Novel Approach using SWCNT as a Mechanism of Toxicity on Fungal and Bacterial Cells

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ABSTRACT

Antimicrobial resistance has developed into a global public health issue. If a microbe is resistant to many drugs, treating the infection can become difficult or even impossible leading to serious disability or even death. The decline in effectiveness of existing drugs is due to a complex interaction among natural selection, environment, and drug use and misuse. Previous research has revealed that single-walled carbon nanotubes (SWCNTs) interact with bacteria and exhibit antibacterial activity [1]. However, to date, there has been no evidence on the direct effect of SWCNTs on fungi. In addition, the bactericidal mechanism is still not well understood. Some reports claim that severe cell membrane damage by direct contact with SWCNTs is a plausible mechanism [2].

The overall goal of this project was to characterize the potential use of SWCNTs as an effective alternative to present antimicrobial agents in dealing with drug-resistance. In this study, we look at possible mechanisms to explain the antimicrobial property of SWCNTs. We propose that toxicity due to the uptake of SWCNTs by the microorganisms is the most likely mechanism for the antimicrobial quality of SWCNTs. After incubating different concentrations of SWCNTs with bacterial and fungal cells, we noticed that interference with binary fission, budding and other cellular processes is what causes the organism to eventually die and not by a direct contact with the SWCNTs. In fact, we noticed that the organisms were able to live for a couple of hours with the SWCNTs inside before they started to die. This observation was true for both the bacterial and fungal models used. However, there were some technical challenges we had to address to determine the potential benefits of SWCNTs to fight microbial infections. First, was how the SWCNTs were presented to the organism, and second the purity and concentration of SWCNTs used. In this study we also describe a novel method to disperse the tubes using a biological agent. This method of dispersion showed very effective in that it made the tubes hydrophilic and easy to work with and it improved their purity. This work will advance both the fields of biomedical engineering and microbiology by providing a novel method for fighting infectious diseases that is nondrug related. It will also provide a foundation for further exploring the use of SWCNTs as building blocks for future antimicrobial materials.

Keywords: Single walled carbon nanotubes, bacteria, fungal cells, *Aspergillus fumigatus*, antimicrobial, treatment.

1 INTRODUCTION

Infectious diseases have been an important cause of morbidity and mortality throughout our history [4]. Over the years, antimicrobials have saved the lives and eased the suffering of millions of people by helping to bring many serious infectious diseases under control [5]. These gains are now seriously jeopardized by another recent development: the emergence and spread of microbes that are resistant to these drugs. We are now faced with a growing population of persistent bacteria that threaten to move us into what some consider the "post antimicrobial era" of infectious diseases [5]. 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. Some of the more problematic drug-resistant pathogens encountered today include methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus*, multidrug-resistant *E. coli*, *Pseudomonas aeruginosa*, and multi-drug resistant *Aspergillus fumigatus* [8, 10, 11].

Resistance to a single drug can spread rapidly through a microbial population. When antimicrobials are used incorrectly such as for too short of a time, at a low dose, or for the wrong disease, the likelihood that bacteria, fungi and other microbes will adapt and replicate rather than be killed is greatly enhanced [6]. 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 [12]. 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. The combination of highly susceptible patients, intensive and prolonged antimicrobial use, and cross-infection has resulted in nosocomial infections with highly resistant bacterial pathogens [7]. Resistant hospital acquired infections are expensive to control and extremely difficult to eradicate [13]. In addition

to bacteria, fungi are also becoming tolerant to antifungal treatments, either through biofilm formation or genetic tolerance [13]. Patients in ICU are often very susceptible to fungal infection, and as we have gained the ability to sustain the lives of sicker people, we have noticed an increase in fungal infections in the ICU [14]. Clearly, antifungal drug resistance is becoming a major problem in the field of pathogenic mycology [14].

Aspergillus is a genus of intense biological, industrial, agricultural and medical importance *Aspergillus fumigatus* is a saprophytic fungus that plays an essential role in recycling environmental carbon and nitrogen [4]. This genus represents a large family of fungi with over 185 species. The natural niche of *A.fumigatus* is soil, however, this species is also an important opportunistic human pathogen which may cause several diseases such as bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis, a fatal disease in immunocompromised individuals [15]. The mortality rate of invasive aspergillosis remains high approaching 80-90% [10]. Antifungal drug resistance of *Aspergillus* might partially account for treatment failure [10].

