no degradation of the carbon nanotube is observed. The black dots are due to imaging artifacts.

To further probe the role of ROS in the mechanism of MWCNT degradation, *in situ* liquid TEM using Protochips' Poseidon system was employed. Using the electron beam as an ionization source, researchers induced the radiolysis of water to produce ROS including hydroxyl radicals, hydronium ions, and hydroperoxyl radicals, as well as other reactive species such as hydrated electrons. Thus they were able to observe the dynamic process of MWCNT degradation in real time. Samples of MWCNTs were enclosed between pairs of sample supports called E-chips which contain electron transparent windows for observation in the TEM. The liquid thickness in which the MWCNTs were contained was 150 nm. Images were obtained using a JEOL ARM 200F equipped with a CEOS corrector and operated at 80 KV in scanning TEM (STEM) mode. Controlling the electron dose enabled the researchers to control the concentration of ROS that were produced and mimic the physiological environment of the macrophage cells.

The top row of Figure 2 shows the progression of hole formation in the graphite walls of the carbon nanotube as the concentration of ROS species increases with exposure to the electron beam. The rate of thinning increased with the dose rate and thus correlated with the rate of ROS concentration.

As a control, *in situ* irradiation of MWCNTs was performed in ethanol. Ethanol, when irradiated by the electron beam, does not form the same radiolysis products that water does. Specifically, substituting ethanol for water results in the formation of peroxyl radicals and eliminates the formation of hydroxyl radicals in solution. To probe the role that hydroxyl radicals play in biodegradation of MWCNT, a sample of MWCNT was imaged immersed in ethanol with the Poseidon system. After several hours of irradiation *in situ* with the electron beam, no change in the structure of the MWCNTs was observed, as shown in the bottom column of Figure 2. In addition to the formation of perforations in the wall of the MWCNTs, both the outer and inner carbon walls were also thinned as shown in Figure 3. Thus, the same two types of structural breakdown, perforation formation and graphitic wall thinning, were observed both *ex situ* after incubation with macrophage cells and *in situ* in the simulated ROS-rich environment of the Poseidon system.

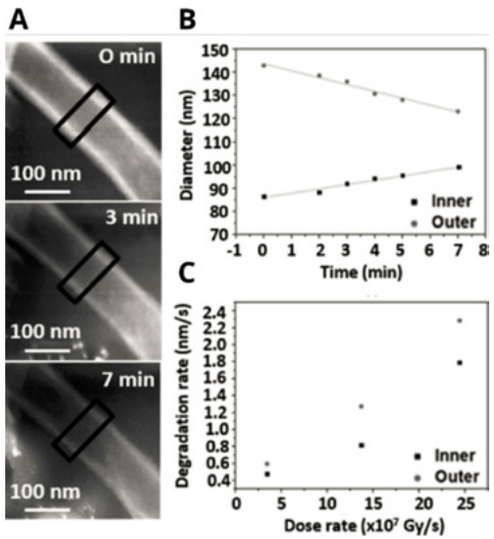

Figure 3: Thinning of the inner and outer carbon wall as a function of electron beam dose. **A:** *In situ* liquid STEM images of a MWCNT during exposure to the electron beam. **B:** Thinning of the carbon wall as a function of time. **C:** Degradation rate of the carbon wall as a function of electron dose.

Discussion

The interaction of the electron beam with water molecules during *in situ* electron microscopy is a well-studied process. When the electron dose is too high, the beam can act as a reducing agent and induce radiolysis of water molecules, generating a variety of highly reactive species such as hydrated electrons, free radicals, and other ROS. Once formed, these reactive chemicals can react with other nearby molecules in solution leading to chain reactions and the formation of more ROS. These reactions are often observed in the form of hydrogen gas generation or the nucleation of nanoparticles from metallic salt solutions. Rather than eliminating the formation of such reactive chemical species, researchers in Alloyeau's group used it to their advantage. They realized that the ROS generated in water by the electron beam are identical to those found inside a macrophage cell. Thus, they were able to exploit the process of radiolysis of water by the electron beam to simulate the chemical environment found inside a macrophage cell. This enabled them to study the mechanism used by macrophage cells to break down foreign material in real time. Because degradation only occurred when the MWCNTs were contained in water, and not ethanol, the researchers were able to pinpoint that hydroxyl radicals were responsible for the MWCNT damage that they observed *in situ* and in the macrophage cells. Once hydroxyl radicals were identified as having a primary role in the mechanism of wall thinning and hole formation during MWCNT biodegradation, the researchers explored the biological pathways that produce hydroxyl radicals *in vivo*. *In vitro* studies showed that the MWCNT degraded within the phagosomes of

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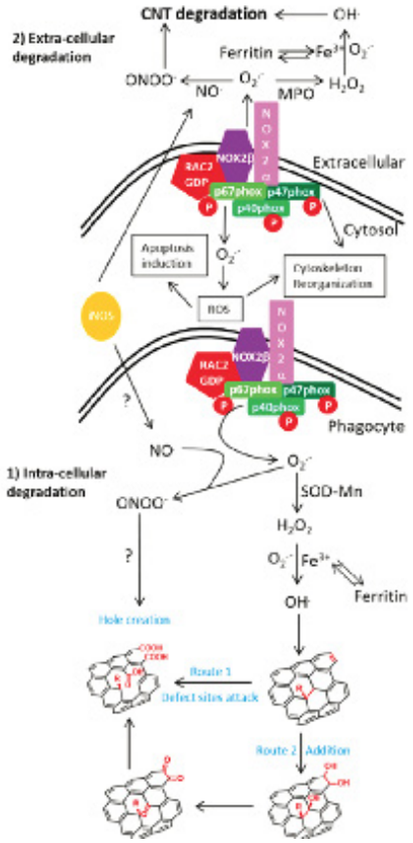

Figure 4: Mechanism of MWCNT degradation in macrophage cells via formation of hydroxyl radicals.

the macrophage cells. Phagosome membranes are known to contain the NOX_2 enzyme complex. Active NOX_2 catalyzes the formation of ROS, eventually forming hydroxyl radicals. The researchers postulated that once MWCNTs are engulfed by the macrophage cells and taken up into phagosome vesicles within the cell, the NOX_2 pathway catalyzes the formation of hydroxyl radicals via the Haber-Weiss reaction as shown in Figure 4. These hydroxyl radicals can then attack defect sites on the surface of the MWCNT resulting in the formation of perforations and holes in the graphitic structure, such as those seen in both the *in vitro* studies and the *in situ* TEM studies.

Conclusions

Researchers were able to elucidate the biodegradation mechanism of MWCNT in macrophage cells using the Poseidon Liquid TEM system. This work also demonstrates the utility of *in situ* liquid TEM studies in complementing and corroborating traditional approaches to *in vitro* TEM analysis of biological specimens. This work represents some of the first examples of graphitic nanostructures imaged at high magnification in liquid with electron microscopy. It also highlights the use of non-aqueous solvents, such as ethanol, as a strategy for eliminating damage to nanomaterials due to ROS, which may expand the utility of *in situ* liquid TEM studies for many applications.

For information about how Poseidon Select can be utilized for your applications, please contact Protochips at 919-377-0800 or visit us at www.protochips.com.

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