Iron-sequestering molecules play a pivotal role on the mechanism of toxicity of SWNTs on fungal and bacterial cells

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ABSTRACT

For all plants and animals, and for virtually all microbes, life without iron is impossible. Even though iron is the fourth most abundant element in the Earth's crust, it is present in the form of extremely insoluble minerals, which severely restrict the bioavailability of this metal. Microorganisms require iron for a variety of metabolic processes. In response to this, microorganisms secrete high-affinity iron-binding compounds called siderophores. Previous research has revealed that single-walled carbon nanotubes (SWNTs) containing iron particles, interact with bacteria and fungi and exhibit antimicrobial activity. The bactericidal mechanism is still not well understood. Some reports claim that severe cell membrane damage by direct contact with SWNTs is a plausible mechanism. However, in this study we described that iron-sequestering molecules are responsible for the toxicity of SWNTs to microorganisms. After incubating different concentrations of functionalized SWNTs with bacteria and fungal cells, it was found that these microorganisms produced siderophores. These molecules were seen in the medium as early as ten minutes after the addition of SWNTs. After 30 minutes we were able to see some bacterial and fungal cells saturated with siderophore-containing SWNTs. In addition, in the culture medium by itself, we noticed siderophores filled with SWNTs. Further analysis of the bacterial and fungal cells saturated with siderophore-containing SWNTs revealed that those bacterial cells were dead due to the fact that too many SWNTs present in the bacterial cells interfered with processes such as binary fission, and electron transport. Direct contact with the SWNTs is not a possible mechanism in this case since the microorganisms had enough time to produce siderophores and still thrived for a few minutes after the endocytosis of the siderophores. In fact, we noticed that the organisms were able to live for a couple of hours with the SWNTs inside before they started to die. This observation was true for both the bacterial and fungal models used. In addition, we noticed that the effect of siderophore-containing SWNTs in plants had the opposite effect. Instead of dying, the plants were able to grow better. We were able to conclude that high levels of metals such as Al, Cu, and Ni in the soil inhibit Fe acquisition and thus plant growth. Siderophores supply plants with Fe from the SWNTs, which enhanced chlorophyll content and lowered the formation of free radicals. This work provides more insight on the toxicity effects of SWNTs in different organisms and the potential use of SWNTs as a new method to treat bacterial and fungal diseases.

Keywords: Single walled carbon nanotubes, iron, siderophores, bacterial cells, fungal cells, toxicity.

1 INTRODUCTION

Infectious diseases have been a major cause of morbidity and mortality throughout our history [4]. Antimicrobial drugs have saved the lives and eased the suffering of millions of people by bringing many serious infectious diseases under control [5]. These gains are now seriously jeopardized by the emergence and spread of microbes that are resistant to these drugs. The decline in effectiveness of existing drugs is a consequence of a complex interaction between natural selection, the environment, and patterns of drug use and misuse [6]. Infections caused by resistant microbes fail to respond to treatment, resulting in prolonged illness and greater risk of death [7]. Most alarming of all, are diseases where resistance is developing for almost all currently available drugs. In the past, medicine and science were able to stay ahead of this natural phenomenon through the discovery of potent new classes of antimicrobials, a process that flourished from 1930-1970 and has since slowed to a virtual standstill [10]. This is a result of misplaced confidence that infectious diseases had been conquered, at least in the industrialized world [7]. Hospitals are a critical component of the antimicrobial resistance problem worldwide. Resistant hospital acquired infections are expensive to control and extremely difficult to eradicate [11].

Iron is an essential mineral used by many enzymes for its ability to trap small molecules like oxygen and to act as a conduit of electron transfer [1]. Humans get the supply of iron from the diet. The bacteria and fungi that inhibit the human bodies need to steal the iron that they require from us [1]. However, the human body has mechanisms to sequester iron tightly and deliver it to our cells. Therefore, the amount of free iron left for bacteria and other microorganisms is extremely small, and successful bacteria have to find ways to gather it efficiently [1]. One of the major ways that microorganisms gather iron is secreting siderophores. These are small, flexible, specialized molecules that contain oxygen atoms that surround and trap individual iron atoms in the environment [3, 2,8]. Siderophores gather up iron ions when they find them, efficiently solubilize iron due to their high binding

capacities for this element, and by a specialized transport system delivers the siderophores into the bacterial or fungal cells [1].