2 ANTIBACTERIAL EFFECTS OF CARBON NANOTUBES

2.1 Early studies have indicated that the size and surface area of the CNT are important material characteristics from a toxicological perspective [2]. The antimicrobial properties of CNTs have been documented with a dependence on carbon nanotube diameter [13, 2]. It was found by Kang et al., that oxidized and acid-treated SWCNTs in suspension had a stronger antimicrobial activity to E. coli cells than large diameter MWCNTs [1]. These observations led them to conclude that the diameter of the nanotube determined the amount of stress and damaged caused to the bacteria [2]. Furthermore, Kang et al. suggested that the toxicity mechanism of SWCNTs had to do with direct contact of the bacterial membrane with the SWCNT [2]. However, despite the general agreement about the antimicrobial properties of SWCNTs, a mechanistic explanation of SWCNTs toxicity is still elusive. In one of our previous studies, we found that the SWCNTs can be internalized by *Pseudomonas aeruginosa* [16]. In addition, the bacterial cell does not die right away as it can be noted by the fact that multiple flagella are produced (Fig. 1). *P. aeruginosa* produces one single polar flagellum, therefore, this observation indicates that the organism has time to up regulate the multiple genes involved in flagellum production. This observation indicates that the bacteria ingest the tubes and once the tubes are inside, they interfere with the microbe cellular processes causing the bacteria to stress. Results from toxicity studies with SWCNTs are often contradictory, likely because of the use of SWCNTs of different purities and functionalization, different cell culture media, and the type of cells used [2, 17]. Such observations highlight the need for a standard purification and physiochemical characterization methods for SWCNTs before testing their toxic effects.

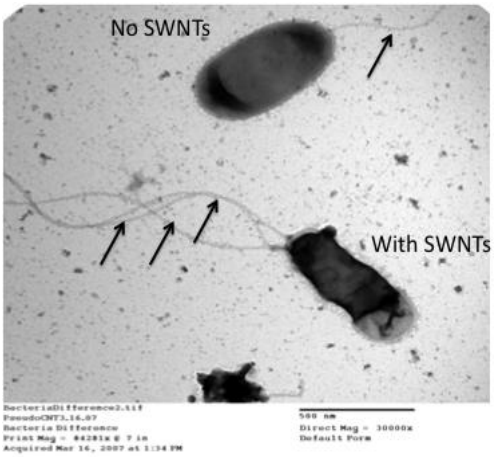

Fig. 1.Effect of SWCNTs in bacteria. The number of flagella produced (see arrows) by both bacterium is different indicating that SWCNTs cause the bacterium to undergo genetic mutations.

2.2 Effect of SWCNTs on fungal cells

Aspergillus fumigatus spores were grown on RPMI 1640 media containing 2% glucose. The spores were inoculated at an estimated concentration of 1000 spores per flask. At the time of initial inoculation, 10 µg of SWCNTs were added to one of the flasks. The flasks were incubated at 37 ºC shaking at 250 rpm. After 12 hours, it was noticed that the spores from the flasks that did not contain SWCNTs had germinated and *A. fumigatus* mycelia were starting to attach to the sides of the flasks. However, no growth was noticed in the flask that was treated with SWCNTs. After 48 hrs, the flasks that were not treated with SWCNTs showed increase mycelial growth, and growth was noticed in the flask treated with SWCNTs (Fig. 2). In addition, after 48 hours figure 2B shows that in the SWCNT treated group the *A. fumigatus* colonies that were floating in the media, as well as the mycelia attached to the flasks, had a black pigmentation as opposed to the colonies and the mycelia mat on the control group (Figure 2A).

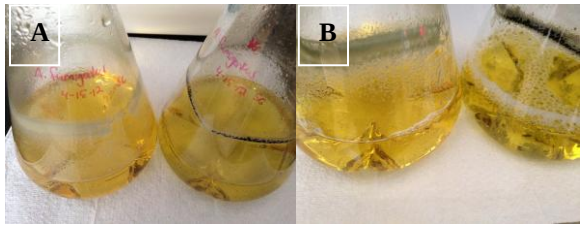

Figure 2: Mycelia mat comparison. In both figures left flask is the control.

A sample from each control and treated group was taken and further examined under the microscope. It was clear that each colony of *A. fumigatus* that was growing in the SWCNTs treated group had a clump of nanotubes in the middle of the colony (Fig. 2C, 2D). This observation is of extreme interest since it means that the spores that germinated to create the fungal colony had to sence some sort of signal to clump the nanotubes in the middle of the colony. Our hypothesis is that the fungi are sensing the iron in the SWCNTs by a siderophore mechanism, and they sequester that iron in the middle of the colony to supply them of this very important nutrient. However, after 60 hours it was pretty visible that the fungi from the SWCNT treated group lost their ability to attach further to the flask.

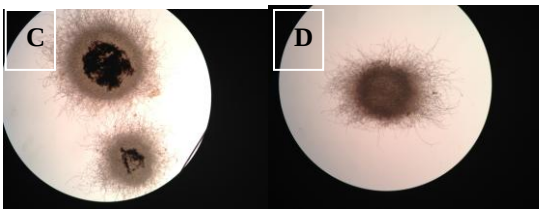

Figure 2: Left Fungi treted with SWCNT.Right, control.

As shown in figure 3 A and 3B, the fungi from the control group not only was still able to attach but also they were still growing since the mycelia mat kept on growing past 2cm (figure 3C). On the other hand, the SWCNTs treated group lost their ability to attach and the fungi also stopped growing (Fig. 3D) since the mycelial mat on the side of the flask stayed at 0.5 cm, which was the same measurement obtained after 48 hours. This is an important observation since the ability to attach to a substrate is a virulence factor used by fungi to invade the host.

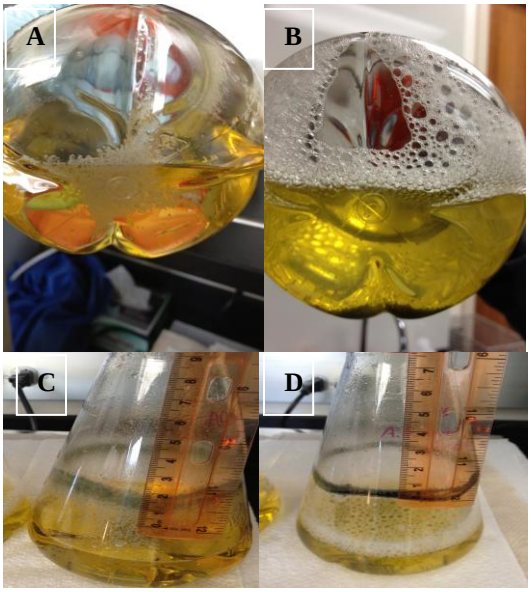

Figure 3: Left images are control. Right images sample treated with SWCNT.

Furthermore, we wanted to confirm that in fact the reason why the mycelial mat from the SWCNTs treated group did not grow was because after 60 hours those cells were dead. Therefore, we took samples from the mycelial mat that remained attached to the flask plus a sample of the cells that were floating in the media, and treated those samples with the dye trypan blue. Trypan blue is a vital stain use to selectively color dead cells blue. Healthy live cells with intact cell membranes cannot internalize the dye. It was clearly shown that those cells from the SWCNTs treated group were dead (Fig. 4). In addition, the pH of the media from both SWCNTs treated and untreated group were measured, and it was found that the pH of the SWCNTs group was more acidic (pH 4) than the media from the control group (pH 5.5). This indicates that the fungal cells from the SWCNTs treated group were more stressed, which caused them changes in their metabolism as more fermentation occured in those samples.

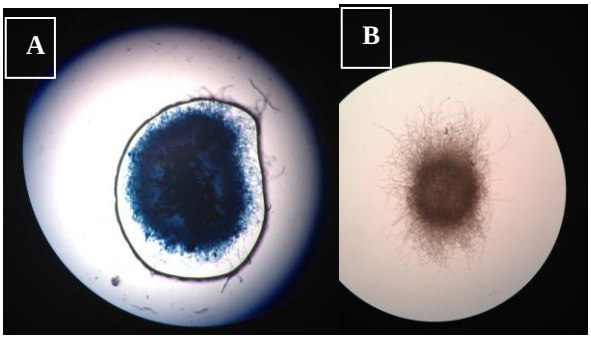

Figure 4: Left, fungi treated with SWCNT exposed to Trypan blue. Right control exposed to Trypan blue.

By-products of fermentation are acid and gas production, as more bubbles (indicative of gas production) as visible in that sample as opposed to the control. This observation is proof-of-principle that direct contact of the nanotube with the cell membrane of the fungi is not a likely mechanism of toxicity. As with bacteria, fungi are able to internalize the nanotubes and once inside the cell, the nanotubes cause a series of metabolic changes inside the cell. In this paper we show that the fungal cells have time to upregulate pathways that deal with the fermentation process of the sugar in the media. This indicates that cell death of the fugi does not occur immediately after contact with the nanotube. The fungi has time to sense something is wrong and the fungal cells start to stress causing their metabolism to go hay-wire. This observation is also noticeable in the media of the treated group as more bubbles are visible in that sample as opposed to the control (Fig. 5).

Figure 5: Controls on the side and the fungi treated with SWCNT on the middle.

3 CONCLUSIONS

The results presented here, bring us closer to the possibility of using SWCNTs as potential antimicrobial agents. More experiments are being conducted currently that will explain more in detail what is going on inside the cells. The advantage of using SWCNTs as antimicrobial agents is that the possibility to develop antimicrobial resistance is not likely since SWCNTs are not chemicals or drugs, therefore, genetic information cannot be transferred from one organism to the next. However, in order for SWCNTs to be used for this purpose, the tubes must be dispersed and in a pristine condition. Different preparation processes could not only produce different impurities, but also different lengths, different sizes, and different surface defects of SWCNTs [18]. All of these will influence the consequence of toxicity tests using microbes and SWCNTs.

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