In this study, we focused on the mechanism that microbes utilize to get iron from the environment , and to see if that mechanism played a pivotal role on the mechanism of toxicty of iron-containing SWNTs on these microorganisms.

2 EFFECTS OF CARBON NANOTUBES ON MICROBIAL AND PLANT CELLS

Previous studies have shown that the size and surface area of the CNT are important characteristics that play a role in the toxicity of carbon nanotubes [12]. It was found by Kang et al, that the diameter of the of the nanotubes used determined the amount of stress and damaged cause to bacteria [12]. In addition, that study suggested that the toxicity mechanism of SWNTs was due to direct contact of the bacteria membrane with the SWNTs. However, in one of our previous studies, we found that SWNTs were internalized by bacteria and the bacterial cell did not die right away [13]. As a matter of fact, we noticed that various bacterial cellular processes were ffected a few hours after the addition of the functionalized SWNTs to the media [13]. Furthermore, in another one of our studies, we noticed that SWNTs had similar toxic effects on fungal cells after 48 hours [14]. Both of these studies show that the toxic effects of SWNTs on bacterial and fungal cells are not due to direct contact with the cells creating the cell to lyse, since the organisms are able to thrive for a few more hours after exposure.

2.1 Siderophore production by bacterial cells

To study in more detail what is the mechanism of toxicity of SWNTs to the microbial cells, *Escherichia. coli* and *Pseudomonas aeruginosa* were grown on TSA medium containing 1 and 2 μg of functionalized SWNTs. A sample of the culture medium and the actual bacterial growth were taken every 10 minutes for two hours for microscopic analysis using a transmission electron microscope (TEM) (JEOL JEM 2100). It was found that after 10 minutes, in the culture medium as well as in the bacterial cell sample of the group treated with SWNTs, we were able to observe small structures of about 20-50nm in size (Fig. 1). In addition, by taking a closer look, we were able to see that there were diffrences between those structures. Some of the structures look darker than others. In the group that was not treated with SWNTs we did not observed those sturctures until after a day or two. This was true for both *E. coli* and *P. aeruginosa* (Fig. 1).

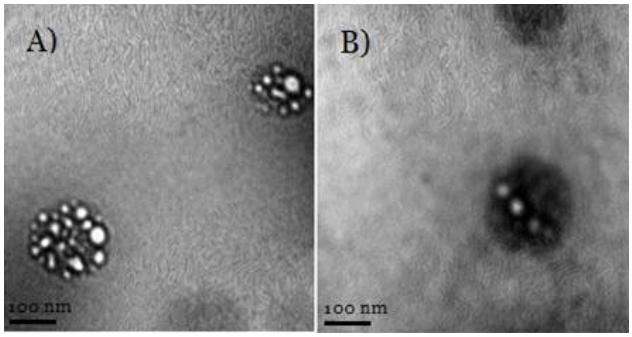

Figure 1. Culture medium treated with SWNTs. A) Empty siderophores found 10 min after addition of SWNTs. B) Siderophores containing SWNTs found 30 min after addition of SWNTs to the bacterial cultures. Both images taken with JEOL JEM 2100.

Furthermore, taking a look at the bacterial cells, we noticed not only the structures inside the bacterial cells, but also a difference between the cells that had structures associted with them and those that did not (Fig. 2). The ones that had structures associated with them look more dense inside, as if they had deposits of some material versus the ones that were not associated with those molecules (Fig. 3). To determine what material was accumulating inside those bacterial cells, we performed an Energy-Dispersive X-Ray analysis (EDS), and we were able to determine that those bacterial cells had iron. This observation led us to believe that the bacterial cells had an accumulation of SWNTs containing iron inside of them, and that's the reason why the bacterial cells die. The bacterial and fungal cells are able to sense the iron trapped in the SWNTs, that is why they start to sinthesized siderophores and those siderophores trapped the SWNTs. Once the nanotubes are inside the siderophores, the nanotubes get inside the bacterial cell using the normal receptor-ligand way that the siderophores utilize to get inside the cell. A continous accumulation of SWNTS inside the microbial cells causes metal toxicity in those organisms and prevented them from dividing, and realizing other metabolic processes such as electron transfer, etc.

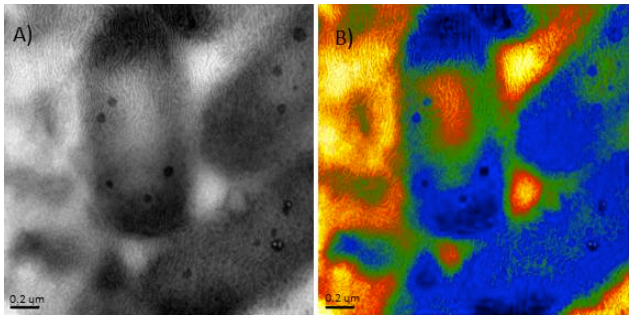

Figure 2. Bacterial cells containing siderophores 30 min after addition of SWNTs. A) Bacteria cell after endocytosis of siderophores containing SWNTs. B) Thermal image of panel A. Both images taken with JEOL JEM 2100.

2.2 Effect of SWNTs on plants

To test the toxic effects of SWNTs on higher eukaryotes, the medium to grow berry plants was treated with SWNTs containing-iron. Different concentrations of SWNTs were used in the medium to grow the plants. A significant difference was found between the plants that received no functionalized SWNTs and those that received 10 µg. It was evident that instead of dying, the plants were able to grow better. These results could be due to the fact that high levels of metals such as Al, Cu, and Ni in the soil inhibit Fe acquisition and thus plant growth. Siderophores supply plants with Fe from the SWNTs, which enhanced chlorophyll content and lowered the formation of free radicals, which makes the plants grow better [Fig. 4]. In addition, due to the toxic effects of SWNTs on bacterial and fungal cells, it can be elucidated that if there are any microorganism that affects plant growth, they will die after ingestion of SWNTs. In addition, it was found that just as with bacterial and fungal cells, nematodes that affect plants are also susceptible to the toxic effects of SWNTs [Fig. 5]. Therefore, by getting rid of plant pathogens, the plants can also grow better.

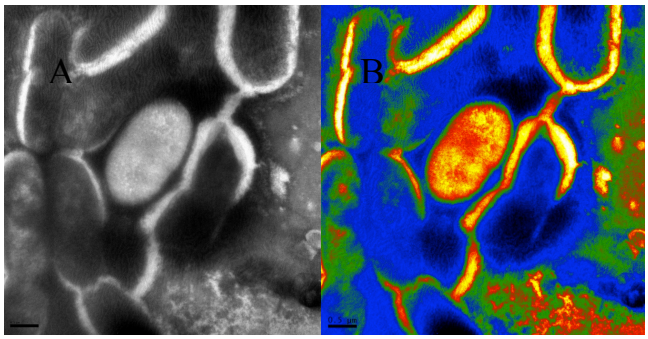

Figure 3. Bacterial cells filled with SWNTs containing iron. A) Bacteria cell after ingestion of siderophores containing SWNTs. Note the bacterium in the middle that doe not contain SWNTs B) Thermal image of panel A. Both images taken with JEOL JEM 2100

3 CONCLUSIONS

The results presented here allow us to get closer and closer to elucidate the mechanism of toxicity of SWNTs on microbial organisms. In addition, in this study we were able to observe the potential toxic effects of SWNTs on higher organisms such as plants. Furthermore, this study, allowed us to conclude that the toxic mechanism of SWNTs on bacterial and fungal cells is actually beneficial to the plants. This observation allowed us to get closer to understanding the toxic effects of SWNTs in different organisms. Toxicity studies using SWNTs are often contradictory. Some of the reasons for the variation in those results could be due to the different purities and functionalization of the SWNTs, as well as the difference in cell culture media, or the types of cells used. These observations highlight the importance for a standard production, purification and characterization methods for carbon nanotubes before testing their toxic effects on mammalian cells.

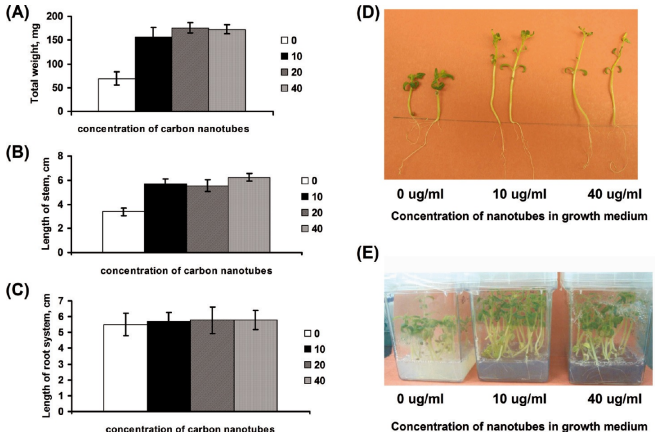

Figure 4. Effect of SWNTs on berry plants. Different concnetrations of SWNTs were used to treat the medium in which the plants were grown. A significant difference in gorwth was noticed between those plants that were not treated with SWNTs and those that were treated with 10 µg of functionalized SWNTs.

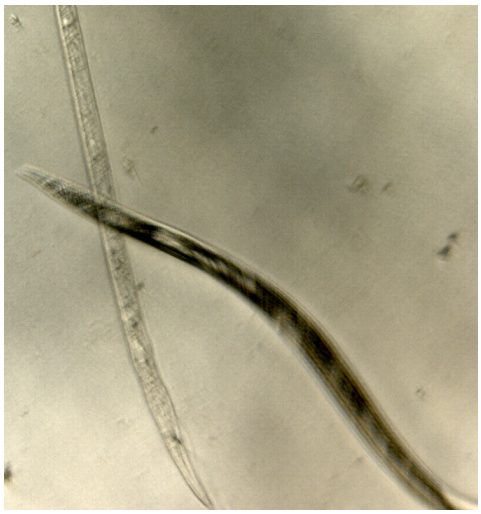

Figure 5. Effect of SWNTs on Nematodes. Nematode that ingested SWNTs (dark one) vs. one that did not. SWNTs were toxic and killed those nematodes that ingested the tubes after a 3-4 days.

REFERENCES

[1] K. Hotta, C.Y. Kim, D.T Fox, A.T. Koppisch, "Siderophore-mediated iron acquisition in Bacillus

anthracis and related strains", Microbiology, 156, 1918-1925, 2010.
[2] R. Trskova, P. Rychlovsky, I. Nemcova, A. Jegorov, "Development of a spectrophotometric determination of siderophores using flow-injection analysis", Talania, 42, 837-843, 1995.
[3] J.B. Neilands, "Microbial iron compounds", Annual review of Biochemistry, 36, 285-309, 1982.
[4] M.B. Avison, "New approaches to combating antimicrobial drug resistance", Genome Biology, 6, 243, 2005.
[5] P.D Lister, D.J Wolter, N.D Hanson, "Antibacterial-resistant Pseudomonas aeruginosa: Clinical Impact and complex regulation of chromosomally encoded resistance mechanisms", Clinical Microbiology Reviews, 4, 582-610, 2009.
[6] N.K Peters, D.M Dixon, S.M. Holland, A.S. Fouci "The research agenda of he National Institute of Allergy and Infectious Diseases for antimicrobial resistance", Perspective, 197, 1087-1093, 2008.
[7] J. Gums, S. Ranka, C. Jearmine, "Significant heterogeneity found in resistance trends between hospitals: Results of the antimicrobial resistance management program. 47th Annual Meeting of the Infectious Diseases Society of America (IDSA)", 47, 2007.
[8] B.F. Matzanke, G. Muller-Matzanke, K.N. Raymond, "Siderophore-mediated iron transport", Iron Carriers and Iron Proteins, 1-121, 1989.
[9] J.M. Meyer, "Exogenous siderophore-mediated iron uptake in Pseudomonas aeuruginosa: possible involvement of porin OprF in iron translocation", Journal of General Microbiology, 138, 951-958, 1992.
[10] G.S. Simonsen, J.W. Tapsall, B. Allegranzi, E.A. Talbot, S. Lazzari, "The antimicrobial resistance containment and surveillance approach – a public health tool", Bulletin of World Helath Organization, 82, 928-934, 2004.
[11] E. Taylor, T.J. Webster, "Reducing infections through nanotechnology and nanoparticles", International Journal of Nanomedicine, 6, 1463-1473, 2011.
[12] S. Kang, M. Herzberg, D>F Rodrigues, M. Elimelech, "Antibacterial Effects of Carbon nanotubes: Size does Matter!", Langmuir, 13, 6409-6413, 2008.
[13] T. Mangir, J. Chaves, S. Chaves, ' Impact of CNT Ingestion on In-vitro cells', Nanotechnology, 2, 168-171, 2008.
[14] J.S. Chaves, S.J. Chaves, " Novel Approach using SWCNT as a mechanism of toxicity on fungal and bacterial cells', Nanotechnology, 3, 300-303, 2012.